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Importance of metamorphosis in coral-reef fish larval recruitment facing anthropogenic pressures

Marc Besson

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THÈSE DE DOCTORAT

de l'Université de recherche Paris Sciences et Lettres
PSL Research University

Préparée à l'École Pratique des Hautes Études

Importance of metamorphosis in coral-reef fish larval recruitment
facing anthropogenic pressures

Ecole doctorale de l'EPHE – ED 472

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Soutenue par :
Marc BESSON
le 9 octobre 2017

Dirigée par :
Dr. David LECCHINI
Pr. Vincent LAUDET

COMPOSITION DU JURY :

Pr. Stuart SANDIN
*Scripps Institution of Oceanography,
UCSD*
Rapporteur

Pr. Stephen SWEARER
School of Biosciences, UMELB
Rapporteur

Dr. Jennifer CASELLE
Marine Science Institute, UCSB
Membre du jury

Pr. Jodie RUMMER
*ARC Centre of Excellence for Coral Reef
Studies, JCU*
Membre du jury

Dr. Serge PLANES
*CRIOBE, EPHE-CNRS-UPVD, PSL
Research University*
Président du jury

Dr. David LECCHINI
*CRIOBE, EPHE-CNRS-UPVD, PSL
Research University*
Directeur de thèse

Pr. Vincent LAUDET
*BIOM, OOB, Université Pierre et Marie
Curie*
Codirecteur de thèse



École Pratique
des Hautes Études



« L'important n'est pas de prendre des poissons mais d'essayer de les attraper ! »

René Barjavel, 13 octobre 1984

Mais d'aucuns disent que cela a également été prononcé par D. Lecchini et V. Laudet

Summary

Abstract

The persistence and sustainability of coral-reef fish populations depends on the continued larval recruitment, *i.e.* successful settlement by pelagic larvae into adult reef habitats and post-settlement survival through metamorphosis to a juvenile stage. There is growing evidence that changes to water conditions due to global change and waterborne pollution can impair coral-reef fish sensory abilities to locate settlement habitats that maximize growth while minimizing mortality risk. However, the inner mechanisms of such impairments remain less well studied. In this thesis, I have examined the recruitment phase of the convict surgeonfish *Acanthurus triostegus*, and determined that the ecological, morphological, physiological, and behavioral changes occurring at recruitment correspond to a metamorphosis mediated by thyroid hormones (TH). Then, I investigated whether this metamorphosis is prone to endocrine disruption under anthropogenic disturbances such as elevated sea water temperature and agricultural pesticide pollution. I demonstrated that such pressures can reduce TH levels at a critical developmental stage in coral-reef fishes, impairing their metamorphic processes such as intestine remodeling, sensory organ maturation, and sensory abilities acquisition, further increasing their mortality rates. Overall, this thesis is a holistic analysis that addresses molecular, histological, anatomical, and behavioral assays of multiple stressors affecting coral-reef fish recruitment. It indicates the importance of a proper endocrine function during coral-reef fish recruitment, highlighting the need for a better understanding of these processes for coral-reef conservation.

Résumé (FR)

Le maintien et le renouvellement des populations de poissons coralliens dépendent en grande partie du recrutement larvaire, c'est-à-dire de l'installation des larves pélagiques dans les habitats récifaux adultes, et de leur survie après s'être métamorphosées en juvéniles. De plus en plus d'études révèlent que les changements de composition de l'eau, causés par le changement climatique et la pollution, peuvent altérer les capacités sensorielles des poissons coralliens, diminuant leurs aptitudes à localiser des habitats propices (maximisant leur croissance et diminuant leur mortalité) lors de l'installation. Cependant, les mécanismes internes à l'origine de ces phénomènes sont méconnus. Lors de cette thèse, j'ai examiné le recrutement larvaire du poisson chirurgien bagnard *Acanthurus triostegus* et mis en évidence que les changements écologiques, morphologiques, physiologiques et comportementaux qui s'y déroulent correspondent à une métamorphose contrôlée par les hormones thyroïdiennes (HT). J'ai ensuite analysé comment des stress d'origine anthropique, tels que l'élévation des températures de surface et la pollution par un pesticide d'origine agricole, peuvent perturber sa métamorphose. Lors de cette étape clé de leur cycle de vie, ces perturbations diminuent les taux d'HT, altérant la maturation de leurs organes sensoriels, leurs capacités sensorielles, et augmentant leur mortalité. Cette thèse est donc une analyse holistique de l'impact des perturbations anthropiques sur les processus moléculaires, et les changements histologiques, anatomiques et comportementaux du recrutement larvaire des poissons coralliens. Elle souligne l'importance du système thyroïdien, et invite à une meilleure compréhension des processus endocriniens du recrutement larvaire, dans l'optique d'une amélioration de la conservation des récifs coralliens.

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Figure I.1 | Coral reef types in French Polynesia

A: Barrier reef (BR) and fringing reef (FR) in Maupiti Island. Fringing reefs can develop in shallow waters along the coasts while barrier reef corresponds to a coral reef growing parallel to the shoreline and separated from it by a lagoon. **B:** Tetiaroa Atoll. Atolls are rings of coral reefs and small islets surrounded by the ocean and surrounding a sandy lagoon where a volcano was standing before being submerged. **Pictures: M. Besson.** p. 40

Figure I.2 | Fishes in coral reefs

A: Blacktail snappers (*Lutjanus fulvus*) on the foreslope reef of Fakarava Atoll (Tuamotu Archipelago, French Polynesia). **B:** Hawaiian bigeyes (*Priacanthus meeki*) on the foreslope reef of Rangiroa Atoll (Tuamotu Archipelago, French Polynesia). **C:** Yellowfin surgeonfishes (*Acanthurus xanthopterus*) in the channel pass of Rangiroa Atoll (Tuamotu Archipelago, French Polynesia). **D:** Convict tangs (*Acanthurus triostegus*) on patch reefs near the barrier reef of Tetiaroa Atoll (Society Archipelago, French Polynesia). **E:** Blue-green damselfishes (*Chromis viridis*) and Whitetail dascyllus (*Dascyllus aruanus*) in the lagoon of Moorea (Society Archipelago, French Polynesia). **F:** Blue-green damselfishes (*C. viridis*) around their *Pocillopora* shelter in a fringing reef of Moorea Island (Society Archipelago, French Polynesia). **Pictures: L. Thiault.** p. 41

Figure I.3 | Herbivorous fishes grazing on algae in a bleached coral reef

A: Two herbivorous fishes: *Chaetodon ephippium* (front) and *Acanthurus nigricans* (back) feeding on turf algae that have recently grown on dead *Pocillopora* following a bleaching event in Moorea Island (French Polynesia). **B:** same observation with the line surgeonfish (*Acanthurus lineatus*). **Pictures: L. Hedouin.** p. 41

Figure I.4 | Ten coral reef fishes from iconic coral reef fish families

A: *Acanthurus triostegus* (Acanthuridae). **B:** *Myripristis berndti* (Holocentridae). **C:** *Chaetodon humeralis* (Chaetodontidae). **D:** *Dascyllus trimaculatus* (Pomacentridae). **E:** *Salarias fasciatus* (Blenniidae). **F:** *Apogonichthyoides pseudotaeniatus* (Apogonidae). **G:** *Stethojulis bandanensis* (Labridae). **H:** *Scarus spinus* (Scaridae). **I:** *Mulloidichthys flavolineatus* (Mullidae). **J:** *Carangoides ferdau* (Carangidae). **Adapted from FAO species identification guides (FAO 2001).** p. 42

Figure I.5 | The convict tang *Acanthurus triostegus* as a model for teleost coral reef fish characteristics

n.: nostril, e.: eye, s.: scales, l.l.: lateral line, a.: anus, op.: operculum, p.op.: pre-operculum, m.: mouth. d.f.: dorsal fin, c.f.: caudal fin, a.f. : anal fin, pel.f. : pelvic fins, pec.f. : pectoral fin. SL: standard length, BH: body height, SnL: snout length. **Picture: M. Besson.** p. 43

Figure I.6 | Life cycle of the convict tang *Acanthurus triostegus*

A. triostegus is a pelagic egg spawning species. This life cycle is representative of all coral reef fish species with this spawning mode (around 2/3 of all coral reef fishes). In benthic egg laying species, pelagic dispersal only concerns larvae, as hatching occurs within the reefs. Larval recruitment is different from the 'Recruitment' presented in this figure, and encompasses all the steps from the end of larval dispersal to the survival of juveniles in nursery areas. **Pictures: M. Besson.** p. 45

Figure I.7 | Settlement patterns or larval coral reef fishes in French Polynesia

Green and dark green lines indicate relative abundance of settlers captured in two crest nets in Moorea, while light blue, blue, and dark blue lines indicate relative abundance of settlers captured in three nets in Nengo Nengo. Circular points indicate a crest net with a westward orientation, triangular points indicate a northward orientation and square points indicate a south-eastward orientation. Black and white circles above each panel indicate the lunar phases: new moon (black circle), first quarter, full moon (white circle) and last quarter. Adapted from Besson (2017).

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Figure I.8 | Thyroid hormone levels and development in anurans

Dph: days post hatching. Circular structure on the 'guts remodeling' axis correspond to schematic representation of the intestine cross-sections. Adapted from Brown and Cai (2007) and Hasebe et al. (2013).

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Figure I.9 | Thyroid hormones and deiodinase enzymes

Schematic representation of the biological role of deiodinase enzymes. T4 and T3 are the two active forms of thyroid hormones (TH) that can bind thyroid hormone receptors (TR). Adapted from Bianco and Kim (2006) and Holzer (2015).

Figure I.10 | Thyroid hormone signaling

Environmental and metabolic cues can either induce or repress the release of TRH in the hypothalamus. TRH binds its receptors on the anterior pituitary, which then releases TSH. TSH binds its receptors on the thyroid, which then secretes TH. TH are transported to target cells where the deiodinase metabolism determines their faith. When T3 reaches TR in the nucleus, this activates the expression of metamorphosis genes (*e.g.*, *Klf9* gene, involved in brain development).

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Figure I.11 | Changes in TH levels (A) and TR expressions (B) during the development of the Pacific Bluefin tuna (*Thunnus orientalis*)

Peaks of TH levels and TR expressions are observed around the N and O developmental stages, which correspond to the stage where most changes are observed in the metamorphosing larvae. Adapted from Kawakami (2008a)

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Figure I.12 | Changes in TH levels (A) and TR expressions (B) during the development of the African clawed frog (*Xenopus laevis*) and the Japanese flounder (*Paralichthys olivaceus*)

TH (dotted lines) levels and TR expressions (solid and dashed lines) peak during metamorphosis of *X. laevis* and *P. olivaceus*. Contrarily, *TR α -B* in *P. olivaceus* (alternating dash-dot line) does not peak. Adapted from Shi (2000), Yamano and Miwa (1998) and figure originating from Schreiber (2013).

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Figure I.13 | Thyroid follicles in the sub-pharyngeal region of *Acanthurus triostegus*

Schiff reagent and hematoxylin staining of a 5 μ m histological section of *A. triostegus* sub-pharyngeal region (primarily fixed in Liquide de Bouin). The numerous thyroid follicles are revealed in indigo. Scale bar indicates 50 μ m.

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Figure I.14 | *Acanthurus triostegus* metamorphosis

Immediately after their entry in the reef at night, metamorphosing and transparent-larvae (left) and metamorphosed and pigmented-juveniles (right) can be encountered in the same nursery areas. The vertical bars of black pigmentation can appear in less than two hours.
Picture: M. Besson.

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Figure I.15 | Location of Moorea Island in French Polynesia

A: Location of French Polynesia within the Pacific Ocean. **B:** Location of Moorea Island within French Polynesia. **C:** Moorea Island. Scale bar indicates 2 km. Adapted from Besson (2017).

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Figure II.1 | Changes in *Acanthurus triostegus* during larval recruitment.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. **(A)** Schematization of the reef colonization by a larva (left) turning into a juvenile (right). The background color shading centered into the reef crest is re-used in following figures. **(B)** Weight variation during larval recruitment. n = 3 biological replicates for 5-to-10 km offshore fish (far), n = 5 for 2 km offshore fish (near) and n = 297 for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). **(C-G)** Variation in pigmentation and morphology at different sampling points, (C) crest larva, (D) day 2 juvenile, (E) day 3 juvenile, (F) day 5 juvenile and (G) day 8 juvenile; scale bars indicate 0.5 cm. The white square represents the magnified region shown in the line below. These pictures are representative of n > 50 fish (biological replicates) per developmental stage, sampled at n > 20 different times during the year (technical replicates). **(H-L)** Close-up on the pigmented middle body band. Scale bars indicate 0.2 cm. These pictures are representative of n > 50 fish (biological replicates) per developmental stage, sampled at n > 20 different times during the year (technical replicates). **(M-Q)** X-Ray microtomography of larva, dentition A, B or C are highlighted respectively in red, blue or green. Scale bars indicate 0.5mm. These pictures are representative of n = 2 fish (biological replicates) per developmental stage. **(R-V)** Histological sections (5 µm width) of intestines with haematoxylin/eosin staining. Scale bars indicate 200µm. These pictures are representative of n = 6 sectioning technical replicates, performed twice (sampling technical replicates), on n = 2 fish (biological replicate). **(W)** Intestine length variation of *A. triostegus* during its larval recruitment. n = 9 to 14 biological replicates for each developmental stage (from n = 2 technical sampling replicates). Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). **(X)** Heatmap clustering of the 18S sequences identified in the intestine content of crest individual (n = 3, left panel), day 8 juveniles (n = 3, middle panel) and adults (n = 3, right panel). Each panel is made of three columns corresponding to one individual. Each line corresponds to one taxonomic group found in the intestines: *Spermatophyta* (dark green), *Conoidasisa* (cyan), *Dinophyceae* (light yellow), *Protostomia* (light pink), *Phaeophyceae* (brown), *Floriodeophyceae* (dark pink), *Chlorophyta* (light green), *Stramenopiles* (orange) and *Ascomycetes* (grey).

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Figure II.2: Thyroid hormone signaling during *Acanthurus triostegus* metamorphosis.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. **(A)** T4 and **(B)** T3 dosage: Y-axis: T4 and T3 levels in pg.g⁻¹; X-axis: sampling point. n = 3 for 5 to 10 km offshore fish (far), n = 5 for 2 km offshore fish (near), n = 297 for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). **(C)** Expression level of *TRα-A*, *TRα-B*, *TRβ* and *Klf9* by qPCR, normalized with *Pold2* and *Rpl7*. Y-axis; Relative

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normalized expression X-axis: gene and sampling point. Far ocean larvae are split in two groups: Far a (black stripes) and Far b (white). For each panel, the color shading is the same one as in Figure II.1A. For each sampling point 3 biological replicates and 3 technical replicates were performed. Statistics: comparisons of each mean to the Crest condition mean (Student's t cumulative distribution function, which was automatically computed by *qPCR software CFX Biorad Manager*, CFX Manager 2017).

Figure II.3 | Impact of the environment and thyroid hormone signaling on *Acanthurus triostegus* metamorphic processes.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001.

(A) Upper panel: schematization of the external slope experiment with control fish, in the lagoon (grey circle), and relocated fish, on the external slope (orange circle). Lower panel: comparison of the pigmentation between the relocated fish (left) and the control lagoon fish (right) at day 2. (B) T4 and (C) T3 dosage of crest captured larvae and day 2, 5 and 8 juveniles, in control (grey shade) or external slope (orange shade) conditions. n = 4 fish for each experimental condition. X-axis: sampling point, Y-axis: T4 or T3 level in pg.g^{-1} . Statistics: mean comparisons between control and external slope (Wilcoxon-Mann-Whitney U-test). (D) Expression level of *TR α -A*, *TR α -B*, *TR β* and *Klf9* by qPCR, normalized with *pold2* and *rpl7* housekeeping genes, in control (grey shade) or external slope (orange shade) conditions. Y-axis: Relative normalized expression. X-axis: gene and stages. For each point 3 biological replicates and 3 technical replicates were performed. Statistics: comparisons of "external slope" means to their "control" counterparts (Student's t cumulative distribution function). (E) Intestine length in cm of fish at crest (C), day 2 (d2) or day 5 (d5) under different treatment conditions: solvent-control (grey shade), T3+IOP treated at 10^{-6} M (blue shade), NH3 treated at 10^{-6} M (green shade), T3+IOP+NH3 treated at 10^{-6} M (blue and green dashed shade) or located to the external slope (orange shade). n > 10 fish for each experimental condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the respective solvent-control condition are indicated. (F) Histological cross section (5 μm width) of intestine stained by haematoxylin/eosin. Scale bars indicate 200 μm . These pictures are representative of n = 2 microtome section and staining technical replicates, performed on n = 3 fish (biological replicate) per treatment and per developmental stage. Two technical replicates of this experiment were performed. (G) Mean number of bites (on turf algae) per fish in a batch of 18 fish at day 2 (d2) and day 5 (d5) under different treatment conditions: solvent control (DMSO, grey shade), T3+IOP at $10^{-6}\mu\text{M}$ (blue shade), NH3 at $10^{-6}\mu\text{M}$ (green shade) or relocated to the external slope (orange shade). n = 3 to 6 independent bite counts (technical replicates) for each condition. Bite counting was not performed on crest captured individuals as *A. triostegus* larvae do not feed for at least 12 hours after colonizing the reef at night (MB pers. obs.). Statistics: same as in (E).

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Figure II.4 | Thyroid hormone levels in distant coral reef fish at larval recruitment.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. TH levels of: *Rhinecanthus aculeatus* (upper), *Chromis viridis* (middle up), *Chaetodon lunula* (middle low) and *Ostorhinchus angustatus* (lower). n = 3 to 5 biological replicates for each condition. First column is T4, second column is T3. Y-axis: T4 or T3 level in pg.g^{-1} . X-axis: sampling point (developmental stage). The phylogenetic relationship between the species is plotted on the far left of the figure. Statistics: for each species, comparisons of each mean to the day 1 (d1) mean (Wilcoxon-Mann-Whitney U-test).

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Figure II.5 | Influence of chlorpyrifos exposure on *Acanthurus triostegus* thyroid hormone signaling, intestine length, grazing activity, and algal removal efficiency.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. **(A)** T3 level in pg.g^{-1} of day 2 (d2) and day 5 (d5) fish exposed to different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g.l}^{-1}$ (1, light yellow bars), $5\mu\text{g.l}^{-1}$ (5, yellow bars) or $30\mu\text{g.l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. n = 10 fish in each condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the solvent-control condition are indicated. **(B)** Intestine length in cm of fish at day 2 (d2) or day 5 (d5) under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g.l}^{-1}$ (1, light yellow bars), $5\mu\text{g.l}^{-1}$ (5, yellow bars) or $30\mu\text{g.l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. n = 5 to 8 fish for each experimental condition. Statistics: same as in (A). **(C)** Mean number of bites (on turf algae) per fish in a batch of 18 fish on reef turf at day 2 (d2) or day 5 (d5), under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g.l}^{-1}$ (1, light yellow bars), $5\mu\text{g.l}^{-1}$ (5, yellow bars) or $30\mu\text{g.l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. n = 3 independent bite counts for each condition. Statistics: same as in (A). **(D)** Mean turf biomass removed per fish in a batch of 10 fish, in 5 days following reef colonization, under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g.l}^{-1}$ (1, light yellow bars), $5\mu\text{g.l}^{-1}$ (5, yellow bars) or $30\mu\text{g.l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. n = 3 independent measurements of grazed turf for each condition. Statistics: comparisons of each mean to the solvent-control (C) mean (Wilcoxon-Mann-Whitney U-test).

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Figure II.6 | Model of *Acanthurus triostegus* metamorphosis and disruption by chlorpyrifos.

(A) Schematic representation of coral reef fish larval recruitment. TH level is plotted on the Y axis and development on the X axis. The onset of metamorphosis is in the open ocean and environmental influence upon development is plotted on the X-axis. **(B)** Consequences of undisturbed (upper panel) and CPF disturbed (lower panel) metamorphosis on grazing activity, and subsequent algal spreading and potential coral survival. Solid red bar indicates inhibition; dotted red line indicates lack of inhibition.

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Figure II.1 - figure supplement 1 | Changes in *Acanthurus triostegus* intestine and teeth during larval recruitment.

(A-O) Complete guts histology of *Acanthurus triostegus* after reef colonization. First column: anterior part of the intestine (first cm), middle column: medium part (variable), last column: posterior part of the intestine (1 cm), **(A-C)**: crest individual, **(D-F)**: 2 days juvenile, **(G-I)**: 3 days juvenile, **(J-L)**: 5 days juvenile, **(M-O)**: 8 days juvenile. Scale bars represent $200\mu\text{m}$. These pictures are representative of n = 6 section technical replicates for each of the proximal, middle and distal intestine, and were performed twice (sampling technical replicates), on n = 2 fish (biological replicates). **(P)** Heat map of bacterial gut community. Each column is one individual. Each row is bacterial taxa. Only the taxa with differential representations between the samples are shown. Sampling stages: day 2, day 5, day 8 and adult are indicated below the heat map. The shape of each of this developmental stage is schematized over the heat map. Each column is highlighted by a circle, the difference in shade between the circles emphasizes the stage difference. The color of each square in the heat map is proportional to the abundance of the taxa (row) in the sample (column). **(Q)** μCT scan of a crest larva, lateral view. Dental family C are highlighted in red and dental family B in blue. **(R)** μCT scan of an adult, frontal

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view (mouth open). **(Q-R)** The scale bar indicates 0.5 mm. These pictures are representative of $n = 2$ fish (biological replicates)

Figure II.2 – figure supplement 1 | Characterization of *Acanthurus triostegus* thyroid hormone receptors.

(A) Schematic representations of TR α -A, TR α -B and TR β from upper to lower rows respectively. The amino acid numbering of the DBD (light gray) and LBD (dark grey) appear above each TR. The 9 amino acid insertion of the TR β long isoform is indicated below the gray dashed area. **(B)** Phylogenetic reconstruction of TR amino acid sequenced by maximum likelihood. Bootstrap over supporting each branches are indicated when $> 700/1000$. *A. triostegus* genes are highlighted in red. **(C)** Transactivation assay of TRs in HEK293T cells with a luciferase reporter. X-axis: Tested genes and treatment condition. T4: blue bars, T3: red bars, T2: green bars, Tetrac: purple bars and Triac: orange bars. The concentrations range from 10^{-9} M to 10^{-7} M which corresponds to the light and dark shade of each color. Tested genes are indicated on the bottom line. Y-axis: arbitrary luciferase unit. 3 technical replicates were performed. **(D)** Transactivation assay of TRs in HEK293T cells with a luciferase reporter. X-axis: tested genes and treatment conditions. Light blue to dark blue bars: T3 treatments at 10^{-9} , 10^{-8} , and 10^{-7} M respectively. Light blue to dark blue dashed bars: T3+NH₃ treatments with T3 at 10^{-8} M and NH₃ at 10^{-8} , 10^{-7} and 10^{-6} M respectively. Y-axis: arbitrary luciferase unit. 3 technical replicates were performed.

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Figure II.2 – figure supplement 2 | Primers used in this study.

(A) Degenerated primers used for the cloning of the *A. triostegus* genes in this study. **(B)** qPCR primers used in this study. **(C)** Primers used for 18S mass sequencing. The overlapping nucleotides between 18SR and the blocking primer are indicated in bold. The mean annealing temperature of each primer is indicated in the last column.

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Figure II.2 – figure supplement 3 | List of thyroid hormone receptors sequences used for the phylogeny.

Species, genes, databases and accession numbers are indicated

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Figure II.3 – figure supplement 1 | Thyroid hormone signaling and environment impact on the dentition and guts histology of *Acanthurus triostegus* at metamorphosis

First column: X-Ray microtomography of larva, dentition A, B or C are highlighted respectively in red, blue or green. Scale bars indicate 0.5mm. These pictures are representative of $n = 3$ fish (biological replicates) per developmental stage and per treatment condition. **Second to fourth columns:** complete gut histology (5 μ m wide microtome sections) of *A. triostegus* at day 2 and day 5 following reef colonization. First column: anterior part of the intestine (first cm), middle column: medium part (variable), last column: posterior part of the intestine (last cm). Haematoxylin and eosin staining. Scale bars indicate 200 μ m. These pictures are representative of $n = 2$ treatment technical replicates, and $n = 2$ microtome section and staining technical replicates, on $n = 3$ fish (biological replicates) per treatment and per day

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Figure II.3 – figure supplement 2 | Pigmentation patterns and thyroid hormone signaling in *Acanthurus triostegus* at day 2 (d2) and day (d5) during larval recruitment

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These pictures are representative of $n > 10$ treatment technical replicates performed on $n > 100$ fish (biological replicates) per treatment and per developmental stage.

Figure II.5 – figure supplement 2: Pigmentation patterns and chlorpyrifos exposure in *A. triostegus* at metamorphosis

(A) T4 level in pg.g^{-1} of day 2 (d2) and day 5 (d5) fish exposed to different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1 \mu\text{g.l}^{-1}$ (1, light yellow bars), $5 \mu\text{g.l}^{-1}$ (5, yellow bars) or $30 \mu\text{g.l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 10$ fish in each condition.. See Figure 5 – source data 5 for detailed data and statistics. (B) Pigmentation patterns and in *A. triostegus* exposed to different conditions: solvent control, or CPF at $1 \mu\text{g.l}^{-1}$, $5 \mu\text{g.l}^{-1}$, or $30 \mu\text{g.l}^{-1}$ at day 2 (d2) and day (d5) during larval recruitment. These pictures are representative of $n = 2$ chlorpyrifos CPF exposure technical replicates performed on $n = 10$ fish (biological replicates) per condition and per day.

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Figure III.1 | Metamorphosis and sensory system changes at recruitment in *Acanthurus triostegus*

a, Morphological and pigmentation changes in *A. triostegus* from day 0 (d0) to day 8 (d8) following their entry in the reefs. Scale bar indicates 1 cm. **b**, Cross section in the eye retina stained in hematoxylin/eosin, presenting the dorsal side (ds) the ventral side (vs) and the optical nerve (on). The dotted gray rectangle represents the magnified region shown in (c). Scale bar indicates $100 \mu\text{m}$. **c**, Cross section in the eye retina stained in hematoxylin/eosin, presenting the ganglionar cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL) that contain ganglionar cells (ggc), bipolar cells (bpc) and photoreceptors nuclei (prn). In the anterior part of the retina, photocone external segments (pes) are identified. Scale bar indicates $50 \mu\text{m}$. Schematic representation of the four cell types are presented leftward to their legends. **d**, SEM picture localizing the right nasal cavity (dotted white rectangle) between the eye and the snout, where the right nostril is located. Scale bar indicates 1 mm. **e**, SEM picture of a right nostril rosetta, with 11 lamellae (light green dots). Scale bar indicates $100 \mu\text{m}$. **f**, SEM picture of the right body flank where lateral line (LL) and LL canals are visible (light blue arrows). The dotted white rectangle represents the magnified region shown in (g). Scale bar indicates 1 mm. **g**, SEM picture of a lateral line canal (blue arrow) and its communication pore. Scale bar indicates $100 \mu\text{m}$. **h-m**, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. **h**, Changes in ggc density along recruitment (no change from d0 to d8, $p=0.986$, $n=57$, ANOVA). **i**, Changes in bpc density along recruitment. $A < B$ ($p < 0.001$) and $B < C$ ($p = 0.011$) ($n=57$, ANOVA+TukeyHSD). **j**, Changes in prn density along recruitment. $A < B$ ($p = 0.014$) and $B < C$ ($p = 0.025$) ($n=57$, ANOVA+TukeyHSD). **k**, Changes in pes density along recruitment $A < B$ ($p = 0.028$) ($n=57$, ANOVA+TukeyHSD). **h-k**, Densities are expressed in $10^{-3} \text{ cell.}\mu\text{m}^{-2}$ except for pes whose density corresponds to the number of pes in a $50 \mu\text{m}$ wide area. **l-m**, Changes in nostril lamellae numbers along recruitment. $A < B$ ($p = 0.010$), $B < C$ ($p = 0.005$) ($n=42$, ANOVA). **m**, Changes in the numbers of LL canal along recruitment. $A < B$ ($p < 0.001$), $B < C$ ($p = 0.033$), $C < D$ ($p = 0.025$) ($n=54$, ANOVA+TukeyHSD). (Methods).

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Figure III.2 | Importance of thyroid hormone signaling in sensory organ development at recruitment in *Acanthurus triostegus*

a-f, Impacts of T3 (i.e. promoted TH signaling), N3 (i.e. disrupted TH signaling) pharmacological treatments on sensory organ development at day 2 (d2) and day 5 (d5) compared to control (CT) and outer slope relocated (OS, i.e. environmentally delayed recruitment) individuals. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. **a**, No difference between treatments in ggc density at d2 ($p=0.582$, $n=51$, ANOVA) and d5 ($p=0.666$, $n=43$, Kruskal-Wallis). **b**, $A < B$ ($p=0.018$) and $B < C$ ($p < 0.001$) at d2 ($n=51$, ANOVA+TukeyHSD) and $A < B$ ($p=0.045$) at d5 ($n=43$, ANOVA+TukeyHSD). **c**, $A < B$ ($p=0.002$) at d2 ($n=51$, ANOVA+TukeyHSD) and no change at d5 ($p=0.242$, $n=43$, ANOVA+TukeyHSD). **d**, $A < B$ ($p=0.022$) at d2 ($n=47$, Kruskal-Wallis+Nemenyi) and $A < B$ ($p=0.033$) at d5 ($n=43$, Kruskal-Wallis+Nemenyi). **e**, $A < B$ ($p=0.005$) at d2 ($n=80$, Kruskal-Wallis+Nemenyi) and $A < B$ ($p=0.044$) at d5 ($n=86$, Kruskal-Wallis+Nemenyi). **f**, $A < B$ ($p < 0.001$) and $B < C$ ($p < 0.001$) at d2 ($n=44$, ANOVA+TukeyHSD) and $A < B$ ($p=0.002$) at d5 ($n=42$, ANOVA+TukeyHSD). (Methods).

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Figure III.3 | Impacts of elevated water temperature and chlorpyrifos exposure on thyroid hormone levels and sensory organ development during recruitment in *Acanthurus triostegus*

a-n, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. d2 and d5 = day 2 and day 5 post-settlement, respectively. **a**, T3 level variations under different temperatures (d2 fish at 28.5°C as standards). $A < B$ ($p=0.021$) at d2 ($n=42$, Kruskal-Wallis+Nemenyi), no difference at d5 ($p=0.105$, $n=30$, Kruskal-Wallis). **b**, T4 levels variations under different temperatures (d2 fish at 28.5°C as standards). $A < B$ ($p=0.001$) at d2 ($n=29$, Kruskal-Wallis+Nemenyi), no difference at d5 ($p=0.074$, $n=27$, Kruskal-Wallis). **c**, bpc density variations under different temperatures. $A < B$ ($p=0.034$) at d2 ($n=20$, ANOVA+TukeyHSD) and no difference at d5 ($p=0.375$, $n=20$, ANOVA). **d**, prn density variations under different temperatures. No difference at d2 ($p=0.637$, $n=20$, ANOVA) and d5 ($p=0.983$, $n=20$, ANOVA). **e**, pes density variations under different temperatures. No difference at d2 ($p=0.291$, $n=19$, ANOVA) and d5 ($p=0.177$, $n=20$, Kruskal-Wallis). **f**, nostril lamellae number variations under different temperatures. No difference at d2 ($p=0.609$, $n=29$, Kruskal-Wallis) and d5 ($p=0.626$, $n=20$, Kruskal-Wallis). **g**, lateral line canal number variations under different temperatures. $A < B$ ($p=0.025$) at d2 ($n=29$, ANOVA+TukeyHSD) and $A < B$ ($p=0.002$) at d5 ($n=20$, ANOVA+TukeyHSD). **h**, T3 level variations under CPF exposure (d2 fish at 28.5°C as standards). $A < B$ ($p=0.039$) at d2 ($n=47$, ANOVA+TukeyHSD), no difference at d5 ($p=0.698$, $n=50$, ANOVA). **i**, T4 levels variations under CPF exposure (d2 fish at 28.5°C as standards). No difference at d2 ($p=0.255$, $n=42$, ANOVA) d5 ($p=0.578$, $n=50$, ANOVA). **j**, bpc density variations under CPF exposure. $A < B$ ($p=0.049$) at d2 ($n=50$, ANOVA+TukeyHSD) and $A < B$ ($p=0.004$) at d5 ($n=50$, ANOVA+TukeyHSD). **k**, prn density variations under CPF exposure. No difference at d2 ($p=0.993$, $n=50$, ANOVA) and d5 ($p=0.436$, $n=50$, Kruskal-Wallis). **l**, pes density variations under CPF exposure. No difference at d2 ($p=0.978$, $n=50$, ANOVA) and $A < B$ ($p=0.022$) at d5 ($n=50$, Kruskal-Wallis+Nemenyi). **m**, nostril lamellae number variations under CPF exposure. No difference at d2 ($p=0.669$, $n=50$, Kruskal-Wallis) and $A < B$ ($p=0.018$) at d5 ($n=49$, Kruskal-Wallis+Nemenyi). **n**, lateral line canal number variations under CPF exposure.

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A<B (p=0.034) at d2 (n=93, ANOVA+TukeyHSD) and A<B (p=0.041) at d5 (n=56, ANOVA+TukeyHSD). **h-j**, adapted from Holzer (2017). (Methods).

Figure III.4 | Impacts of thyroid hormone signaling, elevated water temperature and chlorpyrifos exposure on *Acanthurus triostegus* behavioral responses and survival when facing predation cues and predators

a, Design of the two-channel Atema choice flume apparatus used for olfactory preferences assessment in this study. Total length: 25 cm (laminating channel area = 15 cm, choice arena = 5 cm and drained exit area = 5 cm). Width: 5 cm. **b**, Olfactory preferences and TH signaling when facing olfactory cues from control seawater (Ø) vs seawater with predator *Lutjanus fulvus* odor (P). d2 and d5 = day 2 and day 5 post-settlement, respectively. *** indicates p<0.001 (n = 8 to 13 per treatment and per cue, Wilcoxon-Mann-Whitney). **c**, Design of the choice tank apparatus used for visual preference assessment in this study. Total length: 60 cm (release area = 30 cm, each choice area = 15 cm). Width: 15 cm. The tank is equipped with opaque walls except for the two opposite walls where visual stimuli are presented. **d**, Impact of TH signaling, water temperature and CPF exposure (results in september) on visual preferences when facing visual cues from a 'fish-empty' tank (Ø) vs tank containing a *L. fulvus* live predator (P). ** indicates p<0.01 and *** indicates p<0.001 (n = 10 to 20 per treatment and per cue, Wilcoxon-Mann-Whitney). **e**, Design of the survival arena apparatus for measuring the impact of TH signaling on *A. triostegus* survival rates at recruitment when facing direct predation by *L. fulvus*. Size: 1mx1mx1m. **f**, Relative survival and TH signaling. A<B (p<0.001), B<C (p<0.001), C<D (p<0.001) at d2 (n=20, ANOVA+TukeyHSD) and A<B (p<0.001), B<C (p=0.006) at day 5 (n=20, ANOVA+TukeyHSD). (Methods). **g**, Similar experiment as in f, has yet to be performed. **b,d,f**, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. (Methods)

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III. Extended Data Fig 1 | Body depth variation along recruitment in *Acanthurus triostegus*

Body depth was assessed from day 0 (d0) to day 30 (d30) post-settlement. Body depth was calculated as follows: Body Height / Standard length. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. A<B (p<0.001) and B<C (p<0.001) (n=320, Kruskal-Wallis+Nemenyi).

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III. Extended Data Fig 2 | Fulton's K condition factor variation along recruitment in *Acanthurus triostegus*

Fulton's K condition factor was assessed from day 0 (d0) to day 30 (d30) post-settlement. Fulton's K condition factor was calculated as follows: $K = 100 * (W/SL)^3$ (with SL, in cm, and W, in g) (Fulton 1904). Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. A<B (p=0.024) and B<C (p<0.001) (n=320, Kruskal-Wallis+Nemenyi)

p. 139

III. Data Fig 3 | Lateral line (LL) length variation along recruitment in *Acanthurus triostegus*

LL length was assessed from day 0 (d0) to day 8 (d8) post-settlement. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. No difference in lateral line length was observed from d0 to d8 ($p=0.977$, $n=60$, ANOVA) p. 140

III. Extended Data Fig 4 | Fulton's K condition factor variation along with thyroid hormone (TH) signaling, temperature exposure (Temp), and chlorpyrifos (CPF) exposure during recruitment in *Acanthurus triostegus*

Fulton's K condition factor was calculated as follows: $K = 100 \cdot (W/SL)^3$ (with SL, in cm, and W, in g) (Fulton 1904). Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. At day 2 (d2) post-settlement, neither TH ($p=0.976$, $n=62$, ANOVA), temperature ($p=0.849$, $n=129$, Kruskal-Wallis), nor CPF ($p=0.529$, $n=75$, Kruskal-Wallis) affect Fulton's K condition factor in *A. triostegus*. Same observations were made at day 5 (d5) post-settlement: TH ($p=0.715$, $n=92$, Kruskal-Wallis); Temp ($p=0.733$, $n=135$, Kruskal-Wallis), and CPF ($p=1$, $n=76$, ANOVA). The exact same patterns have been observed for standard length, weight, height, and body depth. (Methods) p. 140

Fig. IV.1 | Schematic representation of the test apparatus for eye lateralization determination

The three dark gray walls represent the opaque wall of the aquarium, while the light blue wall corresponds to the transparent wall where the visual stimuli are presented. No eye preference was recorded when the fish was perpendicular to the transparent wall (*i.e.* binocular stimulation) or when it formed an angle larger than 180° with respect to the transparent wall (*i.e.* fish looking in the opposite direction). p. 155

Fig. IV.2 | Left-right eye preference of *A. triostegus* larvae during the inspection of visual stimuli in an adjacent aquarium

Figure represents LI mean (\pm SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, *: $p < 0.05$; **: $p < 0.01$) between Laterality Index (LI) values and the theoretical 50 % value (dotted line). 13 to 14 replicates (one fish per replicate) were conducted for each visual stimulus. p. 156

Fig. IV.3 | Ecological importance of brain lateralization in larval survival facing direct predation

Survival index (SI) was calculated as follow: survival rate of the group minus the overall survival rate in the *in situ* cage. Figure represents SI mean (\pm SE) values. The asterisk indicates a significant difference in survival index among the three groups (Kruskal-Wallis test, $\chi^2 = 7.064$, $df = 2$, $p\text{-value} = 0.029$) with 'left-eye dominant' experiencing high survival than both 'right-eye dominant' (Nemenyi post-hoc test with Tukey-Dist approximation, $p\text{-value} = 0.01$) and 'no eye dominant' larvae (Nemenyi post-hoc test with Tukey-Dist approximation, $p\text{-value} = 0.03$). Six replicated were conducted. In each replicate, each group was made up with the same amount (four to six) of fish. p. 157

Fig. IV.4 | Effect of pesticides on the visual preference of *A. triostegus* larvae

Figure represents LI mean (\pm SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, *: $p < 0.05$; **: $p < 0.01$) between Laterality Index (LI) values and the theoretical 50 % value (dotted line). Black asterisks above bars indicate a significant right-eye preference, while white asterisks below bars indicate a significant left-eye preference. 10 to 11 replicates (one fish per replicate) were conducted for each visual stimulus in both the pesticide exposure experiment (black bars) and the solvent-control exposure experiment (grey bars).

p. 158

Fig. V.1 | Body color patterns and community organization of *Acanthurus triostegus* juveniles

A: 'Classical color pattern' (CP) of a convict tang juvenile at day 7 post-settlement. **B:** 'Reversal of the color pattern' (RP) of two juveniles also erecting their dorsal fin. **C:** Shoal and community organization of CP (top) and RP (bottom-central) juveniles.

p. 181

Fig. V.2 | Color morph ontogenetic stage, prevalence, and behavior of CP and RP juveniles at day-7 post-settlement

Light gray: CP juveniles; Dark gray: RP juveniles. **A:** Color morph relative prevalence. **B:** Body depth (body height / standard length) of *A. triostegus* along settlement (day 0 to day 11), and of day 7 post-settlement (bold lines) CP and RP juveniles. **C:** Percentage of time spent with the dorsal fin erected in CP and RP juveniles. Errors bars indicate standard errors. ***: p -value < 0.001 .

p. 181

V. Supplementary Fig. 1 | Stress and nocturnal color pattern in *Acanthurus triostegus* juveniles

p. 183

I. General introduction

I.1. Foreword on coral reef fish ecology

I.1.1. Tropical coral reef ecosystems

Darwin (1842) was one of the first to describe the structure and distribution of modern tropical coral reefs. He started his description by quoting François Pyrard de Laval, who claimed in 1605: « C'est une merveille de voir chacun de ces atollons, environné d'un grand banc de pierre tout autour, n'y ayant point d'artifice humain », which translates to "It is wonderful to see each of these atolls entirely surrounded by a great bank of stone, of no human construction". This reveals how fascinating coral reefs have always been for naturalists since their discovery (Darwin 1842; Dana 1875; Yonge 1930). The reproductive strategies, formation and evolution processes, economical benefits and values of coral reef ecosystems have intrigued and enthralled geologists, biologists, and even economists, and their mysteries will probably never be completely elucidated (Duce et al. 2016; Rapuano et al. 2016; Brumbaugh 2017). These iconic tropical reef systems have been present since the Ordovician (around 485 to 444 million years ago) (Adachi et al. 2011) and today they constitute one of the most biologically diverse, productive, and economically important ecosystems worldwide (Odum and Odum 1955; Connell et al. 1978; Moberg and Folke 1999; Brumbaugh 2017). Tropical coral reef edifices dominate many shallow-water environments and harbor more than a third of the ocean's biodiversity while only covering 0.1 to 0.5 % of the ocean floor (Copper 1994; Spalding and Grenfell 1997; McCulloch et al. 2017). With more than 100 countries having coastlines with coral reefs, these ecosystems supply half a billion of people with goods and services such as food, coastal protection from storm and erosion, new medicines, tourism and cultural benefits (Salvat 1992; Jameson et al. 1995). The ecosystem services driven by coral reefs have been estimated at \$36 billion per year (Brumbaugh 2017).

Most tropical coral reefs are dominated by scleractinian corals, which generally only grow at depths shallower than 50 meters, and in a zone extending from approximately 30° N to 30° S of the equator (Dubinsky 1990). Scleractinian corals accumulate and deposit calcium carbonate (CaCO₃) to build their limestone exoskeleton (Zoccola et al. 2015). This bio-accumulation of calcium carbonate is often referred to as calcification, and is promoted by the mutualist partnership between scleractinian corals and their endosymbiotic and photosynthetic dinoflagellates: *Symbiodinium* (zooxanthellae) (Pearse and Muscatine 1971; Furla et al. 2000; Zoccola et al. 2015). There are three main types of coral reefs: fringing reefs, barrier reefs and atolls, which can all be encountered in French Polynesia (Figure I.1). Scleractinian corals are the foundation species of all these tropical reef types, as they produce the biogenic matrix on which these ecosystems depend for structure, function, and biodiversity (Spalding et al. 2002; Alvarez-Filip et al. 2009). The physical structure of coral reefs provides a large diversity of habitats that facilitate species coexistence (*e.g.*, corals, fish, algae, and invertebrates, Figure I.2) through niche partitioning and by providing, for instance, refuges from predators and environmental stressors (Roberts

and Ormond 1987; Almany 2004; Gratwicke and Speight 2005). The great diversity of ecological niches provided by coral reefs has been acknowledged as one of the drivers of the teleost fish diversification (Alfaro et al. 2007; Frédérick et al. 2013).

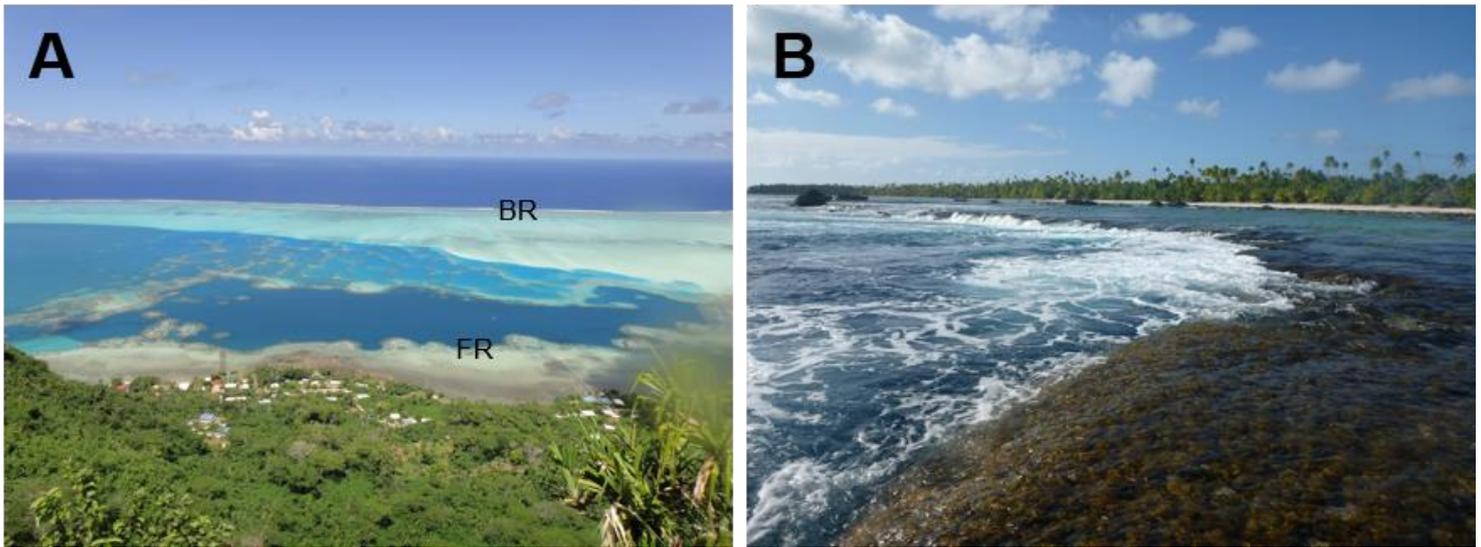


Figure I.1 | Coral reef types in French Polynesia

A: Barrier reef (BR) and fringing reef (FR) in Maupiti Island. Fringing reefs can develop in shallow waters along the coasts while barrier reef corresponds to a coral reef growing parallel to the shoreline and separated from it by a lagoon. **B:** Tetiaroa Atoll. Atolls are rings of coral reefs and small islets surrounded by the ocean and surrounding a sandy lagoon where a volcano was standing before being submerged. **Pictures: M. Besson.**

However, coral reefs are also among the most rapidly changing environments globally. Over the past three decades, they have faced increasingly frequent and severe natural (*e.g.*, cyclones and *Acanthaster planci* outbreaks) and anthropogenic perturbations (*e.g.*, pollution from agricultural runoff, rising sea-water temperatures, overfishing), leading to global declines in habitat quality, biodiversity and associated ecosystem services (Moberg and Folke 1999, Hughes et al., 2003, Hoegh-Guldberg et al., 2007, Hughes et al., 2014, Belwood et al., 2004, Hughes et al., 2017). Many tropical reefs phase shift from coral-dominated to macroalgal-dominated habitats (Hoegh-Guldberg et al. 2007; Hughes et al. 2017). In this context, algal-herbivore fish are considered keystone species for their ability to maintain or rescue coral-dominated habitats, in particular facing algal invasions that follow bleaching events (Figure I.3) (Hughes et al. 2007; Ledlie et al. 2007). Along with other reef-fish functional groups, they play important roles in maintaining reef habitats, preserving their associated biodiversity, and increasing their resilience (*e.g.*, through the regulation of coral-algal interactions or by maintaining trophic sustainability) (Russ 1984, Hughes et al., 2007). Furthermore, while fisheries yield from coral reefs are estimated to be worth at least US\$ 5 billion annually, with dozens of millions of people directly depending on tropical reefs for food and livelihood, most of them are already over-exploited and may have little resilience to additional impacts from global change (Moberg and Folke 1999; Hoegh-Guldberg et al. 2007; Munday et al. 2008)

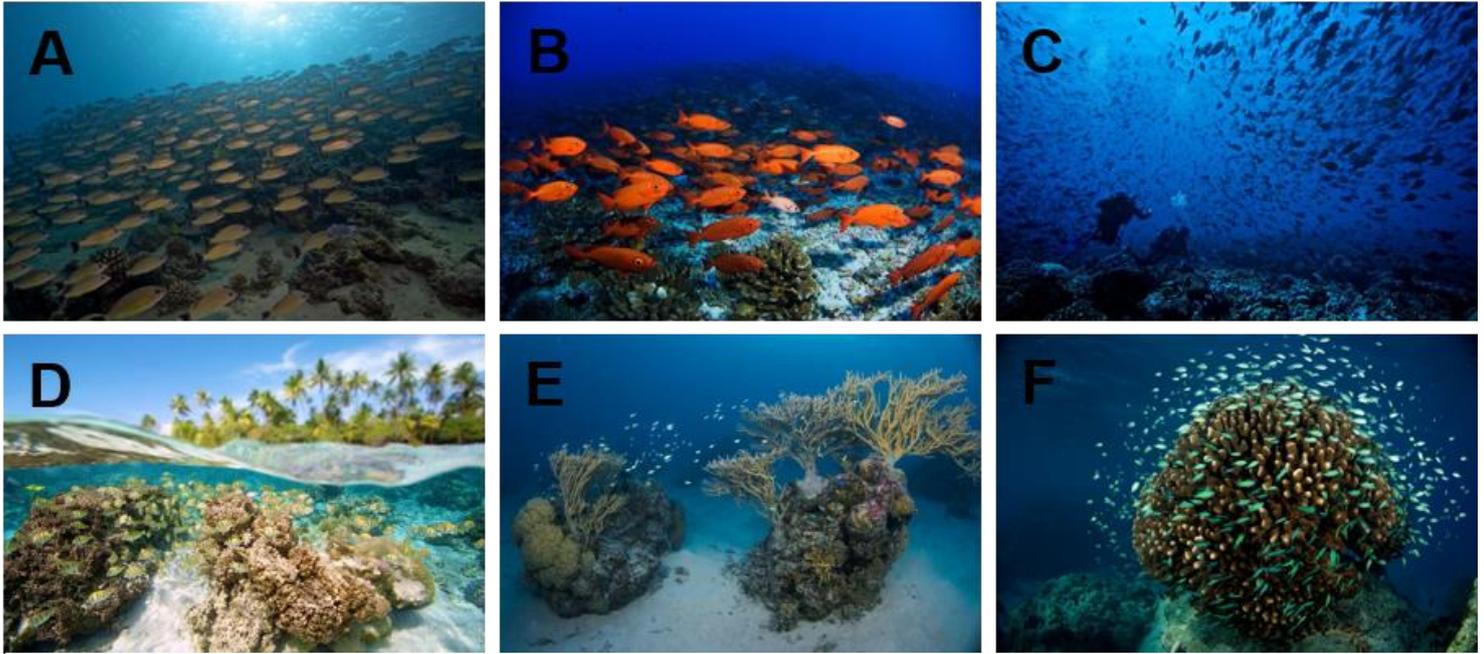


Figure I.2 | Fishes in coral reefs

A: Blacktail snappers (*Lutjanus fulvous*) on the foreslope reef of Fakarava Atoll (Tuamotu Archipelago, French Polynesia). **B:** Hawaiian bigeyes (*Priacanthus meeki*) on the foreslope reef of Rangiroa Atoll (Tuamotu Archipelago, French Polynesia). **C:** Yellowfin surgeonfishes (*Acanthurus xanthopterus*) in the channel pass of Rangiroa Atoll (Tuamotu Archipelago, French Polynesia). **D:** Convict tangs (*Acanthurus triostegus*) on patch reefs near the barrier reef of Tetiaroa Atoll (Society Archipelago, French Polynesia). **E:** Blue-green damselfishes (*Chromis viridis*) and Whitetail dascyllus (*Dascyllus aruanus*) in the lagoon of Moorea (Society Archipelago, French Polynesia). **F:** Blue-green damselfishes (*C. viridis*) around their *Pocillopora* shelter in a fringing reef of Moorea Island (Society Archipelago, French Polynesia). **Pictures:** L. Thiault.

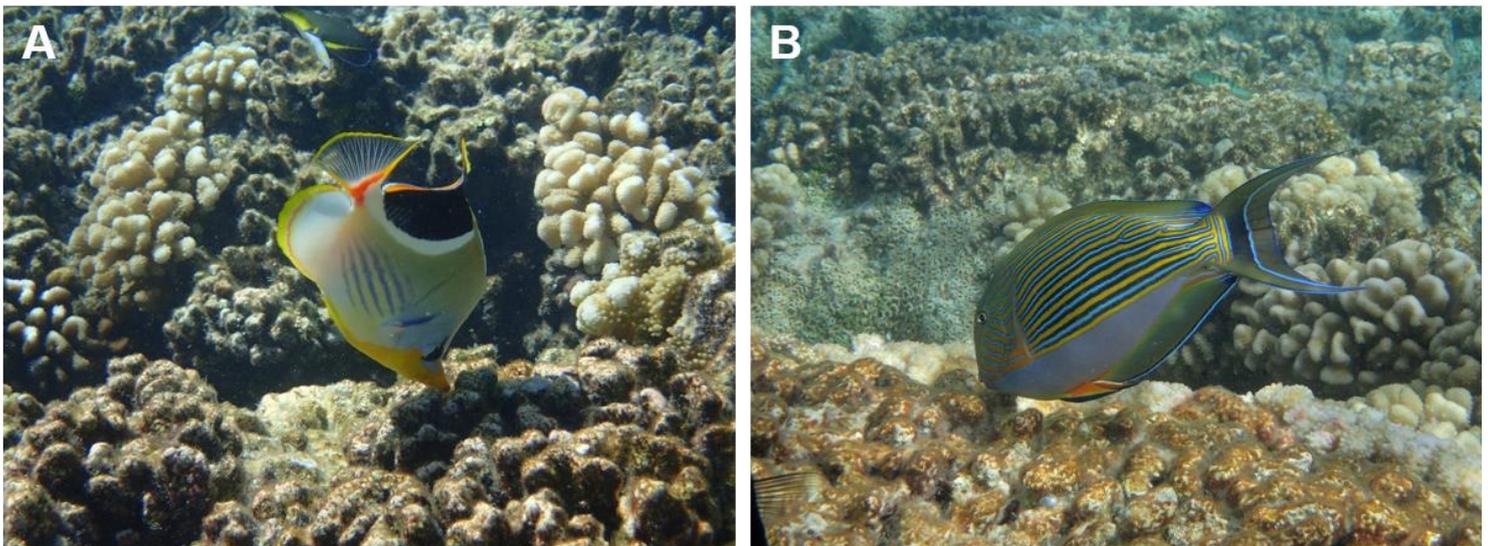


Figure I.3 | Herbivorous fishes grazing on algae in a bleached coral reef

A: Two herbivorous fishes: *Chaetodon ephippium* (front) and *Acanthurus nigricans* (back) feeding on turf algae that have recently grown on dead *Pocillopora* following a bleaching event in Moorea Island (French Polynesia). **B:** same observation with the line surgeonfish (*Acanthurus lineatus*). **Pictures:** L. Hedouin.

I.1.2. Coral reef fishes

Among vertebrates (around 45.000 species), teleost fishes constitute the most biodiverse group (almost 25.000 species). There are at least 4.000 species of fishes inhabiting coral reefs, and, apart from some species of sharks and rays associated with coral reefs, nearly all fishes found in coral reefs are teleost fishes. Coral reef fish hotspot of biodiversity is located in Indonesian coral reefs, with biodiversity decreasing eastward in the Pacific (Sale 2002). Bellwood (1996) established a list of coral reef fish families regarded as “characteristic reef fish families, the essence of a reef fish fauna; all abundant and speciose on coral reefs” (Bellwood 1996; Bellwood and Wainwright 2002). This family list therefore provides no diagnostic, but covers most species that one would always find on a coral reef irrespective of its biogeographic location: Acanthuridae, Apogonidae, Blenniidae, Carangidae, Chaetodontidae, Holocentridae, Labridae, Mullidae, Pomacentridae and Scaridae (Figure I.4).

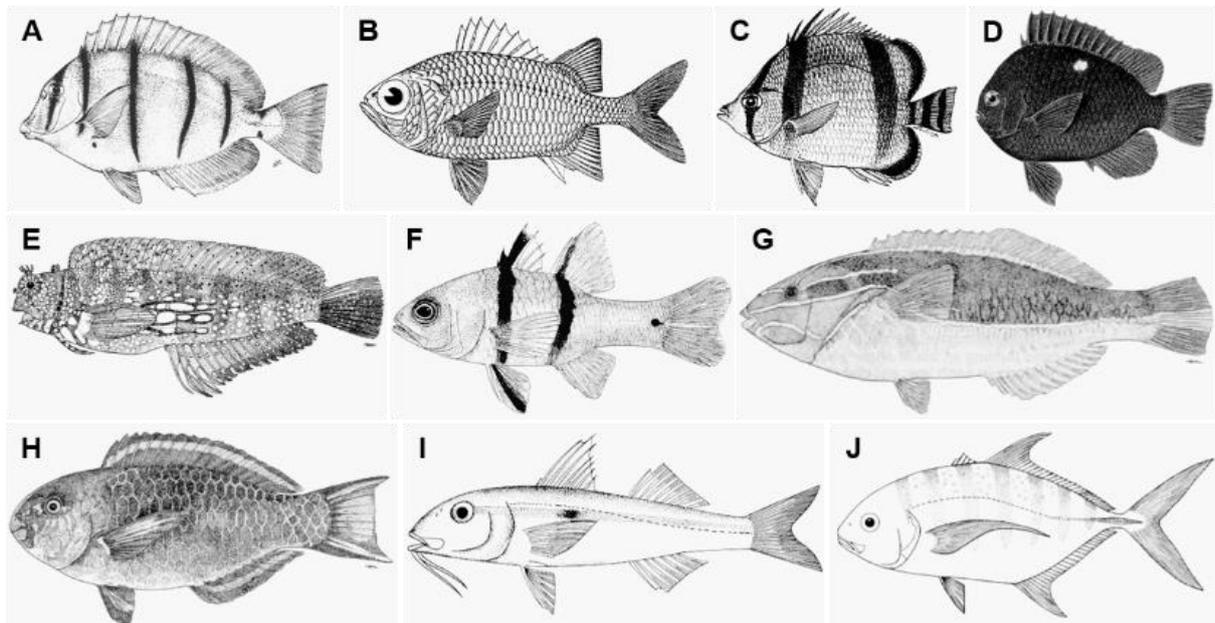


Figure I.4 | Ten coral reef fishes from iconic coral reef fish families

A: *Acanthurus triostegus* (Acanthuridae). **B:** *Myripristis berndti* (Holocentridae). **C:** *Chaetodon humeralis* (Chaetodontidae). **D:** *Dascyllus trimaculatus* (Pomacentridae). **E:** *Salarias fasciatus* (Blenniidae). **F:** *Apogonichthyoides pseudotaeniatus* (Apogonidae). **G:** *Stethojulis bandanensis* (Labridae). **H:** *Scarus spinus* (Scaridae). **I:** *Mulloidichthys flavolineatus* (Mullidae). **J:** *Carangoides ferdau* (Carangidae). **Adapted from FAO species identification guides** (FAO 2001).

All teleost coral reef fishes share anatomical similarities, for example, a dorsal fin, a symmetrical caudal fin, an anal fin and two pairs of bilateral fins: pelvic and pectoral fins (Figure I.5). Breathing functions *via* the circulation of water from the mouth to the gills, which are located in a cavity protected by opercular bones (*e.g.*, pre-operculum and operculum, Figure I.5). Some classical morphological measurements can be conducted for studies at the individual or population levels (*e.g.*, standard length - distance between the extremity of mouth and the end of the notochord, body height - maximum height between the bases of the dorsal and anal fins, perpendicular

to the standard length, and snout length - distance between the extremity of the mouth and the base of the eye, Figure I.5). These fish also possess several sensory organs such as the eyes for vision, the nostrils for olfaction, the lateral lines for the mechanical perception of water movements (e.g., predator surging or sounds), and otoliths for hearing and orientation (Myrberg and Fuiman 2002). Some fishes also exhibit some external gustatory (chemosensory) organs such as barbels, or have the ability to detect magnetic fields (McCormick 1993; O'Connor and Muheim 2017).

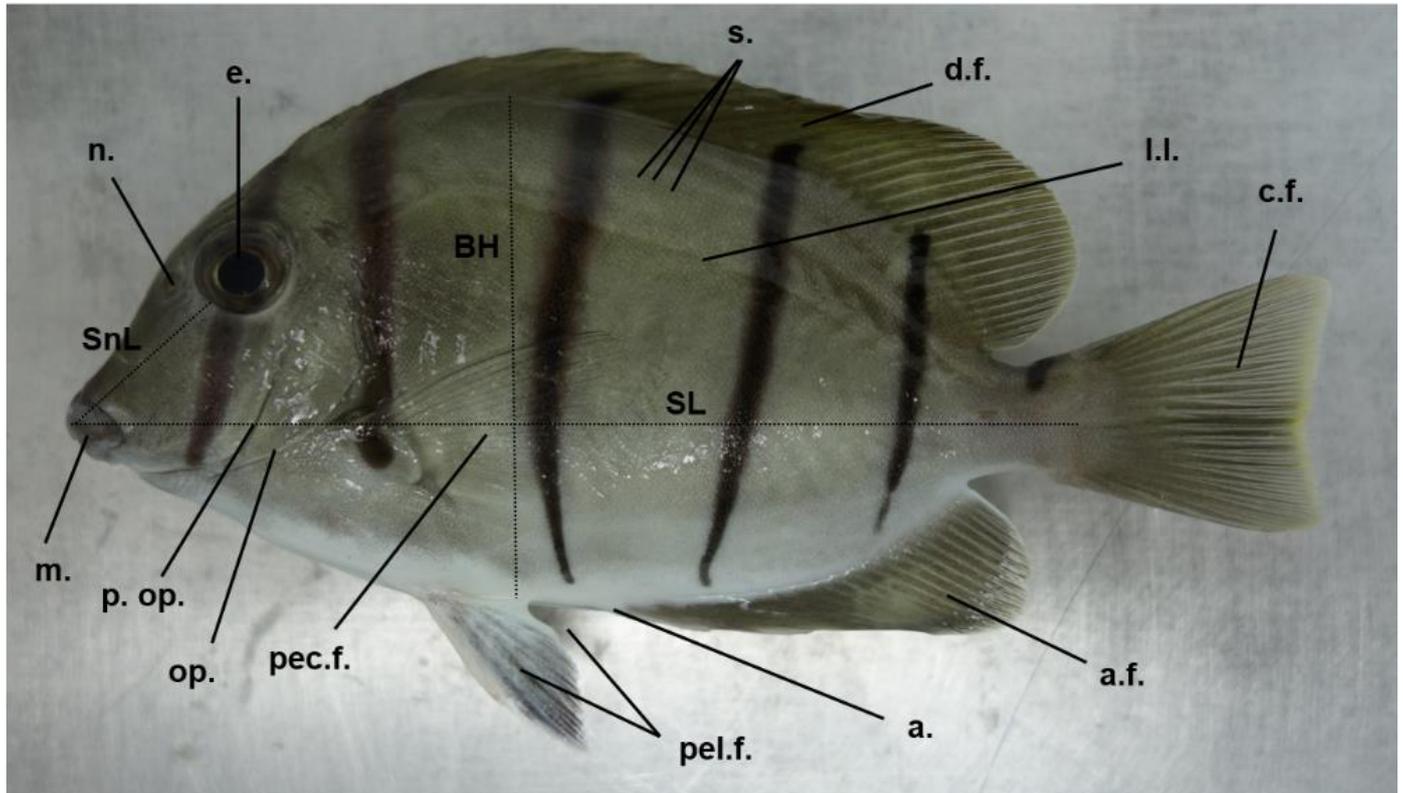


Figure I.5 | The convict tang *Acanthurus triostegus* as a model for teleost coral reef fish characteristics
n.: nostril, e.: eye, s.: scales, l.l.: lateral line, a.: anus, op.: operculum, p.op.: pre-operculum, m.: mouth. d.f.: dorsal fin, c.f.: caudal fin, a.f. : anal fin, pel.f. : pelvic fins, pec.f. : pectoral fin. SL: standard length, BH: body height, SnL: snout length. **Picture: M. Besson.**

In the wide variety of habitats provided by coral reefs, the vertical and horizontal distributions of coral reef fishes depend on several habitat factors such as the amount of algae, plankton, invertebrates and prey available, but also exposure to wave and currents, light levels, and lastly shape and assemblages of coral species (Lieske and Myers 2002). Each coral reef niche exhibits its own set of characteristic species. For example, small and vulnerable planktivores like the blue-green damselfish (*Chromis viridis*) and the whitetail dascyllus (*Dascyllus aruanus*) only feed above their coral shelter (Figure I.2E-F). Larger planktivores such as the yellowfin surgeonfish (*Acanthurus xanthopterus*) and fusiliers school in large groups in deeper and nutrient-rich waters (e.g., channel passes) or above fore slope reefs, where they can roam large areas (Figure I.2C) (Lieske and Myers 2002). Large grazers, such as the majority of surgeonfishes and parrotfishes, also occur in groups, swimming at a short distance above reefs (Figure I.2D). Higher in the trophic chain, some piscivores can be bottom-

dwellers and school, such as blacktail snappers (*Lutjanus fulvous*) and Hawaiian bigeyes (*Priacanthus meeki*) (Figure I.2A-B), but others also live solitary, in pairs, or in small groups occurring in mid-waters such as jacks (Lieske and Myers 2002). However, these examples are not universal, as there are almost as many ecological and social interactions in coral reef fishes as there are species. While most coral reef fishes are diurnal, some of them (*e.g.*, soldier-, squirrel- and cardinal-fishes) only emerge from caves and crevices at night to feed outside from their shelter (Lieske and Myers 2002). Furthermore, most coral reefs fishes do not change sex after reaching sexual maturity, with only a few reef fish species changing sex along their adult growth. For example, some Scaridae, Labridae, and Serranidae first reach sexual maturity as a female, and turn male later in life, while some Pomacentridae, such as clownfish, are first immature juveniles and then males before turning into females when reaching the highest hierarchical position in the anemone) (Lieske and Myers 2002).

Coral reef fish reproduction events can be particularly remarkable, such as the massive spawning aggregations encountered in Acanthuridae and Serranidae (Y. Sadovy de Mitcheson and P.L. Colin 2012). The majority of coral reef fishes emit their gametes (eggs and sperms) directly into the water column (pelagic eggs species), while a minority of species deposit and fertilize their eggs on a reef substratum (benthic eggs species, *e.g.*, Pomacentridae and Balistidae) (Lieske and Myers 2002). Eggs generally hatch in a couple of days (pelagic eggs) to a few weeks (benthic eggs) following fertilization, and the emerging larvae are initially swept away from the reefs to the open ocean by oceanic currents (Leis and McCormick 2002). Larvae can drift for as little as a week (clownfish) (Thresher et al. 1989) to more than 64 weeks (porcupine fish) (Ogden and Quinn 1984) before migrating towards the reefs.

The majority of reef fishes have such a bipartite life cycle, consisting of a pelagic larval stage followed by a largely sedentary reef-associated juvenile and adult stage (Figure I.6) (Leis and McCormick 2002; Cowen and Sponaugle 2009; Clobert et al. 2012). In these species, the pelagic stage (*i.e.* larvae) primarily facilitates survival (as predation pressure on early life stages is lower in the open ocean) but also dispersal, driven by both physical (*e.g.* oceanic currents) and biological (*e.g.* directional swimming and sensory perception) processes (Cowen and Sponaugle 2009; Green et al. 2015; Brooker and Dixson 2016). These processes act at a large spectrum of spatial and temporal scales, with their relevance to dispersal varying depending on larval development and pelagic larval duration. For example, young and poorly developed larvae are usually ineffective swimmers, with initial dispersal largely a result of hydrodynamic processes (Leis and McCormick 2002). However, larvae rapidly develop the ability to orientate themselves within the water column, to swim against the current, and to control their trajectories (Fisher et al. 2000; Fisher 2005). Thus, while larvae often have the potential to travel over vast distances during the pelagic phase, many also have the potential to remain close to their native reefs (Leis and McCormick 2002; Cowen et al. 2005; Irisson and Lecchini 2008), leading to well-connected populations on scales of 0–30 km (Almany et al. 2007; Leahy et al. 2015). While in the

ocean, coral reef fish larvae experience rapid development and growth before reaching a developmental stage called 'competence', after which they usually exhibit reduced growth and body maintenance (Cowen and Sponaugle 1997). In the pre-competent stage, larval growth is usually genetically and environmentally mediated (*e.g.* temperature and food availability) (Victor 1986; Sale 1991; Paris and Cowen 2004). These factors therefore mediate the duration of the pre-competent stage. Once competent, usually near the end of the pelagic phase, larvae are ready to leave the open ocean and rejoin the demersal reef populations (Doherty 2002). The transition between the pelagic and the demersal stages corresponds to settlement (Figure I.6) (Kaufman et al. 1992). This step will be introduced in more details in the next sub-chapter.

In the few days following their colonization of the reef, larval fishes turn into juveniles and select a juvenile reef habitat facilitating their growth and survival (*i.e.*, larval recruitment). Larval recruitment is a generic term used to describe a variety of processes in the reef fish life cycle, usually encompassing all the steps from larval dispersal in the pelagic environment, to the arrival, settlement, and survival in the reef ecosystem (Figure I.6) (Richards and Lindeman 1987). Following larval recruitment, individuals will grow before joining the adult population when sexually mature (*i.e.*, recruitment, in its broad sense, but one should note that larval recruitment is also very often simply called recruitment by scientists when only focusing on the period of larval return to the reefs) (Figure I.6).

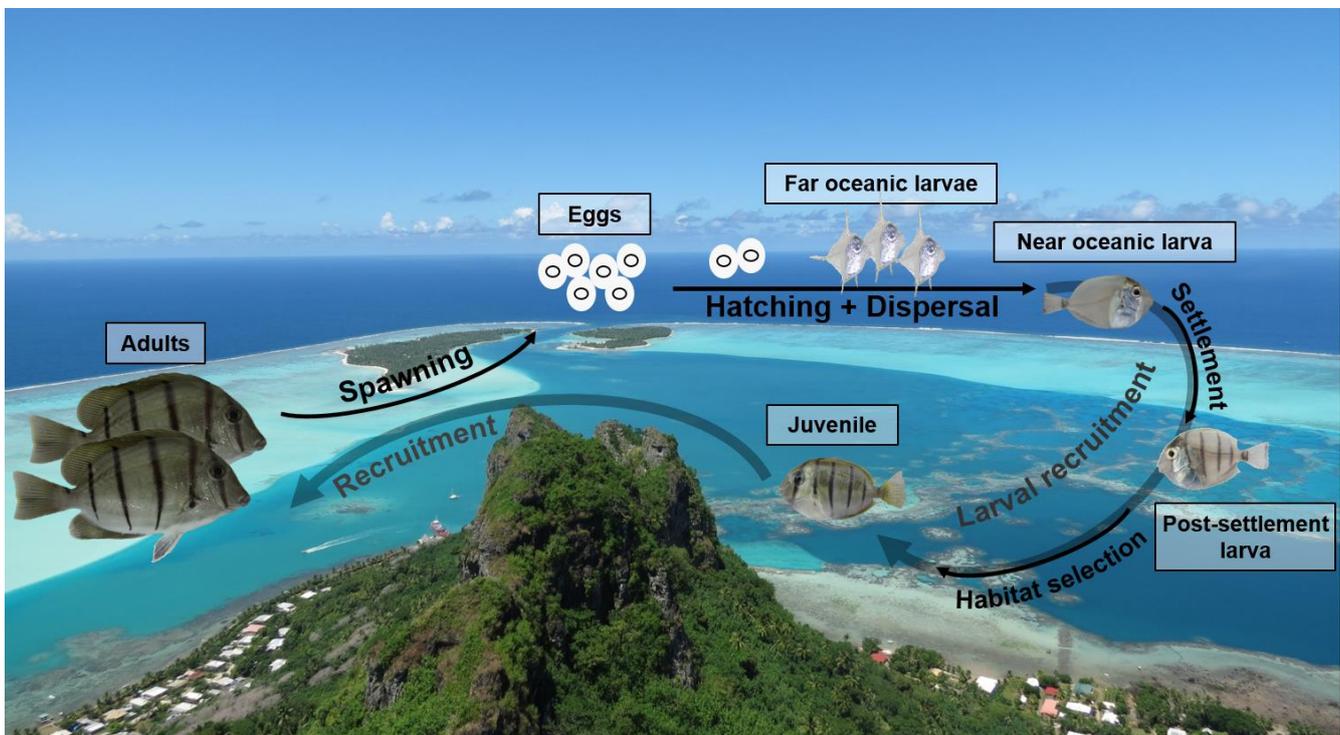


Figure I.6 | Life cycle of the convict tang *Acanthurus triostegus*

A. triostegus is a pelagic egg species. This life cycle is representative of all the pelagic egg coral reef fish species (around 2/3 of all coral reef fishes). In benthic egg species, pelagic dispersal only concerns larvae, as hatching occurs within the reefs. Larval recruitment is different from the 'Recruitment' presented in this figure, and encompasses all the steps from the end of larval dispersal to the survival of juveniles in nursery areas. **Pictures: M. Besson.**

I.1.3. Coral reef fish settlement and recruitment on the reefs

How larval fishes manage to locate coral reefs in the vast ocean has always fascinated ecologists and oceanologists. For example, in French Polynesia, the overall emerged island surface has been estimated as approximately equal to half the area of Corsica island (*i.e.*, half the area of the island of Hawaii), while the oceanic waters around those territories are as vast as Europe (L'ORSTOM 1993). Arriving to the reefs and subsequent return to the benthic environment involves species-specific perception of environmental cues such as reef odors (chemical cues) (Paris et al. 2013; Lecchini et al. 2014b), reef noises (acoustic cues) (Vermeij et al. 2010), sun compass (visual cues) (Berenshtein et al. 2014), and magnetic fields (O'Connor and Muheim 2017) (for review, see Leis (2011) and Barth et al. (2015)). I participated to the latter study, which has been written and published during this PhD, contributing as third author (Barth et al. 2015). This study is available in this thesis (Appendix 1).

Coral larval fish settlement patterns are often species-specific, dependent on nocturnal, lunar, seasonal and inter-annual factors. Some factors appear to be particularly important, with the abundance of larvae settling to reefs often peaking during the night, new moon, summer, and La Niña periods (Dufour and Galzin 1993; Leis and McCormick 2002; Lecchini et al. 2004; Lecchini 2005; Irisson and Lecchini 2008; Lo-Yat et al. 2011; Besson et al. 2017b). I participated to the latter study, which has been written and published during this PhD, contributing as first author (Besson et al. 2017b). This study is available in this thesis (Appendix 2).

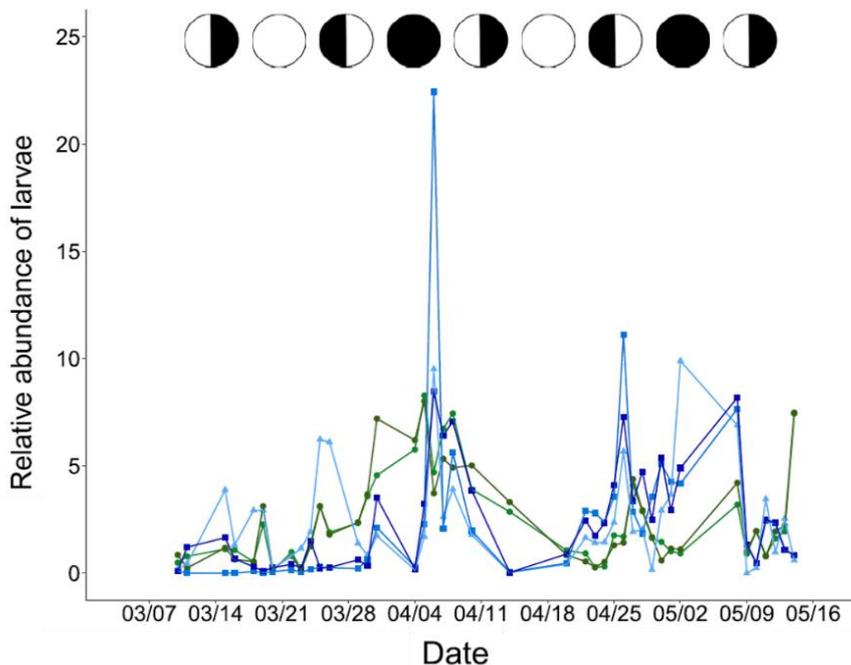


Figure I.7 | Settlement patterns of larval coral reef fishes in French Polynesia

Green and dark green lines indicate relative abundance of settlers captured in two crest nets in Moorea, while light blue, blue, and dark blue lines indicate relative abundance of settlers captured in three nets in Nengo Nengo. Circular points indicate a crest net with a westward orientation, triangular points indicate a northward orientation and square points indicate a south-eastward orientation. Black and white circles above each panel indicate the lunar phases: new moon (black circle), first quarter, full moon (white circle) and last quarter. Adapted from Besson (2017).

While experiencing this ecological transition, coral reef fishes often lose many of their planktonic characteristics and develop other features adapted to their new demersal or benthic lifestyle (see fish pictures in Figure I.6). Indeed, after reaching competency, coral reef fish larvae usually encounter a key species-specific period of adaptive changes, which was first described in lizard-fishes (Breder 1944; Gibbs 1959) and in the convict surgeonfish *Acanthurus triostegus* (Randall 1961). In this context, nearly all studies have focused on morphological transformations (*e.g.*, morphometrical remodeling, fin spine shortening, pigmentation and skeletal development) (McCormick and Makey 1997; McCormick 1999; McCormick et al. 2002; Parmentier et al. 2004a, 2004b, Fr  d  rich et al. 2008, 2010a, 2012), sensory organ development (*e.g.*, evolution of the retina and the barbel structures) (McCormick and Shand 1992; McCormick 1993; Shand 1993, 1994, 1997), and intestine remodeling (*e.g.*, elongation and coiling) (Randall 1961; Labelle and Nursall 1985; Nursall and Turner 1985; Green and McCormick 2001). All these changes can occur either **(i)** in the open ocean (*e.g.* in some pomacentrids, which undergo most of their developmental changes after hatching, before entering the reef and settling) (McCormick et al. 2002), **(ii)** along with settlement (*e.g.* acanthurids, with morphological changes starting a few days before and continuing a few days after settlement in *A. triostegus*) (Randall 1961; McCormick 1999), or **(iii)** later on: up to three weeks after larvae settled in the reef habitat (*e.g.* in some labrids, dartfishes, carapids and scarids) (McCormick and Makey 1997; Parmentier et al. 2004a, 2004b). This diversity of time scales among species is also observed at the species level, as this massive period of change can, in some cases, be delayed until favorable environmental cues are encountered (Randall 1961; Victor 1986; McCormick 1994, 1999; Cowen and Sponaugle 1997). Regardless of whether these changes at the end of the larval stage coincide precisely with settlement or not, they occur very rapidly in most species (Leis and Carson-Ewart 2000). For instance, the pigmentation of the transparent *A. triostegus* larva can occur within 2 hours after entering the reef (McCormick 1999).

Overall, the development of a coral reef fish larva into a juvenile, which occurs at or around settlement (*i.e.*, along larval recruitment), after reaching developmental competency under (currently unknown) environmental signals, involves rapid post-embryonic morphological, behavioral, physiological and ecological changes. This is why this transition is called a 'metamorphosis', as classically defined in the literature (Shi 2000; Bishop et al. 2006; Heyland and Moroz 2006; Laudet 2011).

I.2. Metamorphosis and thyroid hormone signaling

Metamorphosis has been fascinating scientists, philosophers and poets for centuries, with many novels relating humans and gods transforming into something else, inspirations probably originating from the observation of the well-known and spectacular tadpole-to-frog and caterpillar-to-butterfly metamorphoses (Ovide 1925; George 2000). Metamorphosis corresponds to a post-embryonic life transition event that is commonly observed in many animal species. Indeed, 80 % of all animal species associate metamorphosis (not always as spectacular as in frogs and butterflies) and niche shift during their life cycle (Werner 1988), including vertebrates (Paris and Laudet 2008; Laudet 2011). Ecological, physiological, molecular and hormonal proceedings and triggers of metamorphosis have been well described in insects, amphibians and teleost fishes, but remain largely unknown in other arthropods and vertebrates species, as well as in other phyla (Gilbert et al. 1996; Hall and Wake 1999; Nielsen 2000; Shi 2000; Bishop et al. 2006; Heyland and Moroz 2006). Most of our knowledge, from which we build our definition of vertebrate metamorphosis, comes from the study of amphibians.

I.2.1. Metamorphosis in amphibians

Amphibians, particularly the anurans, are the best known models of vertebrate metamorphosis. Their metamorphosis corresponds to the transition of a tadpole, which is herbivorous, and living and breathing underwater, into a carnivorous land-living frog that have essentially a lung-based air respiration. Some environmental parameters such as food availability, temperature, and water volume, trigger metamorphosis by affecting cortisol levels and thyroid hormones (TH) production and release (Denver 1998; Newman 1998; Tata 2006). Thyroxine (T4) and triiodothyronine (T3) are the two TH that endocrinologically trigger metamorphosis (Gudernatsch 1912; Buchholz et al. 2006). At the climax, when the major metamorphic changes occur, a peak of TH levels is observed in the organism (Leloup and Buscaglia 1977; Tata 2006). Also, early treatments with TH can induce early metamorphosis (Gudernatsch 1912). For example, in *Xenopus*, many organs and tissues undergo an extensive remodeling under TH control. Certain structures like the tail and gills undergo a controlled apoptosis whereas other are protected (Brown et al. 1996; Coen et al. 2001). The intestine shifts from a long coiled and villi free structure (typical of an herbivorous diet) towards a short, uncoiled and structurally complex carnivorous-like intestine (Figure I.8) (Su et al. 1997; Schreiber et al. 2005). Other organs such as the eyes also undergo such remodeling processes (Mann and Holt 2001). The limbs (initially nonexistent) develop, with all the bones, muscles and neuronal remodeling it implies (Figure I.8) (Brown et al. 2005). It is particularly spectacular to consider that all these major and complex processes such as cell differentiation, cell proliferation and cell

death, implying a variety of organ responses, are under the control of a single hormonal peak: the thyroid hormone signaling (Laudet 2011).

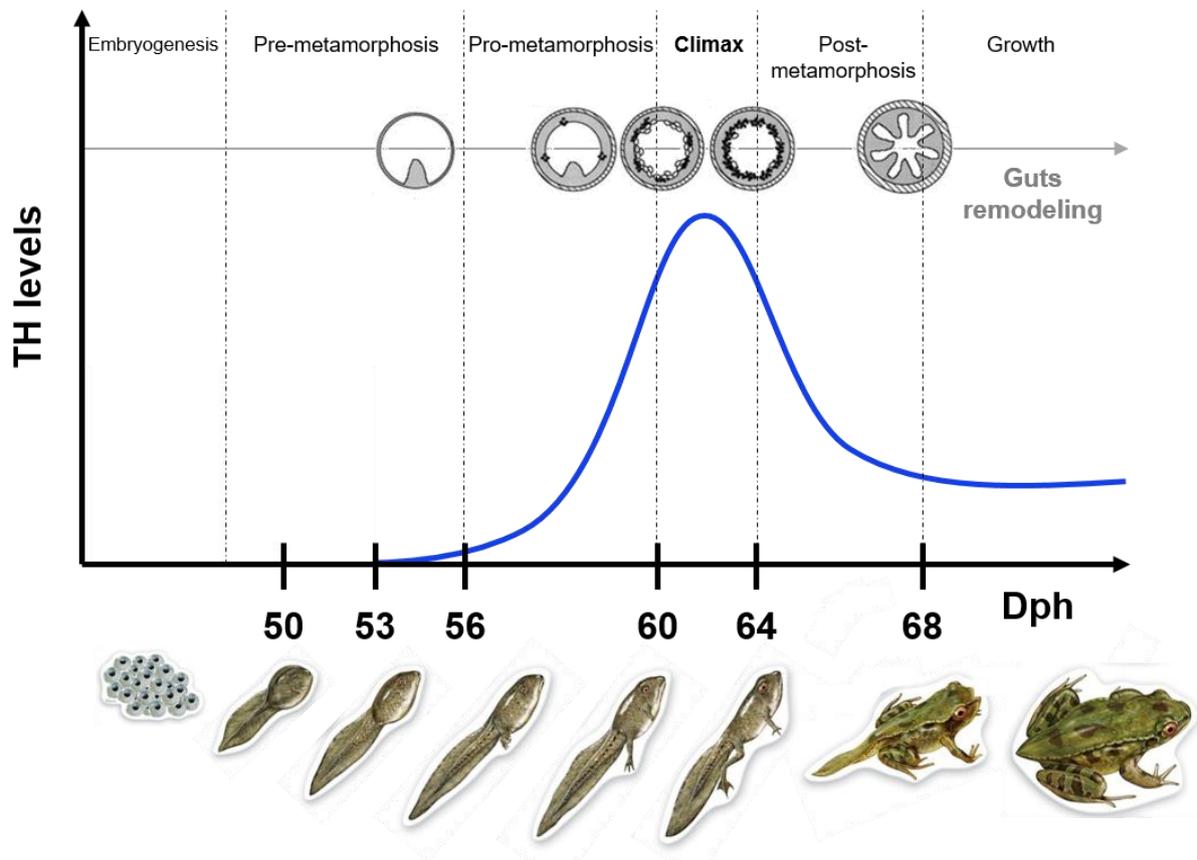


Figure I.8 | Thyroid hormone levels and development in anurans

Dph: days post hatching. Circular structure on the 'guts remodeling' axis correspond to schematic representation of the intestine cross-sections. Adapted from Brown and Cai (2007) and Hasebe et al. (2013).

I.2.2. Thyroid hormone signaling

Thyroid Hormones (TH) have a pleiotropic role in animal organisms. They are associated with the catabolism of fat and sugar (Mullur et al. 2014), thermoregulation in mammals, and birds (Lebon et al. 2001; Ikegami et al. 2015), and thermal-acclimation in zebrafish (Little et al. 2013). The TH signaling pathway is also involved in the physiological response to day lengthening in a wide range of species from fish (*e.g.*, in salmon) (Lorgen et al. 2015) and birds (*e.g.*, in ducks) (Jacquet 1997) to mammals (*e.g.*, in sheep) (Bentley 2008). Moreover, TH are also known for their involvement in developmental processes and organ maturation. This was shown in the previous sub-chapter with regards to metamorphosis, but is also true for less spectacular, but nonetheless important, developmental processes. For example, in humans, there is a peak of TH at birth, and TH are one of the first hormones dosed in new-born infants as any defect in TH levels at this time definitely impairs brain development later in life (Raiti and Newns 1971; Erenberg et al. 1974; Delange 2001; Lopez-Espinosa et al. 2011; Morvan-Dubois et al. 2013). Other studies in mice have

confirmed this importance of TH in the post-embryonic development of the intestine, the bones and the brain (Flamant et al. 2002; Morte et al. 2002; Flamant and Samarut 2003).

TH are synthesized in the thyroid. This gland mainly synthesizes T4, while the conversion of T4 into T3, which has a higher biological activity (*i.e.*, T3 is often referred to as the main metamorphosis hormone), as well as the degradation of both T4 and T3 in inactive forms, are performed by deiodinase enzymes (Figure I.9) (Bianco and Kim 2006a). These enzymes are essentially expressed in peripheral and target tissues, and their expression is highly controlled. This ensures a fine tuned regulation of the T3 level in each tissue in response to any environmental and physiological conditions (Bianco and Kim 2006a). The brain, and more precisely the Hypothalamus-Pituitary-Thyroid (HPT) axis, is the central regulator of TH signaling. HPT integrates metabolic and environmental variations and consequently adjusts TH synthesis through the secretion of thyrotropin releasing hormone (TRH) and thyroid secretory hormone (TSH) (Figure I.10). This centralization allows interconnection and cross-talk with other signaling pathways (*e.g.*, adrenal and gonadal signaling pathways) (Pierantoni et al. 2002; Denver 2009) and feedback loops (Denver 1997; Nakao et al. 2008). Thus, it is not surprising to find that the regulation of such an important hormonal pathway is operated by one of the oldest and most conserved structure of the vertebrate brain (Sower et al. 2009).

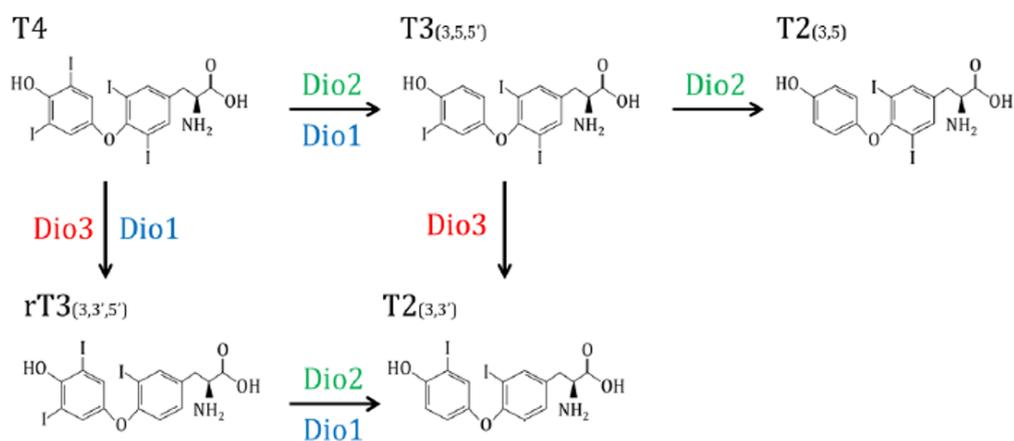


Figure I.9 | Thyroid hormones and deiodinase enzymes

Schematic representation of the biological role of deiodinase enzymes. T4 and T3 are the two active forms of TH that can bind TR. Dio: deiodinase enzyme. Adapted from Bianco and Kim (2006) and Holzer (2015)

Following TH production, the TH signaling pathway involves the binding of TH to the thyroid hormone receptors (TR), which are ligand dependent transcription factors belonging to the superfamily of nuclear receptors. In the absence of TH, TR repress their targeted gene expression by binding on their regulatory sequences and actively recruiting co-repressors (Figure I.10) (Buchholz et al. 2006). In the presence of TH, TH/TR binding triggers the removal of repressor- and the recruitment of activator-cofactors, which in turn enhances the expression of target

metamorphosis genes (Figure I.10) (Buchholz et al. 2006). Therefore, in the context of metamorphosis, the surge of TH during climax induces an important transcriptional response that is responsible for the previously described morphological and physiological changes (Buchholz et al. 2006). There are actually two TR in tetrapods: TR α and TR β , which are the products of the vertebrate-specific double whole-genome duplication (Escriva et al. 2002; Dehal and Boore 2005). In anurans, TR α is expressed early in the larval development, even before TH synthesis, and is responsible, in part, for blocking the metamorphic processes while waiting for optimal conditions to metamorphose (e.g., favorable environmental conditions, sufficient larval growth) (Buchholz et al. 2006). Unlike TR α , TR β expression is controlled directly by TH (Buchholz et al. 2006). Therefore, peak expression of TR β transmits the TH peak signal, particularly as TR β is one of its own targeted genes (positive feedback loop) (Tata 2000). In teleost fishes, there are two TR α : TR α -A and TR α -B, issuing from recent lineage-specific whole-genome duplications (Escriva et al. 2002). At the end of the regulation loop, a negative feedback on both TRH and TSH syntheses, enables the system to stop after peaking (Figure I.10).

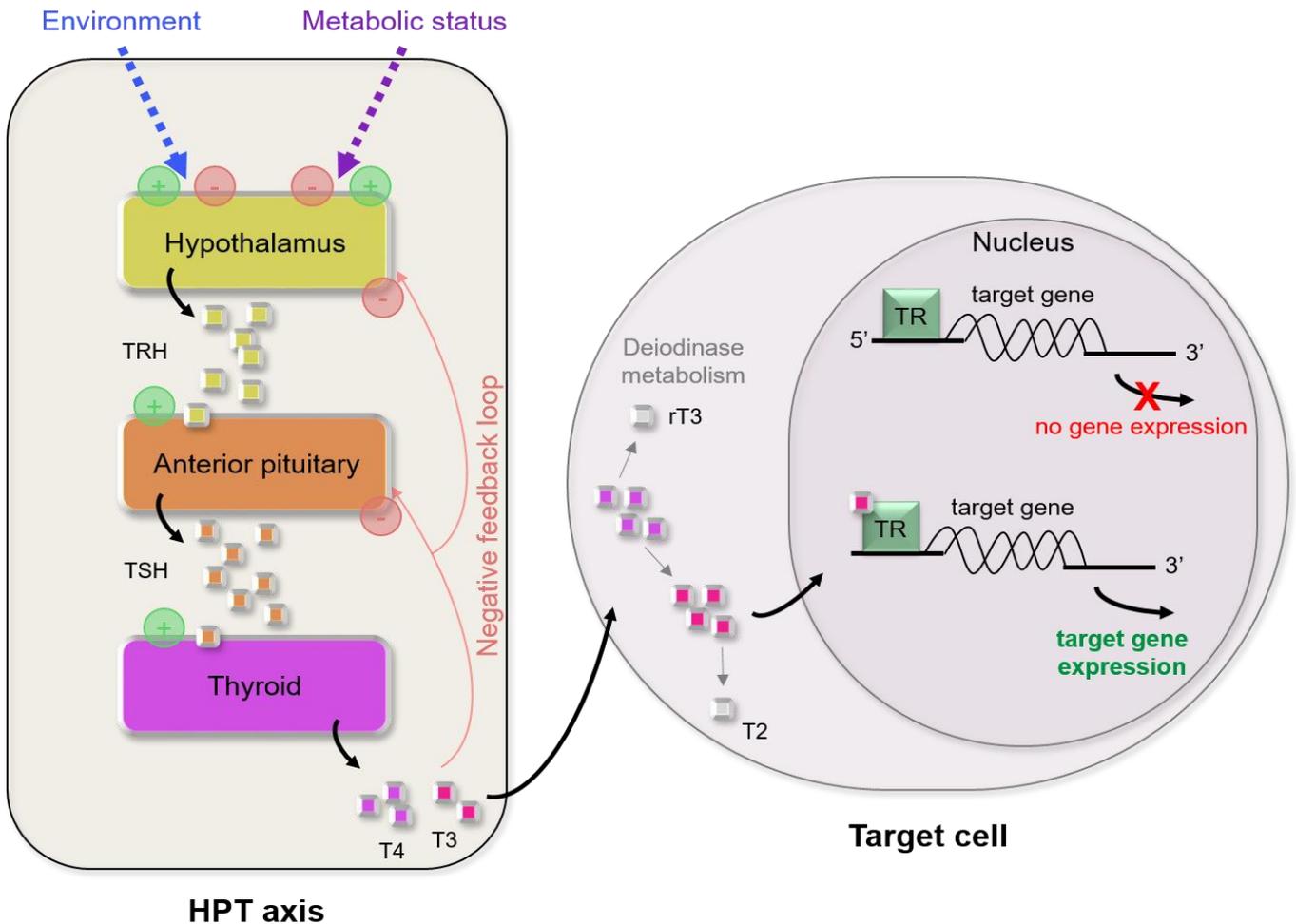


Figure I.10 | Thyroid hormone signaling

Environmental and metabolic cues can either induce or repress the release of TRH in the hypothalamus. TRH binds its receptors on the anterior pituitary, which then releases TSH. TSH binds its receptors on the thyroid, which then secretes TH. TH are transported to target cells where the deiodinase metabolism determines their fate. When T3 reaches TR in the nucleus, this activates the expression of metamorphosis genes (e.g., *Klf9* gene, involved in brain development).

I.2.2. Metamorphosis in teleost fishes

In teleost marine fishes, it is now widely accepted that larvae metamorphose into morphologically, physiologically and ecologically distinct juvenile stages *via* the previously cited hormonally controlled processes (Pechenik et al. 1998; Youson 1998; Paris and Laudet 2008; Laudet 2011; Dufour et al. 2012). However, for farming purposes, research has been almost exclusively conducted on temperate and commercial species, such as sea breams (Campinho et al. 2010; Nikolioudakis et al. 2010, 2014), sea basses (Pérez-Domínguez and Holt 2006), lampreys (Manzon et al. 1998), tunas (Kawakami et al. 2008a, 2008c), flounders (Keefe and Able 1993; Tanaka et al. 1995; Huang et al. 1998; Schreiber and Specker 1998; Soffientino and Specker 2003) and other flatfishes (Markle et al. 1992; Tanaka et al. 1995; Marchand et al. 2004; Ortiz Delgado et al. 2006; Galay-Burgos et al. 2008; Klaren et al. 2008; Manchado et al. 2008). For example, peaks of TH levels and TR expressions are observed during the metamorphosis of the Pacific Bluefin tuna (*Thunnus orientalis*) (Figure I.11) and during the metamorphosis of the Japanese flounder (*Paralichthys olivaceus*) (Figure I.12).

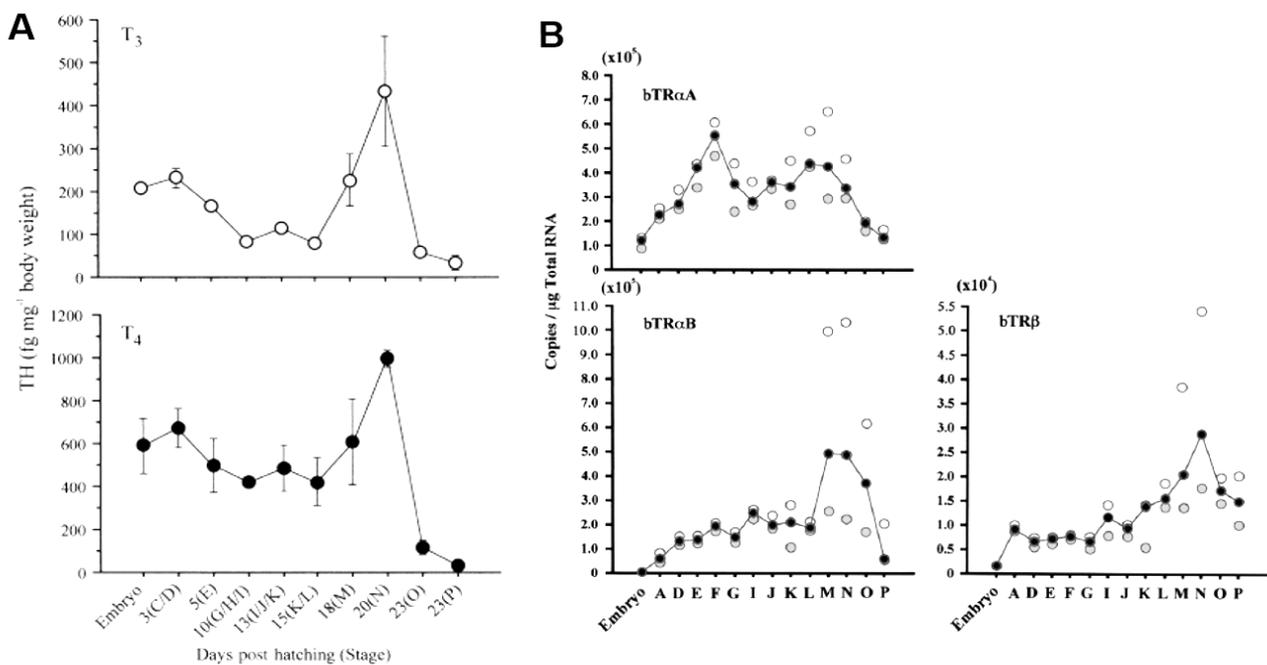


Figure I.11 | Changes in TH levels (A) and TR expressions (B) during the development of the Pacific Bluefin tuna (*Thunnus orientalis*)

Peaks of TH levels and TR expressions are observed around the N and O developmental stages, which correspond to the stage where most changes are observed in the metamorphosing larvae. Adapted from Kawakami (2008a)

In addition to the previously described metamorphic processes, studies in teleost fishes revealed some differences in TR expressions (Marchand et al. 2004; Galay-Burgos et al. 2008; Manchado et al. 2009). For instance, in the Atlantic halibut, TRα-A expression profile is similar to TRβ, while TRα-B expression profile is similar to the TRα expression profile observed in anurans (Galay-Burgos et al. 2008). Figure I.12

also demonstrates another example in *P. olivaceus*. Additionally, the thyroid structure in teleost fish is actually not a true gland, but rather numerous and scattered follicles (but each with the same structure as the ones found in true glands) located in the sub-pharyngeal region (Figure I.13) (Matthews 1948; Kawakami et al. 2008a).

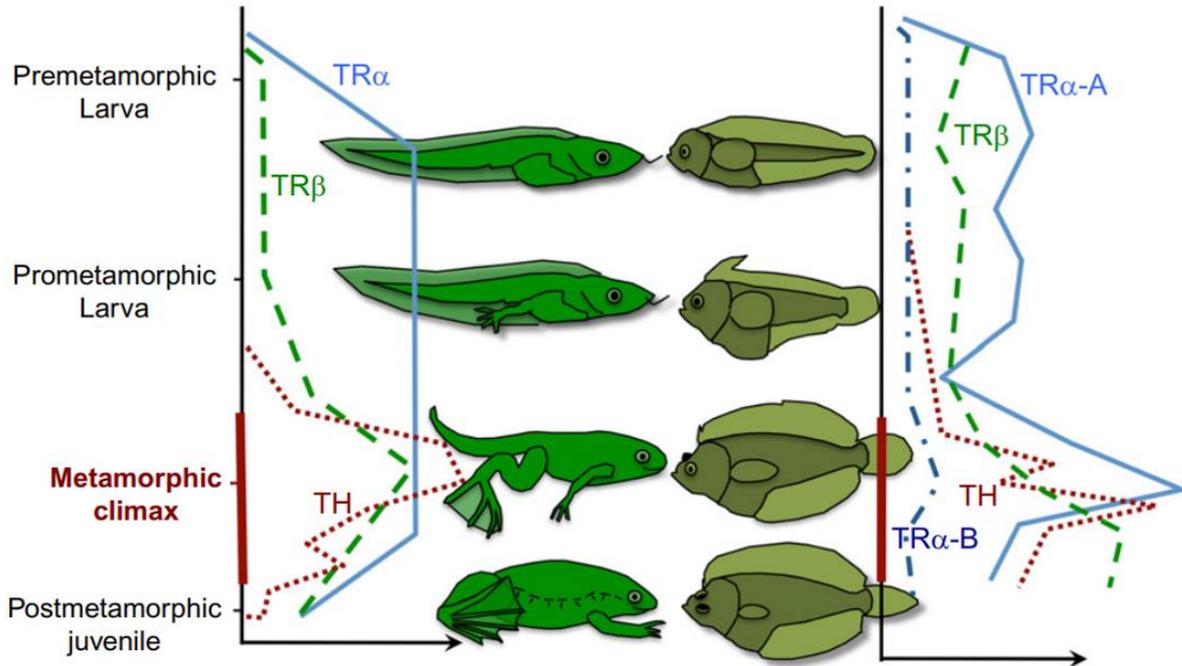


Figure I.12 | Changes in TH levels (A) and TR expressions (B) during the development of the African clawed frog (*Xenopus laevis*) and the Japanese flounder (*Paralichthys olivaceus*)
 TH (dotted lines) levels and TR expressions (solid and dashed lines) peak during metamorphosis of *X. laevis* and *P. olivaceus*. Contrarily, *TRα-B* in *P. olivaceus* (alternating dash-dot line) does not peak. Adapted from Shi (2000), Yamano and Miwa (1998) and figure originating from Schreiber (2013).



Figure I.13 | Thyroid follicles in the sub-pharyngeal region of *Acanthurus triostegus*
 Schiff reagent and hematoxylin staining of a 5 μm histological section of *A. triostegus* sub-pharyngeal region (primarily fixed in Liquide de Bouin). The numerous thyroid follicles are revealed in indigo (see arrows). Scale bar indicates 50 μm .

I.2.3. Metamorphosis in coral reef fishes

In coral reef fishes, metamorphosis at the larva/juvenile transition has been acknowledged by researchers for more than 70 years (Breder 1944; Wenger et al. 2014). However, our knowledge on what characterizes metamorphosis in coral reef fishes is scattered and biased toward morphological features (*e.g.*, pigmentation, shape, skeletal development in *A. triostegus* metamorphosis, Figure I.14) (Breder 1944; Randall 1961; McCormick and Shand 1992; McCormick 1999; McCormick et al. 2002; Frédérich et al. 2008, 2010a; McLeod et al. 2013). We still lack a precise description of the physiological triggers and molecular proceedings of metamorphosis in coral reef fishes. Indeed, there has been surprisingly little research conducted on metamorphosis triggering and the molecular pathway. To date and to my knowledge, only two studies have focused on the physiology of this process, by providing the first evidence that thyroid hormones may trigger metamorphosis in coral reef fishes (de Jesus et al. 1998; Trijuno et al. 2002), as expected from the literature on other non-reef teleost fishes. Moreover, the structure in follicles of the thyroid has been confirmed in the Bermuda parrot fish (*Pseudoscarus guacamaia*) (Matthews 1948) and during this PhD in *A. triostegus* (Figure I.13). Nevertheless, the paucity of information concerning metamorphosis is particularly striking when considering the fact that this transformation occurs during the oceanic to reef transition (*i.e.*, along settlement and larval recruitment), a step often referred to as a “critical period” in the life cycle of coral reef fishes (McCormick et al. 2002; Almany and Webster 2006; Hamilton 2008). Indeed, mortality can reach up to 95 % during this transition (Caselle 1999; Planes and Lecaillon 2001; Steele and Forrester 2002; Doherty et al. 2004; Hamilton et al. 2008) demonstrating that metamorphosis can be a mortality bottleneck that shapes population dynamics.



Figure I.14 | *Acanthurus triostegus* metamorphosis

Immediately after their entry in the reef at night, metamorphosing and transparent-larvae (left) and metamorphosed and pigmented-juveniles (right) can be encountered in the same nursery areas. The vertical bars of black pigmentation can appear in less than two hours.

Picture: M. Besson.

I.3. Coral reef fish metamorphosis and larval recruitment facing anthropogenic pressures

I.3.1. Anthropogenic pressures can degrade reefs and impair larval recruitment

In the current context of world-wide coral decline due to climate change and water pollution (Gardner et al. 2003; Bellwood et al. 2004; Bruno and Selig 2007; Hughes et al. 2010), pressures upon coral reef fish populations and assemblages is no longer solely attributed to overfishing stressors but also replenishment issues (Wilson et al. 2006, 2008, 2010a, 2010b; Munday et al. 2008; Pratchett et al. 2008). Indeed, the loss and degradation of coral habitats has been known for years to reduce the potential for attracting coral reef fish settlers (Lecchini 2005; Lecchini et al. 2007b, 2011; Irisson and Lecchini 2008; Wilson et al. 2008, 2010b; Lecchini and Nakamura 2013; Dixon et al. 2014). Moreover, it has been recently emphasized that stressors such as oceanic acidification, sea level rise, boat noise and soil pollution could also directly affect coral reef fish larval development, growth, behavior and ability to settle. For instance, ocean acidification has been shown to disrupt olfactory, auditory and visual perception of cues usually involved in habitat selection in coral reef fishes (Munday et al. 2009, 2014; Simpson et al. 2011; Ferrari et al. 2012b; Leduc et al. 2013). Also, elevated sea temperature (according to predictions from (Ganachaud et al. 2011)) and subsequent reduced food availability to pelagic larvae (Hays et al. 2005; Richardson 2008; Barton et al. 2013) have been proven to lower larval development rate, body condition at metamorphosis, and survival after metamorphosis, therefore diminishing larval recruitment success (Hamilton 2008; Lo-Yat et al. 2011; McLeod et al. 2013). Furthermore, water pollutants such as anthropogenic boat noise and increasing events of soil and pesticide pollution (*e.g.* near river mouths, because of landclearing and increasing extreme climatic events, which cause massive rains and extensive erosion), but also oil derivatives exposure, have been implicated in reducing larval recruitment (Holles et al. 2013; Wenger et al. 2014; O'Connor et al. 2016; Johansen et al. 2017; Lecchini et al. 2017). I participated to the latter study, which has been written and published during this PhD, contributing as sixth author (Lecchini et al. 2017). This study is available in this thesis (Appendix 3).

All these studies describe the impacts of climate change and water pollutants on larval life history traits and replenishment potential. When considering the extreme mortality experienced by coral reef fish at larval recruitment, small changes in body condition, growth, metamorphose success, settlement success and subsequent mortality rates, could have important replenishment consequences. Given that the replenishment of coral reef fish population is essentially dependent on larval recruitment (Doherty 2002), it is now well established that in degraded habitats, the decline in adult populations of coral reef fishes has more to do with larval recruitment failure than adult mortality (Jones et al. 2004).

I.3.2. Importance of metamorphosis in coral reef fish larval recruitment

The development of a larva into a juvenile, through metamorphosis, occupies a central role in larval recruitment. For instance, metamorphic changes are involved in **(i)** evading predators (*e.g.*, by pigmentation change to the benthic coloration) (McCormick 1999; McCormick et al. 2002), **(ii)** diet shifts (*e.g.*, through neo-orientation of the mouth and remodeling of the guts) (Randall 1961; Labelle and Nursall 1985; Nursall and Turner 1985; Green and McCormick 2001; McCormick et al. 2002) and **(iii)** finding a suitable microhabitat in the reef ecosystem (*e.g.*, *via* the development of new sensory organs that promote greater awareness of the new environment and its broad range of novel sensory stimuli and selective forces) (McCormick and Shand 1992; McCormick 1993; Lecchini et al. 2007a; Mitchell et al. 2011; Frédérich et al. 2012). In fishes that metamorphose in the pelagic environment and settle to reef as a fully developed juvenile, such as the damselfish *Abudefduf saxilitis* (McCormick et al. 2002), metamorphic development of sensory organs is also likely to be required for the perception of multiple sensory cues enabling the return to the reef (Leis et al. 2011).

When facing the novel reef environment at settlement, coral reef fishes display innate preferences in their investigation for a suitable reef habitat (*e.g.*, live coral odors, conspecifics visual cues, avoidance of predator odors) (Lecchini et al. 2007b; Vail and McCormick 2011; Roux et al. 2016). I participated to the latter study, which has been written and published during this PhD, contributing as sixth author study (Roux et al. 2016). This study is available in this thesis (Appendix 4). Yet, larvae are also capable of learning and therefore adapting their behavioral responses to novel cues (*e.g.*, recognition of a novel predator) (Holmes and McCormick 2010; Manassa and McCormick 2013). This sensitive learning at the larval stage is called 'imprinting', and it has been recognized in limiting dispersal and facilitating the return to the reef in coral reef fish larvae (Gerlach et al. 2007; Paris et al. 2013). In clownfish, this imprinting process has been described in the context of the species-specific recognition between the fish and its anemone host (Arvedlund et al. 1999, 2000). In another teleost fish species such as the salmon *Oncorhynchus kisutch*, the TH are involved in the cellular proliferation within the olfactory epithelium during the time of imprinting (responsible for their homing behavior) (Dittman and Quinn 1996; Lema and Nevitt 2004). This hormonally regulated process occurs during the surge of TH levels at the parr-smolt metamorphosis, which corresponds to the morphological and physiological changes supporting the freshwater to seawater transition. Since this phenomenon has been confirmed in other salmon species (Scholz et al. 1985; Hino et al. 2007) and also in a (non-teleost) sturgeon species (Boiko and Grigor'yan 2002), it would be particularly relevant to evaluate the impact of TH in the imprinting process of coral reef fishes. When considering the crucial role played by TH in neurodevelopment, particularly in auditory, olfactory and visual systems (Forrest et al. 2002), and the fact that **(i)** imprinting (Gerlach et al. 2007; Paris et al. 2013), **(ii)** neurodevelopment (Lecchini et al. 2014a), and **(iii)** integration of multiple sensory cues

towards habitat selection (McCormick and Shand 1992; McCormick 1993; Lecchini et al. 2007a; Mitchell et al. 2011; Frédérich et al. 2012) occur at or around metamorphosis in coral reef fishes, we hypothesize that TH might play a key role in the recruitment success of coral reef fishes. A recent study demonstrated the disrupting impact of climate change, and in particular oceanic acidification, on coral reef fish ability to learn and imprint (Ferrari et al. 2012a). Additionally, several studies conducted on other teleost fishes and marine invertebrates illustrated the toxicity of endocrine disruptors such as pesticides, which affected not only survival and reproduction, but development and metamorphosis (Menendez and Ishimatsu 1993; Brown et al. 2004; Delgado et al. 2013). This is of particular interest when considering the substantial anthropogenic impacts on reefs through the worldwide increasing influx of chemical pollution (*e.g.*, from pesticides) resulting from coastal agriculture and river runoff (Carvalho et al. 2002; Islam and Tanaka 2004; Fabricius 2005; Laabs et al. 2007; Leong et al. 2007; Lewis et al. 2009; Packett et al. 2009; Shaw et al. 2010; Roche et al. 2011; Kroon et al. 2012). Such pollutants are known to severely impact reef biodiversity and the biology and ecology of coastal marine organisms (Fabricius 2005; O'Connor et al. 2016). Among these sources of pollution, the Chlorpyrifos (CPF), an organophosphate insecticide widely used on tropical coastal crops (*e.g.*, sugar-cane and rice crops in Australia and south-east Asia respectively (Cavanagh et al. 1999; Leong et al. 2007; Botté et al. 2012)), is one of the most common waterborne chemical pollutants encountered in coral reefs (Cavanagh et al. 1999; Botté et al. 2012). While several studies have demonstrated CPF neurotoxicity and endocrine disruption characteristics (Kavitha and Rao 2008; Botté et al. 2012; Deb and Das 2013; Slotkin et al. 2013), the negative impact on coral reef larval fish sensory abilities has also recently been acknowledged (Botté et al. 2012). Moreover, pesticides belonging to the same family have been discovered in considerable amounts in several fish and crustacean species in French Polynesia (Roche et al. 2011; Salvat et al. 2012). However, whether this pollutant affects metamorphosis itself remains unknown (Botté et al. 2012).

I.4. PhD project presentation

I.4.1. Background, problematics, and objectives

Altogether, climate change and water pollution thoroughly affect coral reef fish populations and assemblages. In particular, they can impact larval and metamorphic stages and subsequently impair larval recruitment success. The processes affected by such stressors are highly diversified and complex and may have a variety of molecular implications. However, due to their involvement in a broad range of developmental processes, TH appear to be powerful mediators, which could help us to characterize the interactions between environmental factors and metamorphic processes. In turn, given metamorphosis plays a central role in larval recruitment, characterizing metamorphic processes and how they impinge on the ecology of coral reef fishes during this important transition period is critical to understanding larval recruitment. This would enhance our understanding of coral reef fish replenishment, resilience and dynamics in regard to climate change and water pollution. This raises many questions:

Is coral reef fish metamorphosis triggered and controlled by TH, as it is in temperate and commercial non-reef teleost fishes?

How are larval recruitment and TH signaling coupled?

Are there specific sensory organs and sensory abilities that develop at metamorphosis and what are their endocrine determinants?

Are there environmental factors that act as endocrine disruptors of TH signaling during coral reef metamorphosis and how would it subsequently affect larval recruitment success?

To answer these questions, this PhD thesis aims to:

- (i) decipher the hormonal proceedings of coral reef fish metamorphosis;
- (ii) better characterize the importance of TH and metamorphosis in larval recruitment (*e.g.*, 1/ development of sensory organs such as the eye retina, nostrils and lateral line, 2/ acquisition of sensory abilities such as the olfactory and visual recognition of predators and conspecifics, and 3/ avoidance of predation); and
- (iii) assess how environmental factors such as sea water temperature and CPF exposure can affect this key transition of the coral reef fish life cycle

To achieve this, the convict tang *Acanthurus triostegus* (Figure I.5, Figure I.6) (Linnaeus, 1758) was used as a model species, and all sampling and field experiments were conducted at the CRIOBE research station on Moorea Island (French Polynesia).

I.4.2. Model species and study site

A. triostegus is, to date, the most studied coral reef fish species in terms of metamorphosis (Figure I.13, Figure I.14) (Randall 1961; McCormick 1999; McCormick et al. 2002; Frédéricich et al. 2010a, 2012). Following their 44-60 day pelagic larval duration (Lo-Yat et al. 2011), the transparent *A. triostegus* larvae metamorphose into pigmented juveniles in a few days (Figure I.14) (McCormick 1999). During this transition, larval planktonic feeders turn into herbivorous juveniles, changing shape as their body height shortens, while their mouth migrates to a more vertical position (as in grazers), and their scales develop (McCormick 1999; McCormick et al. 2002; Frédéricich et al. 2010a, 2012). However, the molecular determinants of such spectacular processes remain unknown. Juveniles and adults feed on filamentous algae (*i.e.*, turf) (Randall 1961). After reef colonization, young settlers are usually encountered in small groups of dozens of individuals, in shallow and quiet waters along the coastline. Contrarily, adults can be solitary but mostly form large aggregations (up to hundreds of individuals) around reef patches close to the barrier reef (Randall 1961).

A. triostegus is one of the most abundant and representative species in the Indo-Pacific (Figure I.6) (Randall 1961), and is particularly abundant in Moorea (French Polynesia) (Lecchini et al. 2004; Besson et al. 2017b). The island of Moorea lies 25 km to the North-West of Tahiti, at 17°30' South latitude, and 149°50' West longitude (Galzin and Pointier 1985; L'ORSTOM 1993). This volcanic island formed less than two millions years ago, originating from an oceanic hotspot (Guillou et al. 2005). It belongs to the Windward Islands of the Society Archipelago. Moorea has a triangular shape with 61 km of coastline surrounded by lagoon of 500 to 1.500 meters wide (Galzin and Pointier 1985). The lagoon is delimited by a barrier reef that opens the lagoon to the ocean in twelve passes. Tidal fluctuations are very low in this region of the world, with tidal differences of a maximum of 40 cm (L'ORSTOM 1993).

I.4.3. Methodology

To achieve the objectives of this PhD, *A. triostegus* was sampled along its different metamorphic stages, from oceanic larvae to metamorphosed juveniles. Sampling was carried out using different techniques, from light-traps to hand nets and crest nets. All these fishing techniques are detailed in the next chapters, as well as in Appendix 2. After capture, fish were either transferred to aquaria at the CRIOBE research station, or kept in the lagoon in *in situ* cages, to study their metamorphosis under specific and controlled conditions. Different hormonal treatments, as well as differential temperature and CPF exposures were applied to metamorphosing individuals, to address the scientific questions above. I also generalized some of the

observations made on *A. triostegus*, to other coral reef fish species belonging to other families, such as *Chromis viridis*, *Chaetodon lunula*, *Ostorhinchus angustatus* and *Rhinecanthus aculeatus*. Lastly, laboratory experiments resulting and following these *in vivo* experiments were conducted at the CRIOBE research station itself, but also at the Institut de Génomique Fonctionnelle in Lyon (France) and at the Observatoire Océanologique in Banyuls (France).

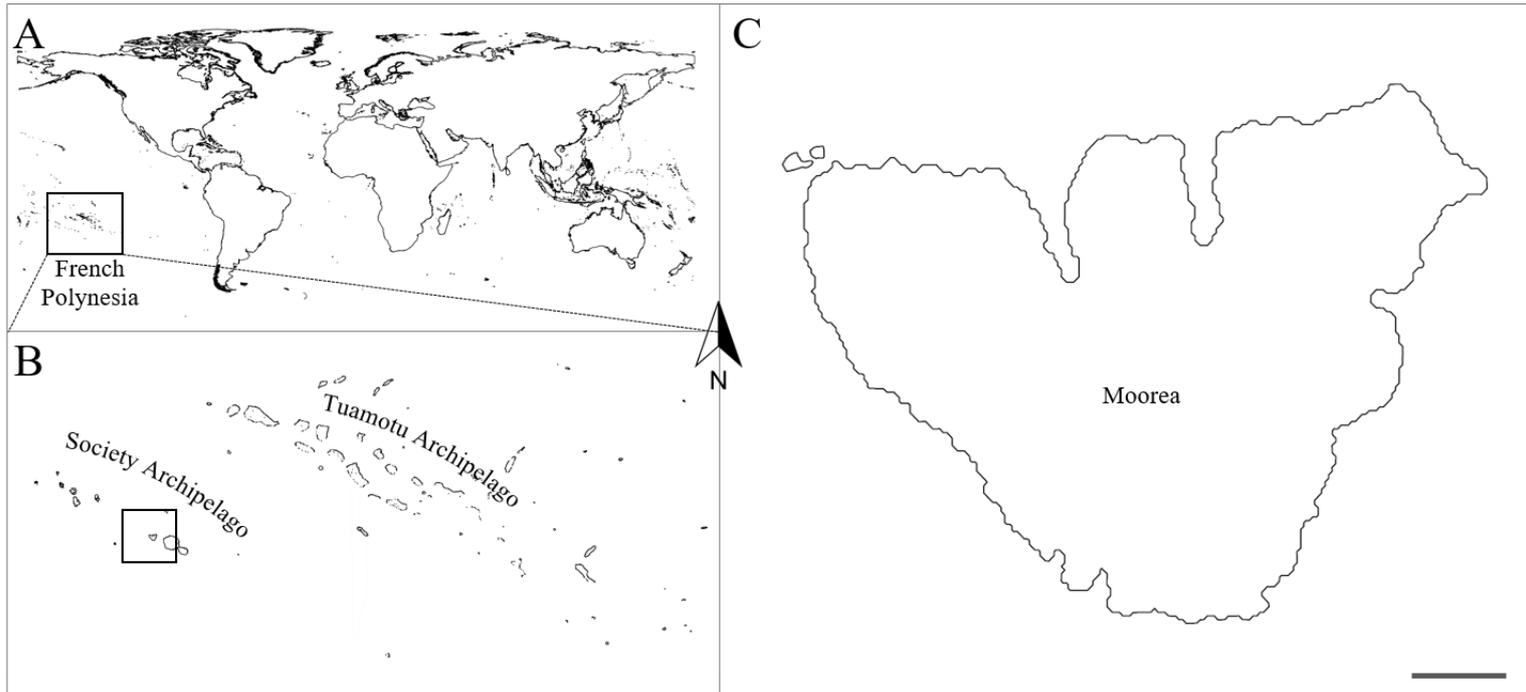


Figure I.15 | Location of Moorea Island in French Polynesia

A: Location of French Polynesia within the Pacific Ocean. **B:** Location of Moorea Island within French Polynesia. **C:** Moorea Island. Scale bar indicates 2 km. Adapted from Besson (2017).

This study was carried out in accordance with the guidelines of the French Polynesia committee for animal ethics, and the experiments were approved by the CRIOBE-IRCP animal ethics committee. This study did not involve endangered or protected species.

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II. Fish larval recruitment to reefs is a thyroid hormone-mediated metamorphosis sensitive to the reef pollutant chlorpyrifos

II.1. Foreword

This chapter aims at answering to the two first questions of my PhD, which are:

- 1 | Is coral reef fish metamorphosis triggered and controlled by TH, as it is in temperate and commercial non-reef teleost fishes?
- 2 | How are larval recruitment and TH signaling coupled?

To achieve this, the fieldwork and analyzes presented in this chapter have been performed in close collaboration with Dr. G. Holzer. He was finishing his PhD while I was starting mine. Understanding the endocrine determinants that mediate larval recruitment in coral-reef fishes was somehow our common project, and the fruit of the collaboration between Dr. D. Lecchini and pr. V. Laudet. At least two years of my PhD were devoted to this chapter. Dr. G. Holzer and I ended up by writing a manuscript gathering all the results he obtained before I started my PhD, and all the results I obtained after he finished his. This manuscript has been submitted to *eLife* in April 2017, as a research article. To enlarge this work and to obtain a chance to get published in such journal, we have added some preliminary results on how metamorphosis could be prone to endocrine disruption in coral-reef fishes, with first clues about such a threat for coral-reef ecosystems. Consequently, this chapter also bring a few first answers to the two other questions of my PhD, which are:

- 3 | Are there specific sensory organs and sensory abilities that develop at metamorphosis and what are their endocrine determinants?
- 4 | Are there environmental factors that act as endocrine disruptors of TH signaling during coral reef metamorphosis and how would it subsequently affect larval recruitment success?

This manuscript has been published in October 2017, after my PhD defense, and is available in Appendix 5.

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II.2. Manuscript information

Title: Fish larval recruitment to reefs is a thyroid hormone-mediated metamorphosis sensitive to the pesticide chlorpyrifos

Authors: Guillaume HOLZER^{1*}, Marc BESSON^{1,2,3*} (equal first author), Anne LAMBERT¹, Loïc FRANÇOIS², Paul BARTH¹, Benjamin GILLET¹, Sandrine HUGHES¹, Gwenaél PIGANEAU³, François LEULIER¹, Laurent VIRIOT¹, David LECCHINI^{2,4,#} and Vincent LAUDET^{1,3,#,£}

Affiliations:

1 Institut de Génomique Fonctionnelle de Lyon; Université de Lyon; Université Claude Bernard Lyon1; UMR CNRS 5242; Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France.

2 PSL Research University: EPHE-UPVD-CNRS, USR3278 CRIOBE, BP 1013, 98729 Papetoai, Moorea, French Polynesia

3 Observatoire Océanologique de Banyuls-sur-Mer, UMR CNRS 7232; Université Pierre et Marie Curie Paris;1, avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France

4 Laboratoire d'Excellence «CORAIL», BP 1013, 98729 Papetoai, Moorea, French Polynesia

* Equal first authors

Equal last authors

£ To whom correspondence should be addressed: Observatoire Océanologique de Banyuls-sur-Mer, 1 avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France. mail: vincent.laudet@obs-banyuls.fr

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II.3. Abstract

Larval recruitment, the transition of pelagic larvae into reef-associated juveniles, is a critical step for the resilience of marine fish populations but its molecular control is unknown. Here, we investigate whether thyroid-hormones (TH) and their receptors (TR) coordinate the larval recruitment of the coral-reef-fish *Acanthurus triostegus*. We demonstrate an increase of TH-levels and TR-expressions in pelagic-larvae, followed by a decrease in recruiting juveniles. We generalize these observations in four other coral reef-fish species. Treatments with TH or TR-antagonist, as well as relocation to the open-ocean, disturb *A. triostegus* larvae transformation and grazing activity. Likewise, chlorpyrifos, a pesticide often encountered in coral-reefs, impairs *A. triostegus* TH-levels, transformation, and grazing activity, hence diminishing this herbivore's ability to control the spread of reef-algae. Larval recruitment therefore corresponds to a TH-controlled metamorphosis, sensitive to endocrine disruption. This provides a framework to understand how larval recruitment, critical to reef-ecosystems maintenance, is altered by anthropogenic stressors.

II.4. Introduction

Life history transitions are critical for many animal species and often correspond to concomitant developmental and ecological shifts (Bishop et al. 2006). Unfortunately, little is known about how internal and external cues act in concert during these events. The life cycle of most teleost reef fish include a major developmental and ecological transition. Adults reproduce in the vicinity of the reef, emitting eggs that disperse and hatch in the ocean, where the larvae grow (Leis and McCormick 2002). Larvae migrate back and enter reefs where they become juveniles, a step called larval recruitment (Leis and McCormick 2002). This step involves the perception of environmental cues for larvae to localize and settle in the reef, and is accompanied by major morphological changes (McCormick et al. 2002; Lecchini et al. 2005a, 2005b, 2013; Barth et al. 2015). This transition of pelagic larvae into reef-associated juveniles is often referred to as metamorphosis and is critical for the maintenance of reef fish populations, but its molecular control remains largely unknown (Dufour and Galzin 1993; Doherty 2002; Leis and McCormick 2002; McCormick et al. 2002; Dixon et al. 2011; Barth et al. 2015). Since larval recruitment is an ecological event coupled to a morphological transformation, reminiscent of the situation in amphibians, it may correspond to a thyroid hormones (TH) controlled metamorphosis (Bishop et al. 2006; Brown and Cai 2007).

TH and their receptors (TR) trigger and coordinate metamorphosis of many species such as *Xenopus* (Brown and Cai 2007) or flatfish (Solbakken et al. 1999). The thyroid gland produces mainly the thyroxine hormone (T₄), which is peripherally transformed into triiodothyronine (T₃), the active form (Chopra 1996). The transformation of T₄ into T₃, and the degradation of T₄ and T₃ are controlled by a family of enzymes called deiodinases (Bianco and Kim 2006b). TH levels peak at the climax of metamorphosis. Precocious treatment with TH triggers metamorphosis whereas goitrogen (TH synthesis inhibitors) treatment blocks it (Tata 2006). Similar mechanisms have been described in several teleost fishes, but in contexts disconnected from the natural environment (Brown 1997; de Jesus et al. 1998; Yamano and Miwa 1998; Kawakami et al. 2003; Marchand et al. 2004; McMenamin and Parichy 2013).

In the convict surgeonfish *Acanthurus triostegus*, larvae have a planktonic diet while juveniles and adults are herbivorous, and external morphological changes occurring at recruitment have been previously described (Randall 1961; McCormick 1999; McCormick et al. 2002; Frédérich et al. 2012). Interestingly, crest-captured larvae can delay their metamorphosis when relocated to the external slope of the reef (open ocean), showing an influence of the environment on their morphological transformation (McCormick 1999). Nevertheless, again, the molecular mechanisms remain unknown.

Here we report that *A. triostegus* larval recruitment to the reefs corresponds to metamorphosis with major pigmentation changes, remodeling of the digestive tract and behavioral modifications. Our results show that TH levels and TR expressions control this remodeling process. Furthermore, recruiting larvae can freeze their TH signaling when relocated on the external slope, demonstrating an influence of the

environment on metamorphosis. Chlorpyrifos (CPF), a known reef pollutant and endocrine disruptor (Roche et al. 2011; Botté et al. 2012; Juberg et al. 2013; Slotkin et al. 2013), affects *A. triostegus* metamorphosis by impairing TH signaling, preventing intestine lengthening, and inhibiting fish grazing activity. CPF therefore impairs *A. triostegus* control on algae spreading, which is a major threat for reef conservation. We extend our observation upon TH levels to four other coral reef fish species. Our work provides a unifying framework that integrates the developmental, ecological and evolutionary perspectives of vertebrate life history transitions. The involvement of the TH signaling pathway in this key post-embryonic step, thus prone to endocrine disruption, provides an obvious entry point to study how anthropogenic stressors may affect reef fish populations.

II.5. Results

Acanthurus triostegus larvae undergo a major and rapid remodeling at recruitment

A. triostegus crest captured individuals (n = 297) experience an important weight loss between day 1 (0.71 ± 0.01 g) and day 8 (0.53 ± 0.01 g) following their entry in the reef (Figure II.1A-B). Individuals also undergo extensive pigmentation changes (Figure II.1C-L), as exemplified by the very rapid appearance of black stripes (less than four hours, MB pers. obs.) after entering the reef (Figure II.1C-D, Figure II.1H-I). This is followed by the widening of these vertical black stripes and by the onset of the body's white pigmentation (Figure II.1D-G, Figure II.1I-L).

Furthermore, using conventional X-Ray microtomography, we identified three different dental generations in crest larvae to day 8 juveniles (red, blue and green, Figure II.1M-Q). Crest larvae present tiny and poorly mineralized pointed teeth at the distal ends of the jaws (red, Figure II.1M, Figure II.1-figure supplement 1R) that are likely remnants of an oceanic larval dentition (dentition A). Other medium-sized and more mineralized teeth are in function in medial areas of the jaws (blue, Figure II.1M). These teeth display pointed cusps and belong to a more advanced dental generation (dentition B). Lastly, much larger teeth of the next generation (green, dentition C) are about to erupt from jaws in crest larvae (Figure II.1M) and replace A and B previous dentitions in day 2 to day 8 juveniles (Figure II.1N-Q). These teeth are highly similar to the shovel-shaped adult teeth (Figure II.1 - figure supplement 1S) that serve for grazing algae on hard substrata. The rapid formation of dentition C is consistent with a diet shift from planktivorous to herbivorous at recruitment. This shift is precisely organized, since it begins in the sagittal area of all individuals, and secondarily extends to the distal parts of the jaw (Figure II.1M-Q). The fact that crest captured larvae already have teeth of the C dentition about to erupt (Figure II.1M) indicates that fish are prepared for reef life conditions before reef entry.

The intestine also goes through a drastic remodeling at recruitment. In particular, the gastro-intestinal tract lengthens from about 2.52 ± 0.07 cm in crest captured larvae to 7.38 ± 0.14 cm in day 8 juveniles (Figure II.1W). To characterize this change, we performed histological sections on the middle part of the intestine during the larval recruitment process (Figure II.1R-V). Intestines of crest larvae and day 2 juveniles have a thick wall with many muscle fibers and regular villi (Figure II.1R and S). At day 3, intestines undergo a spectacular remodeling with the disappearance of the epithelium, leaving only the muscular layer (Figure II.1T), while epithelium reformation occurs later at day 5 (Figure II.1U). In day 8 individuals, the intestine is fully remodeled: the epithelium is thicker and exhibits villi again (Figure II.1V). Such spectacular remodeling events are also observed in the proximal and distal portion of the intestines (Figure II.1 - figure supplement 1A-O).

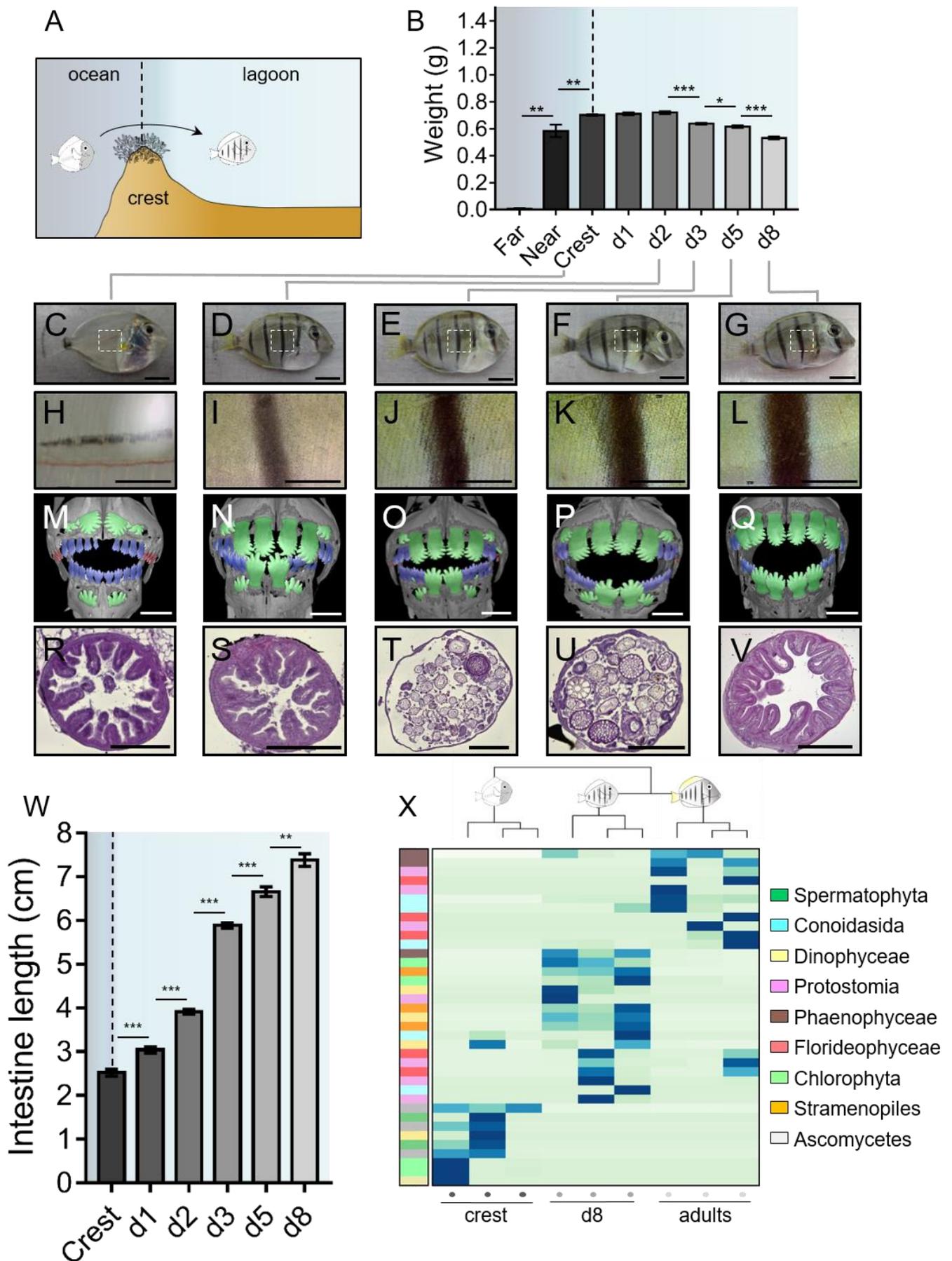


Figure II.1 | Changes in *Acanthurus triostegus* during larval recruitment.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. (A) Schematization of the reef colonization by a larva (left) turning into a juvenile (right). The background color shading centered into the reef crest is re-used in following figures. (B) Weight variation during larval recruitment. n = 3 biological replicates for 5-to-10 km offshore

fish (far), $n = 5$ for 2 km offshore fish (near) and $n = 297$ for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). **(C-G)** Variation in pigmentation and morphology at different sampling points, (C) crest larva, (D) day 2 juvenile, (E) day 3 juvenile, (F) day 5 juvenile and (G) day 8 juvenile; scale bars indicate 0.5 cm. The white square represent the magnified region shown in the line below. These pictures are representative of $n > 50$ fish (biological replicates) per developmental stage, sampled at $n > 20$ different times during the year (technical replicates). **(H-L)** Close-up on the pigmented middle body band. Scale bars indicate 0.2 cm. These pictures are representative of $n > 50$ fish (biological replicates) per developmental stage, sampled at $n > 20$ different times during the year (technical replicates). **(M-Q)** X-Ray microtomography of larva, dentition A, B or C are highlighted respectively in red, blue or green. Scale bars indicate 0.5mm. These pictures are representative of $n = 2$ fish (biological replicates) per developmental stage. **(R-V)** Histological sections (5 μm width) of intestines with haematoxylin/eosin staining. Scale bars indicate 200 μm . These pictures are representative of $n = 6$ sectioning technical replicates, performed twice (sampling technical replicates), on $n = 2$ fish (biological replicate). **(W)** Intestine length variation of *A. triostegus* during its larval recruitment. $n = 9$ to 14 biological replicates for each developmental stage (from $n = 2$ technical sampling replicates). Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). **(X)** Heatmap clustering of the 18S sequences identified in the intestine content of crest individual ($n = 3$, left panel), day 8 juveniles ($n = 3$, middle panel) and adults ($n = 3$, right panel). Each panel is made of three columns corresponding to one individual. Each line corresponds to one taxonomic group found in the intestines: *Spermatophyta* (dark green), *Conoidasisa* (cyan), *Dinophyceae* (light yellow), *Protostomia* (light pink), *Phaeophyceae* (brown), *Florideophyceae* (dark pink), *Chlorophyta* (light green), *Stramenopiles* (orange) and *Ascomycetes* (grey).

To assess whether and to what extent this intestine remodeling was correlated to a diet shift at larval recruitment, we sequenced *A. triostegus* digestive tract content (18S mass sequencing) to identify its eukaryotic composition. We investigated crest captured individuals, day 8 juveniles and adults (Figure II.1W) using a blocking primer to minimize amplification of host sequences. Crest larval intestines are largely empty although we identified sequences of fungi and algae. In day 8 juveniles, the intestinal content is different with more brown and red algae, as well as few protostome sequences. This indicates a shift in the eukaryotic gut content between crest individuals and day 8 juveniles. Adults gut content is less diversified and is mainly composed of brown algae, red algae and alveolates, different from those found in day 8 juveniles (Figure II.1W). Using 16S metagenomic sequencing, we found differences in the relative quantity of bacterial community between the juveniles at day 2, 5 and 8 (Figure II.1 - figure supplement 1P). The 16S profile of the adult is also different from the juvenile profile, with the notable presence of *Lachnospiraceae*, among which the giant bacteria *Epulopiscium fishelsoni* is a well-known symbiont of Acanthuridae (Clements and Bullivant 1991).

Thyroid hormone signaling peaks at larval recruitment

Given the role of TH in vertebrate metamorphosis (Laudet 2011) and the spectacular transformations that *A. triostegus* undergoes at larval recruitment, we analyzed TH levels in larvae captured in the far ocean (from 5 to 10 km offshore, n = 3), near ocean (2 km offshore, n = 5), crest-captured larvae and juveniles (raised *in situ* in lagoon cages, n = 297) up to day 8 after recruitment (Figure II.2A-B).

T4 levels are low in far ocean larvae and rise in fish located closer to the reef (near ocean larvae), before dropping 6-fold between the near ocean larvae and the day 8 juveniles (p-value = 0.001, Figure II.2A). This indicates a peak of T4 in near ocean and in crest larvae. Far ocean larvae exhibit a high level of T3 but with an important variability (Figure II.2B). Although these larvae live in the same ecological niche (*i.e.* open ocean) and are roughly of the same size (4.7, 5.5, and 6.3 mm), they were captured in different locations and are probably not at the same developmental stage. Near ocean and crest captured larvae have similar levels of T3 that are 6-fold higher than the level observed in day 8 juveniles (p-value < 0.001 Figure II.2B).

We characterized the three TR of *A. triostegus*: TR α -A, TR α -B and TR β . These receptors have between 86 % (TR α -A) and 96 % (TR β) sequence identity with their zebrafish orthologs (Figure II.2 - figure supplement 1A). The phylogenetic analysis indicates that each of the three TR clusters with its respective teleosts orthologs (Figure II.2 - figure supplement 1B). Moreover, transactivation assays show that these receptors behave as genuine TR (Figure II.2 - figure supplement 1C). We thus assessed the expression levels of TR α -A, TR α -B, and TR β during *A. triostegus* development (Figure II.2C), as well as *Klf9*, a conserved TR regulated gene (Denver and Williamson 2009). Far ocean larvae have two profiles: one with a very low TR and *Klf9* expressions ("Far a" ocean) and one with very high levels of expression ("Far b" ocean) (Figure II.2C). This suggests that there are, at least, two distinct situations in far ocean larvae: larvae with low TH/TR levels and larvae in which TH/TR levels surge. Far b ocean, near ocean, and crest larvae have high expression levels of the all the TR as well as *Klf9*, indicating a strong activation of the TH signaling at these stages. We observed a drop of all TR expression between crest larvae and day 8 juveniles (p-values = 0.001, 0.005 and 0.002 for TR α -A, TR α -B and TR β respectively, Figure II.2C).

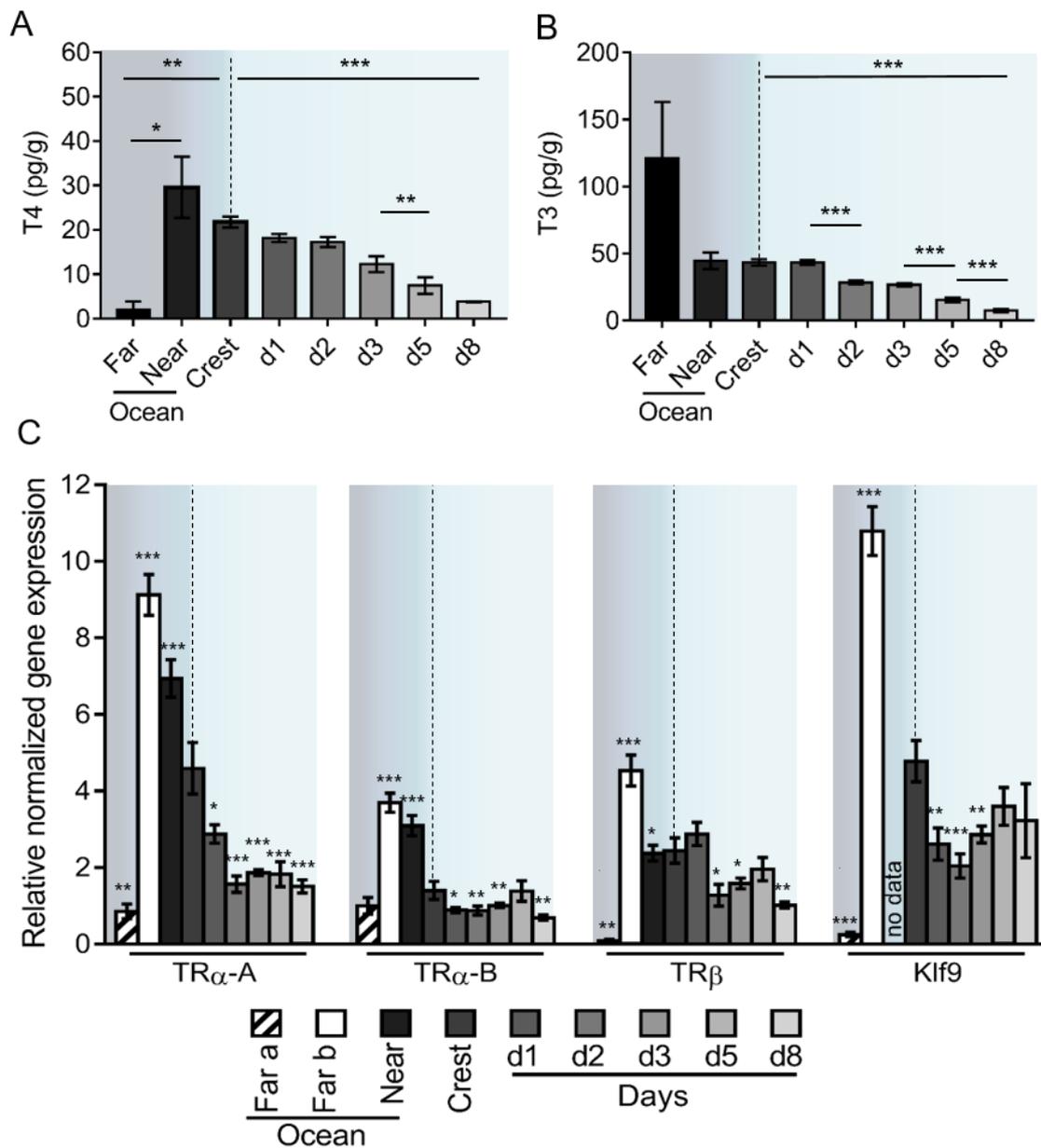


Figure II.2 | Thyroid hormone signaling during *Acanthurus triostegus* metamorphosis.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. (A) T4 and (B) T3 dosage: Y-axis: T4 and T3 levels in $\text{pg}\cdot\text{g}^{-1}$; X-axis: sampling point. $n = 3$ for 5 to 10 km offshore fish (far), $n = 5$ for 2 km offshore fish (near), $n = 297$ for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). (C) Expression level of *TR α -A*, *TR α -B*, *TR β* and *Klf9* by qPCR, normalized with *Pold2* and *Rpl7*. Y-axis; Relative normalized expression X-axis: gene and sampling point.. Far ocean larvae are split in two groups: Far a (black stripes) and Far b (white). For each panel, the color shading is the same one as in Figure II.1A. For each sampling point 3 biological replicates and 3 technical replicates were performed. Statistics: comparisons of each mean to the Crest condition mean (Student's t cumulative distribution function, which was automatically computed by qPCR software CFX Biorad Manager, CFX Manager 2017).

Thyroid hormone signaling and the environment control larvae transformation

To link the TH pathway, the environment and the metamorphic changes occurring at larval recruitment, we disturbed the TH signaling and the location of crest-captured larvae during their metamorphosis (from capture to up to day 8 after reef colonization).

In order to study how the environment controls the metamorphosis processes, we first relocated crest-captured larvae back to the external slope immediately after their reef entry. Indeed, mimicking oceanic condition has been acknowledged to delay metamorphosis (McCormick 1999) (Figure II.3A, upper panel). Similar to this earlier study, we observed a striking delay in the white pigmentation appearance between the vertical black stripes in day 2 relocated fish compared to their lagoon raised counterparts (Figure II.3A, lower panel). The levels of T4 and T3 in fish relocated on the external slope at day 2, day 5 and day 8 are overall higher than the levels of their control counterparts in the lagoon (Figure II.3B-C). The levels of T4, in particular, remain similar in day 2 to day 8 juveniles to the levels in the crest condition (Figure II.3B). The expressions of *TRs* and *Klf9* in the relocated juveniles were also higher than in control juveniles at the same age (Figure II.3E). This suggests that the environment effectively controls the metamorphosis by modulating the TH signaling.

To assess the implication of the TH signaling pathway on metamorphic processes, we then raised crest-captured larvae *in situ* in the lagoon and injected them daily, in their ventral cavity, with 20 μ l of different drug solutions that activate or disrupt their TH molecular pathway. Four drug treatments were applied: **(i)** solvent control (DMSO diluted 10.000 times in Phosphate Buffer Saline 1X, as all drugs were made soluble in DMSO and diluted 10.000 times in PBS 1X); **(ii)** T3 + iopanoic acid (IOP) both at 10^{-6} M, IOP being used as an inhibitor of the deiodinase enzymes that therefore prevents the degradation of injected T3 (Galton 1989; Little et al. 2013; Lorgen et al. 2015); **(iii)** NH₃ at 10^{-6} M, NH₃ being used as an antagonist of TR that prevents the binding of TH on TR (Lim et al. 2002; Renko et al. 2012) therefore disrupting *A. triostegus* TH pathway (Figure II.2 – figure supplement 1D); and **(iv)** T3 + IOP + NH₃ all at 10^{-6} M to ensure the non-toxicity of NH₃.

We used intestine total length, internal structure of guts (intestinal microvilli), dentition and pigmentation as markers of the advancement of metamorphosis since they strongly change after reef recruitment (Figure II.1). T3 + IOP treatment increases the length of the intestine in day 2 and day 5 juveniles (p-values < 0.001, Figure II.3E, blue). On the contrary, NH₃ treatment partially prevents the intestines lengthening occurring after recruitment (p-values < 0.001, Figure II.3E, green). Injections of T3 + IOP + NH₃ result in an intestine length similar to the control individuals at day 2 and day 5 (p-values = 0.486 and 0.444 respectively, Figure II.3E). This shows that T3 and NH₃ effectively compete, and confirms the non-toxicity of

NH₃. In parallel, intestines of fish relocated on the external slope are shorter than those of their lagoon counterparts at day 2 and day 5 (p-value < 0.001 and p-value = 0.042 respectively, Figure II.3E, orange). This shows that their development has been slowed down, similarly to what we observed with the NH₃ treatment. A similar pattern was observed concerning the microvilli remodeling within guts. In the anterior part of the guts, while T3+IOP treatment accelerates the loss of the intestinal microvilli at day 2 and accelerates the development and the thickening of a new epithelium at day 5 (Figure II.3F, second column), NH₃ treatment and external slope relocation prevent microvilli renewal at both day 2 and day 5 (Figure II.3F, third and fourth column). These observations can be extended to the anterior, medium and posterior sections of the guts (see Figure II.3 – figure supplement 1). These changes are consistent with the notion that TH control remodeling of the guts at metamorphosis in *A. triostegus*. We did not observe any effect on teeth development neither with T3+IOP or NH₃ treatment, nor with external slope relocation (Figure II.3 – figure supplement 1). Given that teeth remodeling is a lengthy process that starts very early on in oceanic larvae and therefore before reef colonization, it is not surprising that the treatments performed on crest captured larvae do not affect (or are too late to affect) teeth development. Also, contrarily to individuals relocated on the outer slope, we did not observe any effect of T3+IOP nor NH₃ treatments on skin pigmentation at day 2 and day 5 (Figure II.3 – figure supplement 2). This suggests that pigmentation during the larva to juvenile transition is strongly coupled to the environment but is not directly under TH control that may not sustain such rapid pigmentation changes. Taken together: (i) intestine lengthening and remodeling are mediated by TH signaling; and (ii) disturbing the TH pathway disrupts the normal development of fish after larval recruitment, showing that TH effectively controls some major aspects of *A. triostegus* metamorphosis.

Finally, we tested how TH signaling influences fish behavior. To achieve this, we used grazing activity (number of bites on algal turf), as *A. triostegus* larvae start to graze a couple of days after colonizing the reef, to investigate how TH signaling and metamorphosis completion influence the behavior of recruited fish. T3 + IOP treatment increases the grazing activity by around 50% in day 5 juveniles when compared to the control individuals (p-value = 0.004, Figure II.3G, blue lanes). On the contrary, NH₃ treatment significantly decreases the number of bites at day 2 by 33% (p-value < 0.001, Figure II.3G, green lane). The grazing activity of fish relocated on the external slope (Figure II.3A) is also diminished by more than 4-fold at day 2 (p-value < 0.001, Figure II.3G, orange lanes). These data indicate that TH signaling promotes the biting activity of *A. triostegus* and subsequent grazing behavior adopted at larval recruitment. This shows that TH controls the transformations of the feeding process not only at the morphological and physiological levels but also at the behavioral level.

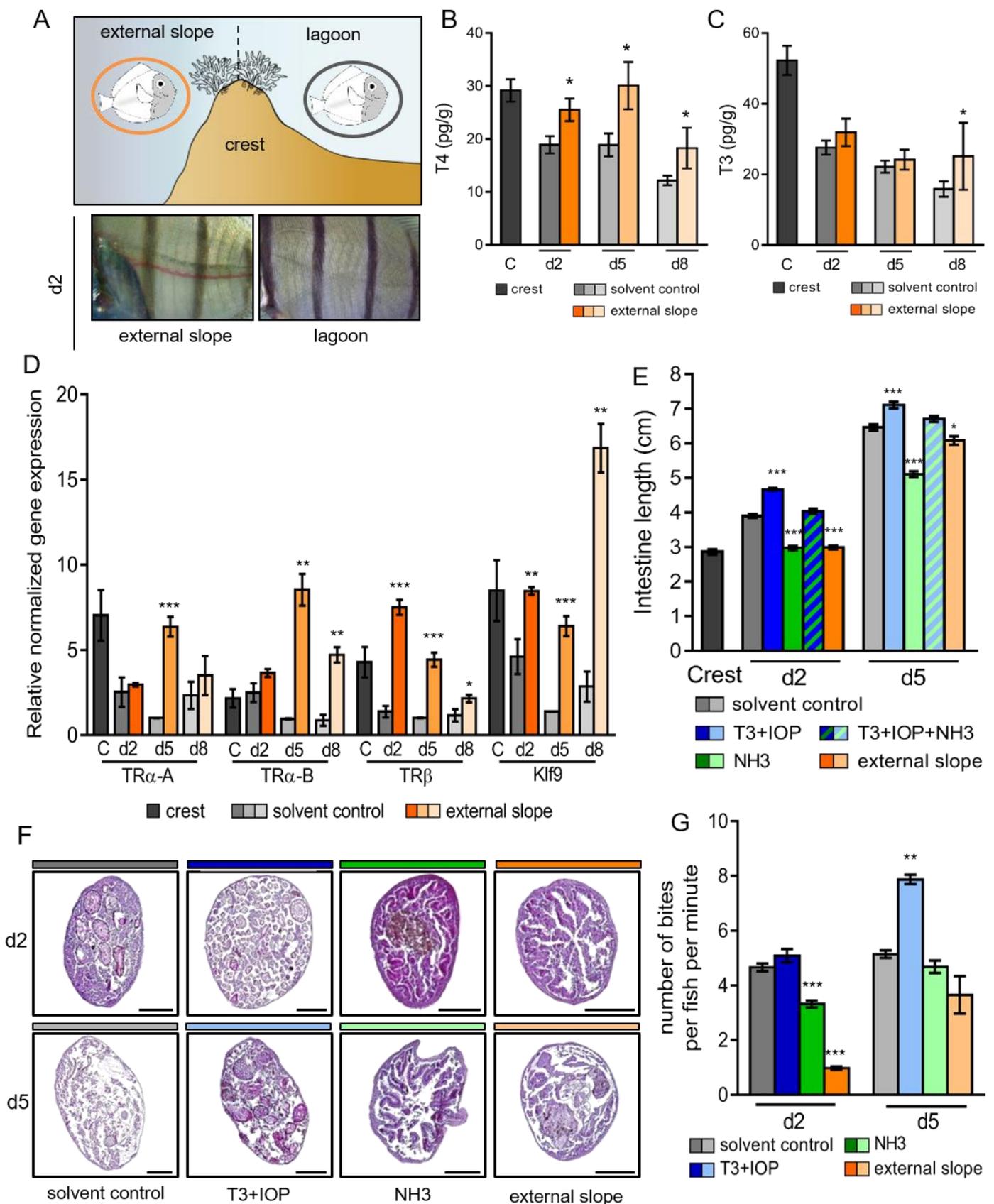


Figure II.3 | Impact of the environment and thyroid hormone signaling on *Acanthurus triostegus* metamorphic processes.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. (A) Upper panel: schematization of the external slope experiment with control fish, in the lagoon (grey circle), and relocated fish, on the external slope (orange circle). Lower panel: comparison of the pigmentation between the relocated fish (left) and the control lagoon fish (right) at day 2. (B) T4 and (C) T3 dosage of crest captured larvae and day 2, 5 and 8 juveniles,

in control (grey shade) or external slope (orange shade) conditions. $n = 4$ fish for each experimental condition. X-axis: sampling point, Y-axis: T4 or T3 level in pg.g^{-1} . Statistics: mean comparisons between control and external slope (Wilcoxon-Mann-Whitney U-test). **(D)** Expression level of *TR α -A*, *TR α -B*, *TR β* and *Klf9* by qPCR, normalized with *pold2* and *rp17* housekeeping genes, in control (grey shade) or external slope (orange shade) conditions. Y-axis: Relative normalized expression. X-axis: gene and stages. For each point 3 biological replicates and 3 technical replicates were performed. Statistics: comparisons of “external slope” means to their “control” counterparts (Student's t cumulative distribution function). **(E)** Intestine length in cm of fish at crest (C), day 2 (d2) or day 5 (d5) under different treatment conditions: solvent-control (grey shade), T3+IOP treated at 10^{-6} M (blue shade), NH₃ treated at 10^{-6} M (green shade), T3+IOP+NH₃ treated at 10^{-6} M (blue and green dashed shade) or located to the external slope (orange shade). $n > 10$ fish for each experimental condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the respective solvent-control condition are indicated. **(F)** Histological cross section (5 μm width) of intestine stained by haematoxylin/eosin. Scale bars indicate 200 μm . These pictures are representative of $n = 2$ microtome section and staining technical replicates, performed on $n = 3$ fish (biological replicate) per treatment and per developmental stage. Two technical replicates of this experiment were performed. **(G)** Mean number of bites (on turf algae) per fish in a batch of 18 fish at day 2 (d2) and day 5 (d5) under different treatment conditions: solvent control (DMSO, grey shade), T3+IOP at $10^{-6}\mu\text{M}$ (blue shade), NH₃ at $10^{-6}\mu\text{M}$ (green shade) or relocated to the external slope (orange shade). $n = 3$ to 6 independent bite counts (technical replicates) for each condition. Bite counting was not performed on crest captured individuals as *A. triostegus* larvae do not feed for at least 12 hours after colonizing the reef at night (MB pers. obs.). Statistics: same as in (E).

Thyroid hormone-mediated metamorphosis is conserved in coral reef fishes

To widen our understanding of coral reef fish metamorphosis, we investigated the TH signaling during larval recruitment in four other coral fish species (*Rhinecanthus aculeatus*, *Chromis viridis*, *Chaetodon lunula* and *Ostorhinchus angustatus*) from distant families (Near et al. 2012). In these species, both T4 and T3 levels drop between day 1 and day 8, up to 3-fold for T4 (in *O. angustatus*, p-value = 0.029) and up to 4-fold for T3 (in *R. aculeatus*, p-value = 0.05) (Figure II.4). This drop of TH levels after reef entry strongly suggests that TH are also key determinants for the metamorphosis of these species. T4 levels in *R. aculeatus* and T3 levels in *C. viridis* also rise between near ocean and day 1 individuals (Figure II.4), further confirming the model of a TH-mediated metamorphosis during coral reef fish larval recruitment. However, T4 levels are 3-fold higher in near ocean individuals than in day 1 individuals in *C. viridis* (Figure II.4). This suggests some potential species-specific variation in coral reef fish TH profiles at recruitment that would be extremely interesting to decipher in future studies on additional species and with regards to other aspects of coral reef fish ecology (e.g., diets, size and pigmentation status at recruitment).

Reef pollutant disrupts the transformation of *Acanthurus triostegus*

Chlorpyrifos (CPF) is an agricultural insecticide that is widely used on tropical coastal crops, therefore one of the most common waterborne chemical pollutants encountered in coral reef surrounding waters (Cavanagh et al. 1999; Leong et al. 2007; Roche et al. 2011; Botté et al. 2012). Given the endocrine determinants of coral reef fish metamorphosis (Figure II.2-3) and the CPF endocrine disruption characteristics (Juberg et al. 2013; Slotkin et al. 2013), we assessed the impact of CPF on *A. triostegus* metamorphosis. To achieve this, we used exposure doses previously reported in coral reef fish (1, 5 or 30 $\mu\text{g.l}^{-1}$ of seawater) (Botté et al. 2012). CPF treatment at 30 $\mu\text{g.l}^{-1}$ decreases the T3 levels by 50 % in day 2 juveniles (p-value = 0.021, Figure II.5A). No significant effect was observed on T4 levels regardless of CPF concentration and duration (p-value = 0.205 for day 2 and p-value = 0.496 for day 5, Figure II.5 – figure supplement 1A). We did not observe any effect of CPF treatments on fish pigmentation at day 2 and day 5 (Figure II.5 – figure supplement 1B). However, CPF exposure at 30 $\mu\text{g.l}^{-1}$ prevents guts' lengthening at day 2 (p-value = 0.024, Figure II.5B left panel), and both exposure at 5 and 30 $\mu\text{g.l}^{-1}$ prevent intestines' lengthening at day 5 (p-values = 0.047 and 0.029 respectively, Figure II.5B right panel). Strikingly, CPF exposure at 30 $\mu\text{g.l}^{-1}$ also decreases the grazing activity of day 2 juveniles with 2.63 ± 0.09 bites per fish per min contrasting with 3.62 ± 0.28 bites per fish per min in the solvent control condition (p-value = 0.014, Figure II.5C). In day 5 juveniles, exposure at 5 and 30 $\mu\text{g.l}^{-1}$ decreases the grazing activity with respectively 2.68 ± 0.18 and 2.63 ± 0.05 bites per fish per min compared to 4.43 ± 0.33 bites per fish per min for the solvent control fish (p-values = 0.001, Figure II.5C). Such a behavior in fish treated with

CPF results in up to a 14-fold reduction in the biomass of turf grazed after 5 days (p-value = 0.05, Figure II.5D).

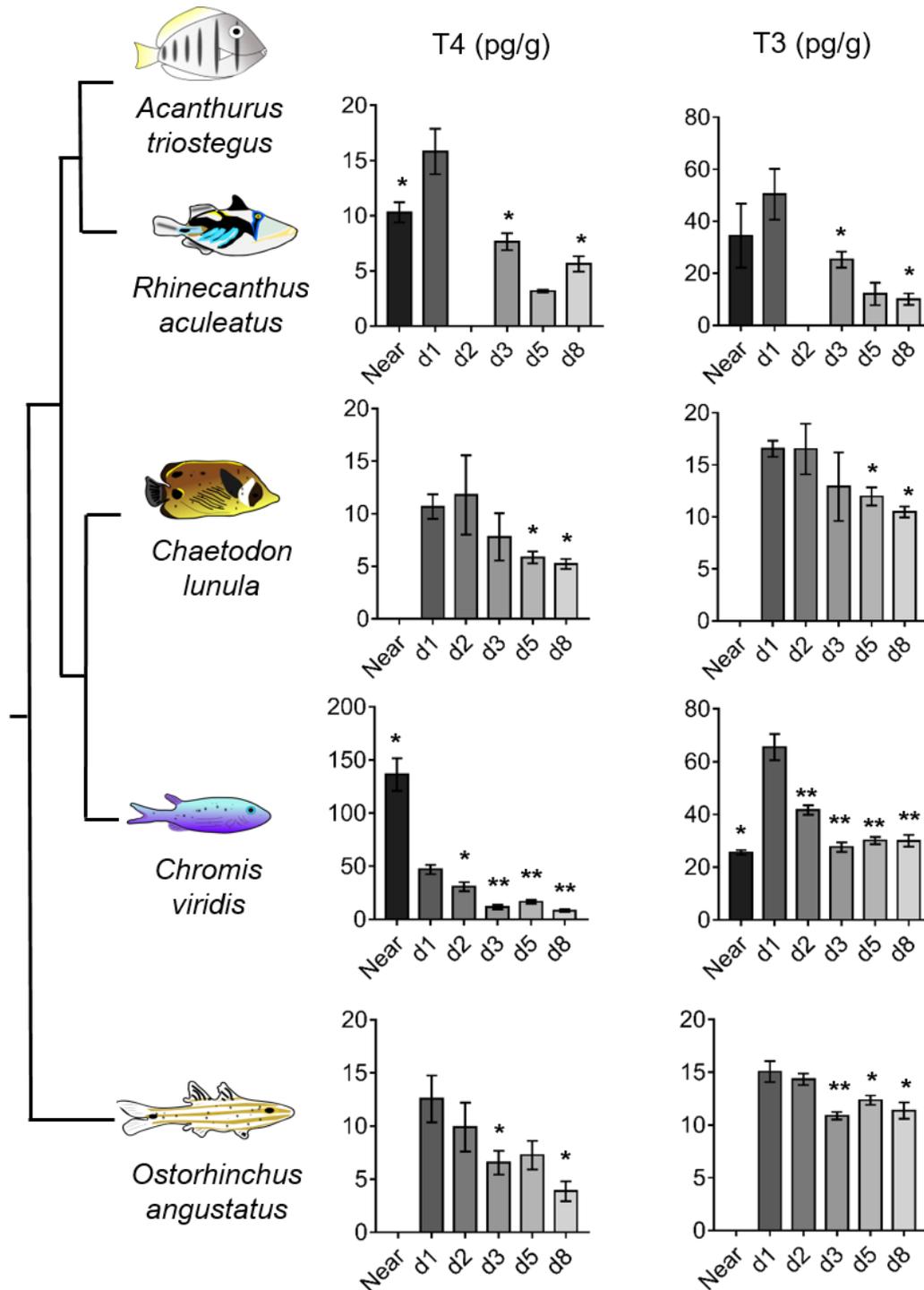


Figure II.4 | Thyroid hormone levels in distant coral reef fish at larval recruitment.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. TH levels of: *Rhinecanthus aculeatus* (upper), *Chromis viridis* (middle up), *Chaetodon lunula* (middle low) and *Ostorhinchus angustatus* (lower). n = 3 to 5 biological replicates for each condition. First column is T4, second column is T3. Y-axis: T4 or T3 level in pg.g⁻¹. X-axis: sampling point (developmental stage). The phylogenetic relationship between the species is plotted on the far left of the figure. Statistics: for each species, comparisons of each mean to the day 1 (d1) mean (Wilcoxon-Mann-Whitney U-test).

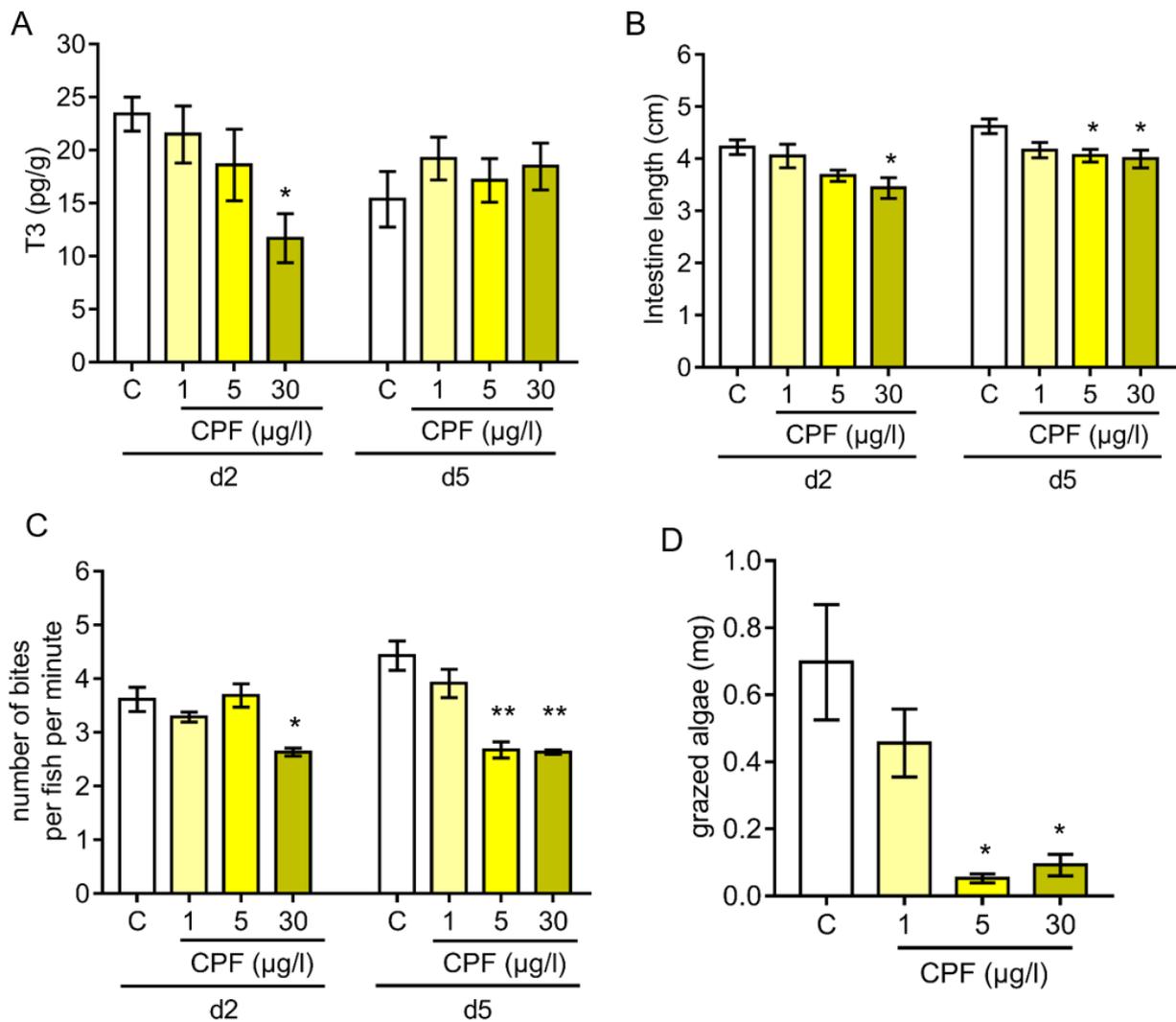


Figure II.5 | Influence of chlorpyrifos exposure on *Acanthurus triostegus* thyroid hormone signaling, intestine length, grazing activity, and algal removal efficiency.

Error bars represent standard errors. *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001. **(A)** T3 level in $\text{pg}\cdot\text{g}^{-1}$ of day 2 (d2) and day 5 (d5) fish exposed to different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 10$ fish in each condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the solvent-control condition are indicated. **(B)** Intestine length in cm of fish at day 2 (d2) or day 5 (d5) under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 5$ to 8 fish for each experimental condition. Statistics: same as in (A). **(C)** Mean number of bites (on turf algae) per fish in a batch of 18 fish on reef turf at day 2 (d2) or day 5 (d5), under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 3$ independent bite counts for each condition. Statistics: same as in (A). **(D)** Mean turf biomass removed per fish in a batch of 10 fish, in 5 days following reef colonization, under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 3$ independent measurements of grazed turf for each condition. Statistics: comparisons of each mean to the solvent-control (C) mean (Wilcoxon-Mann-Whitney U-test).

II.6. Discussion

In this study, we investigated the morphological, physiological and behavioral changes occurring during *A. triostegus* larval recruitment. These results highlight profound anatomical and physiological transformations of the skin and the whole digestive tract (Figure II.1B-W). In particular, the differences in 16S gut content are likely the consequence of a diet shift (carnivorous to herbivorous) and an environmental shift occurring during larval recruitment (Sullam et al. 2009; Frédérich et al. 2012; Baldo et al. 2015). We hypothesize that intestine remodeling (*i.e.* the loss of the villi and internal structures) accelerates the shift of the gut microbiota by allowing the establishment of a new bacterial community fitting the diet change. The presence of *Lachnospiraceae* in the adult but not in the larvae is consistent with the hypothesis that this family of bacteria is acquired through the environment in surgeonfishes (Miyake et al. 2016). Altogether, these observations reinforce the notion that the fish bacterial community depends on their diet and environment. These shifts of 16S and 18S gut contents, occurring between crest individuals and day 8 juveniles, strikingly coordinate with both the changes of the intestine and teeth morphologies observed during larval recruitment (Figure II.1M-V). All these changes from teeth to microbiota are consistent with *A. triostegus* change of diet after entering the reefs, conforming to previous observations (Randall 1961; McCormick 1999; Frédérich et al. 2012). This complete remodeling is associated with a severe weight loss (Figure II.1B), suggesting that larval recruitment corresponds to a real metabolic challenge.

The molecular investigation of TH signaling in the recruiting larvae indicates a surge of TH levels and *TR* expressions in near ocean larvae and crest individuals, and a drop in juveniles, after reef entry (Figure II.2A-C). This indicates that migration towards the reefs marks the onset of metamorphosis and starts very early on (Figure II.6A). Consequently, the transformation process is already well advanced when larvae reach the reef, suggesting that this event is triggered in the open ocean by, yet unknown, environmental cues (Figure II.6A). After reef entry, T3 treatment increases gut length while external slope relocation and NH₃ treatment delay the metamorphosis, although they could not completely interrupt it (Figure II.3A-B). These results are consistent with the model of metamorphosis in amphibians (Brown and Cai 2007) and other fish species (McMenamin and Parichy 2013). It is interesting to relate the morphological and physiological changes we have observed in *A. triostegus* with the TH-controlled post-embryonic process known in zebrafish (the major teleost fish developmental model, one of the few teleost fishes in which we have a functional knowledge of the metamorphosis process). Indeed, in both species, as well as in flatfish, another classical teleost model of metamorphosis, major pigmentation and craniofacial changes occur at metamorphosis (McMenamin and Parichy 2013; McMenamin et al. 2014). However, the appearance of dark melanophore stripes in zebrafish and *A. triostegus* is very likely to be controlled by different mechanisms. In *A. triostegus* this event is extremely rapid (as fast as 2 hours after reef entry, MB pers. obs.), strongly environmentally coupled, and probably not under direct TH control

(Figure II.3 – figure supplement 2). Contrarily, in zebrafish, this pigmentation process takes days and is part of a TH-controlled developmental program in which the triggering role of the environment is unknown (McMenamin et al. 2014). Altogether, our findings indicate that the onset and coordination of metamorphosis are two distinct events, both coordinated by TH and under environmental control. They further demonstrate that *A. triostegus* larval recruitment is a TH-controlled metamorphosis, as in amphibians and flatfishes (Brown and Cai 2007; Laudet 2011; McMenamin and Parichy 2013).

We demonstrated that the decrease of both T4 and T3 after reef entry can be generalized to phylogenetically distant coral fish species (Figure II.4). We propose that this decrease is valid for most if not all coral reef fishes, even if species-specific variations on this general theme can be observed, and that the larval recruitment of coral reef fish corresponds to a TH-controlled metamorphosis (Figure II.6A). Interestingly, variations in the magnitude of TH changes among the five coral reef fish species (Figure II.4) are reminiscent of the situation in stickleback, where differences in TH signaling between two ecotypes support life history trait variation (Kitano et al. 2010; Kitano and Lema 2013). We anticipate that this post-embryonic developmental step is shaped by evolution and varies according to species-specific life history traits such as pelagic larval duration, size at reef colonization and pigmentation status (*i.e.*, metamorphic state of advancement) at recruitment. Given the dramatic diversity of life history strategies they propose, coral reef fish could be excellent models to decipher how these changes are elicited by changes in hormonal systems.

Among the endocrine disruptors already evidenced and encountered in coral reefs, CPF is known to be a thyroid disruptor with adverse effects on fish behavior (Sandahl et al. 2005; Kavitha and Rao 2008; Roche et al. 2011; Yang et al. 2011; Botté et al. 2012; Deb and Das 2013; Juberg et al. 2013; Slotkin et al. 2013). We demonstrated that CPF exposure reduces *A. triostegus* physiological level of T3 at larval recruitment (Figure II.5A). Consistently with our observations on NH₃ treated fish, exposure to CPF impairs metamorphosis, as evidenced with the repression of guts' lengthening (Figure II.5B), and the decrease in the juveniles' grazing activity (Figure II.5C). This latter point led to a reduced algal removal efficiency (Figure II.5D). As *A. triostegus* is one of the major herbivorous fish in coral reefs, this can severely impair the ability of coral reefs to recover after an algal invasion (*e.g.*, after a bleaching event) and therefore contributes to the worldwide degradation of reef health (Russ 1984; Almany et al. 2007; Hughes et al. 2007, 2014; Ledlie et al. 2007) (Figure II.6B).

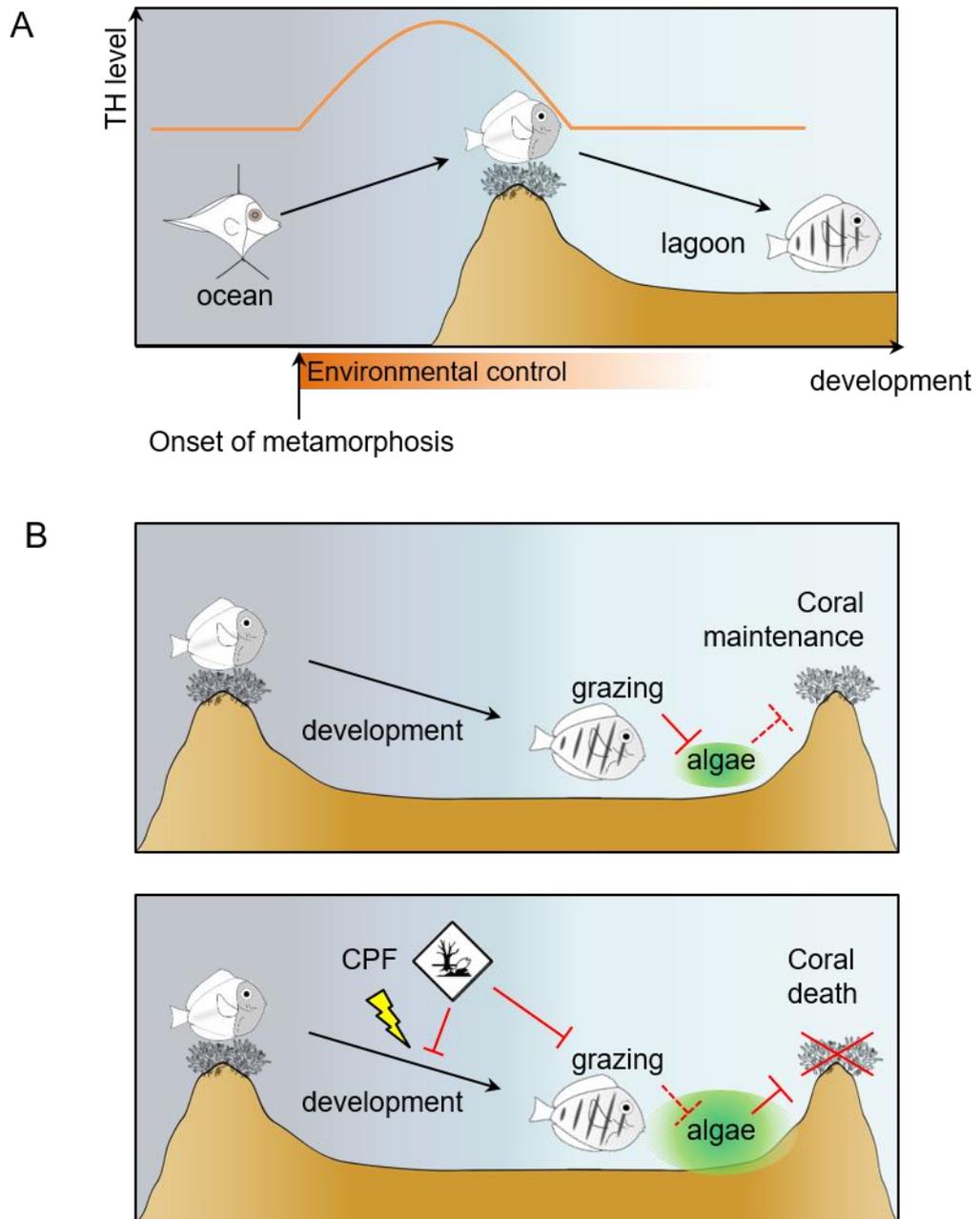


Figure II.6 | Model of *Acanthurus triostegus* metamorphosis and disruption by chlorpyrifos.

(A) Schematic representation of coral reef fish larval recruitment. TH level is plotted on the Y axis and development on the X axis. The onset of metamorphosis is in the open ocean and environmental influence upon development is plotted on the X-axis. (B) Consequences of undisturbed (upper panel) and CPF disturbed (lower panel) metamorphosis on grazing activity, and subsequent algal spreading and potential coral survival. Solid red bar indicates inhibition; dotted red line indicates lack of inhibition.

II.7. Conclusion

Our results highlight how coral reef fish larval recruitment, the transition between the open ocean and the reef, is a TH-regulated metamorphosis at the crossroads of ecological, developmental, physiological and behavioral transformations (Holzer and Laudet 2015). These findings also demonstrate how coral reef fish TH signaling and larval recruitment processes can be altered by reef pollution. Since metamorphosis and larval recruitment are essential for the maintenance of fish populations and subsequent coral reef resilience, this study provides a general framework to better understand, at the molecular level, how global changes, water pollution and human activities can threaten reef ecosystems.

II.8. Materials and Methods

Fish capture, husbandry and conservation

Acanthurus triostegus, *Ostorinchus angustatus*, *Chaetodon lunula*, *Chromis viridis* and *Rhinecanthus aculeatus* larvae were captured at Moorea Island, French Polynesia. Larvae were captured during the night while colonizing the reef crest, using a crest net and hand nets (Dufour and Galzin 1993; Besson et al. 2017b). Larvae were then kept in cages located *in situ* in the lagoon and provided with continuous food from the water column supplemented with coral rubble and algal turf. For the clarity of this study, the age in day of the juvenile corresponds to the number of day spend in the reef in cages since capture, not the absolute age. Near ocean *A. triostegus* larvae were sampled at 1-2 km away from the reef crest using drifting light trap and hand nets from 0.5 m to 5 m depth. Far ocean *A. triostegus* larvae were also captured offshore by trawl haul more than 10 km away from the reef between 1 and 60 m deep. Larvae were euthanized in MS222 at 0.4 mg.ml⁻¹ in filtered seawater. For TH dosage, larvae were frozen dry and conserved at -20°C prior to extraction. For RNA extraction, larvae were lacerated and kept in RNAlater (*Sigma*) 1 hour on ice and stored at -20°C. For histology, larvae were rinsed in PBS 1X, dissected and the intestines were kept in Bouin's fixative at room temperature. For μ CT scan and metagenomics, Larvae were kept in 70 % ethanol at room temperature.

Fish treatments

Acanthurus triostegus captured at the crest were injected with 20 μ l of drugs in the ventral cavity. Drugs tested were as follows: **(i)** solvent control (DMSO diluted 10.000 times in Phosphate Buffer Saline 1X, as all drugs were made soluble in DMSO and diluted 10.000 in PBS 1X); **(ii)** T3 + iopanoic acid (IOP) both at 10⁻⁶ M; **(iii)** NH₃ at 10⁻⁶ M; and **(iv)** T3 + IOP + NH₃ all at 10⁻⁶ M. IOP was used as an inhibitor of

deiodinase enzymes, as evidenced in mammals and amphibians (Galton and Hiebert 1987, 1988; Galton 1989; Simonides et al. 2008; Medina et al. 2011; Renko et al. 2012), and as routinely used in fish to prevent the immediate degradation of injected T3 (Little et al. 2013; Lorgen et al. 2015). NH₃ is a known TR antagonist in vertebrates (Lim et al. 2002), a notion we confirmed in *A. triostegus* (Figure II.2 – figure supplement 1D). NH₃ prevents the binding of TH on TR and impairs the binding of transcriptional coactivators to TR, which therefore remain in inactive conformation (Lim et al. 2002; Figueira et al. 2011). Fish were then kept in the lagoon in *in situ* cages and injected daily following the same protocol to maintain pharmacological treatment. Fish were sampled on the 2nd and 5th day after the beginning of this protocol. DMSO non toxicity was tested by comparing the pigmentation, the teeth development, the intestine length and internal structures between solvent-control individuals (treated with DMSO diluted 10.000 times in PBS 1X), and control individuals (simply raised *in situ* in lagoon cages as mentioned in the previous paragraph) (Figure II.3 – figure supplement 1, Figure II.3 – figure supplement 2).

Chlorpyrifos (CPF) exposure

After crest capture, *A. triostegus* larvae were transferred in aquaria (30 L x 20 W x 20 cm H) in groups of n = 10 fish. Each aquarium was filled with 9 liters of UV-sterilized and filtered - 10 µm filter, and was equipped with an air stone. Fish were then immediately exposed to: nothing (control), acetone at a final concentration of 1:1.000.000 (solvent control, as CPF was made soluble in acetone) or CPF at a final concentration of 1, 5, and 30 µg.l⁻¹, as previously done in the literature in another coral reef fish (Botté et al. 2012). All water was replaced every day, ensuring the maintenance of water quality as well as a continuous concentration of the pesticide or solvent. Fish were sampled the 2nd and 5th day after the beginning of this protocol. Acetone non toxicity was tested by comparing the TH levels, the pigmentation, and the intestine length between solvent-control and control individuals (Figure II.5A-B, Figure II.5 – figure supplement 1A-B).

Cloning of *Acanthurus triostegus* genes

Muscle from adult fish and whole larvae were used. Samples were cut using sterile scalpel blades and crushed in a Precellys with Qiagen extraction buffer. RNA extractions were performed using the Macherey-Nagel RNA extraction kit following the manufacturer's instructions. Total pooled RNAs were retro-transcribed with the Invitrogen Super Script III enzyme following the manufacturer's instructions. For *TR* cloning, actinopterygian universal primers (Figure II.2 - figure supplement 2) were designed for *TRα-A*, *TRα-B* and *TRβ* to retrieve the full-length sequences of the genes. For *polD2* and *rpl7*, actinopterygian universal primers were designed to retrieve partial sequences including at least one exon-exon junction. PCR amplicon were

cloned in the Invitrogen PCR II plasmid following the manufacturer instruction for sequencing. *TR α -A*, *TR α -B* and *TR β* were subcloned in pSG5 between EcoRI sites for *in cellulo* expression.

qPCR assay

Far ocean to day 8 juveniles were assayed. For each larvae 1 μ g of RNA were used for retro-transcription using the *Invitrogen Super Sript III* following the manufacturer's instructions, including a DNase I treatment. qPCR primers were designed to anneal on different exons, *Rpl7* and *Pold2* were used as normalization genes (Figure II.2 - figure supplement 2). qPCRs were performed in 96 well plate with the *BioRad IQ Syber Green Super Mix* in 10 μ l of final reaction per well following the manufacturer's ratios. qPCRs were assayed in a BioRad thermocyclers and analysed on *BioRad CFX Manager software*. Assays were performed on duplicates in at least two independent RNA extractions and retro-transcriptions. *Klf9*, which is known to respond to TH signaling, was used as a TH signaling reporter gene.

Phylogenetic analysis

The amino acid sequences of *A. triostegus* cloned TRs as well as available TR sequences (Figure II.2 - figure supplement 3) were aligned using *MUSCLE* software. Trees were generated using the Maximum Likelihood method with the *Seaview 4* software under the *JTT model* with estimated gamma shape and eight rate categories (RRID:SCR_015059) (Gouy et al. 2010). Bootstrap analysis of 1000 replicates was carried out to support the tree.

Functional characterization of the receptors

Human embryonic kidney 293 cells (ATCC:CRL_11268, RRID:CVCL_0045) (Iwema et al. 2007; Gutierrez-Mazariegos et al. 2014; Sadier et al. 2015) were grown in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum and penicillin/streptomycin at 100 μ g.ml⁻¹. Cells were maintained at 37°C, 5 % CO₂ and tests for mycoplasma contamination were negative. The transient transfection assays were carried out in 96-well plate with 30 000 cells per plate using *Exgen500* according to the manufacturer's instructions. For each well, cells were transfected with 50ng of total DNA: 12,5 ng of full-length receptor encoding plasmid, 12,5 ng of reporter plasmid with four DR4 repeat in the luciferase promoter, 12,5 ng of β -galactosidase encoding plasmid and 12,5 ng of pSG5 empty plasmid. Drugs were incubated for 48 hours and cells were harvested using a passive lysis buffer and frozen at -20°C. On half of the lysate, luciferase activities were assayed with the luciferase reagent buffer from *Promega* on a *Veritas Turner Biosystem luminometer*.

On the other half of the lysate, the β -galactosidase activity was measured using ONPG substrate and absorbance at 420 nm for normalization. Each assay was performed at least 3 times independently on well triplicates. Drugs from *Sigma-Aldrich* were diluted in DMSO à 10^{-2} M then in sterile PBS1X prior treatment.

Thyroid hormone quantification

TH were extracted from dry-frozen fish following an extraction protocol adapted from previous publications relating TH level variations in teleost fish (Tagawa and Hirano 1989; Einarsdottir et al. 2006; Kawakami et al. 2008a). Far ocean larvae to day 8 juveniles were assayed. At least three individuals per sampling point were used. Each fish was first crushed with a *Precellys* in 500 μ l methanol, centrifuged at 4°C and supernatant reserved, three times. Pooled supernatant were dried at 70°C. Hormones were re-extracted with 400 μ l methanol, 100 μ l chloroform and 100 μ l barbital buffer twice from the first dried extract. Pooled supernatant was dried out and extract was reconstituted in 2 ml of PBS1X for quantification. The quantification was performed following the *Roche ELICA* kit on a Cobas analyser by a medical laboratory according to the manufacturer's standardized method.

Intestine histology

The intestines in Bouin's fixative were embedded in paraffin. Sections of 5 μ m were performed in the proximal, medial and distal part using a microtome (*Leica*). The histological sections were stained with Hematoxylin and Eosin. Photography were taken on a Leica microscope and the mosaic reconstructed using Image J software.

Gut Metagenomics

For 16S mass sequencing, juveniles of 2, 5, 8 days after reef entry and adult *A. triostegus* gut were dissected in triplicates for a total of twelve samples. Total DNA was extracted with a *Macherey-Nagel* DNA extraction kit following the manufacturer's recommendations. 16S library were constructed for each individual using the *Ion 16S Metagenomic* kit from *Life technologies* following the manufacturer's recommendations. Sequencing of bacterial 16S was performed using a PGM Ion Torrent. Three controls for contaminations were performed. The environment control consisted of an open tube during the dissection. The extraction control monitored the DNA extraction process. The blank control monitored the library construction. The sequencing results were then analyzed using the *Life technologies* 16S pipeline. 18S mass sequencing was performed on crest larvae, 8 days juveniles and adults in triplicates. Intestine contents were extracted in the same condition as 16S. 18S library were

constructed with the pre-amplified V7 region of the eukaryotic 18S (Figure II.2 - figure supplement 2). A blocking primer was designed to prevent the amplification of host sequences (Figure II.2 - figure supplement 2). The blocking primer was designed to partially overlap the 18S reverse primer and was modified with a C3 spacer. Sequencing was performed using a *PGM Ion Torrent*. The sequences were assembled and blasted against the PR2 database, completed with 18S sequenced from multi-cellular organisms for taxonomic affiliation.

μCT scan analysis

Fish samples were conserved in 70 % ethanol, dehydrated in successive baths of 95 % ethanol, twice 100 % ethanol and in vacuum arena for at least 4 hours. X-ray microtomographies were performed on a *Phoenix Nanotom (General Electric)* at 70 kV of tension, 100 mA of intensity with a tungsten filament. 3000 images per sample were taken at 500 ms of exposure per image and at a resolution ranging from 2.5 to 2.8 μm. 3D volumes were reconstructed and analysed with VGI studiomax software.

Grazing activity

For each condition (e.g. hormonal treatments, pesticide conditionings), 18 fish were placed in a 5 liter tank with coral rubbles covered with turf algae. After an acclimation period of 1 hour, we recorded the total number of bites made on turf algae during 10 min. This experiment was replicated with a new batch of fish 3 to 6 times depending on the condition.

Grazed turf biomass

For each pesticide exposition conditioning, we weighed (underwater) 3 pieces of coral rubble prior the introduction of a group of 20 crest captured fish (weight A), and after 5 days of grazing by this group of fish (weight B). For each piece of coral rubble, the weight of grazed algae was estimated through the difference weight B minus weight A.

Statistical information

All statistical analyses were conducted using the *R-Cran* project free software (<http://www.rproject.org/>, *R-3.3.1*). Mean comparisons were performed using Wilcoxon-Mann-Whitney U-test when comparing two means, and using univariate analysis of variance (ANOVA) followed by Tukey post-hoc test (should a significant

difference be detected) for multiple comparisons. Prior ANOVA, normality of values (or residuals) and variance homogeneity were assessed using Shapiro and Bartlett tests. In qPCR analyses, comparisons of gene expression were performed through Student's t cumulative distribution functions, automatically computed by *qPCR software CFX Biorad Manager*, (CFX Manager 2017).

II.9. Additional information

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Author contributions

G.H., M.B., D.L. and V.L. designed the experiments and wrote the article. M.B., L.F. and D.L. captured the fish and performed the *in vivo* and *in situ* experiments. G.H., M.B., L.F. and P.B. performed the hormone dosage and the molecular biology experiments. G.H., B. G., S. H., G. P and F.L. designed, performed and analyzed the metagenomics experiments. M.B. and A.L. performed the histological analyses. G.H. and L.V. performed the X-Ray microtomography.

Competing financial interests

The authors declare no competing financial interests.

II.10. Figure supplements

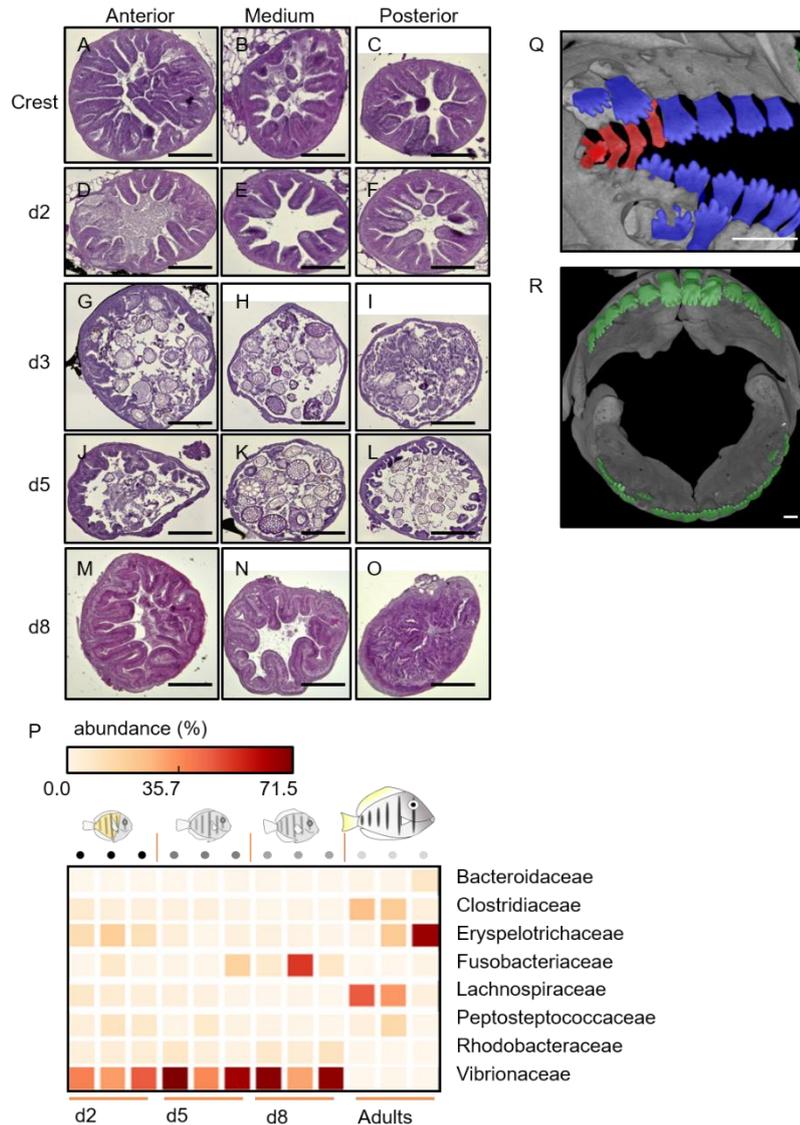


Figure II.1 - figure supplement 1 | Changes in *Acanthurus triostegus* intestine and teeth during larval recruitment.

(A-O) Complete guts histology of *Acanthurus triostegus* after reef colonization. First column: anterior part of the intestine (first cm), middle column: medium part (variable), last column: posterior part of the intestine (1 cm), (A-C): crest individual, (D-F): 2 days juvenile, (G-I): 3 days juvenile, (J-L): 5 days juvenile, (M-O): 8 days juvenile. Scale bars represent 200 μm . These pictures are representative of $n = 6$ section technical replicates for each of the proximal, middle and distal intestine, and were performed twice (sampling technical replicates), on $n = 2$ fish (biological replicates). (P) Heat map of bacterial gut community. Each column is one individual. Each row is bacterial taxa. Only the taxa with differential representations between the samples are shown. Sampling stages: day 2, day 5, day 8 and adult are indicated below the heat map. The shape of each of this developmental stage is schematized over the heat map. Each column is highlighted by a circle, the difference in shade between the circles emphasizes the stage difference. The color of each square in the heat map is proportional to the abundance of the taxa (row) in the sample (column). (Q) μCT scan of a crest larva, lateral view. Dental family C are highlighted in red and dental family B in blue. (R) μCT scan of an adult, frontal view (mouth open). (Q-R) The scale bar indicates 0.5 mm. These pictures are representative of $n = 2$ fish (biological replicates)

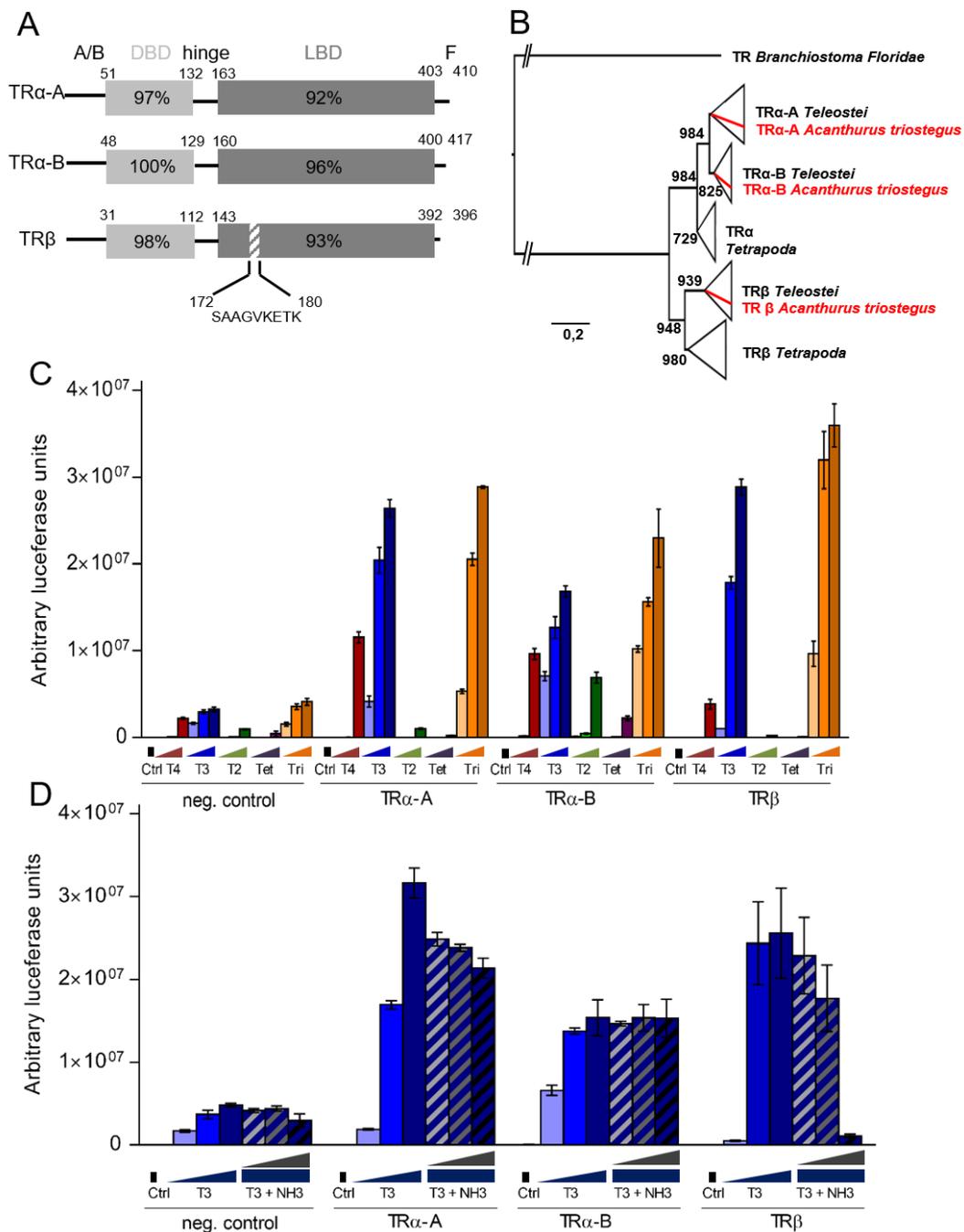


Figure II.2 – figure supplement 1 | Characterization of *Acanthurus triostegus* thyroid hormone receptors.

(A) Schematic representations of TR α -A, TR α -B and TR β from upper to lower rows respectively. The amino acid numbering of the DBD (light gray) and LBD (dark grey) appear above each TR. The 9 amino acid insertion of the TR β long isoform is indicated below the gray dashed area. (B) Phylogenetic reconstruction of TR amino acid sequenced by maximum likelihood. Bootstrap over supporting each branches are indicated when > 700/1000. *A. triostegus* genes are highlighted in red. (C) Transactivation assay of TRs in HEK293T cells with a luciferase reporter. X-axis: Tested genes and treatment condition. T4: blue bars, T3: red bars, T2: green bars, Tetrac: purple bars and Triac: orange bars. The concentrations range from 10^{-9} M to 10^{-7} M which corresponds to the light and dark shade of each color. Tested genes are indicated on the bottom line. Y-axis: arbitrary luciferase unit. 3 technical replicates were performed. (D) Transactivation assay of TRs in HEK293T cells with a luciferase reporter. X-axis: tested genes and treatment conditions. Light blue to dark blue bars: T3 treatments at 10^{-9} , 10^{-8} , and 10^{-7} M respectively. Light blue to dark blue dashed bars: T3+NH3 treatments with T3 at 10^{-8} M and NH3 at 10^{-8} , 10^{-7} and 10^{-6} M respectively. Y-axis: arbitrary luciferase unit. 3 technical replicates were performed.

Figure II.2 – figure supplement 2 | Primers used in this study.

(A) Degenerated primers used for the cloning of the *A. triostegus* genes in this study. (B) qPCR primers used in this study. (C) Primers used for 18S mass sequencing. The overlapping nucleotides between 18SR and the blocking primer are indicated in bold. The mean annealing temperature of each primer is indicated in the last column.

	Target gene	Sens	Sequence (5' to 3')	Annealing temperature
(A) Cloning primers	<i>TRα-A</i>	F	CCTCAKTSTCCBGTGATTGGC	51°C
	<i>TRα-A</i>	R	TCABACYTCCTGGTCCTCRAASACC	56°C
	<i>TRα-B</i>	F	ATGGMACAMATGCCCRARGAGSAGG	56°C
	<i>TRα-B</i>	R	TCABACYTCCTGGTCCTCRAASACC	56°C
	<i>TRβ</i>	F	ATGTCAGAGCMAGSAGAVAAATGC	51°C
	<i>TRβ</i>	R	TCAGTCYTCRAASACYTCYAGGAAG	53°C
	<i>rpl7</i>	F	CAGATCTTCAAYGGWGTNTTYGT	51°C
	<i>rpl7</i>	R	GAADGGCCACAGGAAGTTGTTG	58°C
	<i>pold2</i>	F	AGCTTCARYCGBCAGTATGC	51°C
	<i>pold2</i>	R	CTGCTGTACYTCTVDATGTC	46°C
	<i>klf9</i>	F	GVRCYHTRHRATGGYBGCVATGAT	60°C
	<i>klf9</i>	R	TKVAKCATRCTKGGVTGGAAGYC	59°C
(B) qPCR primers	<i>TRα-A</i>	F	CAAAAGCCTCGCTCAGTTTAACTG	60°C
	<i>TRα-A</i>	R	CTTGGCACTGCTCAATCTTCTCC	59°C
	<i>TRα-B</i>	F	GAAGAGGAAGAACAGCCAATGTTCG	60°C
	<i>TRα-B</i>	R	GTCACCACACACCACACATGG	60°C
	<i>TRβ</i>	F	GTCGCTGTCCTCCTTTAACCTGG	61°C
	<i>TRβ</i>	R	CCTCTTGGCAACGTTCCGATTCG	60°C
	<i>rpl7</i>	F	CAGGATTGCTGAGCCTTACATCG	59°C
	<i>rpl7</i>	R	AATACGCTGTTTCCTCATCCTGC	60°C
	<i>pold2</i>	F	CACCAAGAAGACTCAGGCTGG	59°C
	<i>pold2</i>	R	GTGTAGTTGGTGGGGTCATACTGG	60°C
	<i>klf9</i>	F	GCAAGTCGTCACCTCAAGG	59°C
	<i>klf9</i>	R	GGAGAACTTCTTGCCACAGTCCG	61°C
(C) Mass-seq	18S	F	GGCTTAATTTGACTCAACACG	53°C
	18S	R	GGGCATCACAGACCTG	52°C
	blocking	R	ACCTGTTATTGCTCAATCTCGTGTGGCTG-C3-spacer	64°C

Figure II.2 – figure supplement 3 | List of thyroid hormone receptors sequences used for the phylogeny.

Species, genes, databases and accession numbers are indicated.

Species	Protein	Accession number	Database	Species	Protein	Accession number	Database
<i>Acanthopagrus schlegelii</i>	TR α	ABQ96861.1	NCBI	<i>Monodelphis domestica</i>	TR α	NP_001184135.1	NCBI
<i>Acanthopagrus schlegelii</i>	TR β	ABQ96862.1	NCBI	<i>Monodelphis domestica</i>	TR β	XP_007505233.1	NCBI
<i>Ambystoma mexicanum</i>	TR α	AY174871.1	NCBI	<i>Mus musculus</i>	RAR α	NP_033050.2	NCBI
<i>Ambystoma mexicanum</i>	TR β	AY174872.1	NCBI	<i>Mus musculus</i>	TR α	NM_178060.3	NCBI
<i>Anolis carolinensis</i>	TR α	XP_008111558.1	NCBI	<i>Mus musculus</i>	TR β	NM_001113417.1	NCBI
<i>Anolis carolinensis</i>	TR β	XP_0081117165.1	NCBI	<i>Necturus maculosus</i>	TR α	Y16623.2	NCBI
<i>Branchiostoma floridae</i>	TR	EF672344.1	NCBI	<i>Necturus maculosus</i>	TR β	AY168331.1	NCBI
<i>Canis lupus familiaris</i>	TR α	NP_001273791.1	NCBI	<i>Oreochromis niloticus</i>	TR α -A	ENSONIT00000008144	Ensembl
<i>Canis lupus familiaris</i>	TR β	XP_862690.2	NCBI	<i>Oreochromis niloticus</i>	TR α -B	ENSONIT00000022999	Ensembl
<i>Conger myriaster</i>	TR α -A	AB183396.1	NCBI	<i>Oreochromis niloticus</i>	TR β	ENSONIT00000012974	Ensembl
<i>Conger myriaster</i>	TR α -B	AB183397.1	NCBI	<i>Oryzias latipes</i>	TR α	AB114860	NCBI
<i>Conger myriaster</i>	TR β -A	AB183394.1	NCBI	<i>Oryzias latipes</i>	TR β	AB114861	NCBI
<i>Conger myriaster</i>	TR β -B	AB183395.1	NCBI	<i>Paralichthys olivaceus</i>	TR α -A	D16461.1	NCBI
<i>Danio rerio</i>	TR α -A	NM_131396.1	NCBI	<i>Paralichthys olivaceus</i>	TR α -B	D16462.1	NCBI
<i>Danio rerio</i>	TR α -B	XM_001920978	NCBI	<i>Paralichthys olivaceus</i>	TR β	D45245.1	NCBI
<i>Danio rerio</i>	TR β	NM_131340.1	NCBI	<i>Rattus norvegicus</i>	TR α	NP_001017960.1	NCBI
<i>Epinephelus coioides</i>	TR α -A	ABP62960.1	NCBI	<i>Rattus norvegicus</i>	TR β	NM_012672	NCBI
<i>Epinephelus coioides</i>	TR α -B	ABP62961.1	NCBI	<i>Salmo salar</i>	TR α	NM_001123628	NCBI
<i>Epinephelus coioides</i>	TR β	ABP62962.1	NCBI	<i>Salmo salar</i>	TR β	NM_001123700	NCBI
<i>Eublepharis macularius</i>	TR α	AB204861	NCBI	<i>Sus scrofa</i>	TR α	NM_214190	NCBI
<i>Eublepharis macularius</i>	TR β	AB204862	NCBI	<i>Sus scrofa</i>	TR β	F1RS47_PIG	Ensembl
<i>Gallus gallus</i>	TR α	NM_205313.1	NCBI	<i>Xenopus laevis</i>	TR α	NP_001081595	NCBI
<i>Gallus gallus</i>	TR β	NM_205447.1	NCBI	<i>Xenopus laevis</i>	TR β	NP_001090182	NCBI
<i>Homo sapiens</i>	TR α	NM_199334.3	NCBI	<i>Xenopus tropicalis</i>	TR α	NM_001045796.1	NCBI
<i>Homo sapiens</i>	TR β	NM_000461.4	NCBI	<i>Xenopus tropicalis</i>	TR β	NM_001045805.1	NCBI

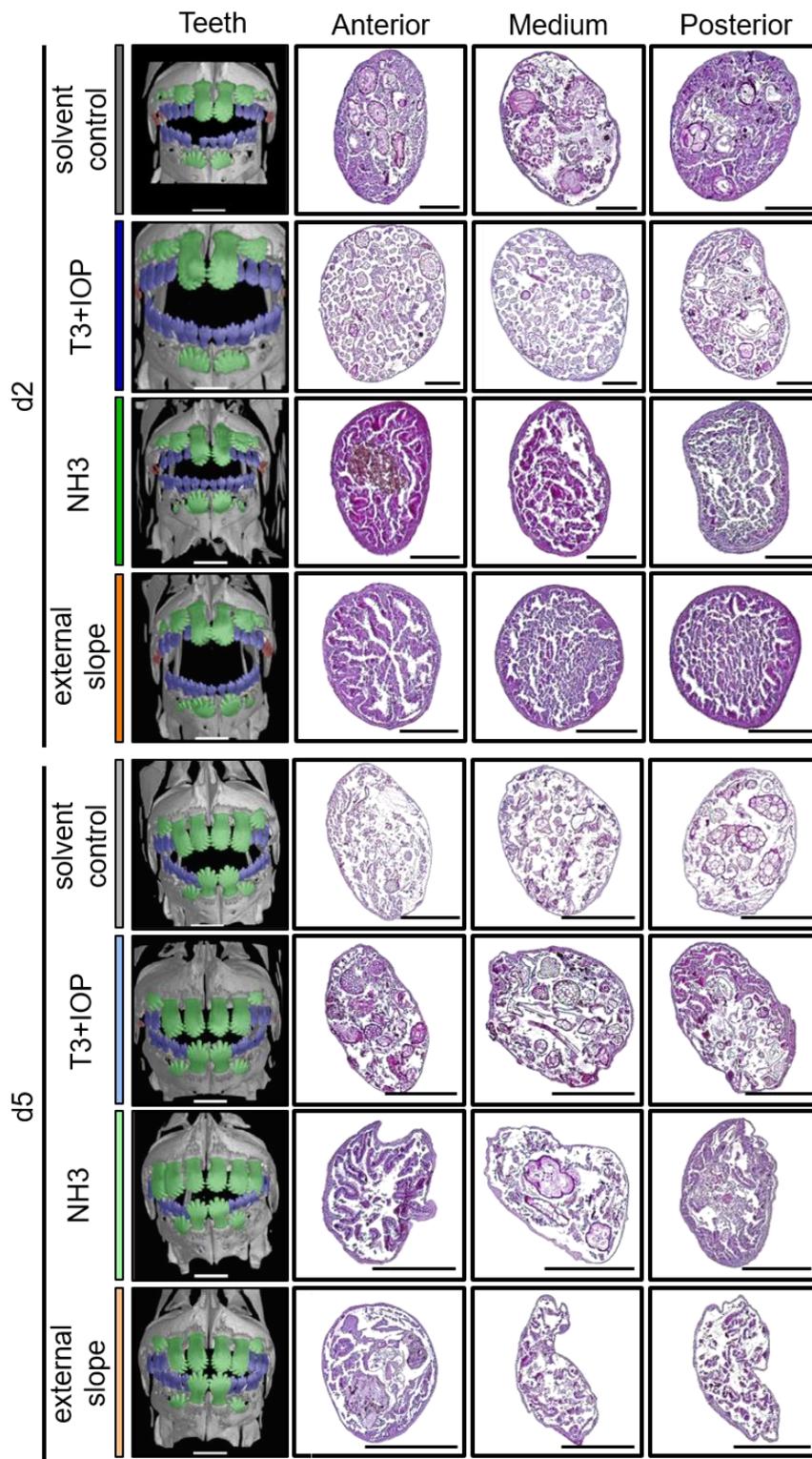


Figure II.3 – figure supplement 1 | Thyroid hormone signaling and environment impact on the dentition and guts histology of *Acanthurus triostegus* at metamorphosis

First column: X-Ray microtomography of larva, dentition A, B or C are highlighted respectively in red, blue or green. Scale bars indicate 0.5mm. These pictures are representative of n = 3 fish (biological replicates) per developmental stage and per treatment condition. **Second to fourth columns:** complete guts histology (5 μ m wide microtome sections) of *A. triostegus* at day 2 and day 5 following reef colonization. First column: anterior part of the intestine (first cm), middle column: medium part (variable), last column: posterior part of the intestine (last cm). Haematoxylin and eosin staining. Scale bars indicate 200 μ m. These pictures are representative of n = 2 treatment technical replicates, and n = 2 microtome section and staining technical replicates, on n = 3 fish (biological replicates) per treatment and per day

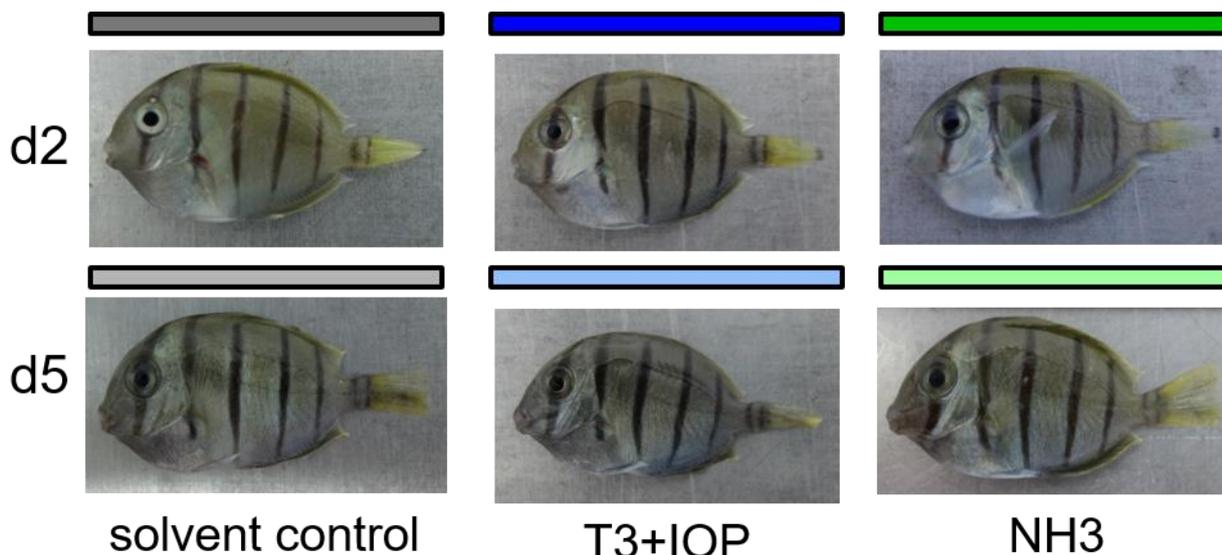


Figure II.3 – figure supplement 2 | Pigmentation patterns and thyroid hormone signaling in *Acanthurus triostegus* at day 2 (d2) and day (d5) during larval recruitment

These pictures are representative of $n > 10$ treatment technical replicates performed on $n > 100$ fish (biological replicates) per treatment and per developmental stage.

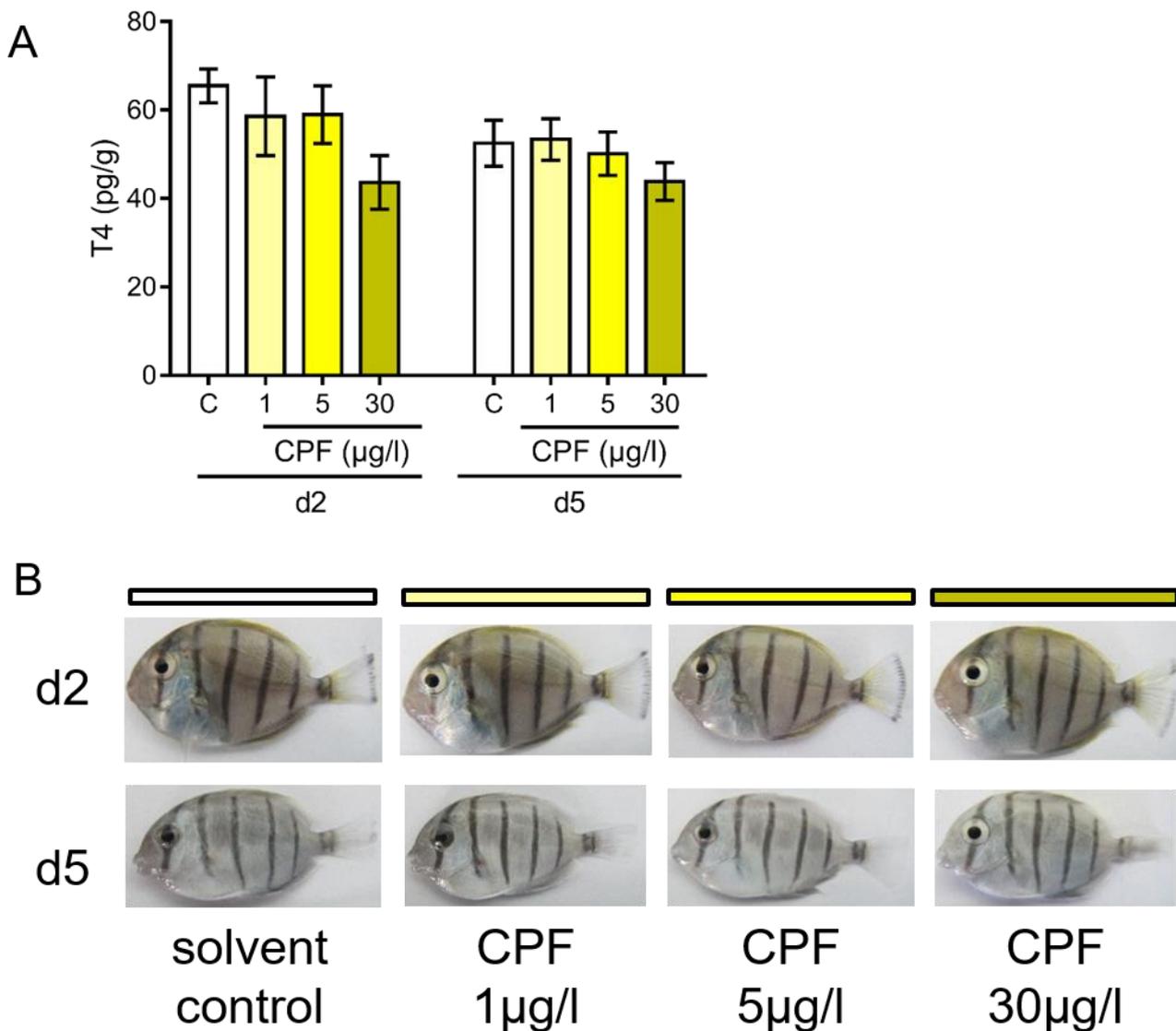


Figure II.5 – figure supplement 2: Pigmentation patterns and chlorpyrifos exposure in *A. triostegus* at metamorphosis

(A) T4 level in $\text{pg}\cdot\text{g}^{-1}$ of day 2 (d2) and day 5 (d5) fish exposed to different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\ \mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\ \mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 10$ fish in each condition.. See Figure 5 – source data 5 for detailed data and statistics. (B) Pigmentation patterns and in *A. triostegus* exposed to different conditions: solvent control, or CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$, $5\ \mu\text{g}\cdot\text{l}^{-1}$, or $30\ \mu\text{g}\cdot\text{l}^{-1}$ at day 2 (d2) and day (d5) during larval recruitment. These pictures are representative of $n = 2$ chlorpyrifos CPF exposure technical replicates performed on $n = 10$ fish (biological replicates) per condition and per day.

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III. Endocrine disruption impairs sensory organ maturation and survival in a coral reef fish

III.1. Foreword

This chapter aims at answering to the two last questions of my PhD, which are:

3 | Are there specific sensory organs and sensory abilities that develop at metamorphosis and what are their endocrine determinants?

4 | Are there environmental factors that act as endocrine disruptors of TH signaling during coral reef metamorphosis and how would it subsequently affect larval recruitment success?

To achieve this, the intense fishing and fish husbandry efforts presented in this chapter have been performed in close collaboration with two interns that I supervised with Dr. D. Lecchini: I. Moniz and L. François. I then performed all the histological work, other analyses, and writing, during my last year. At least two years of my PhD were devoted to this chapter (one year in common with the previous chapter). Given the variety of processes addressed by this study, many other collaborators have been involved in the study elaboration as well as in the writing (see next sub-chapter).

This chapter corresponds to a comprehensive and holistic analysis that tackle the two questions mentioned above, at the molecular, anatomical, behavioral and ecological levels. Given the novelty of the results conveyed by this work, we have decided (with the future manuscript co-authors) that a few additional experiments should be performed in order to maximize the chance to publish this research in a high impact-factor journal. More precisely, two small but important experiments have yet to be performed (see next sub-chapters). These experiments are currently being done, and hopefully part of the results should be available by September, and a first submission of this research should be done by the end of the year. To date, I wrote a first version of this manuscript, for a future submission to *Nature* (Letters). This explains the particular format of this chapter, with a short referenced summary of the study, followed by a short description and discussion of the results.

III.2. Manuscript information

Title: Endocrine disruption impairs sensory system and survival in a coral-reef fish

Authors: Besson M.^{1,2}, Brooker R.M.³, O'Connor J.⁴, Moniz I.¹, François L.¹, Salis P.², Jacob H.^{1,5}, Gilson A.^{1,2}, Holzer G.⁶, Antoine M.¹, Iwankow G.¹, Latry L.⁷, Flamant F.⁶, Besseau L.², Laudet V.² and Lecchini D.^{1,8}

Affiliations:

1 PSL Research-University: EPHE-UPVD-CNRS, USR3278-CRIOBE, 98729 Moorea, French Polynesia

2 UPMC, CNRS-UMR7232-BIOM OOB, 1 Avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France

3 School Marine Science and Policy, University of Delaware, Lewes, DE, United States of America

4 School of BioSciences, University of Melbourne, Australia

5 International Atomic Energy Agency, Environment Laboratories, Principality of Monaco, Monaco

6 Institut de Génomique Fonctionnelle de Lyon, UMR CNRS 5242 ENS Lyon, 69007 Lyon, France.

7 CRESCO, UMR BOREA 7208, Muséum National d'Histoire Naturelle, Dinard, France

8 Laboratoire d'Excellence ^aCORAIL^o, Moorea, French Polynesia

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III.3. Referenced summary

The persistence and sustainability of coral-reef fish populations depends on the continued recruitment, *i.e.* arrival (settlement) and post-settlement survival of pelagic larvae into adult reef habitats (Jones et al. 2004; Almany et al. 2007). Due to the importance of recruitment, there is strong selective pressure for traits that increase an individual's likelihood of locating settlement habitats that maximize subsequent growth and fitness while minimizing mortality risk (Doherty 1987; McCormick and Hoey 2004). There is growing evidence that changes to water conditions due to global change and waterborne pollution can impair the sensory abilities of recruitment-stage fish, impacting habitat selection and decreasing survival rates (Besson et al. 2017a; Johansen et al. 2017). Recent research has identified that recruitment timing corresponds to a metamorphosis mediated by thyroid-hormones (TH) (Holzer et al. 2017). However, the role of TH signaling in sensory system maturation and the impact of environmental perturbations on this process remains largely unknown (Holzer et al. 2017). Here we show that the eye (vision), lateral-line (mechanosensory perception), and nostril (olfaction), all undergo rapid TH-controlled changes during recruitment in the convict surgeonfish *Acanthurus triostegus*. However, a +3°C temperature shift as well as exposure to chlorpyrifos (a common pesticide detected in coastal reef waters (Shaw et al. 2010)), decrease TH levels at a critical developmental stage, limiting sensory system maturation, impairing visual and olfactory abilities, and decreasing survival. Even small declines in survival during this sensitive recruitment stage could impair population replenishment (Jones et al. 2004; Almany et al. 2007). A reduced abundance of keystone algal herbivores, such as *A. triostegus*, may impair their ability to maintain or rescue coral-dominated habitats, further threatening the ecological and economic resilience of coral-reef ecosystems (Ledlie et al. 2007; Munday et al. 2008). In other vertebrate species, THs also integrate ecological, physiological, morphological and behavioral changes during critical life history stages (Brown and Cai 2007; Lorgen et al. 2015). As TH appear highly sensitive to ambient ecological conditions, identifying how they are affected by current and predicted disturbance is essential.

III.4. Results and discussion

Settlement, the transition from a pelagic larvae to a benthic-dwelling juvenile, is a particularly perilous step in the life history of most coral-reef fishes, with ~90 % mortality during this stage (Doherty et al. 2004). Any additional factors that increase this mortality rate could have dramatic consequences for population replenishment (Jones et al. 2004; Almany et al. 2007). One of the key factors influencing survival at recruitment is settlement-site selection, with larval fishes using acute sensory systems to locate appropriate benthic habitats (Lecchini et al. 2007a; Mitchell et al. 2011; Brooker and Dixon 2016). As coral-reefs are rapidly degrading globally due to anthropogenic activities (Hughes et al. 2003; Hoegh-Guldberg et al. 2007), a large number of studies have examined how larval sensory abilities (*e.g.*, recognition of olfactory cues from conspecifics or predators) are impaired by factors such as global change and water pollution (Dixon et al. 2014; Johansen et al. 2017; Lecchini et al. 2017). However, to date no study has integrated molecular, anatomical, and behavioral approaches to identify the processes that impair larval sensory ecology and subsequent survival. Recent evidence that metamorphosis and recruitment in coral-reef fishes are mediated by thyroid hormones (TH) prone to endocrine disruption (Holzer et al. 2017) provides a novel framework for examining how environmental perturbations could threaten this life history transition (Holzer et al. 2017). Thyroxine (T₄), and more particularly its derivative triiodothyronine (T₃) are the two vertebrates TH, acting as key regulators of brain, olfactory, and visual system development in a number of teleost fishes and amphibians during metamorphosis (Forrest et al. 2002; Brown and Cai 2007; Hino et al. 2007; Lorgen et al. 2015). Given the similarities between the metamorphoses of coral-reef fishes and these other species (Holzer et al. 2017), TH appear to be likely candidates as controllers of sensory organ maturation in coral-reef fishes. If this is the case, endocrine disruption during recruitment could have unforeseen impacts on larval coral-reef fish sensory ecology and coral-reefs as a whole.

We used the convict surgeonfish, *Acanthurus triostegus*, as a model to determine the role of TH in sensory organ maturation in larval coral-reef fishes at recruitment. Larvae were collected just prior to settlement (*i.e.* 0 days post-settlement = d0) from reefs surrounding Moorea Island, French Polynesia. Larvae were then raised in cages placed *in situ* in a nursery area in the Moorea lagoon, until their second (d2), fifth (d5), eighth (d8), and thirtieth (d30) day post-settlement (Methods). Recruits completed metamorphosis within eight days post-settlement, as indicated by rapid pigmentation changes (Fig. III.1a), and decrease in body depth (III. Extended Data Fig 1). Fulton's condition factor (Fulton 1904) rapidly declined from d0 to d8 (III. Extended Data Fig 2), indicating that metamorphosis constitutes a considerable metabolic challenge for fish. Histological analyses of the visual (retina, Fig. III.1b-c), olfactory (nostril, Fig. III.1d-e) and mechanosensory (lateral line (LL), Fig. III.1f-g) organs revealed rapid maturation post-settlement (d0 to d8) (Methods). Within the retina, the density of ganglionar cells (ggc) remained stable post-settlement (Fig. III.1h), but increased for bipolar cells (bpc), photoreceptor nuclei (prn), and photocone external segments (pes)

(Fig. III.1i-k). The number of nostril lamellae and LL canals also increased, with a twofold surge in LL canals density between d0 and d2 (Fig. III.1l-m, III. Extended Data Fig 3).

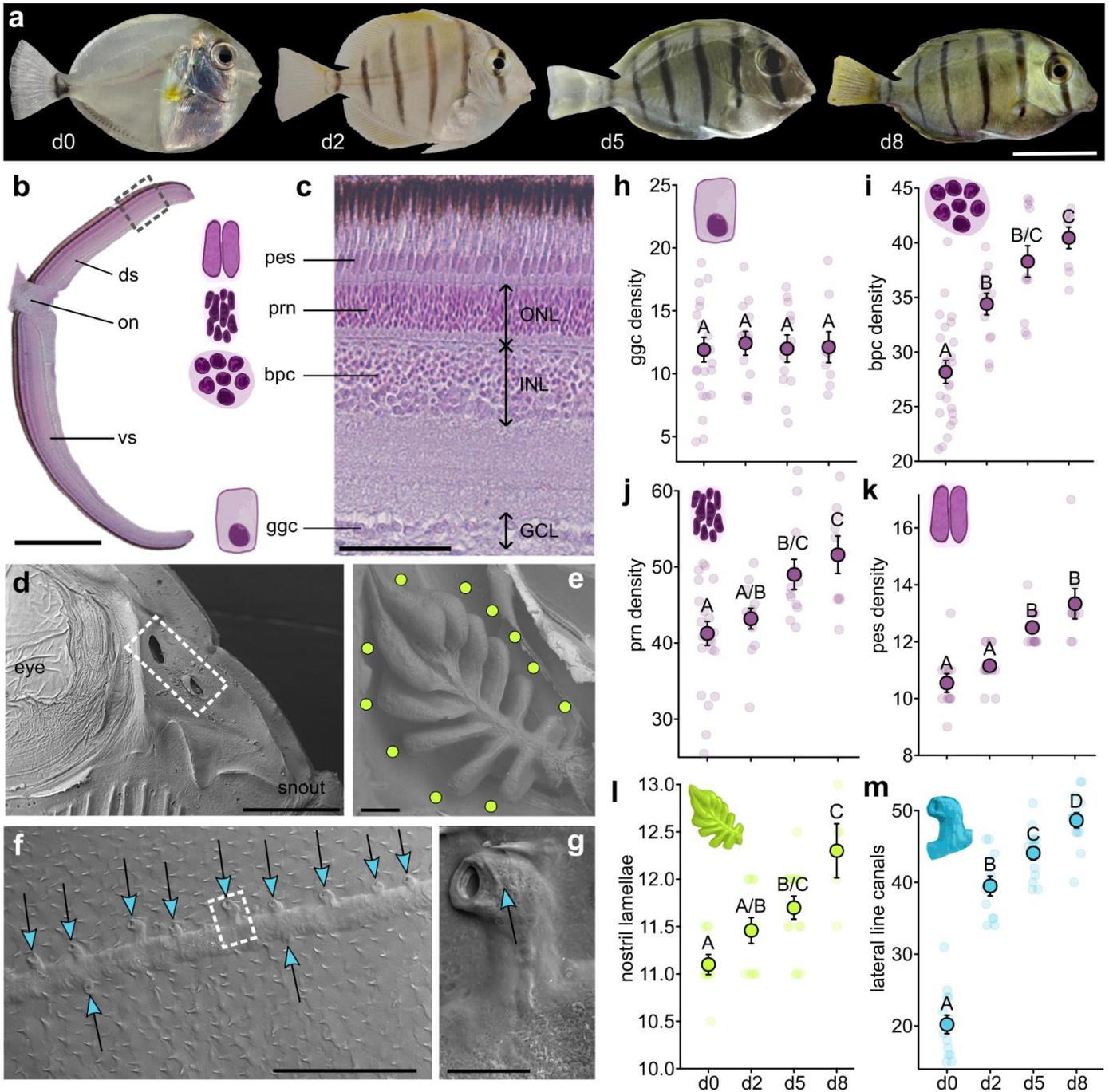


Figure III.1 | Metamorphosis and sensory system changes at recruitment in *Acanthurus triostegus*

a, Morphological and pigmentation changes in *A. triostegus* from day 0 (d0) to day 8 (d8) following their entry in the reefs. Scale bar indicates 1 cm. **b**, Cross section in the eye retina stained in hematoxylin/eosin, presenting the dorsal side (ds) the ventral side (vs) and the optical nerve (on). The dotted gray rectangle represents the magnified region shown in (c). Scale bar indicates 100 μ m. **c**, Cross section in the eye retina stained in hematoxylin/eosin, presenting the ganglionar cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL) that contain ganglionar cells (ggc), bipolar cells (bpc) and photoreceptors nuclei

(prn). In the anterior part of the retina, photocone external segments (pes) are identified. Scale bar indicates 50 μm . Schematic representation of the four cell types are presented leftward to their legends. **d**, SEM picture localizing the right nasal cavity (dotted white rectangle) between the eye and the snout, where the right nostril is located. Scale bar indicates 1 mm. **e**, SEM picture of a right nostril rosetta, with 11 lamellae (light green dots). Scale bar indicates 100 μm . **f**, SEM picture of the right body flank where lateral line (LL) and LL canals are visible (light blue arrows). The dotted white rectangle represents the magnified region shown in **(g)**. Scale bar indicates 1 mm. **g**, SEM picture of a lateral line canal (blue arrow) and its communication pore. Scale bar indicates 100 μm . **h-m**, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. **h**, Changes in ggc density along recruitment (no change from d0 to d8, $p=0.986$, $n=57$, ANOVA). **i**, Changes in bpc density along recruitment. $A < B$ ($p < 0.001$) and $B < C$ ($p = 0.011$) ($n=57$, ANOVA+TukeyHSD). **j**, Changes in prn density along recruitment. $A < B$ ($p = 0.014$) and $B < C$ ($p = 0.025$) ($n=57$, ANOVA+TukeyHSD). **k**, Changes in pes density along recruitment $A < B$ ($p = 0.028$) ($n=57$, ANOVA+TukeyHSD). **h-k**, Densities are expressed in 10^{-3} cell. μm^{-2} except for pes whose density corresponds to the number of pes in a 50 μm wide area. **l-m**, Changes in nostril lamellae numbers along recruitment. $A < B$ ($p = 0.010$), $B < C$ ($p = 0.005$) ($n=42$, ANOVA). **m**, Changes in the numbers of LL canal along recruitment. $A < B$ ($p < 0.001$), $B < C$ ($p = 0.033$), $C < D$ ($p = 0.025$) ($n=54$, ANOVA+TukeyHSD). (Methods).

To investigate the role of TH signaling in sensory organ maturation, we disturbed the TH pathway by injecting fish with different pharmacological drugs: (i) TH signal promotion = T3 treatment (T_3 + iopanoic acid (IOP) both at 10^{-6} M), and (ii) TH signal disruption = N3 treatment (NH_3 at 10^{-6} M). A third injection condition (TH signal undisturbed = CT control treatment) was performed to allow comparison with the maturation of sensory organs observed in control conditions at recruitment (Fig. III.1h-k). In addition, we also conducted another treatment where larvae were maintained from d0 in cages on the outer reef slope (OS treatment) (*i.e.* as opposed to the lagoon), as preventing access to inner reef is known to environmentally delay metamorphosis (Holzer et al. 2017) (Methods). The density of ggc did not vary between treatments or the control (Fig. III.2a), indicating that it is not controlled by TH. However, the T3 treatment induced an increased rate of maturation in the retina (elevated densities of prn and pes at d2, Fig. III.2c-d), and in the nostril and LL (elevated number of lamellae at d5, Fig. III.2e, and a greater number of LL canals at both d2 and d5, Fig. III.2f). In contrast, N3-treated fish experienced repressed sensory organ maturation, particularly at d5 for the retina (Fig. III.2b-d), at d2 for the nostril (Fig. III.2e), and at both d2 and d5 for LL (Fig. III.2f). Finally, while the OS treatment caused delayed nostril maturation at d2 (Fig. III.2e), it had no effect on retina or LL (Fig. III.2a-d,f), suggesting environmental relocation had a relatively limited impact on their maturation.

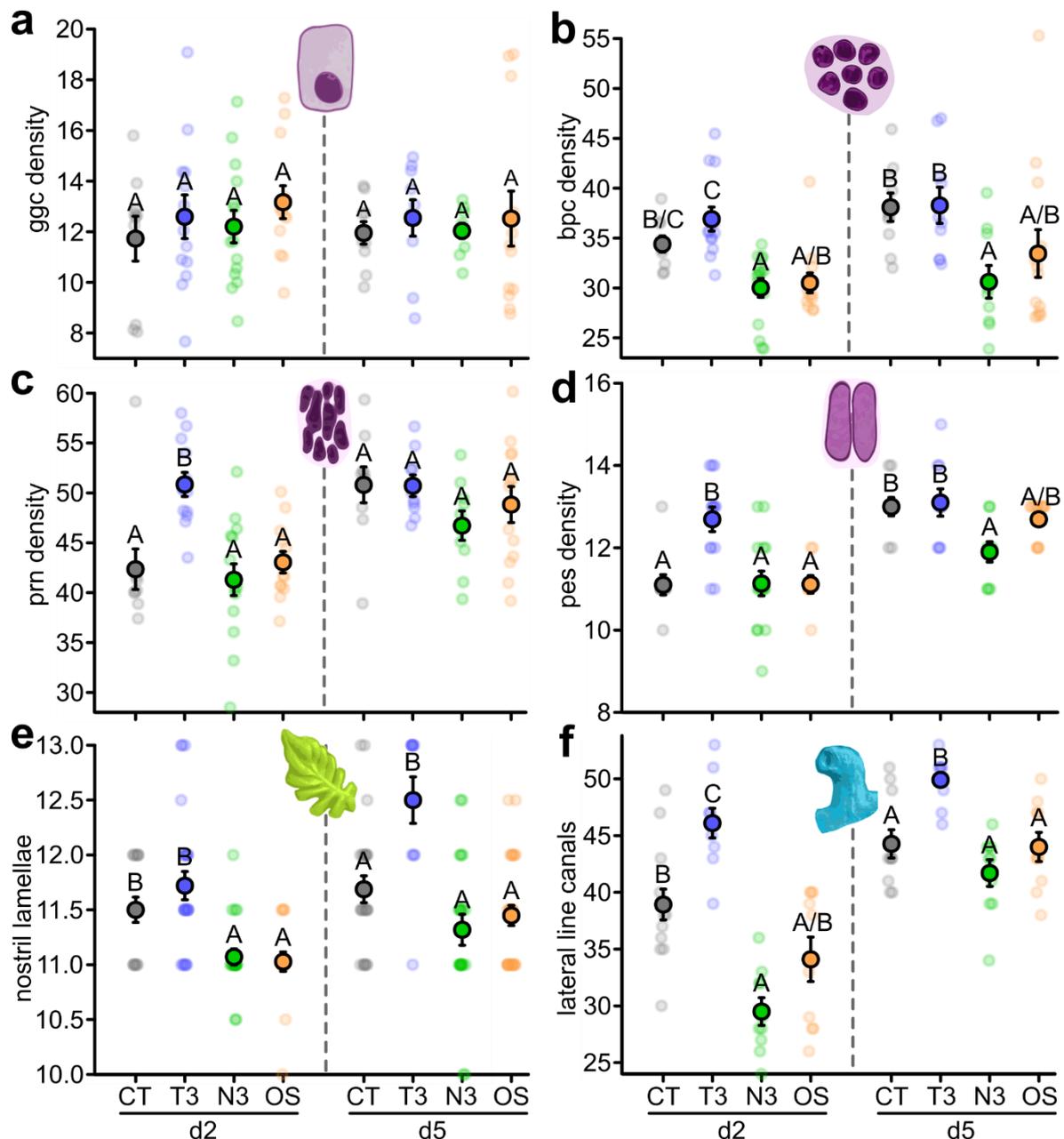


Figure III.2 | Importance of thyroid hormone signaling in sensory organ development at recruitment in *Acanthurus triostegus*

a-f, Impacts of T3 (i.e. promoted TH signaling), N3 (i.e. disrupted TH signaling) pharmacological treatments on sensory organ development at day 2 (d2) and day 5 (d5) compared to control (CT) and outer slope relocated (OS, i.e. environmentally delayed recruitment) individuals. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. **a**, No difference between treatments in ggc density at d2 ($p=0.582$, $n=51$, ANOVA) and d5 ($p=0.666$, $n=43$, Kruskal-Wallis). **b**, $A < B$ ($p=0.018$) and $B < C$ ($p < 0.001$) at d2 ($n=51$, ANOVA+TukeyHSD) and $A < B$ ($p=0.045$) at d5 ($n=43$, ANOVA+TukeyHSD). **c**, $A < B$ ($p=0.002$) at d2 ($n=51$, ANOVA+TukeyHSD) and no change at d5 ($p=0.242$, $n=43$, ANOVA+TukeyHSD). **d**, $A < B$ ($p=0.022$) at d2 ($n=47$, Kruskal-Wallis+Nemenyi) and $A < B$ ($p=0.033$) at d5 ($n=43$, Kruskal-Wallis+Nemenyi). **e**, $A < B$ ($p=0.005$) at d2 ($n=80$, Kruskal-Wallis+Nemenyi) and $A < B$ ($p=0.044$) at d5 ($n=86$, Kruskal-Wallis+Nemenyi). **f**, $A < B$ ($p < 0.001$) and $B < C$ ($p < 0.001$) at d2 ($n=44$, ANOVA+TukeyHSD) and $A < B$ ($p=0.002$) at d5 ($n=42$, ANOVA+TukeyHSD). (Methods).

Among the many stressors affecting coral-reefs, elevated sea water temperature likely represents the greatest current threat to these ecosystems, with impacts ranging from habitat degradation, altered community composition, reduced biodiversity, and behavioral and physiological impairments in associated organisms (Nilsson et al. 2009; McLeod et al. 2013; Hughes et al. 2017). In addition to rising sea surface temperature, coral-reefs have also experienced increasing levels of chemical pollution (*e.g.*, agricultural pesticides and petrogenic compounds) following intensive agricultural and industrial developments (Fabricius 2005; Burns 2014). Among these pollutants, the agricultural pesticide chlorpyrifos (CPF) is one of the most common encountered in coral-reef waters (Shaw et al. 2010). As CPF acts as an endocrine disruptor, coral-reef fishes are high sensitive to it during metamorphosis (Holzer et al. 2017). To determine the relative impact of thermal and chemical stressors on sensory organ maturation, we exposed d0 larvae to either a +0 (*i.e.* 28.5°C, ambient lagoon temperature), +1.5 (30.0°C), or +3.0 (31.5°C) temperature shift (following end-of-century projections for the tropical Pacific (IPCC 2014)), or to CPF at 1, 5, and 30 $\mu\text{g.l}^{-1}$. In addition, we also conducted a control (C) and solvent-control (SC) exposure, to control for aquarium and CPF-solvent (*i.e.* acetone) impacts (Methods). A temperature shift of +3°C reduced both T₃ and T₄ levels around twofold at d2 in comparison to fish exposed at 28.5°C (Fig. III.3a-b). Moreover, this +3°C increase also reduced bpc density by 7 % at d2 (Fig. III.3c), and LL canal number by 10 % at both d2 and d5 (Fig. III.3g). However, it had no impact on prn density, pes density, and nostril development (Fig. III.3d-f). CPF produced a twofold decrease in T₃ at the highest concentration, but did not affect T₄ levels at any concentration (Fig. III.3h-i, adapted from Holzer, Besson, et al. (2017)). At both d2 and d5, reduced bpc densities and LL canals were observed in fish exposed to 30 $\mu\text{g.l}^{-1}$ CPF (Fig. III.3j, Fig. III.3n), while reduced pes density and nostril lamellae were observed at d5 in individuals exposed to 30 $\mu\text{g.l}^{-1}$ CPF (Fig. III.3l-m). Densities of prn exhibited no difference between CPF concentrations (Fig. III.3k-l).

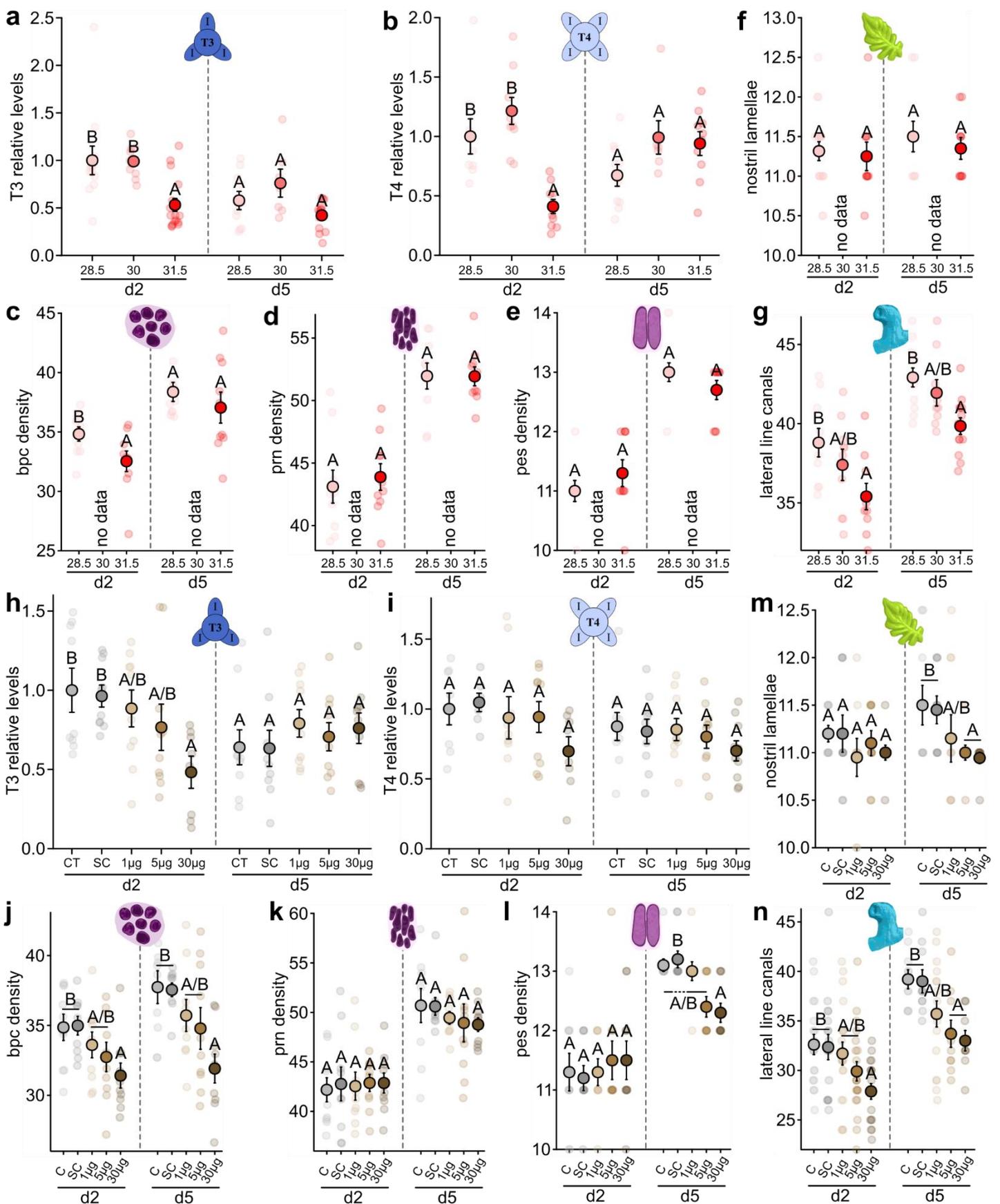


Figure III.3 | Impacts of elevated water temperature and chlorpyrifos exposure on thyroid hormone levels and sensory organ development during recruitment in *Acanthurus triostegus*

a-n, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. d2 and d5 = day 2 and day 5 post-settlement, respectively. **a**, T3 level variations under different temperatures (d2 fish at 28.5°C as standards). A<B (p=0.021)

at d2 (n=42, Kruskal-Wallis+Nemenyi), no difference at d5 (p=0.105, n=30, Kruskal-Wallis). **b**, T4 levels variations under different temperatures (d2 fish at 28.5°C as standards). A<B (p=0.001) at d2 (n=29, Kruskal-Wallis+Nemenyi), no difference at d5 (p=0.074, n=27, Kruskal-Wallis). **c**, bpc density variations under different temperatures. A<B (p=0.034) at d2 (n=20, ANOVA+TukeyHSD) and no difference at d5 (p=0.375, n=20, ANOVA). **d**, prn density variations under different temperatures. No difference at d2 (p=0.637, n=20, ANOVA) and d5 (p=0.983, n=20, ANOVA). **e**, pes density variations under different temperatures. No difference at d2 (p=0.291, n=19, ANOVA) and d5 (p=0.177, n=20, Kruskal-Wallis). **f**, nostril lamellae number variations under different temperatures. No difference at d2 (p=0.609, n=29, Kruskal-Wallis) and d5 (p=0.626, n=20, Kruskal-Wallis). **g**, lateral line canal number variations under different temperatures. A<B (p=0.025) at d2 (n=29, ANOVA+TukeyHSD) and A<B (p=0.002) at d5 (n=20, ANOVA+TukeyHSD). **h**, T3 level variations under CPF exposure (d2 fish at 28.5°C as standards). A<B (p=0.039) at d2 (n=47, ANOVA+TukeyHSD), no difference at d5 (p=0.698, n=50, ANOVA). **i**, T4 levels variations under CPF exposure (d2 fish at 28.5°C as standards). No difference at d2 (p=0.255, n=42, ANOVA) d5 (p=0.578, n=50, ANOVA). **j**, bpc density variations under CPF exposure. A<B (p=0.049) at d2 (n=50, ANOVA+TukeyHSD) and A<B (p=0.004) at d5 (n=50, ANOVA+TukeyHSD). **k**, prn density variations under CPF exposure. No difference at d2 (p=0.993, n=50, ANOVA) and d5 (p=0.436, n=50, Kruskal-Wallis). **l**, pes density variations under CPF exposure. No difference at d2 (p=0.978, n=50, ANOVA) and A<B (p=0.022) at d5 (n=50, Kruskal-Wallis+Nemenyi). **m**, nostril lamellae number variations under CPF exposure. No difference at d2 (p=0.669, n=50, Kruskal-Wallis) and A<B (p=0.018) at d5 (n=49, Kruskal-Wallis+Nemenyi). **n**, lateral line canal number variations under CPF exposure. A<B (p=0.034) at d2 (n=93, ANOVA+TukeyHSD) and A<B (p=0.041) at d5 (n=56, ANOVA+TukeyHSD). **h-j**, adapted from Holzer (2017). (Methods).

Impaired sensory system maturation could reduce survival if it limits an individual's ability to identify and distinguish between critical sensory cues during recruitment (Johansen et al. 2017). We examined this by testing if the metamorphosis-, temperature-, and CPF-induced defects observed impacted the ability of *A. triostegus* larvae to identify predator sensory cues. Firstly, responses to chemical predator cues were assessed by comparing *A. triostegus* preferences for control seawater vs. seawater containing the odor of a common predator (*Lutjanus fulvus*) in two-channel Atema choice flume (Fig. III.4a, Methods). While *A. triostegus* showed no preference for either water source at d0, a clear preference for control seawater was observed in d2 and d5 CT-fish (Fig. III.4a). The same pattern was observed for T3 treated fish; however, N3- and OS-treated fish showed no preference for control seawater over the predator treated seawater at d2 (Fig. III.4a). Secondly, responses to visual predator cues were examined by comparing reactions to *L. fulvus* in a choice aquarium (Fig. III.4c, Methods). At d2, T3 fish, along with CT fish and those held at ambient temperature (*i.e.* 28.5°C) avoided predator visual cues; however, N3 and OS individuals showed no particular attraction or aversion (Fig. III.4d). Thus, individuals whose sensory maturation was impaired by disruption of the TH pathway, either via pharmacological means or environmental conditions, are less able to identify important sensory cues that could increase survival.

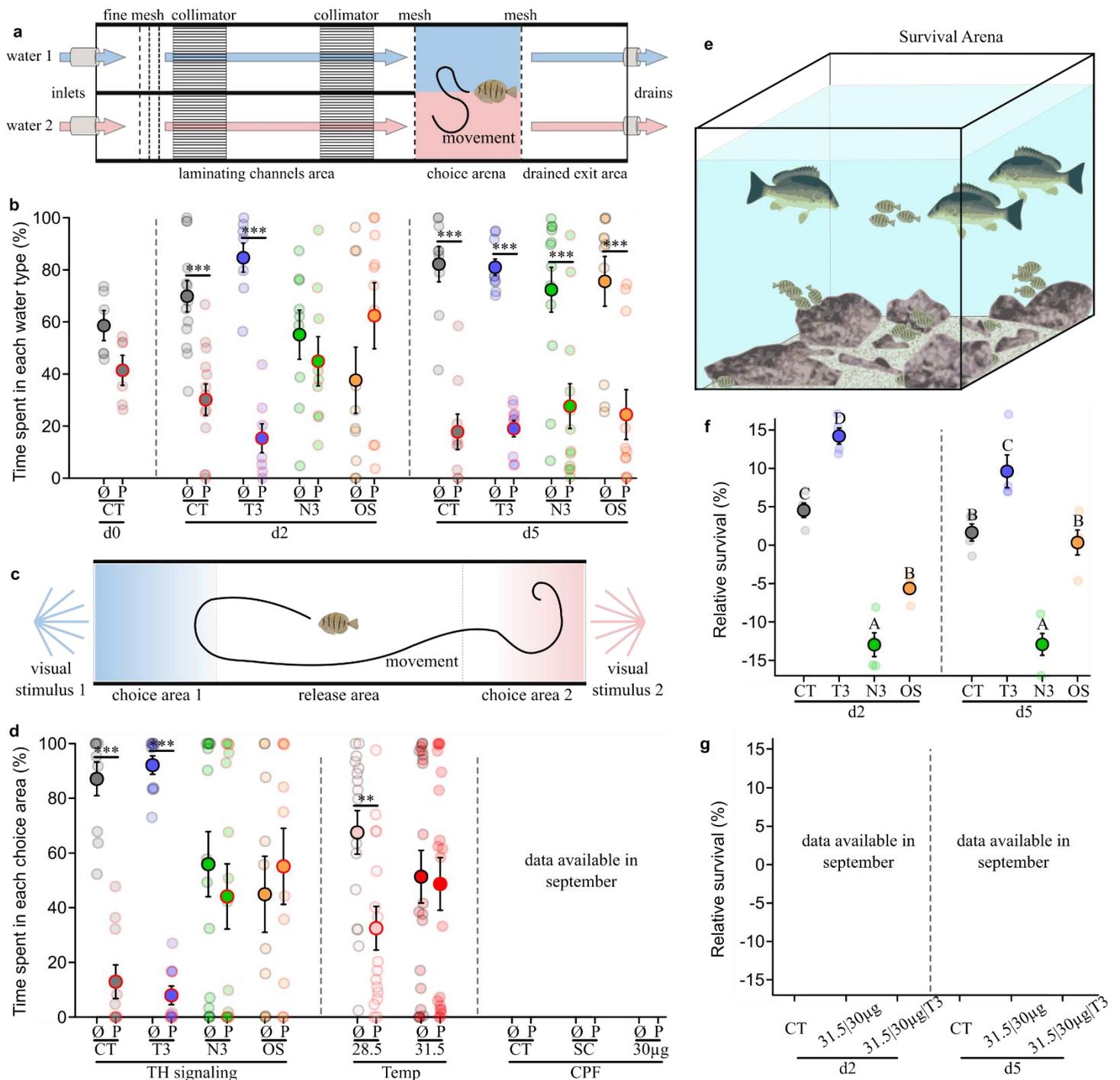


Figure III.4 | Impacts of thyroid hormone signaling, elevated water temperature and chlorpyrifos exposure on *Acanthurus triostegus* behavioral responses and survival when facing predation cues and predators

a, Design of the two-channel Atema choice flume apparatus used for olfactory preferences assessment in this study. Total length: 25 cm (laminating channel area = 15 cm, choice arena = 5 cm and drained exit area = 5 cm). Width: 5 cm. **b**, Olfactory preferences and TH signaling when facing olfactory cues from control seawater (Ø) vs seawater with predator *Lutjanus fulvus* odor (P). d2 and d5 = day 2 and day 5 post-settlement, respectively. *** indicates $p < 0.001$ ($n = 8$ to 13 per treatment and per cue, Wilcoxon-Mann-Whitney). **c**, Design of the choice tank apparatus used for visual preference assessment in this study. Total length: 60 cm (release area = 30 cm, each choice area = 15 cm). Width: 15 cm. The tank is equipped with opaque walls expect for the two opposite walls where visual stimuli are presented. **d**, Impact of TH signaling, water temperature and CPF exposure (results in september) on visual preferences when facing visual cues from a 'fish-empty' tank (Ø) vs tank containing a *L. fulvus* live

predator (P). ** indicates $p < 0.01$ and *** indicates $p < 0.001$ ($n = 10$ to 20 per treatment and per cue, Wilcoxon-Mann-Whitney). **e**, Design of the survival arena apparatus for measuring the impact of TH signaling on *A. triostegus* survival rates at recruitment when facing direct predation by *L. fulvus*. Size: $1\text{m} \times 1\text{m} \times 1\text{m}$. **f**, Relative survival and TH signaling. $A < B$ ($p < 0.001$), $B < C$ ($p < 0.001$), $C < D$ ($p < 0.001$) at d2 ($n=20$, ANOVA+TukeyHSD) and $A < B$ ($p < 0.001$), $B < C$ ($p=0.006$) at day 5 ($n=20$, ANOVA+TukeyHSD). (Methods). **g**, Similar experiment as in **f**, has yet to be performed. **b,d,f**, Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. (Methods)

Finally, to test whether endocrine disruption could reduce recruitment success by increasing mortality rates, we conducted survival experiments in an *in situ* arena (Fig. III.4e, Methods). CT, T3, N3, and OS fish all exhibited different mortality rates when exposed to predators simultaneously. T3 individuals had higher survival rates than their control counterparts (CT) at both d2 and d5, while N3 fish exhibited an opposite pattern (Fig. III.4f). OS fish exhibited greater mortality rates than CT fish at d2, but similar rates to the control at d5 (Fig. III.4f). These differences in survival cannot be attributed to variation in fish size or condition between treatments, as these were similar between groups (III. Extended Data Fig 4).

This research is a holistic analysis of molecular, morphological, histological and behavioral assays of multiple stressors affecting coral-reefs. It builds a mechanistic theory of the impacts of elevated temperature and water pollution during the critical recruitment phase in coral-reef fishes. This indicates the importance of proper endocrine function during coral-reef fish recruitment. A final experiment should test whether exposure to both $+3.0^\circ\text{C}$ and $30 \mu\text{g.l}^{-1}$ CPF also increase mortality rates, and whether performing T3 treatment on those fish improve their survival (*i.e.* by rescuing the TH signaling) (Fig. III.4g). This research highlights the need for a considerable diminution of anthropogenic pressures on coral-reefs, both locally (water pollution) and globally (climate change), as well as for a better understanding of the interactions between such stressors (Hale et al. 2017), more particularly at the brain level (Forrest et al. 2002).

III.5. Methods

Ethics statement

This study was carried out in accordance with the guidelines of the French Polynesia committee for animal ethics, and all experiments were approved by the CRIOBE-IRCP animal ethics committee. This study did not involve endangered or protected species.

Study site and species sampling

This study was conducted from February 2015 to August 2017, at Moorea Island, French Polynesia (S17°32'16.4589", W149°49'48.3018"). The convict surgeonfish *Acanthurus triostegus* (Linnaeus, 1758) has a pelagic larval duration of 53 ± 8 days (Randall 1961) after which oceanic larvae settle back to the reefs, essentially during new moon periods (Besson et al. 2017b). *A. triostegus* larvae were collected nightly (d0), while colonizing the reefs near *Tema'e*, on the north-east coast of the island (S17°29'49.7362", W149°45'13.899"). Sampling was performed using crest nets (Dufour and Galzin 1993). This species was chosen as our model species due to the high number of settling larvae available, and because of the previous studies that have started to tackle endocrine disruption at recruitment in this species (Besson et al. 2017a; Holzer et al. 2017).

Fish husbandry and measurements

Following their capture, young fish were immediately transferred to a nursery area on the north coast of the island (S17°29'26.5378", W149°53'29.2252") where they were raised in *in situ* cages (cylindrical cages, diameter: 50 cm, height: 70 cm, maximum of 10 fish per cage), permitting the study of their development in 'undisturbed' conditions. Cages were equipped with coral rubbles that were replaced daily, ensuring shelter and constant turf availability for fish (as *A. triostegus* feeds on algal turf after settlement (Randall 1961; Holzer et al. 2017)). Fish were then sampled on their second (d2), fifth (d5), eighth (d8), and thirtieth (d30) day of living within the reefs for the study of their pigmentation, behavior, and development. When necessary, fish were euthanized in freshly prepared MS222 at 0.4 mg.ml^{-1} in filtered seawater at 4°C, and instantly placed on ice to minimize animal suffering. Following euthanasia, all fish, except the ones reserved for histological analyses (to preserve tissue quality) were measured for weight (W), height (H) and standard length (SL), allowing the further calculation of the Fulton's condition factor (Fulton 1904) $K = 100 \cdot (W/SL)^3$

(with SL, in cm, and W, in g), as well as body depth $BD = H/SL$. Fish were also scaled and pictured for further measurements such as lateral line (LL) length.

Fish treatments

When tested for the role of their thyroid hormone signaling, *A. triostegus* at d0 were immediately injected after their capture, in their ventral cavity, each with 20 μ l of different pharmacological treatments: **(i)** solvent control (CT, corresponding to DMSO diluted 10.000 times in Phosphate Buffer Saline (PBS) 1X, as the two pharmacological drugs were made soluble in DMSO and diluted 10.000 times in PBS 1X), **(ii)** triiodothyronine (T_3) + iopanoic acid (IOP) both at 10^{-6} M (T_3 treatment), and **(iii)** NH_3 at 10^{-6} M (N_3 treatment). IOP was used as an inhibitor of deiodinase enzymes, as evidenced in mammals and amphibians (Galton and Hiebert 1987, 1988; Galton 1989; Simonides et al. 2008; Medina et al. 2011; Renko et al. 2012), and as routinely used in fish to prevent the immediate degradation of injected T_3 (Little et al. 2013; Lorgen et al. 2015). T_3 treatment was therefore applied to promote TH signaling. NH_3 is a known TR antagonist in vertebrates (Lim et al. 2002) and *A. triostegus* in particular (Holzer et al. 2017). NH_3 prevents the binding of thyroid hormones (THs) such as T_3 on nuclear receptor (TR), therefore impairing the binding of transcriptional coactivators to TR, which therefore remain in an inactive conformation (Lim et al. 2002; Figueira et al. 2011). N_3 treatment was thus applied to repress TH signaling by disrupting the TH pathway. Following these injections, fish were transferred to *in situ* cages in the same nursery area mentioned above. Before their sampling at d2 and d5, these fish were re-injected every morning to maintain the pharmacological treatments active. DMSO and NH_3 non toxicity have been ensured in a previous study (Holzer et al. 2017).

Outer slope relocation

Immediately after their capture at d0, some *A. triostegus* larvae were transferred into *in situ* cages on the outer slope. Cages were not equipped with coral rubbles nor food to mimic oceanic conditions. This environmental relocation has been shown to delay some aspects of larval coral-reef-fish metamorphosis (McCormick 1999; Holzer et al. 2017). Outer slope relocation was therefore used as an additional 'delayed' metamorphosis condition, allowing comparisons with N_3 treatment that pharmacologically represses TH signaling and hence represses metamorphosis processes more importantly.

Elevated temperature and chlorpyrifos (CPF) exposure

After capture, d0 fish were immediately transferred to the CRIOBE marine research station. For elevated temperature exposure, fish were maintained in groups of 10 individuals in 70Lx24Wx45H cm aquaria filled with UV-sterilized and filtered (10 μm filter) seawater in a closed circulating system under 12:12 LD cycle (06:00, onset – 18:00, offset). Aquaria were daily renewed with fresh coral rubble, providing shelter and constant turf availability for fish. Water temperature was maintained at either 28.5°C, 30.0°C or 31.5°C depending on the exposure condition. 28.5°C was chosen as the basal temperature as this was the mean temperature in Moorea lagoon at the time of the study, and as it corresponds to the mean annual lagoon temperature in this region. + 1.5°C and + 3.0°C temperature shifts were selected following projections for the end of the century for the tropical Pacific waters (IPCC 2014). For CPF exposure, fish were maintained in 20Lx11Wx15H cm aquaria filled with 3 liters of UV-sterilized and filtered (10 μm filter) seawater, and oxygenated with an air stone. Five different exposure conditions were applied: nothing (C, control condition), acetone at a final concentration of 1:1.000.000 (SC, solvent control condition, as CPF was made soluble in acetone), or CPF at a final concentration of 1, 5, or 30 $\mu\text{g.l}^{-1}$ (1 μg , 5 μg , and 30 μg conditions) as previously done in another study (Holzer et al. 2017). These concentrations are ecologically relevant and reflect appropriately the change of pesticide concentration larval fishes can encounter when settling, from the open ocean, in coral-reef coastal areas (NRA 2000; Leong et al. 2007; Bigot et al. 2016). Aquaria were not equipped with coral rubbles and all water was replaced every day, ensuring the maintenance of water quality as well as a constant concentration of the pesticide or solvent. The inability of fish to feed in this CPF exposure experiment may be one of the reasons explaining the developmental delays observed between control individuals of the CPF exposure experiment and the control individuals of the pharmacological treatment experiment. Comparison between CPF exposed fish with their relative control counterpart of this CPF exposure experiment allows for CPF-specific developmental effects to be quantified.

Eye retina histology

The retina is the light-sensitive tissue of the eye, and is composed of different cell-layers organized ventrally (vs) and dorsally (ds) around the optic nerve (on) (Fig. III.1b). To analyze retina cell layers, we first dissected fish around their eye region in a way allowing us to distinguish the right eye from the left eye, and the dorsal and ventral sides. Tissues were then fixed in Liquide de Bouin before being embedded in paraffin for microtome sectioning (5 μm). We chose cross sections of the retina that were done at similar depth by selecting sections performed at the level of the optic

nerve (Fig. III.1b). Sections were then stained using Hematoxylin and Eosin. In *A. triostegus* from d0 to d8, these sections enabled us to identify different cell segments, types, and layers, among which (i) photocone-external segments (pes, perceiving light signals), (ii) photo-receptor-nuclei (prn, which are, in part, the internal and nuclear segments of the pes), (iii) bipolar cells (bpc, which integrate the synaptic signals originating from the photoreceptors) and (iv) ganglionar cells (ggc, which integrate signals from bpc and create action potential towards the optic nerve) were easily distinguishable (Fig. III.1c). In this study, we only scrutinized the right eye of *A. triostegus* fish, as this species was evidenced to be lateralized at recruitment, using mostly its right eye to examine predator stimuli (Besson et al. 2017a). Also, we only examined the dorsal side of the retina, as the ventral side undergoes no change at recruitment in another coral reef fish species (McCormick and Shand 1992), and we selected the peripheral area of this dorsal side (Fig. III.1b) for comparison and post-treatment matters (densities were sometimes too high and thus impossible to count in the retina central area). Cell counting was performed in a 50 μm wide area perpendicular to the retina cell layers. The measure of density of photocone external segments corresponds to the number of counts in this 50 μm wide area, while the measures of densities of photoreceptors nuclei, bipolar and ganglionar cells correspond to the actual density per μm^2 after measuring the mean height of each cell layer (3 random measurements for each cell layer: GCL, INL and ONL, Fig. III.1c).

Nostril histology

In fish, the olfactory organ is a paired structure composed of a left and a right nostril, which are each located on the bottom of their respective nasal cavity (Kasumyan 2004). In *A. triostegus*, these two nasal cavities are located on the dorsal surface, between the eye and the snout edge, and water circulates through them from an anterior to a posterior opening (Fig. III.1d). Each nostril is covered by an olfactory epithelium that can detect water chemicals, and is constituted of several olfactory lamellae forming an olfactory rosette (Kasumyan 2004) (Fig. III.1e). These pictures were obtained using SEM microscopy after a specific tissue preparation involving the dissection of the nostril cavities, fixation in a sodium cacodylate + glutaraldehyde solution for a week before washing and dehydrating samples, and lastly using HMDS to replace critical point drying. Samples were not metalized. SEM pictures were obtained using MiniMEB Hitachi TM3030 (University of French Polynesia). Following these observations and given the macroscopic size of nostrils in *A. triostegus*, lamellae counting was performed on tissues samples identically prepared, but observed later under a binocular stereomicroscope.

Lateral line histology

In fish, the lateral line (LL) enables individuals to detect weak water motions and pressure gradients, and is mainly composed of superficial and canal neuromasts, which are ciliary sensory structures located respectively on the fish skin or embedded in lateral line canals (Bleckmann and Zelick 2009). Using the same SEM protocol as for nostrils histology, we evaluated the LL system of *A. triostegus* essentially by scrutinizing its two curved lines starting from behind the eye to the base of the tail (Fig. III.1a), each located on the left and right body flanks. Most of the time, no superficial neuromasts were present on body flanks in d0 to d8 *A. triostegus*, whereas several LL canals, opening to the environment through a pore, were easily distinguished (Fig. III.1f-g). As for nostrils, LL canal counting was then performed under a binocular stereomicroscope. Counting was performed on either the right or left side of the body, as preliminary experiments revealed no significant difference between the numbers of LL canals present on the left or right flanks of the body. Given that fish body generally curves when critically dried in HMDS, this permitted us to select the best conserved and accessible side of the body for LL canal counting.

Thyroid hormone quantification

TH were extracted from frozen-dry fish, following an extraction protocol adapted from previous studies relating TH levels in teleost fish (Tagawa and Hirano 1989; Einarsdottir et al. 2006; Kawakami et al. 2008b) and particularly in coral-reef fishes (Holzer et al. 2017). Fish were individually crushed in 500 µl of methanol using a *Minilys* and glass beads (3*30 secs, 5.000 rpm), centrifuged at 4°C and supernatant reserved, twice. Fish were grinded one last time with 400 µl of methanol, 100 µl of chloroform, and 100 µl of barbital buffer, centrifuged and supernatant reserved. Pooled supernatant were then dried at 70°C. TH were re-extracted with 400 µl of methanol, 100 µl of chloroform, and 100 µl of barbital buffer twice from the first dried extract. Pooled supernatant was re-dried and the extract reconstituted in 2 ml of PBS 1X for quantification (*Roche Elica* kit on a Cobas analyser, following the manufacturer's standardized method). TH levels in pg/g of fish were then transformed into relative levels, by selecting d2 fish exposed at the control temperature of 28.5°C as the baseline in the elevated temperature experiment, and by selecting control fish at d2 as the baseline in the CPF exposure experiment.

Behavioral tests

A two-channel Atema choice flume (Atema et al. 2002; Gerlach et al. 2007; Jutfelt et al. 2017) (25 x 5 cm) was used to assess preference of *A. triostegus* between two water inlets: control seawater (5 liters of UV-sterilized and filtered - 10 µm filter – seawater from the collection site, Ø) vs ‘predator seawater’ (5 liters of UV-sterilized and filtered - 10 µm filter – seawater from the collection site in which five *Lutjanus fulvus* predators had been soaked for two hours prior to the experiment, P). Using flow meters, a slow 100 ml.min⁻¹ flow rate was maintained in each water inlet (Fig. III.4a), allowing fish to swim without struggle against the current and ensuring that fish movements within the choice arena effectively reflect cue preference (Lecchini et al. 2017). Prior to each fish observation, dye tests were conducted to confirm laminar flow, without eddies or areas of water mixing, within the choice arena. After releasing the fish in the choice arena, a 2 min acclimation period was observed, then fish position was recorded every 2 sec for 5 min. Water inlets were then switched and another 2 min acclimation period followed by a 5 min period, during which fish position was again assessed every 2 sec, was observed. Preliminary experiments were conducted to ensure the absence of unanticipated biases for the two channel flume (e.g., fish preference for one side over the other, irrespective of water inlets). Fish preference for a water inlet was also ensured by comparing the first and second 5 min recorded periods, before and after water inlet switch. These experiments were performed under red light, to prevent potential visual perturbations such as the observer presence, and to allow comparison with d0 fish, whose preferences were tested at night, also under red light, right after their arrival and capture.

A double choice tank (Fig. III.4a) was used to assess preference of *A. triostegus* between two visual stimuli: an adjacent aquarium, equipped with an air stone, and containing a 10 cm (standard length) *L. fulvus* (P condition, Fig. III.4d) vs and adjacent aquarium, equipped with an air stone, and containing no fish (Ø condition, Fig. III.4d). Fish were first placed in the release area, without the ability to access and visualize the adjacent choice areas (opaque panels were positioned at the edges of the release area), for a 2 min acclimation period. Then, opaque panels were removed, leaving the fish able to visualize, through the two opposed transparent walls of the choice tank, the Ø and P visual stimuli, and to access the choice areas. Fish position was then assessed every two seconds during 10 min, using a camera to prevent any external visual disturbances such as an observer presence. Location of visual stimulus (left or right side of the double choice tank) were switched between each fish to ensure the absence of unanticipated biases for the double choice tank (e.g., fish global preference for one side over the other, irrespective of the presented visual stimuli).

Survival arena

Survival tests (Fig. III.4e-g) were conducted in an arena set up *in situ* in a quiet nursery lagoon area along the north-coast (S17°29'7.0272", W149°49'51.1166"). It consisted of a cubic meter cage (1mx1mx1m) with a hard bottom covered with sand and coral rubbles and four lateral walls equipped with fine mesh (5mm). Each group of fish was tagged separately with a specific color, using visible implant elastomer (Northwest Marine Technology), at least one hour before being released into the arena. All evaluated groups of fish were released simultaneously (8 to 16 fish per group) and allowed to acclimate for 30 min before the introduction of 5 to 8 *L. fulvus* (minimum 10 cm SL). After 4 hours, predators were removed, and survivors were identified to each initial group using their color tag. Overall survival rate in the arena (total number of survivors/total number of fish initially introduced) and survival rate in each group (number of survivor in group A/number of fish from group A initially introduced) were then calculated, permitting the final calculation of the relative survival rate of each group (survival rate of the group – overall survival rate in the arena). This standardization permitted a better comparison of survival rates among replicate runs that could have been influenced by differences due to the predators (*e.g.*, numbers, behavior, hunger, etc.).

Statistical analyses

All statistical analyses were conducted using the *R-cran* project free software (R-3.3.1). Multiple means comparisons among fish groups were performed using univariate analysis of variance (ANOVA) followed by Tukey post hoc tests (should a significant difference be detected), when data (or linear model residuals) met assumptions of normality (Shapiro test) and homogenous variance (Bartlett test). When normality and homogenous variance were not verified, Kruskal-Wallis followed by Kruskal-Nemenyi post hoc tests were performed. Mean to mean comparisons were performed using Wilcoxon-Mann-Whitney U-test when $n < 15$.

III.6. Additional information

Acknowledgment

We thank T. Lorin, and H. Dore for critical reviewing, and for their fruitful advice on the design of the figures. We thank the association *Te mana o te moana* for the accommodation of our cages.

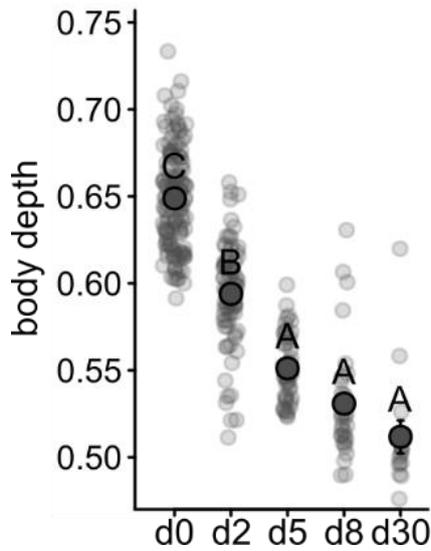
Author Contributions

M.B. conceived the study with G.H., V.L., and D.L.; M.B., I.M., L.F., H.J., A.G., L.L., and D.L. collected the data; M.B., I.M., L.F., P.S., G.H., M.A., G.I. and L.L. implemented the analyses; M.B. and L.L. built the figures; M.B. led the manuscript with R.M.B., J.O., F.F., L.B., V.L., and D.L.

Competing financial interests

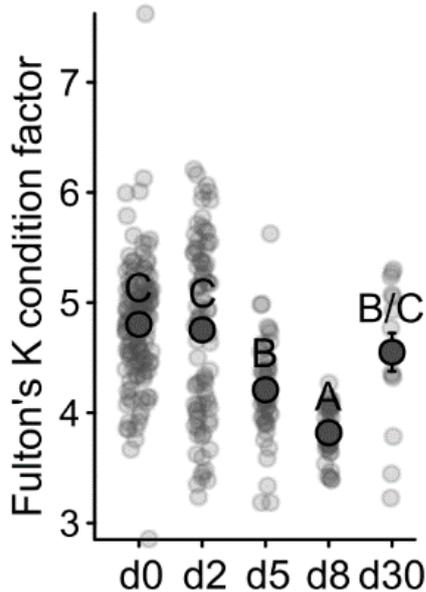
The authors declare no competing financial interests.

III.7. Extended data



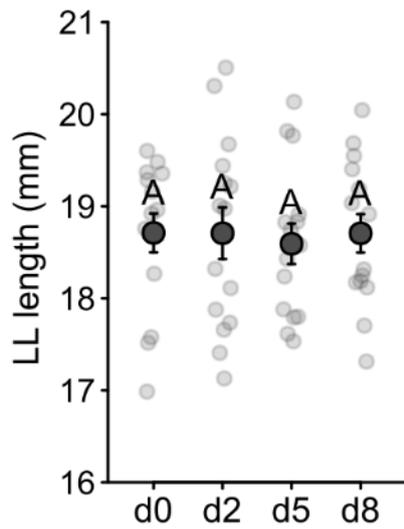
III. Extended Data Fig 1 | Body depth variation along recruitment in *Acanthurus triostegus*

Body depth was assessed from day 0 (d0) to day 30 (d30) post-settlement. Body depth was calculated as follows: Body Height / Standard length. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. A<B (p<0.001) and B<C (p<0.001) (n=320, Kruskal-Wallis+Nemenyi).



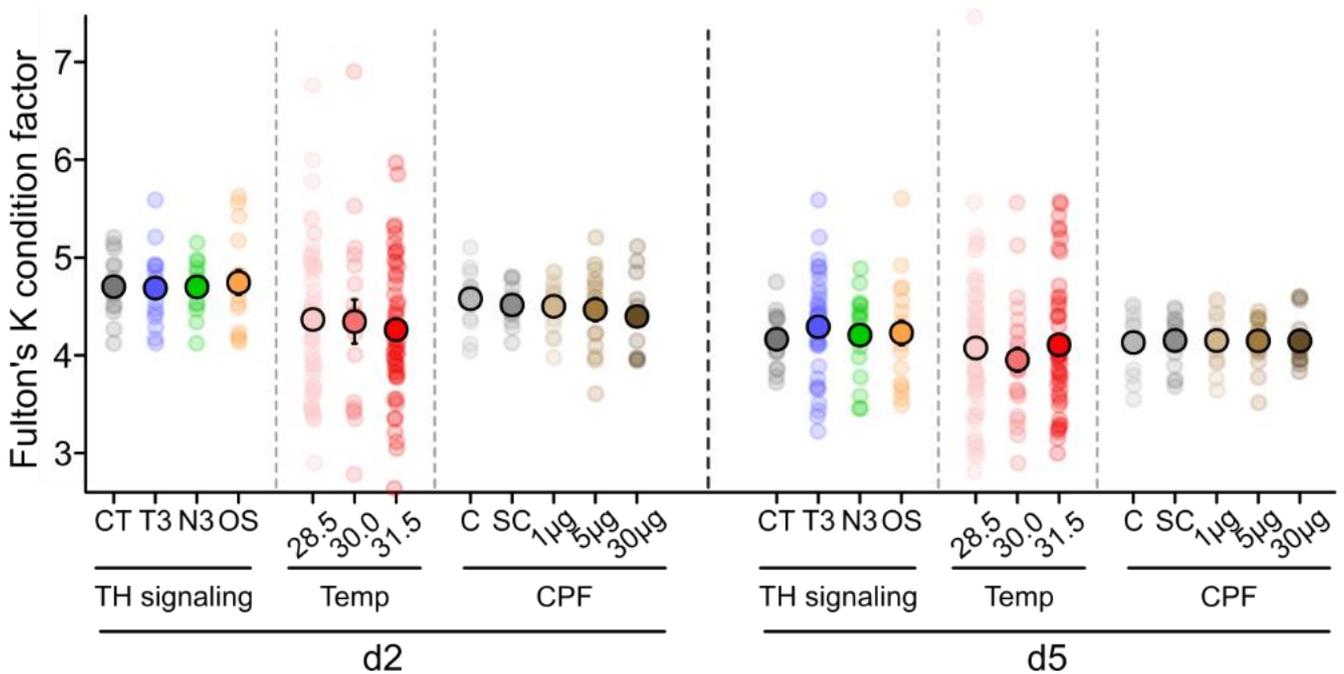
III. Extended Data Fig 2 | Fulton's K condition factor variation along recruitment in *Acanthurus triostegus*

Fulton's K condition factor was assessed from day 0 (d0) to day 30 (d30) post-settlement. Fulton's K condition factor was calculated as follows: $K = 100 \cdot (W/SL)^3$ (with SL, in cm, and W, in g) (Fulton 1904). Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. A<B (p=0.024) and B<C (p<0.001) (n=320, Kruskal-Wallis+Nemenyi)



III. Data Fig 3 | Lateral line (LL) length variation along recruitment in *Acanthurus triostegus*

LL length was assessed from day 0 (d0) to day 8 (d8) post-settlement. Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. No difference in lateral line length was observed from d0 to d8 ($p=0.977$, $n=60$, ANOVA)



III. Expanded Data Fig 4 | Fulton's K condition factor variation along with thyroid hormone (TH) signaling, temperature exposure (Temp), and chlorpyrifos (CPF) exposure during recruitment in *Acanthurus triostegus*

Fulton's K condition factor was calculated as follows: $K = 100 \cdot (W/SL)^3$ (with SL, in cm, and W, in g) (Fulton 1904). Error bars indicate standard errors (error bars are not visible when standard errors are too small). Circles bordered in black represent means while alpha transparent circles in the background represent each data. At day 2 (d2) post-settlement, neither TH ($p=0.976$, $n=62$, ANOVA), temperature ($p=0.849$, $n=129$, Kruskal-Wallis), nor CPF ($p=0.529$, $n=75$, Kruskal-Wallis) affect Fulton's K condition factor in *A. triostegus*. Same observations were made at day 5 (d5) post-settlement: TH ($p=0.715$, $n=92$, Kruskal-Wallis); Temp ($p=0.733$, $n=135$, Kruskal-Wallis), and CPF ($p=1$, $n=76$, ANOVA). The exact same patterns have been observed for standard length, weight, height and body depth. (Methods).

III.8. References

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IV. Exposure to agricultural pesticide impairs visual lateralization in a larval coral reef fish

IV.1. Foreword

The two previous chapters emphasized the importance of behavioral changes at recruitment in coral-reef fishes, underpinning the potential critical role of brain and sensory systems during recruitment. The study presented in Appendix 4 also demonstrates how brain lateralization processes are at play during metamorphosis and recruitment in a soldier coral-reef fish. However, such processes remain unknown in the model species of this PhD, *Acanthurus triostegus*. Moreover, the impacts of reef chemical pollutants such as the agricultural pesticide chlorpyrifos on such lateralized brain processes in coral reef fish have yet to be elucidated.

Consequently, this third chapter aims at bringing new information and new perspectives to the two last questions of my PhD, which are:

3 | Are there specific sensory organs and sensory abilities that develop at metamorphosis and what are their endocrine determinants?

4 | Are there environmental factors that act as endocrine disruptors of TH signaling during coral reef metamorphosis and how would it subsequently affect larval recruitment success?

To achieve this, the fieldwork and analyzes presented in this chapter have been performed in close collaboration with the former Master student C. Gache that I supervised with Dr. D. Lecchini during my PhD. I performed with C. Gache most of the field work, from fishing to fish husbandry, while he performed all the lateralization measures. I then conducted all the final analyzes and wrote the manuscript presented in this chapter. This study has been published in *Scientific Reports* on August, 22nd 2017. This chapter corresponds to the final manuscript of this study, which is also available in its final pdf format in Appendix 6.

IV.2. Manuscript information

Title: Exposure to agricultural pesticide impairs visual lateralization in a larval coral reef fish

Authors: Marc Besson^{1,2,*,#}, Camille Gache^{1,3*}, Frédéric Bertucci^{1,4}, Rohan M. Brooker⁵, Natacha Roux^{1,2}, Hugo Jacob^{1,6}, Cécile Berthe^{1,3}, Valeria Anna Sovrano⁷, Danielle L. Dixon⁵, David Lecchini^{1,3}

Affiliations:

1 PSL Research University: EPHE-UPVD-CNRS, USR 3278 CRIOBE, BP 1013, 98729 Papetoai, Moorea, French Polynesia

2 Université Pierre et Marie Curie, UMR CNRS 7232 OOB, 1 Avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France

3 Laboratoire d'Excellence "CORAIL", BP 1013, 98729 Papetoai, Moorea, French Polynesia

4 Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH Research Center, Institut de Chimie B6c, Université de Liège, Liège, Belgium

5 School Marine Science and Policy, University of Delaware, 111 Robinson Hall, Newark DE 19716 USA.

6 International Atomic Energy Agency, Environment Laboratories (IAEA-EL), Principality of Monaco, 98000 Monaco

7 Center for Mind/Brain Sciences (CIMEC) and Department of Psychology and Cognitive Sciences, University of Trento, Piazza Manifattura 1, 38068 Rovereto (TN), Italy

*: Equal first authors

Correspondence: Email: marc.besson@ens-lyon.org, Tel: +33(0)4 30 19 24 05

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IV.3. Abstract

Lateralization, *i.e.* the preferential use of one side of the body, may convey fitness benefits for organisms within rapidly-changing environments, by optimizing separate and parallel processing of different information between the two brain hemispheres. In coral reef-fishes, the movement of larvae from planktonic to reef environments (recruitment) represents a major life-history transition. This transition requires larvae to rapidly identify and respond to sensory cues to select a suitable habitat that facilitates survival and growth. This ‘recruitment’ is critical for population persistence and resilience. In aquarium experiments, larval *Acanthurus triostegus* preferentially used their right-eye to investigate a variety of visual stimuli. Despite this, when held in *in situ* cages with predators, those larvae that previously favored their left-eye exhibited higher survival. These results support the “brain's right-hemisphere” theory, which predicts that the right-eye (*i.e.* left-hemisphere) is used to categorize stimuli while the left-eye (*i.e.* right-hemisphere) is used to inspect novel items and initiate rapid behavioral-responses. While these experiments confirm that being highly lateralized is ecologically advantageous, exposure to chlorpyrifos, a pesticide often inadvertently added to coral-reef waters, impaired visual-lateralization. This suggests that chemical pollutants could impair the brain function of larval fishes during a critical life-history transition, potentially impacting recruitment success.

IV.4. Introduction

Brain asymmetry and the preference to use one side of the body over the other to accomplish actions (termed lateralization), has been identified in a variety of vertebrate and invertebrate species (Bisazza et al. 1998; Vallortigara et al. 1999, 2011; Frasnelli et al. 2012; Rosa Salva et al. 2012). Lateralization processes have been characterized in adult organisms but also in early-life-stages, such as fish larvae (Barth et al. 2005; Dadda et al. 2010), fish juveniles (Roussigné et al. 2012; Roux et al. 2016), chicks (Vallortigara and Andrew 1991) (but see (Cooke 2004; Levin 2005; Güntürkün and Ocklenburg 2017) for reviews on embryonic and post-embryonic development of lateralized organs and acquisition of lateralized behaviors in several vertebrate and invertebrate species). Processes of lateralization can therefore be expected along the whole ontogeny of an animal organism. Brain lateralization is thought to increase cognitive abilities, in particular for decision making when facing novel multi-sensory signals, by enabling individuals to cope with divided attention and to partition, and optimize, the parallel processing of different types of information into the two separate brain hemispheres (Rogers 2000; Vallortigara 2000; Rogers et al. 2004; Sovrano et al. 2005; Vallortigara and Rogers 2005; Rosa Salva et al. 2012). With regards to habitat exploration and response to sensory cues, there are two main theories concerning brain lateralization in animals. The “**brain's right hemisphere theory**” assumes that the right hemisphere is predominantly used to process information regarding novel items and/or items requiring a rapid behavioral response, while the left hemisphere is used for categorizing stimuli and/or processing information that requires consideration of alternatives (MacNeilage et al. 2009; Rogers et al. 2013). Alternatively, the “**valence theory**” proposes that the right hemisphere is preferentially used to process information regarding negative stimuli, while the left hemisphere is used for the processing of information regarding positive stimuli (Hook-Costigan and Rogers 1998; Quaranta, A., Siniscalchi, M., Vallortigara 2007; Siniscalchi et al. 2013). While empirical evidence supports both theories, previous studies have generally only considered lateralized responses to social sensory stimuli under normal environmental conditions, and only in a few species of amphibians, birds, freshwater fishes, and mammals (Vallortigara and Andrew 1991; Sargent et al. 1992; Hook-Costigan and Rogers 1998; Sovrano et al. 1999, 2001; Bisazza et al. 2002; Dadda et al. 2003; Sovrano 2004). Therefore, we still lack a comprehensive understanding of the ecological importance of sensory lateralization in marine fishes, as well as in the context of rapidly changing environments.

Among the most rapidly changing environments globally are coral reefs, one of the most biologically diverse, productive, and economically important ecosystems worldwide (Odum and Odum 1955; Connell et al. 1978; Moberg and Folke 1999). Over the past three decades, coral reefs have faced increasingly frequent and severe natural (*e.g.*, cyclones, *Acanthaster planci* outbreaks) and anthropogenic perturbations (*e.g.*, pollution from agricultural runoff, rising sea-water temperatures, overfishing) leading to global declines in habitat quality, biodiversity and associated ecosystem

services (Moberg and Folke 1999; Hughes et al. 2003, 2014, 2017; Bellwood et al. 2004; Hoegh-Guldberg et al. 2007). In the context of these rapidly changing conditions, reef fishes often play an important role in maintaining habitat structure, preserving associated biodiversity, and increasing ecosystem resilience (*e.g.*, through the regulation of coral-algal interactions or by maintaining trophic chain sustainability) (Russ 1984; Hughes et al. 2007; Lecchini et al. 2007b). However, these species are often highly vulnerable to changing conditions, with individual health and population persistence impaired by both localized and global impacts (Hughes et al. 2014). The majority of reef fishes have a bipartite life cycle, consisting of a pelagic larval stage that facilitates dispersal followed by a largely sedentary reef-associated juvenile and adult stage (Leis and McCormick 2002). At the conclusion of the larval phase, the transition from the planktonic to reef environments is referred to as settlement (Leis and McCormick 2002). During this transition, reef-naïve larvae metamorphose into benthic dwelling juveniles and must rapidly identify and respond appropriately to a host of novel multi-sensory signals in order to select a suitable habitat that will facilitate their survival and growth (Planes and Lecaillon 2001; McCormick et al. 2002; Doherty et al. 2004; Lecchini et al. 2005a, 2005b; Almany and Webster 2006; Frédérick et al. 2012; Lecchini and Nakamura 2013). The selection of, and persistence in, an appropriate habitat is termed recruitment (Leis and McCormick 2002). Continued and consistent recruitment is essential to maintain reef fish populations and assist with the resilience and recovery of degraded reef fish communities (Doherty 2002). The ability to identify visual cues that indicate the identity and location of predators has shown to increase survival rates during recruitment in a number of coral reef fish species (Lecchini et al. 2005a, 2005b; Huijbers et al. 2012; Dixson et al. 2014; O'Connor et al. 2016).

The relationship between brain morphology, lateralization, social interactions, and anti-predator behaviors in early-life-stage coral reef fishes remains largely unknown (Lecchini et al. 2005a, 2005b; Dixson et al. 2014; Barth et al. 2015; Ferrari et al. 2015a, 2015b; Roux et al. 2016), with the role of behavioral lateralization during recruitment only initially assessed (Ferrari et al. 2015a, 2015b). These recent experiments have primarily used detour tests, which examine potential turning bias of individuals in a context of vigilance, and revealed that such lateralized behaviors can be associated with anti-predator response phenotypes and survival benefits (Nilsson et al. 2012; Ferrari et al. 2015a, 2015b). However, these studies were conducted on captive-bred larvae, larvae caught in light traps (which may selectively attract certain behavioral phenotypes), or wild caught post-settlement stage juveniles. Experimental use of individuals that have been reared, are from mixed larval, pre-settlement, and post-settlement stages, or are non-naïve and have past reef experience potentially adds biases to these findings. This prevents a complete understanding of the ecological importance of coral reef fish lateralization at the critical time of recruitment. In addition, current research has yet to examine the contribution of either brain hemisphere in accomplishing a specific task.

Short-term exposure to environmental stressors, such as high predation risk or elevated CO₂ levels, has been shown to alter lateralized anti-predator behaviors, which can lead to greater mortality rates (Nilsson et al. 2012; Ferrari et al. 2015b). These findings highlight the critical role of lateralization during the recruitment in coral reef fishes, and point towards potential population or ecosystem-level impacts when an environmental stressor is applied (Domenici et al. 2012, 2014; Nilsson et al. 2012; Ferrari et al. 2015a). In particular, anthropogenic impacts on reefs, such as the worldwide increasing influx of chemical pollution (*e.g.*, from pesticides) resulting from coastal agriculture and river runoff (Carvalho et al. 2002; Islam and Tanaka 2004; Fabricius 2005; Laabs et al. 2007; Leong et al. 2007; Lewis et al. 2009; Packett et al. 2009; Shaw et al. 2010; Roche et al. 2011; Kroon et al. 2012), are known to severely impact reef biodiversity and the biology and ecology of coastal marine organisms (Fabricius 2005; O'Connor et al. 2016). Among these sources of pollution, the Chlorpyrifos (CPF), an organophosphate insecticide widely used on tropical coastal crops (*e.g.*, sugar-cane and rice crops in Australia and south-east Asia respectively (Cavanagh et al. 1999; Leong et al. 2007; Botté et al. 2012)), is one of the most common waterborne chemical pollutant encountered in coral reefs (Cavanagh et al. 1999; Botté et al. 2012). While several studies have demonstrated its neurotoxicity and endocrine disruption characteristics (Kavitha and Rao 2008; Botté et al. 2012; Deb and Das 2013; Slotkin et al. 2013), the negative impact on coral reef larval fish sensory abilities has also recently been acknowledged (Botté et al. 2012). Moreover, pesticides belonging to the same family have been discovered in important amounts in several fish and crustacean species in French Polynesia (Roche et al. 2011; Salvat et al. 2012). However, whether this pollutions affect or not behavioral lateralized processes remains unknown (Botté et al. 2012).

Here, we examined visual lateralization in the larvae of a common coral reef fish species, the convict surgeonfish *Acanthurus triostegus* (Randall 1961), during its recruitment phase. If settlement-stage larvae preferentially use their right eye to examine and categorize positive stimuli (*e.g.*, conspecifics, such as in *Myrispristis pralinia*, a coral reef fish (Roux et al. 2016), or in freshwater fishes (Sovrano et al. 1999, 2001, 2005; Sovrano and Andrew 2006)) and their left eye for negative stimuli or to inspect novel items and execute rapid responses (*e.g.*, potential predators, such as in freshwater fishes (Sovrano et al. 1999, 2001, 2005; Sovrano and Andrew 2006)), this would be good evidence for both the “**brain's right hemisphere**” and the “**valence**” theories. Using a range of aquarium based (Fig. IV.1) and *in situ* experiments we tested whether larvae exhibit a naïve preference for their left or right eye when inspecting both positive (mirror self-image, conspecific) or negative (predator) stimuli (**Exp. 1**) and whether visual lateralization increased or decreased vulnerability to predation (**Exp. 2**). Lastly, to examine the potential for coastal development to affect this mechanism we determined whether exposure to CPF, at ecologically relevant concentration (NRA 2000; Leong et al. 2007; Botté et al. 2012), would alter the degree of lateralization larvae exhibit (**Exp. 3**).

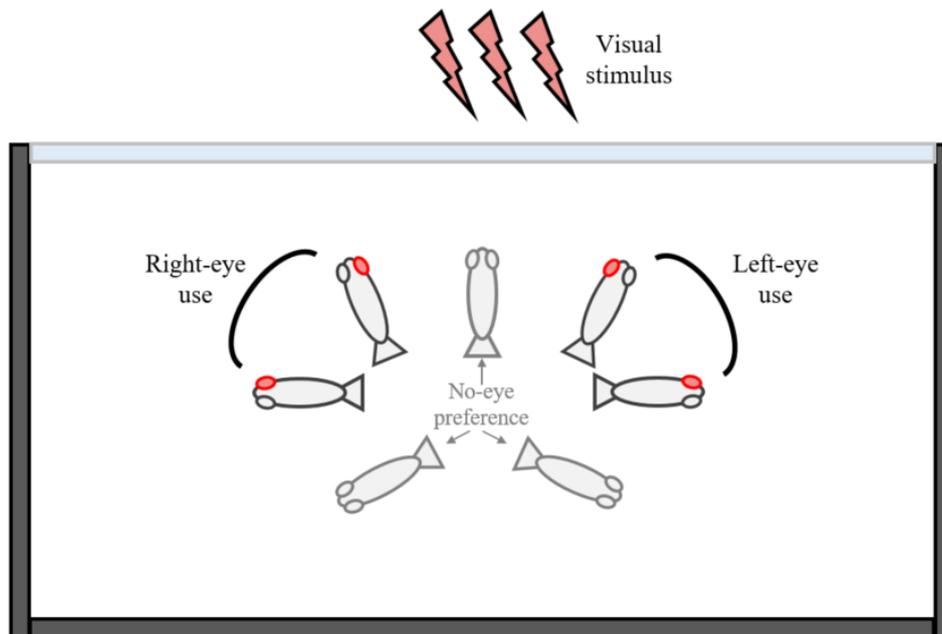


Fig. IV.1 | Schematic representation of the test apparatus for eye lateralization determination.

The three dark gray walls represent the opaque wall of the aquarium, while the light blue wall corresponds to the transparent wall where the visual stimuli are presented. No eye preference was recorded when the fish was perpendicular to the transparent wall (*i.e.* binocular stimulation) or when it formed an angle larger than 180° with respect to the transparent wall (*i.e.* fish looking in the opposite direction).

IV.5. Results

For each individual within each experiment, a lateralization index (LI), reflecting the degree of preferential eye use, was calculated as: $[(\text{frequency of right eye use}) / (\text{frequency of right eye use} + \text{frequency of left eye use})] \times 100$. Values significantly higher than 50% indicate preference for right eye use while values significantly lower than 50% indicate preference for left eye use. Subsequent statistical tests were conducted using these values.

Exp. 1 tested whether larvae exhibit a naïve eye preference when inspecting visual cues from positive (conspecific) or negative (predator) stimuli. In the absence of any visual stimulus, *A. triostegus* larvae exhibited no eye preference (LI = 41.8 ± 4.6 %, Mann-Whitney U test, $U = 32$, $n = 14$, $p\text{-value} = 0.364$) (Fig. 2). However, larvae showed a significant preference when inspecting test stimuli (Kruskal-Wallis rank sum test, $\chi^2 = 14.173$, $df = 3$, $p\text{-value} = 0.003$), with LI values significantly higher when examining both conspecifics (Nemenyi post-hoc test with Tukey-Dist approximation, $p\text{-value} = 0.006$) or predator cues ($p\text{-value} = 0.006$). With regards to the eye preferred, larvae displayed a significant preference for their right eye when inspecting themselves in a mirror (LI = 57.3 ± 2.4 %, Mann-Whitney U test, $U = 93$, $n = 14$, $p\text{-value} = 0.009$), when examining a group of five adult conspecifics (LI = 62.5 ± 5.5 %, $U = 85$, $n = 14$, $p\text{-value} = 0.009$).

value = 0.042), or when examining a predator (LI = 62.2 ± 3.6 %, U = 83, n = 13, p-value = 0.006) (Fig. 2).

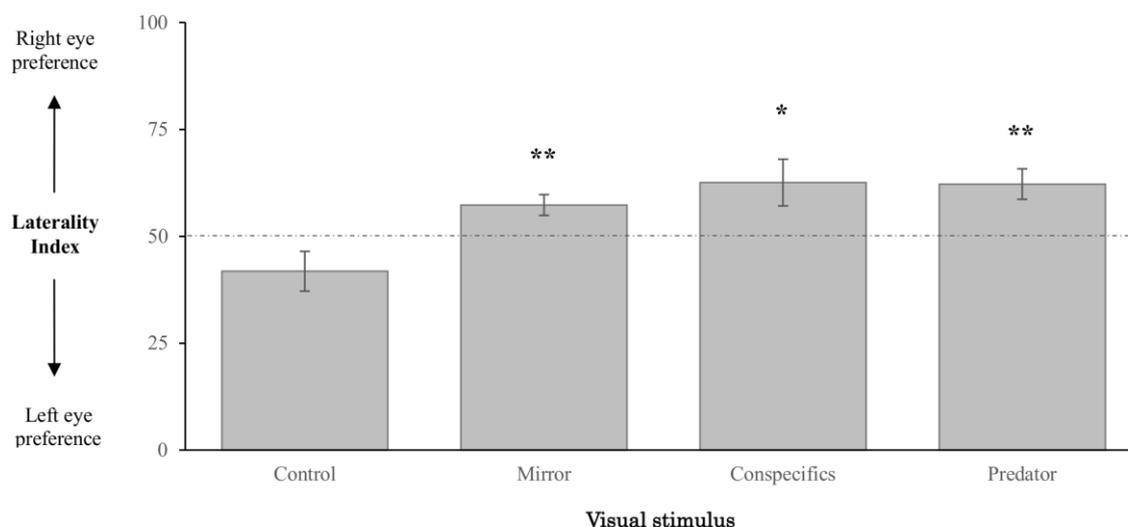


Fig. IV.2 | Left-right eye preference of *A. triostegus* larvae during the inspection of visual stimuli in an adjacent aquarium.

Figure represents LI mean (\pm SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, *: $p < 0.05$; **: $p < 0.01$) between Laterality Index (LI) values and the theoretical 50 % value (dotted line). 13 to 14 replicates (one fish per replicate) were conducted for each visual stimulus.

Exp. 2 tested whether visual lateralization increased or decreased vulnerability to predation. Individual larvae from Exp. 1 were classified and grouped as either ‘no eye dominant’, ‘right-eye dominant’, or ‘left-eye dominant’ based on the behavior displayed when exposed to the predator in Exp. 1. All three groups experienced significantly different survival rates (Kruskal-Wallis rank sum test, $\chi^2 = 7.064$, $df = 2$, p-value = 0.029) with ‘left-eye dominant’ larvae having a higher survival rate than both ‘right-eye dominant’ (Nemenyi post-hoc test with Tukey-Dist approximation, p-value = 0.01) and ‘no eye dominant’ larvae (p-value = 0.03) (Fig. 3).

Exp. 3 utilized the same protocol as in Exp. 1 and tested whether exposure to a waterborne organophosphate pesticide often encountered in coral reefs: chlorpyrifos (CPF), would alter the degree of lateralization larvae exhibit. Larvae exposed to CPF at $1 \mu\text{g}\cdot\text{l}^{-1}$ (black bars in Fig. 4) and larvae only exposed to the CPF solvent (grey bars in Fig. 4) again showed no eye preference in the absence of visual stimuli (respectively LI = 52.5 ± 6.1 % and 52.3 ± 2.7 %, Mann-Whitney U test with respectively U = 33 and 35, n = 10 and 11, p-value = 0.625 and 0.476) (Fig. 4). However, a preference was seen when these larvae inspected a mirror (Wilcoxon rank sum test, $W = 106$, n = 10, p-value < 0.001), conspecifics ($W = 87$, n = 10, p-value = 0.004) or a predator ($W = 85$, n = 10, p-value = 0.009). Similar to the trends seen in Exp. 1 (Fig. 2), solvent control larvae preferentially used their right eye when inspecting a mirror (LI = 59.2 ± 2.9 %, Mann-Whitney U test, U = 61.5, n = 11, p-value = 0.013), conspecifics (LI = 58.9 ± 2.1 %, U = 54,

n = 10, p-value = 0.004) or a predator (LI = 58.8 ± 2.4 %, U = 45, n = 10, p-value = 0.009) (Fig. 4). However, when exposed to CPF at $1 \mu\text{g.l}^{-1}$ for five days, larvae no longer exhibited any eye preference when inspecting either the conspecifics or predator (respectively LI = 49.0 ± 1.9 % and 44.9 ± 3.7 %, U = 25 and 16, n = 10 and 10, p-value = 0.846 and 0.262). In addition, when inspecting themselves in a mirror, CPF treated larvae now preferentially used of their left eye (LI = 43.3 ± 2.2 %, U = 1, n = 10, p-value = 0.004) (Fig. 4).

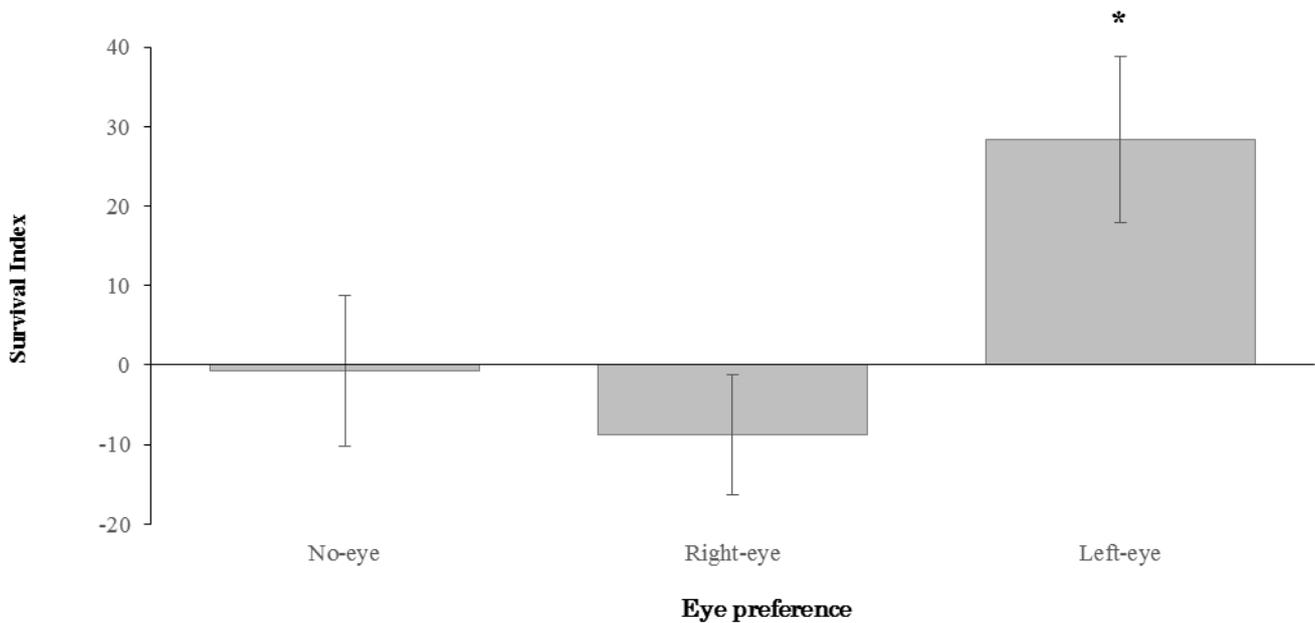


Fig. IV.3 | Ecological importance of brain lateralization in larval survival facing direct predation.

Survival index (SI) was calculated as follow: survival rate of the group minus the overall survival rate in the *in situ* cage. Figure represents SI mean (\pm SE) values. The asterisk indicates a significant difference in survival index among the three groups (Kruskal-Wallis test, $\chi^2 = 7.064$, df = 2, p-value = 0.029) with 'left-eye dominant' experiencing high survival than both 'right-eye dominant' (Nemenyi post-hoc test with Tukey-Dist approximation, p-value = 0.01) and 'no eye dominant' larvae (Nemenyi post-hoc test with Tukey-Dist approximation, p-value = 0.03). Six replicated were conducted. In each replicate, each group was made up with the same amount (four to six) of fish.

IV.6. Discussion

This study identified a strong preference for the right eye when inspecting stimuli from either a mirror, conspecifics, or a predator. While a preference for the using the right eye, or the left hemisphere of the brain, to categorize familiar stimuli (*e.g.* same size conspecifics) has also been seen in another coral reef fish: *Myrispristis pralinia* (Roux et al. 2016), our results suggest that this preference also extends to unfamiliar stimuli. Larvae used in this experiment were naïve without any prior experience of non-larval reef fishes, therefore adult conspecifics and predators are

considered unfamiliar stimuli while mirror-images are considered as the image of a familiar conspecific (*e.g.* larva from the same larval cohort) (Sovrano 2004). The preference for the right eye is intriguing since it is opposite to the preference for the left eye generally observed during the inspection of mirror self-images in freshwater fishes and amphibians, and which conforms with the key role the right telencephalon hemisphere plays in social cognition (Vallortigara and Andrew 1994, 1991; Sargent et al. 1992; Hook-Costigan and Rogers 1998; Sovrano et al. 1999, 2001, 2016; Bisazza et al. 2002; Dadda et al. 2003; Sovrano 2004; Sovrano and Andrew 2006; Zucca and Sovrano 2008). The difference suggests that patterns of laterality could be associated with habitat preference (*e.g.* freshwater *vs.* sea water) or evolutionary history, and thus driven by ecological pressures or phylogenetic constraints. For instance, the preferential use of the right eye could reflect a greater need for settlement-stage *A. triostegus* to initially categorize new sensory cues upon recruitment rather than establishing social interactions and aggregations (Lecchini et al. 2007a).

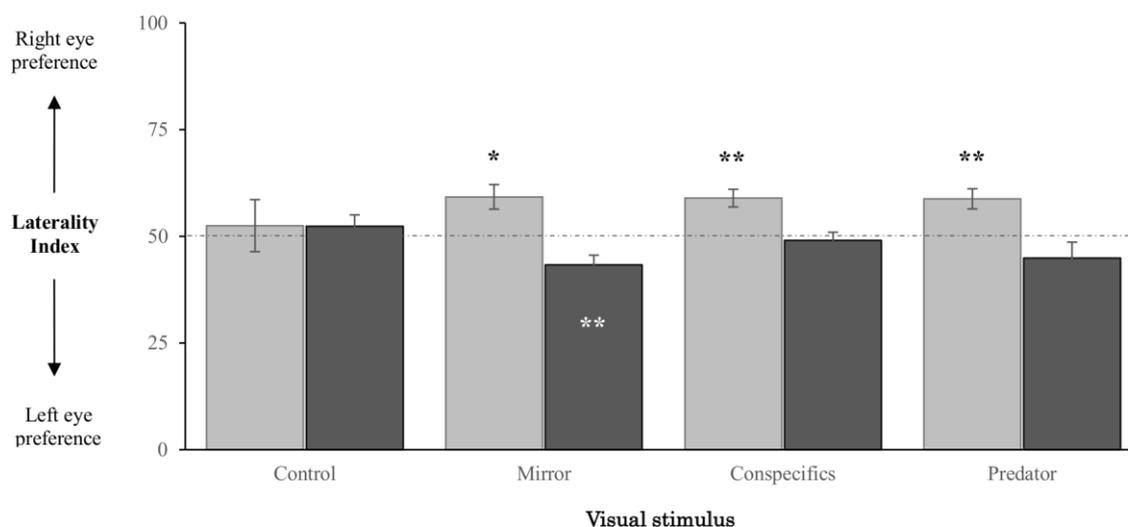


Fig. IV.4 | Effect of pesticides on the visual preference of *A. triostegus* larvae.

Figure represents LI mean (\pm SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, *: $p < 0.05$; **: $p < 0.01$) between Laterality Index (LI) values and the theoretical 50 % value (dotted line). Black asterisks above bars indicate a significant right-eye preference, while white asterisks below bars indicate a significant left-eye preference. 10 to 11 replicates (one fish per replicate) were conducted for each visual stimulus in both the pesticide exposition experiment (black bars) and the solvent-control exposition experiment (grey bars).

We also observed that larvae that displayed a left-eye preference when inspecting a predator held in an adjacent aquarium in Exp. 1, experienced higher survival when exposed to predation risk (Exp. 2). This result could be attributed to a learning process, with right-brain lateralized individuals able to recognize previously-experienced threats faster than left-brain lateralized individuals when this has been examined in other fishes (Bibost and Brown 2014). Left-eye dominant larvae could potentially escape predation more efficiently. Even if it is unknown if the right or left

eye dominated in Exp. 2 itself, it has been shown that an eye preference displayed during the first observation of a predator (as in Exp. 1) can influence behavioral responses (e.g. subsequent eye preference, turning rates) when facing for a second time that same, no longer unknown, stimulus (Cantalupo et al. 1995; Miklosi et al. 1997). Therefore, after the Exp. 1 conditioning, the left-eye users may adopt a behavioral response to predation that differs from the majority of their right-eye user and not-lateralized conspecifics and that could favor their survival (e.g. by disorienting predators or by moving differently within their environment such as seen in mosquitofish (De Santi et al. 2001)). Overall, our results strongly support the “brain's right hemisphere” theory that predicts that the right-eye (i.e. left-hemisphere) is predominantly used to categorize stimuli, while the left-eye (i.e. right hemisphere) is dedicated to attending to events that require rapid behavioral responses, such as predation (MacNeilage et al. 2009; Rogers et al. 2013). In the context of larval settlement, where individuals face a variety of novel sensory cues and constantly high predation risk, our study suggests that there is a survival advantage to being lateralized, with the ability to optimize survival overcoming the obvious disadvantages of displaying predictable asymmetric behaviors. Future experiments brain lateralization in coral reef fishes should include juveniles that have been allowed to contact with older conspecifics, or that are no longer predator naïve, in comparison to others held in solitary confinement, to better understand how asymmetric behavioral patterns are adopted by a population and the importance of learning and selection in lateralization processes.

Our results show that the presence of chlorpyrifos (CPF), one of the most common waterborne pesticides encountered in coral reefs (Botté et al. 2012), can completely diminish or reverse critical lateralization patterns (Fig 4). Oceanic levels of chlorpyrifos contamination have only been assessed, to our knowledge, in the Southern Ocean, revealing levels up to 0.54 pg.l⁻¹ (Bigot et al. 2016). This is much lower than the levels observed in Australian reef surface waters, where contamination have been shown to reach 1 µg.l⁻¹ (NRA 2000), or than in certain Australian and Malaysian river mouths, where contamination levels can reach up to 26 µg.l⁻¹ (NRA 2000; Leong et al. 2007). The concentration of 1 µg.l⁻¹ is therefore ecologically relevant and reflects appropriately the change of pesticide concentration larval fishes can face when settling, from the open ocean, in coral reef coastal areas (NRA 2000; Leong et al. 2007; Botté et al. 2012; Bigot et al. 2016). This loss and reversal of lateralization in fish exposed to chlorpyrifos may further increase larval mortality and impact the subsequent recruitment and maintenance of reef ichthyologic diversity, as evidenced in Exp. 2 (Fig 3). Similar findings have been documented in the coral reef fish *Pomacentrus wardi* when juveniles were exposed to future ocean acidification conditions (Domenici et al. 2012). In addition, a turning bias, shifting from the right to the left, was observed in *P. wardi* populations exposed to elevated-CO₂ concentrations (930 µatm) (Domenici et al. 2014). Lastly, the same study also revealed how increased temperatures (+ 3°C higher than ambient temperatures) can have a dampening effect on lateralization processes (Domenici et al. 2014). In the context of global degradation

of coral reefs, it is striking to observe that pesticide pollution, oceanic acidification, and elevated water temperatures could have similar and potentially interactive effects in impairing lateralized behaviors of marine organisms and subsequent survival to predation, particularly in early developmental stages. With this study, we demonstrate how the worldwide increasing influx of pesticide onto coral reefs, due to expanding coastal agriculture, as well as changing patterns of land use upstream (Carvalho et al. 2002; Islam and Tanaka 2004; Laabs et al. 2007; Leong et al. 2007; Lewis et al. 2009; Packett et al. 2009; Shaw et al. 2010; Roche et al. 2011; Kroon et al. 2012), may lead to a loss or reversal of visual lateralization during a critical step of coral reef fish life cycle. This may potentially lead to greater larval mortality rates and reduce subsequent recruitment success. In addition, any potential reduction in the abundance of major herbivorous fishes in coastal marine ecosystems, such as *A. triostegus* in coral reefs (Randall 1961), due to chemical pollution from organophosphate components (Fulton and Key 2001), could impair the ability of reefs to cope with increasing macro-algal abundance and impact ecosystems recovery and resilience (Hughes et al. 2007; Ledlie et al. 2007).

While coral reefs are experiencing increasing perturbations (Hughes et al. 2014), persistence of species in degraded areas rely on the 'rescue' effect of recruitment (Doherty 2002). The potential for a population to be supplemented by recruits depends largely on pelagic larvae detecting an appropriate habitat, towards which they orientate in order to settle and persist (Doherty 2002). Successful recruitment of individuals could be partly jeopardized by the alteration of lateralized cognitive abilities of larvae caused by local stressors such as pesticides. An understanding of the underlying mechanisms of these physiological and behavioural disturbances requires further investigations.

IV.7. Materials and Methods

Sampling and study site

Fish larvae were collected nightly from January to June 2016 using a crest net set on the north-east coast of Moorea Island, French Polynesia (17°29'52.19"S, 149°45'13.55"W). This crest net had a mesh size of 1 mm and was equipped with a rectangular mouth (width: 2 m, height: 1 m) oriented perpendicular to the water flow. Crest nets retain all settlement-stage larvae at the site as they move over the reef crest (Dufour and Galzin 1993; Dufour et al. 1996; Lecchini et al. 2004; Lo-Yat et al. 2011). This sampling technique ensures a non-selective capture (as opposed to light-traps) of wild larval fishes, precisely at the settlement stage, with no prior reef experience (as opposed to the capture of post-settlement juveniles within reefs using light traps or nets) (Besson et al. 2017b). While this method also permits the study of wild individuals caught *in situ* (as opposed to aquarium reared larval fish), it however does not ensure that the oceanic experience was the same for all larvae. As larval fish primarily recruit to reefs at night (Leis and McCormick 2002), the net was set up at 7 p.m. and larvae were collected at 6 a.m. the following day.

The convict surgeonfish, *Acanthurus triostegus* (Linnaeus, 1758) has a pelagic larval duration of 53 ± 8 days, after which larvae metamorphose and recruit in shoals on shallow sandy and rubble reef areas (Randall 1961; McCormick 1999; Frédérick et al. 2012). This species was chosen as our model due to the high number of larvae collected in the crest net ($n = 278$ larvae, size: 23 ± 2 mm). After removal from the crest net, larvae were transferred to CRIOBE research station where they were maintained in individual aquaria filled with UV-sterilized and filtered (10 μ m filter) seawater before being tested. Water temperature was maintained at 28.5°C, under 12:12 LD cycle (06:00, onset – 18:00, offset). For Exp. 1 and Exp. 2, tests were conducted immediately following collection (within 12 hours). Exp. 3 tests were conducted on the fifth day post-collection. Throughout their entire time in captivity fish were fed a commercial dry food twice daily.

Exp. 1: Do *A. triostegus* larvae exhibit a left-right visual preference when inspecting either negative (predator or positive (mirror self image, conspecific) stimuli?

Preferences for using the left or right eye to inspect visual stimuli was assessed using a protocol adapted from studies of brain lateralization in freshwater fishes (Sovrano et al. 1999, 2001; Sovrano 2004; Sovrano and Andrew 2006). The experimental aquarium was a rectangular glass tank (20 L x 11 W x 15 cm H) with all sides covered with white opaque screen except one of the long sides where stimuli were presented. The tank was lighted with a neon lamp (45 W) and filled with UV-

sterilized, filtered (10 µm filter) seawater to 15 cm depth. A video camera was mounted above the experimental tank to record the fish position.

Four types of visual stimuli were presented to *A. triostegus* larvae:

(i) Control: a white opaque screen was displayed on the transparent wall, so that all walls of the experimental tank were covered with white opaque screens. This control was conducted to account for any effect of the experimental procedure on fish behavior.

(ii) Mirror: a mirror was placed along the transparent wall, presenting larvae with a visual stimulus of their own reflection.

(iii) Conspecific: an aquarium (30 x 20 x 20 cm) containing juvenile conspecifics (n = 5, size: 80 ± 4 mm) was placed against the transparent wall. The conspecifics were collected with hand nets in shallow sandy areas surrounded by corals at Moorea Island.

(iv) Predator: similarly to (iii), a common reef predator of *A. triostegus* (Besson pers. obs.), *Lutjanus fulvus*, (n = 1, size: 162 mm) was presented in an aquarium placed against the transparent wall of the experimental aquarium.

For each stimulus, 14 biological replicates were conducted (one larva per replicate). Between each replicate, all water in the experimental tank was replaced. For each replicate, the use of the right or left monocular visual field (*i.e.* eye use) was assessed every two seconds for a total of 10 min. Discrimination between the use of the right or the left eye was determined by examining the angle between larva's body orientation and the transparent wall (where the visual stimulus was presented, Fig. 1), following the method established by Sovrano et al. (Sovrano et al. 1999) and used in various eye lateralization studies since (Sovrano et al. 2001, 2005, 2016; Sovrano 2004; Sovrano and Andrew 2006). If the fish was perpendicular to the transparent wall (*i.e.* binocular stimulation), or if its body orientation to the transparent wall formed an angle larger than 180° with respect to the transparent wall (*i.e.* fish looking in the opposite direction), no eye preference was recorded (Sovrano et al. 2001, 2016) (Fig. 1). In this study, binocular stimulation never occurred (in all three experiments). Moreover, when a visual stimulus was presented, the times when an eye use preference could not be established (because of the fish looking in the opposite direction of the stimulus) only represented 24.01 ± 2.25 % of the observations in Exp. 1, 23.78 ± 5.82 % of the observations in Exp. 2, and 24.20 ± 2.02 % in Exp. 3. Therefore, when examining a visual stimulus, fishes spent 75 % of the time examining this visual stimulus with either their left or their right eye, and the rest of the time looking in the opposite direction, but never examining the stimulus with both eyes. Observations of the left-eye or right-eye use (Fig. 1) resulted in a lateralization index (LI), reflecting relative eye use: $LI = [(frequency\ of\ right\ eye\ use) / (frequency\ of\ right\ eye\ use + frequency\ of\ left\ eye\ use)] \times 100$ for each stimulus tested (Sovrano et al. 1999). Values significantly higher than 50 % indicate a preference for the right eye, whereas values significantly lower than 50 % indicate a preference for the left eye. For each stimulus, the difference between the measured LI and a 'no eye preference' case (LI = 50 %) was tested using a two-tailed Mann-Whitney U test, in order to identify any potential eye

preference. Further statistical analyses to compare LI values between each stimulus were carried out by non-parametric analysis of variance (Wilcoxon rank sum test and Kruskal-Wallis test). For all stimuli, no significant difference was observed between the first 5 min of the trial and the last 5 min of the trial (Kruskal-Wallis test, $\chi^2 = 92.8$, $df = 91$, $p\text{-value} = 0.42$). To verify that our modified protocol (one single transparent window) did not introduce any behavioral bias compared to the more commonly used protocol (two transparent windows equipped with two mirrors), we performed an additional two-mirror stimulus where an aquarium was equipped with two transparent walls and two mirrors (Sovrano et al. 1999, 2001; Sovrano 2004). No significant difference was found when comparing LI values obtained in this two-mirror protocol and the values obtained with the use of the one-mirror protocol (respectively $LI = 59.8\%$ and 57.3% , Wilcoxon rank sum test, $W = 105$, $n = 14$, $p\text{-value} = 0.769$), therefore we assume that our adapted method provides results comparable to conventional methods. All statistical analyses were conducted using the R-Cran software (R-3.3.1).

Exp. 2: Ecological importance of brain lateralization in larvae facing predation

Using a similar protocol as Exp. 1, larvae were tested individually to determine if they preferentially used the right eye, left eye, or displayed no lateralization when presented with a predator. Larvae were then classified and pooled into three groups according to their visual preference: 'left-eye dominant', 'right-eye dominant' and 'no eye dominant' individuals. Each group was tagged with a different color, using visible implant fluorescent filament (Northwest Marine Technology), at least one hour before being released simultaneously into a caged patch reef (1x1x1 m), built from rubble and live coral, and located in an open sandy area at 2 m depth. After a one hour habituation period, three predators, *Lutjanus fulvous* ($n = 3$, size: 165 ± 14 mm), were introduced into the cage. Prior to their introduction, the predators were held for 48 h in aquaria without food to ensure that they were motivated to feed. The experiment was stopped after two hours, predators were removed and the remaining larvae were counted and identified according to their color tag. The overall survival rate was recorded, as well as a survival rate for each group. For each group, a survival index (SI) was calculated: $SI = \text{survival rate of the group} - \text{overall survival rate in the cage}$. SI values allowed a comparison between the survivals of each replicate, regardless of the relative predation rate in each technical replicate. This experiment was replicated 6 times, and each time a different tag color was attributed to the groups to account for any potential predation bias towards one color. For each replicate, all larval groups contained the same number of fish (between 4 and 6 larvae per group depending on the replicate). Statistical analyses to compare SI values were conducted by using non-parametric analysis of variance (Wilcoxon rank sum test and Kruskal-Wallis test, followed by Nemenyi post-hoc test should a significant difference be detected).

Exp. 3: Effect of chemical pollution on the visual lateralization of *A. triostegus* larvae

Preliminary experiments were conducted on three groups of $n = 10$ fish maintained in 30 L x 20 W x 20 cm H aquaria, which were exposed, after collection in the crest net, to chlorpyrifos (CPF) at 1, 30 and 100 $\mu\text{g.l}^{-1}$, as previously done in the literature in another coral reef fish (Botté et al. 2012). After 10 days of exposure, larvae at 30 $\mu\text{g.l}^{-1}$ presented an irregular swimming behavior and most larvae at 100 $\mu\text{g.l}^{-1}$ died after only one day of exposure. Larvae exposed to the 1 $\mu\text{g.l}^{-1}$ concentration exhibited no behavioral abnormality and no mortality. Consequently, we tested the effect of a CPF pollution on larvae visual lateralization through two experimental conditions: **(A) solvent control** (9 liters of UV-sterilized and filtered - 10 μm filter – seawater + 9 μl of acetone) and **(B) 1 $\mu\text{g.l}^{-1}$ of CPF** (9 liters of UV-sterilized and filtered - 10 μm filter – seawater + 9 μg of CPF diluted in 9 μl of acetone).

For each condition, larvae were tested for their eye preference as in Exp. 1, when presented with the same visual stimuli as in Exp. 1 (control, mirror, conspecifics and predator), after a five day exposure (to CPF or to the solvent). Consequently, we performed $n = 8$ (2×4) experiments (control, mirror, conspecifics and predator visual stimulus with fish exposed to solvent *vs.* fish exposed to CPF at 1 $\mu\text{g.l}^{-1}$). For each of those 8 experiments, *A. triostegus* larvae were maintained, after collection in the crest net, in groups of $n = 5$ to 6 fish in 30 L x 20 W x 20 cm H aquaria. Each aquarium was equipped with an air stone. All water was replaced every day, ensuring the maintenance of water quality as well as a continuous concentration of the pesticide or solvent. A second technical replicate for these 8 experiments, with significantly similar results as in the first replicate, was performed. Consequently $n = 10$ to 11 biological replicates were conducted for each of these 8 experiments. Statistical tests for LI values analyses were performed as described in Exp. 1.

Ethical approval

All the experiments were approved by the CRIOBE-IRCP animal ethics committee and performed in accordance with the guidelines of the French Polynesia committee for publication and animal ethics.

IV.8. Additional information

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Author Contributions

M.B., C.G., V.A.S. and D.L. designed the experiments. M.B., C.G., F.B., R.M.B., V.A.S., D.L.D. and D.L wrote the article. M.B., C.G., N.R., F.B., H.J. and C.B. captured the fish and performed the in situ and in aquaria experiments. M.B and C.G. performed the statistical analyses.

Competing financial interests

The authors declare no competing financial interests.

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V. Complete and rapid reversal of the body color pattern in juveniles of the surgeonfish *Acanthurus triostegus* at Moorea Island (French Polynesia)

V.1. Foreword

I think one of the best part of science, and ecology in particular, is to have the chance to observe *in situ* an intriguing and original phenomenon, especially when it goes against our beliefs.

The convict surgeonfish *Acanthurus triostegus* is the model species of my PhD and has been largely described in the four first chapters. This species is remarkable for its gregarious behavior as juveniles and adults, and exhibit a relatively simple pigmentation pattern: black vertical bars on a whitish body. One of the most striking parts of *A. triostegus*'s metamorphosis is the rapid pigmentation of the initially transparent larva. I have been looking at thousands of *A. triostegus* early-life-stage individuals while observing their pigmentation along metamorphosis, with always this consistent acquisition of black vertical bars in the first few hours after settlement, followed by the whitening of the rest of the body in a couple of days post-settlement. Therefore, my surprise was considerable when I observed in some of my aquaria young juveniles with the complete opposite pigmentation pattern: white vertical bars on a blackish body, and exhibiting an unusual aggressive and territorial behavior. I couldn't find anything related to that in the literature, and I made this observation several times, on different individuals, from different larval cohorts. I also checked and confirmed that such a pigmentation pattern can be found *in situ*, allowing me to think that this observation is somehow not specifically related to an aquarium stress.

To me, the other best part of science is probably to try to elucidate such intriguing phenomena, for example by deciphering their ecological relevance and understanding their mechanism. I really see that as the perfect mix between discovery, science, and ecological relevance, resulting in some sort of exciting challenges.

This fifth chapter is therefore different from the three previous ones, as it is not a greatly elaborated research related to metamorphosis or pollutants during recruitment, but rather an observation of a counterintuitive and original phenomenon during larval recruitment in *A. triostegus*. This led me to discuss with Dr. P. Salis about the potential cellular mechanisms underlying this particular patterning and the fact that individuals can switch from one pigmentation to the other in less than a second. This collaboration resulted in a first description of this original phenomenon, and we have published it in *Coral Reefs* (2017-10-22). Three videos accompany this manuscript in the version submitted to *Coral Reefs*. These videos are available on demand by contacting me. This manuscript is available in Appendix 7.

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V.2. Manuscript information

Title: Complete and rapid reversal of the body color pattern in juveniles of the surgeonfish *Acanthurus triostegus* at Moorea Island (French Polynesia)

Authors: Marc Besson ^{1,2,*,#}, Pauline Salis ^{2,*}, Vincent Laudet ^{2,£}, David Lecchini ^{1,3,£}

Affiliations:

1 PSL Research-University: EPHE-UPVD-CNRS, USR3278-CRIOBE, BP1013, 98729 Papetoai, Moorea, French Polynesia

2 UPMC, CNRS-UMR7232-OOB, 1 Avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France

3 Laboratoire d'Excellence "CORAIL", BP 1013, 98729 Papetoai, Moorea, French Polynesia

* equal first authors

£ equal last authors

corresponding author, email: marc.besson@ens-lyon.org, Tel: +33(0)4 30 19 24 05

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V.3. Abstract

Color polymorphism is widespread in animals and can be associated with temporary adjustments to environmental variables (polyphenism). In teleost fishes, one of the most remarkable color polyphenism is the background-adaptation in flatfishes. However, such rapid and complete body color and pattern changes remain relatively unexplored in other species. In coral reef fish, *Acanthurus triostegus* is a gregarious species whose body color pattern consists of black vertical stripes on a whitish body. Here, we describe an entirely reverse body color pattern (white vertical stripes on a blackish body) that we observed in some *A. triostegus* juveniles in a nursery area at Moorea Island, French Polynesia. In aquaria, we determined that change from one color morph to the other is rapid, reversible, and corresponds to a color polyphenism associated with an unexpected aggressive and territorial behavior.

V.4. Introduction

The occurrence of two or more distinct and genetically influenced color morphs within a single interbreeding population, *i.e.* color polymorphism, is widespread across the animal kingdom (Endler 1980; Olendorf et al. 2006; Gray and McKinnon 2007). At the individual level, body colors and patterns (*i.e.* distributions of at least two colors across the body) can also be adjusted in response to environmental factors such as habitat background, temperature, food availability, population density and level of predation risk (for review, see Leimar 2007). This environmentally-influenced color plasticity within individuals of the same genotype is termed color polyphenism, and is highly adaptive when facing changing and selective environments (Gray and McKinnon 2007).

Color polyphenism has been particularly acknowledged in teleost fishes, and is well exemplified by the background-adaptation (camouflage) in flatfishes (Ramachandran et al. 1996; Höglund et al. 2002; Kelman et al. 2006). Body color and pattern adjustments can also be associated with temporary social status (Beeching 1995; Höglund et al. 2002), sexual display (Kodric-Brown 1998; Rhodes and Schlupp 2012), or response to a predation risk (Fuller and Berglund 1996). Changes in body colors and patterns are regulated by a variety of genetic, endocrine and cellular processes such as distribution, morphology, and density of pigment organelles and chromatophores (Fujii and Novalés 1969; Sugimoto 2002; Olendorf et al. 2006; Miller et al. 2007). These changes can be fixed or reversible, and can occur within a few weeks or a few seconds, representing long-term or rapid adaptations to the environment (Sugimoto 2002; Sköld et al. 2013). For example, the dusky dottyback (*Pseudochromis fuscus*) can change its body coloration from yellow to brown and vice versa within two weeks, depending on the reef location and the type of prey available (Messmer et al. 2005; Cortesi et al. 2015), while the paradise whiptail (*Pentapodus paradiseus*) can switch its head and body reflective stripes from blue to red in less than one second for presumed communication matters (Mäthger et al. 2003).

Apart from the complex body color and pattern changes observed during the background-adaptation in flatfishes and agonistic interactions in cichlid fishes (Beeching 1995), rapid changes (within a few seconds) are generally discrete (*e.g.* apparition or change of a stripe's color) and relatively unexplored in other fish species (Mäthger et al. 2003; Rhodes and Schlupp 2012; Watson et al. 2014). In coral reef fishes, while long-term (within a few days or weeks) color and pattern changes have been described during ontogeny (Frédérich et al. 2010b), sexual maturation (Kodric-Brown 1998), and mimicry strategies (Cortesi et al. 2015), rapid changes remain largely unknown (Mäthger et al. 2003; Watson et al. 2014).

The convict surgeonfish *Acanthurus triostegus* is a common herbivorous reef-fish in the Indo-Pacific region (Randall 1961). Adults are encountered around reef patches close to the barrier reef where they live in large aggregations mostly feeding on turf

algae (Randall 1961). The color pattern of reef-associated juveniles and adults consists of black vertical stripes on a whitish body (referred here as 'classical color pattern', CP, Fig V.1A) (Randall 1961; Frédérich et al. 2012). Here, in a nursery area of Moorea Island (Lecchini et al. 2005b), in French Polynesia, we observed some *A. triostegus* juveniles exhibiting a complete reversal of their color pattern: white vertical stripes on a blackish body (referred here as 'reversal of the color pattern', RP, Fig V.1B) and chasing their conspecifics. The aim of this study is to better characterize this RP color morph and to determine if it is associated with behavioral and/or developmental characteristics and corresponds to a color polyphenism. To do so, we collected large cohorts ($n > 50$) of reef-naïve *A. triostegus* larvae while they colonized the reef at Moorea Island, and raised them, in aquaria, until the appearance of the RP pattern. We demonstrated that the CP to RP color morph change is rapid and reversible, and corresponds to a color polyphenism associated with an unexpected territorial behavior.

V.5. Materials and Methods

Fish sampling and raising

Fish larvae were collected nightly in March 2015 using a crest net on the north-east coast of Moorea Island, French Polynesia (17°29'52.19"S, 149°45'13.55"W), close to the nursery area where RP juveniles were observed. The crest net had a mesh size of 1 mm and was equipped with a rectangular mouth (width: 2 m, height: 1 m) oriented perpendicular to the water flow. The crest net retains all settlement-stage larvae as they move over the reef crest and enter the net (Dufour and Galzin 1993). As larval fish primarily recruit to reefs at night, the net was set up at 7 p.m. and larvae were collected at 6 a.m. the following day. Three large cohorts of *A. triostegus* larvae were collected consecutively ($n = 53, 60$ and 69 respectively). After removal from the crest net, individuals were transferred to the CRIOBE research station where they were maintained, with only their respective larval cohort, in a 200 liters aquarium in an open water circuit (sea water was pumped from the lagoon area on the north shore of Moorea). Water temperature was maintained at 28.5°C, under 12:12 LD cycle (06:00, onset – 18:00, offset), and fish were fed turf algae using live rocks and rubbles that were changed daily. At the end of the study, all CP and RP fish were released in the nursery area close to their collection site.

Juveniles' body color pattern, developmental stage, and behavior

CP pattern was defined as black vertical stripes on a whitish body (Fig V.1A) (Randall 1961; McCormick 1999), while RP pattern was defined as the opposite color pattern: white vertical stripes on a blackish body (Fig V.1B). In the three cohorts studies, color morph respective prevalence (*i.e.* percentage of CP and RP fish in each

of the three cohort) was assessed when RP juveniles were first observed, at day 7 post-settlement (Fig V.2A). The developmental stage of CP (n = 14) and RP (n = 10) fish was also assessed at that time, by measuring individual body depth (body height divided by standard length), as it is a good indicator of post-settlement development in *A. triostegus* (McCormick 1999; McCormick et al. 2002). Body depth measurements were then compared between RP and CP, as well as with measurements on *A. triostegus* juveniles from day 0 to day 11 post-settlement obtained in parallel to this study (raised in smaller groups of 20 individuals in lagoon *in situ* cages) (Fig V.2B). Lastly, aggressiveness of CP (n = 24) and RP (n = 24) fish was assessed at day 7 post-settlement, by measuring the percentage of time each spent with their dorsal fin erected (Fig V.2C), as evidenced in zebrafish and in a cod species (Brawn 1960; Gerlai 2003). A high percentage of time with the dorsal fin erected was considered as an aggressive and territorial behavior.

V.6. Results and Discussion

At day 7 post-settlement, 13.3 ± 2.5 % of *A. triostegus* juveniles had turned their color pattern from CP to RP (Fig V.1A-B, Fig V.2A). This color morph change can occur within a few seconds (Supplementary Video 1), and is also reversible, as RP juveniles turned instantly to CP and mixed up with the CP shoal when disturbed by a net or by the addition of a new rubble in the aquarium (Besson pers. obs.). This confirms that the CP to RP transition corresponds to a reversible, complete and rapid color pattern change. No difference in body depth was observed between CP and RP juveniles (Wilcoxon rank sum test, $W = 67$, p-value = 0.886) (Fig. V.2B). For both color morphs, the body depth did not exhibit any delay or advance compared to the body depth variations observed in *A. triostegus* juveniles that we raised *in situ* in smaller groups in parallel to this study (Fig V.2B). This suggests that the differences between the two morphs are not related to an ontogenetic difference. Actually, individuals turning from CP to RP were not predictable or did not show any prior detectable phenotypic difference. RP color morph was initially observed *in situ* in a dense nursery area of Moorea Island (Lecchini 2005), and was confirmed in aquaria in large juvenile groups (originating from the same larval cohort) during periods of intense foraging and decreasing resource availability. Introducing large amounts of turf algae and diminishing the number of juveniles in the aquarium delayed and even prevented the apparition of the RP color morph (Besson pers. obs.). Altogether, this suggests that this color plasticity corresponds to a color polyphenism, and that conditions of high juvenile density and low resource availability may be responsible for the apparition of the RP color morph.

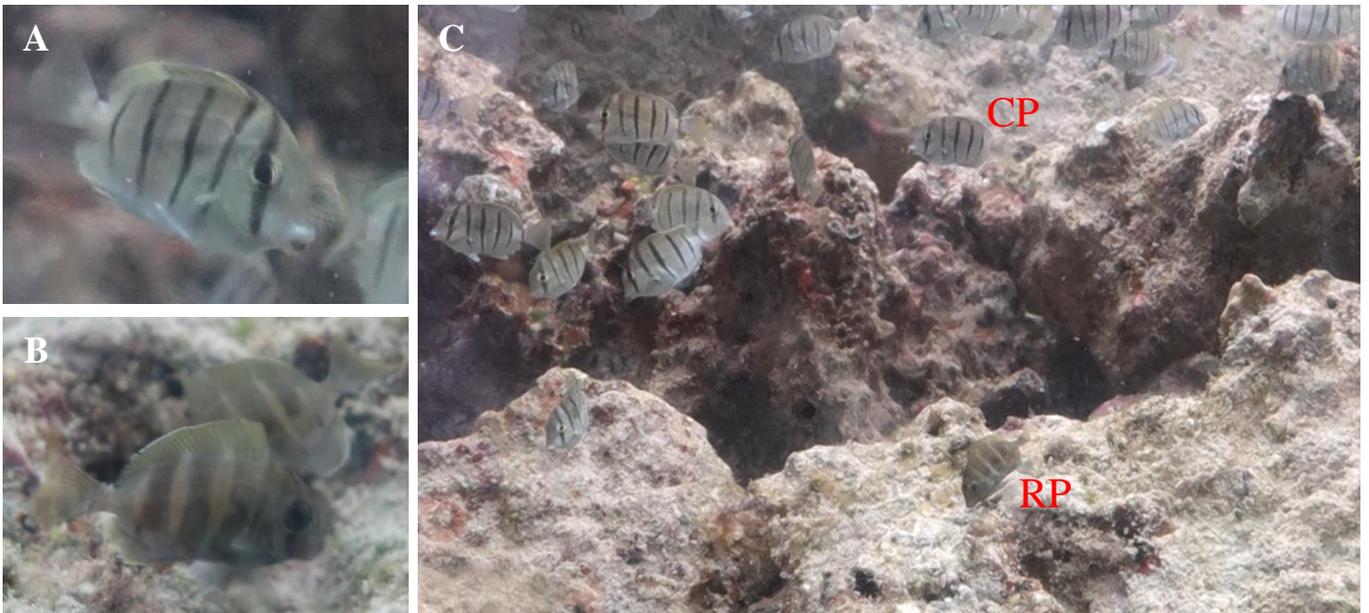


Fig. V.1 | Body color patterns and community organization of *Acanthurus triostegus* juveniles.

A: 'Classical color pattern' (CP) of a convict tang juvenile at day 7 post-settlement. **B:** 'Reversal of the color pattern' (RP) of two juveniles also erecting their dorsal fin. **C:** Shoal and community organization of CP (top) and RP (bottom-central) juveniles.

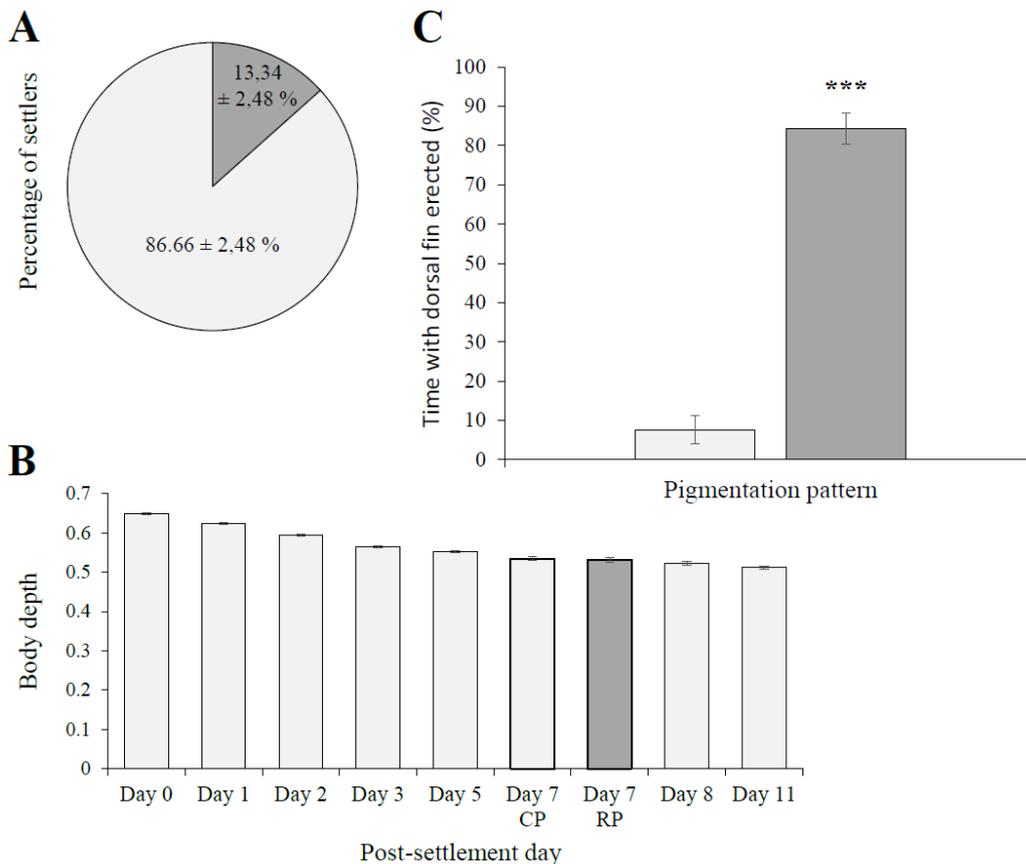


Fig. V.2 | Color morph ontogenetic stage, prevalence, and behavior of CP and RP juveniles at day-7 post-settlement.

Light gray: CP juveniles; Dark gray: RP juveniles. **A:** Color morph relative prevalence. **B:** Body depth (body height / standard length) of *A. triostegus* along settlement (day 0 to day 11), and of day 7 post-settlement (bold lines) CP and RP juveniles. **C:** Percentage of time spent with the dorsal fin erected in CP and RP juveniles. Errors bars indicate standard errors. ***: p-value < 0.001.

Interestingly, RP color morph was associated with a unique solitary, aggressive, and territorial behavior, with RP juveniles chasing their conspecifics (Supplementary Videos 1-3). This was exemplified by the significant difference between CP and RP juveniles in the percentage of time spent with the dorsal fin erected (7.6 ± 3.6 vs. 84.3 ± 4.0 % respectively, Wilcoxon rank sum test, $W = 569$, p -value < 0.001 , Fig V.2C), which illustrates the aggressive behavior of RP individuals (Brawn 1960; Gerlai 2003). Consequently, the presence of the RP color morph resulted in a newly-organized fish community, with RP juveniles defending their territory while CP juveniles swam or foraged in groups above these guarded areas (Fig. V.1C, Supplementary Videos 2-3). Therefore, the CP to RP transition is a rare observation of a color morph change involving a rapid and complete reversal of the body color pattern that is not associated with camouflage (Ramachandran et al. 1996; Höglund et al. 2002; Kelman et al. 2006; Sköld et al. 2013; Watson et al. 2014). Besides, the RP color morph is also not related to a stressed or nocturnal pigmentation, which actually consists of the CP pattern with black rectangles between each stripes (see V. Supplementary Fig. 1). Actually, this is reminiscent of the situation in the cichlid fish *Astronotus ocellatus*, where agonistic interactions between males can lead to a rapid color pattern change (lightening of the body color and apparition of white vertical stripes) in individuals defeated in combat (Beeching 1995).

At the cellular level, body color and pattern changes can be divided in two categories: morphological and physiological color changes (MCC and PCC) (Fujii and Novalés 1969; Leclercq et al. 2010). MCC often result from long-term changes in the distribution, morphology, and density of chromatophores (pigment cells) while PCC originate from rapid motile movements of pigment organelles (*e.g.* melanosomes) within the chromatophores (Sugimoto 2002; Leclercq et al. 2010; Sköld et al. 2013). Here, the rapidity of the CP to RP color morph change suggests that the color and pattern changes result from a PCC, and that condensation/dispersion of melanosome in the melanophores may be responsible for the rapid and reversible black/white color reversal (Zhu and Thomas 1997). However, a cellular study of this process needs to confirm this hypothesis, supposing that one can sample the RP juveniles before they turn back their pattern to CP.

This study demonstrates color polyphenism in juveniles of *A. triostegus*, revealing fish ability to switch from one color pattern to its complete opposite within a few seconds. RP color morph was not observed in adults, suggesting that this color polyphenism may result from a juvenile-specific adaptive behavior to force some individuals to quit the cohort in case of low resource availability.



V. Supplementary Fig. 1 | Stress and nocturnal color pattern in *Acanthurus triostegus* juveniles.

V.7. Additional information

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Author Contributions

M.B. designed the experiments, and collected and analyzed the data. M.B., P.S., V.L., and D.L wrote the article.

Competing financial interests

The authors declare no competing financial interests.

V.8. References

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VI. General discussion, perspectives and conclusion

VI.1. Coral-reef fish larval recruitment is a TH-controlled metamorphosis

In this thesis, I have investigated the morphological, physiological, and behavioral changes occurring during *A. triostegus* recruitment. In particular, this research brings a new understanding of the changes encountered in the whole digestive system, from the dentition to the intestine remodeling, along with a precise description of the shifts in 16S and 18S gut content. Furthermore, this thesis also provides a first characterization of *A. triostegus* sensory system and their maturation during recruitment. There is a substantial amount of studies that have described other and complementary morphological features in *A. triostegus* recruitment (McCormick 1999; McCormick et al. 2002; Frédérick et al. 2010a, 2012). However, none of them have related these transformations and developmental processes to any endocrine system. Coral-reef fish metamorphosis has been known for more than seventy years (Breder 1944). Given the similarities between the metamorphoses of coral-reef fish and other non-reef teleost fishes (McMenamin and Parichy 2013), the involvement of thyroid hormones (TH) was expected. Moreover, first evidences of their role was provided through the study of pigmentation and growth in larval coral reef fishes in aquaculture (de Jesus et al. 1998; Trijuno et al. 2002). However, these studies did not permit deciphering the potential coupling between recruitment to the reefs (ecological event) and metamorphosis (endocrinological event), using wild captured larvae. Moreover, data from other teleosts are scarce and there is no general framework to understand the role of environment in teleost metamorphosis and how this event integrates in the fish ecology. Here, this research demonstrates that TH levels surge in larvae prior to reef entry, and decrease once they settle in the reef. This pattern has been observed not only in *A. triostegus*, but also in phylogenetically distant coral-reef fish species (e.g., *Chromis viridis* and *Rhinecanthus aculeatus*). As these TH profiles are reminiscent of the situation in amphibians and other non-reef teleost fishes, and given that the changes undergone by *A. triostegus* larvae at recruitment are controlled by these TH, this work provides the first evidence that larval recruitment of coral-reef fish corresponds to a TH-controlled metamorphosis.

Species-specific variations on this general theme could be observed, as this research did not demonstrate this pattern in all coral-reef fish families. To date, this project has examined TH profiles at recruitment in six species: *A. triostegus*, *C. viridis*, *R. aculeatus*, *Chaetodon lunula*, *Ostorynchus angustatus* and *Platax orbicularis*. TH level patterns were similar in these six species (i.e. TH surge before reef entry, TH decrease after reef entry), although the capture of oceanic larvae was not possible for some of them. The batfish species, *P. orbicularis*, was not sampled in the wild but in an aquaculture facility in Tahiti. While a peak of TH was observed at day 12 post hatching (corresponding to the time when most morphological changes are observed), further confirming the involvement of TH in *P. orbicularis* metamorphosis, this result was not included in this thesis as it could not be related to larval recruitment (as animals were raised and fed artificially). In a near future, TH profiles at recruitment of at least five more species (*Zebrasoma scopas*, *Zebrasoma veliferum*, *Ctenochaetus striatus*, *Canthigaster*

bennetti and *Epinephelus socialis*) will be available. This will provide an extraordinary opportunity to study how variations in the magnitude of TH changes can be shaped by evolution and species-specific life history traits such as pelagic larval duration, pigmentation status at settlement (as some species' larvae colonize the reef fully pigmented while others are still transparent) and size at settlement (as some species are very small at recruitment, compare to the size of the adults, *e.g.* Serranids, while others are much bigger, *e.g.* Mullids) (McCormick et al. 2002). Given the considerable diversity of life history strategies they propose, coral reef fish could be excellent models to decipher how recruitment strategies are elicited by changes in hormonal systems. Interestingly, this is reminiscent of the situation in stickleback, where differences in TH signaling between two ecotypes support life history trait variations (Kitano et al. 2010; Kitano and Lema 2013).

VI.2. Coral-reef fish sensory ecology at recruitment

One of the key factors influencing survival at recruitment is settlement-site selection, with larval fishes using acute sensory systems to locate appropriate benthic habitats (Lecchini et al. 2007a; Mitchell et al. 2011; Brooker and Dixson 2016). As coral-reefs are rapidly degrading globally due to anthropogenic activities (Hughes et al. 2003; Hoegh-Guldberg et al. 2007), a large number of studies have examined how larval sensory abilities (*e.g.*, recognition of olfactory cues from conspecifics or predators) are impaired by factors such as global change and water pollution (Dixson et al. 2014; Johansen et al. 2017; Lecchini et al. 2017). However, to date no study has integrated molecular, anatomical, and behavioral approaches to identify the processes that impair larval sensory ecology and subsequent survival. In this context, this thesis brings first insights on the potential coupling between sensory organ maturation and subsequent sensory abilities. It demonstrates how an impaired sensory system maturation is correlated with **(i)** a limitation in individual's ability to identify and distinguish between critical sensory cues during recruitment, and **(ii)** reduced survival rates when facing predation. This is a very high topic in coral reef fish sensory ecology, as many efforts are devoted to examine the mechanisms by which larvae locate and select their settlement reef habitat (Dixson et al. 2014; Brooker and Dixson 2016; Johansen et al. 2017; Jutfelt et al. 2017; Lecchini et al. 2017). This thesis has evidenced that TH are key mediators for the maturation of sensory organs in *A. triostegus*. Further investigations are required to properly examine these maturation processes and their relevance in sensory abilities. For example, only variations among the different retina cell densities were examined during this thesis, while it would be more informative to compare ratios of densities between cells perceiving the visual signals (*e.g.* photoreceptors) and those that integrate these signals (*e.g.* bipolar cells) (Shand 1997; Lara 2001). Such comparisons should therefore be performed in the analyses presented in chapter II, before proceeding to its submission.

Histological analyzes of sensory organs represent a tremendous amount of work, and reaching the necessary conditions to properly assess larval fish sensory abilities can sometimes be difficult (Hale et al. 2017; Jutfelt et al. 2017). Therefore, I propose that simply monitoring TH levels in coral-reef fish recruits could be an easy and rapid alternative to these fastidious analyzes for characterizing the sensory states of coral-reef fish populations, for example when comparing a degraded *vs.* a pristine habitat.

VI.3. Coral-reef fish metamorphosis is prone to endocrine disruption

Among the many stressors affecting coral-reefs, increasing levels of chemical pollution (*e.g.*, agricultural pesticides and petrogenic compounds), which followed the intensive agricultural and industrial developments (Fabricius 2005; Burns 2014), are sometimes neglected in comparison to the elevated sea water temperature threat (Nilsson et al. 2009; McLeod et al. 2013; Hughes et al. 2017). However, this chemical pollution is most often local, and should therefore be theoretically easier to control. Among these pollutants, the agricultural pesticide chlorpyrifos (CPF) is one of the most common encountered in coral-reefs waters (Shaw et al. 2010). CPF is known to be a thyroid disruptor with adverse effects on fish behavior (Sandahl et al. 2005; Kavitha and Rao 2008; Roche et al. 2011; Yang et al. 2011; Botté et al. 2012; Deb and Das 2013; Juberg et al. 2013; Slotkin et al. 2013). In this thesis, I demonstrated that CPF exposure causes a variety of developmental and behavioral defects in *A. triostegus* at recruitment. Overall, CPF exposure reduces TH levels, delays intestine lengthening and remodeling, delays sensory organs maturation, inhibits grazing activity and impairs visual lateralization. As *A. triostegus* is one of the major herbivorous fish in coral reefs, this can severely impair the ability of coral reefs to recover after an algal invasion (*e.g.*, after a bleaching event). Indeed, a reduced abundance of keystone algal herbivores, may impair their ability to maintain or rescue coral-dominated habitats, further threatening the ecological and economic resilience of coral-reef ecosystems (Russ 1984; Almany et al. 2007; Hughes et al. 2007, 2014; Ledlie et al. 2007; Munday et al. 2008; Pratchett et al. 2008). Oceanic levels of chlorpyrifos contamination have only been assessed, to my knowledge, in the Southern Ocean, revealing levels up to 0.54 pg.l⁻¹ (Bigot et al. 2016). This is much lower than the levels observed in Australian reef surface waters, where contamination have been shown to reach 1 µg.l⁻¹ (NRA 2000), or than in certain Australian and Malaysian reefs near river mouths, where contamination levels can reach up to 26 µg.l⁻¹ (NRA 2000; Leong et al. 2007). The range of concentrations used in this thesis (1 to 30 µg.l⁻¹) are therefore ecologically relevant and reflects appropriately the change of pesticide concentration larval fishes can face when settling, from the open ocean, in coral reef coastal areas (NRA 2000; Leong et al. 2007; Botté et al. 2012; Bigot et al. 2016). Altogether, this indicates that ambient levels of CPF pollution are already high enough to severely disrupt essential processes of coral-reef fish ecology. Considerable efforts should be made to reduce this pollution, especially as this could be handle locally, and with short-term effects.

Lastly, this thesis highlights the need for a better understanding of the combined effects of all the disturbances that affect coral-reef ecosystems. Assessing the impact of combined elevated temperature and CPF exposure on *A. triostegus* recruitment process is something that is already planned, as mentioned in chapter III. In particular, given that both elevated temperature and CPF exposures separately repress metamorphic processes, I expect that their combination would amplify their impacts. Additionally, it would be particularly interesting to combine these stressors with acidification, another considerable threat to coral-reef ecosystems (Hoegh-Guldberg et al. 2007). Oceanic acidification has been evidenced to severely impair coral reef fish sensory abilities and brain function (Munday et al. 2009; Simpson et al. 2011; Ferrari et al. 2012a, 2012b; Leduc et al. 2013; Rossi et al. 2016; Lecchini et al. 2017). Unfortunately, I did not initially focus on the role of oceanic acidification on *A. triostegus* metamorphic processes during this thesis. While a few trials were performed at the end of my thesis, with preliminary results showing an interesting and counterintuitive trend with relatively increased TH levels in *A. triostegus* at day 2 and day 5 post-settlement, this topic definitely requires further investigations in a near future. While this thesis has shed some light on the importance of metamorphosis and TH in coral-reef fish larval recruitment facing anthropogenic pressures and endocrine disruption, most coral reef fish recruitment processes have yet to be elucidate. Additionally, given that coral organisms and marine invertebrates also possess pelagic larval stages that undergo metamorphosis when settling into the reefs (Burke 1983; Grasso et al. 2011), deciphering how ambient and predicted environmental conditions could disrupt their respective endocrine system during their vulnerable recruitment-stage, is essential.

VI.4. Conclusion

In this thesis, I have examined the recruitment phase of the convict surgeonfish *Acanthurus triostegus*, and determined that the ecological, morphological, physiological and behavioral changes occurring at recruitment correspond to a metamorphosis mediated by thyroid hormones (TH). Then, I investigated whether this metamorphosis is prone to endocrine disruption under anthropogenic disturbances such as elevated sea water temperature and agricultural pesticide pollution. I demonstrated that such pressures can reduce TH levels at a critical developmental stage in coral-reef fishes, impairing their metamorphic processes such as sensory organ maturation and sensory abilities acquisition, further increasing their mortality rates. Overall, this thesis is a holistic analysis that addresses molecular, morphological, histological, and behavioral assays of multiple stressors affecting coral-reef fish recruitment. It indicates the importance of a proper endocrine function during coral-reef fish recruitment, highlighting the need for a better understanding of these processes for coral-reef conservation.

VI.5. References

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Appendices

Appendix 1: Manuscript – Larval coral fishes sensory ecology review

Barth P., Berenshtein I., **Besson M.**, Roux N., Parmentier E., Banaigs B., Lecchini D. (2015). From the ocean to a reef habitat: how do the larvae of coral reef fishes find their way home? A state of art on the latest advances. *Life and Environment*, 2015, 65(2): 91-100.

FROM THE OCEAN TO A REEF HABITAT: HOW DO THE LARVAE OF CORAL REEF FISHES FIND THEIR WAY HOME? A STATE OF ART ON THE LATEST ADVANCES

P. BARTH^{1,2}, I. BERENSHTEIN^{3,4}, M. BESSON^{1,2}, N. ROUX^{1,2}, E. PARMENTIER⁵,
B. BANAIGS⁶, D. LECCHINI^{1,7,*}

¹ USR 3278 CNRS-EPHE-UPVD, CRIOBE, BP1013 Papetoai, 98729 Moorea, French Polynesia

² Molecular Zoology Team, Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Lyon 1, CNRS UMR 5242; École Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon, France

³ Marine Biology and Biotechnology Program, Eilat Campus, Department of Life Sciences, Ben-Gurion University of the Negev, P.O. Box 653, Beer-Sheva 84105, Israel

⁴ The Interuniversity Institute for Marine Sciences of Eilat, PO Box 469, Eilat 88103, Israel

⁵ Laboratoire de Morphologie Fonctionnelle et Évolutive, Institut de chimie, Bât. B6, Université de Liège, B-4000 Liège, Belgium

⁶ USR 3278 CNRS-EPHE-UPVD, CRIOBE, Université de Perpignan, 66100 Perpignan, France

⁷ Laboratoire d'Excellence "CORAIL", BP1013 Papetoai, 98729 Moorea, French Polynesia

* Corresponding author: lecchini@univ-perp.fr

PERCEPTION OF INFORMATION
CORAL REEF FISH
LARVAL SETTLEMENT
HABITAT SELECTION
CORAL REEF DEGRADATION

ABSTRACT. – As it is unlikely that successful settlement is solely a matter of chance (i.e. to find a suitable habitat), one of the greatest challenges facing the fish larvae is how to locate the relatively rare patches of coral reef habitat on which they settle and ultimately reside as adults. The answer must lie partly in the sensory modalities of fishes at settlement. Habitat selection is only possible if fish larvae could detect some environmental cues to select a suitable reef habitat at settlement. The present review aims at providing the latest works dealing with information perception in coral reef fish larvae at settlement. Two decades ago, it was generally assumed that larval behaviors and sensory abilities at settlement were considered too feeble to significantly affect dispersal outcomes. Several recent studies showed that recognition of suitable reef habitats by fish larvae at settlement is based on a combination of visual, chemical and acoustic cues. The first part of our review shows the main advances in the knowledge of visual, chemical and acoustic cues used by fish larvae to detect an island, a reef, a micro-habitat, a conspecific or some predators. The second part of our review deals with the effect of imprinting and/or innate capabilities. The third part focuses on the different cues used at different scales and underlines some contradictory results about the distance of transmission and detection of chemical and acoustic cues in coral reefs. Finally, as global and regional environmental changes have stressed coral reefs to such an extent that they are either destroyed or in decline, the fourth part presents the effects of both anthropogenic and environmental stressors on information perception and response capacities in coral reef fish larvae. If polluted seawater disrupts the larval abilities to find a suitable reef habitat, fish larvae may spend more time in the planktonic environment, resulting in increased energetic costs and predation risk, and consequently a lower larval settlement. We hypothesize that as the stability of fish communities is dependent, in part, on the stability of social interactions, the disruption of "larvae-habitat relationships" can have major consequences for larval settlement into adult population with further repercussions for the ecosystem as a whole. Overall, larval settlement of coral reef fish is an excellent example of the complexity of interactions between an organism and its environment as without perceiving environmental cues, fish larva would have very little chance of selecting a suitable reef habitat. Moreover, understanding the relationship between reef state and settlement potential will allow management planning for the maintenance of coral reefs that are increasingly degraded.

INTRODUCTION

Animals constantly make decisions: they choose mates, select a place to live or forage, and they decide whether to engage in activities which expose them to risk of predation. These decisions necessitate the perception of some environmental cues in order to optimize the social interactions (Cassier *et al.* 2000). Thus, results of simple decisions based on the perception of some cues may influence an individual's life span, a species' population biology,

and community structure (Blumstein & Bouskila 1996). While there is a lot of information about decision-making in breeding, foraging and habitat selection, little is known on habitat selection for fish species at settlement, especially in coral reefs (Lecchini *et al.* 2015).

The life cycle of most coral reef fish species includes a planktonic larval phase which usually lasts from three to six weeks, followed by a sedentary reef phase for the juveniles and adults (Leis & McCormick 2002). During the pelagic phase, the larvae may move far from their

native reef due to currents and/or their swimming abilities. Then larvae return to the reef (natal or not) to continue their development into juveniles, then to adults. At the end of its pelagic phase, a species-specific change in morphology and physiology, called metamorphosis, occurs during which fish lose many of the characteristics that enhance survival in the pelagic environment and develop other features suited to their new reef environment (transformation from larval to juvenile stage – McCormick *et al.* 2002). Moreover, a fish larva must choose a suitable reef habitat to settle, habitat that will facilitate post-settlement survival and growth (Doherty 2002). As fish larvae are subjected to strong selective pressures to choose the suitable reef habitat, many fish species show specific preference of habitat at settlement based on the presence/absence of specific substrates, conspecifics, predators and competitors for food and space (Doherty 2002). After several weeks or months in the settlement habitat, juveniles move into the adult population and become sexually mature (Lecchini & Galzin 2003).

As it is unlikely that successful settlement is solely a matter of chance (i.e. to find a suitable habitat), one of the greatest challenges faced by the fish larvae is how to locate the relatively rare patches of coral reef habitat on which they will settle and ultimately reside as adults (Myrberg & Fuiman 2002). The answer must lie partly in the sensory modalities of fishes at settlement. Habitat selection is only possible if fish larvae could detect some environmental cues to select the suitable habitat at settlement. Since 2000s, studies have highlighted the role of larval sensory and swimming mechanisms in habitat selection, including the detection of visual, chemical and sound cues from conspecifics, habitats, or predators (see next paragraphs). Thus, the perception of information by coral reef fish larvae resulting in successful settlement is a key process in population biology, shaping the characteristic texture of populations, communities and ecosystems in space and time.

The present review aims at providing the results of recent works dealing with information perception in coral reef fish larvae at settlement without going back to the historical background that is provided by the five excellent previous reviews (Kingsford *et al.* 2002, Myrberg & Fuiman 2002, Montgomery *et al.* 2006, Arvedlund & Kavanagh 2009, Leis *et al.* 2011). We tried to answer some of the questions raised by these syntheses using the latest works in the topic. In the first part, we present the main advances in the knowledge of visual, chemical and acoustic cues used by coral reef fish larvae at settlement. The second part deals with the effect of imprinting and/or innate capabilities. The third part focuses on the different cues used at different scales. Lastly, the fourth part presents the effects of both anthropogenic and environmental changes on information perception and response capacities in coral reef fish larvae.

The sensory cues used by coral reef fish larvae at settlement... Fifteen years after

At the end of their pelagic phase, fish larvae undergo metamorphosis and choose settling habitats, based mainly on the presence or absence of conspecifics, as well as other species (Doherty 2002). Until two decades ago, it was generally assumed that larval behaviors and sensory abilities at settlement were considered too feeble to significantly affect dispersal outcomes (Williams *et al.* 1984). In fact, only three main studies have explored the sensory abilities of coral reef fish larvae at settlement before 2000s (Sweatman 1988, Booth 1992, Elliott *et al.* 1995). Since then, much evidence have demonstrated the developed behavioral and sensory abilities of larvae, rejecting the traditional paradigm that fish larvae could be regarded as passive plankton (Leis 2015). Today, we know that visual, olfactory and auditory senses are used by fish larvae to settle on their first reef habitat.

New insights on chemical cues

Coppock *et al.* (2013) used choice plumes to show that the presence of conspecifics and the coral species attract settlement-stage larvae of *Dascyllus melanurus*, *Chrysiptera paraseuna*, *Chaetodon octofasciatus*. Several recent studies have thus demonstrated that chemical cues are used by fish larvae to find a suitable reef habitat (e.g., Atema *et al.* 2002, Lecchini *et al.* 2005a, b, 2013, Dixon *et al.* 2011, 2014a), to find conspecifics (e.g., Lecchini *et al.* 2005a, b, 2007b), or to avoid predators (e.g., Dixon *et al.* 2012). However, few studies have explored the relative importance of the different types of chemical cues (predator, conspecifics, vs. habitat). For example, Lecchini & Nakamura (2013) explored the effect of chemical cues at different spatial scales (reef patch vs. micro-habitat or conspecifics) at Ishigaki Island (Japan). They showed with choice flume experiments that three of the eight fish species detected olfactory cues at large spatial scales (Exp. 1: reef patch), whereas six of the eight species detected olfactory cues at small spatial scales (Exp. 2: conspecifics). Moreover, when fish larvae had the choice between chemical cues of reef patch, coral habitat and conspecifics, they preferred the conspecific cues above other types of information for habitat selection. A multitude of other animals also use conspecific information more often than habitat information to find a suitable habitat (Stamps & Krishnan 2005). Conspecific attraction generally occurs when animals use social cues as positive proximate stimuli during decision-making, including foraging, movement, and settlement decisions (Danchin *et al.* 2004). Social aggregation of fish larvae with older conspecifics may be the result of individuals using conspecific “guides” to potentially find beneficial resources (availability of resources and low mortality; Ben-Tzvi *et al.* 2009). However, the importance of chemical cues

of conspecifics vs. predators has not been explored. Yet, some recent studies have tackled the question of chemical cues from the perspective of risk assessment. Vail & McCormick (2011) showed that *Pomacentrus nagasienkii* and *P. amboinensis* larvae (damselfishes) settled 24–43 % less to reefs with predator scent.

The relative effectiveness of chemical cues originated from the reef is dependent on the spatial scale, and the hydrological conditions at which fish larvae could detect each cue. No study has directly addressed this point. However, some studies have estimated the distance of detection of fish larvae. Paris *et al.* (2013a), using *in situ* techniques, showed that some pomacentrids and apogonids larvae changed speed and direction when placed in an odour plume, even at a distance of several kilometres from the reefs. Atema *et al.* (2002) estimated that the transmission distance of odour plume from a coral lagoon in the ocean could range between 1.6 to 3 km, using temperature measurements and visual observations of turbidity. Lecchini *et al.* (2014a) explored the distance of transmission of chemical cues emitted by live vs. dead coral reefs (High Pressure Liquid Chromatography analyses of water sampling stations at 0, 1 and 2 km away from the reef) at Ishigaki Island, Japan. The results highlighted that a live coral reef produced different and distinct molecules from a dead coral reef, and some of these molecules could be transported to a distance of at least 2 km from the reef with a 14–17-fold reduction in concentration. However, *Chromis viridis* larvae detected the chemical cues only at a distance of 1 km away from the live coral reef.

Overall, several recent studies have demonstrated that chemical cues are mainly used by fish larvae to find a suitable reef habitat and/or conspecifics, or to avoid predators. Three fruitful research avenues should be conducted in the future on the chemical cues at settlement: (1) Test the interaction, the hierarchy, and the relative importance of habitat, conspecifics and predator cues to understand the decision making dynamics when choosing a habitat for settlement. (2) Identify the distance of detection of chemical cues in different environments; and (3) Test the importance of chemical cues integrated with visual and acoustic cues. (4) Chemical cue orientation is normally assumed to involve swimming along chemical concentration gradients; however mixing processes in the ocean may break a chemical plume into smaller disconnected patches. Larvae which use chemical cues for orientation need to behaviorally overcome this problem using special ranging strategies (Vergassola *et al.* 2007). It will be interesting to examine how coral reef fish larvae behaviorally cope with this problem.

New insights on acoustic cues

Acoustic cues are crucial in most marine organisms for mating, feeding, orientation, territory defence and prey-predator detection (*e.g.*, Tolimieri *et al.* 2004, Mont-

gomery *et al.* 2006, Parmentier *et al.* 2010, Simpson *et al.* 2010). They have the advantage of propagation in all directions, are not stream-dependent as are the chemical cues, are effective independently on the light and turbidity, and propagate far beyond the visual cues. The underwater acoustic environment is characterized by loud and various noises (Cato 1992), including from abiotic (*e.g.*, surf, wind and waves) and biotic sources (*e.g.*, crustaceans, fishes and mammals). In coral reefs, sound level is known to be greater close to the reef due to a high density of sound sources (*e.g.*, breaking waves, snapping shrimps and vocalizing fishes), and reef noise has been shown to vary during day periods, moon phases and seasons (Cato 1992, Montgomery *et al.* 2006, Radford *et al.* 2008). Several studies have thus highlighted that the reef sound attracted fish larvae at settlement (*e.g.*, Tolimieri *et al.* 2004, Simpson *et al.* 2005, Montgomery *et al.* 2006, Radford *et al.* 2007, Holles *et al.* 2013, Parmentier *et al.* 2015a).

In this review we will focus on a few recent studies which examine in detail the response of coral reef fish larvae to sound. Parmentier *et al.* (2015a) explored the attraction of fish larvae to underwater sounds from different reef habitats (barrier reef, fringing reef and mangrove) using the choice chamber experiment at Moorea Island (French Polynesia). Sixteen of the twenty species tested have significantly changed their behavior under at least one of the habitat playback conditions, and a various range of responses was observed: fishes were a) attracted by a single sound but repelled by none (*e.g.*, *Ostorhinchus angustatus*), b) repelled by one or more sounds but attracted by none (*e.g.*, *Pristiapogon fraenatus*), c) attracted by all sounds (*e.g.*, *Ctenochaetus striatus*), d) attracted and repelled by several sounds (*e.g.*, *Dascyllus aruanus*), and e) not influenced by any sound (*e.g.*, *Acanthurus triostegus*). These results confirmed the previous studies that fish larvae are attracted by acoustic cues of coral habitats at settlement. The use of sound to locate habitat has also been recently shown at Lizard Island, Australia, where Pomacentridae larvae were significantly more attracted to patch reefs with lagoon or fringing reef sounds than to patch reefs with no playback sound (Radford *et al.* 2011b). However, the originality of Parmentier study is the highlighting of two settlement strategies: a direct selection of habitats using sound (45 % of the species), or a selection by default with the avoidance of certain sound habitats (35 %). Avoidance of reef noise by pelagic crustacean larvae was previously described by Simpson *et al.* (2011). Thus, sound will not only give information on the attractiveness of a habitat, but also on habitat quality (Kennedy *et al.* 2010) and appropriateness as a site for settlement. Avoidance of inappropriate habitat sounds should thus be included in the future studies. Indeed, acoustic, but also visual and chemical cues of habitat and conspecifics could have an attractive effect, but in some cases, they could repulse fish larvae at settlement. Unfortunately, not

enough studies take into consideration this possible repulsive effect (Simpson *et al.* 2011, Vail & McCormick 2011, Parmentier *et al.* 2015a).

As for chemical cues, the spatial scale over which the signal exceeds background noise and the nature of propagation of sound away from reefs into deeper water remain unknown. The few studies estimating the distances that marine larvae could potentially detect coastal reef noise provide estimates ranging from 500 m to ~100 km (*e.g.*, Mann *et al.* 2007, Wright *et al.* 2010, Radford *et al.* 2011a). Based on fish hearing abilities and ambient sound level, Mann *et al.* (2007) has estimated the maximum detection distance of reef by fish larvae to less than 1 km. Radford *et al.* (2011a), basing the estimation on sound pressure level recorded in the field, estimated this distance could be several kilometers due to a “reef effect” which would increase the propagation distance of reef noise. A recent study conducted by Parmentier *et al.* (2015b) based on field measurement in the ocean around Moorea Island and at various day periods, estimates the distance at which the reef sound gradient overlapped with ocean sound. For all frequencies between 0 and 5000 Hz, which correspond to auditory capacities of most coral reef fishes, field data fit the cylindrical model of sound attenuation up to a distance between 750 m and 1000 m. At longer distances, the sound level recorded along the transect is higher than expected by the model for frequencies below 2000 Hz. Therefore, the reef sound gradient would only be detectable by the fish larvae at distances below one kilometer.

Overall, future studies should continue to explore the propagation distance of sound and hearing capacities of fish larvae at settlement, as discussed in recent papers (*e.g.*, Simpson *et al.* 2011, Parmentier *et al.* 2015a, b). Nevertheless, one fruitful research avenue is to explore “soundscapes and living communities in coral reefs” (Staaterman *et al.* 2012a, Nedelec *et al.* 2015). Soundscape analysis in air has proved very useful for determining how habitat structure may predict soundscapes (Pekin *et al.* 2012) and for assessing species present in habitats where other survey methods may be logistically difficult (Pijanowski *et al.* 2011). Soundscapes are now considered an important part of landscape ecology (Pijanowski *et al.* 2011). Yet, although underwater soundscapes have likely driven the evolution of hearing (Fay 2009), our knowledge of underwater soundscapes is still in its infancy (Staaterman *et al.* 2012b).

New insights on visual cues

Among the sensory cues used by coral reef fish larvae, visual cues are the most discussed and their importance is the least understood (Lecchini *et al.* 2014b). Some works have shown that coral reef fish have a highly developed sensory system during the larval stage (*e.g.*, Kotrschal *et al.* 1990, Fisher *et al.* 2000, Lara 2001). The visual abili-

ties of fish larvae increase during their pelagic life and reach a maximum around metamorphosis (Lara 2001). Fish larvae therefore have the sensory abilities to perceive visual information emitted by conspecifics, heterospecifics and predators. However, only seven studies have explored the importance of visual cues during habitat selection of coral reef fish larvae (Booth 1992, Leis & Carson-Ewart 1999, Lecchini *et al.* 2005a,b, Igulu *et al.* 2011, Huijberg *et al.* 2012, Lecchini *et al.* 2014b).

Igulu *et al.* (2011) showed that fish larvae of *Lutjanus fulviflamma* preferred seagrass and coral above mangrove roots. Fish larvae were more attracted towards visual cues of a combination of conspecifics or heterospecifics and seagrass microhabitats than to seagrass microhabitats alone, but showed a significantly stronger preference for visual cues of conspecifics than of heterospecifics when placed in preferred seagrass or non-preferred mangrove microhabitats. However, vision of fish larvae could be significant over only short ranges in coral reefs (up to 5-10 m) even if water transparency is high (Myrberg & Fuiman 2002). Moreover, coral reef fish larvae usually settle during the night which questions the actual use of visual cues by fish larvae at settlement. Only one study explored the importance of visual cues at night. Lecchini *et al.* (2014b) showed that *Acanthurus triostegus* larvae was attracted to the visual cues of conspecifics and not that of heterospecifics during the day and during bright nights; but did not show such behavior during dark nights. This study highlights the trade-off between “bright night” settlement which favors the visual recognition of conspecifics and predators, and “dark night settlement” which reduces the risk of predation.

Overall, more research is needed in the field of spectral capabilities and differential sensitivities to light intensity of fish species (*e.g.*, Kotrschal *et al.* 1990, Fisher *et al.* 2000, Lara 2001) in order to better understand the temporal patterns of settlement between bright and dark nights. Some species with high spectral and/or light intensity sensitivity could settle during dark night, while others could settle during bright nights and then would be subjected to a stronger predation. Additionally, more work is needed to understand which specific visual factor (shape, color, etc.) is used by larvae to recognize conspecifics, habitats and predators; and how far could fish larvae see under different light intensities.

New insights on the other cues

Some recent studies showed the use of another cue for the fish larvae at settlement: the sun (*e.g.*, Leis & Carson-Ewart 2003, Leis *et al.* 2014). Mouritsen *et al.* (2013) showed that *Ostorhinchus doederleini* larvae oriented using the sun and strikingly, microsatellite markers indicated that fish larvae came from either their natal reef or from a reef 12 km away from it. Similarly, Berenshtein *et al.* (2014) showed that *Premnas biaculeatus* larvae could

orient depending on the sun and polarized light. During sunny conditions larvae oriented in a more directional manner compared with cloudy conditions. However, settlement has been shown to occur mainly during the night, so light might be used at a certain scale but be less useful or even unused during the night. Information about the use of other potential cues for habitat selection such as: rheotactic, magnetic, wave motion and thermal cues is still unknown, except Paris *et al.* (2013b) showing that larval behavior differed in a water mass of different temperature.

Imprinting and/or innate capabilities at settlement?

Many studies conducted on fish settlement and their perception of information used naive larvae captured either with light traps or crest nets (*e.g.*, Lecchini *et al.* 2005a, b, 2013, Lecchini & Nakamura 2013). Some studies used also fish larvae reared in aquaria, especially for Amphiprion species (*e.g.*, Dixon *et al.* 2010, 2012). For example, using the crest net, fish larvae were captured just before they entered the lagoon to settle (Lecchini *et al.* 2004, 2006). Obviously, these fish larvae had no prior experience of settlement (*i.e.*, naive larvae). Therefore, how could these naive individuals distinguish between conspecifics (vs. heterospecifics) or predators (vs. non-predatory fish)?

Arvedlund & Nielsen (1996) were the first to highlight the imprinting process in coral reef fish. *Amphiprion ocellaris* eggs were maintained with either the host anemone *Heteractis magnifica* or without anemone. They showed that larvae from treated eggs settled in less than five minutes to the anemone whereas larvae from non-treated eggs remained indifferent to the anemone during 48 hours of experiment. Similarly, Arvedlund (1999) showed that the attraction of *Amphiprion melanopus* larvae was innate recognition for the natural host anemone (*Entacmaea quadricolor*) and it was an imprinting process for the *Heteractis malu* (host of another Amphiprion species). Dixon *et al.* (2012) showed that *A. percula* larvae innately recognize predatory fish based on chemical cues. Lastly, Dixon *et al.* (2014b) showed that Amphiprion larvae imprinted themselves to their host and their environment. *Amphiprion percula* larvae recognized innately their anemone host, but this recognition was stronger when larvae were in contact with the anemone during their development.

Overall, these studies conducted only on clownfish suggest that both processes, imprinted and innate capabilities, could be used at settlement to detect the presence of anemones or predators. Apogonids are mouth brooders (Job & Bellwood 2000) with the male carrying the fertilized eggs and in some apogonid species also the hatched embryos. This close contact during embryogenesis may enable such species to imprint to conspecific cues. However, imprinting can happen only in oviparous

species which lay demersal eggs (40 % of coral reef fish species). For the other species called egg-laying fish or pelagic spawners, the spermatozooids and oocytes are carried away from parental environment by currents. In that case, how can we explain the chemical preference for conspecifics of pelagic spawners without an imprinting process? This is a fruitful research avenue for future studies: do oviparous species with demersal eggs recognize their settlement habitat better than pelagic spawning species? For the latter, it seems that these capacities must be innate (Leis *et al.* 2011). However, a third process could explain these capacities: "pelagic learning". During the oceanic phase, fish larvae could learn to recognize their conspecifics and to avoid some predators. Indeed, fish larvae swim in the plankton along with many other taxa; but conspecific fish larvae which share similar characteristics and preferences may very well group and form cohorts inside the multi-taxa plankton. Thus, within their group, conspecifics could lead to recognize themselves and some predators via vision, odor and sound. Additionally, mixing conditions could form prevailing patches of eggs or young larvae which "stick together", resulting in conspecifics-imprinting during the first post-spawning days. Overall, innate behaviors, imprinting process and/or pelagic life experience of fish larvae are still in debate and necessitate more studies.

Orienting in a multiple cues system: sequential and hierarchical use at different spatial scales

Most marine animals dwell in a dynamic environment providing variable chemical, physical and biological factors which could potentially be used as behavioral cues. For example, magnetic fields are used by adult salmon and tuna to perform a homing in combination with a chemical imprinting (Dittman & Quinn 1996). Thus, the animals, in order to move, migrate or find a suitable habitat may take into account a combination of several cues. For coral reef fish larvae at settlement, research has been done mainly on of each sensory cue alone (Leis *et al.* 2011). Yet, fish larvae undergo their settlement in a multifactorial environment, and therefore a few cues are present at any given time (Lecchini *et al.* 2014b).

Orienting in a multifactorial system

One of the first studies using a multiple-cue approach was Lecchini *et al.* (2005b) in Moorea Island. In field experiments, they showed that *Chromis viridis* larvae detected reefs containing conspecifics using visual and/or acoustic cues at distances < 75 cm; detection distances increased to < 375 cm when olfactory information was available (particularly for reefs located up-current). Similarly, Huijbers *et al.* (2012) showed that *Haemulon flavolineatum* larvae only responded to sound from coral reefs and to chemical cues from mangroves/seagrass beds,

while visual cues of conspecifics were prioritized over olfactory cues from mangrove/seagrass water. Igulu *et al.* (2013) showed that fish *Lutjanus fulvivlamma* preferred chemical cues from seagrass leaves over those from conspecifics when both were offered at the same time. Visual habitat cues were prioritized over chemical cues only when it concerned preferred cues (i.e. seagrass as opposed to mangrove cues). Overall, these studies (e.g., Lecchini *et al.* 2005b, Huijbers *et al.* 2012, Igulu *et al.* 2013) underline that fish at settlement possess the capacity of using multiple reliable chemical cues to locate suitable habitats, although the ranking of these cues is context-dependent. This flexibility in choice behavior would probably be an adaptive strategy to enhance fitness by increasing successful habitat selection at settlement. Future studies conducted will require simultaneous examination of different cues because, in most cases, several cues are present at settlement.

Different cues for different scales

In the previous section, we reviewed the use of multiple cues by fish larvae; yet there is a need to understand which cue is used at which stage. Successful settlement requires actions at a range of spatial scales: (1) identifying an island or a continental shelf within the seemingly boundless oceanic matrix, (2) locating an appropriate reef patch within this island or this continental shelf, and (3) choosing a suitable microhabitat within this patch. Some studies have highlighted the visual, chemical, and/or acoustic abilities of fish larvae to recognize an island or a continental shelf (e.g., Gerlach *et al.* 2007, Dixon *et al.* 2008), a suitable reef patch (e.g., Huijbers *et al.* 2008, Lecchini *et al.* 2013), a suitable settlement micro-habitat (e.g., Igulu *et al.* 2011, Devine *et al.* 2012), predators (Dixon *et al.* 2012), or conspecifics (e.g., Lecchini *et al.* 2005a, b, Lecchini & Nakamura 2013). However, it is still needed to determine the effective spatial scale of each of these cues. This depends on the location and the physical and biological factors related to each. For example, in turbid waters, visual cues will operate over a smaller scale than in oligotrophic tropical waters.

Paris *et al.* (2013a) suggested that reefs produce plumes of odor all around them which may provide the first hint of the reef's vicinity for larvae. Once in an odor plume, they could navigate directionally towards a preferential reef patch and micro-habitat. However, Lecchini *et al.* (2014a) showed that fish larvae could detect the chemical cues emitted by a live coral reef only up to 1 km away from the reef. Staaterman *et al.* (2012a) modelled larval dispersal of *Stegastes partitus* taking into account orientation behaviors. A threshold of sound detection at 1-5 km gave the highest settlement results. However, Parmentier *et al.* (2015b) suggested that the reef sound gradient would only be detectable by fish larvae at distances below one kilometer. Thus, these different studies showed

some contradictory results about the distance of transmission and of detection of chemical and acoustic cues. A recent synthesis of Staaterman & Paris (2014) suggested a theoretical framework showing the various cues available to fish larvae as they move throughout space. At 100 km away from the reefs, fish larvae would orient by magnetic cues, polarized light, or sun compass, or simply swim towards a particular cardinal direction maintaining an angle relative to the mean direction of surface capillary waves (although no study has demonstrated it yet). At 10 km from the reefs, fish larvae could detect acoustic pressure of a reef/island. Lastly, close to the reef (at less than 5 km), fish larvae could detect odour plumes and may swim upstream along the gradient towards the odor origin. We add to this model the use of visual cues for habitat-selection once fish larvae entered the reef patch, although this would be computationally challenging as it would require an increase of the model's spatial resolution from a few hundreds of meters to a few meters only.

Overall, despite these recent studies presented above, the sensory and behavioral mechanisms by which larvae disperse are still largely unknown (Montgomery *et al.* 2006, Arvedlund & Kavanagh 2009, Leis *et al.* 2011). The detection distance of fish larvae depends on the intensity and frequency components of the soundscape, the concentration of chemicals in the reef, the magnitude of turbulence, the mean current speed and direction near the reef, as well as detection abilities of the larva (Staaterman & Paris 2014). Future studies should be conducted in the field in order to test simultaneously and separately the different cues and at different spatial scales.

The use of bio-physical models

Studying the cues used by fish larvae during settlement is highly important by itself. However if we wish to synthesize all this accumulated information and realistically examine the consequences of the larvae behavior, we must use bio-physical models. Individual Based Lagrangian stochastic bio-physical models have been already used for a few decades to simulate larval dispersal. These models usually transport thousands of virtual larvae according to currents data (advection) normally extracted from General Circulation Models, turbulent diffusion, and the behavioral traits (e.g., swimming speeds, diel vertical migration and mortality rates) of the simulated organism (e.g., Paris *et al.* 2013b). Only recently, larvae's orientation capacities were incorporated in such models providing an essential assessment of the ecological consequences of these capacities, namely their effect on dispersal and connectivity (Staaterman *et al.* 2012a). Wolanski *et al.* 2014 showed that the degree of self seeding in swimming virtual larvae (fish) is significantly higher (> 20 %) than that of passive (coral planulae) virtual larvae (< 2 %), and that the dispersal outcome, specifically self-seeding, are highly affected by the larvae response to auditory and olfactory cues.

Perceiving the information in a changing world

Coral reef ecosystems are among the most biologically diverse and complex marine ecosystems worldwide. Coral reefs harbour 25 % of the biodiversity in the seas while only constituting 0.02 % of the ocean, thus they are critical for conservation biology and resources management. In the early 90's, questions were raised concerning the resilience of coral reefs to global changes (Grigg & Dollar 1990). Over the past three decades climate change and increasing anthropogenic pressure lead to the destruction of 20 % of the coral reefs, another 25 % are in great immediate threat and another 25% will be threatened by 2050 (Hughes *et al.* 2007, Chin *et al.* 2011). Jones *et al.* (2004) showed that the decline in adult populations of coral reef fish in degraded habitats had more to do with settlement failure than adult mortality. They suggested that the rescue effect of settlement might be completely ineffective in a degraded habitat. Despite the strong impact of natural and human stressors on coral reef biodiversity, the mechanisms that determine how marine larvae respond to different stages of coral stress and the extent of coral loss during larval settlement remain poorly understood (for exception see: Gleason *et al.* 2009, Munday *et al.* 2009, Lecchini *et al.* 2013, Dixon *et al.* 2014b, Siebeck *et al.* 2015).

Lecchini *et al.* (2013) explored how chemical cues emitted from coral vs. algal reefs attract fish larvae at Rangiroa Atoll (French Polynesia). They showed with 2-channel choice chambers that at settlement, seven of the 10 fish species studied preferred water from reefs dominated by coral compared to reefs dominated by algae (*e.g.*, *Aulostomus chinensis*, *Chromis viridis*, *Ptereleotris microlepis*, *Sargocentron spiniferum*). The authors suggested that fish larvae could respond to: (1) many different types of chemical cues associated with coral or algae directly, (2) chemical cues of conspecifics whose presence changes in response to coral vs. algal cover, or (3) the concentration of settlement cues emitted from the environment. In addition to the natural stressors, several anthropogenic stressors could also modify the level of taxis towards suitable habitat or away from predators. For example, Holles *et al.* (2013), using choice chambers, showed that boat noise induced a lower settlement rate in coral reef fish. Thus, if boat noise disrupts the settlement process (Tolimieri *et al.* 2004, Radford *et al.* 2011b, 2014), nearby reefs will suffer from depletion in larval supply, whereas fish larvae may remain in the open ocean after metamorphosis, thus increasing their risk of predation. Another anthropogenic stressor widely studied is the ocean acidification. Munday *et al.* (2009) showed that acidification in the ocean could disrupt the olfactory mechanism by which clownfish larvae discriminate between cues which may be used in locating suitable adult habitat. Dixon *et al.* (2010) showed that when eggs and larvae of *Amphiprion percula* were exposed to seawater with lowered pH (simulat-

ing ocean acidification), settlement-stage larvae became strongly attracted to the smell of predators, and the ability to discriminate between predators and non-predators was lost. Similarly, Munday *et al.* (2013) reared *Plectropomus leopardus* at settlement in different CO₂ concentrations during four weeks (490, 570, 700 and 900 μ atm). They showed that *P. leopardus* reared in acidified water were attracted to olfactory cues from potential predators, rather than seeking shelter, whereas fish reared under natural conditions exhibited the predicted sheltering response.

Overall, all the studies above demonstrate negative effects of natural and anthropogenic stressors on the larval sensory abilities. Although fish larvae do not die in presence of algal reef water, acidified water or noisy environment, the indirect consequences are behavioral responses which reduce the larval fitness (Leis *et al.* 2011, Siebeck *et al.* 2015). Thus, if polluted seawater disrupts the settlement process, fish larvae may spend more time in the planktonic environment, resulting in increased energetic costs and predation risk, and consequently a lower larval settlement. As the stability of fish communities is dependent, in part, on the stability of social interactions, the disruption of "larvae-habitat relationships" can have major consequences for larval settlement into adult population with further repercussions for the ecosystem as a whole. Indeed, if the settlement potential of coral reefs declines due to these natural and anthropogenic stressors, the populations of reef organisms (fish, cephalopods, and crustaceans) may also decline due to a reduction in larval supply. Understanding the relationship between reef status and settlement potential will allow management planning for the maintenance of coral reefs that are increasingly degraded.

CONCLUSION AND PERSPECTIVES

We have reviewed some of the latest researches on orientation, early sensory development, and the role of specific environmental cues in the settlement process of coral reef fish. We highlighted that fish larvae use a variety of cues and senses at different distances from the reef, in order to find a suitable reef habitat that will facilitate post-settlement survival and growth. At present, we will use some papers already published to point out particular gaps and to stimulate further research.

• *Using naive fish larvae* – For example, Igulu *et al.* (2013) used recently settled fish to study the importance of sensory cues at settlement. The authors suggested that it is unlikely that fish lose their attraction to preferred chemical cues directly after settlement. Some studies showed that recently settled fish had the same cue preference even if they had been collected from different settlement habitats (Grol *et al.* 2011, Huijbers *et al.* 2012). However, Danilowicz (1996) showed that preferences for coral species may differ depending on whether fish

are tested at or after settlement. Similarly, Lecchini *et al.* (2005a) showed that four of the five species tested used the same sensory modalities at larval and juvenile stages to detect their reef habitat. But, *Chrysiptera leucopoma* used different senses at different developmental stages, with larvae recognizing conspecifics by visual cues and juveniles by chemical cues. Overall, these studies showed different results depending on the sensory abilities of fish at and after settlement; therefore, it is necessary to further investigate these abilities throughout ontogeny.

- **Using several heterospecifics as control cues** – For example, Lecchini *et al.* (2005a) showed that *Abudefduf sordidus* larvae were more attracted by the visual cues of conspecifics than the ones of heterospecifics. However, the authors used *Lutjanus fulvus* juveniles as heterospecifics. Would the authors get the same result if they use *A. sexfasciatus*, species having a color pattern relatively similar to *A. sordidus*? The future studies on sensory abilities of fish larvae should use more than one species as heterospecifics with heterospecific species belonging to same and different families in order to know until which taxonomic level fish larvae could distinguish cues between conspecifics and heterospecifics.

- **Conduct the experiments on several fish species** – For example, Holles *et al.* (2013) showed that boat noise induced a lower settlement rate in coral reef fish at Moorea. The study was only conducted on one species, *Apogon doryssa*. Yet, Parmentier *et al.* (2015a) tested the larval attraction to sound cues of different Moorea reefs. They showed that among the 21 species tested, there was a great variation in the larval attraction inside and among fish families. It is impossible to conclude that one family is attracted, for example, by the sound of barrier reef. Therefore, future studies should ideally be conducted on several fish species belonging to the same and to different families in order to generalize the results.

- **Conduct mainly some field experiments** – Leis & McCormick (2002) indicated the importance of finding ‘innovative means’ to study the behavior of larvae at night. Today, this issue is still relevant because of the difficulty to work directly in the field (see for exception, Lecchini *et al.* 2005b, Paris *et al.* 2013a). Paris *et al.* (2013a) tried to tackle the problem of *in situ* observations using a “Drifting In Situ Chamber”. Therefore, the main priority in future studies should be focused on innovative *in situ* methods testing, at night, the sensory abilities of naïve larvae to cues of fish conspecifics vs. fish heterospecifics, of different coral species, or of different predator species.

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Appendix 2: Manuscript – Larval coral reef fish settlement patterns

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RESEARCH ARTICLE

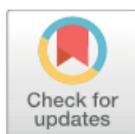
Consistency in the supply of larval fishes among coral reefs in French Polynesia

Marc Besson^{1,2*}, Camille Gache¹, Rohan M. Brooker³, Rakamaly Madi Moussa¹, Viliame Pita Waqalevu^{4,5}, Moana LeRohellec¹, Vincent Jaouen¹, Kévin Peyrusse¹, Cécile Berthe¹, Frédéric Bertucci¹, Hugo Jacob^{1,6}, Christophe Brié¹, Bruno Wan⁷, René Galzin^{1,8}, David Lecchini^{1,8}

1 PSL Research University: EPHE-UPVD-CNRS, USR 3278 CRIOBE, BP, Moorea, French Polynesia, **2** UMR 7232, CNRS-UPMC, Observatoire Océanologique de Banyuls-sur-Mer, Banyuls-sur-Mer, France, **3** School Marine Science and Policy, University of Delaware, Lewes, DE, United States of America, **4** School of Marine Studies, Institute of Marine Resources University of the South Pacific, Suva, Fiji, **5** Institute for Pacific Coral Reefs, IRCP, Moorea, French Polynesia, **6** International Atomic Energy Agency, Environment Laboratories (IAEA-EL), Principality of Monaco, Monaco, **7** Tahiti Perles, Papeete, Tahiti, French Polynesia, **8** Laboratoire d'Excellence "CORAIL", Moorea, French Polynesia

© These authors contributed equally to this work.

* marc.besson@ens-lyon.org



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Abstract

For marine fishes with a bipartite life cycle, pelagic larval dispersal can shape the distribution, connectivity, composition and resilience of adult populations. Numerous studies of larval dispersal, and associated settlement and recruitment processes, have examined the relationship between population connectivity and oceanographic features. However, relatively little is known about spatial and temporal variation in the abundance of larvae settling among different reefs and the extent to which the species assemblage of larvae settling at one location is reflective of the assemblage in neighbouring areas. Here, using crest nets, which provide a non-selective measure of the total abundance and assemblage of larvae settling to a reef (*i.e.* larval supply), we collected larval coral reef fishes at five locations surrounding two spatially disparate French Polynesian islands: Moorea and Nengo-Nengo. Overall, larval settlement patterns were correlated with the lunar cycle, with larval abundance peaking during the new moon. Although there were some spatial differences in larval supply among the five monitored sites, settlement patterns were largely consistent, even at the species level, irrespective of factors such as coastline orientation or distance between sites. This study provides further insights into the mechanisms driving patterns of dispersal and settlement of larval fishes over large spatial scales.

Introduction

Identifying the factors that determine patterns of distribution and abundance is a fundamental goal of ecology and conservation biology [1]. In species that undergo ontogenetic shifts in habitat use, patterns of early life-stage dispersal, return (*i.e.* settlement), and persistence in adult habitats (*i.e.* recruitment) can impact population dynamics and connectivity [2].

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Consequently, deciphering these patterns is essential for understanding population persistence and predicting resilience to environmental change [1,3,4].

The majority of coral reef fishes have a bipartite life cycle, consisting of a pelagic larval stage followed by a largely sedentary reef-associated juvenile/adult stage [3,5]. In these species, the pelagic stage (*i.e.* larvae) primarily facilitates dispersal, driven by both physical (*e.g.* oceanic currents) and biological (*e.g.* directional swimming and sensory perception) processes [4–6]. These processes act at a variety of spatial and temporal scales, with their relevance to dispersal varying depending on larval development and pelagic larval duration. For example, young larvae are usually poorly developed and mostly ineffective swimmers, with initial dispersal largely a result of hydrodynamic processes [7]. However, many larvae develop the capability to orientate themselves within the water column, to swim against the current, and to control their trajectories [8,9]. Thus, while larvae often have the potential to move over vast distances during the pelagic phase, many also have the potential to remain close to their native reefs [7,10,11], leading to well-connected populations on scales of 0–30 km [12,13]. After this planktonic larval phase, which usually lasts from 1 to 64 weeks, larval fishes settle into benthic reef habitats. Settlement patterns are often species-specific, dependent on nocturnal, lunar, seasonal and inter-annual factors. Some factors appear to be particularly important, with the abundance of larvae settling to reefs often peaking during the night, new moon, summer, and La Niña periods [7,14,15].

While a substantial amount is known regarding larval transport, dispersal, aggregations, movement patterns, and their relationship to oceanographic features [7,16–19], relatively little research has examined how closely settlement patterns at one reef reflect settlement to neighbouring reefs [20–22]. Indeed, most research has sampled relative larval abundance using light traps [17,23,24] or towed nets [17,25,26], while studies that have examined connectivity, dispersal and recruitment patterns have mainly done so via *in situ* surveys of larvae in pelagic or reef environments [27–31], or through genetics analyses [19,32] and otolithometry [13,16,33]. However, deciphering precisely where and when these larvae settled out of the plankton is often either not possible, or prohibitively difficult and labour intensive, using these techniques.

Where the reef structure is appropriate, crest nets allow for the collection of settlement-stage larval assemblages, with almost no sampling bias due to larval behaviour (such as occurs with light traps) and almost no net avoidance due to the continuous current flow over the reef crest and the turbulence of the surf zone in front of the net [15,20–22,34,35]. Crest nets therefore provide an accurate measure of the abundance and assemblage of settlement-stage larvae (*i.e.* larval supply) on a given reef. The few previous studies that have examined geographic patterns of larval supply have identified minimal spatial variation between reef sites located within the same island, whether they were separated by only 200 m and had the same coastline orientation [20,22], or by more than 10 km with different coastline orientations [21]. However, these studies have generally only compared a limited number of locations and further work is needed to quantify the degree to which oceanographic features (*e.g.* currents and diffusion) and biological processes (*e.g.* spawning, swimming potential, behaviour, and mortality) do or do not synchronize the arrival of larvae across spatially separated reefs [5,18]. Identifying the degree of spatial and temporal consistency or variation in larval supply between disparate reefs could also provide important spatial information regarding larval patch sizes in the ocean [16,36].

In the present study, we used crest nets to measure the variation in larval supply at five reef sites located on two spatially-disparate French Polynesian Archipelagos (Moorea Island in the Society Archipelago, and Nengo-Nengo Atoll in the Tuamotu Archipelago) over a 41 day period. This study aimed to identify spatial and temporal variability in patterns of larval settlement (using abundance and assemblage of settlement-stage larvae as proxies) and the

relationship between these patterns and external factors including lunar cycle, coastline orientation, and relative proximity.

Materials and methods

Ethics statement

This study was carried out in accordance with the guidelines of the French Polynesia committee for animal ethics, and the experiments were approved by the CRIOBE-IRCP animal ethics committee. This study did not involve endangered or protected species.

Study sites

This study was conducted on the reef crests surrounding two islands in French Polynesia: along the west coast of Moorea Island (Tetaiuo sector: 17°31'7.38"S, 149°55'20.89"W) in the Society Archipelago, and along the north and south-east coasts of Nengo-Nengo Atoll (18°42'38.37"S, 141°49'6.20"W; 18°46'32.52"S, 141°45'43.57"W) in the Tuamotu Archipelago (Fig 1A and 1B). The distance between the Moorea and Nengo-Nengo sites is approximately 778 km. The supply of larval fishes at each site was recorded using crest nets: two at Moorea and three at Nengo-Nengo. At Moorea, both crest nets were set up along the west coast, spaced 100 m apart, and designated M_W1 and M_W2 (Moorea west crest net 1 and 2) (Fig 1C). At Nengo-Nengo, two crest nets were set up on the south-east coast, also spaced 100 m apart, and designated NN_SE1 and NN_SE2 (Nengo-Nengo south-east crest net 1 or 2), and one crest net was set up on the north coast, 7 km from the south-east nets, and designated NN_N (Nengo-Nengo north crest net) (Fig 1D). These five sites were chosen as they were: (i) accessible in most weather and tidal conditions, and (ii) had the correct reef crest structure for crest net installation. Replicating sites with the same coastline orientation at Moorea and Nengo-Nengo was not possible due to logistical difficulties.

Sampling

The crest nets used to collect larval fishes followed a design used previously by Dufour and Galzin [15]. The body of each net was made of 2 mm mesh, allowing retention of all incoming larvae. Larvae entered the net via its rectangular mouth (width: 1.8 m; height: 1 m), oriented parallel to the reef crest, *i.e.* against the water flow and wave direction. For each net, the capture area was enlarged to a total of 5 m width through the addition of two hinged panels (width: 2m, height: 1 m) oriented at 40° on each side of the net entry. Using this method, all larvae entering the reef at this point were channelled down the net where they were collected in a cod-end for subsequent sampling. Cod-ends were only attached to the nets in the late afternoon to minimise the catch of debris during daylight hours when larval arrival onto the reef is minimal [15], and detached following sunrise to remove the larvae captured during the night. Following collection, all larval fishes were transferred to aquaria, where they were identified to the lowest taxonomic level possible following the key by Leis and Carson-Ewart [37], before being released. Larvae were collected from each of the five nets each morning between 10th March and 14th May 2011.

Statistical analyses

All statistical analyses were conducted using the R-Cran project free software (<http://www.rproject.org/>, R-3.3.1). To determine if the total and daily abundance of settlement-stage larvae differed over the study period between Moorea Island and Nengo-Nengo Atoll, we performed Welch two-sample t-tests, when data met assumptions of normality (Shapiro test) and

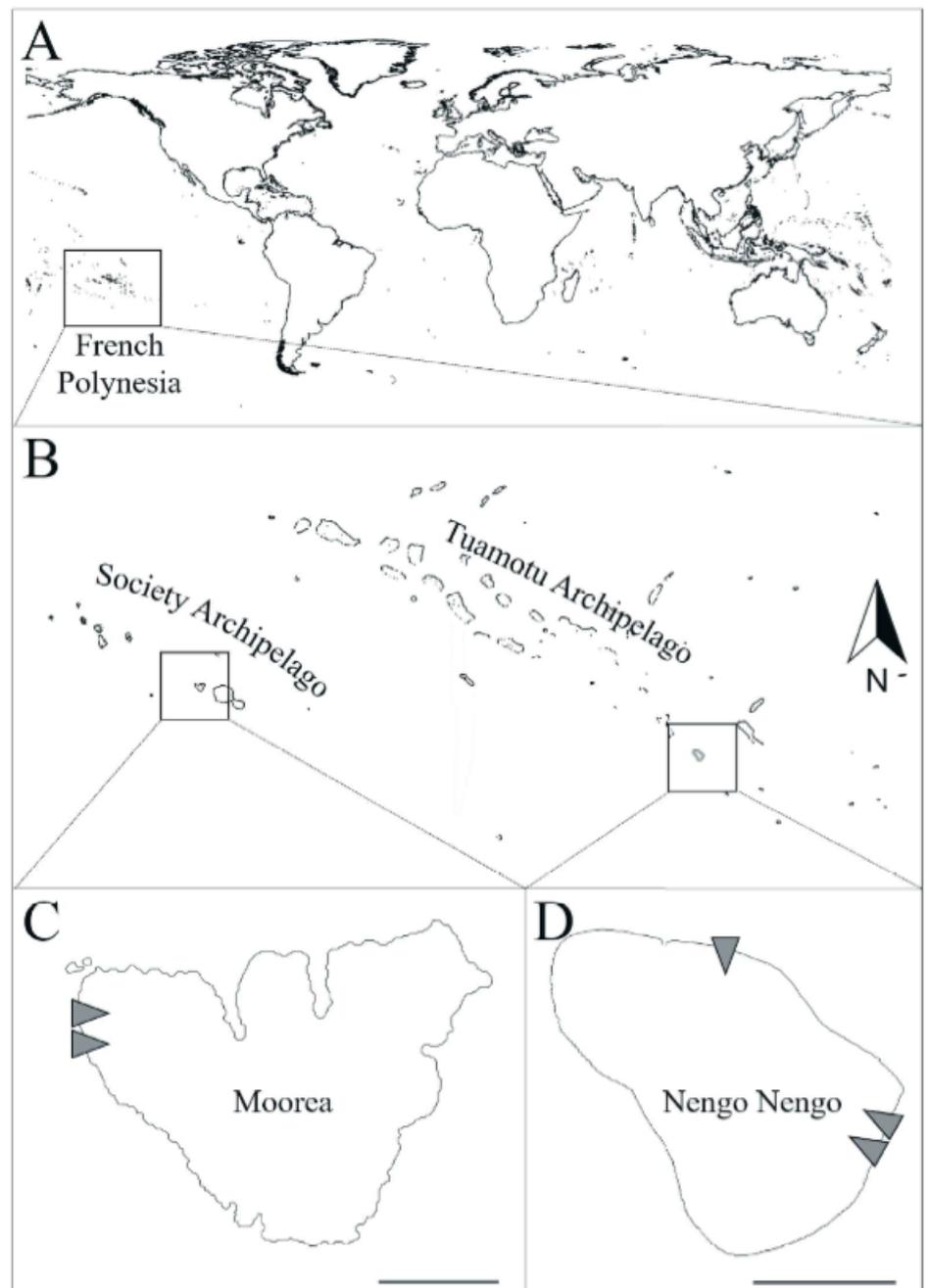


Fig 1. Study sites and crest net locations. (A) Location of French Polynesia within the Pacific Ocean. (B) Location of Moorea Island and Nengo-Nengo Atoll within French Polynesia. (C) Location of crest net sites. Grey triangles indicate crest net locations (M_W1 and M_W2 along the west coast of Moorea, NN_N on the north coast of Nengo-Nengo, and NN_SE1 and NN_SE2 along the south-east coast of Nengo-Nengo), as well as respective inflow orientations.

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homogenous variance (Bartlett test). Wilcoxon rank sum tests were conducted when data did not meet these assumptions. We also determined if differences occurred between Moorea Island and Nengo-Nengo for the four most abundant species collected: *Acanthurus triostegus*, *Bothus mancus*, *Chromis viridis* and *Pristiapogon fraenatus*, by conducting the same statistical tests after normalizing species abundances to the abundance of all larvae captured at each site. We then compared within-site differences in daily abundance of settlement-stage larvae (*i.e.* among the five sites: both Moorea sites and the three Nengo-Nengo sites) using a Kruskal-Wallis test followed by a Kruskal-Nemenyi post hoc test.

To investigate if temporal variations in the supply of larval fishes (*i.e.* settlement patterns) were correlated with the lunar cycle, we performed cross correlation function (CCF) analyses using the *stats* package. These analyses compared the total and species-specific daily larval abundance at each site with the percentage of the moon that was illuminated (Table 1). Ranked

Table 1. Larval settlement patterns in relation to lunar phases.

Species	CCF	lag	Spearman ρ
All species	M_W1	1 [-2:2]	-0.48 (**)
	M_W2	1 [-1:2]	-0.49 (**)
	NN_N	NS	NS
	NN_SE1	-1 [-2:1]	-0.58 (***)
	NN_SE2	1 [-1:2]	-0.57 (***)
A. triostegus	M_W1	-1 [-2:1]	-0.58 (***)
	M_W2	0 [-2:2]	-0.62 (***)
	NN_N	NS	NS
	NN_SE1	-1 [-2:0]	-0.50 (**)
	NN_SE2	NS	NS
B. mancus	M_W1	2 [1:4]	-0.53 (***)
	M_W2	NS	NS
	NN_N	NS	NS
	NN_SE1	-1 [-2:1]	-0.56 (***)
	NN_SE2	NS	NS
C. viridis	M_W1	-8 [-9:-7]	0.46 (**)
	M_W2	-8 [-11:-8]	0.42 (**)
		-1 [-2:0]	-0.47 (**)
	NN_N	0 [-1:0]	-0.43 (**)
	NN_SE1	0 [-2:1]	-0.54 (***)
P. fraenatus	NN_SE2	0 [-1:1]	-0.47 (**)
	M_W1	-4	0.41 (**)
	M_W2	-4	0.40 (**)
	NN_N	-3 [-5:-2]	-0.44 (**)
	NN_SE1	-2 [-2:-1]	-0.42 (**)
NN_SE2	NS	NS	

CCF analyses were performed with total and species-specific daily larval abundance as the first (X) variable, and percentage of the moon that was illuminated as the second (Y) variable. In the "lag" column, α [β : γ ; δ] indicates that lags β to γ , as well as lag δ , are the lags of X for which the correlations between X and Y are significant, and that among all these lags α is the lag with the highest absolute Spearman ρ value. Spearman ρ coefficient is only indicated for lag α and *, ** and *** indicate that this correlation coefficient is significantly different from 0 with a p-value inferior to 0.05, 0.01 and 0.001 respectively. NS indicates that no significant correlation was sorted out the CCF analysis.

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daily larval abundance data were used as raw data did not meet the assumption of normality. CCF analyses resulted in a variety of “lags” (*i.e.* delay days), and their associated Spearman ρ , for which the daily larval abundance and the percentage of the moon that was illuminated were correlated. A negative “lag” indicates that variations in larval abundance (*e.g.* a peak or a minimum) occurred a certain number of days prior to variations in lunar illumination (*e.g.* full moon or new moon), while a positive “lag” indicates that variations in larval abundance occurred a certain number of days following variations in lunar illumination. For example, a “lag” value of “-2”, associated with a negative value Spearman ρ indicates that larval abundance and the percentage of the moon that is illuminated are correlated, with larval abundance peaking two days before minimum lunar illumination (*i.e.* new moon). Lags were sorted and removed if their associated Spearman ρ was $< |0.40|$ (with an associated p -value > 0.01), and pooled if consecutive. For example, if lags “-2”, “-1”, “0”, “1” and “6” were sorted from a CCF analysis, with the highest correlation (highest absolute value of Spearman ρ) between the two series at lag = “-1”, results were presented the following way: -1 [-2:1;6], with Spearman ρ only given for the lag maximum (“-1” in this example) (see Tables 1–3).

To investigate if variations in temporal supply of larval fishes were correlated among the five sites, we compared the total and species-specific daily larval abundances between each site (Table 2). To do so, we used CCF analyses in a similar way as described above. To investigate species-specific variations in temporal supply of fish larvae at each site (*i.e.* species-specific settlement patterns), we compared the daily larval abundances of each of the four dominant species with the total daily larval abundance of the associated site (Table 3). Again, we used CCF analyses in a similar way as described above.

Results

A total of 83,915 larval fishes belonging to 112 species were collected and identified from the five crest nets between March 10th and May 14th, 2011. In Moorea, the two crest nets collected 4,892 and 4,225 larvae from 104 species (including 75 also found in the Nengo-Nengo catches), respectively. In Nengo-Nengo, 16,970 larval fishes were collected at the crest net located on the north coast, and the south-east coast crest nets collected 29 206 and 28 622 larval fishes from 95 species, respectively. Overall, the total larval abundance was 5 times higher in Nengo-Nengo than Moorea (Welch two sample t -test, $t = -5.09$, $df = 2.03$, $p < 0.05$), as was the daily larval abundance (Kruskal-Wallis $\chi^2 = 42.13$, $df = 4$, $p < 0.001$). Within each location (Moorea and Nengo-Nengo), no significant difference in larval abundance was observed between nets (Kruskal-Wallis $\chi^2 = 4$, $df = 4$, $p = 0.41$). The relative abundance of larvae collected peaked at the beginning of April and in late April- early May (Fig 2A).

Among the 112 species captured, the four most abundant were: *Acanthurus triostegus* (0.2 to 23% of net catches), *Bothus mancus* (1.1 to 4.3%), *Chromis viridis* (4.4 to 28.3%) and *Pristiapogon fraenatus* (1.2 to 42.4%). The abundance of *A. triostegus* larvae was almost 34 times higher in Moorea compared to Nengo-Nengo (Welch two sample t -test, $t = 10.78$, $df = 1.16$, $p < 0.05$), while the abundance of *C. viridis* larvae was almost 5 times higher in Nengo-Nengo compared to Moorea (Welch two sample t -tests, $t = -6.95$, $df = 2.14$, $p < 0.01$). The abundance of *B. mancus* and *P. fraenatus* larvae were not significantly different between islands (Welch two sample t -test, $t = -1.01$, $df = 2.42$, $p > 0.40$, and Wilcoxon rank sum test, $W = 0$, $p = 0.2$). Except for *P. fraenatus*, the relative abundance of all species peaked at the beginning of April and May (Fig 2B–2F).

Peaks in total larval abundance occurred one day before or one day after the new moon, in both nets at Moorea and both south-east nets at Nengo-Nengo (Table 1). At the species level, the same temporal settlement pattern was observed for *A. triostegus* at M_W1, M_W2 and

NN_SE1, *B. mancus* at M_W1 and NN_SE1, and *C. viridis* in all sites except M_W1 (Table 1). At M_W1 and M_W2, peaks in *C. viridis* larval abundance occurred 8 days before the full moon (Table 1). For *P. fraenatus*, peaks in larval abundance occurred 4 days before the full moon at M_W1 and M_W2, and several days before the new moon at NN_N and NN_SE1 (Table 1).

Table 2. Comparisons of larval settlement patterns between nets.

Species	CCF	lag	Spearman ρ
All species	M_W1 ~ M_W2	0 [-2:2]	0.96 (***)
	M_W1 ~ NN_N	NS	NS
	M_W1 ~ NN_SE1	NS	NS
	M_W1 ~ NN_SE2	NS	NS
	M_W2 ~ NN_N	NS	NS
	M_W2 ~ NN_SE1	NS	NS
	M_W2 ~ NN_SE2	NS	NS
	NN_N ~ NN_SE1	0	0.42 (**)
	NN_N ~ NN_SE2	0	0.52 (***)
	NN_SE1 ~ NN_SE2	0 [-4:1]	0.83 (***)
<i>A. triostegus</i>	M_W1 ~ M_W2	1 [-2:2]	0.79 (***)
	M_W1 ~ NN_N	13	0.46 (**)
	M_W1 ~ NN_SE1	NS	NS
	M_W1 ~ NN_SE2	NS	NS
	M_W2 ~ NN_N	NS	NS
	M_W2 ~ NN_SE1	3	0.43 (**)
	M_W2 ~ NN_SE2	NS	NS
	NN_N ~ NN_SE1	NS	NS
	NN_N ~ NN_SE2	NS	NS
	NN_SE1 ~ NN_SE2	NS	NS
<i>B. mancus</i>	M_W1 ~ M_W2	0	0.49 (**)
	M_W1 ~ NN_N	NS	NS
	M_W1 ~ NN_SE1	NS	NS
	M_W1 ~ NN_SE2	NS	NS
	M_W2 ~ NN_N	NS	NS
	M_W2 ~ NN_SE1	NS	NS
	M_W2 ~ NN_SE2	NS	NS
	NN_N ~ NN_SE1	0	0.67 (***)
	NN_N ~ NN_SE2	0 [0:5]	0.53 (***)
	NN_SE1 ~ NN_SE2	NS	NS
<i>C. viridis</i>	M_W1 ~ M_W2	-6 0 [-1:0]	-0.47 (**) 0.62 (***)
	M_W1 ~ NN_N	NS	NS
	M_W1 ~ NN_SE1	NS	NS
	M_W1 ~ NN_SE2	NS	NS
	M_W2 ~ NN_N	NS	NS
	M_W2 ~ NN_SE1	1	0.40 (**)
	M_W2 ~ NN_SE2	NS	NS
	NN_N ~ NN_SE1	0	0.81 (***)
	NN_N ~ NN_SE2	0	0.73 (***)
	NN_SE1 ~ NN_SE2	0 [-1:1;3]	0.84 (***)

(Continued)

Table 2. (Continued)

Species	CCF	lag	Spearman ρ
<i>P. fraenatus</i>	M_W1 ~ M_W2	0 [0:1]	0.73 (***)
	M_W1 ~ NN_N	NS	NS
	M_W1 ~ NN_SE1	-3	-0.43 (**)
	M_W1 ~ NN_SE2	NS	NS
	M_W2 ~ NN_N	NS	NS
	M_W2 ~ NN_SE1	-3	-0.41 (**)
	M_W2 ~ NN_SE2	NS	NS
	NN_N ~ NN_SE1	0 [0:1]	0.84 (***)
	NN_N ~ NN_SE2	0 [-1:0]	0.75 (***)
NN_SE1 ~ NN_SE2	0 [-1:0]	0.73 (***)	

Net1 ~ Net2 indicates that the CCF analyses was performed with total or species-specific daily larval abundance at site 1 as the first (X) variable, and at site 2 as the second (Y) variable. In the "lag" column, α [β ; γ ; δ] indicates that lags β to γ , as well as lag δ , are the lags of X for which the correlations between X and Y are significant, and that among all these lags α is the lag with the highest absolute Spearman ρ value. Spearman ρ coefficient is only indicated for lag α and *, ** and *** indicate that this correlation coefficient is significantly different from 0 with a p-value inferior to 0.05, 0.01 and 0.001 respectively. NS indicates that no significant correlation was sorted out the CCF analysis.

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Table 3. Comparisons of species versus total settlement patterns.

	CCF	lag	Spearman ρ
M_NW1	<i>A. triostegus</i>	0 [-2:2]	0.60 (***)
	<i>B. mancus</i>	NS	NS
	<i>C. viridis</i>	-8	-0.45 (**)
		-1 [-1;1]	0.43 (**)
<i>P. fraenatus</i>	NS	NS	
M_NW2	<i>A. triostegus</i>	-9	-0.41 (**)
		-1 [-1:0]	0.68 (***)
	<i>B. mancus</i>	NS	NS
	<i>C. viridis</i>	0 [-2:1;7]	0.44 (**)
	<i>P. fraenatus</i>	NS	NS
NN_N	<i>A. triostegus</i>	NS	NS
	<i>B. mancus</i>	NS	NS
	<i>C. viridis</i>	NS	NS
	<i>P. fraenatus</i>	-1	0.43 (**)
NN_SE1	<i>A. triostegus</i>	-1 [-1:6]	0.67 (***)
	<i>B. mancus</i>	0 [-1:1;4:5]	0.66 (**)
	<i>C. viridis</i>	0 [-2:3]	0.73 (***)
	<i>P. fraenatus</i>	0 [-3:1;4]	0.71 (***)
NN_SE2	<i>A. triostegus</i>	NS	NS
	<i>B. mancus</i>	-1 [-1:0]	0.58 (***)
	<i>C. viridis</i>	0 [-1:2]	0.82 (***)
	<i>P. fraenatus</i>	NS	NS

CCF analyses were performed with species daily larval abundance at a site as the first (X) variable, and total daily larval abundance of the same site as the second (Y) variable. In the "lag" column, α [β ; γ ; δ] indicates that lags β to γ , as well as lag δ , are the lags of X for which the correlations between X and Y are significant, and that among all these lags α is the lag with the highest absolute Spearman ρ value. Spearman ρ coefficient is only indicated for lag α and *, ** and *** indicate that this correlation coefficient is significantly different from 0 with a p-value inferior to 0.05, 0.01 and 0.001 respectively. NS indicates that no significant correlation was sorted out the CCF analysis.

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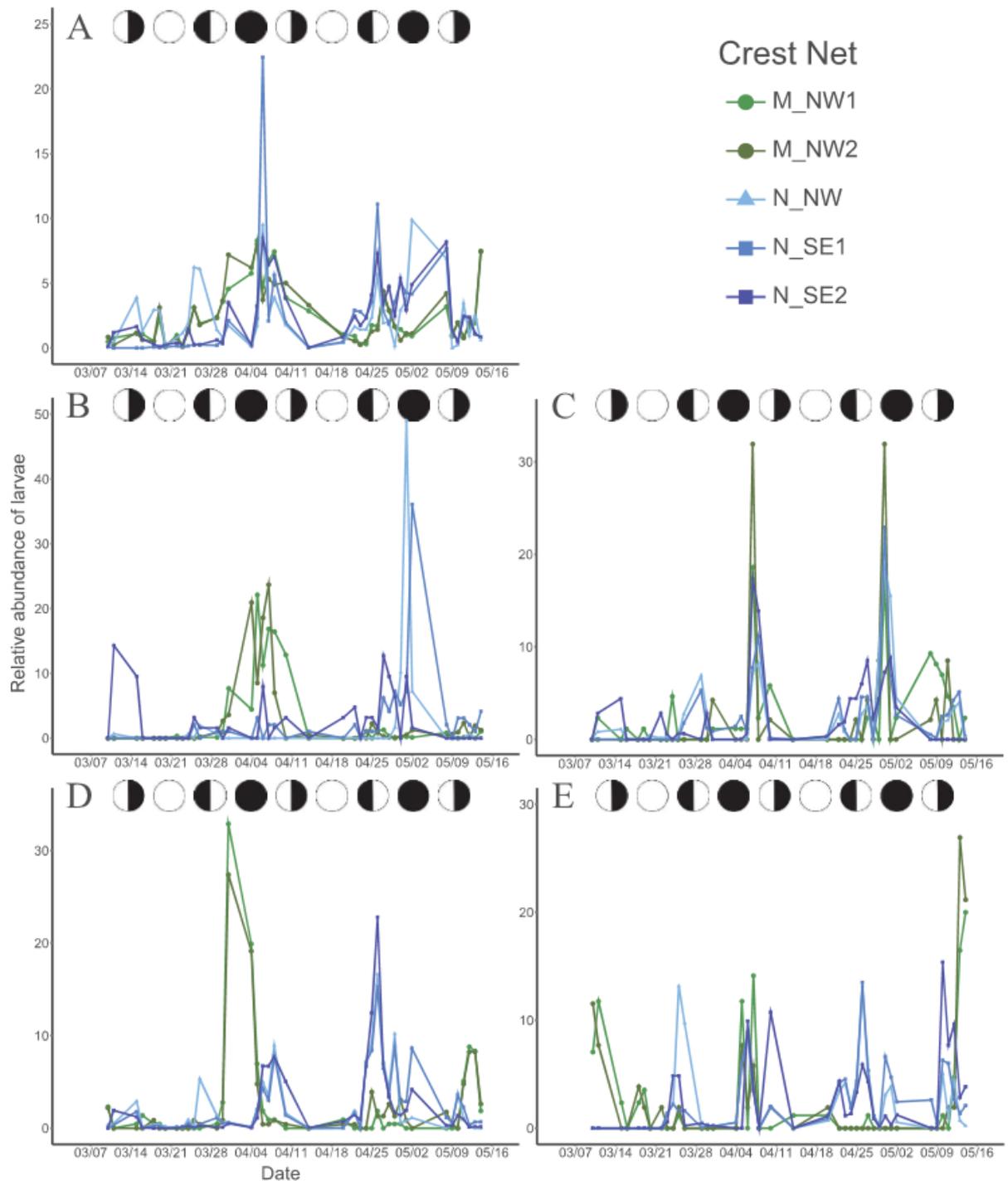


Fig 2. Relative abundances of larvae in each crest net. Relative abundance of (A) all larval fishes, (B) *Acanthurus triostegus* larvae, (C) *Bothus mancus* larvae, (D) *Chromis viridis* larvae, (E) *Pristiapogon fraenatus* larvae. Green and dark green lines indicate M_NW1 and M_NW2 nets respectively while light blue, blue, and dark blue lines indicate N_NW, N_SE1 and N_SE2. Circular points indicate a crest net with a westward orientation, triangular points indicate a northward orientation and square points indicate a south-eastward orientation. Black and white circles above each panel indicate the lunar phases: new moon (black circle), first quarter, full moon (white circle) and last quarter.

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When considering all larvae, settlement patterns were similar between the two nets at Moorea and among the three nets at Nengo-Nengo (Table 2). At the species level, the larval settlement patterns of *A. triostegus*, *B. mancus*, *C. viridis* and *P. fraenatus* were similar between the two nets at Moorea (Table 2), while only the larval settlement patterns of *C. viridis* and *P. fraenatus* were consistent between the three nets at Nengo-Nengo (Table 2). While larval settlement patterns of *B. mancus* were not correlated between NN_SE1 and NN_SE2, both were found to be similar to NN_N individually (Table 2).

Finally, at M_W1 and M_W2, only *A. triostegus* and *C. viridis* exhibited larval settlement patterns that were similar to the total pattern of their associated net (Table 3), while the larval abundance of all four key species peaked at the same time at NN_SE1 (Table 3). At NN_SE2 this was also the case for *B. mancus* and *C. viridis* (Table 3), while at NN_N, only *P. fraenatus* exhibited a larval settlement pattern that was correlated with the total pattern of its associated net, with a peak of abundance occurring one day before the peak of total abundance (Table 3).

Discussion

Although spatial differences in larval abundance (total and daily) and assemblage structure were observed among the five monitored sites (Fig 2 and Table 2), temporal patterns of larval settlement were broadly similar in that peaks of larval supply occurred at the new moon at four of the five sites (Table 1). This finding conforms with previously observed patterns of larval settlement in this region as well as other reef areas [14,15,38]. This result also reveals that, even among sites with different coastline orientations (*i.e.* each submitted to different wind and swell inputs) and separated by almost 800 km, the lunar cycle had a greater influence than local habitat and hydrodynamic conditions in determining patterns of reef fish settlement.

At the species level, while an especially high abundance of settlement-stage *A. triostegus* in Moorea has been acknowledged previously along almost all coastlines [22,39], this is the first study to identify a high abundance of settlement-stage *C. viridis* (Pomacentridae), *P. fraenatus* (Apogonidae) and *B. mancus* (Bothidae) in an atoll in the Tuamotu Archipelago [38]. With regards to temporal settlement patterns, the abundance of *A. triostegus* larvae was correlated between both Moorea sites and one Nengo-Nengo site (NN_SE1), while the other dominant species exhibited distinct trends in larval abundance between the two islands (Tables 1 & 2). Patterns of *C. viridis* larval abundance were similar in both Moorea sites (Table 1), with peaks occurring approximately one week before the full moon; a period equivalent to that described by Dufour and Galzin [15], but different to that observed in the Nengo-Nengo sites, where larval abundance peaked around the new moon (Table 1).

Differences between Moorea and Nengo-Nengo with regards to both larval abundances and species-specific settlement patterns may be explained by the different coastline orientations of the crest nets at each location, if associated sampling sites were subject to different hydrographic pressures (*e.g.* currents, eddies, swells and winds). For example, both islands are located within the westward moving 'Southern Equatorial Current' [40], but Nengo-Nengo nets were oriented towards this current while those in Moorea were not. These differences in water influx could have affected larval supply at each location. However, Dufour et al. [21] demonstrated that patterns of larval settlement in Moorea can be homogeneous, even between sites separated by 8 to 12 km with different coastline orientations. This suggests that orientation to prevailing current does not play a central role in determining relative larval abundance. Although this study used a small number of crest net replicates, similar homogeneous patterns of larval settlement were observed at Nengo-Nengo, with larval abundances peaking simultaneously for three of the dominant species at all three sites (Table 2). Consequently, what is more likely is that differences in larval abundance between islands is a consequence of factors

such as differences in the adult density [41], the relative size of each island's lagoon (Nengo-Nengo lagoon area is 108 km² against 61 km² for Moorea), the topographic variation (high island vs. atoll environments), and the relative geographical proximity of other islands (several other atolls surround Nengo-Nengo, while Moorea is comparatively relatively isolated with only Tahiti high island and small Tetiaroa atoll nearby) [11,40]. In addition, as coral reef fish larvae use a variety of marine and land-based sensory cues to locate, differentiate between, and orientate towards settlement sites [42–45], the environmental differences that exist between the two islands could potentially affect their relative ability to attract settlement-stage larval fish. Moorea is a high island with rivers, bays, a developed coastline, twelve passes between the ocean and the lagoon that are distributed on all coastlines, and almost no tides [40]. Therefore, Moorea potentially emits sensory cues, such as waterborne chemicals from terrestrial vegetation, homogeneously and over limited spatial scales. In contrast, Nengo-Nengo is a remote, relatively undeveloped, atoll subject to a strong tidal influence and with only one pass [40]. Therefore, important cues, such as chemicals that indicate the location of the lagoon, are likely released heterogeneously (in the direction of the pass) and spread over a larger spatial area. Further work that compares patterns of larval settlement between spatially disparate sites that have the same coastline orientation will determine if an island's environmental characteristics are more important than coastline orientation in determining the associated larval supply.

Spatial consistency in larval supply could also reflect the size of the larval patch in the ocean [16,17,22]. A substantial body of research suggests that the presence of certain oceanographic processes, such as wake eddies behind reefs, can promote the retention of larvae in patches around islands [12,13,18,46–49]. From these patches, larvae may then swim towards nearby reef habitats to settle following a pelagic waiting period [15], with the timing determined by broad-scale environmental conditions (e.g. the lunar phase), as well as local processes that affect recruitment (e.g. temporal patterns of spawning, narrow ranges in pelagic larval duration, variations in reef-associated noise, wind direction, or local dynamism of the water column) [7,50]. The results of this study do not provide definitive evidence for either the 'large larval patch' or 'dilution of a smaller larval patch at the time of settlement' hypotheses [16,17]. Nevertheless, the occurrence of synchronous larval settlement events with identical temporal patterns, even at the species level, at sites with different coastline orientations, were seen here at Nengo-Nengo and in a previous study at Moorea [21]. At sites with the same coastline orientation, previous studies have also identified consistency in temporal patterns of larval settlement among six sites separated by 200 m on the west coast of Moorea [22], and also between sites separated by 200 m on the west coast of Australia [20]. All these results point towards the presence of a large oceanic larval patch surrounding islands. Indeed it is unlikely that, in spatially disparate areas with different coastline orientations, larvae from small patches would exhibit precise synchronization with regards to settlement. Moreover, among multiple sites located within the same coastline, settlement of small patches would have resulted, at some sites, in an absence of settlers. However, this was not the case here or in other studies [20–22].

Examination of variation in larval supply in Moorea and Nengo-Nengo identified that larval abundance is generally higher during new moon periods, even between sites separated by up to 800 km. This result is in line with other studies, on other reef fish species, from the Caribbean [51,52], Mediterranean [53] and Pacific [15,20,21,38]. This study also highlights the value of crest nets as a mean for gathering accurate and unbiased estimations of larval supply in comparison to light traps (selective catch), otolithometry and *in situ* surveys (*i.e.* which reveal more recruitment than settlement patterns). Although we did observe differences between both islands, in particular regarding relative abundances at the species level (*i.e.* assemblage structure), there was a strong consistency in larval settlement patterns within each island, even among sites located along different coastlines. The reasons for this consistency are still unclear,

but are likely linked to a variety of physical and biological processes, interacting with a large and diluted larval patch surrounding each island.

Supporting information

SI File. Collected larval fishes and percentage of the moon that was illuminated. Raw data of the larval fishes that were collected during the study and data of the percentage of the moon that was illuminated.
(XLSX)

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Author Contributions

Conceptualization: MB RG DL.

Data curation: MB CG DL.

Formal analysis: MB CG DL.

Funding acquisition: VPW RG DL.

Investigation: MB CG RMM VPW ML VJ KP C. Berthe FB HJ C. Brié BW RG DL.

Methodology: MB.

Project administration: DL.

Resources: C. Brié BW RG DL.

Software: MB CG.

Supervision: RG DL.

Validation: MB CG DL.

Visualization: MB CG DL.

Writing – original draft: MB CG DL.

Writing – review & editing: MB CG RMB DL.

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Appendix 3: Chemical pollution and marine larvae sensory abilities

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Habitat selection by marine larvae in changing chemical environments

D. Lecchini^{a,b,*}, D.L. Dixon^c, G. Lecellier^{b,d}, N. Roux^{a,e}, B. Frédérick^f, M. Besson^{a,g}, Y. Tanaka^h, B. Banaigs^a, Y. Nakamuraⁱ^a EPHE, PSL Research University, UPVD-CNRS, USR3278 CRIOBE, F-66360 Perpignan, France^b Laboratoire d'Excellence CORAIL, Moorea, French Polynesia^c School Marine Science and Policy, University of Delaware, Newark, USA^d University of Versailles-Saint, Versailles, France^e Equipe Biologie Intégrative de la Métamorphose BIOM UMR7232 CNRS-UPMC Observatoire Océanologique de Banyuls sur mer, Banyuls sur mer, France^f Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH Research Center, Liege, Belgium^g Molecular Zoology Team, Institut de Génétique Fonctionnelle de Lyon, Université Lyon 1, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Lyon, France^h Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Sesoko, Japanⁱ Graduate School of Kuroshio Science, Kochi University, Kochi, Japan

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ABSTRACT

The replenishment and persistence of marine species is contingent on dispersing larvae locating suitable habitat and surviving to a reproductive stage. Pelagic larvae rely on environmental cues to make behavioural decisions with chemical information being important for habitat selection at settlement. We explored the sensory world of crustaceans and fishes focusing on the impact anthropogenic alterations (ocean acidification, red soil, pesticide) have on conspecific chemical signals used by larvae for habitat selection. Crustacean (*Stenopus hispidus*) and fish (*Chromis viridis*) larvae recognized their conspecifics via chemical signals under control conditions. In the presence of acidified water, red soil or pesticide, the ability of larvae to chemically recognize conspecific cues was altered. Our study highlights that recruitment potential on coral reefs may decrease due to anthropogenic stressors. If so, populations of fishes and crustaceans will continue their rapid decline; larval recruitment will not replace and sustain the adult populations on degraded reefs.

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1. Introduction

Coral reefs are home to approximately 25% of the ocean's biodiversity while only accounting for 0.02% of the ocean's surface area (Spalding et al., 2001). Due to the economic and environmental importance, unique assemblage of organisms, and high species diversity found on coral reefs, protective measures are critical (Chin et al., 2011; de Groot et al. 2012). Beginning in the early 1990's, questions have been raised concerning the resilience of coral reefs to global changes (Grigg and Dollar, 1990). Today, it is estimated that 20% of coral reefs have been destroyed, 25% are under great immediate threat, and a further 25% will be under threat by 2050 (Chin et al., 2011). Several studies have shown that coral reefs exposed to a disturbance event often exhibit a decline in adult populations. This decline leads to accelerated rates of extirpation compared to "non-degraded" habitats (Hughes et al., 2003; Munday et al., 2009). For example, the decline in adult populations of coral reef fishes on degraded reefs in Papua New Guinea was a reflection of larval recruitment failure rather than adult mortality.

Results suggest the "rescue" effect of recruitment may be ineffective in degraded habitats (Jones et al. 2004). Unfortunately, the mechanisms that determine how pelagic larvae respond to different environmental stressors and the role anthropogenic induced change plays during settlement site selection by recruiting organisms remains poorly understood (Hanski and Gilpin, 1997; Lecchini et al., 2013; Dixon et al., 2014).

Most species of coral reef fishes and crustaceans have stage-structured life histories: a relatively sedentary benthic stage (juveniles and adults) produces highly dispersive pelagic larvae (Kingsford et al., 2002). The transition from the pelagic environment to a benthic reef (i.e., recruitment process) represents a key period in the ontogeny of marine organisms (Lecchini, 2005). At recruitment, fish and crustacean larvae are subjected to strong selective pressure to choose a suitable reef habitat that will promote post-recruitment survival and growth of individuals (Doherty, 2002). Up to 90% of fish larvae may be removed by predation during the first week post-recruitment if suitable habitat was not selected (Doherty et al., 2004). Thus, many fish and crustacean species show high selectivity in suitable reef habitat; basing decisions on the presence of specific substrates and/or conspecifics, as well as the absence of predators and/or competitors (Kingsford et al., 2002; Lecchini et al., 2010; Barth et al., 2015). Chemical cues are often used

* Corresponding author at: EPHE, PSL Research University, UPVD-CNRS, USR3278 CRIOBE, F-66360 Perpignan, France.

E-mail address: lecchini@univ-perp.fr (D. Lecchini).

by fish and crustacean larvae to locate suitable reefs (Leis et al., 2011; Lecchini et al., 2013; Dixon et al., 2014), to identify conspecifics (Dixon et al., 2011), and to avoid predators (Dixon et al., 2012). For example, settling fish larvae are capable of olfactory discrimination among reefs, preferring the water-borne odors of their home reefs compared to neighboring reef habitats (Gerlarch et al., 2007). The understanding of how animals make decisions is a fundamental question in behavioural ecology (Feely et al., 2009; Barth et al., 2015). While evidence is mounting that larval organisms are active participants in the process of dispersal and recruitment (Lecchini et al., 2010), the sensory and behavioural mechanisms by which larvae disperse and return to appropriate habitat remain unknown. Understanding the recruitment process is especially important in the context of global reef decline due to natural and/or anthropogenic stressors (Munday et al., 2009; Lecchini et al., 2013; Dixon et al., 2014).

We investigated the behavioural response towards conspecific chemical stimuli by fish and crustacean larvae used in habitat selection during recruitment at two reef locations (Moorea Island, French Polynesia; Sesoko Island, Ryukyu Archipelago, Japan). Research was conducted in the context of anthropogenic-induced change, specifically focusing on 1) ocean acidification, 2) red soil pollution and 3) pesticide pollution.

Since the beginning of the industrial revolution, atmospheric concentrations of carbon dioxide (CO₂) have risen dramatically. As atmospheric CO₂ passively diffuses into the ocean's surface waters, seawater pH decreases, and in turn increasing water acidity (Feely et al., 2009). Several studies have shown that changes to seawater chemistry are predicted to impact the health and function of fishes (Munday et al., 2012; Leduc et al., 2013). We investigated how acidified seawater (700 and 1000 ppm) could alter the chemical cues of conspecifics available to fish and crustacean larvae.

Turbidity is one of the biggest sources of habitat degradation. The elevated amount of sediment suspended in the water column is generated by increased urbanisation and agricultural development run-off (Fabricius, 2005). We investigated the effects of red soil pollution (50 and 200 mg/L of red soil in clear or turbid water) on the chemical abilities of fish and crustaceans to recognize their conspecifics. The term "red soil" refers to a laterite soil prominent in Ryukyu Islands, Japan (Omija, 2004). The geographic features and rainfall patterns combined with surges in land development since the 1970s on Ryukyu Islands has resulted in the periodic erosive run-off and re-suspension of red soil. The increase in red soil runoff pollutes the surrounding coral reefs with high levels of silt and turbidity (Omija, 2004; O'Connor et al., 2016).

In contrast to the large numbers of studies testing the influence of pesticides on food and human health, few studies have explored its influence on reef biodiversity (Fabricius et al., 2005; Botte et al., 2012). In French Polynesia, Roche et al. (2011) illustrated the contamination of marine organisms (fish, green algae, mollusk, coral and holothurian) by several herbicides (chloroacetamide and triazine derivatives) and several insecticides (organophosphates and organochlorines). We examined the effect of the organophosphorus pesticide (1 and 100 µg/L of chlorpyrifos) on the chemical abilities of marine larvae to detect conspecific cues.

We hypothesized that conspecifics emit chemical cues that larvae are able to recognize in un-polluted seawater. However in the presence of anthropogenic pollution, the ability to chemically recognize conspecifics will be altered. Thus, if the recruitment potential of coral reefs has decreased due to these anthropogenic stressors (i.e. larvae unable to detect important chemical cues in polluted environment), the populations of fishes and crustaceans will continue to rapidly decline, as larval recruitment will not sustain adult populations on degraded ecosystems.

2. Materials and methods

2.1. Sampling locations and target species

Habitat naïve larval fish (*Chromis viridis*) and crustaceans (*Stenopus hispidus*) were collected just before settlement using light traps

(Nakamura et al., 2009a,b) set 300 m off of the fringing reefs on the south-east side of Sesoko Island (26°38'08.94"N, 127°51'55.04"E). The acidification and red soil experiments were conducted at the Sesoko research station in August 2012. To conduct the pesticide experiment, habitat naïve larvae were collected just before settlement using crest nets (Lecchini et al., 2004, 2006; Lo-Yat et al., 2011) set off the west coast of Moorea Island (17°30'58.85"S, 149°55'26.77"W) in March–April 2013. The capture of *S. hispidus* was low, therefore the pesticide experiment focused on *C. viridis*. All collected larvae were transferred and maintained in habitat free individual aquaria (0.3 × 0.3 × 0.2 m; water temperature: 26–27 °C) supplied with flow-through ocean seawater. Laboratory experiments were performed within 24 h of larval capture (Dixon et al., 2011; Lecchini et al., 2013). Conspecifics and heterospecifics, used as cues transmitters, were *C. viridis* and *S. hispidus* larvae reared in aquaria for 7 days post capture. *C. viridis* was used as the heterospecific cue for *S. hispidus* and vice versa. This was done to represent a post-settled juvenile stage. Larvae, conspecifics and heterospecifics were fed three times per day (live *Artemia* sp. Nauplii - *C. viridis* and dead fish - *S. hispidus*).

2.2. Behavioural experiments in choice flume

A two-channel choice flume described in Gerlach et al. (2007) was used to test larval preferences between olfactory cues in present day and acidified seawater conditions (Exp. 1), water treated with or without red soil (Exp. 2), and pesticides (Exp. 3). Briefly, a flow rate of 100 mL/min was maintained using flow meters. The low flow allowed larvae to swim without struggle against the current, ensuring movement patterns were a result of cue preference (Leis et al., 2011). Dye tests were conducted to confirm laminar flow within the chamber without eddies or areas of water mixing.

A larva was placed in the center of the downstream end of the flume during a 2 min habituation period. During this time the larva could explore the chamber and swim between the two parallel flowing water sources. Individuals that did not actively swim or explore both sides of the chamber during the habituation period were discarded (<2% of fish and <6% of crustacean). After the habituation period, the position of the larva, in either the right or left water channel, was recorded at five-second intervals for a two-minute test period. The water sources entering the chamber were then switched, with a one-minute rest period allocated to ensure the flushing of both channels (verified by dye tests). This was done to control for any side preferences individuals may display. After water sources were switched, another two-minute habituation period was given, followed by a second two-minute test period.

Preliminary experiments were conducted to 1) ensure no unanticipated biases existed for the two channel choice chamber; 2) the use of chemical cues in the recognition of conspecific and heterospecific cues; and 3) to ensure repeated measurements did not interfere with larval responses (Supp. Mat).

2.3. Experiment 1: Anthropogenic ocean acidification

The partial pressure of CO₂ (pCO₂) in seawater was adjusted with a high-precision pCO₂ control system (Kimoto Electric). Using methods described in Tanaka et al. (2014), fresh filtered seawater (pore size, 1 µm) was treated with a gas mixture of CO₂ and ambient air in a bubbling tank. pCO₂ of the seawater flowing from the bubbling tank was directly measured and maintained at the desired level by continuously regulating pCO₂ in the gas mixture. Three types of seawater were simultaneously prepared using this system: 400 ppm (pH value = 8.11), 700 ppm (pH value = 7.96) and 1000 ppm (pH value = 7.84). These concentrations were chosen to reflect a present day control (400 ppm), and two future scenarios expected by 2050 (700 ppm) and 2100 (1000 ppm) (Feely et al., 2009; Munday et al., 2012; Leduc et al., 2013). The standard deviation of pCO₂ and pH values were <15%

and <1% of target values, respectively, which were monitored for 10 days before the experiment (Tanaka et al., 2014). The temperature of the seawater was 27.4 °C (SD = 0.2).

To test the effect of acidification on the behavioural response towards conspecifics cues, *C. viridis* or *S. hispidus* larvae were given a choice between conspecifics and heterospecific treated seawater adjusted to one of three acidification levels. Seawater was first acidified to the desired level. Cue treatments were then made by soaking either 5 conspecific or 5 heterospecific individuals in 5 L of filtered seawater

for 3 h. Cue preferences were determined using the 30 *C. viridis* and 15 *S. hispidus* larvae. Trials run consisted of conspecific vs. heterospecific cue in seawater at: i) 400 ppm, ii) an acidified condition (700 or 1000 ppm), and again iii) 400 ppm. Between each consecutive test, the larva was maintained in an individual tank of untreated control seawater (400 ppm) for 60 min (Fig. 1).

A z-test with the null hypothesis that larvae would spend 50% of their time in either water source was used to determine if marine larvae were significantly i) attracted to conspecifics chemical cues in control

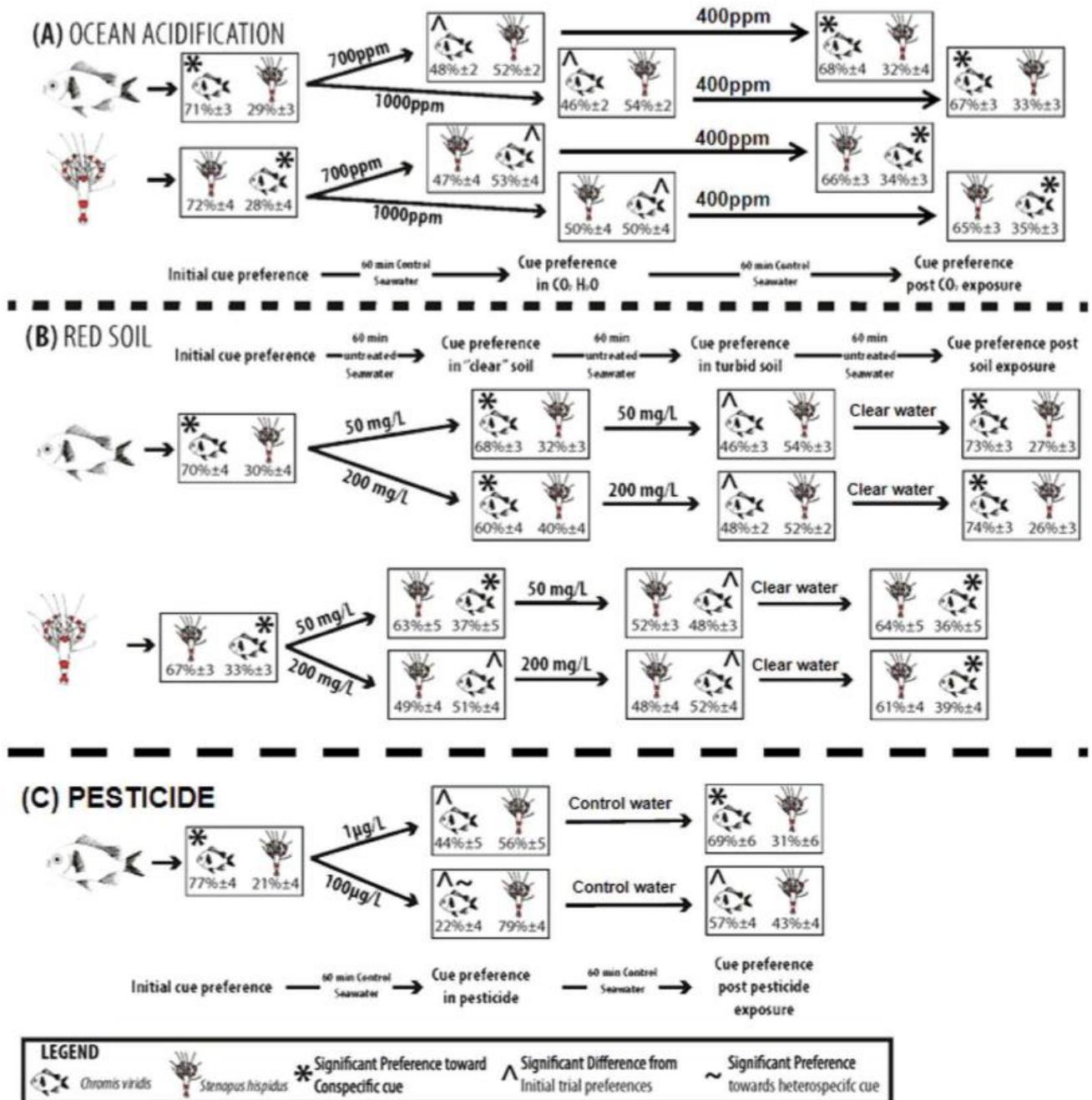


Fig. 1. Infogram of the experimental methods to test for the effect of A) ocean acidification (Sesoko, n = 30 *C. viridis*, n = 15 *S. hispidus* for each acidified seawater concentration: 700 or 1000 ppm), B) red soil pollution (Sesoko, n = 30 *C. viridis*, n = 15 *S. hispidus* for each red soil concentration: 50 or 200 mg/L) and C) pesticide chlorpyrifos (Moorea, n = 15 *C. viridis* for each pesticide concentration: 1 or 100 µg/L) on the larval ability to discriminate between conspecific and heterospecific chemical cues before, during and after exposure to the pollutants. Chemical cues from conspecifics were tested against the chemical cues of heterospecifics. Percent values indicate the mean percentage of time (± SE) larvae spent in one water source opposed to the other. * indicate a significant preference towards the conspecific chemical cue; ^ indicate a significant difference in preference compared to the initial trial; ~ indicate a significant preference towards the heterospecific chemical cue.

conditions, ii) lost the attraction towards the conspecifics cues in acidified seawater (700 or 1000 ppm), and iii) remained attracted towards conspecifics cues in control conditions after exposure to the acidified water. The pairwise comparison between all treatments was analyzed by a generalized linear model (GLM) of fish distribution, followed by post hoc pairwise comparison Dunnett test. Dunnett's test, a post-hoc multiple comparison procedure, is designed to hold the family wise error rate at or below alpha when performing multiple comparisons of treatment group with control. For the same individual repeatedly, the GLM used a repeated measures design. In this case, a correction factor like Bonferroni may be unnecessary.

2.4. Experiment 2: Red soil treatment

Two concentrations (50 and 200 mg/L) of unpolluted red soil (*Kunigami mahgi*), obtained from the Okinawa Prefectural Agricultural Research Centre, were tested. The suspended sediment concentration of <50 mg/L is regarded as low level pollution in marginal near shore reefs. While >100 mg/L is considered a high state of pollution worldwide (Omija, 2004; Fabricius et al., 2005; Wenger et al., 2012). Red soil pollution alters marine systems in two ways; first by introducing additional chemical cues emitted from the red soil into the water column and second, by increasing water column turbidity. Therefore, it was important to experimentally separate the impact of turbidity from the additional of red soil chemical cues.

To test the effect of red soil on the behavioural response towards conspecifics, *C. viridis* or *S. hispidus* larvae were given a choice between conspecifics seawater and heterospecifics seawater. The same larva was tested four consecutive times i) untreated seawater (control trial), ii) seawater treated with either 50 or 200 mg/L of settled red soil (testing chemical cues and removing turbidity), iii) seawater treated with either 50 or 200 mg/L of red soil in turbid water (testing both chemical cues and turbidity) and, iv) untreated seawater (a second control for the exposure to turbidity and red soil chemical cues). Water treatments were created by dissolving red soil into 5 L of filtered ocean seawater with either 5 conspecifics or 5 heterospecifics for 3 h. The conspecifics and heterospecifics were removed from the tank, and the water sources were left for a further two hours. This allowed the sediment to settle out of solution for the isolation of red soil chemical cues or remain in suspension by adding aeration to test turbidity. Between each test, the larva was maintained in an individual tank supplied with flow-through ocean seawater for 60 min before being re-tested. The experiment was conducted on 30 *C. viridis* larvae and 20 *S. hispidus* larvae for each red soil concentration (50 or 200 mg/L).

2.5. Experiment 3: Pesticide treatment

The effect pesticides had on conspecific recognition of *C. viridis* was tested using a seawater control, a solvent control (acetone) and two chlorpyrifos concentrations (1 and 100 µg/L). Chlorpyrifos are one of the most common pesticides found in coral reefs. The concentrations tested reflect levels previous research had identified to inflict negative effects on fish biology, ecology and behavior (Botte et al., 2012). A total of 50 mg of chlorpyrifos was first dissolved into 125 mL acetone at a final concentration of 400 mg/L (Botte et al., 2012). To minimize potential solvent effects, stock concentrations were diluted 10 L tanks, ensuring acetone volume did not exceed 0.02% (v/v).

To test the effect of chlorpyrifos on the behavioural response towards conspecifics cues, 15 *C. viridis* larvae per concentration level were given a choice between conspecifics treated seawater and heterospecifics treated seawater. The same larva was tested three consecutive times i) using untreated filtered seawater (control), ii) using filtered seawater with 1 or 100 µg/L of pesticides, and iii) using untreated filtered seawater (control for previous pesticide exposure). Between each test, the larva was maintained in an individual tank of untreated filtered seawater for 60 min. Additionally, to determine any effect

exposure to acetone may have had on fish behavioural preferences, 10 *C. viridis* larvae were given the choice between untreated filtered seawater and filtered seawater containing acetone (125 mL of acetone in 10 L of sea-water; z-test).

2.6. Sample preparation and analysis of seawater chemical fingerprints

High performance liquid chromatography (HPLC) was used to acquire the profiles of seawater metabolites. This allowed the comparison of the chemical fingerprints of conspecifics between untreated seawater and the different experimental treatments (acidification, red soil, and pesticides). Water samples were prepared by holding 10 juvenile *C. viridis* for 3 h in 10 L of: i) filtered untreated seawater at each location (Moorea and Sesoko), ii) seawater treated to either 400, 700 or 1000 ppm CO₂, iii) "clear" seawater made with either 50 or 200 mg/L settled red soil, iv) turbid seawater made with either 50 or 200 mg/L⁻¹ of red soil and lastly, v) seawater with 1 or 100 µg/L of chlorpyrifos. Each water sample (10 L) was vacuum filtered through solid phase extraction (SPE) cartridges containing C₁₈ silica-gel-based bonded phase sorbent, washed with 50 mL distilled water, and subsequently desorbed with 50 mL methanol. The organic phase of each sample was freeze-dried leaving a powdery organic residue. The organic extracts were dissolved in 1 mL methanol before analysis. The data were processed to create a max plot chromatogram that plotted the maximum spectral absorbance measured at each time point. Max plot chromatogram detects all UV-absorbing components in a sample. The absorbance of each component (computed as the area of peak and expressed in absorbance units, AU) was used to identify the molecular diversity of conspecifics odor in presence or absence of acidified seawater, red soil or pesticides.

3. Results

3.1. Experiment 1: Anthropogenic ocean acidification

Both *C. viridis* and *S. hispidus* displayed a significant attraction to the chemical cues of conspecifics when tested against the chemical cues of heterospecifics under current day conditions (400 ppm; z test, *p*-value < 0.001 for both taxa; Fig. 1A). Larvae spent ~70% of their time in the chemical cue containing the conspecific cue opposed to seawater containing heterospecific cues (mean % ± SE; *C. viridis*: 71 ± 3.2%; *S. hispidus*: 70 ± 3.5%). However, when seawater was treated to either 700 ppm or 1000 ppm CO₂, this attraction was significantly reduced. When compared to the initial present day control trial, *C. viridis* displayed a >23% decreased attraction and *S. hispidus* decreased conspecific attraction by >25% (GLM, *p* < 0.001; Dunnett test, *p* < 0.001 for both taxa). Furthermore, at both 700 ppm or 1000 ppm CO₂, marine larvae lost their attraction towards the conspecific cue, spending equal time in water containing either the conspecific or heterospecific signal (z test, *p* > 0.19 for both taxa).

Larvae of both species, previous exposed to either 700 ppm or 1000 ppm CO₂, regained their preference for conspecific chemical signals when tested for a third time in present day (400 ppm) conditions (*C. viridis*: 68 ± 3.5%; *S. hispidus*: 66 ± 3.0%; z test, *p* < 0.003 for both taxa; Fig. 1A). *C. viridis* and *S. hispidus* larvae displayed no significant difference between their initial 400 ppm trial and the final 400 ppm experimental trial (Dunnett test, *p* = 0.97 for *C. viridis*, *p* = 0.94 for *S. hispidus*).

3.2. Experiment 2: Red soil treatment

C. viridis and *S. hispidus* larvae displayed significant preferences towards the chemical cues of conspecifics opposed to heterospecifics prior to red soil exposure (*C. viridis*: 70 ± 3.6% and *S. hispidus*: 67 ± 3.1%; Fig. 1B).

Preferences for the conspecific chemical signal persisted in both species when experiment trials were run in water treated with 50 mg/L of red soil that was allowed to precipitate out of solution (Fig. 1B). The

attraction towards conspecific cues was reduced by 2% for *C. viridis* and 4% for *S. hispidus* (GLM $p < 0.001$; Dunnett test *C. viridis* $p = 0.98$; *S. hispidus* $p = 0.55$). *C. viridis* was able to maintain a significant preference for the conspecific chemical cue when the red soil concentration was increased to 200 mg/L (z test, $p = 0.03$). However, the preference for the conspecific cue decreased from 70% to 60% (Fig. 1B). In contrast, *S. hispidus* lost their preference for the conspecific chemical cue when the amount of red soil was increased to 200 mg/L (z test, $p = 0.16$) with the preference for the conspecific cue decreased from 67% to 49% (Fig. 1B).

In the turbid water treatment containing both the chemical cues and turbidity of the red soil, regardless of soil concentration 50 mg/L or 200 mg/L, the attraction towards conspecific cues was significantly reduced in both species compared to the initial trial with no red soil (Fig. 1B). The turbidity associated with 50 mg/L red soil significantly reduced the time spent in conspecific chemical cues by 24% for *C. viridis* and 19% for *S. hispidus* (Dunnett test, $p < 0.001$ for both taxa). Resulting in a loss of chemical attraction behavior in both *C. viridis* and *S. hispidus* larvae at 50 mg/L (z test, $p > 0.43$ for both taxa) and 200 mg/L red soil (z test, $p > 0.17$ for both taxa).

Larvae were retested for their chemical preferences of conspecific vs. heterospecific cues post exposure to either levels of red soil (50 or 200 mg/L) and exposure types (chemical cue only and turbid red soil water). Both species significantly regained attraction to conspecific chemical cues ($p < 0.02$ for all tests). There were no significant differences in the strength of their cue preference between the initial control and final post exposure control trials ($p > 0.90$ for all tests).

3.3. Experiment 3: Pesticide treatment

Exposure to acetone had no effect on the choice behavior of *C. viridis* larvae (mean \pm SE percentage of time spent in the control flow: $54 \pm 2.1\%$ - z test, $p = 0.85$). As expected by previous results, initial preferences demonstrated a significant attraction towards the chemical cues of conspecifics compared to heterospecifics (Fig. 1C).

Exposure to seawater treated with 1 μ g/L of chlorpyrifos reduced the attraction towards conspecific cues by 33%, resulting in *C. viridis* larvae no longer spending significantly more time in the conspecific cue choice (z test, $p = 0.29$). Time spent in the conspecific cue was further reduced in seawater treated with 100 μ g/L of chlorpyrifos. *C. viridis* initially spent 77% of their time in the seawater containing chemical cues of the conspecific. However, 100 μ g/L of chlorpyrifos reduced the amount of time spent in conspecific water to only 22%. Unlike all other anthropogenic experimental trials, exposure to 100 μ g/L altered the chemical preference of *C. viridis* so greatly that fish in this treatment displayed a significant preference for heterospecific opposed to conspecific chemical cues (z test, $p < 0.001$).

In the post exposure control trial (without pesticide), larvae previously exposed to 1 μ g/L chlorpyrifos were significantly attracted to the conspecific chemical cues (z test, $p = 0.01$) and no significant difference was found in the strength of their preference between the initial and post exposure control trials (Dunnett test, p -value = 0.36). Recovery of chemical preference was not shown in *C. viridis* that had been exposed to 100 μ g/L, individuals did not significantly prefer the chemical cues of conspecifics, spending only 57% of their time this cue (z test, $p = 0.06$). As a result, cue preference was significantly different when comparing pre and post exposure control trials (Dunnett test, $p = 0.04$).

3.4. Analysis of seawater chemical fingerprints

HPLC determined the chemical fingerprints of *C. viridis* in presence or absence of acidified seawater, red soil and pesticides (Figs. 2,3,4). Three control seawater samples (no anthropogenic treatments or conspecifics) were characterized by very few minor peaks. Therefore, each peak found was due to the odor of conspecifics rather than the seawater itself.

In acidification treatments, the chemical fingerprint of conspecifics at 400 ppm was characterized by two major peaks (noted 1 and 2 in Fig. 2) and one peak complex (noted 3 in Fig. 2). At 700 ppm and 1000 ppm CO₂, the fingerprint still contained one major peak (noted 2) and one peak complex (noted 3). However, the absorbance of each peak decreased: from 5.37 AU at 400 ppm to 5.44 AU at 1000 ppm for the peak 2; and from 7.28 AU at 400 ppm to 6.54 AU at 1000 ppm. No significant variation was highlighted between the absorbance of each peak and acidified water (no significant regression, slope = 0.0001, $p = 0.97$ for peak 2; slope = -0.001, $p = 0.72$ for peak 3). Interestingly, the peak noted 1 at 400 ppm (0.56 AU) disappeared in seawater treated to 700 ppm and 1000 ppm; and a new minor peak appeared at 1000 ppm (0.08 AU; noted A in Fig. 2).

The chemical fingerprint of conspecific cues in the absence of red soil was characterized by three major peaks (noted 1 to 3 in Fig. 3) and one peak complex (noted 4 in Fig. 3). The fingerprint of unpolluted red soil (*Kunigami mahgi*) did not show any peaks. In presence of red soil, the chemical fingerprint of conspecific cues was still characterized by the one major peak and one peak complex (noted 2 and 4). No significant variation was found between the peak absorbance and red soil concentration. The first major peak (noted 1, 0.18 AU) disappeared when red soil was added at any concentration and the third peak (noted 3) was only present on the chromatograms of 50 mg/L red soil (0.98 AU without red soil, 0.28 AU in clear water, and 0.85 AU in turbid water). Two peaks appeared in presence of red soil: a minor peak (noted A, from 0.13 to 0.33 AU) on all chromatograms and a major peak (noted B, 2.22 AU) on the chromatogram of 50 mg/L red soil in clear water (Fig. 3).

The chemical fingerprint of conspecifics in the absence of a pesticide was characterized by seven major peaks (noted 1 to 7 in Fig. 4) and one peak complex (noted 8 in Fig. 4). The fingerprint of chlorpyrifos was characterized by one major peak at 18'31 min (4.79 AU). This peak was present on the fingerprint of conspecifics at 1 μ g/L (0.28 AU) and 100 μ g/L (5.10 AU). In presence of 1 μ g/L pesticides, the fingerprint of conspecific chemical cues was characterized by a decrease in absorbance of all but one peak (peak 7) and the disappearance of 1 peak (noted 6) and the peak complex (noted 8). For example, the absorbance of peak 1 was reduced 10-fold (from 12.8 AU to 1.2 AU). One minor peak appeared in seawater treated with 1 μ g/L pesticides (noted A in Fig. 4). In presence of 100 μ g/L pesticides, the chemical fingerprint of conspecific cues was characterized by virtually no peaks, with the exception of two (noted 1 and 7), both had low absorbance reduced by 11 and 18 fold respectively (Fig. 4).

4. Discussion

Anthropogenic induced stressors, such as ocean acidification, sedimentation/turbidity, and pesticide runoff result in direct consequences for coral reef habitats. These anthropogenic alterations also impact the ability of larvae during the recruitment stage to recognize and respond to the chemical cues used for habitat identification. The use of chemical cues in habitat selection (Kingsford et al., 2002; Dixson et al. 2014; Barth et al., 2015) and the importance of conspecific chemical cues has been widely accepted (Kingsford et al., 2002; Dixson et al., 2011; Coppock et al. 2013; Lecchini and Nakamura 2013; Barth et al., 2015). Results collected here, confirm an attraction towards conspecific opposed to heterospecific chemical cues, in fish (*C. viridis*) and crustacean (*S. hispidus*) larvae. However, when chemical cues were exposed to anthropogenic induced stressors (ocean acidification, sedimentation/turbidity, and pesticide runoff), waterborne chemical information is altered. This change results in fish and crustacean larvae no longer displaying an attraction towards important conspecific chemical signatures. The reduced attraction could result in decreased recruitment to reefs experiencing said stressors.

Fish and shrimp larvae lost their chemical attraction to conspecifics cues in seawater treated to mimic conditions expected to occur in 50–100 years (Fig. 1A). The larval attraction was reduced by 23% in

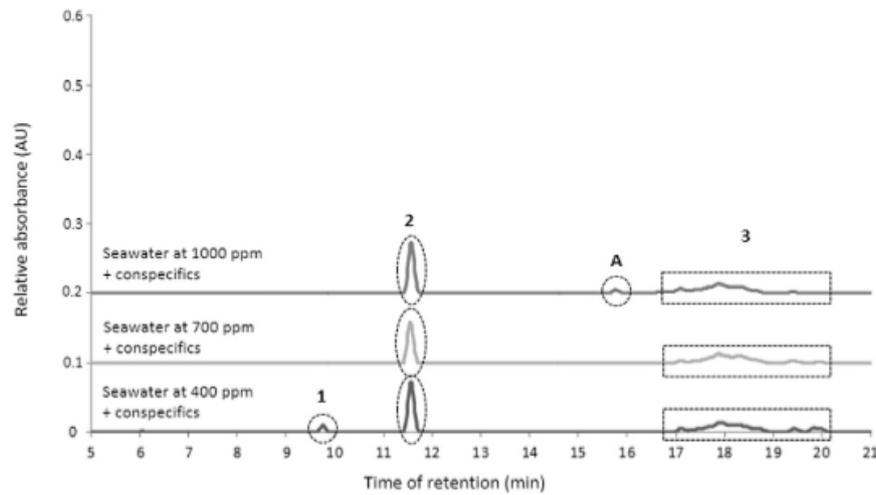


Fig. 2. High performance liquid chromatography chromatograms (HPLC) of conspecific fingerprint different levels of acidified water (400, 700, 1000 ppm). The first 5 min of chromatograms were removed, as it corresponded to dead volume in the column, and to maximize sensitivity, the data were processed to create max plot chromatograms, which plots the maximum spectral absorbance measured at each time point. The specific peaks of conspecific fingerprint in absence of stressor were noted 1 to 3. The alarm peak of conspecifics emitted in polluted environment was noted A.

either 700 ppm or 1000 ppm CO₂ seawater when compared to a present day control condition (400 ppm). Similarly, *Amphiprion percula* (clownfish) larvae reared in seawater simulating future ocean acidification conditions, resulted in the inability of larvae to discriminate between predators and non-predators (Dixon et al. 2010) and parents and non-parents (Munday et al. 2009). Our results are consistent with previous studies, demonstrating an acidification induced effect on fish behavior and the perception of chemical cues (Dixon et al. 2010, Munday et al. 2012; Heuer and Grosell, 2014).

Red soil also affected the chemical abilities of marine larvae to recognize their conspecifics (Fig. 1B). When red soil was suspended in water and allowed to precipitate from the water column, leaving red soil chemical cues but removing turbidity, the larval attraction to conspecifics was only lost for *S. hispidus* at high concentrations (200 mg/L) of red soil. Both *C. viridis* and *S. hispidus* were able to recognize and respond to conspecific cues at the lower concentration tested (50 mg/L).

While the “clear” red soil treatment no longer contains the red soil particulates, it does contain the waterborne chemical components produced by each concentration level of red soil within the water column. In contrast, *C. viridis* and *S. hispidus* larvae lost their chemical attraction to conspecific cues in turbid water at both 50 mg/L and 200 mg/L of red soil. A reduction in fish abundance, biomass and species diversity has been documented at inshore sites and sites highly impacted by sediment compared to offshore or low sediment sites (Fabricius et al., 2005; Mallela et al., 2007). Our results indicate that crustacean and fish larvae are more chemically sensitive to water turbidity (i.e. sedimentation) than the chemical pollution of red soil (Fig. 1B). Similarly, an increase in turbidity (suspended sediment levels >45 mg/L) impaired habitat choice and foraging success of coral reef fish recruits due to reduced ability to distinguish visual and chemical cues (Wenger et al., 2012, 2015; O’Connor et al., 2016). Importantly, the rainy season corresponds with a high recruitment levels on the Great

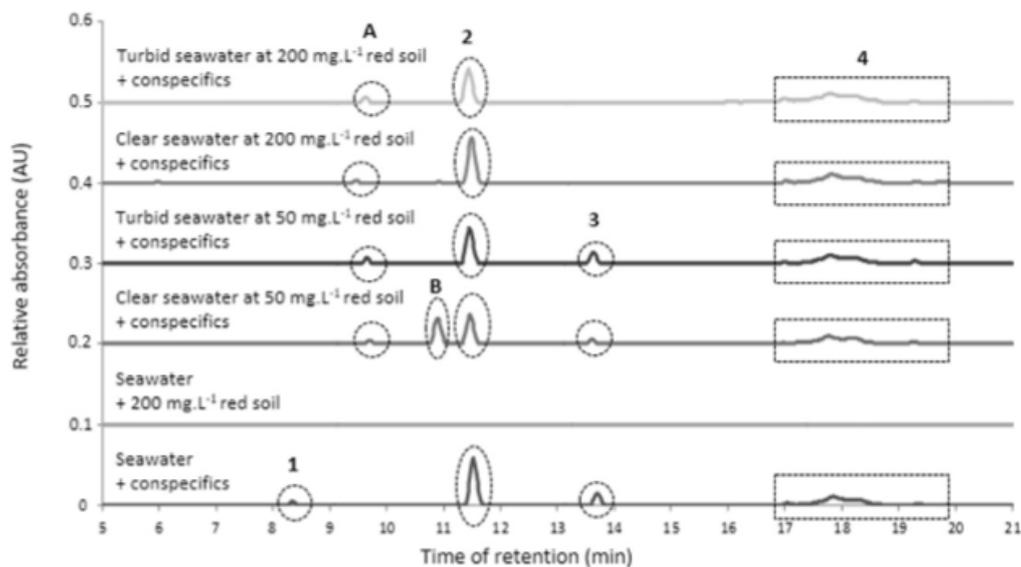


Fig. 3. HPLC of conspecific fingerprint in different levels of concentrations of red soil pollution (0, 50 or 200 mg/L of red soil in clear or turbid water) and of unpolluted red soil *Kunigami mahi*. The specific peaks of conspecific fingerprint in absence of stressor were noted 1 to 4. The alarm peaks of conspecifics emitted in polluted environment was noted A and B.

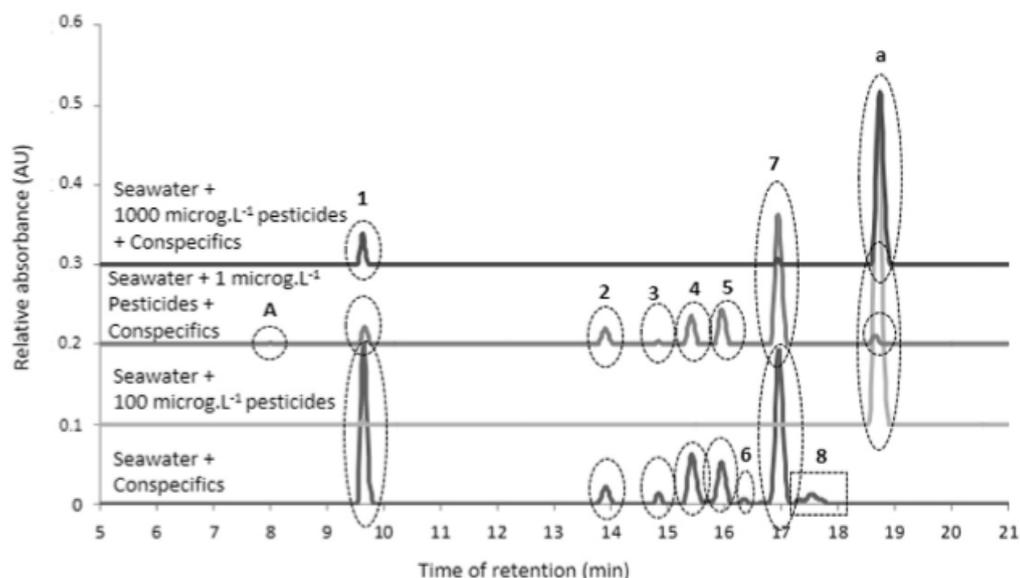


Fig. 4. HPLC of conspecific fingerprint in different concentrations of pesticide (0, 1 or 100 $\mu\text{g/L}$) and of pure chlorpyrifos. The specific peaks of conspecific fingerprint in absence of stressor were noted 1 to 8. The alarm peak of conspecifics emitted in polluted environment was noted A. The specific peak of chlorpyrifos was noted 'a'.

Barrier Reef, Ryukyu Island, and French Polynesia (Nakamura et al., 2009a; Lo-Yat et al., 2011); meaning fish and crustacean larvae experience sub-optimal conditions during recruitment as sedimentation is increased due to precipitation induced runoff.

In addition to terrestrial runoff, some pesticides, used in agriculture, are carried into coral lagoons, as in Moorea Island (Roche et al., 2011). Toxicology research in coral reefs have focused on the pesticide concentration found the tissue of different marine organisms (Roche et al., 2011). Few studies highlighted the influence of pesticides on biology, ecology and ontogenetic development of coral and fishes (Fabricius et al., 2005; Botte et al., 2012). No studies to date have tested the impact of pollutants on recruitment cues used by settlement stage larvae. *C. viridis* larvae significantly lost their ability to chemically recognize conspecific cues at 1 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ chlorpyrifos (Fig. 1C). Moreover, among all anthropogenic treatments studied, exposure to the 100 $\mu\text{g/L}$ chlorpyrifos pesticide had lasting effects on *C. viridis*. Fish were unable to recognize conspecific chemical cues after pesticide exposure when tested in untreated filtered seawater.

Although several studies suggest the presence of alarm cues emitted as a signal of stress (Barth et al., 2015), no studies in coral reefs have explored the effects of human pollution on social interactions (e.g., reproduction, recruitment, foraging activities – Leis et al., 2011). Coral reef fish and crustaceans are exposed to an enormous diversity of chemical cues that are mixed and dispersed by waves, currents, and tides. Therefore, extracting useful information from multiple cues is crucial to the survival of marine organisms (Barth et al., 2015). It has been shown that the chemical cues from specific reef components can impact recruitment, with coral acting as a positive cue and seaweed acting as a negative source of chemical stimuli (Dixson et al. 2014). Lecchini et al. (2013) found, during the recruitment stage, 70% of the fish species preferred water from reefs dominated by live coral compared to reefs dominated by algae. It was suggested that fish larvae could respond to the quantity of chemical cues emitted by conspecifics whose abundance changes in response to coral and algal cover, or the chemical cues varied based on the environment. Here, we demonstrate the latter, showing a modification of the chemical fingerprints of conspecifics according to the environment (Figs. 2,3,4). In acidification and red soil treatments, conspecific fingerprints were modified with a disappearance of some peaks and the appearance of some 'alarm' peaks (i.e. emission of metabolic disturbance factors). For example, at 700 ppm CO_2 , there was a

disappearance of peak noted 1 (Fig. 2), which suggest that conspecific behavior is altered by acidified water. At 1000 ppm CO_2 , there was the appearance of one peak noted A (Fig. 2), suggesting the production of alarm cues by conspecifics. Similarly, two peaks appeared in the presence of red soil, a minor peak A (from 0.13 to 0.33 AU) on all chromatograms and a major peak B (2.22 AU) on the chromatogram of 50 mg/L in water containing the chemical cues of red soil alone (Fig. 3). The presence of alarm peaks in the red soil experiments did not inhibit the larval attraction towards conspecific cues, suggesting alarm signals are minor compared to the cue of conspecific juveniles. In the 1 $\mu\text{g/L}$ pesticide treatment, the conspecific chemical fingerprint was modified with a decrease in absorbance of nearly all peaks, the disappearance of two peaks and the appearance of one alarm peak (Fig. 4). At a concentration of 100 $\mu\text{g/L}$ pesticide, the conspecific chemical fingerprint contained only two peaks at low absorbance (Fig. 4). These results suggest that conspecific behavior was altered by the presence of 1 $\mu\text{g/L}$ pesticides with the release of alarm cues. At 100 $\mu\text{g/L}$ pesticides, conspecific cues were so strongly altered that almost no chemical signal was emitted into the seawater. Consequently, the larval attraction for conspecific chemical signals was decreased. Overall, these results highlight, for the first time, that polluted seawater modified the specific chemical signature of conspecifics. A weak stressor evoked the production of alarm cues, a strong stressor resulted in reduced cue emission.

Our results highlight that both global (ocean acidification) and local stressors (red soil and pesticides) to coral reef ecosystems could have significant effects on the sustainability of reefs by altering the behavior of fish and crustacean populations during a critical life-history transition (i.e. recruitment phase). In chemical polluted environments, marine larvae displayed a reduced or no attraction to conspecifics, thus larvae may not locate suitable settlement habitat, resulting higher predation and lower recruitment levels. Predicting how anthropogenic changes will affect connectivity and recruitment in coral reef ecosystems is important. The dispersal of larvae between reefs is a key component in population dynamics of reef organisms and reef connectivity (Munday et al., 2009). Our study investigated non-lethal behavioural effects of chemical cues in changing environments. Although the levels of ocean acidification, pesticide pollution and terrestrial runoff did not result in mortality among the exposed organisms, indirect consequences were found. Thus, if polluted seawater disrupts the recruitment process, fish larvae may misinterpret important chemical signals resulting in lower

recruitment levels to impacted reefs. Stability of marine organisms is dependent, in part, on the stability of social interactions. Therefore disruption to larvae-conspecifics relationships could have major consequences for recruitment and adult population with further repercussions for the ecosystem. Understanding the relationship between reef health and recruitment potential could allow management planning for the maintenance of coral cover and biodiversity on reefs that are increasingly degraded.

Competing interests

The authors have declared no competing interests exist.

Author contributions

D.L., Y.T., B.B. and Y.N. designed research; D.L., G.L., D.L.D., N.R., B.F., M.B. and Y.N. collected and analyzed data; D.L., G.L., D.L.D. and Y.N. wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.marpolbul.2016.08.083>.

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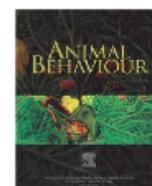
Appendix 4: Brain lateralization and conspecific recognition in a juvenile coral reef fish

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Brain lateralization involved in visual recognition of conspecifics in coral reef fish at recruitment

Natacha Roux^{a, b, *}, Emilio Duran^c, Rynae G. Lanyon^{d, e}, Bruno Frédérick^f, Cécile Berthe^a, Marc Besson^{a, g}, Danielle L. Dixson^h, David Lecchini^{a, i}^a USR 3278, CNRS-EPHE-UPVD, CRIOBE, Moorea, French Polynesia^b UMR 7232, CNRS-UPMC, Observatoire Océanologique de Banyuls-sur-Mer, Banyuls-sur-Mer, France^c Laboratorio de Psicobiología, University of Sevilla, Campus Santiago Ramon y Cajal, Sevilla, Spain^d School of Marine Studies, Institute of Marine Resources, University of the South Pacific, Suva, Fiji^e Institute for Pacific Coral Reefs, IRCP, Moorea, French Polynesia^f Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH Research Center, Institut de Chimie B6c, Université de Liège, Liège, Belgium^g Molecular Zoology Team, Institut de Génomique Fonctionnelle de Lyon, Université Lyon 1, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Lyon, France^h School of Marine Science and Policy, University of Delaware, Newark, DE, U.S.A.ⁱ Laboratoire d'Excellence 'CORAIL', Paris Sciences Lettres (PSL), Moorea, French Polynesia

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In vertebrates, brain functional asymmetries are widespread and increase brain performance. Some species of fishes are known to have brain asymmetries; however, little information is available on brain lateralization in coral reef fishes and the impact this could have during the recruitment phase. In this study, soldierfish, *Myripristis pralinia*, at the larval and juvenile stage recognized conspecifics through visual cues. Larvae with the ablation of either the right or left telencephalic hemisphere lost the attraction towards conspecific cues. In contrast, juveniles with the ablation of the right (but not left) telencephalic hemisphere still displayed a preference towards conspecific visual cues. These results suggest the left telencephalic hemisphere is responsible for the lateralization process used in the visual recognition of coral reef fish juveniles. The determinism of lateralized perception of conspecifics during fish ontogeny may be a consequence of genetic factors, linked with the metamorphosis processes and/or environmental factors such as predation at recruitment.

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Lateralization, the preference for one side of the body over the other, is a common phenomenon in vertebrate (for a review see Salva, Regolin, Mascalzoni, & Vallortigara, 2012) and invertebrate species (for a review see Frasnelli, Vallortigara, & Rogers, 2012). Brain lateralization is thought to increase cognitive abilities, behavioural complexity or behavioural laterality, leading to advantages in brain function (Vallortigara & Rogers, 2005). Several studies have explored the role lateralization plays in emotion or cognitive decisions. For cognition, the brain's right hemisphere is responsible for processing novel items and/or items requiring a rapid response. The brain's left hemisphere is used to categorize stimuli and/or process information requiring consideration of alternatives (Rogers, Vallortigara, & Andrew, 2013). It has been

suggested that the brain's ability to process positive and negative emotions utilizes the right hemisphere for both positive and negative emotional responses. Alternatively, the valence theory proposes that the right hemisphere is dominant for negative emotions and the left hemisphere is primarily used with positive emotions (e.g. Hook-Costigan & Rogers, 1998; Quaranta, Siniscalchi, & Vallortigara, 2007; Siniscalchi, Lusito, Vallortigara, & Quaranta, 2013). Empirical evidence supports the cognition and emotion theories; however, it can be argued that additional species from a more diverse group must be studied to come to any general conclusion (e.g. Jozet-Alves et al., 2012; McManus, 2005).

Most species of fishes in coral reefs have a stage-structured life history. A relatively sedentary benthic stage (juveniles and adults) produces a highly dispersive pelagic larval stage (Leis, Siebeck, & Dixson, 2011). At the end of the larval phase, organisms are required to enter the benthic reef environment, termed recruitment (Lecchini & Galzin, 2003). During this time period, species-specific

* Correspondence: N. Roux, UMR 7232, CNRS-UPMC, Observatoire Océanologique de Banyuls-sur-Mer, 1 avenue du Fontaulé, 66650 Banyuls-sur-Mer, France.

E-mail address: natacha.roux@obs-banyuls.fr (N. Roux).

changes in morphology and physiology, referred to as metamorphosis, occur (Leis & McCormick, 2002). Larvae lose many of the characteristics that enhanced survival in the pelagic environment, such as transparency (McCormick, 1999). In turn, once settlement occurs newly settled recruits rapidly develop features better suited for the reef habitat, such as pigmentation (Dufour, Lecaillon, & Romans, 2000). Moreover, during the recruitment phase, fish larvae are subjected to strong selective pressures to choose a suitable reef habitat that will facilitate survival and growth. Studies have documented up to 90% larval mortality in the first 7 days on the reef (Doherty et al., 2004; Lecchini, Osenberg, Shima, St Mary, & Galzin, 2007). Recruitment of new individuals is a critical process in the maintenance and recovery of marine communities (Lecchini & Galzin, 2003). Correct habitat identification depends strongly on the ability of marine larvae to recognize and respond to sensory signals from conspecifics, habitat components and predators (Barth et al., 2015). Many coral reef fish species recognize their conspecifics using visual cues (Huijbers et al., 2012; Lecchini, Peyrusse, Lanyon, & Lecellier, 2014; O'Connor et al., 2015). Despite the importance of visual, acoustic and chemical cues during the recruitment process (Leis et al., 2011), very little information is available on the relationship between brain morphology or lateralization and the behavioural and social interactions of coral reef fishes (Barth et al., 2015). A link between brain area and life history traits has been found. Indeed, nocturnally active fish species that spend less than 30 days in the pelagic environment before settling have a larger optic tectum and telencephalon than diurnal fish species that spend less than 15 days in the ocean (Lecchini, Lecellier et al., 2014). Additionally, behavioural lateralization in coral reef fish larvae is impaired by ocean acidification (Domenici et al., 2014, 2012; Nilsson et al., 2012). Few studies have explored whether lateralization could vary across the development of an individual (Concha, Bianco, & Wilson, 2012; Rogers et al., 2013; Skiba, Diekamp, & Güntürkün, 2002). Zebrafish, *Danio rerio*, 6–21 days posthatching show a significant left-eye preference when examining their reflection in a mirror (Sovrano & Andrew, 2006). Cuttlefish, *Sepia officinalis*, 3–45 days posthatching progressively develop a left turning bias (Jozet-Alves et al., 2012). Both studies indicate the development of the lateralization process during an early life history stage. Coral reef fish offer an important group of organisms to study the development of lateralization. Selection favours individuals on reefs that chose an ideal location, and the visual recognition of conspecifics provides an indication of optimal settlement sites. The use of visual cues could be favoured by the lateralization process in cognition, with larvae recognizing familiar conspecifics compared to unfamiliar heterospecifics.

Overall, understanding the ontogeny of lateralization is essential for complete comprehension of the larval recruitment processes. This is especially important as changing climate conditions impact lateralization's function (Nilsson et al., 2012). Here, we tested coral reef fish at the larval (premetamorphosis) and juvenile (metamorphosed) stage to better understand the role that lateralization plays in the cognitive recognition of visual conspecific cues. The importance of telencephalic hemispheres in the visual recognition of conspecifics was tested using behavioural assays conducted on fish with and without one or other hemisphere of the telencephalon. This determined whether the lateralization of the brain is present at the larval and/or juvenile stages during recruitment.

METHODS

Specimen Collection

Fish larvae were collected nightly from February to June 2011 and September 2014 using crest nets set on the west coast of

Moorea Island, French Polynesia (17°31'03.56"S, 146°55'21.53"W). This sampling technique collects fish larvae in the process of recruitment (Lecchini, Dufour, Stand, & Galzin, 2004; Lecchini et al., 2006; Lo-Yat et al., 2011). A rectangular mouth (1 m wide, 2 m high) net (1 mm mesh) was oriented perpendicular to the water flow, designed to retain all incoming larvae (for methods see Lecchini et al., 2004). Larvae were collected between 1700 and 0600 hours, to maximize the capture of nocturnal recruiting larvae while minimizing the amount of debris collected during daylight hours (Lecchini et al., 2006). Crest nets do not target specific species.

The soldierfish, *Myripristis pralinia*, was chosen due to the high number of individuals collected (total: 148 larvae) and the biology of the species. *Myripristis pralinia* live in shoals that include all sessile ontogenetic stages: newly settled recruits, juveniles and adults (Lecchini & Galzin, 2005). Fish larvae and juveniles use vision to recognize conspecifics during recruitment (Barth et al., 2015; Lecchini, Shima, Banaigs, & Galzin, 2005). To determine the difference in lateralization between life history stages, both larvae (size: 5.8 ± 0.2 cm) and juveniles (size: 6.2 ± 0.1 cm) were tested. Juveniles were obtained by rearing collected larvae in aquaria for 7 days, which ensured that metamorphosis was finished.

Brain Surgery

To determine the importance of brain lateralization in conspecific recognition, surgery was necessary. Surgical procedures followed modified methods from Salas, Broglio, Duran, Gomez, and Rodriguez (2008) and Durán, Ocaña, Broglio, Rodríguez, and Salas (2010). Surgery on larvae was conducted within 24 h of collection. Surgery on juveniles was conducted 7 days after larval collection. An individual was first anaesthetized in 0.07 g/litre of MS222 (m-aminobenzoic acid ethyl ester, methanesulphate salt) dissolved in sea water. Once unconscious, the fish was placed in a surgical chamber. Two PVC side plates kept the fish stable during surgery. The fish was maintained alive and unconscious using a tube inserted into the mouth continuously flushing sea water containing MS222 (0.07 g/litre). The telencephalic hemispheres were exposed through four incisions on the top of the skull using conventional dissection equipment. The removed piece of the cranium was placed on wet cotton until the end of the surgery. The left or right telencephalic hemisphere was removed using a micropipette connected to a syringe. Regular verifications were made through a binocular microscope to avoid surgical mistakes. After hemisphere removal, the piece of cranium was replaced in its original position and fixed with cyanoacrylate glue. To ensure that the surgical procedure did not affect fish behaviour, the surgery was conducted on 10 individuals of each stage and the skull was closed without ablation, termed a sham operation. After surgery, the fish was woken by circulating untreated sea water through the tube inserted into the mouth. All surgeries were conducted in less than 10 min. Overall, four experimental groups of fishes were considered: nonoperated (NS), sham-operated (Sh), right telencephalic hemisphere removed (RT) and left telencephalic hemisphere removed (LT).

Visual Recognition of Conspecifics by Fish Larvae and Juveniles

The fish were behaviourally assessed using a three-compartment test chamber (60 × 12 cm and 10 cm high (Lecchini, Peyrusse, et al., 2014; Fig. 1). The side compartments consisted of two transparent Plexiglas panels separated by 1 cm placed to create barriers at 8 cm from each end, resulting in a central compartment (length: 32 cm). The central compartment was separated into three

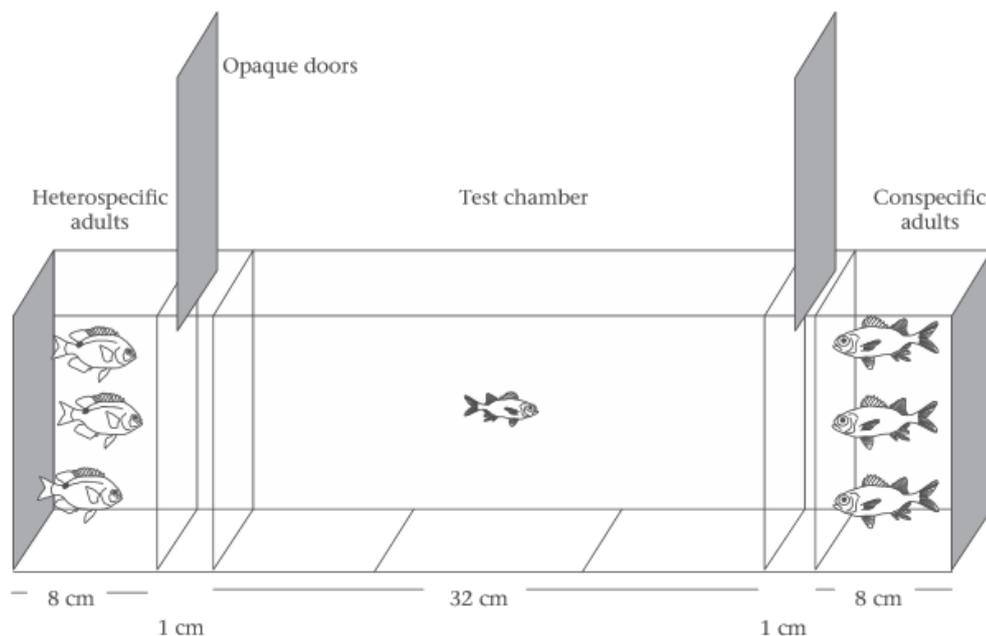


Figure 1. The two-choice aquarium (60 × 12 cm and 10 cm high) used to behaviourally assess visual recognition of conspecific cues in larval and juvenile *M. pralinia*. Three of the chamber sides were opaque to limit distractions to the test fish.

equal parts (length: 10.6 cm) using two lines drawn on the bottom of the aquaria to identify the three equal compartments.

This experimental system isolated the larva or juvenile, placed into the central compartment, from chemical and acoustic cues emitted by the conspecifics and heterospecifics used as visual cue transmitters (Lecchini, Lecellier et al., 2014; Lecchini, Osenberg et al., 2007). A hydrophone placed in the centre of the aquarium (HTI-96-MIN with inbuilt preamplifier; sensitivity 165 dB re 1 V/μPa; frequency range 2 Hz–30 kHz; High Tech Inc., Gulfport, MS, U.S.A.) connected to a solid-state recorder (Edirol R-09HR 16-bit recorder; sampling rate 44.1 kHz; Roland Systems Group, Bellingham, WA, U.S.A.) verified auditory stimuli were not being transmitted from visual cue sources. Conspecifics and heterospecifics were, respectively, *M. pralinia* (range size: 7.1 ± 0.3 cm) and *Stegastes nigricans* (range size: 6.9 ± 0.2 cm) adults captured in the Moorea lagoon (see Appendix). Adults used for visual cue transmission, regardless of species, did not display any type of aggressive behaviour (quick movements, charging towards the test fish, etc.). Instead, adults remained fairly stationary in the chambers, allowing larvae and juveniles to freely move within the test chamber uninhibited by potential aggressive interactions. The lack of aggressive displays by adults towards test subjects was further verified by the movement patterns of the fish. Larvae and juveniles were not seen to approach either the heterospecific or conspecific side and quickly retreat. Preliminary field observations in Moorea showed no competition for space or food between *S. nigricans* and *M. pralinia* (Lecchini & Galzin, 2005; Lecchini, Nakamura, Tsuchiya, & Galzin, 2007).

For each fish group (NS, Sh, RT and LT), the following protocol was applied: one individual (71 larvae or 66 juveniles) was introduced into the middle of the central compartment for a 1 min habituation period. Prior to the fish being introduced into the test aquaria, opaque screens were placed between the Plexiglas barriers in order to avoid visual contact between the tested individual and fish placed in adjacent compartments. Following the 1 min habituation period, the opaque screens were removed. The location of fish in each of the three parts of the aquarium was recorded every second for 1 min. After each trial, the aquarium was emptied and

washed with freshwater. To exclude a possible side bias, compartments containing conspecifics and heterospecifics were randomized (see also methods in Lecchini, Peyrusse et al., 2014; O'Connor et al., 2015).

Two separate one-way analyses of variance (ANOVA) with the fixed factor of surgical treatment were run on each ontogenetic stage (larval and juvenile) to determine whether the mean percentage time spent near the conspecific differed between brain ablation treatments. A post hoc Tukey test was conducted to compare the results from individuals receiving no operation to other experimental time (i.e. Sh, RT, LT). A significant difference indicated that the individual spent either significantly more time or significantly less time near conspecifics. Last, a two-way ANOVA was conducted to compare the results between the larval and juvenile stages.

Preliminary blank experiments were conducted to ensure no potential side biases existed. Blank experiments recorded the position of an individual *M. pralinia*, as described above, but with empty adjacent compartments. Another set of preliminary experiments was conducted to determine the use of visual cues in the recognition of both conspecifics and heterospecifics. The position of an individual *M. pralinia* placed in the centre of the central compartment was recorded using methods described above. One adjacent compartment contained heterospecific *S. nigricans* adults and the other remained empty (see Appendix).

Ethical Note

To minimize adverse impacts on the welfare of fish during this experiment, *M. pralinia* larvae or juveniles were anaesthetized using MS222. Surgery began only once the fish was fully unconscious. The ethical guidelines of French Polynesia and the surgery protocol of Salas et al. (2008) and Durán et al. (2010) were followed. All fish tested were kept in aquaria for 15 days after the experiment to ensure survival. Of the 148 fish used in this study only 5% died (3% larvae and 2% juveniles). Data of deceased fish were removed from the analysis. After 15 days, fish were euthanized using a long exposure in MS222.

RESULTS

Visual Recognition of Conspecifics by Fish Larvae

Surgical treatments significantly impacted conspecific recognition in *M. pralinia* larvae (ANOVA: $F_{3,67} = 4.49$, $P = 0.003$). Both control treatments, *M. pralinia* larvae without surgery (NS) and those whose skull was opened (Sh), significantly preferred conspecific visual cues to heterospecific visual cues (Tukey test: NS: $P = 0.02$; Sh: $P = 0.002$). The larvae without surgery spent 56% of their trial time in the compartment closest to the conspecifics. Individuals that had their skull opened but no ablation spent 69% of their time near the conspecifics (Fig. 2a). In contrast, larvae with right or left telencephalon ablation (RT and LT) did not show an attraction to conspecifics (RT: $P = 0.80$; LT: $P = 0.73$), spending 33% and 34% of their time near the conspecific cue, respectively (Fig. 2a).

Visual Recognition of Conspecifics by Fish Juveniles

As seen with the larval *M. pralinia*, a significant difference was found between surgical treatments in juveniles (ANOVA: $F_{3,62} = 3.76$, $P = 0.009$). Juveniles without surgery (NS), individuals whose skull was opened (Sh) and those whose right telencephalon was removed (RT) spent significantly more time in the section nearest conspecifics (NS: $P = 0.006$; Sh: $P = 0.04$; RT: $P = 0.03$). In

contrast, juveniles whose left telencephalon was removed (LT) displayed no significant preferences for conspecifics, spending 42% of their time in the compartment closest to the heterospecifics and 25% of their time in the compartment closest to the conspecifics ($P = 0.84$; Fig. 2b).

No significant difference was found in the amount of time spent near conspecifics when comparing the two ontogenetic stages of the control experiment; both larvae and juveniles preferred to spend time near the conspecific cue (two-way ANOVA: $P = 0.81$). However, when the larval and juvenile results from the right ablated telencephalon treatment were compared, the larval stage spent significantly less time near the conspecific than the juvenile stage (two-way ANOVA: $P = 0.009$).

DISCUSSION

At both larval and juvenile stages, *M. pralinia* was able to recognize their conspecifics through visual cues. Juveniles with right telencephalon ablations (but not left) were still able to recognize the conspecifics. However, larvae with either right or left telencephalon ablations lost the attraction to conspecific visual cues (Fig. 2). Therefore, our results indicate that juvenile *M. pralinia* are lateralized, preferentially using the left telencephalic hemisphere to recognize conspecifics. Both larvae and juveniles showed no interaction (neither repulsion nor attraction) towards the

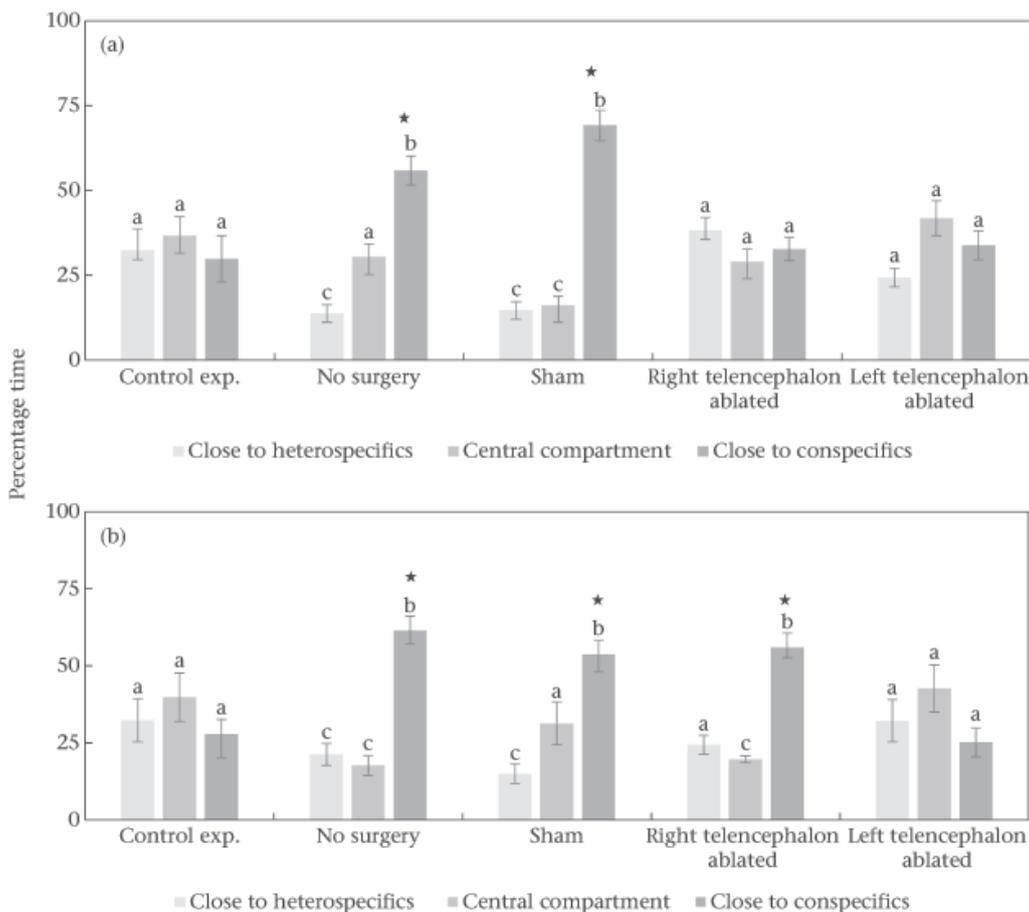


Figure 2. Mean percentage time spent \pm SE in the three compartments of the choice aquarium by *M. pralinia* (a) larvae or (b) juveniles. Results are separated by experimental treatment: blank control: without conspecifics and heterospecifics ($N = 10$ larvae, 10 juveniles); no surgery ($N = 17$ larvae, 16 juveniles); sham surgery ($N = 10$ larvae, 10 juveniles), right telencephalon ablation ($N = 12$ larvae, 10 juveniles) and left telencephalon ablation ($N = 12$ larvae, 10 juveniles). \star indicates a significant attraction towards the conspecifics (ANOVA: $P < 0.05$). Letters (a, b, c) above the bars indicate significant groupings by a post hoc Tukey test.

heterospecific cue, suggesting that *S. nigricans* was considered unfamiliar by *M. pralinia* larvae and juveniles.

Lateralization in response to social stimuli is often dominated by the right telencephalon hemisphere in response to visual cues provided by conspecifics (humans: [Sergent, Signoret, Bruce, & Rolls, 1992](#); monkeys: [Hook-Costigan & Rogers, 1998](#); birds: [Vallortigara, Rogers, Bisazza, Lippolis, & Robins, 1998](#); freshwater fishes: [Sovrano, Bisazza, & Vallortigara, 2001](#)). Our results suggest that *M. pralinia* juveniles use the left hemisphere of the brain to recognize their conspecifics and support existing evidence that some vertebrate taxa (mainly freshwater fish and birds) may be able to differentiate, using either the left or right hemisphere, between familiar and unfamiliar conspecifics ([Concha et al., 2012](#); [Kaarthigeyan & Dharmaretnam, 2005](#); [Sovrano, 2004](#)). Chicks, *Gallus gallus domesticus*, for example, use the right hemisphere for the recognition of familiar objects or conspecifics and the left hemisphere for objects that are unfamiliar ([Vallortigara & Andrew, 1991, 1994](#)). The fish *Xenopoeilus sarasinorum* show preferential use of the monocular field from the left eye when presented with familiar conspecifics and a slight preferential use of the right eye with unfamiliar conspecifics ([Sovrano, 2004](#)). *Myripristis pralinia* larvae were captured before recruiting into a reef. As a result, the larvae and juveniles were naïve, having had no previous experience with adult fishes. Juveniles used in this study were held individually for 7 days prior to experimental testing, without any contact with other fishes including conspecifics of the same cohort. *Myripristis pralinia* larvae, juveniles and adults have different colour patterns at Moorea island ([Maamaatuaiahutapu, Remoissenet, & Galzin, 2006](#)). The conspecific presented in the chamber (adults) would be considered unfamiliar for both larvae and juveniles individually tested, justifying the need to use the left hemisphere ([Culum Brown, Western, & Braithwaite, 2007](#); [Concha et al., 2012](#)). Further experiments should be conducted on juveniles that have been allowed contact with older conspecifics compared to those held in solitary confinement. This would determine whether *M. pralinia* are able to distinguish between familiar and unfamiliar conspecifics, and which telencephalon part is used for this differentiation.

The use of sensory cues during the recruitment process is widely accepted in larval ecology ([Kingsford et al., 2002](#); [Leis et al., 2011](#)). Many studies have found that recruiting larvae utilize the cues emitted by conspecifics for habitat recognition ([Booth, 1992](#); [Huijbers et al., 2012](#); [Lecchini et al., 2005](#)). Visual and chemical cues appear to be especially important during recruitment ([Devine, Munday, & Jones, 2012](#); [Dixson et al., 2008](#); [Dixson, Munday, Pratchett, & Jones, 2011](#); [Lecchini, Nakamura et al., 2007](#); [Lecchini, Osenberg et al., 2007](#)). The indifference shown by *M. pralinia* towards the visual cues of heterospecifics and preference towards the visual cues of conspecifics strongly suggests the importance of conspecific recognition. However, it is plausible that the fish tested here were responding to information other than conspecific identification, such as body shape or colour. Adult *M. pralinia* have a characteristic red body whereas the heterospecific, *S. nigricans* are predominantly black; therefore larvae and juveniles may have displayed a colour preference rather than conspecific preference. Given the known importance of conspecific recognition, this seems unlikely, but further experiments are required to disprove the idea. However, the significant difference found between experimental treatments in our study indicates the importance of the left hemisphere when juveniles process visual information. This finding is independent of the specific preference used by test subjects for position within the test chamber.

Overall, our results suggest that coral reef fish juveniles potentially use the left telencephalic hemisphere in the lateralization process of visual cue recognition. The extrapolation of results to other species should be done with caution, as this study was

conducted on a single species. The determinism of lateralized perception of conspecifics during fish ontogeny was not tested in our study. Determinism may be a consequence of genetic factors controlling the metamorphosis process and/or environmental factors such as predation at recruitment. In the freshwater fish *Brachyraphis episcopi*, individuals collected in areas of high and low predation pressure differ in both the strength and direction of the lateralized responses ([Brown, Gardner, & Braithwaite, 2004](#)). Settling coral reef fish recruits are subjected to strong predation pressure. Up to 90% of newly settled fish are removed by predation during the first week postrecruitment ([Doherty et al., 2004](#); [Lecchini, Nakamura et al., 2007](#); [Lecchini, Osenberg et al., 2007](#)). Moreover, during the recruitment phase, reef fish juveniles are subjected to additional intra- and interspecific interactions compared to fish larvae in open ocean ecosystems ([Barth et al., 2015](#); [Lecchini & Galzin, 2003](#); [Leis et al., 2011](#)). Thus, the brain lateralization of coral reef fish occurring at metamorphosis, from an oceanic larva to a reef juvenile, could increase their chance of survival and favour intraspecific interactions. A similar hypothesis was suggested in cuttlefish: [Jozet-Alves et al. \(2012\)](#) suggested that the development of lateralized response could allow juvenile cuttlefish to assess visual information faster and thus better deal with predation pressure. Additional experiments are necessary to determine whether brain lateralization established during ontogeny is a consequence of a genetic factor linked with the metamorphosis process and/or environmental factors such as predation. The diversity found on coral reefs and differences seen here with other vertebrate groups indicate that this ecosystem should be an area of future research on the evolution of brain lateralization.

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APPENDIX. CONTROL TREATMENT OF AQUARIA BIAS AND HETERO-SPECIFIC TREATMENT TO CHOOSE THE SPECIES

A control treatment was conducted to test potential bias of the apparatus. Here, one *M. pralinia* individual was introduced into the centre of the central compartment, and no fishes were introduced into the two adjacent compartments. A chi-square test was carried out to check that the control distribution was not significantly different to the theoretical distribution (i.e. fish spending 33% of their time in each part of the aquarium). The results showed no significant differences between the control distribution and the theoretical ones for both larvae and juveniles ($P > 0.05$). Considering our control distributions homogeneous, they were used to compare the experimental distributions.

To determine that both larvae and/or juveniles were able to distinguish and display a preference towards the visual cues of conspecifics, heterospecifics were used as cue transmitters. However, heterospecifics should not attract/repel both *M. pralinia* larvae and juveniles. To control for this, one individual was introduced into the centre of the central compartment and heterospecifics (in our study *S. nigricans*) were placed into one of the two adjacent compartments, the other one remaining empty. A chi-square test was carried out to confirm the distribution was not significantly different to the control distribution. The results showed no significant differences to the control distribution ($P > 0.05$) proving that both larvae and juveniles are indifferent to *S. nigricans*, confirming our choice. Moreover, visual observations of the aquaria showed no competition between *M. pralinia* at larval or juvenile stages and *S. nigricans*. Thus we can be sure that it is recognition that occurs rather than avoidance of a particular trait of heterospecifics such as larger size or vigorous locomotion. Last, some field surveys at Moorea showed, first, the presence of *M. pralinia* schools in which larvae, juveniles and adults live together, and, second, no competition for space or food between *S. nigricans* and *M. pralinia* (Lecchini, Nakamura, et al., 2007).

Appendix 5: Fish larval recruitment is a metamorphosis sensitive to reef pollutants

Holzer G., **Besson M. (equal first author)**, Lambert A., François L., Barth P., Gillet B., Hughes S., Piganeau G., Leulier F., Viriot L., Lecchini D., Laudet V. 2017. Fish larval recruitment is a metamorphosis sensitive to reef pollutants. *eLife* 2017;6:e27595.



Fish larval recruitment to reefs is a thyroid hormone-mediated metamorphosis sensitive to the pesticide chlorpyrifos

Guillaume Holzer^{1†*}, Marc Besson^{1,2,3†*}, Anne Lambert¹, Loïc François², Paul Barth¹, Benjamin Gillet¹, Sandrine Hughes¹, Gwenaël Piganeau³, Francois Leulier¹, Laurent Viriot¹, David Lecchini^{2,4‡}, Vincent Laudet^{1‡*}

¹Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, UMR CNRS 5242, Ecole Normale Supérieure de Lyon, Lyon, France; ²CRILOBE USR3278 EPHE-UPVD-CNRS, PSL Research University, Moorea, French Polynesia; ³Observatoire Océanologique de Banyuls-sur-Mer, UMR CNRS 7232, Université Pierre et Marie Curie Paris, Paris, France; ⁴Laboratoire d'Excellence CORAIL, Moorea, French Polynesia

Abstract Larval recruitment, the transition of pelagic larvae into reef-associated juveniles, is a critical step for the resilience of marine fish populations but its molecular control is unknown. Here, we investigate whether thyroid-hormones (TH) and their receptors (TR) coordinate the larval recruitment of the coral-reef-fish *Acanthurus triostegus*. We demonstrate an increase of TH-levels and TR-expressions in pelagic-larvae, followed by a decrease in recruiting juveniles. We generalize these observations in four other coral reef-fish species. Treatments with TH or TR-antagonist, as well as relocation to the open-ocean, disturb *A. triostegus* larvae transformation and grazing activity. Likewise, chlorpyrifos, a pesticide often encountered in coral-reefs, impairs *A. triostegus* TH-levels, transformation, and grazing activity, hence diminishing this herbivore's ability to control the spread of reef-algae. Larval recruitment therefore corresponds to a TH-controlled metamorphosis, sensitive to endocrine disruption. This provides a framework to understand how larval recruitment, critical to reef-ecosystems maintenance, is altered by anthropogenic stressors.

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*For correspondence: guillaume.holzer@ens-lyon.fr (GH); marc.besson@ens-lyon.org (MB); vincent.laudet@obs-banyuls.fr (VL)

†These authors contributed equally to this work

‡These authors also contributed equally to this work

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Introduction

Life history transitions are critical for many animal species and often correspond to concomitant developmental and ecological shifts (Bishop et al., 2006). Unfortunately, little is known about how internal and external cues act in concert during these events. The life cycle of most teleost reef fish include a major developmental and ecological transition. Adults reproduce in the vicinity of the reef, emitting eggs that disperse and hatch in the ocean, where the larvae grow (Leis and McCormick, 2002). Larvae migrate back and enter reefs where they become juveniles, a step called larval recruitment (Leis and McCormick, 2002). This step involves the perception of environmental cues for larvae to localize and settle in the reef, and is accompanied by major morphological changes (McCormick et al., 2002; Lecchini et al., 2005b, 2005a; Lecchini et al., 2013; Barth, 2015). This transition of pelagic larvae into reef-associated juveniles is often referred to as metamorphosis and is critical for the maintenance of reef fish populations, but its molecular control remains largely unknown (Dufour and Galzin, 1993; Doherty, 2002; Leis and McCormick, 2002; McCormick et al., 2002; Dixon et al., 2011; Barth, 2015). Since larval recruitment is an ecological

eLife digest Many animals go through a larval phase before developing into an adult. This transformation is called metamorphosis, and it is regulated by hormones of the thyroid gland in vertebrates. For example, most fish found on coral reefs actually spend the first part of their life as free-swimming larvae out in the ocean. The larvae usually look very different from the juveniles and adults. When these fish become juveniles, the larvae undergo a range of physical and behavioral changes to prepare for their life on the reef. Yet, until now it was not known what hormones control metamorphosis in these fish.

To address this question, Holzer, Besson et al. studied the convict surgeonfish *Acanthurus triostegus*. This herbivorous coral-reef fish lives in the Indo-Pacific Ocean, and the results showed that thyroid hormones do indeed regulate the metamorphosis of its larvae. This includes changing how the larvae behave and how their adult features develop. Further, Holzer, Besson et al. found that this was also true for four other coral-reef fish, including the lagoon triggerfish and the raccoon butterflyfish. In *A. triostegus*, thyroid hormones controlled the changes that enabled the juveniles to efficiently graze on algae growing on the reef such as an elongated gut.

When the fish larvae were then exposed to a pesticide called chlorpyrifos, a well-known reef pollutant, their hormone production was disturbed. This in turn affected their grazing behavior and also their metamorphosis. These fish had shortened, underdeveloped guts and could not graze on algae as effectively.

Herbivorous fish such as *A. triostegus* play a major role in supporting coral reef ecosystems by reducing algal cover and therefore promoting coral recruitment. These new findings show that pollutants from human activities could disturb the metamorphosis of coral-reef fish and, as a consequence, their ability to maintain the reefs. A next step will be to test what other factors can disrupt the hormones in coral-reef fish and thus pose a threat for fish populations and the coral-reef ecosystem.

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event coupled to a morphological transformation, reminiscent of the situation in amphibians, it may correspond to a thyroid hormones (TH) controlled metamorphosis (Bishop et al., 2006; Brown and Cai, 2007).

TH and their receptors (TR) trigger and coordinate metamorphosis of many species such as *Xenopus* (Brown and Cai, 2007) or flatfish (Solbakken et al., 1999). The thyroid gland produces mainly the thyroxine hormone (T4), which is peripherally transformed into triiodothyronine (T3), the active form (Chopra, 1996). The transformation of T4 into T3, and the degradation of T4 and T3 are controlled by a family of enzymes called deiodinases (Bianco and Kim, 2006). TH levels peak at the climax of metamorphosis. Precocious treatment with TH triggers metamorphosis whereas goitrogen (TH synthesis inhibitors) treatment blocks it (Tata, 2006). Similar mechanisms have been described in several teleost fishes, but in contexts disconnected from the natural environment (Brown, 1997; de Jesus et al., 1998; Yamano and Miwa, 1998; Kawakami et al., 2003; Marchand et al., 2004; McMenamin and Parichy, 2013).

In the convict surgeonfish *Acanthurus triostegus*, larvae have a planktonic diet while juveniles and adults are herbivorous, and external morphological changes occurring at recruitment have been previously described (Randall, 1961; McCormick, 1999; McCormick et al., 2002; Frédérick et al., 2012). Interestingly, crest-captured larvae can delay their metamorphosis when relocated to the external slope of the reef (open ocean), showing an influence of the environment on their morphological transformation (McCormick, 1999). Nevertheless, again, the molecular mechanisms remain unknown.

Here we report that *A. triostegus* larval recruitment to the reefs corresponds to metamorphosis with major pigmentation changes, remodeling of the digestive tract and behavioral modifications. Our results show that TH levels and TR expressions control this remodeling process. Furthermore, recruiting larvae can freeze their TH signaling when relocated on the external slope, demonstrating an influence of the environment on metamorphosis. Chlorpyrifos (CPF), a known reef pollutant and endocrine disruptor (Roche et al., 2011; Botté et al., 2012; Juberg et al., 2013; Slotkin et al.,

2013), affects *A. triostegus* metamorphosis by impairing TH signaling, preventing intestine lengthening, and inhibiting fish grazing activity. CPF therefore impairs *A. triostegus* control on algae spreading, which is a major threat for reef conservation. We extend our observation upon TH levels to four other coral reef fish species. Our work provides a unifying framework that integrates the developmental, ecological and evolutionary perspectives of vertebrate life history transitions. The involvement of the TH signaling pathway in this key post-embryonic step, thus prone to endocrine disruption, provides an obvious entry point to study how anthropogenic stressors may affect reef fish populations.

Results

Acanthurus triostegus larvae undergo a major and rapid remodeling at recruitment

A. triostegus crest captured individuals ($n = 297$) experience an important weight loss between day 1 (0.71 ± 0.01 g) and day 8 (0.53 ± 0.01 g) following their entry in the reef (Figure 1A–B). Individuals also undergo extensive pigmentation changes (Figure 1C–L), as exemplified by the very rapid appearance of black stripes (less than four hours, MB pers. obs.) after entering the reef (Figure 1C–D, H–I). This is followed by the widening of these vertical black stripes and by the onset of the body's white pigmentation (Figure 1D–G, Figure 1I–L).

Furthermore, using conventional X-Ray microtomography, we identified three different dental generations in crest larvae to day 8 juveniles (red, blue and green, Figure 1M–Q). Crest larvae present tiny and poorly mineralized pointed teeth at the distal ends of the jaws (red, Figure 1M, Figure 1—figure supplement 1R) that are likely remnants of an oceanic larval dentition (dentition A). Other medium-sized and more mineralized teeth are in function in medial areas of the jaws (blue, Figure 1M). These teeth display pointed cusps and belong to a more advanced dental generation (dentition B). Lastly, much larger teeth of the next generation (green, dentition C) are about to erupt from jaws in crest larvae (Figure 1M) and replace A and B previous dentitions in day 2 to day 8 juveniles (Figure 1N–Q). These teeth are highly similar to the shovel-shaped adult teeth (Figure 1—figure supplement 1S) that serve for grazing algae on hard substrata. The rapid formation of dentition C is consistent with a diet shift from planktivorous to herbivorous at recruitment. This shift is precisely organized, since it begins in the sagittal area of all individuals, and secondarily extends to the distal parts of the jaw (Figure 1M–Q). The fact that crest captured larvae already have teeth of the C dentition about to erupt (Figure 1M) indicates that fish are prepared for reef life conditions before reef entry.

The intestine also goes through a drastic remodeling at recruitment. In particular, the gastrointestinal tract lengthens from about 2.52 ± 0.07 cm in crest captured larvae to 7.38 ± 0.14 cm in day 8 juveniles (Figure 1W). To characterize this change, we performed histological sections on the middle part of the intestine during the larval recruitment process (Figure 1R–V). Intestines of crest larvae and day 2 juveniles have a thick wall with many muscle fibers and regular villi (Figure 1R and S). At day 3, intestines undergo a spectacular remodeling with the disappearance of the epithelium, leaving only the muscular layer (Figure 1T), while epithelium reformation occurs later at day 5 (Figure 1U). In day 8 individuals, the intestine is fully remodeled: the epithelium is thicker and exhibits villi again (Figure 1V). Such spectacular remodeling events are also observed in the proximal and distal portion of the intestines (Figure 1—figure supplement 1A–O).

To assess whether and to what extent this intestine remodeling was correlated to a diet shift at larval recruitment, we sequenced *A. triostegus* digestive tract content (18S mass sequencing) to identify its eukaryotic composition. We investigated crest captured individuals, day 8 juveniles and adults (Figure 1W) using a blocking primer to minimize amplification of host sequences. Crest larval intestines are largely empty although we identified sequences of fungi and algae. In day 8 juveniles, the intestinal content is different with more brown and red algae, as well as few protostome sequences. This indicates a shift in the eukaryotic gut content between crest individuals and day 8 juveniles. Adults gut content is less diversified and is mainly composed of brown algae, red algae and alveolates, different from those found in day 8 juveniles (Figure 1W). Using 16S metagenomic sequencing, we found differences in the relative quantity of bacterial community between the juveniles at day 2, 5 and 8 (Figure 1—figure supplement 1P). The 16S profile of the adult is also different from

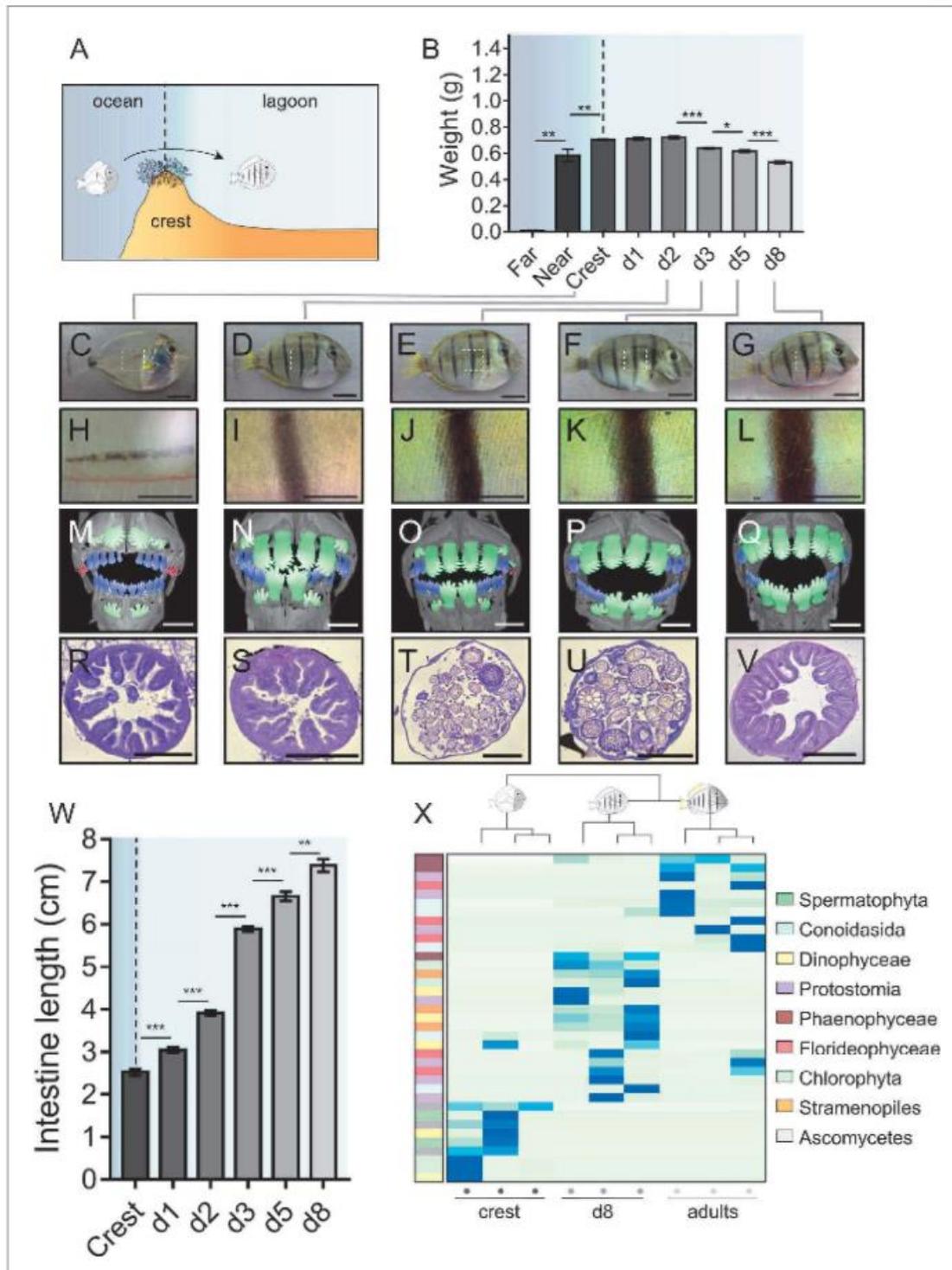


Figure 1. Changes in *Acanthurus triostegus* during larval recruitment. Error bars represent standard errors. *p-value<0.05; **p-value<0.01; ***p-value<0.001. (A) Schematization of the reef colonization by a larva (left) turning into a juvenile (right). The background color shading centered into the reef crest is re-used in following figures. (B) Weight variation during larval recruitment. n = 3 biological replicates for 5-to-10 km offshore fish (far), n = 5 for 2 km offshore fish (near) and n = 297 for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). See **Figure 1—Figure 1 continued on next page**

Figure 1 continued

source data 1 for detailed data and statistics. (C–G) Variation in pigmentation and morphology at different sampling points, (C) crest larva, (D) day 2 juvenile, (E) day 3 juvenile, (F) day 5 juvenile and (G) day 8 juvenile; scale bars indicate 0.5 cm. The white square represent the magnified region shown in the line below. These pictures are representative of $n > 50$ fish (biological replicates) per developmental stage, sampled at $n > 20$ different times during the year (technical replicates). (H–L) Close-up on the pigmented middle body band. Scale bars indicate 0.2 cm. These pictures are representative of $n > 50$ fish (biological replicates) per developmental stage, sampled at $n > 20$ different times during the year (technical replicates). (M–Q) X-Ray microtomography of larva, dentition A, B or C are highlighted respectively in red, blue or green. Scale bars indicate 0.5 mm. These pictures are representative of $n = 2$ fish (biological replicates) per developmental stage. (R–V) Histological sections (5 μm width) of intestines with haematoxylin/eosin staining. Scale bars indicate 200 μm . These pictures are representative of $n = 6$ sectioning technical replicates, performed twice (sampling technical replicates), on $n = 2$ fish (biological replicate). (W) Intestine length variation of *A. triostegus* during its larval recruitment. $n = 9$ to 14 biological replicates for each developmental stage (from $n = 2$ technical sampling replicates). Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). See **Figure 1—source data 2** for detailed data and statistics. (X) Heatmap clustering of the 18S sequences identified in the intestine content of crest individual ($n = 3$, left panel), day 8 juveniles ($n = 3$, middle panel) and adults ($n = 3$, right panel). Each panel is made of three columns corresponding to one individual. Each line corresponds to one taxonomic group found in the intestines: *Spermatophyta* (dark green), *Conoidasida* (cyan), *Dinophyceae* (light yellow), *Protostomia* (light pink), *Phaeophyceae* (brown), *Floriophyceae* (dark pink), *Chlorophyta* (light green), *Stramenopiles* (orange) and *Ascomycetes* (grey).

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The following source data and figure supplement are available for figure 1:

Source data 1. *Acanthurus triostegus* weight at different developmental stages.DOI: <https://doi.org/10.7554/eLife.27595.005>**Source data 2.** *Acanthurus triostegus* intestine length at different developmental stages.DOI: <https://doi.org/10.7554/eLife.27595.006>**Figure supplement 1.** Changes in *Acanthurus triostegus* intestine and teeth during larval recruitment.DOI: <https://doi.org/10.7554/eLife.27595.004>

the juvenile profile, with the notable presence of *Lachnospiraceae*, among which the giant bacteria *Eupoliscium fishelsoni* is a well-known symbiont of Acanthuridae (Clements and Bullivant, 1991).

Thyroid hormone signaling peaks at larval recruitment

Given the role of TH in vertebrate metamorphosis (Laudet, 2011) and the spectacular transformations that *A. triostegus* undergoes at larval recruitment, we analyzed TH levels in larvae captured in the far ocean (from 5 to 10 km offshore, $n = 3$), near ocean (2 km offshore, $n = 5$), crest-captured larvae and juveniles (raised in situ in lagoon cages, $n = 297$) up to day 8 after recruitment (Figure 2A–B).

T4 levels are low in far ocean larvae and rise in fish located closer to the reef (near ocean larvae), before dropping 6-fold between the near ocean larvae and the day 8 juveniles (p -value=0.001, Figure 2A). This indicates a peak of T4 in near ocean and in crest larvae. Far ocean larvae exhibit a high level of T3 but with an important variability (Figure 2B). Although these larvae live in the same ecological niche (i.e. open ocean) and are roughly of the same size (4.7, 5.5, and 6.3 mm), they were captured in different locations and are probably not at the same developmental stage. Near ocean and crest captured larvae have similar levels of T3 that are 6-fold higher than the level observed in day 8 juveniles (p -value < 0.001 Figure 2B).

We characterized the three TR of *A. triostegus*: TR α -A, TR α -B and TR β . These receptors have between 86% (TR α -A) and 96% (TR β) sequence identity with their zebrafish orthologs (Figure 2—figure supplement 1A). The phylogenetic analysis indicates that each of the three TR clusters with its respective teleosts orthologs (Figure 2—figure supplement 1B). Moreover, transactivation assays show that these receptors behave as genuine TR (Figure 2—figure supplement 1C). We thus assessed the expression levels of TR α -A, TR α -B, and TR β during *A. triostegus* development (Figure 2C), as well as *Klf9*, a conserved TR regulated gene (Denver and Williamson, 2009). Far ocean larvae have two profiles: one with a very low TR and *Klf9* expressions ('Far a' ocean) and one with very high levels of expression ('Far b' ocean) (Figure 2C). This suggests that there are, at least, two distinct situations in far ocean larvae: larvae with low TH/TR levels and larvae in which TH/TR levels surge. Far b ocean, near ocean, and crest larvae have high expression levels of the all the TR as well as *Klf9*, indicating a strong activation of the TH signaling at these stages. We observed a drop of all TR expression between crest larvae and day 8 juveniles (p -values=0.001, 0.005 and 0.002 for TR α -A, TR α -B and TR β respectively, Figure 2C).

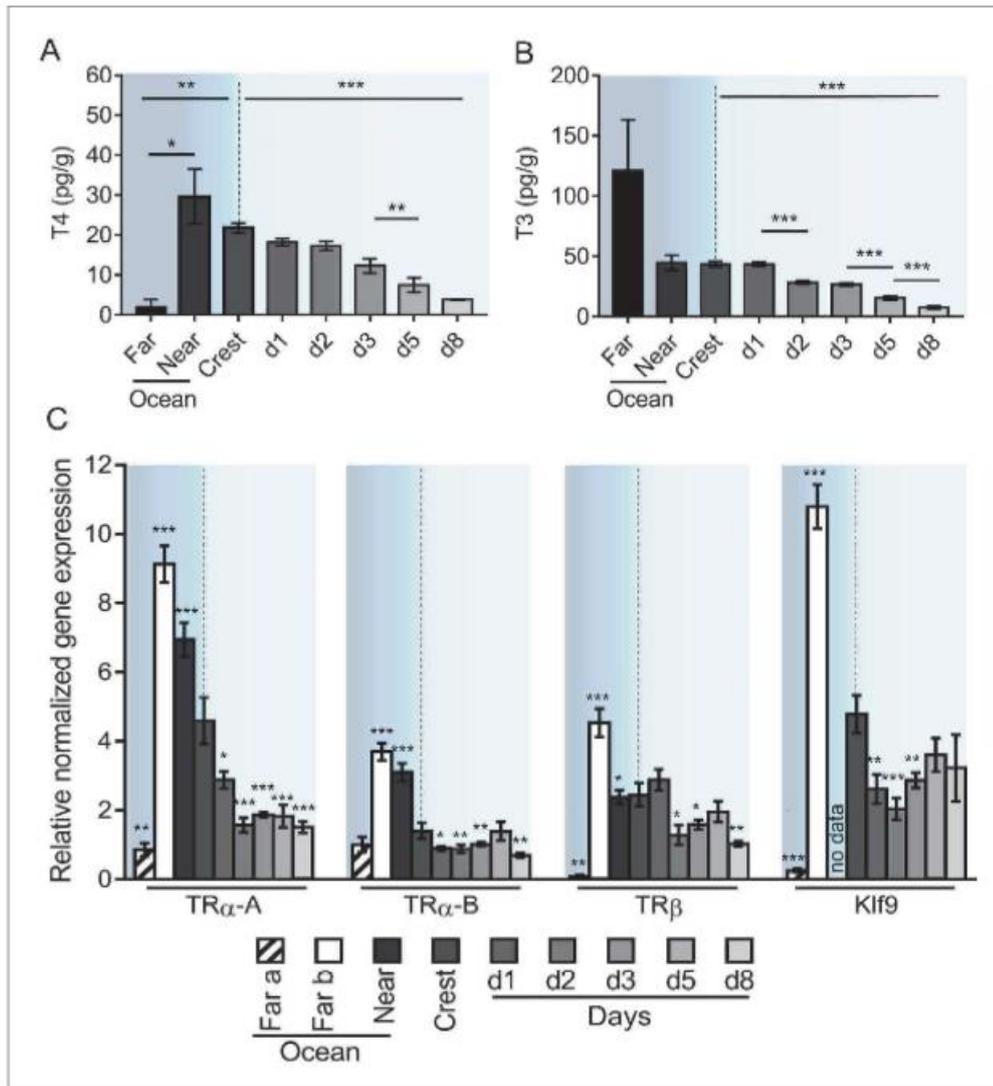


Figure 2. Thyroid hormone signaling during *Acanthurus triostegus* metamorphosis. Error bars represent standard errors. *p-value<0.05; **p-value<0.01; ***p-value<0.001. (A) T4 and (B) T3 dosage: Y-axis: T4 and T3 levels in $\text{pg}\cdot\text{g}^{-1}$; X-axis: sampling point. $n = 3$ for 5 to 10 km offshore fish (far), $n = 5$ for 2 km offshore fish (near), $n = 297$ for crest to day 8 (d8) fish. Statistics: mean comparisons (Wilcoxon-Mann-Whitney U-test). See **Figure 2—source data 1** and **Figure 2—source data 2** for detailed data and statistics. (C) Expression level of *TR α -A*, *TR α -B*, *TR β* and *Klf9* by qPCR, normalized with *Pold2* and *Rpl7*. Y-axis; Relative normalized expression X-axis: gene and sampling point. Far ocean larvae are split in two groups: Far a (black stripes) and Far b (white). For each panel, the color shading is the same one as in **Figure 1A**. For each sampling point three biological replicates and three technical replicates were performed. Statistics: comparisons of each mean to the Crest condition mean (Student's t cumulative distribution function, which was automatically computed by qPCR software CFX Biorad Manager, CFX Manager SH, 2017). See **Figure 2—source data 3** for detailed data and statistics.

DOI: <https://doi.org/10.7554/eLife.27595.007>

The following source data and figure supplements are available for figure 2:

Source data 1. *Acanthurus triostegus* T4 levels in larvae and juveniles.

DOI: <https://doi.org/10.7554/eLife.27595.011>

Source data 2. *Acanthurus triostegus* T3 levels in larvae and juveniles.

DOI: <https://doi.org/10.7554/eLife.27595.012>

Source data 3. *TR* and *Klf9* expression levels in *Acanthurus triostegus* at different developmental stages (qPCR).

DOI: <https://doi.org/10.7554/eLife.27595.013>

Source data 4. Transactivation assay of *Acanthurus triostegus* *TR* for several thyroid hormone derivatives.

Figure 2 continued on next page

Figure 2 continued

DOI: <https://doi.org/10.7554/eLife.27595.014>**Source data 5.** Transactivation assay of *Acanthurus triostegus* TR for the competition of T3 and NH₃.DOI: <https://doi.org/10.7554/eLife.27595.015>**Source data 6.** List of primers used in this study.DOI: <https://doi.org/10.7554/eLife.27595.016>**Source data 7.** List of TR used for phylogenetic reconstruction.DOI: <https://doi.org/10.7554/eLife.27595.017>**Figure supplement 1.** Characterization of *Acanthurus triostegus* thyroid hormone receptors.DOI: <https://doi.org/10.7554/eLife.27595.008>**Figure supplement 2.** Primers used in this study.DOI: <https://doi.org/10.7554/eLife.27595.009>**Figure supplement 3.** List of thyroid hormone receptors sequences used for the phylogeny.DOI: <https://doi.org/10.7554/eLife.27595.010>

Thyroid hormone signaling and the environment control larvae transformation

To link the TH pathway, the environment and the metamorphic changes occurring at larval recruitment, we disturbed the TH signaling and the location of crest-captured larvae during their metamorphosis (from capture to up to day 8 after reef colonization).

In order to study how the environment controls the metamorphosis processes, we first relocated crest-captured larvae back to the external slope immediately after their reef entry. Indeed, mimicking oceanic condition has been acknowledged to delay metamorphosis (McCormick, 1999) (Figure 3A, upper panel). Similar to this earlier study, we observed a striking delay in the white pigmentation appearance between the vertical black stripes in day 2 relocated fish compared to their lagoon raised counterparts (Figure 3A, lower panel). The levels of T4 and T3 in fish relocated on the external slope at day 2, day 5 and day 8 are overall higher than the levels of their control counterparts in the lagoon (Figure 3B–C). The levels of T4, in particular, remain similar in day 2 to day 8 juveniles to the levels in the crest condition (Figure 3B). The expressions of TRs and *Klf9* in the relocated juveniles were also higher than in control juveniles at the same age (Figure 3E). This suggests that the environment effectively controls the metamorphosis by modulating the TH signaling.

To assess the implication of the TH signaling pathway on metamorphic processes, we then raised crest-captured larvae in situ in the lagoon and injected them daily, in their ventral cavity, with 20 μ l of different drug solutions that activate or disrupt their TH molecular pathway. Four drug treatments were applied: (i) solvent control (DMSO diluted 10.000 times in Phosphate Buffer Saline 1X, as all drugs were made soluble in DMSO and diluted 10.000 times in PBS 1X); (ii) T3 + iopanoic acid (IOP) both at 10^{-6} M, IOP being used as an inhibitor of the deiodinase enzymes that therefore prevents the degradation of injected T3 (Galton, 1989; Little et al., 2013; Lorgen et al., 2015); (iii) NH₃ at 10^{-6} M, NH₃ being used as an antagonist of TR that prevents the binding of TH on TR (Lim et al., 2002; Renko et al., 2012) therefore disrupting *A. triostegus* TH pathway (Figure 2—figure supplement 1D); and (iv) T3 + IOP + NH₃ all at 10^{-6} M to ensure the non-toxicity of NH₃.

We used intestine total length, internal structure of guts (intestinal microvilli), dentition and pigmentation as markers of the advancement of metamorphosis since they strongly change after reef recruitment (Figure 1). T3 + IOP treatment increases the length of the intestine in day 2 and day 5 juveniles (p -values < 0.001, Figure 3E, blue). On the contrary, NH₃ treatment partially prevents the intestines lengthening occurring after recruitment (p -values < 0.001, Figure 3E, green). Injections of T3 + IOP + NH₃ result in an intestine length similar to the control individuals at day 2 and day 5 (p -values = 0.486 and 0.444 respectively, Figure 3E). This shows that T3 and NH₃ effectively compete, and confirms the non-toxicity of NH₃. In parallel, intestines of fish relocated on the external slope are shorter than those of their lagoon counterparts at day 2 and day 5 (p -value < 0.001 and p -value = 0.042 respectively, Figure 3E, orange). This shows that their development has been slowed down, similarly to what we observed with the NH₃ treatment. A similar pattern was observed concerning the microvilli remodeling within guts. In the anterior part of the guts, while T3 + IOP treatment accelerates the loss of the intestinal microvilli at day 2 and accelerates the development and the thickening of a new epithelium at day 5 (Figure 3F, second column), NH₃ treatment and external

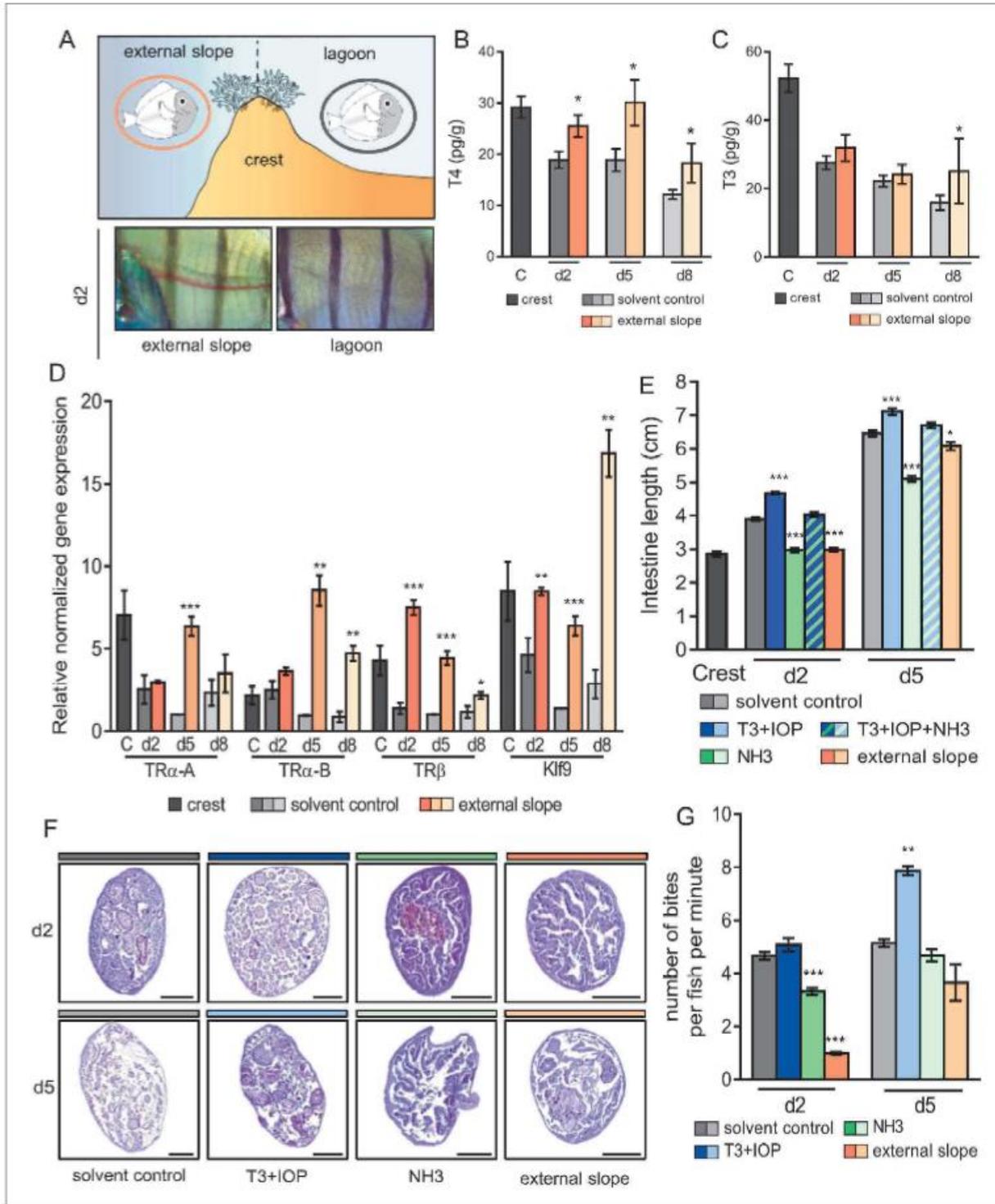


Figure 3. Impact of the environment and thyroid hormone signaling on *Acanthurus triostegus* metamorphic processes. Error bars represent standard errors. *p-value<0.05; **p-value<0.01; ***p-value<0.001. (A) Upper panel: schematization of the external slope experiment with control fish, in the lagoon (grey circle), and relocated fish, on the external slope (orange circle). Lower panel: comparison of the pigmentation between the relocated fish (left) and the control lagoon fish (right) at day 2. (B) T4 and (C) T3 dosage of crest captured larvae and day 2, 5 and 8 juveniles, in control (grey shade) Figure 3 continued on next page.

Figure 3 continued

or external slope (orange shade) conditions. $n = 4$ fish for each experimental condition. X-axis: sampling point, Y-axis: T4 or T3 level in $\text{pg}\cdot\text{g}^{-1}$. Statistics: mean comparisons between control and external slope (Wilcoxon-Mann-Whitney U-test). See **Figure 3—source data 1** and **Figure 3—source data 2** for detailed data and statistics. (D) Expression level of *TR α -A*, *TR α -B*, *TR β* and *Klf9* by qPCR, normalized with *pold2* and *rpl7* housekeeping genes, in control (grey shade) or external slope (orange shade) conditions. Y-axis: Relative normalized expression. X-axis: gene and stages. For each point three biological replicates and three technical replicates were performed. Statistics: comparisons of 'external slope' means to their 'control' counterparts (Student's t cumulative distribution function). See **Figure 3—source data 3** for detailed data and statistics. (E) Intestine length in cm of fish at crest (C), day 2 (d2) or day 5 (d5) under different treatment conditions: solvent-control (grey shade), T3 + IOP treated at 10^{-6} M (blue shade), NH3 treated at 10^{-6} M (green shade), T3 + IOP + NH3 treated at 10^{-6} M (blue and green dashed shade) or relocated to the external slope (orange shade). $n > 10$ fish for each experimental condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the respective solvent-control condition are indicated. See **Figure 3—source data 4** for detailed data and statistics (F) Histological cross section ($5\ \mu\text{m}$ width) of intestine stained by haematoxylin/eosin. Scale bars indicate $200\ \mu\text{m}$. These pictures are representative of $n = 2$ microtome section and staining technical replicates, performed on $n = 3$ fish (biological replicate) per treatment and per developmental stage. Two technical replicates of this experiment were performed. (G) Mean number of bites (on turf algae) per fish in a batch of 18 fish at day 2 (d2) and day 5 (d5) under different treatment conditions: solvent control (DMSO, grey shade), T3 + IOP at $10^{-6}\ \mu\text{M}$ (blue shade), NH3 at $10^{-6}\ \mu\text{M}$ (green shade) or relocated to the external slope (orange shade). $n = 3$ to 6 independent bite counts (technical replicates) for each condition. Bite counting was not performed on crest captured individuals as *A. triostegus* larvae do not feed for at least 12 hr after colonizing the reef at night (MB pers. obs.). Statistics: same as in (E). See **Figure 3—source data 5** for detailed data and statistics.

DOI: <https://doi.org/10.7554/eLife.27595.018>

The following source data and figure supplements are available for figure 3:

Source data 1. T4 levels in external slope relocated *Acanthus triostegus*.DOI: <https://doi.org/10.7554/eLife.27595.021>**Source data 2.** T3 levels in external slope relocated *Acanthus triostegus*.DOI: <https://doi.org/10.7554/eLife.27595.022>**Source data 3.** *TR* and *Klf9* expression levels in external slope relocated *Acanthus triostegus* (qPCR).DOI: <https://doi.org/10.7554/eLife.27595.023>**Source data 4.** *Acanthus triostegus* intestine length under pharmacological treatments or relocation.DOI: <https://doi.org/10.7554/eLife.27595.024>**Source data 5.** *Acanthus triostegus* grazing activity under pharmacological treatments or relocation.DOI: <https://doi.org/10.7554/eLife.27595.025>**Figure supplement 1.** Thyroid hormone signaling and environment impact on the dentition and guts histology of *Acanthus triostegus* at metamorphosis.DOI: <https://doi.org/10.7554/eLife.27595.019>**Figure supplement 2.** Pigmentation patterns and thyroid hormone signaling in *Acanthus triostegus* at day 2 (d2) and day 5 (d5) during larval recruitment.DOI: <https://doi.org/10.7554/eLife.27595.020>

slope relocation prevent microvilli renewal at both day 2 and day 5 (**Figure 3F**, third and fourth column). These observations can be extended to the anterior, medium and posterior sections of the guts (see **Figure 3—figure supplement 1**). These changes are consistent with the notion that TH control remodeling of the guts at metamorphosis in *A. triostegus*. We did not observe any effect on teeth development neither with T3 + IOP or NH3 treatment, nor with external slope relocation (**Figure 3—figure supplement 1**). Given that teeth remodeling is a lengthy process that starts very early on in oceanic larvae and therefore before reef colonization, it is not surprising that the treatments performed on crest captured larvae do not affect (or are too late to affect) teeth development. Also, contrarily to individuals relocated on the outer slope, we did not observe any effect of T3 + IOP or NH3 treatments on skin pigmentation at day 2 and day 5 (**Figure 3—figure supplement 2**). This suggests that pigmentation during the larva to juvenile transition is strongly coupled to the environment but is not directly under TH control that may not sustain such rapid pigmentation changes. Taken together: (i) intestine lengthening and remodeling are mediated by TH signaling; and (ii) disturbing the TH pathway disrupts the normal development of fish after larval recruitment, showing that TH effectively controls some major aspects of *A. triostegus* metamorphosis.

Finally, we tested how TH signaling influences fish behavior. To achieve this, we used grazing activity (number of bites on algal turf), as *A. triostegus* larvae start to graze a couple of days after colonizing the reef, to investigate how TH signaling and metamorphosis completion influence the behavior of recruited fish. T3 + IOP treatment increases the grazing activity by around 50% in day 5

juveniles when compared to the control individuals (p -value=0.004, **Figure 3G**, blue lanes). On the contrary, NH₃ treatment significantly decreases the number of bites at day 2 by 33% (p -value<0.001, **Figure 3G**, green lane). The grazing activity of fish relocated on the external slope (**Figure 3A**) is also diminished by more than 4-fold at day 2 (p -value<0.001, **Figure 3G**, orange lanes). These data indicate that TH signaling promotes the biting activity of *A. triostegus* and subsequent grazing behavior adopted at larval recruitment. This shows that TH controls the transformations of the feeding process not only at the morphological and physiological levels but also at the behavioral level.

Thyroid hormone-mediated metamorphosis is conserved in coral reef fishes

To widen our understanding of coral reef fish metamorphosis, we investigated the TH signaling during larval recruitment in four other coral fish species (*Rhinecanthus aculeatus*, *Chromis viridis*, *Chaetodon lunula* and *Ostorhinchus angustatus*) from distant families (Near et al., 2012). In these species, both T₄ and T₃ levels drop between day 1 and day 8, up to 3-fold for T₄ (in *O. angustatus*, p -value=0.029) and up to 4-fold for T₃ (in *R. aculeatus*, p -value=0.05) (**Figure 4**). This drop of TH levels after reef entry strongly suggests that TH are also key determinants for the metamorphosis of these species. T₄ levels in *R. aculeatus* and T₃ levels in *C. viridis* also rise between near ocean and day 1 individuals (**Figure 4**), further confirming the model of a TH-mediated metamorphosis during coral reef fish larval recruitment. However, T₄ levels are 3-fold higher in near ocean individuals than in day 1 individuals in *C. viridis* (**Figure 4**). This suggests some potential species-specific variation in coral reef fish TH profiles at recruitment that would be extremely interesting to decipher in future studies on additional species and with regards to other aspects of coral reef fish ecology (e.g., diets, size and pigmentation status at recruitment).

Reef pollutant disrupts the transformation of *Acanthurus triostegus*

Chlorpyrifos (CPF) is an agricultural insecticide that is widely used on tropical coastal crops, therefore one of the most common waterborne chemical pollutants encountered in coral reef surrounding waters (Cavanagh et al., 1999; Leong et al., 2007; Roche et al., 2011; Botté et al., 2012). Given the endocrine determinants of coral reef fish metamorphosis (**Figures 2–3**) and the CPF endocrine disruption characteristics (Juberg et al., 2013; Slotkin et al., 2013), we assessed the impact of CPF on *A. triostegus* metamorphosis. To achieve this, we used exposure doses previously reported in coral reef fish (1, 5 or 30 $\mu\text{g.l}^{-1}$ of seawater) (Botté et al., 2012). CPF treatment at 30 $\mu\text{g.l}^{-1}$ decreases the T₃ levels by 50% in day 2 juveniles (p -value=0.021, **Figure 5A**). No significant effect was observed on T₄ levels regardless of CPF concentration and duration (p -value=0.205 for day 2 and p -value=0.496 for day 5, **Figure 5—figure supplement 1A**). We did not observe any effect of CPF treatments on fish pigmentation at day 2 and day 5 (**Figure 5—figure supplement 1B**). However, CPF exposure at 30 $\mu\text{g.l}^{-1}$ prevents guts' lengthening at day 2 (p -value=0.024, **Figure 5B** left panel), and both exposure at 5 and 30 $\mu\text{g.l}^{-1}$ prevent intestines' lengthening at day 5 (p -values=0.047 and 0.029 respectively, **Figure 5B** right panel). Strikingly, CPF exposure at 30 $\mu\text{g.l}^{-1}$ also decreases the grazing activity of day 2 juveniles with 2.63 ± 0.09 bites per fish per min contrasting with 3.62 ± 0.28 bites per fish per min in the solvent control condition (p -value=0.014, **Figure 5C**). In day 5 juveniles, exposure at 5 and 30 $\mu\text{g.l}^{-1}$ decreases the grazing activity with respectively 2.68 ± 0.18 and 2.63 ± 0.05 bites per fish per min compared to 4.43 ± 0.33 bites per fish per min for the solvent control fish (p -values=0.001, **Figure 5C**). Such a behavior in fish treated with CPF results in up to a 14-fold reduction in the biomass of turf grazed after 5 days (p -value=0.05, **Figure 5D**).

Discussion

In this study, we investigated the morphological, physiological and behavioral changes occurring during *A. triostegus* larval recruitment. These results highlight profound anatomical and physiological transformations of the skin and the whole digestive tract (**Figure 1B–W**). In particular, the differences in 16S gut content are likely the consequence of a diet shift (carnivorous to herbivorous) and an environmental shift occurring during larval recruitment (Frédérich et al., 2012; Baldo et al., 2015; Sullam et al., 2012). We hypothesize that intestine remodeling (i.e. the loss of the villi and internal structures) accelerates the shift of the gut microbiota by allowing the establishment of a new bacterial community fitting the diet change. The presence of *Lachnospiraceae* in the adult but not in

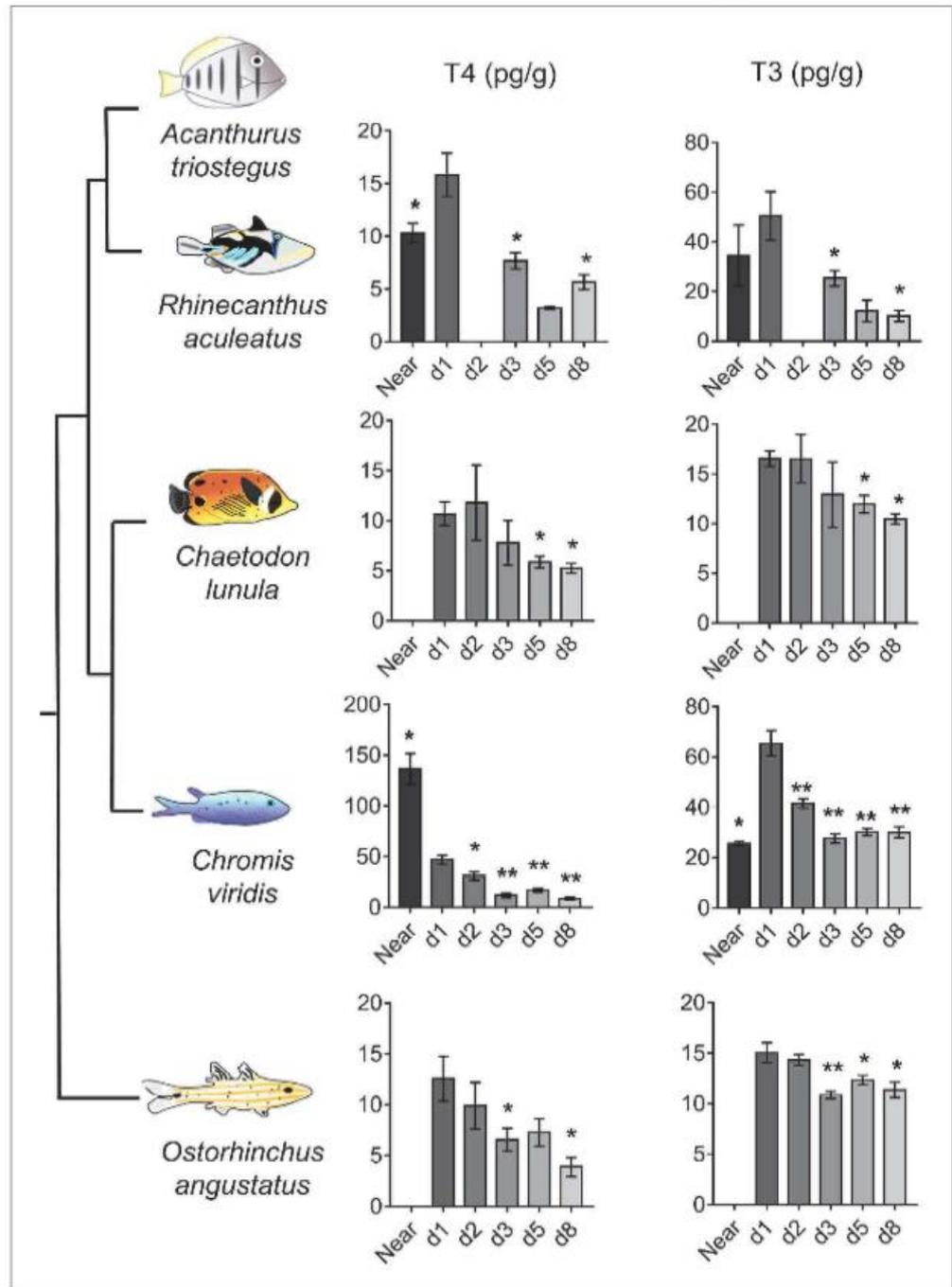


Figure 4. Thyroid hormone levels in distant coral reef fish at larval recruitment. Error bars represent standard errors. *p-value<0.05; **p-value<0.01; ***p-value<0.001. TH levels of: *Rhinecanthus aculeatus* (upper), *Chromis viridis* (middle up), *Chaetodon lunula* (middle low) and *Ostorhinchus angustatus* (lower). n = 3 to 5 biological replicates for each condition. First column is T4, second column is T3. Y-axis: T4 or T3 level in $\text{pg}\cdot\text{g}^{-1}$. X-axis: sampling point (developmental stage). The phylogenetic relationship between the species is plotted on the far left of the figure. Statistics: for each species, comparisons of each mean to the day 1 (d1) mean (Wilcoxon-Mann-Whitney U-test). See **Figure 4—source data 1** for detailed data and statistics.

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Figure 4 continued on next page

Figure 4 continued

The following source data is available for figure 4:

Source data 1. T4 and T3 levels of various coral reef fishes at different development stages.

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the larvae is consistent with the hypothesis that this family of bacteria is acquired through the environment in surgeonfishes (Miyake et al., 2016). Altogether, these observations reinforce the notion that the fish bacterial community depends on their diet and environment. These shifts of 16S and 18S gut contents, occurring between crest individuals and day 8 juveniles, strikingly coordinate with both the changes of the intestine and teeth morphologies observed during larval recruitment (Figure 1M–V). All these changes from teeth to microbiota are consistent with *A. triostegus* change of diet after entering the reefs, conforming to previous observations (Randall, 1961; McCormick, 1999; Frédérick et al., 2012). This complete remodeling is associated with a severe weight loss (Figure 1B), suggesting that larval recruitment corresponds to a real metabolic challenge.

The molecular investigation of TH signaling in the recruiting larvae indicates a surge of TH levels and *TR* expressions in near ocean larvae and crest individuals, and a drop in juveniles, after reef entry (Figure 2A–C). This indicates that migration towards the reefs marks the onset of metamorphosis and starts very early on (Figure 6A). Consequently, the transformation process is already well advanced when larvae reach the reef, suggesting that this event is triggered in the open ocean by, yet unknown, environmental cues (Figure 6A). After reef entry, T3 treatment increases gut length while external slope relocation and NH3 treatment delay the metamorphosis, although they could not completely interrupt it (Figure 3A–B). These results are consistent with the model of metamorphosis in amphibians (Brown and Cai, 2007) and other fish species (McMenamin and Parichy, 2013). It is interesting to relate the morphological and physiological changes we have observed in *A. triostegus* with the TH-controlled post-embryonic process known in zebrafish (the major teleost fish developmental model, one of the few teleost fishes in which we have a functional knowledge of the metamorphosis process). Indeed, in both species, as well as in flatfish, another classical teleost model of metamorphosis, major pigmentation and craniofacial changes occur at metamorphosis (McMenamin and Parichy, 2013; McMenamin et al., 2014). It is not clear if the appearance of the dark melanophore stripes in zebrafish and *A. triostegus* is controlled by similar mechanisms. In *A. triostegus*, this event is extremely rapid (as fast as 2 hr after reef entry, MB pers. obs.), and strongly environmentally coupled, whereas in zebrafish juveniles the pigment pattern changes very gradually and is presumably not under selection for rapid change during metamorphosis (McMenamin et al., 2014). However, melanophores can differentiate rapidly (in less than 12 hr) when appropriately stimulated in juvenile zebrafish. For example, acute stimulation with TH may initiate rapid melanophore differentiation (McMenamin et al., 2014). Therefore, if the melanophore precursors are already in place and poised to differentiate in *A. triostegus*, it is possible that they could become melanized within a few hours upon direct stimulation with TH. Our NH3 treatments suggest that this may not be the case (Figure 3—figure supplement 2), but this remains an open question that requires further investigation. Altogether, our findings indicate that the onset and coordination of metamorphosis are two distinct events, both coordinated by TH and under environmental control. They further demonstrate that *A. triostegus* larval recruitment is a TH-controlled metamorphosis, as in amphibians and flatfishes (Brown and Cai, 2007; Laudet, 2011; McMenamin and Parichy, 2013).

We demonstrated that the decrease of both T4 and T3 after reef entry can be generalized to phylogenetically distant coral fish species (Figure 4). We propose that this decrease is valid for most if not all coral reef fishes, even if species-specific variations on this general theme can be observed, and that the larval recruitment of coral reef fish corresponds to a TH-controlled metamorphosis (Figure 6A). Interestingly, variations in the magnitude of TH changes among the five coral reef fish species (Figure 4) are reminiscent of the situation in stickleback, where differences in TH signaling between two ecotypes support life history trait variation (Kitano et al., 2010; Kitano and Lema, 2013). We anticipate that this post-embryonic developmental step is shaped by evolution and varies according to species-specific life history traits such as pelagic larval duration, size at reef colonization and pigmentation status (i.e., metamorphic state of advancement) at recruitment. Given the

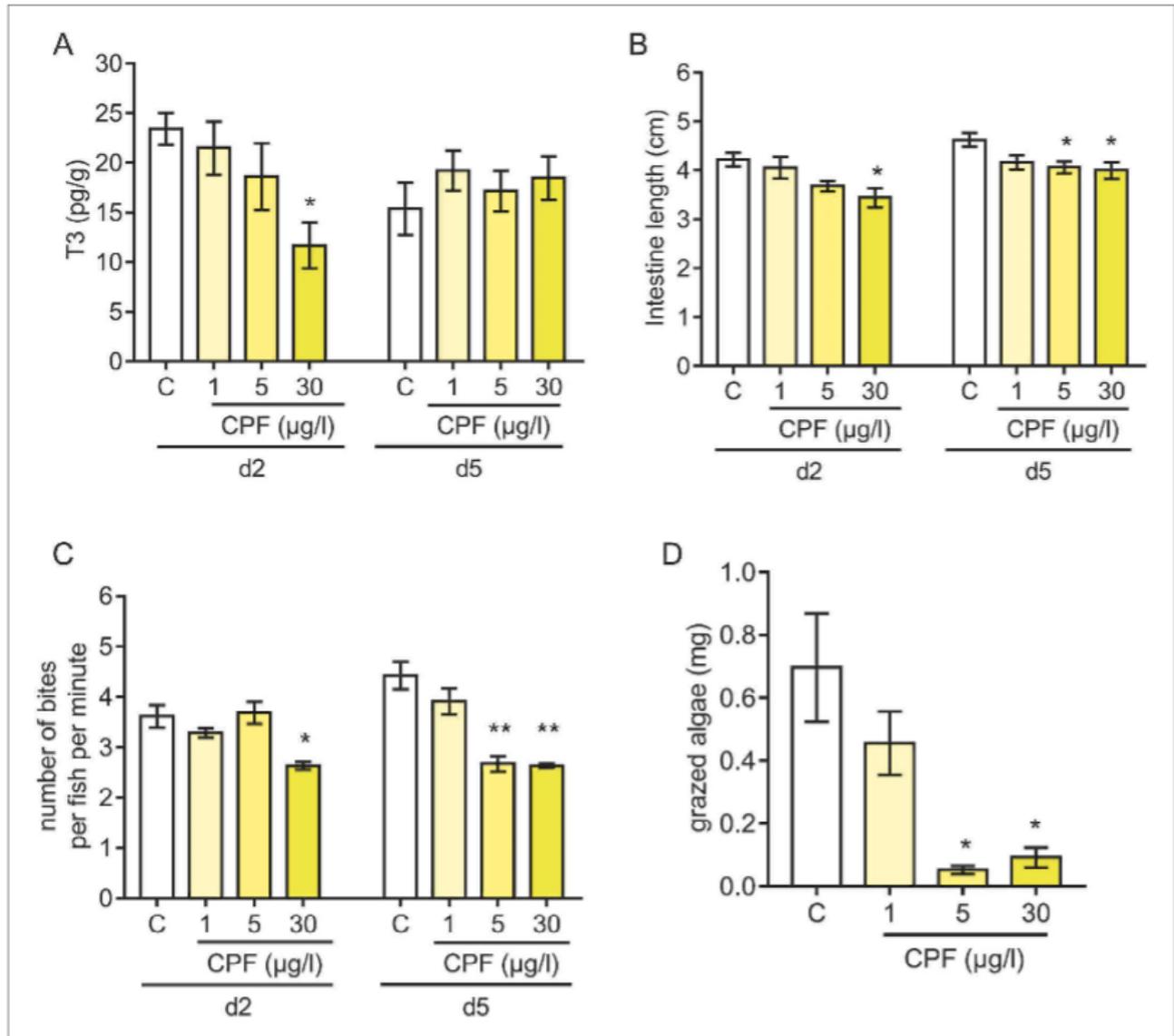


Figure 5. Influence of chlorpyrifos exposure on *Acanthurus triostegus* thyroid hormone signaling, intestine length, grazing activity, and algal removal efficiency. Error bars represent standard errors. *p-value<0.05; **p-value<0.01; ***p-value<0.001. (A) T3 level in $\text{pg}\cdot\text{g}^{-1}$ of day 2 (d2) and day 5 (d5) fish exposed to different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\ \mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\ \mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 10$ fish in each condition. Statistics: ANOVA followed by Tukey posthoc tests, only significant differences with the solvent-control condition are indicated. See **Figure 5—source data 1** for detailed data and statistics. (B) Intestine length in cm of fish at day 2 (d2) or day 5 (d5) under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\ \mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\ \mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 5$ to 8 fish for each experimental condition. Statistics: same as in (A). See **Figure 5—source data 2** for detailed data and statistics. (C) Mean number of bites (on turf algae) per fish in a batch of 18 fish on reef turf at day 2 (d2) or day 5 (d5), under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\ \mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\ \mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 3$ independent bite counts for each condition. Statistics: same as in (A). See **Figure 5—source data 3** for detailed data and statistics. (D) Mean turf biomass removed per fish in a batch of 10 fish, in 5 days following reef colonization, under different conditions: solvent control (C, acetone, white bars), or exposed to CPF at $1\ \mu\text{g}\cdot\text{l}^{-1}$ (1, light yellow bars), $5\ \mu\text{g}\cdot\text{l}^{-1}$ (5, yellow bars) or $30\ \mu\text{g}\cdot\text{l}^{-1}$ (30, dark yellow bars). Exposure started right after crest capture. $n = 3$ independent measurements of grazed turf for each condition. Statistics: comparisons of each mean to the solvent-control (C) mean (Wilcoxon-Mann-Whitney U-test). See **Figure 5—source data 4** for detailed data and statistics.

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Figure 5 continued on next page

Figure 5 continued

The following source data and figure supplement are available for figure 5:

Source data 1. T3 levels in *Acanthurus triostegus* under different chlorpyrifos exposure conditions.

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Source data 2. Intestine length of *Acanthurus triostegus* under different chlorpyrifos exposure conditions.

DOI: <https://doi.org/10.7554/eLife.27595.031>

Source data 3. Grazing activity of *Acanthurus triostegus* under different chlorpyrifos exposure conditions.

DOI: <https://doi.org/10.7554/eLife.27595.032>

Source data 4. Quantity of grazed algae by *Acanthurus triostegus* under different chlorpyrifos exposure conditions.

DOI: <https://doi.org/10.7554/eLife.27595.033>

Source data 5. T4 levels in *Acanthurus triostegus* under different chlorpyrifos exposure conditions.

DOI: <https://doi.org/10.7554/eLife.27595.034>

Figure supplement 1. Pigmentation patterns and chlorpyrifos exposure in *A. triostegus* at metamorphosis.

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dramatic diversity of life history strategies they propose, coral reef fish could be excellent models to decipher how these changes are elicited by changes in hormonal systems.

Among the endocrine disruptors already evidenced and encountered in coral reefs, CPF is known to be a thyroid disruptor with adverse effects on fish behavior (Sandahl et al., 2005; Kavitha and Rao, 2008; Roche et al., 2011; Yang et al., 2011; Botté et al., 2012; Deb and Das, 2013; Juberg et al., 2013; Slotkin et al., 2013). We demonstrated that CPF exposure reduces *A. triostegus* physiological level of T3 at larval recruitment (Figure 5A). Consistently with our observations on NH₃ treated fish, exposure to CPF impairs metamorphosis, as evidenced with the repression of guts' lengthening (Figure 5B), and the decrease in the juveniles' grazing activity (Figure 5C). This latter point led to a reduced algal removal efficiency (Figure 5D). As *A. triostegus* is one of the major herbivorous fish in coral reefs, this can severely impair the ability of coral reefs to recover after an algal invasion (e.g., after a bleaching event) and therefore contributes to the worldwide degradation of reef health (Russ, 1984; Almany et al., 2007; Ledlie et al., 2007; Hughes et al., 2007; Hughes et al., 2014) (Figure 6B).

Conclusion

Our results highlight how coral reef fish larval recruitment, the transition between the open ocean and the reef, is a TH-regulated metamorphosis at the crossroads of ecological, developmental, physiological and behavioral transformations (Holzer and Laudet, 2015). These findings also demonstrate how coral reef fish TH signaling and larval recruitment processes can be altered by reef pollution. Since metamorphosis and larval recruitment are essential for the maintenance of fish populations and subsequent coral reef resilience, this study provides a general framework to better understand, at the molecular level, how global changes, water pollution and human activities can threaten reef ecosystems.

Materials and methods

Fish capture, husbandry and conservation

Acanthurus triostegus, *Ostorinchus angustatus*, *Chaetodon lunula*, *Chromis viridis* and *Rhinecanthus aculeatus* larvae were captured at Moorea Island, French Polynesia. Larvae were captured during the night while colonizing the reef crest, using a crest net and hand nets (Dufour and Galzin, 1993; Besson et al., 2017). Larvae were then kept in cages located in situ in the lagoon and provided with continuous food from the water column supplemented with coral rubble and algal turf. For the clarity of this study, the age in day of the juvenile corresponds to the number of day spend in the reef in cages since capture, not the absolute age. Near ocean *A. triostegus* larvae were sampled at 1–2 km away from the reef crest using drifting light trap and hand nets from 0.5 m to 5 m depth. Far ocean *A. triostegus* larvae were also captured offshore by trawl haul more than 10 km away from the reef between 1 and 60 m deep. Larvae were euthanized in MS222 at 0.4 mg.ml⁻¹ in filtered seawater. For TH dosage, larvae were frozen dry and conserved at –20°C prior to extraction. For RNA

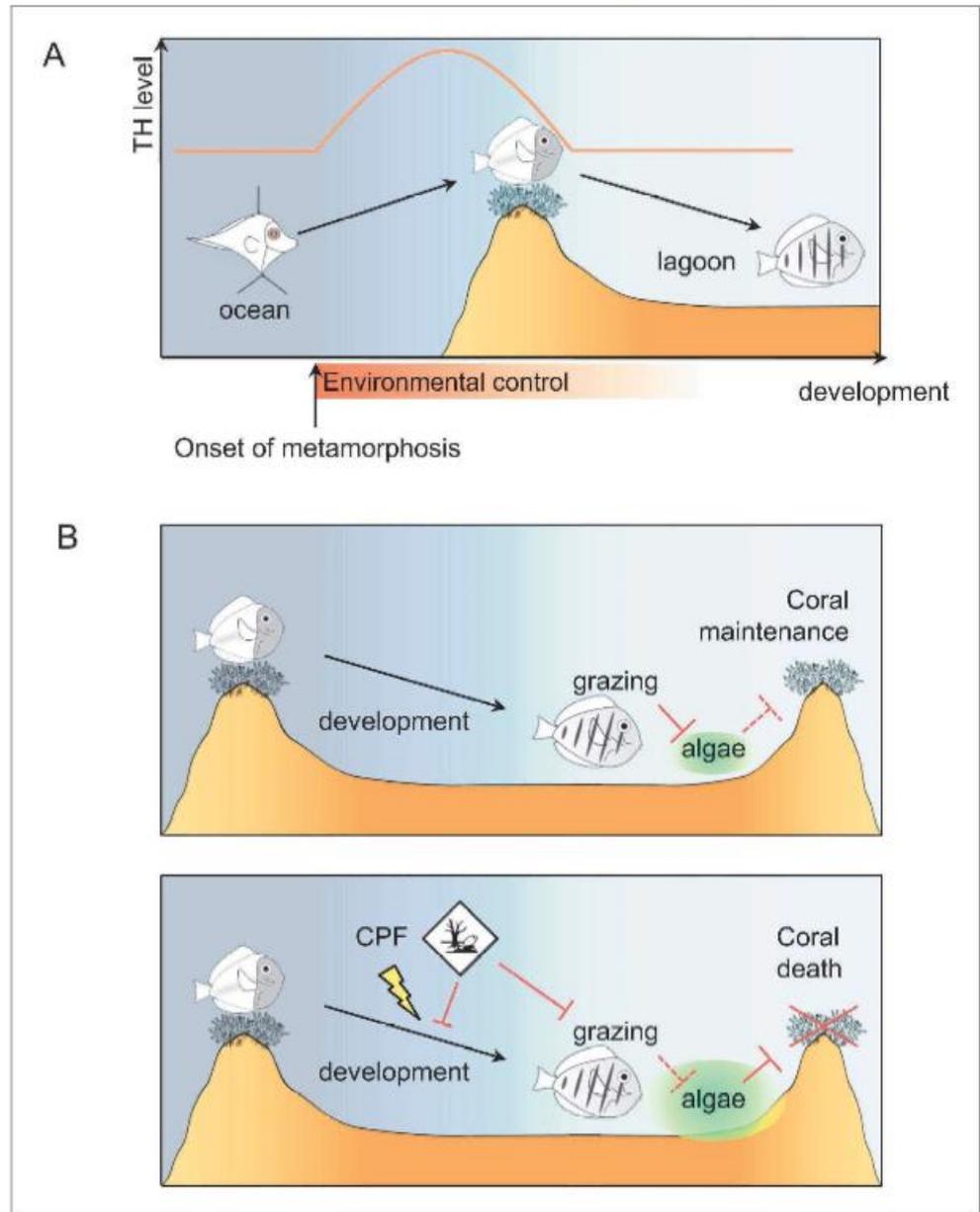


Figure 6. Model of *Acanthurus triostegus* metamorphosis and disruption by chlorpyrifos. (A) Schematic representation of coral reef fish larval recruitment. TH level is plotted on the Y axis and development on the X axis. The onset of metamorphosis is in the open ocean and environmental influence upon development is plotted on the X-axis. (B) Consequences of undisturbed (upper panel) and CPF disturbed (lower panel) metamorphosis on grazing activity, and subsequent algal spreading and potential coral survival. Solid red bar indicates inhibition; dotted red line indicates lack of inhibition.

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extraction, larvae were lacerated and kept in RNAlater (Sigma) 1 hr on ice and stored at -20°C . For histology, larvae were rinsed in PBS 1X, dissected and the intestines were kept in Bouin's fixative at room temperature. For μCT scan and metagenomics, Larvae were kept in 70% ethanol at room temperature.

Fish treatments

Acanthurus triostegus captured at the crest were injected with 20 μ l of drugs in the ventral cavity. Drugs tested were as follows: (i) solvent control (DMSO diluted 10,000 times in Phosphate Buffer Saline 1X, as all drugs were made soluble in DMSO and diluted 10,000 in PBS 1X); (ii) T3 + iopanoic acid (IOP) both at 10^{-6} M; (iii) NH₃ at 10^{-6} M; and (iv) T3 + IOP + NH₃ all at 10^{-6} M. IOP was used as an inhibitor of deiodinase enzymes, as evidenced in mammals and amphibians (Galton and Hiebert, 1987; Galton and Hiebert, 1988; Galton, 1989; Simonides et al., 2008; Medina et al., 2011; Renko et al., 2012), and as routinely used in fish to prevent the immediate degradation of injected T3 (Little et al., 2013; Lorgen et al., 2015). NH₃ is a known TR antagonist in vertebrates (Lim et al., 2002), a notion we confirmed in *A. triostegus* (Figure 2—figure supplement 1D). NH₃ prevents the binding of TH on TR and impairs the binding of transcriptional coactivators to TR, which therefore remain in inactive conformation (Figueira et al., 2011; Lim et al., 2002). Fish were then kept in the lagoon in in situ cages and injected daily following the same protocol to maintain pharmacological treatment. Fish were sampled on the second and fifth day after the beginning of this protocol. DMSO non toxicity was tested by comparing the pigmentation, the teeth development, the intestine length and internal structures between solvent-control individuals (treated with DMSO diluted 10,000 times in PBS 1X), and control individuals (simply raised in situ in lagoon cages as mentioned in the previous paragraph) (Figure 3—figure supplement 1, Figure 3—figure supplement 2).

Chlorpyrifos (CPF) exposure

After crest capture, *A. triostegus* larvae were transferred in aquaria (30 L x 20 W x 20 cm H) in groups of $n = 10$ fish. Each aquarium was filled with 9 liters of UV-sterilized and filtered - 10 μ m filter, and was equipped with an air stone. Fish were then immediately exposed to: nothing (control), acetone at a final concentration of 1:1,000,000 (solvent control, as CPF was made soluble in acetone) or CPF at a final concentration of 1, 5, and 30 μ g.l⁻¹, as previously done in the literature in another coral reef fish (Botté et al., 2012). All water was replaced every day, ensuring the maintenance of water quality as well as a continuous concentration of the pesticide or solvent. Fish were sampled the 2nd and 5th day after the beginning of this protocol. Acetone non toxicity was tested by comparing the TH levels, the pigmentation, and the intestine length between solvent-control and control individuals (Figure 5A–B, Figure 5—figure supplement 1A–B).

Cloning of *Acanthurus triostegus* genes

Muscle from adult fish and whole larvae were used. Samples were cut using sterile scalpel blades and crushed in a Precellys with Qiagen extraction buffer. RNA extractions were performed using the Macherey-Nagel RNA extraction kit following the manufacturer's instructions. Total pooled RNAs were retro-transcribed with the Invitrogen Super Script III enzyme following the manufacturer's instructions. For TR cloning, actinopterygian universal primers (Figure 2—figure supplement 2) were designed for TR α -A, TR α -B and TR β to retrieve the full-length sequences of the genes. For *polD2* and *rpl7*, actinopterygian universal primers were designed to retrieve partial sequences including at least one exon-exon junction. PCR amplicons were cloned in the Invitrogen PCR II plasmid following the manufacturer instruction for sequencing. TR α -A, TR α -B and TR β were subcloned in pSG5 between EcoRI sites for *in cellulo* expression.

qPCR assay

Far ocean to day 8 juveniles were assayed. For each larvae 1 μ g of RNA were used for retro-transcription using the Invitrogen Super Sript III following the manufacturer's instructions, including a DNase I treatment. qPCR primers were designed to anneal on different exons, *Rpl7* and *PolD2* were used as normalization genes (Figure 2—figure supplement 2). qPCRs were performed in 96 well plate with the BioRad IQ Syber Green Super Mix in 10 μ l of final reaction per well following the manufacturer's ratios. qPCRs were assayed in a BioRad thermocyclers and analysed on BioRad CFX Manager software. Assays were performed on duplicates in at least two independent RNA extractions and retro-transcriptions. *Klf9*, which is known to respond to TH signaling, was used as a TH signaling reporter gene.

Phylogenetic analysis

The amino acid sequences of *A. triostegus* cloned TRs as well as available TR sequences (**Figure 2—figure supplement 3**) were aligned using *MUSCLE* software. Trees were generated using the Maximum Likelihood method with the *Seaview four* software under the *JTT model* with estimated gamma shape and eight rate categories (RRID:SCR_015059) (Gouy et al., 2010). Bootstrap analysis of 1000 replicates was carried out to support the tree.

Functional characterization of the receptors

Human embryonic kidney 293 cells (ATCC:CRL_11268, RRID:CVCL_0045) (Iwema et al., 2007; Gutierrez-Mazariegos et al., 2014; Sadier et al., 2015) were grown in Dulbecco's modified Eagle's medium supplemented with 10% of coal stripped foetal bovine serum and penicillin/streptomycin at 100 $\mu\text{g}\cdot\text{ml}^{-1}$. Cells were maintained at 37°C, 5% CO₂ and tests for mycoplasma contamination were negative. The transient transfection assays were carried out in 96-well plate with 30 000 cells per plate using *Exgen500* according to the manufacturer's instructions. For each well, cells were transfected with 50 ng of total DNA: 12,5 ng of full-length receptor encoding plasmid, 12,5 ng of reporter plasmid with four DR4 repeat in the luciferase promoter, 12,5 ng of β -galactosidase encoding plasmid and 12,5 ng of pSG5 empty plasmid. Drugs were incubated for 48 hr and cells were harvested using a passive lysis buffer and frozen at -20°C. On half of the lysate, luciferase activities were assayed with the luciferase reagent buffer from *Promega* on a *Veritas Turner Biosystem luminometer*. On the other half of the lysate, the β -galactosidase activity was measured using ONPG substrate and absorbance at 420 nm for normalization. Each assay was performed at least three times independently on well triplicates. Drugs from *Sigma-Aldrich* were diluted in DMSO to 10⁻² M then in sterile PBS1X prior treatment.

Thyroid hormone quantification

TH were extracted from dry-frozen fish following an extraction protocol adapted from previous publications relating TH level variations in teleost fish (Tagawa and Hirano, 1989; Einarsdóttir et al., 2006; Kawakami et al., 2008). Far ocean larvae to day 8 juveniles were assayed. At least three individuals per sampling point were used. Each fish was first crushed with a *Precellys* in 500 μl methanol, centrifuged at 4°C and supernatant reserved, three times. Pooled supernatant were dried at 70°C. Hormones were re-extracted with 400 μl methanol, 100 μl chloroform and 100 μl barbital buffer twice from the first dried extract. Pooled supernatant was dried out and extract was reconstituted in 2 ml of PBS1X for quantification. The quantification was performed following the *Roche ELICA* kit on a *Cobas* analyser by a medical laboratory according to the manufacturer's standardized method.

Intestine histology

The intestines in Bouin's fixative were embedded in paraffin. Sections of 5 μm were performed in the proximal, medial and distal part using a microtome (*Leica*). The histological sections were stained with Hematoxylin and Eosin. Photography were taken on a *Leica* microscope and the mosaic reconstructed using *Image J* software.

Gut metagenomics

For 16S mass sequencing, juveniles of 2, 5, 8 days after reef entry and adult *A. triostegus* gut were dissected in triplicates for a total of twelve samples. Total DNA was extracted with a *Macherey-Nagel* DNA extraction kit following the manufacturer's recommendations. 16S library were constructed for each individual using the *Ion 16S Metagenomic* kit from *Life technologies* following the manufacturer's recommendations. Sequencing of bacterial 16S was performed using a PGM Ion Torrent. Three controls for contaminations were performed. The environment control consisted of an open tube during the dissection. The extraction control monitored the DNA extraction process. The blank control monitored the library construction. The sequencing results were then analyzed using the *Life technologies* 16S pipeline. 18S mass sequencing was performed on crest larvae, 8 days juveniles and adults in triplicates. Intestine contents were extracted in the same condition as 16S. 18S library were constructed with the pre-amplified V7 region of the eukaryotic 18S (**Figure 2—figure supplement 2**). A blocking primer was designed to prevent the amplification of host sequences (**Figure 2—figure supplement 2**). The blocking primer was designed to partially overlap the 18S reverse

primer and was modified with a C3 spacer. Sequencing was performed using a PGM Ion Torrent. The sequences were assembled and blasted against the PR2 database, completed with 18S sequenced from multi-cellular organisms for taxonomic affiliation.

μCT scan analysis

Fish samples were conserved in 70% ethanol, dehydrated in successive baths of 95% ethanol, twice 100% ethanol and in vacuum arena for at least 4 hr. X-ray microtomographies were performed on a *Phoenix Nanotom (General Electric)* at 70 kV of tension, 100 mA of intensity with a tungsten filament. 3000 images per sample were taken at 500 ms of exposure per image and at a resolution ranging from 2.5 to 2.8 μm. 3D volumes were reconstructed and analysed with VGI studiomax software.

Grazing activity

For each condition (e.g. hormonal treatments, pesticide conditionings), 18 fish were placed in a 5 liter tank with coral rubbles covered with turf algae. After an acclimation period of 1 hr, we recorded the total number of bites made on turf algae during 10 min. This experiment was replicated with a new batch of fish 3 to 6 times depending on the condition.

Grazed turf biomass

For each pesticide exposition conditioning, we weighed (underwater) 3 pieces of coral rubble prior the introduction of a group of 20 crest captured fish (weight A), and after 5 days of grazing by this group of fish (weight B). For each piece of coral rubble, the weight of grazed algae was estimated through the difference weight B minus weight A.

Statistical information

All statistical analyses were conducted using the *R-Cran* project free software (<http://www.rproject.org/>, R-3.3.1). Mean comparisons were performed using Wilcoxon-Mann-Whitney U-test when comparing two means, and using univariate analysis of variance (ANOVA) followed by Tukey post-hoc test (should a significant difference be detected) for multiple comparisons. Prior ANOVA, normality of values (or residuals) and variance homogeneity were assessed using Shapiro and Bartlett tests. In qPCR analyses, comparisons of gene expression were performed through Student's t cumulative distribution functions, automatically computed by qPCR software *CFX Biorad Manager*, (*CFX Manager SH, 2017*). All statistical information and data are available in each Figure source data (see Figure captions).

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Additional information

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Author contributions

Guillaume Holzer, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing, Wrote the paper, Designed the experiments, Performed gene cloning, qPCR, Transactivation assay, μCT scan, Hormone dosage, Assisted in metagenomics and analyzing the data, Assisted with preparing the

manuscript; Marc Besson, Conceptualization, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing, Wrote the paper, Designed the experiments, Performed hormone dosage, Fish capture, Fish treatment, Grazing experiments, Histology and analyzing the data, Assisted with preparing the manuscript; Anne Lambert, Data curation, Investigation, Visualization, Methodology, Assisted with histology and data analysis, Assisted with preparing the manuscript; Loïc François, Conceptualization, Data curation, Investigation, Visualization, Writing—review and editing, Assisted with fish capture, fish treatment and analysis of the data, Assisted with preparing the manuscript; Paul Barth, Data curation, Formal analysis, Assisted in gene cloning, Hormone dosage, Analysis of the data, Assisted with preparing the manuscript; Benjamin Gillet, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—review and editing, Performed the metagenomics, Design of the experiment and assisted in the analysis of the data, Assisted with preparing the manuscript; Sandrine Hughes, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—review and editing, Performed the metagenomics analysis, Assisted in the experiment and the design, Assisted with preparing the manuscript; Gwenaël Piganeau, Data curation, Formal analysis, Visualization, Writing—review and editing, Assisted in the analysis of metagenomics data, Assisted with preparing the manuscript; Francois Leulier, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—review and editing, Assisted in the analysis of metagenomic data and design of the experiment, Assisted with preparing the manuscript; Laurent Viriot, Performed and analyzed X-ray microtomography, Read and commented on the manuscript; David Lecchini, Conceptualization, Resources, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing, Wrote the paper, Designed the experiments, Performed fish capture, Analyzed the data, Assisted with preparing the manuscript; Vincent Laudet, Conceptualization, Resources, Data curation, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing, Wrote the paper, Designed the experiments

Author ORCIDs

Marc Besson,  <https://orcid.org/0000-0003-3381-322X>

Ethics

Animal experimentation: This study was performed in strict accordance with the guidelines of the French Polynesia committee for publication and animal ethics. All the animals were handled in accordance with the guidelines CRIOBE-IRCP animal ethics committee, and every effort was made to minimize suffering. The experiments and protocols were approved by the CRIOBE-IRCP animal ethics committee (Decision letter 02-14-2015)

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Appendix 6: Exposure to agricultural pesticide impairs visual lateralization in a larval coral reef fish

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Exposure to agricultural pesticide impairs visual lateralization in a larval coral reef fish

Marc Besson^{1,2}, Camille Gache^{1,3}, Frédéric Bertucci^{1,4}, Rohan M. Brooker⁵, Natacha Roux^{1,2}, Hugo Jacob^{1,6}, Cécile Berthe^{1,3}, Valeria Anna Sovrano⁷, Danielle L. Dixon⁵ & David Lecchini^{1,3}

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Lateralization, *i.e.* the preferential use of one side of the body, may convey fitness benefits for organisms within rapidly-changing environments, by optimizing separate and parallel processing of different information between the two brain hemispheres. In coral reef-fishes, the movement of larvae from planktonic to reef environments (recruitment) represents a major life-history transition. This transition requires larvae to rapidly identify and respond to sensory cues to select a suitable habitat that facilitates survival and growth. This 'recruitment' is critical for population persistence and resilience. In aquarium experiments, larval *Acanthurus triostegus* preferentially used their right-eye to investigate a variety of visual stimuli. Despite this, when held in *in situ* cages with predators, those larvae that previously favored their left-eye exhibited higher survival. These results support the "brain's right-hemisphere" theory, which predicts that the right-eye (*i.e.* left-hemisphere) is used to categorize stimuli while the left-eye (*i.e.* right-hemisphere) is used to inspect novel items and initiate rapid behavioral-responses. While these experiments confirm that being highly lateralized is ecologically advantageous, exposure to chlorpyrifos, a pesticide often inadvertently added to coral-reef waters, impaired visual-lateralization. This suggests that chemical pollutants could impair the brain function of larval fishes during a critical life-history transition, potentially impacting recruitment success.

Brain asymmetry and the preference to use one side of the body over the other to accomplish actions (termed lateralization), has been identified in a variety of vertebrate and invertebrate species^{1–5}. Lateralization processes have been characterized in adult organisms but also in early-life-stages, such as fish larvae^{6,7}, fish juveniles^{8,9}, chicks¹⁰ (but see refs 11–13 for reviews on embryonic and post-embryonic development of lateralized organs and acquisition of lateralized behaviors in several vertebrate and invertebrate species). Processes of lateralization can therefore be expected along the whole ontogeny of an animal organism. Brain lateralization is thought to increase cognitive abilities, in particular for decision making when facing novel multi-sensory signals, by enabling individuals to cope with divided attention and to partition, and optimize, the parallel processing of different types of information into the two separate brain hemispheres^{1,14–18}. With regards to habitat exploration and response to sensory cues, there are two main theories concerning brain lateralization in animals. The "brain's right hemisphere theory" assumes that the right hemisphere is predominantly used to process information regarding novel items and/or items requiring a rapid behavioral response, while the left hemisphere is used for categorizing stimuli and/or processing information that requires consideration of alternatives^{19,20}. Alternatively, the "valence theory" proposes that the right hemisphere is preferentially used to process information regarding negative stimuli, while the left hemisphere is used for the processing of information regarding positive stimuli^{21–23}. While empirical evidence supports both theories, previous studies have generally only considered lateralized responses to social

¹PSL Research University: EPHE-UPVD-CNRS, USR 3278 CRIOBE, BP 1013, 98729, Papetoai, Moorea, French Polynesia. ²Université Pierre et Marie Curie, UMR CNRS 7232 OOB, 1 Avenue Pierre Fabre, 66650, Banyuls-sur-Mer, France. ³Laboratoire d'Excellence "CORAIL", BP 1013, 98729, Papetoai, Moorea, French Polynesia. ⁴Laboratoire de Morphologie Fonctionnelle et Evolutive, AFFISH Research Center, Institut de Chimie B6c, Université de Liège, Liège, Belgium. ⁵School of Marine Science and policy, University of Delaware, 111 Robinson Hall, Newark, DE, 19716, USA. ⁶International Atomic Energy Agency, Environment Laboratories (IAEA-EL), Principality of Monaco, 98000, Monaco. ⁷Center for Mind/Brain Sciences (CIMEC), University of Trento, Piazza Manifattura 1, 38068, Rovereto (TN), Italy. Marc Besson and Camille Gache contributed equally to this work. Correspondence and requests for materials should be addressed to M.B. (email: marc.besson@ens-lyon.org)

sensory stimuli under normal environmental conditions, and only in a few species of amphibians, birds, freshwater fishes, and mammals^{10, 21, 24–29}. Therefore, we still lack a comprehensive understanding of the ecological importance of sensory lateralization in marine fishes, as well as in the context of rapidly changing environments.

Among the most rapidly changing environments globally are coral reefs, one of the most biologically diverse, productive, and economically important ecosystems worldwide^{30–32}. Over the past three decades, coral reefs have faced increasingly frequent and severe natural (e.g., cyclones, *Acanthaster planci* outbreaks) and anthropogenic perturbations (e.g., pollution from agricultural runoff, rising sea-water temperatures, overfishing) leading to global declines in habitat quality, biodiversity and associated ecosystem services^{31, 33–37}. In the context of these rapidly changing conditions, reef fishes often play an important role in maintaining habitat structure, preserving associated biodiversity, and increasing ecosystem resilience (e.g., through the regulation of coral-algal interactions or by maintaining trophic chain sustainability)^{38–40}. However, these species are often highly vulnerable to changing conditions, with individual health and population persistence impaired by both localized and global impacts⁴¹. The majority of reef fishes have a bipartite life cycle, consisting of a pelagic larval stage that facilitates dispersal followed by a largely sedentary reef-associated juvenile and adult stage⁴¹. At the conclusion of the larval phase, the transition from the planktonic to reef environments is referred to as settlement⁴¹. During this transition, reef-naïve larvae metamorphose into benthic dwelling juveniles and must rapidly identify and respond appropriately to a host of novel multi-sensory signals in order to select a suitable habitat that will facilitate their survival and growth^{42–49}. The selection of, and persistence in, an appropriate habitat is termed recruitment⁴¹. Continued and consistent recruitment is essential to maintain reef fish populations and assist with the resilience and recovery of degraded reef fish communities⁵⁰. The ability to identify visual cues that indicate the identity and location of predators has shown to increase survival rates during recruitment in a number of coral reef fish species^{45, 46, 51–53}.

The relationship between brain morphology, lateralization, social interactions, and anti-predator behaviors in early-life-stage coral reef fishes remains largely unknown^{8, 45, 46, 51, 54–56}, with the role of behavioral lateralization during recruitment only initially assessed^{55, 56}. These recent experiments have primarily used detour tests, which examine potential turning bias of individuals in a context of vigilance, and revealed that such lateralized behaviors can be associated with anti-predator response phenotypes and survival benefits^{55–57}. However, these studies were conducted on captive-bred larvae, larvae caught in light traps (which may selectively attract certain behavioral phenotypes), or wild caught post-settlement stage juveniles. Experimental use of individuals that have been reared, are from mixed larval, pre-settlement, and post-settlement stages, or are non-naïve and have past reef experience potentially adds biases to these findings. This prevents a complete understanding of the ecological importance of coral reef fish lateralization at the critical time of recruitment. In addition, current research has yet to examine the contribution of either brain hemisphere in accomplishing a specific task.

Short-term exposure to environmental stressors, such as high predation risk or elevated CO₂ levels, has been shown to alter lateralized anti-predator behaviors, which can lead to greater mortality rates^{55, 57}. These findings highlight the critical role of lateralization during the recruitment in coral reef fishes, and point towards potential population or ecosystem-level impacts when an environmental stressor is applied^{56–59}. In particular, anthropogenic impacts on reefs, such as the worldwide increasing influx of chemical pollution (e.g., from pesticides) resulting from coastal agriculture and river runoff^{60–69}, are known to severely impact reef biodiversity and the biology and ecology of coastal marine organisms^{33, 62}. Among these sources of pollution, the Chlorpyrifos (CPF), an organophosphate insecticide widely used on tropical coastal crops (e.g., sugar-cane and rice crops in Australia and south-east Asia respectively^{64, 70, 71}), is one of the most common waterborne chemical pollutant encountered in coral reefs^{70, 71}. While several studies have demonstrated its neurotoxicity and endocrine disruption characteristics^{71–74}, the negative impact on coral reef larval fish sensory abilities has also recently been acknowledged⁷¹. Moreover, pesticides belonging to the same family have been discovered in important amounts in several fish and crustacean species in French Polynesia^{68, 75}. However, whether this pollutions affect or not behavioral lateralized processes remains unknown⁷¹.

Here, we examined visual lateralization in the larvae of a common coral reef fish species, the convict surgeonfish *Acanthurus triostegus*⁷⁶, during its recruitment phase. If settlement-stage larvae preferentially use their right eye to examine and categorize positive stimuli (e.g., conspecifics, such as in *Myripristis pralinia*, a coral reef fish⁸, or in freshwater fishes^{17, 24, 25, 77}) and their left eye for negative stimuli or to inspect novel items and execute rapid responses (e.g., potential predators, such as in freshwater fishes^{17, 24, 25, 77}), this would be good evidence for both the “**brain’s right hemisphere**” and the “**valence**” theories. Using a range of aquarium based (Fig. 1) and *in situ* experiments we tested whether larvae exhibit a naïve preference for their left or right eye when inspecting both positive (mirror self-image, conspecific) or negative (predator) stimuli (Exp. 1) and whether visual lateralization increased or decreased vulnerability to predation (Exp. 2). Lastly, to examine the potential for coastal development to affect this mechanism we determined whether exposure to CPF, at ecologically relevant concentration^{64, 71, 78}, would alter the degree of lateralization larvae exhibit (Exp. 3).

Results

For each individual within each experiment, a lateralization index (LI), reflecting the degree of preferential eye use, was calculated as: [(frequency of right eye use)/(frequency of right eye use + frequency of left eye use)] × 100. Values significantly higher than 50% indicate preference for right eye use while values significantly lower than 50% indicate preference for left eye use. Subsequent statistical tests were conducted using these values.

Exp. 1 tested whether larvae exhibit a naïve eye preference when inspecting visual cues from positive (con-specific) or negative (predator) stimuli. In the absence of any visual stimulus, *A. triostegus* larvae exhibited no eye preference (LI = 41.8 ± 4.6%, Mann-Whitney U test, U = 32, n = 14, p-value = 0.364) (Fig. 2). However, larvae showed a significant preference when inspecting test stimuli (Kruskal-Wallis rank sum test, $\chi^2 = 14.173$, df = 3, p-value = 0.003), with LI values significantly higher when examining both conspecifics (Nemenyi post-hoc test

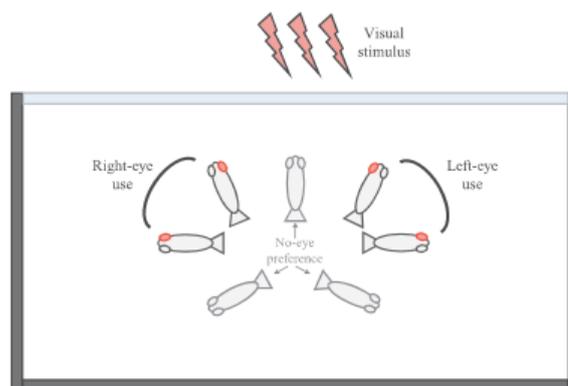


Figure 1. Schematic representation of the test apparatus for eye lateralization determination. The three dark gray walls represent the opaque wall of the aquarium, while the light blue wall corresponds to the transparent wall where the visual stimuli are presented. No eye preference was recorded when the fish was perpendicular to the transparent wall (*i.e.* binocular stimulation) or when it formed an angle larger than 180° with respect to the transparent wall (*i.e.* fish looking in the opposite direction).

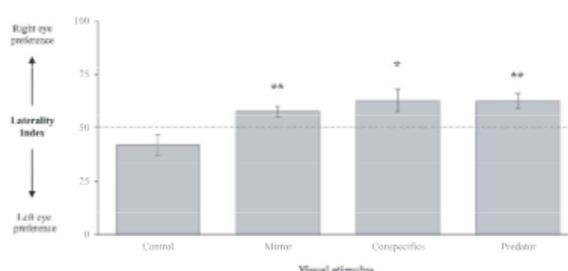


Figure 2. Left-right eye preference of *A. triostegus* larvae during the inspection of visual stimuli in an adjacent aquarium. Figure represents LI mean (±SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, * $p < 0.05$; ** $p < 0.01$) between Laterality Index (LI) values and the theoretical 50% value (dotted line). 13 to 14 replicates (one fish per replicate) were conducted for each visual stimulus.

with Tukey-Dist approximation, p -value = 0.006) or predator cues (p -value = 0.006). With regards to the eye preferred, larvae displayed a significant preference for their right eye when inspecting themselves in a mirror (LI = $57.3 \pm 2.4\%$, Mann-Whitney U test, $U = 93$, $n = 14$, p -value = 0.009), when examining a group of five adult conspecifics (LI = $62.5 \pm 5.5\%$, $U = 85$, $n = 14$, p -value = 0.042), or when examining a predator (LI = $62.2 \pm 3.6\%$, $U = 83$, $n = 13$, p -value = tested whether visual lateralization increased 0.006) (Fig. 2).

Exp. 2 or decreased vulnerability to predation. Individual larvae from Exp. 1 were classified and grouped as either 'no eye dominant', 'right-eye dominant', or 'left-eye dominant' based on the behavior displayed when exposed to the predator in Exp. 1. All three groups experienced significantly different survival rates (Kruskal-Wallis rank sum test, $\chi^2 = 7.064$, $df = 2$, p -value = 0.029) with 'left-eye dominant' larvae having a higher survival rate than both 'right-eye dominant' (Nemenyi post-hoc test with Tukey-Dist approximation, p -value = 0.01) and 'no eye dominant' larvae (p -value = 0.03) (Fig. 3).

Exp. 3 utilized the same protocol as in Exp. 1 and tested whether exposure to a waterborne organophosphate pesticide often encountered in coral reefs: chlorpyrifos (CPF), would alter the degree of lateralization larvae exhibit. Larvae exposed to CPF at $1 \mu\text{g.l}^{-1}$ (black bars in Fig. 4) and larvae only exposed to the CPF solvent (grey bars in Fig. 4) again showed no eye preference in the absence of visual stimuli (respectively LI = $52.5 \pm 6.1\%$ and $52.3 \pm 2.7\%$, Mann-Whitney U test with respectively $U = 33$ and 35 , $n = 10$ and 11 , p -value = 0.625 and 0.476) (Fig. 4). However, a preference was seen when these larvae inspected a mirror (Wilcoxon rank sum test, $W = 106$, $n = 10$, p -value < 0.001), conspecifics ($W = 87$, $n = 10$, p -value = 0.004) or a predator ($W = 85$, $n = 10$, p -value = 0.009). Similar to the trends seen in Exp. 1 (Fig. 2), solvent control larvae preferentially used their right eye when inspecting a mirror (LI = $59.2 \pm 2.9\%$, Mann-Whitney U test, $U = 61.5$, $n = 11$, p -value = 0.013), conspecifics (LI = $58.9 \pm 2.1\%$, $U = 54$, $n = 10$, p -value = 0.004) or a predator (LI = $58.8 \pm 2.4\%$, $U = 45$, $n = 10$, p -value = 0.009) (Fig. 4). However, when exposed to CPF at $1 \mu\text{g.l}^{-1}$ for five days, larvae no longer exhibited any eye preference when inspecting either the conspecifics or predator (respectively LI = $49.0 \pm 1.9\%$ and $44.9 \pm 3.7\%$, $U = 25$ and 16 , $n = 10$ and 10 , p -value = 0.846 and 0.262). In addition, when inspecting themselves in a mirror,

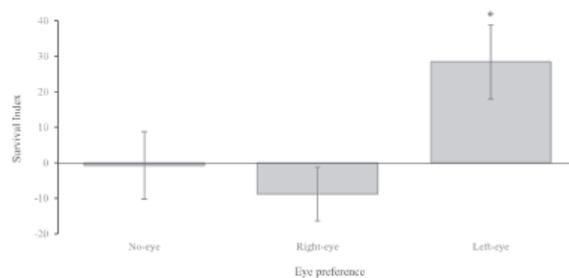


Figure 3. Ecological importance of brain lateralization in larval survival facing direct predation. Survival index (SI) was calculated as follows: survival rate of the group minus the overall survival rate in the *in situ* cage. Figure represents SI mean (\pm SE) values. The asterisk indicates a significant difference in survival index among the three groups (Kruskal-Wallis test, $\chi^2 = 7.064$, $df = 2$, p -value = 0.029) with 'left-eye dominant' experiencing higher survival than both 'right-eye dominant' (Nemenyi post-hoc test with Tukey-Dist approximation, p -value = 0.01) and 'no eye dominant' larvae (Nemenyi post-hoc test with Tukey-Dist approximation, p -value = 0.03). Six replicates were conducted. In each replicate, each group was made up with the same amount (four to six) of fish.

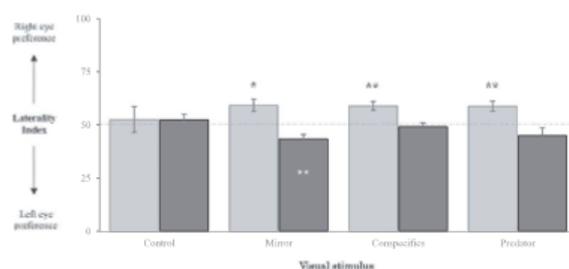


Figure 4. Effect of pesticides on the visual preference of *A. triostegus* larvae. Figure represents LI mean (\pm SE) values. Asterisks indicate significant differences (Mann-Whitney U tests, * $p < 0.05$; ** $p < 0.01$) between Laterality Index (LI) values and the theoretical 50% value (dotted line). Black asterisks above bars indicate a significant right-eye preference, while white asterisks below bars indicate a significant left-eye preference. 10 to 11 replicates (one fish per replicate) were conducted for each visual stimulus in both the pesticide exposition experiment (black bars) and the solvent-control exposition experiment (grey bars).

CPF treated larvae now preferentially used of their left eye (LI = $43.3 \pm 2.2\%$, $U = 1$, $n = 10$, p -value = 0.004) (Fig. 4).

Discussion

This study identified a strong preference for the right eye when inspecting stimuli from either a mirror, conspecifics, or a predator. While a preference for the using the right eye, or the left hemisphere of the brain, to categorize familiar stimuli (e.g. same size conspecifics) has also been seen in another coral reef fish: *Myripristis pralinia*⁸, our results suggest that this preference also extends to unfamiliar stimuli. Larvae used in this experiment were naïve without any prior experience of non-larval reef fishes, therefore adult conspecifics and predators are considered unfamiliar stimuli while mirror-images are considered as the image of a familiar conspecific (e.g. larva from the same larval cohort)²⁷. The preference for the right eye is intriguing since it is opposite to the preference for the left eye generally observed during the inspection of mirror self-images in freshwater fishes and amphibians, and which conforms with the key role the right telencephalon hemisphere plays in social cognition^{10, 21, 24–29, 77, 79–81}. The difference suggests that patterns of laterality could be associated with habitat preference (e.g. freshwater vs. sea water) or evolutionary history, and thus driven by ecological pressures or phylogenetic constraints. For instance, the preferential use of the right eye could reflect a greater need for settlement-stage *A. triostegus* to initially categorize new sensory cues upon recruitment rather than establishing social interactions and aggregations⁸².

We also observed that larvae that displayed a left-eye preference when inspecting a predator held in an adjacent aquarium in Exp. 1, experienced higher survival when exposed to predation risk (Exp. 2). This result could be attributed to a learning process, with right-brain lateralized individuals able to recognize previously-experienced threats faster than left-brain lateralized individuals when this has been examined in other fishes⁸³. Left-eye dominant larvae could potentially escape predation more efficiently. Even if it is unknown if the right or left eye dominated in Exp. 2 itself, it has been shown that an eye preference displayed during the first observation of a predator (as in Exp. 1) can influence behavioral responses (e.g. subsequent eye preference, turning rates) when facing for a second time that same, no longer unknown, stimulus^{84, 85}. Therefore, after the Exp. 1 conditioning, the left-eye

users may adopt a behavioral response to predation that differs from the majority of their right-eye user and not-lateralized conspecifics and that could favor their survival (e.g. by disorienting predators or by moving differently within their environment such as seen in mosquitofish⁸⁶). Overall, our results strongly support the “brain’s right hemisphere” theory that predicts that the right-eye (i.e. left-hemisphere) is predominantly used to categorize stimuli, while the left-eye (i.e. right hemisphere) is dedicated to attending to events that require rapid behavioral responses, such as predation^{19,20}. In the context of larval settlement, where individuals face a variety of novel sensory cues and constantly high predation risk, our study suggests that there is a survival advantage to being lateralized, with the ability to optimize survival overcoming the obvious disadvantages of displaying predictable asymmetric behaviors. Future experiments brain lateralization in coral reef fishes should include juveniles that have been allowed to contact with older conspecifics, or that are no longer predator naive, in comparison to others held in solitary confinement, to better understand how asymmetric behavioral patterns are adopted by a population and the importance of learning and selection in lateralization processes.

Our results show that the presence of chlorpyrifos (CPF), one of the most common waterborne pesticides encountered in coral reefs⁷¹, can completely diminish or reverse critical lateralization patterns (Fig. 4). Oceanic levels of chlorpyrifos contamination have only been assessed, to our knowledge, in the Southern Ocean, revealing levels up to 0.54 pg.l⁻¹⁸⁷. This is much lower than the levels observed in Australian reef surface waters, where contamination have been shown to reach 1 µg.l⁻¹⁷⁸, or than in certain Australian and Malaysian river mouths, where contamination levels can reach up to 26 µg.l⁻¹^{64,78}. The concentration of 1 µg.l⁻¹ is therefore ecologically relevant and reflects appropriately the change of pesticide concentration larval fishes can face when settling, from the open ocean, in coral reef coastal areas^{64,71,78,87}. This loss and reversal of lateralization in fish exposed to chlorpyrifos may further increase larval mortality and impact the subsequent recruitment and maintenance of reef ichthyologic diversity, as evidenced in Exp. 2 (Fig. 3). Similar findings have been documented in the coral reef fish *Pomacentrus wardi* when juveniles were exposed to future ocean acidification conditions⁵⁹. In addition, a turning bias, shifting from the right to the left, was observed in *P. wardi* populations exposed to elevated-CO₂ concentrations (930 µatm)⁵⁸. Lastly, the same study also revealed how increased temperatures (+3 °C higher than ambient temperatures) can have a dampening effect on lateralization processes⁵⁸. In the context of global degradation of coral reefs, it is striking to observe that pesticide pollution, oceanic acidification, and elevated water temperatures could have similar and potentially interactive effects in impairing lateralized behaviors of marine organisms and subsequent survival to predation, particularly in early developmental stages. With this study, we demonstrate how the worldwide increasing influx of pesticide onto coral reefs, due to expanding coastal agriculture, as well as changing patterns of land use upstream^{60,61,63–69}, may lead to a loss or reversal of visual lateralization during a critical step of coral reef fish life cycle. This may potentially lead to greater larval mortality rates and reduce subsequent recruitment success. In addition, any potential reduction in the abundance of major herbivorous fishes in coastal marine ecosystems, such as *A. triostegus* in coral reefs⁷⁶, due to chemical pollution from organophosphate components⁸⁸, could impair the ability of reefs to cope with increasing macro-algal abundance and impact ecosystems recovery and resilience^{40,89}.

While coral reefs are experiencing increasing perturbations³⁴, persistence of species in degraded areas rely on the ‘rescue’ effect of recruitment⁵⁰. The potential for a population to be supplemented by recruits depends largely on pelagic larvae detecting an appropriate habitat, towards which they orientate in order to settle and persist⁵⁰. Successful recruitment of individuals could be partly jeopardized by the alteration of lateralized cognitive abilities of larvae caused by local stressors such as pesticides. An understanding of the underlying mechanisms of these physiological and behavioural disturbances requires further investigations.

Material and Methods

Sampling and study site. Fish larvae were collected nightly from January to June 2016 using a crest net set on the north-east coast of Moorea Island, French Polynesia (17°29′52.19″S, 149°45′13.55″W). This crest net had a mesh size of 1 mm and was equipped with a rectangular mouth (width: 2 m, height: 1 m) oriented perpendicular to the water flow. Crest nets retain all settlement-stage larvae at the site as they move over the reef crest^{90–93}. This sampling technique ensures a non-selective capture (as opposed to light-traps) of wild larval fishes, precisely at the settlement stage, with no prior reef experience (as opposed to the capture of post-settlement juveniles within reefs using light traps or nets)⁹⁴. While this method also permits the study of wild individuals caught *in situ* (as opposed to aquarium reared larval fish), it however does not ensure that the oceanic experience was the same for all larvae. As larval fish primarily recruit to reefs at night⁴¹, the net was set up at 7 p.m. and larvae were collected at 6 a.m. the following day.

The convict surgeonfish, *Acanthurus triostegus* (Linnaeus, 1758) has a pelagic larval duration of 53 ± 8 days, after which larvae metamorphose and recruit in shoals on shallow sandy and rubble reef areas^{48,76,95}. This species was chosen as our model due to the high number of larvae collected in the crest net (n = 278 larvae, size: 23 ± 2 mm). After removal from the crest net, larvae were transferred to CRIOBE research station where they were maintained in individual aquaria filled with UV-sterilized and filtered (10 µm filter) seawater before being tested. Water temperature was maintained at 28.5 °C, under 12:12 LD cycle (06:00, onset – 18:00, offset). For Exp. 1 and Exp. 2, tests were conducted immediately following collection (within 12 hours). Exp. 3 tests were conducted on the fifth day post-collection. Throughout their entire time in captivity fish were fed a commercial dry food twice daily.

Exp. 1: Do *A. triostegus* larvae exhibit a left-right visual preference when inspecting either negative (predator or positive (mirror self image, conspecific) stimuli?. Preferences for using the left or right eye to inspect visual stimuli was assessed using a protocol adapted from studies of brain lateralization in freshwater fishes^{24,25,27,77}. The experimental aquarium was a rectangular glass tank (20 L × 11 W × 15 cm H) with all sides covered with white opaque screen except one of the long sides where stimuli were presented. The tank

was lighted with a neon lamp (45 W) and filled with UV-sterilized, filtered (10 μ m filter) seawater to 15 cm depth. A video camera was mounted above the experimental tank to record the fish position.

Four types of visual stimuli were presented to *A. triostegus* larvae:

- (i). **Control:** a white opaque screen was displayed on the transparent wall, so that all walls of the experimental tank were covered with white opaque screens. This control was conducted to account for any effect of the experimental procedure on fish behavior.
- (ii). **Mirror:** a mirror was placed along the transparent wall, presenting larvae with a visual stimulus of their own reflection.
- (iii). **Conspecific:** an aquarium (30 \times 20 \times 20 cm) containing juvenile conspecifics ($n = 5$, size: 80 ± 4 mm) was placed against the transparent wall. The conspecifics were collected with hand nets in shallow sandy areas surrounded by corals at Moorea Island.
- (iv). **Predator:** similarly to (iii), a common reef predator of *A. triostegus* (Besson pers. obs.), *Lutjanus fulvus*, ($n = 1$, size: 162 mm) was presented in an aquarium placed against the transparent wall of the experimental aquarium.

For each stimulus, 14 biological replicates were conducted (one larva per replicate). Between each replicate, all water in the experimental tank was replaced. For each replicate, the use of the right or left monocular visual field (*i.e.* eye use) was assessed every two seconds for a total of 10 min. Discrimination between the use of the right or the left eye was determined by examining the angle between larva's body orientation and the transparent wall (where the visual stimulus was presented, Fig. 1), following the method established by Sovrano *et al.*²⁴ and used in various eye lateralization studies since^{17,25,27,77,79}. If the fish was perpendicular to the transparent wall (*i.e.* binocular stimulation), or if its body orientation to the transparent wall formed an angle larger than 180° with respect to the transparent wall (*i.e.* fish looking in the opposite direction), no eye preference was recorded^{25,79} (Fig. 1). In this study, binocular stimulation never occurred (in all three experiments). Moreover, when a visual stimulus was presented, the times when an eye use preference could not be established (because of the fish looking in the opposite direction of the stimulus) only represented $24.01 \pm 2.25\%$ of the observations in Exp. 1, $23.78 \pm 5.82\%$ of the observations in Exp. 2, and $24.20 \pm 2.02\%$ in Exp. 3. Therefore, when examining a visual stimulus, fishes spent 75% of the time examining this visual stimulus with either their left or their right eye, and the rest of the time looking in the opposite direction, but never examining the stimulus with both eyes. Observations of the left-eye or right-eye use (Fig. 1) resulted in a lateralization index (LI), reflecting relative eye use: $LI = [(frequency\ of\ right\ eye\ use)/(frequency\ of\ right\ eye\ use + frequency\ of\ left\ eye\ use)] \times 100$ for each stimulus tested²⁴. Values significantly higher than 50% indicate a preference for the right eye, whereas values significantly lower than 50% indicate a preference for the left eye. For each stimulus, the difference between the measured LI and a 'no eye preference' case ($LI = 50\%$) was tested using a two-tailed Mann-Whitney U test, in order to identify any potential eye preference. Further statistical analyses to compare LI values between each stimulus were carried out by non-parametric analysis of variance (Wilcoxon rank sum test and Kruskal-Wallis test). For all stimuli, no significant difference was observed between the first 5 min of the trial and the last 5 min of the trial (Kruskal-Wallis test, $\chi^2 = 92.8$, $df = 91$, p -value = 0.42). To verify that our modified protocol (one single transparent window) did not introduce any behavioral bias compared to the more commonly used protocol (two transparent windows equipped with two mirrors), we performed an additional two-mirror stimulus where an aquarium was equipped with two transparent walls and two mirrors^{24,25,27}. No significant difference was found when comparing LI values obtained in this two-mirror protocol and the values obtained with the use of the one-mirror protocol (respectively $LI = 59.8\%$ and 57.3% , Wilcoxon rank sum test, $W = 105$, $n = 14$, p -value = 0.769), therefore we assume that our adapted method provides results comparable to conventional methods. All statistical analyses were conducted using the R-Cran software (R-3.3.1).

Exp. 2: Ecological importance of brain lateralization in larvae facing predation. Using a similar protocol as Exp. 1, larvae were tested individually to determine if they preferentially used the right eye, left eye, or displayed no lateralization when presented with a predator. Larvae were then classified and pooled into three groups according to their visual preference: 'left-eye dominant', 'right-eye dominant' and 'no eye dominant' individuals. Each group was tagged with a different color, using visible implant fluorescent filament (Northwest Marine Technology), at least one hour before being released simultaneously into a caged patch reef (1 \times 1 \times 1 m), built from rubble and live coral, and located in an open sandy area at 2 m depth. After a one hour habituation period, three predators, *Lutjanus fulvus* ($n = 3$, size: 165 ± 14 mm), were introduced into the cage. Prior to their introduction, the predators were held for 48 h in aquaria without food to ensure that they were motivated to feed. The experiment was stopped after two hours, predators were removed and the remaining larvae were counted and identified according to their color tag. The overall survival rate was recorded, as well as a survival rate for each group. For each group, a survival index (SI) was calculated: $SI = survival\ rate\ of\ the\ group - overall\ survival\ rate\ in\ the\ cage$. SI values allowed a comparison between the survivals of each replicate, regardless of the relative predation rate in each technical replicate. This experiment was replicated 6 times, and each time a different tag color was attributed to the groups to account for any potential predation bias towards one color. For each replicate, all larval groups contained the same number of fish (between 4 and 6 larvae per group depending on the replicate). Statistical analyses to compare SI values were conducted by using non-parametric analysis of variance (Wilcoxon rank sum test and Kruskal-Wallis test, followed by Nemenyi post-hoc test should a significant difference be detected).

Exp. 3: Effect of chemical pollution on the visual lateralization of *A. triostegus* larvae.

Preliminary experiments were conducted on three groups of $n = 10$ fish maintained in $30\text{ L} \times 20\text{ W} \times 20\text{ cm H}$ aquaria, which were exposed, after collection in the crest net, to chlorpyrifos (CPF) at 1, 30 and $100\ \mu\text{g.l}^{-1}$, as previously done in the literature in another coral reef fish⁷¹. After 10 days of exposure, larvae at $30\ \mu\text{g.l}^{-1}$ presented an irregular swimming behavior and most larvae at $100\ \mu\text{g.l}^{-1}$ died after only one day of exposure. Larvae exposed to the $1\ \mu\text{g.l}^{-1}$ concentration exhibited no behavioral abnormality and no mortality. Consequently, we tested the effect of a CPF pollution on larvae visual lateralization through two experimental conditions: (A) solvent control (9 liters of UV-sterilized and filtered $-10\ \mu\text{m}$ filter – seawater + $9\ \mu\text{l}$ of acetone) and (B) $1\ \mu\text{g.l}^{-1}$ of CPF (9 liters of UV-sterilized and filtered $-10\ \mu\text{m}$ filter – seawater + $9\ \mu\text{g}$ of CPF diluted in $9\ \mu\text{l}$ of acetone).

For each condition, larvae were tested for their eye preference as in Exp. 1, when presented with the same visual stimuli as in Exp. 1 (control, mirror, conspecifics and predator), after a five day exposure (to CPF or to the solvent). Consequently, we performed $n = 8$ (2×4) experiments (control, mirror, conspecifics and predator visual stimulus with fish exposed to solvent vs. fish exposed to CPF at $1\ \mu\text{g.l}^{-1}$). For each of those 8 experiments, *A. triostegus* larvae were maintained, after collection in the crest net, in groups of $n = 5$ to 6 fish in $30\text{ L} \times 20\text{ W} \times 20\text{ cm H}$ aquaria. Each aquarium was equipped with an air stone. All water was replaced every day, ensuring the maintenance of water quality as well as a continuous concentration of the pesticide or solvent. A second technical replicate for these 8 experiments, with significantly similar results as in the first replicate, was performed. Consequently $n = 10$ to 11 biological replicates were conducted for each of these 8 experiments. Statistical tests for LI values analyses were performed as described in Exp. 1.

Ethical approval. All the experiments were approved by the CRIOBE-IRCP animal ethics committee and performed in accordance with the guidelines of the French Polynesia committee for publication and animal ethics.

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Author Contributions

M.B., C.G., V.A.S. and D.L. designed the experiments. M.B., C.G., F.B., R.M.B., V.A.S., D.L.D. and D.L. wrote the article. M.B., C.G., N.R., F.B., H.J. and C.B. captured the fish and performed the *in situ* and in aquaria experiments. M.B. and C.G. performed the statistical analyses.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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Appendix 7: Complete and rapid reversal of the body color pattern in juveniles of the surgeonfish *Acanthurus triostegus* at Moorea Island (French Polynesia)

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NOTE

Complete and rapid reversal of the body color pattern in juveniles of the convict surgeonfish *Acanthurus triostegus* at Moorea Island (French Polynesia)

Marc Besson^{1,2} · Pauline Salis² · Vincent Laudet² · David Lecchini^{1,3}

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Abstract Color polymorphism is widespread in animals and can be associated with temporary adjustments to environmental variables (phenotypic plasticity). In teleost fishes, one of the most remarkable examples of color plasticity is background adaptation in flatfishes. However, such rapid and complete changes in body color and pattern remain relatively unreported in other species. The convict surgeonfish *Acanthurus triostegus* is a gregarious species whose body color pattern consists of black vertical bars on a whitish body. Here, we describe an entirely reverse body color pattern (white vertical bars on a blackish body) that we observed in some *A. triostegus* juveniles in a nursery area at Moorea Island, French Polynesia. In aquaria, we determined that change from one color pattern to the other is rapid, reversible, and corresponds to phenotypic plasticity associated with aggressive behavior.

Keywords Color pattern · Phenotypic plasticity · Fish behavior · *Acanthurus triostegus*

Introduction

The occurrence of two or more distinct and genetically influenced color morphs within a single interbreeding population, i.e., color polymorphism, is widespread across the animal kingdom (Endler 1980; Gray and McKinnon 2007). Individuals can also adjust body colors and patterns in response to environmental factors such as habitat background, temperature, food availability, population density and level of predation risk (Leimar 2007). This environmentally influenced color plasticity within individuals of the same genotype is highly adaptive when facing changing and selective environments (Gray and McKinnon 2007).

In teleost fishes, phenotypic plasticity in color patterns is well exemplified by background adaptation (camouflage) in flatfishes (Ramachandran et al. 1996; Höglund et al. 2002; Kelman et al. 2006). Body color and pattern adjustments can also be associated with temporary social status (Beeching 1995; Höglund et al. 2002), sexual display (Kodric-Brown 1998; Rhodes and Schlupp 2012) or response to a predation risk (Fuller and Berglund 1996). Changes in body colors and patterns are regulated by a variety of genetic, endocrine and cellular processes such as distribution, morphology and density of pigment organelles and chromatophores (Fujii and Novales 1969; Sugimoto 2002; Miller et al. 2007). These changes can be fixed or reversible and can occur within a few weeks or a few seconds, representing long-term or rapid adaptations to the environment (Sugimoto 2002; Sköld et al. 2013). For example, the dusky dottyback (*Pseudochromis fuscus*) can

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Marc Besson and Pauline Salis: equal first authors.
Vincent Laudet and David Lecchini: equal last authors.

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✉ Marc Besson
marc.besson@ens-lyon.org

¹ EPHE-UPVD-CNRS, USR3278-CRIOBE, PSL Research-University, BP 1013, 98729 Papetoai, Moorea, French Polynesia

² CNRS-UMR7232-OOB, UPMC, 1 Avenue Pierre Fabre, 66650 Banyuls-sur-Mer, France

³ Laboratoire d'Excellence "CORAIL", BP 1013, 98729 Papetoai, Moorea, French Polynesia

change its body coloration from yellow to brown and vice versa within 2 weeks, depending on habitat and prey availability (Messmer et al. 2005; Cortesi et al. 2015), while the paradise whiptail (*Pentapodus paradiseus*) can switch its head and body reflective stripes from blue to red in less than one second, presumably for communication (Mäthger et al. 2003).

Apart from the complex body color and pattern changes observed during camouflage in flatfishes (Ramachandran et al. 1996) and agonistic interactions in cichlid fishes (Beeching 1995), rapid changes (within a few seconds) are generally discrete (e.g., apparition or change in color of a stripe) and relatively unreported in other fish species (Fujii et al. 1989; Mäthger et al. 2003; Rhodes and Schlupp 2012; Watson et al. 2014). While long-term (within a few days or weeks) color and pattern changes have been described for coral reef fishes during ontogeny (Frédérich et al. 2010), sexual maturation (Kodric-Brown 1998) and mimicry strategies in adults (Cortesi et al. 2015), rapid and complete pattern changes remain relatively little explored (Fujii et al. 1989; Mäthger et al. 2003; Watson et al. 2014), particularly in juveniles, for which most studies have focused on slower ontogenetic changes (McCormick et al. 2002).

The convict surgeonfish *Acanthurus triostegus* is a common herbivorous reef fish in the Indo-Pacific region (Randall 1961). Adults are encountered around reef patches close to the barrier reef where they live in large aggregations mostly feeding on turf algae (Randall 1961). The color pattern of reef-associated juveniles and adults consists of black vertical bars on a whitish body (referred to

here as 'classical color pattern,' CP; Fig. 1a; Randall 1961; Frédérich et al. 2012). Here, in a nursery area of Moorea Island (Lecchini et al. 2005), in French Polynesia, we observed some *A. triostegus* juveniles exhibiting a complete reversal of their color pattern: white vertical bars on a blackish body (referred here as 'reversal of the color pattern,' RP; Fig. 1b) and chasing their conspecifics. The aim of this study was to better characterize the RP and to determine whether it is associated with behavioral and/or developmental characteristics, and corresponds to phenotypic plasticity. We collected large cohorts ($n > 50$) of reef-naïve *A. triostegus* larvae while they colonized the reef at Moorea Island, and raised them in aquaria until the appearance of the RP. We demonstrated that the CP–RP change is rapid and reversible, and that this phenotypic plasticity is associated with aggressive behavior.

Materials and methods

Fish sampling and raising

Fish larvae were collected nightly in March 2015 using a crest net on the northeast coast of Moorea Island, French Polynesia ($17^{\circ}29'52.19''S$, $149^{\circ}45'13.55''W$), close to the nursery area where RP juveniles were observed. The crest net had a mesh size of 1 mm and was equipped with a rectangular mouth (width: 1.5 m, height: 1 m) oriented perpendicular to the water flow. The crest net retains all settlement-stage (reef-naïve) larvae as they move over the



Fig. 1 Body color patterns and community organization of *Acanthurus triostegus* juveniles. a 'Classical color pattern' (CP) of a convict tang juvenile at day 7 post-settlement. b 'Reversal of the color

pattern' (RP) of two juveniles also erecting their dorsal fin. c Shoal and community organization of CP (top) and RP (bottom) juveniles

reef crest and enter the net (Dufour and Galzin 1993). As larval fish recruit to reefs at night, the net was set up at 1900 h and larvae were collected at 0200 h the following day. Three large cohorts of *A. triostegus* larvae were collected consecutively ($n = 53, 60$ and 69). After removal from the crest net, individuals were transferred to the CRIOBE research station where they were maintained, with only their respective larval cohort, in a 200-L aquarium in an open water circuit. Water temperature was maintained at $28.5\text{ }^{\circ}\text{C}$, under a 12:12 LD cycle (0600 h onset to 1800 h offset), and fish were fed turf algae using live rocks and rubble that were changed daily. Fish were released at day 11 post-settlement in the nursery area close to their collection site.

Juvenile body color pattern, developmental stage and behavior

Every day from capture to release, the entire fish cohort was observed at 0800, 1200 and 1600 h for 30 min, to assess the color pattern of each fish. Pigmentation pattern prevalence in each cohort (i.e., percentage of CP and RP fish in each of the three cohorts) was assessed when RP juveniles were first observed, at day 7 post-settlement (Fig. 2a). To ensure that CP ($n = 14$) and RP ($n = 10$) fish were at the same developmental stage, individual body depth (body height divided by standard length) was recorded, as it is a good indicator of post-settlement development in *A. triostegus* (McCormick 1999; McCormick et al. 2002). To ensure that aquaria did not impair fish development, we also measured individual body depth in day 7 post-settlement juveniles that were raised in smaller groups of 20 individuals in in situ cages in the nursery area close to the crest net. Body depth comparisons were made

using ANOVA followed by Tukey's post hoc tests after verification of data normality and homoscedasticity. To compare the behaviors of the two color patterns, aggressiveness of CP ($n = 24$) and RP ($n = 24$) fish was assessed at day 7 post-settlement using camera records (mean \pm SE duration of 5.0 ± 0.9 s for CP and 6.3 ± 1.7 s for RP). The percentage of time each unique individual spent with the dorsal fin erected was assessed from the camera records (Fig. 2c). A high percentage of time with the dorsal fin erected was considered as aggressive behavior (Brawn 1960; Gerlai 2003).

Results and discussion

After observation of *A. triostegus* juveniles exhibiting a RP in a dense nursery area of Moorea Island, we confirmed, in aquaria, the occurrence of the RP in three separate groups of juveniles ($n = 53, 60, 69$, respectively) originating from three larval cohorts that we collected at settlement. At day 7 post-settlement, $13.3 \pm 2.5\%$ of *A. triostegus* juveniles had changed color pattern from CP to RP (Fig. 1a, b; Fig. 2a). This change occurred in 1.33 ± 0.64 s (Electronic supplementary material, ESM, Video S1) and lasted until fish release (day 11 post-settlement). The change was also rapidly reversible, as disturbing the fish by introducing a net in the aquarium lead RP juveniles to turn into CP and to mix with the CP shoal. This confirms that the CP–RP transition corresponds to a reversible, complete and rapid color pattern change in juveniles. The RP has never been encountered in adults (Randall 1961; McCormick 1999).

There was no difference in body depth between RP and CP juveniles raised in aquaria and CP juveniles raised in in situ cages in a lagoon nursery area (ANOVA, $n = 43$,

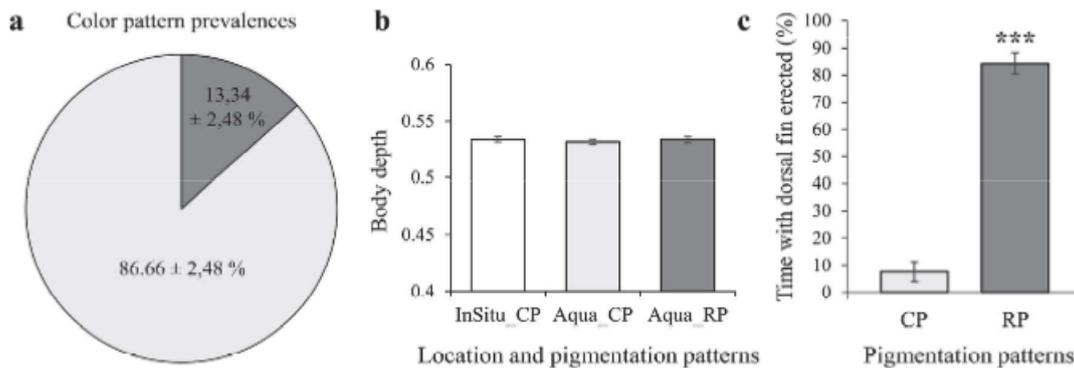


Fig. 2 Color pattern prevalence, ontogenetic stage and behavior of CP and RP juveniles at day 7 post-settlement. **a** Color pattern prevalence of classical color pattern (CP, light gray) and reversal color pattern (RP, dark gray) juveniles at day 7 post-settlement in $n = 3$ juvenile cohorts held in aquaria. **b** Body depth (body height/standard length) of CP individuals held in situ in lagoon cages

(InSitu_CP, white bar, $n = 20$) or held in aquarium (Aqua_CP, $n = 14$) and of RP individuals held in aquarium (Aqua_RP, $n = 10$). **c** Percentage of time spent with the dorsal fin erected in CP (light gray, $n = 24$) and RP (dark gray, $n = 24$) juveniles held in aquaria. Errors bars indicate standard errors. *** p value < 0.001 (Wilcoxon)

$F = 0.063$, $p = 0.939$; Fig. 2b). This suggests that these two patterns are not related to an ontogenetic difference. RP was also different from the stressed/nocturnal pigmentation pattern, which consists of the CP with black rectangles between each bar (ESM Fig. S1). RP was associated with aggressive behavior, with RP juveniles chasing their conspecifics (ESM Video S2). This was exemplified by the significant difference between CP and RP juveniles in the percentage of time spent with the dorsal fin erected ($7.6 \pm 3.6\%$ vs. $84.3 \pm 4.0\%$, respectively; Wilcoxon rank-sum test, $n = 48$, $W = 569$, $p < 0.001$; Fig. 2c), which illustrates the aggressive behavior of RP individuals (Brawn 1960; Gerlai 2003). Consequently, the presence of the RP resulted in a newly organized fish community, with RP juveniles defending their territory, while CP juveniles swam or foraged in groups above the guarded areas (Fig. 1c; ESM Video S3). Therefore, the CP to RP transition in *A. triostegus* juveniles is a rare observation of a rapid and complete reversal of the body color pattern that is not associated with camouflage or mimicry (Ramachandran et al. 1996; Höglund et al. 2002; Kelman et al. 2006; Sköld et al. 2013; Watson et al. 2014). In fact, this behavior is reminiscent of the cichlid fish *Astronotus ocellatus*, where agonistic interactions between males can lead to rapid change in color pattern (darkening of the body color and appearance of white vertical bars) in individuals defeated in combat, with opposite behavioral consequences (i.e., inhibition of aggression; Beeching 1995).

At the cellular level, color and pattern changes can be divided in two categories: morphological and physiological color changes (MCC and PCC; Fujii and Novales 1969; Leclercq et al. 2010). MCC often result from long-term changes in the distribution, morphology and density of chromatophores (pigment cells), while PCC originate from rapid motile movements of pigment organelles (e.g., melanosomes) within the chromatophores (Sugimoto 2002; Leclercq et al. 2010; Sköld et al. 2013). Here, the rapidity of the CP–RP change suggests that this results from PCC and that condensation/dispersion of melanosome in the melanophores may be responsible for the rapid and reversible black/white color reversal (Zhu and Thomas 1997). However, a cellular study of this process is required to confirm this hypothesis.

This study demonstrates phenotypic plasticity in juveniles of *A. triostegus*, revealing an ability to switch from one color pattern to its complete opposite within a few seconds. While CP and RP fish also exhibited distinct behaviors, individuals turning from CP to RP were not predictable and did not show any prior detectable phenotypic difference. Renewing rubble (with fresh turf algae) twice a day and diminishing the number of juveniles in the aquarium delayed and even prevented the appearance of the RP (Besson pers. obs.). Further investigation is

therefore required to test whether this phenotypic plasticity is juvenile specific and only appears in conditions of intense foraging competition, and whether the RP aggressive and solitary behavior is the result of an adaptive strategy to force some individuals to quit the cohort in situations of low resource availability.

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Résumé

Le maintien et le renouvellement des populations de poissons coralliens dépendent en grande partie du recrutement larvaire, c'est-à-dire de l'installation des larves pélagiques dans les habitats récifaux adultes, et de leur survie après s'être métamorphosées en juvéniles. De plus en plus d'études révèlent que les changements de composition de l'eau, causés par le changement climatique et la pollution, peuvent altérer les capacités sensorielles des poissons coralliens, diminuant leurs aptitudes à localiser des habitats propices (maximisant leur croissance et diminuant leur mortalité) lors de l'installation. Cependant, les mécanismes internes à l'origine de ces phénomènes sont méconnus. Lors de cette thèse, j'ai examiné le recrutement larvaire du poisson chirurgien bagnard *Acanthurus triostegus* et mis en évidence que les changements écologiques, morphologiques, physiologiques et comportementaux qui s'y déroulent correspondent à une métamorphose contrôlée par les hormones thyroïdiennes (HT). J'ai ensuite analysé comment des stress d'origine anthropique, tels que l'élévation des températures de surface et la pollution par un pesticide d'origine agricole, peuvent perturber sa métamorphose. Lors de cette étape clé de leur cycle de vie, ces perturbations diminuent les taux d'HT, altérant la maturation de leurs organes sensoriels, leurs capacités sensorielles, et augmentant leur mortalité. Cette thèse est donc une analyse holistique de l'impact des perturbations anthropiques sur les processus moléculaires, et les changements histologiques, anatomiques et comportementaux du recrutement larvaire des poissons coralliens. Elle souligne l'importance du système thyroïdien, et invite à une meilleure compréhension des processus endocriniens du recrutement larvaire, dans l'optique d'une amélioration de la conservation des récifs coralliens.

Mots Clés

Ecologie sensorielle, hormones thyroïdiennes, chlorpyrifos, perturbateurs endocriniens, changement climatique, *Acanthurus triostegus*

Abstract

The persistence and sustainability of coral-reef fish populations depends on the continued larval recruitment, *i.e.* successful settlement by pelagic larvae into adult reef habitats and post-settlement survival through metamorphosis to a juvenile stage. There is growing evidence that changes to water conditions due to global change and waterborne pollution can impair coral-reef fish sensory abilities to locate settlement habitats that maximize growth while minimizing mortality risk. However, the inner mechanisms of such impairments remain less well studied. In this thesis, I have examined the recruitment phase of the convict surgeonfish *Acanthurus triostegus*, and determined that the ecological, morphological, physiological, and behavioral changes occurring at recruitment correspond to a metamorphosis mediated by thyroid hormones (TH). Then, I investigated whether this metamorphosis is prone to endocrine disruption under anthropogenic disturbances such as elevated sea water temperature and agricultural pesticide pollution. I demonstrated that such pressures can reduce TH levels at a critical developmental stage in coral-reef fishes, impairing their metamorphic processes such as intestine remodeling, sensory organ maturation, and sensory abilities acquisition, further increasing their mortality rates. Overall, this thesis is a holistic analysis that addresses molecular, histological, anatomical, and behavioral assays of multiple stressors affecting coral-reef fish recruitment. It indicates the importance of a proper endocrine function during coral-reef fish recruitment, highlighting the need for a better understanding of these processes for coral-reef conservation.

Keywords

Sensory ecology, thyroid hormones, chlorpyrifos, endocrine disruption, climate change, *Acanthurus triostegus*