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Alteration of camouflage and behaviour in two marine invertebrates, *Sepia officinalis* and *Carcinus maenas*, by antidepressant medication

Apolline Chabenat

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Normandie Université

Pour obtenir le diplôme de doctorat

Spécialité **PHYSIOLOGIE ET BIOLOGIE DES ORGANISMES – POPULATIONS - ECOLOGIE**

Préparée au sein de l'Université Le Havre Normandie

Alteration of camouflage and behaviour in two marine invertebrates, *Sepia officinalis* and *Carcinus maenas*, by antidepressant medication

Présentée et soutenue par
Apolline CHABENAT

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devant le jury composé de

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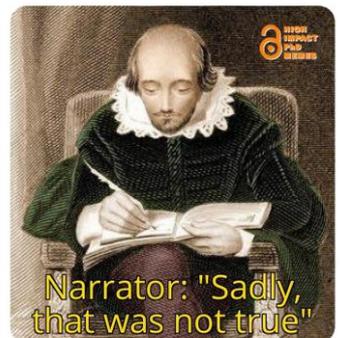
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Me: "I will use this quarantine time to finish my manuscript"





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Introduction

In the vast oceans, countless marine animals are thriving, but also struggling to survive. Indeed, they have to stay hidden from myriads of predators by means of camouflage, one of the most widespread antipredator strategies (Stevens, 2016; Stevens and Merilaita, 2011). Camouflage may be defined as the ability of animals to blend into the environment in order to decrease the probability to be detected or recognized (Merilaita et al., 2017). It can take various forms from colouration to shapes, not to forget matching motion or even remaining immobile (Stevens and Merilaita, 2011). But when thinking of camouflage, complex body patterns might come in mind first.

Animals can wear colours in uniform as well as complex patterns (expression of at least two colours) with the same ultimate goal to be fairly undistinguishable from their environment by predator sight, or preys. Indeed, evolution of camouflage depends on selective survival pressure defined by prey-predator interactions (Merilaita, 2003). Camouflage is known as a very efficient anti-predator technique as well as a powerful predator strategy. Based on the detection abilities and discrimination of the environment (Merilaita et al., 2017), an efficient camouflage may diminish predation pressure (in case of a prey), or increase chances of predation (predator point of view). The most famous example is the rapid evolutionary change of the peppered moth, *Biston betularia*, a population of lepidoptera in Great-Britain in the mid-nineteen century (Kettlewell, 1956). Because of industrial pollution, trunks of white birch turned dark rendering the brighter morphs more visible than the melanic ones. The birds acted ‘as selective agents’ on the light coloured morph since the dark coloured melanic morph displayed a cryptic advantage in those polluted areas. Thus, in each environment, the colours and pattern selected are those that allow animals to blend and fade into the background to keep out of sight of predators, or, otherwise, prey.

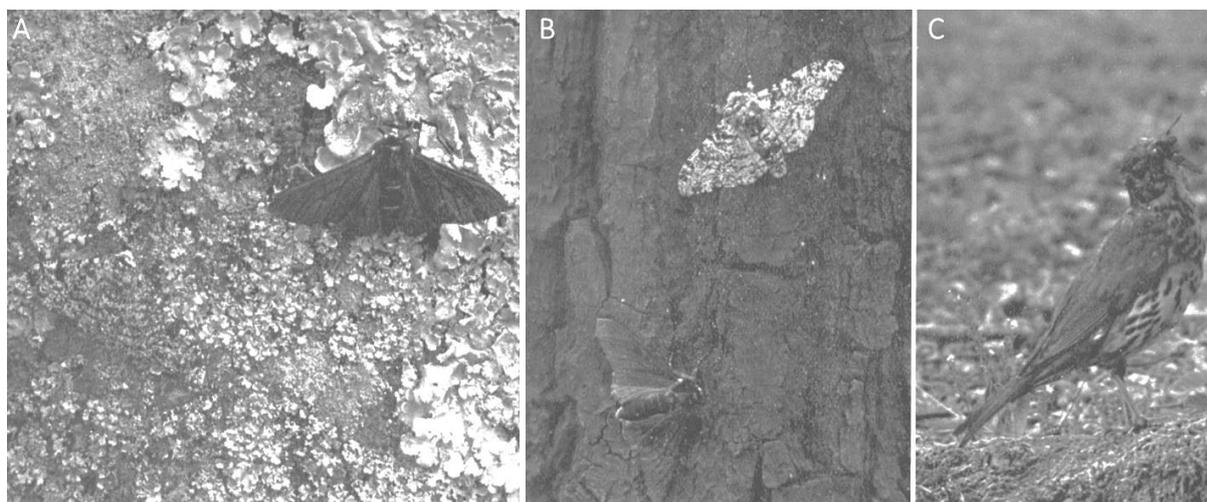


Figure 1. **A**, the peppered moth, *Biston betularia* in its light form (left) and melanic form *carbonaria* (right) on lichenized tree trunk from a pristine area in Deanend Wood, Dorset; **B**, *Biston betularia* in its melanic form (bottom left) and its light form (right) on lichen-free trunk in a polluted area near Birmingham; **C**, a predator, the Song Thrush, *Turdus ericetorum*, with a *carbonaria* in its beak. Adapted from the original publication of Kettlewell (1956).

In a marine environment, attacks may come from any direction (Stevens and Merilaita, 2011). In pelagic environments, most animals will exhibit transparency, mirrors and/or counter-shading as it is a ‘featureless’ milieu (Johnsen, 2014). The countershading colouration presents a light and a dark side, *i.e.*, a dorso-ventral contrast, with the ventral side being generally of a brighter colour (Cuthill et al., 2016; Kelley et al., 2017), as it is the case with penguins, rays or sharks (**Fig.1A-C**). The technique of transparency permits the animals to dissipate their shape within the surrounding body of water (Cronin, 2016; Johnsen, 2001).

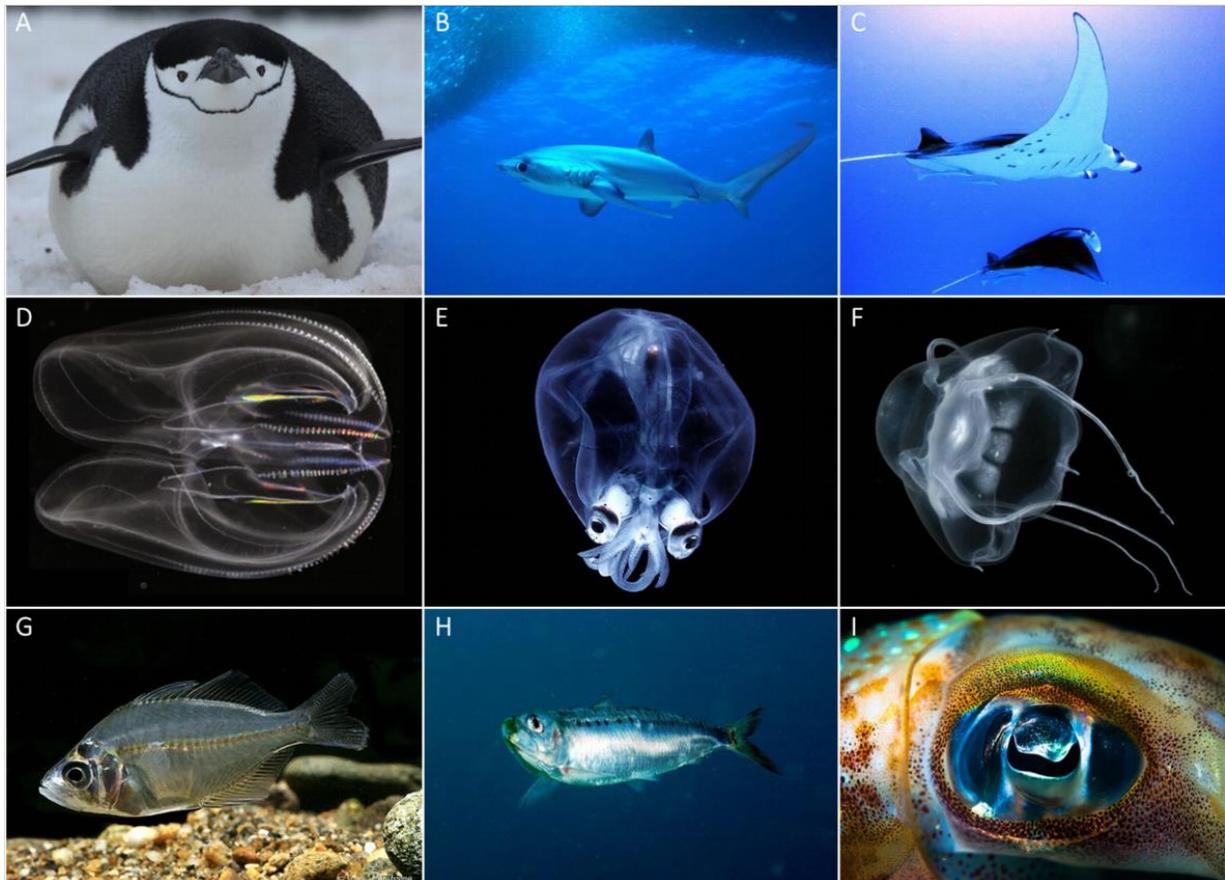


Figure 2. Examples of countershading (**A-C**), transparency (**D-F**) and mirrors camouflage (**G-I**) in several aquatic species. **A**, the chinstrap penguin, *Pygoscelis antarcticus* – ©Carolyn Dankowski; **B** the common thresher, *Alopias vulpinus* – ©Paulo Oliveira; **C**, the manta ray, *Manta birostris*, - ©Valérie Macon; **D**, the ctenophore *Mnemiopsis leidyi* – ©William E. Browne; **E**, the glass squid, *Teuthowenia pellucida* – ©John Foley; **F**, *Bathykorus bouilloni* – ©Kevin Raskoff; **G**, the Indian glassy perch, *Parambassis ranga* – ©Nonn Panitvong; **H**, the European pilchard, *Sardina pilchardus* – ©José Alberto and **I**, the bigfin reef squid, *Sepioteuthis lessoniana* – ©Shawn Miller.

The most compelling example is that of ctenophores displaying an almost perfect transparency, as they are composed over 95 % of water (**Fig.1D**). Hence, they nearly do not modify light diffusion (Bailey et al., 1995; Scolardi et al., 2006). Cnidarians present similar features as well, as do some cephalopods, such as squids or sepioles (**Fig.1E-F**). Silvery camouflage, also called ‘mirror’, is often used by pelagic teleosts, allowing them to keep out of sight any pigmented body part, such as the eyes or the guts (**Fig.1G**) (Johnsen, 2014) or their entire body (**Fig.1H**) (Marshall and Johnsen, 2011). This technique is

also used by cephalopods by a silvery iridescence around their eyes to camouflage them (**Fig.1I**) (Mäthger et al., 2009).

As we dive deeper, we might come across some species buried into the substrate such as the famous flatfish *Solea solea* (**Fig.3A**). Burying is a common behavioural trait, which allows concealing parts (**Fig.3B**) or the entire body (**Fig.3C**) to either stay hidden from predators, or from prey that might pass by (Bellwood, 2002; Mather, 1986; Ryer et al., 2008). This behaviour is a transitory strategy, and is used mainly by species living in environments with moving substrate, such as in the intertidal zones breaking the waves. Burying is, however, not to be confused with burrowing, which designates the digging of a cavity, or ducts into which the animal may retreat and where it can reside for longer periods of time (Bellwood, 2002).



Figure 3. Illustration of the burying behaviour in various species. **A**, the common sole, *Solea solea*, entirely buried in sand – ©Anthony Leydet; **B**, the southern keeled octopus, *Octopus berrima*, half buried in sand – ©John Lewis; **C**, the sand crab, *Ovalipes australiensis*, buried with eyes and antennae above sand – ©Julian Finn

If we focus on coral reef habitats, which may have very diverse colours and substrates, we might pass by the pygmy sea horse *Hippocampus bargibanti* (**Fig.4A**), very well hidden between branches of corals, or an egg shell shrimp, *Hamopontonia fungicola* (**Fig.4B**), without noticing it, because it is tremendously camouflaged by its white and transparent cuticle that perfectly imitates the mushroom coral where it lives. In the same kind of neighbourhood, a flamboyant Gaudy clown crab (*Platypodiella spectabilis*) (**Fig.4C**), which is harbouring the vivid colours of near environment. Those species have in common to wear fixed or very slowly changing cryptic body pattern.



Figure 4. Examples of various colouration and texture camouflage encountered in coral reefs. **A**, the pygmy seahorse *Hippocampus bargibanti* – ©EunJae Im; **B**, the egg shell shrimp, *Hamopontonia fungicola* – ©Véronique Lamare; **C**, the Gaudy clown crab, *Platypodiella spectabilis* – ©Ellen Muller.

Other animals use colour change to provide camouflage in variable environments. In marine habitats, this impressive ability to change colour is represented by a limited number of animals, such as some teleosts (goby, (Aspengren et al., 2009); flatfish, (Burton, 2010), crustaceans (shrimp (Siegenthaler et al., 2018), crabs (Detto et al., 2008; Stevens et al., 2014a)), and unshelled cephalopods (cuttlefish, octopus and squids, (Hanlon et al., 2011)). Amongst those species, the rapidity of colour change spreads from milliseconds to days, weeks or months. The different time scales for the change of colour relate to two types of colour change, referred to as ‘physiological’ and ‘morphological’, whereby rapid and reversible colour changes correspond to ‘physiological colour changes’ involving pigments contained in specialised cells called chromatophores (see **Box 1 and Box 3**). Pigments can rapidly and reversibly disperse or aggregate within the chromatophore and, therefore, induce a change of the animal’s coloration in less than a second to a few hours (Duarte et al., 2017; Stuart-Fox and Moussalli, 2011). The slower and longer-lasting colour changes correspond to ‘morphological colour change’, involving structural changes, such as the density of chromatophores, or the pigments included in the tegument (Stuart-Fox and Moussalli, 2011; Umbers et al., 2014). Animals may express both physiological and morphological changes at the same time. Furthermore, in arthropods, those colour changes may be accompanied with moulting and may thus be associated with a specific developmental stages, such as in crabs (Detto et al., 2008; Todd et al., 2006).

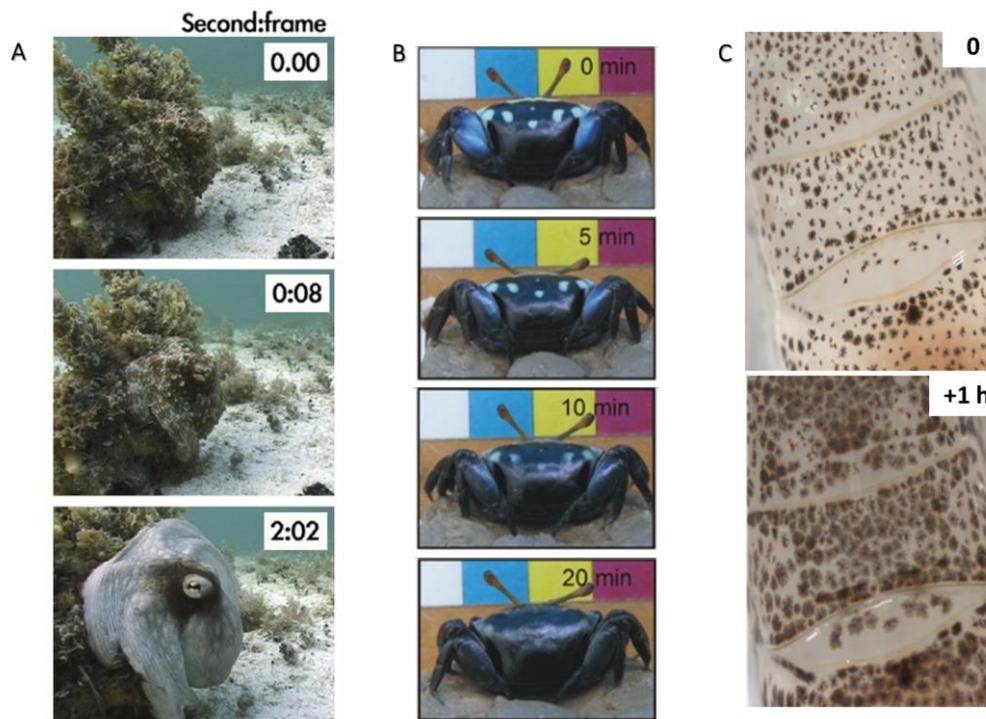
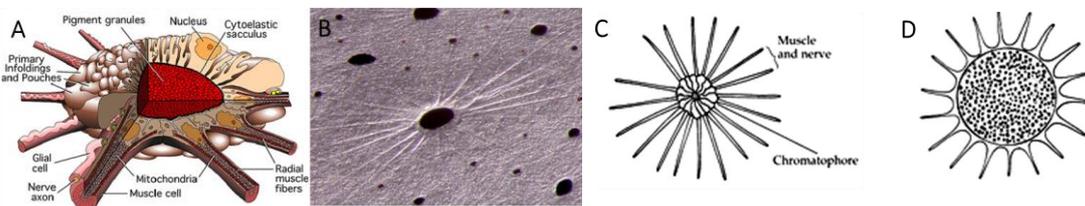


Figure 5. Different timescales of rapid, physiological colour change between species. **A**, common octopus, *Octopus vulgaris*, changing colour and texture of its skin in seconds between the first and the third picture (adapted from Hanlon et al., 2011); **B**, darkening of the carapace of the fiddler crab *Uca capricornis* within 20 min (adapted from Detto et al., 2008); **C**, close-up of dorsal abdominal segments of a shrimp *Crangon crangon*, changing from a light to a dark cuticle within hours (adapted from Siegenthaler et al., 2018).

Box 1 – Colour change in cephalopods

The patterns of cuttlefish are generated by monochromatic chromatophores which can contain dark-brown, red-orange or yellow pigments (Cloney and Florey, 1968). The pigments are contained in a sacculus within the chromatophore surrounded by a neuro-muscular complex (**Fig. A**). A muscular contraction stretches the sacculus, which expands and disperses the pigments in the skin (**Fig. D**), whereas a relaxation will allow the sacculus to retract and concentrate the pigments in one single spot (**Fig. B-C**). In addition, the deeper layers of the epidermis contain iridophores and leucophores (Cloney and Brocco, 1983). The iridophores, located under the chromatophores, are characterised by stacks of thin cells, which reflect light at different wavelengths. Depending on the angle, the colour perceived may be iridescent green to blue (Messenger, 2001a). The leucophores, located even deeper in the epidermis layer, are elongated cells that allow the dispersion of incident light, thus conferring white colour. Both types of cells will develop during post-embryonal stages, and are, therefore, absent in newly-hatched cuttlefish which depend solely on chromatophores for colour changes.



Figures A-D. Cephalopod chromatophores. **A**, schematic of the anatomy of a chromatophore © adapted from Cloney and Florey (1968); **B**, picture of chromatophores in a portion of squid fin skin © George Bell; **C**, relaxed and **D**, contracted chromatophore © adapted from Jerry Wilkinson

When approaching the coasts of Normandy, one can encounter different species keen to stay hidden by an effective camouflage involving either physiological or morphological colour changes. Amongst them are the two species that have been studied in this thesis: the common cuttlefish, *Sepia officinalis*, and the green shore crab, *Carcinus maenas*. Indeed, *S. officinalis* is found in the North-East Atlantic from the North of Europe to North-West Africa as well as in Mediterranean Sea (Bloor, 2013; Gras, 2013). In the English Channel, mature adults are found in shallow waters along the coast where they gather for reproduction from late spring to early autumn. The cuttlefish is a semelparous species with a two-years life cycle and adults die rapidly after laying some 150 up to 2 000 eggs (Boletzky, 1983; Hanley et al., 1998; Zatylny-Gaudin and Henry, 2018). Thus, hatchlings have to survive without parental presence until autumn, when they begin a slow migration into deeper waters (Goff and Daguzan, 1991).

The cuttlefish is a coleoid cephalopod belonging to the phylum of molluscs. The coleoids are distinguished from the Nautiloids by the loss of an external shell, which lets the mantle exposed. The unprotected skin comprises all coloured cellular structures, the iridophores, leucophores and chromatophores (see **Box 1**). The chromatophores of cephalopods are fundamentally different from those of other species and enable very rapid colour changes (Florey, 1969; Messenger, 2001a). In fact, this physiological colour change is under direct control by motoneurons located in the central nervous system that induce contraction or relaxation of the muscle crown. This nervous control of

chromatophores is influenced by neurotransmitters, such as serotonin (5-hydroxytryptamine, 5-HT) or glutamate, and by neuropeptides (Loi and Tublitz, 2000; Messenger, 2001a). Indeed, 5-HT controls rapid relaxation (**Fig. 6A**), while the glutamate causes rapid contraction (**Fig. 6B**). Furthermore, neuropeptides called FMRFamides have a role in a slower and longer lasting contraction (Aroua et al., 2011; Tublitz et al., 2006). Thus, the cuttlefish may display rapid and efficient camouflage due to a direct control of the chromatophores by neural projections from the nervous system that, in addition, can be modified by peptidergic regulation.

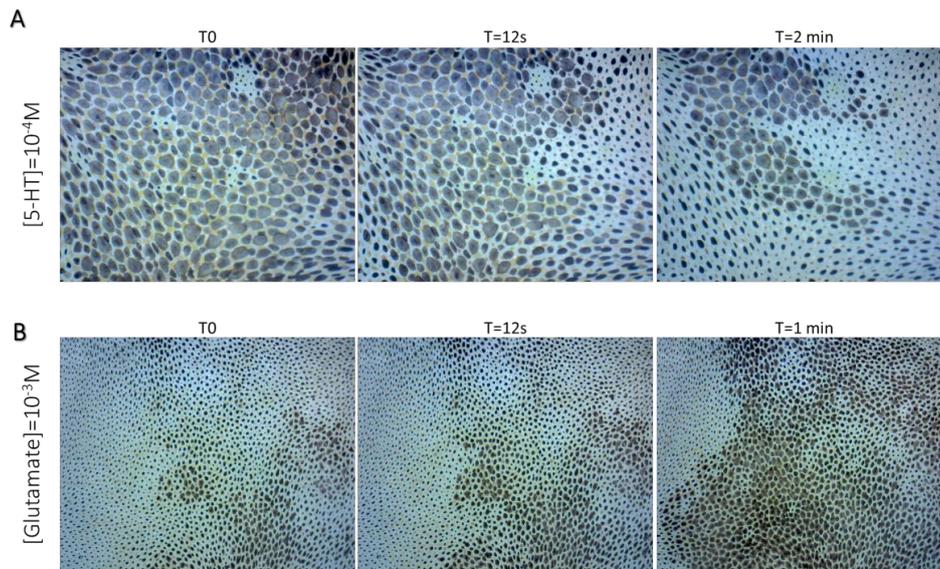


Figure 6. Observations of rapid colour changes on *ex-vivo* skin, *i.e.*, explants of juvenile cuttlefish after application of either serotonin (5-HT) (**A**) or glutamate (**B**). **A**, a solution of 5-HT at the concentration of 10^{-4} M provokes a relaxation of a majority of chromatophores, concentrating pigments into small dots; **B**, a solution of glutamate of 10^{-3} M provokes a contraction of a majority of chromatophores, *i.e.*, dispersing pigments. Preliminary study on the elaboration of chromatophore bioassay – © Apolline Chabenat.

The cuttlefish is an animal known to display different body patterns by changing the colour of its mantle as well as its arms and head, either for camouflage, or for communication. Cuttlefish possess three types of pigments, *i.e.*, yellow-orange, red and dark-brown (Florey, 1969), which permit to express three main patterns: uniform, mottle and disruptive body patterns (**Fig.7**) (Hanlon et al., 2011; Hanlon and Messenger, 2018). A uniform body pattern is characterised as having little or no contrast on the skin, which means the animal is displaying plain colour and brightness all over the mantle, head and arms (see **Box 3**). This pattern is observed in cuttlefish settled on uniform substrates, such as fine sand or uniformly coloured rocks. A mottle body pattern is described as an irregular arrangement of small-to-moderate-scale light and dark patches spread homogeneously over the body. Because of the contrast between the patches, this pattern is preferably displayed by cuttlefish on heterogeneous backgrounds with a small-to-moderate sized light and dark objects, such as coarse sand or fine gravel. Disruptive body patterns break the body shape and contours of the animal with the expression of very contrasted large-scale skin components of multiple shapes and scales (see **Box 4**). Cuttlefish display disruptive

patterns on heterogeneous substrates, such as coloured pebbles or contrasted broken shells. Overall, to adapt to a novel substrate the cuttlefish will be able to switch body patterns in milliseconds for dynamic camouflage (Messenger, 2001a).

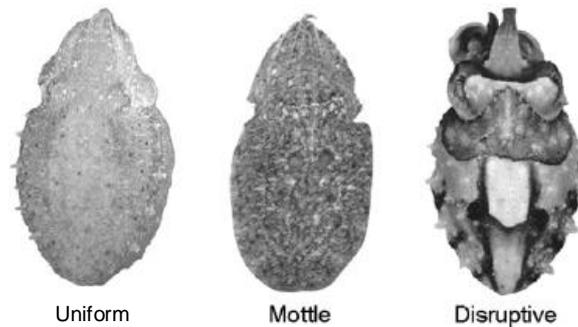


Figure 7. The three main body patterns that cuttlefish can express depending on the substrate. Uniform body pattern displayed on plain background, with minimal contrast variations. Mottle body pattern displayed on little to moderate contrasted background, with homogeneous small to moderate contrasted variation. Disruptive body pattern displayed on very contrasted background, with various contrasted components. – © Adapted from Barbosa et al. (2008).

In addition to the impressive rapid colour changes, cuttlefish may use another cryptic behaviour to stay out of sight: burying themselves into the substrate. This has been referred to as ‘sand-digging behaviour’ in the pertinent literature (Di Poi et al., 2014; Dickel et al., 2006; Mather, 1986; Poirier et al., 2004). Mäther (1986) has characterised this behaviour as a fixed sequence of five seconds duration. When settling on the substrate, the cuttlefish ejects a series of water jets from its funnel, wiggles laterally with its mantle to progressively be covered with sand. This behaviour appears immediately after hatching, but stays flexible and improves with experience (Navet et al., 2014; Poirier et al., 2004). Thus, newly-hatched cuttlefish learn how to perform an even more efficient sand-digging behaviour with age. Furthermore, cuttlefish can also complete the cryptic behaviour with background matching by controlling their skin chromatophores to resemble the colour, brightness, contrast and granularity of the sand.

Because they do not benefit from parental care, newly-hatched cuttlefish are particularly vulnerable to predators. Moreover, they have to learn fast in order to survive, which requires learning to hunt efficiently. At hatching, cuttlefish can subsist with the remainders of their internal yolk that contains energetic reserves (Dickel et al., 1997). This reserve may help to sustain them up to five days post-hatching. Yet, this period is critical, because if the cuttlefish do not eat before the total yolk resorption, their cuttlebone becomes positively buoyant and may interfere with their ability to hunt efficiently, eventually condemning them to perish (Boletzky, 1975; Boucaud-Camou & Roper, 1995). Besides, cuttlefish must grow quickly to become a less endangered prey and a more efficient predator. Growth

is, therefore, positively correlated with food intake (Koueta & Boucaud-Camou, 1999). Indeed, a poor food intake will reduce growth and may have repercussions later in the animal's life.

Box 2 – Sequence of predation behaviour and predator techniques of cuttlefish: the jumping vs. the tentacle ejection

The predatory behaviour of the cuttlefish towards their prey is visually driven. Thus, the cuttlefish will follow a sequence of (i) prey detection, (ii) orientation of the head toward the prey, (iii) pursuit of the prey if necessary, (iv) positioning at appropriate distance with ocular convergence, and (v) prey seizure (Dickel et al., 2013).

Cuttlefish will apprehend their prey differently, depending on prey size and form, estimated escape speed of the prey and personal risk (Chichery and Chichery, 1988; O'Brien et al., 2017). Thus, cuttlefish will attack with the tentacle ejection strategy shrimps, little crabs and small fish, which are relatively small preys and/or are known to be swift (**Fig. A**). The cuttlefish will attack the shrimp by ejecting the two tentacles equipped with large suckers allowing them to bring the prey back to their arms and mouth (Messenger, 1977). With other prey, cuttlefish will prefer to use a 'jumping' technique. For instance, in the case of a large crab with its two dangerous claws, the cuttlefish will apprehend the prey by attacking from behind with partially open arms and without the use of the tentacles (**Fig. B**) (Duval et al., 1984).

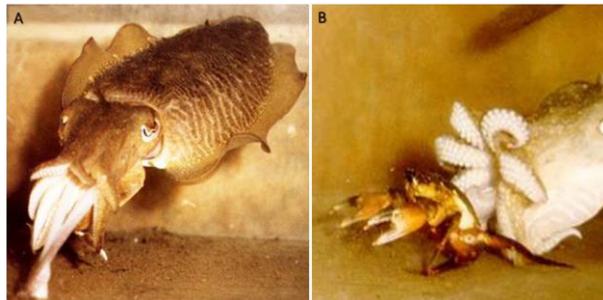


Figure A. Tentacle ejection strategy: the cuttlefish ejects its tentacles to attack a shrimp.

Figure B. Jumping strategy: the cuttlefish opens its arms and jump on a large crab from behind.

Both photographs are from Dickel et al. (2013)

Before becoming a redoubtable predator, hatchlings of cuttlefish will also have to learn and adapt their hunting method depending on whether the prey will be a crab or a shrimp (Hanlon and Messenger, 2018) (see **Box 2**). At hatching, cuttlefish will preferably choose a shrimp over a crab or a small fish and the number, type and size of the prey will grow along with the growth of the cuttlefish (Darmaillacq et al., 2006; Guibé et al., 2012; Koueta and Boucaud-Camou, 1999).

Climbing back on to the intertidal shore, one may come across several ten-legged species, *i.e.*, decapods, such as the green shore crab, *C. maenas*, also called European green crab outside Europe, where it is an invasive species. Indeed, this brachyuran decapod, is found originally along the same coast as the common cuttlefish, *i.e.*, Atlantic Northern European and Northern African coasts and the Mediterranean Sea (Crothers, 1968), but has reached almost every continent except for Antarctica (Compton et al., 2010). The life cycle of the shore crab begins as a planktonic larva developing for 80 days from protozoa to zoea in estuarine and coastal waters before coming back inshore as megalopa stage that

metamorphoses into the juvenile crab (Anger, 2001). Juvenile crabs remain in the upper littoral and will undergo frequent moulting until they reach their adult stage when they moult roughly one to two times per year, depending on the geographical latitude and environmental conditions (Hartnoll, 2001; Styriahave et al., 2004).

Box 3 – Colour change in crustaceans

The colour displayed by crustaceans depends on the number, types and distribution of chromatophores, as well as the types and density of exoskeletal pigments (Davie et al., 2015). Indeed, crustacean pigments may be present in the cuticle as well as in chromatophores and their number may slowly evolve depending on environmental conditions, or because of growth and developmental stage, which corresponds to morphological changes. Thus, a long-term adaptation to a coloured environment may result from an increase of chromatophores and associated pigments; for instance, a dark background may lead to an increase of melanophores, while a light background may lead to an increase of leucophores (Davie et al., 2015). While a rapid change of substrate, illumination, or the presence of a predator will trigger a rapid colour change caused by the distribution of pigments within chromatophores, which either aggregate or disperse, whereby the migration from the cell centre into the ramifications of the chromatophores renders the colouration more intense, the opposite is the case when the pigment granules aggregate within the cell centre. Such changes are typically transient, rapid and reversible. Located in the epidermis, chromatophores can contain one type of pigments such as dark-brown (melanophores), red (erythrophores), white (leucophores) and yellow (xanthophores), or a mix of them in polychromatic chromatophores (**Fig.A, B, D**) (Anger, 2001; Davie et al., 2015). In most cases, monochromatic chromatophores are grouped in cluster, *i.e.*, two or more chromatophores interlocked, forming so called chromatosomes (**Fig.C-E**) (Davie et al., 2015). In *C. maenas*, Powell (1962b) demonstrated that possesses monochromatic chromatophores containing black, red and white pigments. More recently, Nery and Castrucci (2002) and Stevens (2016) presented evidences pointing out the existence of yellow pigments.



Figure A-E. Crustacean chromatophores. **A-C.** Schematic of different types of chromatophores: **A**, monochromatic chromatophore; **B**, polychromatic chromatophore; **C**, chromatosome, *i.e.*, clusters of chromatophores. © Rao (1985). **D-E.** Pictures of chromatophores: **D**, monochromatic chromatosome (red) from the neotropical freshwater shrimp, *Macrobrachium olfersi*; **E**, dichromatic chromatosome, the yellow one fully dispersed the red/brown with partially dispersed pigments, from the shrimp *Palaemon affinis*. © McNamara and Milograna (2015)

Interestingly, juvenile *C. maenas* are highly polymorphic and wear various patterns of different colours on their carapace and the subjacent epidermis, depending on the surrounding environment, which makes them very difficult to spot (Hogarth, 1978; Stevens et al., 2014b; Todd et al., 2006). Furthermore, they

may be able to adapt their colour and brightness to match nearby substrates and elements of the environment. In fact, as most others crustaceans, juveniles of the green shore crab can go through morphological and physiological colour changes (see **Box 3**). Their thin cuticle lets the epidermal colours shine through. Thus, juvenile crabs are able to brighten or darken and to adapt their colouration rapidly in few hours depending on the background (Stevens et al., 2014b). On the contrary, the cuticle of the adult morphs is too thick for the rapid colour change to be seen through the carapace, although the epidermis still contains chromatophores. Overall, both morphological and physiological colour changes allow the crabs to form more or less complex patterns and different colours on their carapace and legs (Stevens et al., 2014a). Thus, they may display uniform to disruptive patterns enabling them to blend into the heterogeneous and variable intertidal zone where they thrive (see **Box 4** and **Box 5**).

Box 4 – Background matching

The **background matching**, also called crypsis, corresponds to the ability of the animal to resemble the colour, contrast, texture and/or pattern of the background to prevent detection (Merilaita and Stevens, 2011). The uniform body pattern displayed by numerous animals belongs to the category of background matching. Indeed, by wearing the same colouration than the substrate, the animals blend into the environment by matching the background.

The **cuttlefish** may display uniform body pattern by expressing one homogeneous colour on their whole body skin, which can go from light white to brown depending on the adjacent substrate (**Fig.A**) (Hanlon and Messenger, 1988).

The **juvenile crabs** can also display a homogenous colouration over their carapace and legs when their habitat is made of sandy substrates or brown algae for instance (**Fig.B**) (Hogarth, 1978). They may express various colours from uniform green to white, as well as dark brown or even orange.

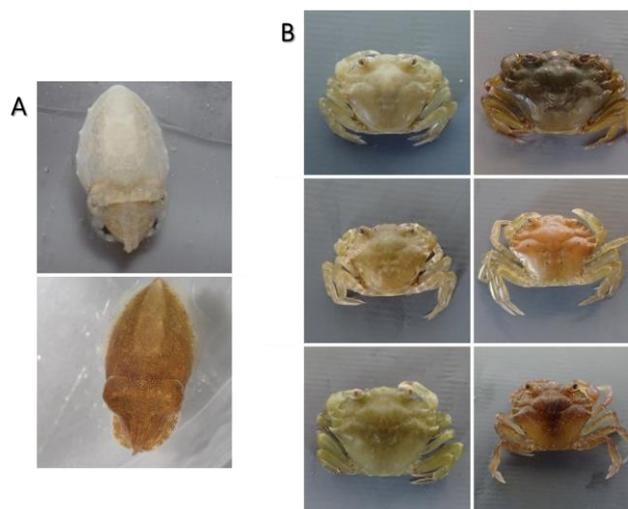


Figure A. Illustration of a uniform body pattern in juvenile *Sepia officinalis*. The two animals exhibit a uniform body colour on their mantle: either plain white or brown. The paler part in the middle of their mantle is caused by their thin skin letting their cuttlebone shine through. ©Apoline Chabenat

Figure B. Variety of uniform colouration in juvenile *Carcinus maenas*. Crabs may display plain white, green, orange, or even brown colour on their carapace. ©Apoline Chabenat

Their variety of patterns and colours allow them to stay out of sight and decrease the chances to be found by terrestrial or aquatic predators. In addition to rapid colour changes, juveniles will moult frequently, which enables them to adapt the colour of their carapace to the successively varying backgrounds they inhabit when descending from the upper to the lower littoral, before attaining the adult stage (Hartnoll, 2001). Finally, the adults become largely monomorphic, with either a more greenish or more reddish morphotype (Baeta et al., 2005; Styrihave et al., 2004).

Box 5 – Disruptive colouration

The **disruptive colouration** is defined by a set of diverse conspicuous and contrasted patches, markings, spots expressed by the animal to break the body shape and let false edges appear making the whole animal look like a bland object in the background, thus being difficult to detect as a prey (or a predator). The major difference between uniform and disruptive colouration is that the latter may be used independently of the background (Schaefer and Stobbe, 2006).

In **cuttlefish**, a disruptive body pattern corresponds to the expression of eleven major components on their dorsal skin as well as their head and arms (**Fig.A**) (Hanlon and Messenger, 1988). The components are divided into five light chromatic components and six dark chromatic components. Barbosa et al. (2007) have developed a method to grade their body pattern: each component is given a grade ranging from 0 (not expressed), 1 (weakly expressed), 2 (moderately expressed) to 3 (strongly expressed), which gives a final grade on 33 points, with 0 equivalent to perfectly uniform and 33 perfectly disruptive.

In **juvenile green shore crabs**, the disruptive pattern may take different forms (**Fig.B**). Indeed, they can display various symmetrical patches of light and dark colours from white, light green or orange to red, dark brown and black, on their carapace which help them to disappear on a substrate made of molluscan shells or algae for instance (Powell 1962 b). In adult crabs, the kind of patterns present in juvenile stages progressively disappears to let a green or a red morph colouration take over (Todd et al. 2005).

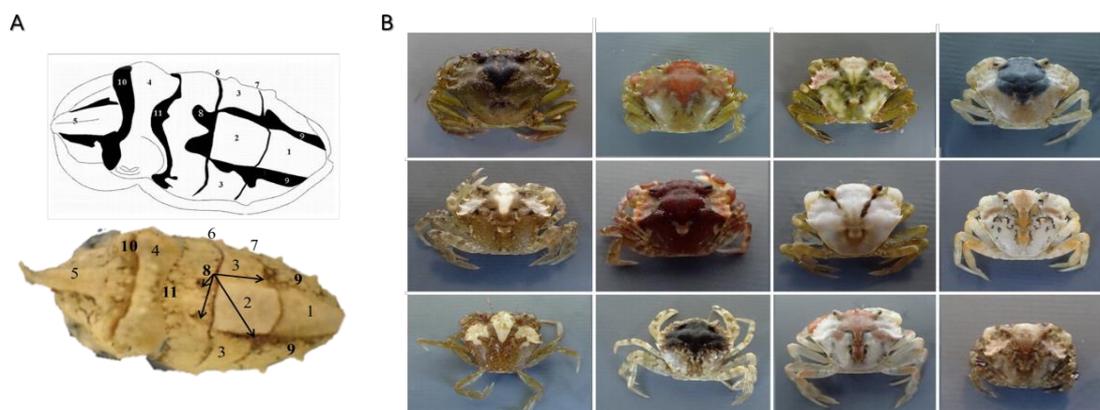


Figure A. Disruptive body pattern in cuttlefish. The drawing shows the eleven components, which may be expressed: n°1, 2, 3, 4 and 5 are light chromatic components, while n°6, 7, 8, 9, 10 and 11 are dark chromatic components (adapted from Barbosa et al., 2007, and Poirier et al., 2005). The picture below refers all the components on a juvenile cuttlefish expressing a disruptive body pattern – ©Apolline Chabenat.

Figure B. Variety of disruptive pattern on the carapace of juvenile *Carcinus maenas* encountered on the shores of Roscoff, France. Juvenile crabs may display various colour combinations of two to four colours in our observations. ©Apolline Chabenat.

Those rapid, but also the slower colour changes, which the juvenile crabs undergo are under the control of hormonal neuropeptides (Powell, 1962a). In crustaceans, those molecules trigger either the dispersion or the concentration of pigments, on the one hand, as well as moulting on the other hand. The physiological colour changes are regulated by neurohormones called chromatophorotropins, such as Red-Pigment Concentrating Hormone (RPCH) and two isoforms of Pigment Dispersing Hormone (PDH), α - and β -PDH (Christie et al., 2010; Fingerman et al., 1998). As its name indicates, the RPCH induces pigment concentration in erythrophores, but might as well do the same in leucophores, melanophores and xanthophores. The PDH will trigger pigment dispersion in all types of chromatophores (Rao, 2001). Other neuropeptides, such as corazonin (Porrás et al., 2003) or crustacean cardioactive neuropeptide (Nery et al., 1999) may also be involved in rapid colour change. Upstream, the neuropeptide hormones are influenced by levels of monoamines, such as 5-HT, norepinephrine (NE) and dopamine (DA). These neurotransmitters act on the release of the chromatophorotropins into the bloodstream *via* neurohaemal organs located in the sinus glands of the eyestalks and the postcommisural organ (Davie et al., 2015). Indeed, 5-HT and NE stimulates the secretion of PDH (Rao and Fingerman, 1970; Hanumante and Fingerman, 1981; Fingerman, 1985), whereas DA stimulates the release of RPCH (Fingerman, 1985).

As for cuttlefish, crabs may choose to bury themselves into the sand, to hide below stones and between pebbles, so as to escape the sight of predators, but also to prevent desiccation at low tide (Bellwood, 2002). Called ‘burying’ or ‘digging behaviour’, *C. maenas* enters the substrate backwards by using its flattened pereopods. To be completely covered, the crab will either slam its body against the substrate to dig itself in deeper, or perform a sternal push thereby brushing aside the substrate laterally to create a depression to settle within (Bellwood, 2002). Depending on their general environment, juvenile crabs may also display background matching or adapt their body colour by brightening or darkening, depending of the type of sand (**Fig.8A**). Indeed, juveniles found in sandy areas will exhibit speckled sandy colours and patterns (**Fig.8B**).



Figure 8. Illustration of different natural sandy substrates and patterns in juveniles of *Carcinus maenas* found in various sandy substrates. **A**, a fine sand substrate (above picture) and a coarse sand substrate mixed with small contrasted coloured gravel (below picture) among which each a juvenile crab presents same colour, contrast and pattern than the background (an arrow points to the crab on the below picture); **B**, examples of juvenile crabs collected in sandy substrates (second picture above is the crab indicated by the arrow in **A**). © Apolline Chabenat

Contrary to pelagic environments, the intertidal rocky shore generally consists of highly heterogeneous habitats composed of algae, sands, chalk, gravel, pebbles and/or broken shells. Furthermore, depending on the tidal level, animals may be the targets of predators. Thus, both juvenile crabs and cuttlefish will have to wear their most appropriate patterns and colours, to behave calmly and move carefully to successfully subsist without being eaten.

In crustaceans as well as in molluscs, monoamines such as 5-HT, NE and DA regulate a wide range of physiological functions and their behavioural aspects as they act as neurotransmitters and/or endocrine messengers. They are known to have roles, for instance, in reproduction (Gibbons and Castagna, 1984; Khotimchenko and Deridovich, 1991), growth (Fingerman, 1997), metabolism (Fingerman, 1997; Sarojini et al., 1995) and colour change (Fingerman, 1997; Messenger, 2001a). But they also are involved in many behaviours, such as aggressive behaviour (Sneddon et al., 2000; Tierney and Mangiamele, 2001a), anxiety-like behaviour (Fossat et al., 2014), locomotor activity (Aonuma et al., 2020; Barthe et al., 1989; Peeke et al., 2000), phototactic behaviour (McPhee and Wilkens, 1989) and appetite (Beiras and Widdows, 1995). Many pollutants discharged in the environment may interact with the synthesis, release and the regulation of monoamine concentrations in non-target organisms, which may affect camouflage and cause alterations of behavioural traits of non-target organisms, such as juvenile crabs and cuttlefish.

Besides accommodating a tremendous number of animal species, the marine environment is, unfortunately, also a receptacle of continental anthropogenic pollution (Costello et al., 2010). Indeed, many pollutants are found in marine waters and many of them are capable of interfering with neuronal, neurohormonal and hormonal regulation of animal physiology (Iqbal et al., 2020; Wayne and Trudeau, 2011). Amongst them, pharmaceuticals are of particular interest, because medication is intended to modify the regulation of physiological functions. Furthermore, the consumption of drugs increases worldwide, leading to the continuous contamination of aquatic compartments (OECD Health Statistics 2019). Consequently, great volumes of pharmaceuticals are excreted per day by humans, or dumped into toilets, and eventually end up in surface waters (Jones et al., 2001). Wastewater treatment plants are not yet fully equipped to remove those molecules. Thus, a fraction of drugs is continuously released into the water bodies (Bueno et al., 2012; Schlüsener et al., 2015). Consequently, hundreds of active molecules have been detected across the globe in effluents, ground water, surface waters, marine waters and even in tap water, ranging from few ng to hundreds of $\mu\text{g}\cdot\text{L}^{-1}$ (Fram and Belitz, 2011; Giebułtowicz and Nałęcz-Jawecki, 2014; K'oreje et al., 2016; McEneff et al., 2014; Minguez et al., 2016; Rodríguez-Navas et al., 2013). The occurrence of pharmaceuticals in virtually every aquatic compartment raises serious concerns about their impact on the biota, especially because they are generally designed to act specifically and at low doses, to have a sufficiently long half-life and a lasting activity (Schloss and Williams, 1998; Sulser, 1983). Yet, non-target organisms may share the same biological mechanisms

the drugs were designed to interfere with in human or veterinary medicine (Gunnarsson et al., 2008). Therefore, they may have an impact on the physiology and/or the behaviour of wildlife.

The antidepressants of the class of selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), with prescriptions still on the rise worldwide (OECD Health Statistics 2019), are particularly worrying because they were designed to act on monoamine levels, especially on 5-HT. They are employed as medication for the treatment of neurological disorders, such as depression, anxiety, binge-eating or obsessive-compulsive disorders. The first SSRI placed on the market was fluoxetine in 1987 (Das, 2011; Huskamp et al., 2008). This family of antidepressants was followed nearly a decade later, in 1995, by the SNRIs, notably venlafaxine.

The SSRIs are supposed to selectively bind and block the serotonin transporters (SERT) that control the reuptake 5-HT from the pre-synaptic cleft, whereas the SNRIs would additionally inhibit the transporters responsible of the reuptake of norepinephrine (NET) (Fig. 9) (Richelson, 1996; Stahl et al., 2013). Both antidepressants have in common to act on the levels of monoamines by increasing the availability of neurotransmitters in the synaptic cleft in patients with a depressive state, for example. In humans, venlafaxine has the particularity to have 10 to 30 times more affinity for the SERTs than for the NETs (Stahl et al., 2005). Thus, at low doses its action should basically be the inhibition of SERTs and act more or less as the SSRIs (Richelson, 2001; Stahl et al., 2005). Furthermore, at even higher dosage, it may have an impact on the recapture of dopamine (Stahl, 2000).

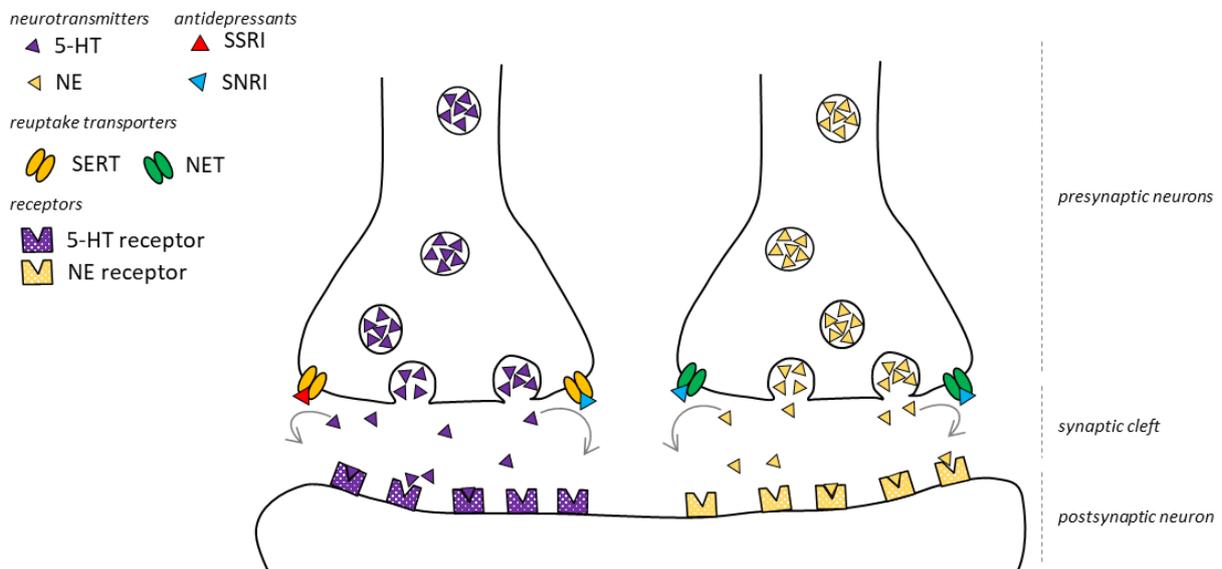


Figure 9. Mode of action of antidepressants from the families of SSRIs (Selective Serotonin Reuptake Inhibitor) and SNRIs (Serotonin Norepinephrine Reuptake Inhibitor). 5-HT: serotonin; NE: norepinephrine; SERT: serotonin transporter; NET: norepinephrine transporter.

For more than three decades, the high consumption of both antidepressants has been contaminating the different aquatic compartments and, therefore, antidepressants are regularly detected in surface waters.

Generally, concentrations reported for running waters are 10 to 100 times higher for venlafaxine than for fluoxetine (Zenker et al., 2014). Indeed, fluoxetine was detected from a few $\text{ng}\cdot\text{L}^{-1}$ to hundreds of $\text{ng}\cdot\text{L}^{-1}$ in freshwater, such as lakes, groundwater or effluents of wastewater treatment plants, whereas venlafaxine can be found at concentrations in the $\mu\text{g}\cdot\text{L}^{-1}$ range in wastewater effluents (K'oreje et al., 2016; Ma et al., 2016; Meador et al., 2016; Schlüsener et al., 2015; Schultz et al., 2010; Valcárcel et al., 2011). Few studies investigated the presence of these compounds in marine waters, but interestingly, Mínguez et al. (2016) have detected both fluoxetine and venlafaxine up to 0.3 and 1.3 $\text{ng}\cdot\text{L}^{-1}$, respectively, in the Bay of Seine, France, where juvenile crabs and cuttlefish can be found.

Furthermore, fluoxetine and venlafaxine possess their own chemical properties making them more or less predestined to bioaccumulate in animal tissues. Indeed, both molecule display relatively high $\log K_{ow}^1$, superior to 4 for fluoxetine (Adlard et al., 1995; DrugBank; Zorita et al., 2007) and around 3 for venlafaxine (Calisto et al., 2015; Li et al., 2010; PubChem). Both substances are considered persistent because of their stability in aqueous solutions (Kwon and Armbrust, 2008, 2006). With a half-life greater than 100 days, being lipophilic and easily adsorbed by organic matter, fluoxetine strongly accumulates in the biota, even if its concentration in the water is low, or below detection limits (Kwon and Armbrust, 2006). While venlafaxine presents a relatively lower $\log K_{ow}$, suggesting a lower potential of bioaccumulation, several studies demonstrated a bioaccumulation of the substance in several aquatic species such as bivalves, gastropods and fish (Maulvault et al., 2018; Moreno-González et al., 2016; Togunde et al., 2012). Overall, the toxicity of antidepressants may not be represented by their water concentrations, but by their persistency and their accumulation in quite a number of aquatic animals. Furthermore, despite the low concentrations, the continuous discharge of residues of medicaments add to their persistency and leads to a chronic exposure, which may be responsible for long-term effects in non-target organisms.

Amongst all ecological concerns, there is a growing interest in research concerning the effect of pollutants on behavioural traits in animals. Not surprisingly, behaviour becomes increasingly important in ecotoxicological research (Brodin et al., 2014; for Brooks, 2014, see discussion). In this context, more and more studies have shown that low environmental concentrations of fluoxetine and/or venlafaxine have altered behaviours of several non-target species. Therefore, behaviour, including camouflage and colour change, represents a particularly sensitive and useful endpoint to investigate the effects of antidepressants at low environmental concentration on wildlife. Nevertheless, there is still a lack of knowledge on the effect of pollutants on camouflage and other behavioural endpoints. To date, only few studies have investigated potential effects of either fluoxetine or venlafaxine on fundamental behavioural traits in crustaceans and molluscs.

¹ The $\log K_{ow}$, *i.e.*, octanol/water partition coefficient, is an indicator of the tendency of the substance to adsorb to soil and living organisms. The $\log K_{ow}$ is often inversely related to the water solubility of the molecule. If $\log > 3$ there is a tendency of bioaccumulation (Zenker et al., 2014)

To begin with, **colour change** provides a particularly interesting mechanism with respect to the effects of antidepressants on wildlife, because colour changes involve neuronal and neurohormonal control, which in turn requires upstream regulation by neurotransmitters. The common cuttlefish, *S. officinalis*, certainly constitutes the reference in the field of colour change when it comes to ecotoxicological studies of molluscs. Di Poi et al. (2014) have demonstrated that perinatal exposure to fluoxetine at $1 \text{ ng}\cdot\text{L}^{-1}$ modified the uniform body pattern in a transient manner: at hatching, animals exposed to fluoxetine displayed a better background matching than the control; this difference was not observed two weeks later. Bidet et al., (2016) could not demonstrate clear changes in the quality of camouflage at hatching after an exposure to either 1 or $10 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ of fluoxetine during the last 15 days of embryonic development. In the case of venlafaxine, Bidet et al. (2016a) demonstrated a decrease of uniform pattern efficiency after an exposure to $100 \text{ ng}\cdot\text{L}^{-1}$ for 19 days post-hatching whereas the lower concentration, *i.e.*, $5 \text{ ng}\cdot\text{L}^{-1}$, did not. Concerning crustaceans, some studies have used the fiddler crab *Uca pugilator* to investigate the effects of pollutants such as PCB, naphthalene or cadmium on colour change in crustaceans (Fingerman et al., 1998), but few studies addressed the issues of antidepressants. However, Fingerman and co-workers have investigated the impact of fluoxetine on chromatophores, because of its known action on 5-HT levels in mammals. In the fiddler crab *U. pugilator* and the shrimp *M. ohione*, fluoxetine accelerated the dispersion of pigments in the erythrophores when animals were transferred from a white to a black background (Fingerman et al., 1981a; Hanumante and Fingerman, 1983a). But when crabs were transferred from a black to a white background, fluoxetine slowed down the concentration of the pigments.

Burying behaviour used by the cuttlefish and the shore crab as a powerful strategy of concealment may as well be modified by the presence of pollutants. In cuttlefish, Di Poi et al. (2014) reported an increase in the frequency of sand-digging at $1 \text{ ng}\cdot\text{L}^{-1}$ of fluoxetine during the two first weeks of life post-hatching. To our knowledge, so far no study investigated the impact of antidepressants on burying behaviour in crustaceans. To date, only three studies used this particular behaviour to demonstrate the impacts of human pollution, *i.e.*, oil spills and an urban sewage accident, on burying behaviour in two different crab species (Boere et al., 2011a; Burger et al., 1991a; Zengel et al., 2016).

Concerning **predatory behaviour**, most of the studies focused on fish models. For instance, Bisesi et al. (2016, 2014) have investigated the effects of fluoxetine and venlafaxine in the range of $\mu\text{g}\cdot\text{L}^{-1}$ on the predatory behaviour of striped seabass and showed an increase in the time to capture their prey for both antidepressants. Regarding molluscs and crustaceans, few studies looked-for potential effect of antidepressants on this key behaviour. Bidet et al. (2015) demonstrated an effect of venlafaxine on the latency before the first attack of the prey at environmental concentration, *i.e.*, $5 \text{ ng}\cdot\text{L}^{-1}$. If predatory behaviour is indeed altered, the food intake may decrease as a result. Indeed, Melnyk-Lamont et al. (2014) showed that after seven days of exposure to venlafaxine at $1 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ rainbow trout presented a

reduced total feed intake per day. Furthermore, as a consequence of reduced food intake, growth may be reduced on the long run.

Locomotor activity is a behaviour quite easy to monitor and to measure for many species. Thus, several studies have investigated potential effects of SSRI and SNRI on this particular endpoint. Di Poi et al. (2014) have reported that an exposure to fluoxetine at 1 or 100 ng·L⁻¹ had no effect on locomotion in cuttlefish. In *C. maenas*, however, Mesquita et al. (2011) have demonstrated an effect of fluoxetine on locomotor behaviour, but very high concentrations ranging from 120 to 750 µg·L⁻¹ were necessary to observe behavioural changes in locomotor activity. In another species of crabs, *Hemigrapsus oregonensis*, Peters et al. (2017) demonstrated an increase of locomotor behaviour after an exposure to fluoxetine at 30 ng·L⁻¹. Furthermore, linked to locomotion, geotaxis and phototaxis have been altered by a three weeks exposure to fluoxetine at concentrations between 10 ng·L⁻¹ and 10 µg·L⁻¹ in the marine amphipod *Echinogammarus marinus* (Bossus et al., 2014). The same authors also demonstrated significant increase of velocity in the same species after one day of exposure to fluoxetine at 1 ng·L⁻¹. In another amphipod, *Gammarus pulex*, an exposure for 14 days to fluoxetine at 100 ng·L⁻¹ equally increased the velocity of this amphipod (De Castro-Català et al., 2017), while an exposure for 35 min at a similar range of concentrations, *i.e.*, 10-100 ng·L⁻¹ caused a decrease of activity in *G. pulex* (De Lange et al., 2006). A decrease in locomotor activity was also observed in the rusty crayfish, *Orconectes rusticus*, after an exposure to fluoxetine at 2 and 500 ng·L⁻¹ for 10 days (Tierney et al., 2016). By contrast, the freshwater crayfish *Procambarus virginalis* exhibited no alteration of locomotor behaviour when exposed to 1 µg·L⁻¹ of venlafaxine (Kubec et al., 2019). In two marine snails, *Urosalpinx cinerea* and *Lithopoma americanum*, fluoxetine and venlafaxine displayed different effects on locomotion, either reducing or increasing locomotor activity after only few hours but at high concentrations of 31 µg·L⁻¹ and 345 µg·L⁻¹, respectively (Fong et al., 2015).

Overall, the majority of the studies cited used rather high concentrations of antidepressants and did not consider cumulated concentrations of several antidepressants. Historically, it may be noticed that fluoxetine was used more frequently compared to venlafaxine in the literature and that the environmental realistic combination of different antidepressants remains undervalued. However, in order to better apprehend the environmental risk from these kinds of molecules there is a need to use low concentrations of contaminants and to combine them in more or less complex cocktails. Furthermore, using similar concentrations and periods of exposure, numerous studies reported very different outcomes for the same endpoints for relatively similar experimental settings. This issue highlights the need to investigate more cautiously, to gather more data and, eventually, to come up with standardised tests to be able to compare behaviours within the same species, but also between several species in a reproducible manner.

Aims of the study

Our goal is to approach environmentally realistic scenarios with accumulated antidepressants on sensitive endpoints shared by *Sepia officinalis* and *Carcinus maenas*. We, thus, investigated the effect of fluoxetine combined, or not, with venlafaxine at low concentrations on key behaviours and cryptic colouration, which are modulated by neurotransmitters regulating either nervous or a neurohormonal control in juveniles of the common cuttlefish and of the green shore crab. It is especially relevant to study juveniles, as these are known to be more sensitive to pollutants. Because they need to adapt to their close environment during their development, they depend highly on camouflage. Moreover, both juvenile species inhabit potentially polluted areas and are particularly vulnerable to predation. Whichever way the antidepressants affect their behaviour or their ability to camouflage themselves from predators, it is assumed that even a minor modification may reduce fitness of populations of both species studied. Hence, we ask whether antidepressants at environmentally realistic concentrations modify or alter behavioural traits and colour change in juvenile cuttlefish and shore crabs?

To study the potential effects of antidepressants, juvenile cuttlefish and crabs were exposed to fluoxetine and venlafaxine during about one month to mimic a sub-chronical contamination. The concentrations were chosen to approximate concentrations of fluoxetine and venlafaxine detected along the Normandy coast by Minguez et al. (2016), *i.e.*, $0.3 \text{ ng}\cdot\text{L}^{-1}$ and $1.3 \text{ ng}\cdot\text{L}^{-1}$ respectively. The impact of this exposure has been assessed on the efficiency of their camouflage either by body color change on standardized background (**Article I** for juvenile crabs and **Article II** for juvenile cuttlefish) or by burying behaviour (**Article III**). Fundamental behaviours, such as predatory behaviour (**Article IV** with newly-hatched cuttlefish) or locomotion (**Article I** with juvenile crabs) have also been studied. Each behaviour was dissected into several parameters in order to evaluate even subtle potential changes caused by an exposure with antidepressants. **Preliminary results** on the bioaccumulation of fluoxetine and venlafaxine in juvenile and adults of both species will be also presented in the form of a poster.

Article I – Effects of environmental antidepressants on colour change and locomotor behaviour in juvenile shore crabs, *Carcinus maenas*

The goal of this study was to evaluate the impacts of an exposure to low concentrations of fluoxetine and venlafaxine in combination on juvenile crabs. Thus, crabs were placed in contrasted background, *i.e.*, black or white, to estimate the efficiency of colour change. Moreover, we investigated a behaviour distantly linked with camouflage allowing them to escape, *i.e.*, locomotion could be affected by the antidepressants. All parameters were analysed on a weekly basis.

Effects of environmental antidepressants on colour change and locomotor behaviour in juvenile shore crabs, *Carcinus maenas*.

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1. Introduction

Camouflage allows animals to stay hidden from predators, or from potential prey. Camouflage has been defined as “body colours and pattern and other morphological adaptations that decrease the probability that an animal will be detected or recognized” (Merilaita et al., 2017). But camouflage can encompass also complex behavioural choices (Stevens and Ruxton, 2019), amongst them, actively choosing substrates depending on their colour, *i.e.*, ‘background matching’ (Marshall et al., 2016; Tyrie et al., 2015), using elements of the environment to help concealment, *i.e.*, decoration camouflage (Guinot and Wicksten, 2015), adopting postural camouflage (Barbosa et al., 2012), moving without rising any suspicion by predators (or preys) by mimicking elements of the environment, *i.e.*, matching environmental motion (Bian et al. 2016), or simply by remaining immobile (Caro, 2005; Lindberg, 1980). Thinking of camouflage—and particularly colour change—chameleon or octopus come first in mind, which change colour within minutes or seconds depending on their surroundings (Hanlon and Messenger, 2018; Stuart-Fox et al., 2008, 2006). But this rapid colour change does not occur frequently in the wild. Indeed, colour change in most animals proceeds much slower, within hours to days, or even in weeks or months to match the background, for example in shrimps (Siegenthaler et al., 2018), juvenile shore crabs (Stevens et al., 2014a) or fish (Sugimoto, 2002), not to mention the well-known seasonal changes of birds and mammals living in snowy environments during winter wearing white fur or feathers (Zimova et al., 2018).

Rapid and reversible colour changes, which are called ‘physiological colour change’, involve pigments contained in the sacculus of specialised cells, named chromatophores, which can reversibly disperse or concentrate (aggregate) within the sacculus (Fingerman, 1965). Slower and long-lasting colour changes, named ‘morphological colour change’, involve structural changes, for instance the density of

chromatophores and in the pigments included in the tegument, such as the cuticle of arthropods (Umbers et al., 2014). In crustaceans, physiological and morphological colour changes can take place simultaneously, allowing animals to blend into their environment (Fingerman, 1985). Nevertheless, the thickness and colouration of the cuticle hampers rapid colour change, even if chromatophores within the epithelial tissues respond quickly to variation of the nearby environment. This holds true for the green shore crab, *Carcinus maenas*, for which colour change is visible throughout early stages (Stevens et al. 2014a; 2014b), when the cuticle is thin enough to let subtle colour change shine through. Powell (1962) showed that *C. maenas* possess three types of monochromatic chromatophores containing black, white and red pigments. In addition, more recent evidence points to the existence of yellow pigments (Nery and Castrucci, 2002; Stevens, 2016). Therefore, juvenile crabs are capable to darken or brighten and to adapt their colouration within hours according to their environment (Stevens et al. 2014b). In addition, juvenile crabs display different colours and various patterns on their carapace and legs (Stevens et al., 2014a, 2014b), ranging from uniform to disruptive colouration. Both, physiological and morphological colour changes allow the crabs to form colours and patterns, which correspond perfectly to the heterogeneous and variable habitats of the intertidal zone where the juveniles thrive (Fig. 1). The complexity of possible colour changes allows them to stay hidden and increase their chances to escape their predators. Eventually, juvenile crabs are moulting quite frequently (Hartnoll, 2001), enabling them to adapt the colour of their carapace to the successively varying backgrounds they inhabit when descending from the upper to the lower littoral before attaining the adult stage. The carapace adults basically display green or red morphs and the cuticle becomes too thick to make any physiological colour change visible.

Both, rapid and slower, *i.e.*, physiological and morphological, colour changes in crustaceans are triggered by hormonal neuropeptides that either act on the dispersion or concentration of pigments, or on moulting and tanning. Several neuropeptide hormones, also called chromatophorotropins (Powell, 1962a), regulate physiological colour changes, amongst them, notably, Red-Pigment Concentrating Hormone (RPCH) and numerous isoforms of Pigment Dispersing Hormone (PDH) (Christie et al., 2010; Fingerman et al., 1998) Depending on the species studied, RPCH induced pigment concentration exclusively in erythrophores as well as in leucophores, melanophores and xanthophores in various combinations, whereas PDH appears to trigger pigment dispersion in all types of chromatophores (reviewed in Rao, 2001). Putatively, other non-classical chromatophorotropins, such as corazonin (Porras et al., 2003) or crustacean cardioactive peptide (Nery et al., 1999) may be involved in rapid colour change as well—Upstream, those hormones are influenced by levels of monoamines, such as serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE) and dopamine (DA). Indeed, 5-HT and NE stimulated the secretion of PDH (Rao and Fingerman, 1970; Hanumante and Fingerman, 1981; Fingerman, 1985), whereas DA stimulated the release of RPCH (Fingerman, 1985).

Monoamines such as 5-HT, NE and DA are also known to have roles in diverse behaviours in crustaceans, such as agonistic behaviour, *i.e.*, aggressiveness (Sneddon et al., 2000; Tierney and Mangiamale, 2001), phototactic behaviour (McPhee and Wilkens, 1989), anxiety-like behaviour (Fossat et al., 2014), and locomotion (Barthe et al., 1989; MCPhee and Wilkens, 1989; Peeke et al., 2000). The latter appears to be essential for survival of juvenile crabs allowing them to escape and to repair to locations with an appropriate background that matches their body colouration, thus hiding from potential predators. Indeed, background matching may appear as very efficient if the animal stays still. Nevertheless, after a short time lag juvenile crabs usually attempt to flee so as to avoid the field of vision of their predators (Siegenthaler et al., 2018), requiring locomotor activity, hence providing a link between camouflage and locomotion. As stated above, monoamines can have an impact on many behavioural traits where locomotion is involved. MCPhee and Wilkens (1989) have demonstrated that elevated 5-HT level increased and altered locomotion, *i.e.* in random directions. Furthermore, neuropeptide hormones from the eyestalk seem to regulate rhythmic locomotory behaviours in crustaceans like shrimps and crabs (Strauss and Dirksen, 2010; Williams et al., 1979). Thus, any changes in the levels of monoamines, either by modulation of their synthesis, release, action or reuptake will impact neurosecretory release of peptide hormones or motor control centers. As a consequence, colour change and/or locomotor behaviour of juvenile crabs may be modified which will eventually may imperil their survival.

Various pollutants are released along the coastal areas where the juvenile crabs develop. Several compounds may interfere with the synthesis, release and the regulation of monoamine concentrations in non-target organisms, which may affect camouflage and cause alterations of behavioural traits. Of particular concern are antidepressant drugs specifically designed to act on serotonergic and norepinephrine systems (Mezzelani et al., 2018). Most of the antidepressant drugs act on the regulation of monoamines levels in the synaptic cleft and their prescription rate is still rising worldwide (OECD Health Statistics 2019). Consequently, great volumes of residues are released each day through human excretion or disposal of unused and expired pills in toilets (Jones et al., 2001). This constant flux of antidepressant residues is discharged in wastewater treatment plants, which are unable to eliminate them efficiently (Bueno et al., 2012; Schlüsener et al., 2015). Cumulated amounts of antidepressants may, therefore, be detected in significant concentrations in freshwater and marine water (Gonzalez-Rey et al., 2015; Meador et al., 2016; Metcalfe et al., 2010; Paíga et al., 2016; Rúa-Gómez and Püttmann, 2012a), and even in tap water (Giebułtowiec and Nałęcz-Jawecki, 2014), despite of single antidepressants being in the low $\text{ng}\cdot\text{L}^{-1}$ range. Amongst the many different antidepressants, we focused on two of the most prescribed antidepressants, the Selective Serotonin Reuptake Inhibitor (SSRI) fluoxetine (FLX) and the Serotonin Norepinephrine Reuptake Inhibitor (SNRI) venlafaxine (VEN) (Fong and Ford, 2014a). Because FLX was the first SSRI distributed worldwide at the end of the 1980's, FLX alone serves as reference antidepressant in the present study. The SNRI VEN is part of the new generation of

antidepressants that were commercialised nearly a decade later. As a result, both are the most commonly detected antidepressants in water compartments. Indeed, the two antidepressants were detected at concentrations up to $0.3 \text{ ng}\cdot\text{L}^{-1}$ for FLX and $1.3 \text{ ng}\cdot\text{L}^{-1}$ for VEN by Minguez et al. (2016) in Northwestern coastal waters of France, where juvenile shore crabs are found abundantly. These molecules are designed to act at low doses (Broekkamp et al., 1995), raising concern about their potential sub-lethal effects on non-target organisms, such as crabs of the intertidal zone.

In this study, we tested the hypothesis that cumulated environmental concentrations of the most prominent antidepressants are sufficiently effective to modulate cryptic behaviour composed of colour change and locomotion in an intertidal crustacean, *Carcinus maenas*. In the last decade, several studies investigated the effects of such antidepressants on different behavioural traits in aquatic species and highlighted impairments of predatory behaviour (Bisesi et al., 2014a; Mennigen et al., 2010), maturation and learning processes (Chabenat et al., 2019; Di Poi et al., 2013a), or reproductive behaviour (Campos et al., 2016). Even if behaviour becomes increasingly important in ecotoxicological research (Brodin et al., 2014), little attention has been given to the effect of pollutants on camouflage associated behaviours such as colour change, and locomotor behaviour. Some studies have shown that colour change was affected by the presence of FLX or VEN in juvenile cuttlefish (Bidel et al., 2016a, 2016b; Di Poi et al., 2014). A number of studies have investigated the effects of pollutants such as PCB, naphthalene or cadmium on colour change in crustaceans, but they were all conducted with *Uca pugnator* (Fingerman et al., 1998). Concerning locomotion, Mesquita et al. (2011) have demonstrated an effect of FLX on locomotor behaviour in *C. maenas*, but at very high concentrations, ranging from 120 to $750 \mu\text{g}\cdot\text{L}^{-1}$. Another study examined the effect of FLX at $30 \text{ ng}\cdot\text{L}^{-1}$ in *Hemigrapsus oregonensis* and demonstrated an increase of locomotor behaviour (Peters et al., 2017). In two marine snails, FLX and VEN displayed different effects on locomotion, either reducing or increasing locomotor activity (Fong et al., 2015). Notwithstanding, only few studies have taken cumulated antidepressant concentrations into account, which, however, represents a more environmentally realistic condition. Previous studies suggested more pronounced effects of antidepressant mixtures on learning processes of sand-digging behaviour in juvenile cuttlefish, or modification of sand-digging behaviour in juvenile shore crabs (Chabenat et al., 2019), on predatory behaviour in striped sea bass (Bisesi et al., 2016) and on predator avoidance behaviour (Painter et al., 2009a).

To test the hypothesis, this study examines —for the first time— the effect of two cumulated antidepressants on colour change and locomotor behaviour in juvenile *C. maenas*. Any modification of colour change ability or locomotion may have an impact on survival of juveniles. Therefore, this study focuses on the effect of environmentally realistic concentrations of antidepressants, FLX alone or in mixture with VEN, on fundamental behavioural endpoints in juvenile shore crabs.

2. Materials and Methods

2.1. Animal keeping and experimental conditions

Juvenile green shore crabs (*C. maenas*, L. 1758) were collected by hand on the rocky shore of Yport, France (between N 49° 43' 47, E 0° 16' 3 and N 49° 44' 25, E 0° 18' 47) on the upper intertidal where the smallest animals reside. The intertidal foreshore of Yport is a heterogeneous habitat composed of chalk and flintstone with several dispatched areas of sand separated by small to large shingles covered with kelp as well as green or red algae. Correspondingly, the crabs collected along this shoreline were quite heterogeneous, displaying various patterns from uniform to highly disruptive (Fig. 1). The crabs ranged from 5-20 mm cephalothorax width. Although they may have strongly pigmented spots (Fig. 1), the cuticle of juveniles of this size is sufficiently thin and semi-transparent for colour changes by the subjacent chromatophores to be detectable. The resulting brightening or darkening also intensifies, or attenuates disruptive colouration (Detto et al., 2008).

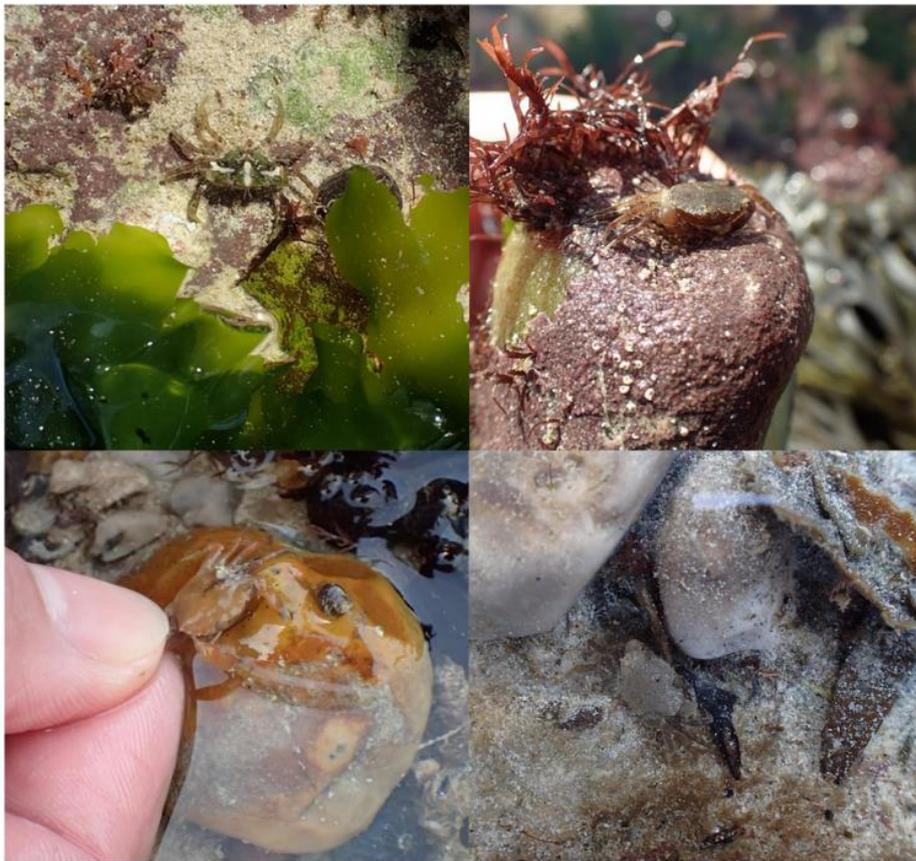


Figure 1. Photography of juvenile crabs, *Carcinus maenas*, that exhibit background matching in various micro-habitats of the intertidal foreshore of Yport (France) with green and red algae as well as sandy and rocky substrates.

Animals were transferred to the laboratory and separated into different tanks according to their size to avoid cannibalism (density = 15 ± 5 crabs/tank of 20 L). The animals were kept in a closed-loop system with filtered and oxygenated artificial seawater (ASW) at a salinity of 35 PSU made from Marine SeaSalt (Tetra, Melle, Germany) with dechlorinated tap water. Temperature and photoperiod were

maintained at $15\pm 1^\circ\text{C}$ and 12h light : 12h dark (8 a.m. to 8 p.m.), respectively. Animals were fed once a day at 9 a.m. with pieces of frozen seabass filet (*Dicentrarchus labrax*) over the entire experiment. Animals were kept in those conditions for eight weeks in order to acclimate and to override their endogenous locomotor rhythmicity (Naylor, 1985; Webb, 1983).

Taking into account the variability between crabs (Stevens et al., 2014a), we evenly distributed the crabs to the different test conditions so as to comprise the varying sizes and patterns as good as possible. In addition, a ‘benchmark week’ was included in which the crabs underwent testing under control condition, *i.e.*, in ASW only (Fig. 2), thus serving as their own individual references. To this end, juvenile crabs were isolated in 250 mL glass beakers (\varnothing 6 cm, height 10 cm) containing 200 mL of ASW during 8 days (*i.e.*, from day -8 to day 0). Each crab was submitted to the behavioural tests as described below (see 2.3 colour change and locomotor behaviour), in order to provide reference values for each individual crab (Fig. 2) (see 2.4 for further explanation). These reference data were then used to normalise the test data of each crab from all treatment groups at all time points of exposure so as to determine the potential effect of antidepressants by minimising individual variability.

After the benchmark week, *i.e.*, at day 0, juvenile crabs were exposed individually to either (i) ASW (control), or (ii) ASW with $5\text{ ng}\cdot\text{L}^{-1}$ of FLX alone (FLX5), or (iii) ASW with $5\text{ ng}\cdot\text{L}^{-1}$ of FLX and $5\text{ ng}\cdot\text{L}^{-1}$ of VEN (FLXVEN5). Juvenile crabs of each treatment group were submitted to the behavioural tests, *i.e.*, colour change and locomotor behaviour, each week at four time points over a period of 25 days (Fig. 2) using the same glass beakers as described above, henceforth designated ‘home tanks’.

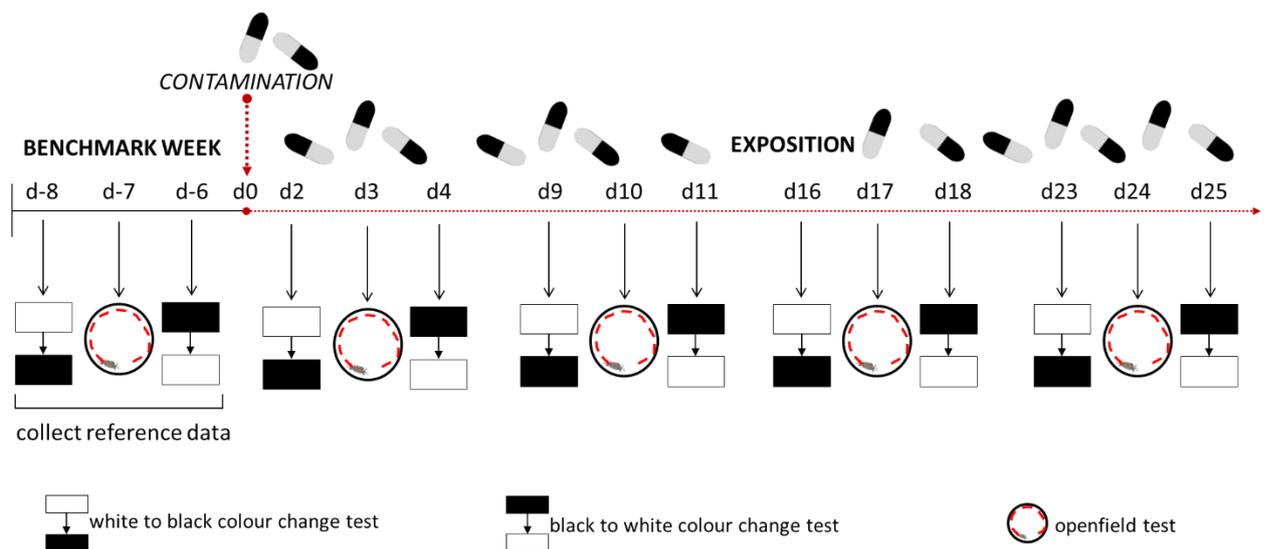


Figure 2. Chronology of behavioural tests. Following a benchmark week providing individual reference data for each individual and test, *i.e.*, colour change (white to black and black to white) and locomotor behaviour (openfield), for all juvenile crabs. The contamination commenced at day 0 (d0) for 25 days. Behavioural tests began at day 2 (d2) and were repeated every week over a period of 25 days.

2.2. Chemical contamination

Stock solutions at $10 \mu\text{g}\cdot\text{L}^{-1}$ of either FLX hydrochloride (CAS 56296-78-7, Sigma Aldrich, St. Louis, USA) or VEN hydrochloride (CAS 99300-78-4, Sigma Aldrich) dissolved in distilled water, were added daily to ASW together with a complete water renewal to obtain final concentrations of either $5 \text{ ng}\cdot\text{L}^{-1}$ of FLX alone (FLX5), or $5 \text{ ng}\cdot\text{L}^{-1}$ of FLX and $5 \text{ ng}\cdot\text{L}^{-1}$ of VEN in mixture (FLXVEN5).

2.3. Behavioural tests

2.3.1. Colour change test on opposite background

Crabs were placed, within their home tanks, in either a white or a black environment composed of painted Plexiglas for underneath and laminated paper for the sides. After 21 hours, each individual crab was photographed before being placed in the opposite colour environment, *i.e.*, black to white, or white to black, for 2 hours, after which a second picture was taken (Fig. 3). All images were taken in a black tent (Neewer®, Guangdong, China) equipped with a circular LED lamp (Neewer®) in order to have a homogeneous light source. Photography was carried out with a Stylus Tough TG-4 digital camera (Olympus, Tokyo, Japan) against a grey medium background (Lastolite, Manfrotto, Cassola, Italy). The images were analysed *a posteriori* using ImageJ® (Bethesda, Maryland, USA) in order to obtain: (i) the absolute colour change, (ii) the percent colour change and (iii) the normalised colour change.

- (i) **Absolute colour change** is defined by the Mean Grey Values (MGV), corresponding to the sum of the grey values of all the pixels in the selected area divided by the number of pixels, *i.e.*, the average brightness/darkness in the selected carapace area. Therefore, a MGV for each crab of each treatment group at each time point in either of the environments (black or white) is obtained after the contours of the crab's carapace were drawn manually using ImageJ®. A MGV of 0 corresponds to absolute black, whereas a MGV of 255 corresponds to absolute white (Fig. 3.A). These data were used to calculate both (ii) absolute colour change and (iii) relative colour change.
- (ii) **Percent colour change** is defined as the percentage of colour change, *i.e.*, amplitude of colour change, at each time point between two opposite environments (black or white). It is calculated at each time point from MGVs at 21h and 2h: $\%cc = [(MGV_{21h} - MGV_{2h}) / MGV_{21h}] \times 100$ (Fig. 3.B).
- (iii) **Normalised colour change**, designated Δcc and expressed as fold change, is defined as the difference between MGV at 21h and at 2h ($MGV_{21h} - MGV_{2h}$), at each time point normalised with the reference data of each crab (benchmark), and expressed as fold-change relative to $\Delta cc_{\text{benchmark week}}$, *i.e.*, $\Delta cc_{\text{fold-change}} = \Delta cc_{\text{experimental time point}} / \Delta cc_{\text{benchmark week}}$. Hence, this $\Delta cc_{\text{fold-change}}$ highlights the variation of colour change during the experimental time course relative to the benchmark value. Thus, after normalisation, a fold-change of relative colour change equal to 1 means that there was no difference between $\Delta cc_{\text{experimental time point}}$

and $\Delta cc_{\text{benchmark week}}$, whereas inferior or superior to 1 means a decrease or an increase of the capacity to brighten or darken respectively, depending on the condition (Fig. 3.C).

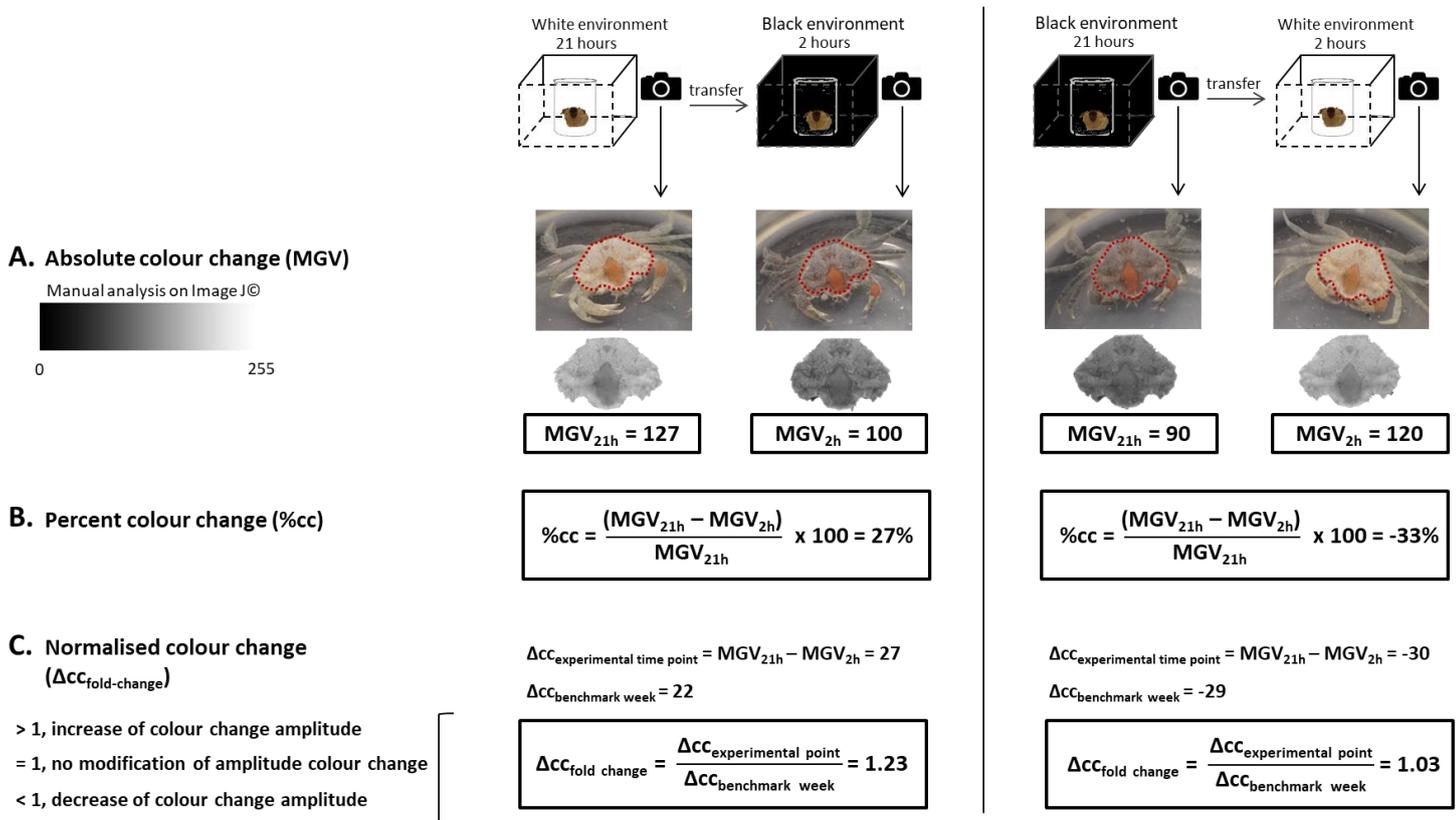


Figure 3. Procedure for colour change test: Crabs are placed with their home tanks in either a white or black environment for 21 hours. A first picture is taken before the crab is transferred to the opposite environment, either black or white, for 2 hours, after which a second picture is taken. Both pictures are analysed in ImageJ© to obtain the raw colour change and then calculate the absolute and relative colour change.

2.3.2. Locomotor behaviour

Crabs were gently transferred from their tanks to the test arena placed under a black tent (Neewer®) to avoid any external stimuli. The test arena was a circular openfield arena (Ø 11 cm, height 5 cm) with white walls and grey floor. The circular shape avoided that crabs place themselves in corners and stop moving, which would prevent measuring locomotor behaviour. The test began when the crab was placed in the centre of the test arena (Ø 4.5 cm) and filmed for 5 min with a HDR-CX240E camcorder (Sony, Tokyo, Japan) installed at 40 cm above the test arena, placed in the middle of the circular LED lamp (Neewer®) (as described in 2.3.1.). Video-recordings were analysed *a posteriori* with Ethovision XT® (Noldus, Wageningen, The Netherlands). Locomotor behaviour was assessed through different parameters: the total distance moved (cm), the mean velocity (cm/s), the time spent at the periphery of

the arena (s and the total time spent moving (s). The different parameters of Ethovision XT® were set to: sample rate = 5/s; smoothing = 3; minimum distance moved = 0.15 cm; maximum distance moved = 3 cm; threshold = 0.75 – 1; averaging interval = 3 samples.

2.4. Statistical analysis

Data were analysed with R Studio (Version 1.1.453 – © RStudio, Inc., Boston, USA, <https://www.r-project.org/>) and Statistics Calculators (<https://www.socscistatistics.com/>). Results are expressed as means \pm standard error of the means (SEM). Non-parametric permutation test was used, as data which did not meet normality and homoscedasticity. We performed a permutation test for paired samples as the same crab was tested at each experimental time point, using the function `ezPerm` from “ez” package. Mixed within-and-between Ss designs were used (*i.e.*, repeated factor: exposure time; independent factor: experimental groups) to analyse absolute and relative colour change, and locomotor behaviour (*i.e.*, normalised velocity; distance moved; time spent in periphery; total time spent moving). The number of iterations was set to 1 000. If the null hypothesis was rejected ($p \leq 0.05$), post-hoc permutation test for paired samples from “RVAidememoire package” (function `pairwise.perm.t.test`) was performed to reveal any differences between experimental groups and between exposure time. For post-hoc tests, false discovery rate (fdr) was applied using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). The permutation tests were complemented with Friedman test to analyse absolute colour change and to test for effects of exposure time within treatment groups, followed by sign test as post-hoc analysis as well as Kruskal-Wallis one-way non-parametric ANOVA to test differences between treatments at each time point, followed by Dunn’s test as post-hoc analysis.

3. Results

3.1. Colour change on opposite background

The sample sizes for juvenile crabs tested for colour change were as follows: (i) from white to black environment: control, $n = 16$; FLX5, $n = 15$; FLXVEN5, $n = 16$; (ii) from black to white environment: control, $n = 12$; FLX5, $n = 11$; FLXVEN5, $n = 11$. The number of crabs were unequal between the different groups and test conditions, *i.e.*, (i) and (ii), because of the loss of individual crabs and data.

3.1.1. White to black environment

Absolute colour change

Within the control group, crabs had a mean MGCV of 108.4 ± 35.1 over the benchmark week, *i.e.*, d-8, whereas during the experimental time course mean MGCV progressively increased to a maximum of 127.4 ± 37.3 at d16 (Table. 1). Indeed, the MGCV of the carapaces after 21 hours adaptation to a white background turned out to differ significantly over the experimental time course ($p = 0.004$). Post-hoc permutation test showed a difference of MGCV between d-8, *i.e.*, the benchmark week, and each of the experimental time points (d-8-d2, $p = 0.02$; d-8-d9, d-8-d16 and d-8-d23, $p = 0.04$, respectively).

Consequently, the control crabs were displaying a brighter carapace over time. Following the 21 hours on white background, crabs from the control groups were able to get darker when placed on black background for 2 hours, but they were displaying a less dark carapace over time. After 2 hours on black background, crabs had a mean MGV of 90.0 ± 26.8 at d-8 whereas at d2, d9, d16 and d23 mean MGV increased progressively (Table. 1). As a matter of consequence, the MGV on black background after 2 hours changed significantly over the experimental time course ($p = 0.001$). Post-hoc permutation test showed a difference of MGV between d-8 and d16 as well as d23 (d-8-d16, $p = 0.04$; d-8-d23, $p = 0.05$). In summary, the control group improved its capacity to display a high MGV, *i.e.*, paler carapace, over time. On the other hand, the capacity to adapt to a darker background with a lower MGV, *i.e.*, darker carapace, diminished throughout the entire experimental time course as a result of the general trend for the crabs to become brighter during the experimental period.

Concurrently, the FLX5 group showed the same pattern as the control with an average MGV of 105.5 ± 30.4 at d-8, while it increased at d2, d9, d16 and d23 (Table. 1). The MGV on white background after 21 hours changed significantly over time ($p < 0.001$). Post-hoc permutation tests demonstrated a significant difference of MGV between d-8 and each of the experimental time points, *i.e.*, d2, d9, d16, d23, (d-8-d2, $p = 0.01$; d-8-d9, $p = 0.007$; d-8-d16, $p = 0.007$; d-8-d23, $p = 0.007$). As for control group, the carapaces of crabs from FLX5 group were becoming brighter over time. Furthermore, the MGV on black background after 2 hours was significantly different over the experimental time course ($p = 0.002$). Post-hoc permutation test showed a difference of MGV between d-8 and d16, d23 (d-8-d16, $p = 0.048$; d-8-d23, $p = 0.04$), but also between d2 and d16, d23 (d2-d16, $p = 0.04$; d2-d23, $p = 0.04$). Alike the control group, the increasingly paler carapace after 21 hours in a white environment reduced the capacity of the FLX5 group to adapt to a darker background.

Within FLXVEN5 group crabs displayed a more stable MGV over time on either white or black environment (Table. 1). Incidentally, neither did the MGV on white background after 21 hours change significantly over time ($p = 0.918$), nor did the MGV on black background after 2 hours ($p = 0.419$). This means that the evolution of a brightening carapace observed over experimental time course in the control and FLX5 groups did not occur in the FLXVEN5 group.

Overall, the control and FLX5 group showed a capacity to brighten after 21 hours on white background, with an improved capacity to get paler whereas FLXVEN5 group showed stable changes of colour over time (Table. 1).

Table 1. Mean Grey Values (MGVs) of juvenile crabs' carapace from the three experimental groups: control, FLX at $5 \text{ ng}\cdot\text{L}^{-1}$ (FLX5) and FLX combined with VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ (FLXVEN5) each after being placed in a white environment for 21 hours and subsequently transferred to a black environment for two hours. Benchmark week (d-8) and experimental time points d2, d9, d16 and d23. MGVs are expressed as means \pm SEM ($n = 15-16$); * indicates a significant difference between the benchmark week and

experimental time points; ** indicates a significant difference between d2 and other experimental time points (permutation test, pairwise post-hoc permutation test with fdr correction)

21h in white environment

	d-8	d2	d9	d16	d23
CONTROL	108.4 ± 35.1	121.1 ± 37.1 *	123.2 ± 38.8 *	127.4 ± 37.3 *	127.2 ± 37.1 *
FLX	105.5 ± 30.4	125.8 ± 32.3 *	127.6 ± 35.2 *	133.7 ± 25.9 *	132.9 ± 29.0 *
FLXVEN	110.0 ± 32.8	122.0 ± 29.8	117.5 ± 36.8	109.7 ± 31.9	112.4 ± 32.9

2h in black environment

	d-8	d2	d9	d16	d23
CONTROL	90.0 ± 26.8	91.9 ± 30.2	93.6 ± 23.4	102.7 ± 24.6 *	103.4 ± 23.7 *
FLX	89.5 ± 23.5	89.5 ± 20.1	98.2 ± 25.1	103.7 ± 20.7 **	102.6 ± 20.4 **
FLXVEN	85.0 ± 20.9	102.0 ± 20.1	97.4 ± 19.1	94.0 ± 17.5	94.7 ± 16.8

Percent colour change

Untreated white adapted animals from both the benchmark week and the controls from d2 to d23 became about 18% darker when being placed for 2h in a black environment, but the individual variances were quite high (18.5%±9.51 SEM). During the benchmark week, the capacity of colour change was similar for all treatment groups (Kruskal-Wallis, p=0.910). In the FLX5 group, colour change varied significantly between the benchmark week and two of the experimental time points (Friedman, p = 0.048): the MGV increased by 14 percentage points to 27,8% on d2 and by 10 percentage points on d23 to 23.81% relative to the benchmark week, leading to significant differences between d-08, d2 and d23 (sign test, p=0.020 for d-08-d23 and d2-d23; table S1). A slight increase could be also observed for the control group, but without being statistically significant (Friedman, p = 0.338). No increase, as compared to the benchmark week, was discernable for the FLXVEN5 group (Friedman, p = 0.663). The strong increase in colour change following FLX5 exposure and the largely unchanged coloration of the animals treated with FLXVEN5 lead to significant differences between the treatment groups (Kruskal Wallis, p = 0.009). Dunn's post hoc test revealed a significant difference in the capacity to change colour between the FLX5 and the FLXVEN5 group at the beginning of the exposure, *i.e.*, d2 (14.05% *vs.* 27.80%, respectively; p = 0.0125). The average colour change also differed between the treatment groups at the end of the experiment, *i.e.*, d23, with 19.71%±0.86 SEM for the controls, 23.81%±2.62 SEM for the FLX5 animals and 15.39%±11.78 SEM for the FLXVEN5 group (Dunn's test, C *vs.* FLX5, p = 0.004 and FLX5 *vs.* FLXVEN5, p = 0.032, respectively).

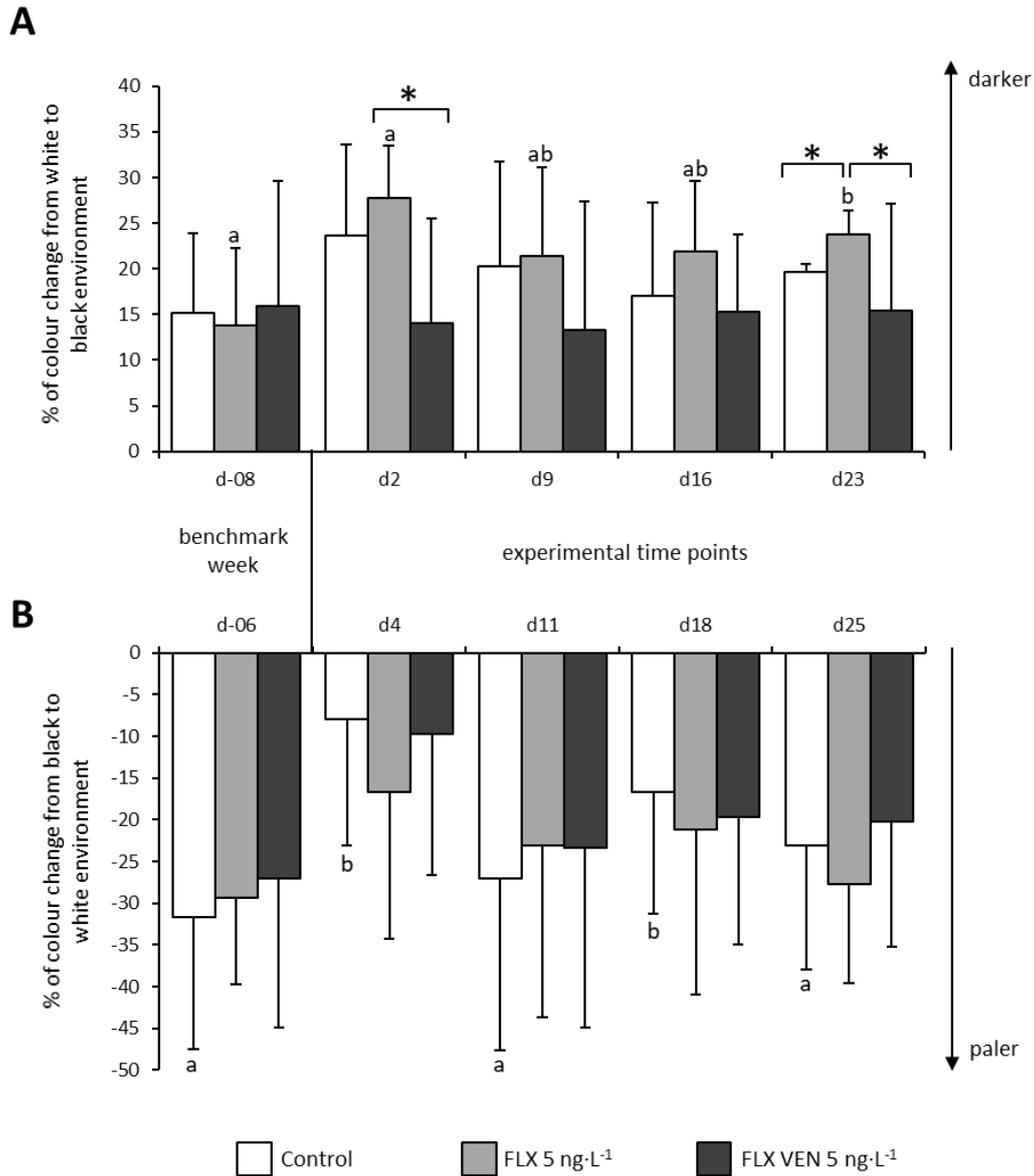


Figure 4. Percent colour change of juvenile crabs' carapace, expressed as a percentage of colour change, from the three experimental groups: control, FLX at 5 ng·L⁻¹ (FLX) and FLX combined with VEN at 5 ng·L⁻¹ (FLXVEN) each after being placed either (A) from white to black, and (B) from black to white environment. Means ± SEM; n = 11-16; * indicate significant differences between experimental groups at p ≤ 0.05; the letters a and b indicate a difference between time points at p ≤ 0.05 (permutation test, pairwise post-hoc permutation test with fdr correction)

Normalised colour change

Overall, the normalised colour change between white and black environment did differ over the entire exposure time (p = 0.785) (**Fig 5.A**). Albeit a constantly lower normalised colour change was observed for the FLXVEN5 group, in the majority of cases no significant difference between the treatments could be obtained, except for d16 (Kruskal Wallis, C_{d16} vs. FLX_{d16} vs. FLXVEN_{d16}, p = 0.046). At this time

point, the difference between the FLX5 and the FLXVEN5 group, which could be observed throughout the experiment, became statistically significant (Dunn's test, $p = 0.039$). The generally high variability in the data sets, effectively prevented statistical significances for most of the obvious differences in the colour change of animals exposed to FLX5 on the one hand, and FLXVEN5 on the other hand.

3.1.2. Black to white environment

Absolute colour change

In the control group, the MGV after 21h in black environment was 85.3 ± 21.5 during the benchmark week, *i.e.*, d-6, and kept increasing up to 102.1 ± 23.0 at d25 (Table. 2). Therefore, the MGV on black background after 21 hours changed significantly over time ($p = 0.002$). Post-hoc permutation tests showed a difference of MGV between d-6 and d18 as well as d25 (d-6-d18, d-6-d25, $p = 0.02$, respectively), as well as between d11, d18 and d25 (d11-d18, d11-d25, $p = 0.02$, respectively). A trend to become brighter may be noticed between d4, d18 and d25 (d4-d18, $p = 0.056$; d4-d25, $p = 0.067$). Thus, crabs from control group were exhibiting poorer colour change on black background, *i.e.*, getting less dark over time. Correspondingly, the MGV on white background after 2 hours changed significantly over time ($p = 0.002$). Post-hoc permutation tests only highlighted a trend between d-6 and d18 as well as d26 (d-6-d18, $p = 0.08$; d-6-d25, $p = 0.07$). Because the crabs adapted to a black background over 21 hours became brighter over time, alike the crabs that were adapted to a white background after 21 hours, the crabs from control group were able to improve their MGV when placed for 2 hours on a white background, *i.e.*, they were getting brighter during the experiment. Generally, the values corresponded to those that were observed when crabs were kept for 21 hours on a white background (Table. 2).

Although the groupwise permutation test indicated a significant change of the MGV in the FLX5 group after 21 hours over time ($p = 0.002$), the post-hoc permutation tests did not demonstrate any significant difference between the experimental time points. Similarly, the MGV on white background after 2 hours was significantly different over time ($p < 0.001$) across all experimental time points, but post-hoc permutation test failed to reveal which of the time points were significantly different.

For the FLXVEN5 group, the MGV on black background after 21 hours was not significantly different ($p = 0.057$), even if a trend towards an increased MGV over time may be noticed. The MGV on white background after 2 hours was significantly different ($p = 0.008$), but post-hoc permutation test failed to highlight any significant difference between time points.

Overall, crabs from the control group expressed a higher MGV over time, *i.e.*, displaying a paler carapace, even when placed on black background. Tendentiously this was the case in the exposed crabs as well. For all groups, the MGV at d4 was comparatively low, which is likely to be responsible for the significant over time differences.

Table 2. Mean Grey Values (MGV) of juvenile crabs' carapace from the three experimental groups: control, FLX at 5 ng·L⁻¹ (FLX5) and FLX combined with VEN at 5 ng·L⁻¹ (FLXVEN5) after being placed in a black environment for 21 hours and then transferred to a white environment for 2 hours. Benchmark week, *i.e.*, d-6, and the experimental time points, *i.e.*, d4, d11, d18 and d25. MGV are expressed as mean ± SEM (n=11-12). * indicates a significant difference between the benchmark week and experimental time points; ** indicates a significant difference between d11 and other experimental time points (permutation test, pairwise post-hoc permutation test with fdr correction)

21h in black environment

	d-6	d4	d11	d18	d25
CONTROL	85.3 ± 21.5	72.9 ± 45.0	91.5 ± 21.6 *	105.4 ± 20.5 * / **	102.1 ± 23.0 * / **
FLX	94.7 ± 23.1	68.5 ± 42.2	101.3 ± 25.5	106.7 ± 18.6	99.9 ± 18.6
FLXVEN	82.5 ± 22.7	78.4 ± 41.5	92.0 ± 21.9	99.6 ± 23.0	95.3 ± 20.8

2h in white environment

	d-6	d4	d11	d18	d25
CONTROL	113.3 ± 33.1	82.4 ± 57.5	117.0 ± 37.4	125.2 ± 36.6 *	127.0 ± 38.0 *
FLX	122.8 ± 33.9	85.0 ± 58.6	122.8 ± 30.5	128.8 ± 30.7	127.8 ± 28.8
FLXVEN	106.0 ± 36.8	86.7 ± 51.9	114.1 ± 40.0	119.9 ± 36.6	115.6 ± 34.7

Percent colour change

In average, all black adapted animals became about 18% brighter when being placed for 2h in a white environment (18.5% ± 3,16 SEM; fig.4). Some instability in the capacity of colour change from black to white could be noted in all treatment groups: there was a notable depression in their ability of becoming brighter at d4 as well as a slightly reduced amplitude at d18. However, these fluctuations over time were statistically significant in the control group only (Friedman, p = 0.003; sign test, d-06 vs. d4 and d-06 vs. d18, p = 0.004, respectively). No significant effects of the antidepressant treatments on the capacity of becoming brighter when adapted to a black background could be detected for any of the time points.

Normalised colour change

The experimental time course had a significant effect on colour change between white and black environment (p = 0.001), but without any effect of treatment (p = 0.984) (**Fig 5. B**). Pairwise post-hoc permutation test highlighted a difference between d4 and d11, d18, d25 (d4-d11, p = 0.006; d4-d18, p = 0.036; d4-d25, p = 0.006). Hence, the crabs of all experimental groups were continuously getting paler

over time relative to the benchmark week. The antidepressant treatment, notably in the FLXVEN5 group, may have advanced this process slightly, but the variability in colour change was much too high to obtain any statistical significance in the differences between the groups.

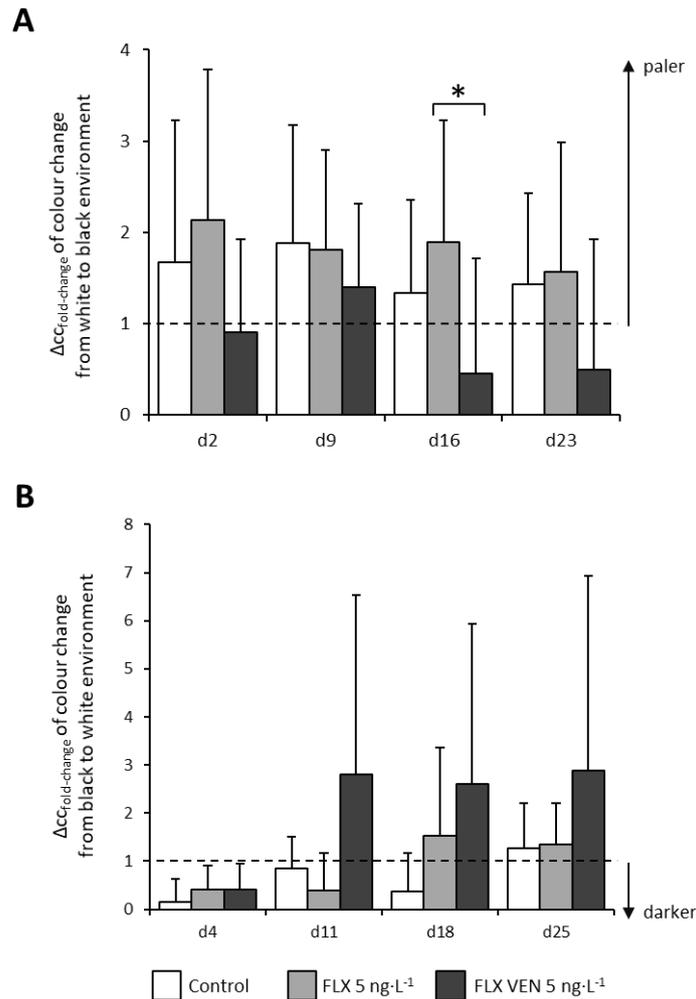


Figure 5. Relative colour change expressed as $\Delta cc_{\text{fold-change}}$ between two opposite backgrounds by juvenile crabs exposed to FLX alone at 5 ng·L⁻¹ (FLX) or in mixture with VEN at 5 ng·L⁻¹ each (FLXVEN), or in control condition; (A) from white to black, and (B) from black to white environment. Colour change test was done over four time points. The dotted line represents the baseline corresponding to the averaged individual reference data to which colour change values were normalised with the values obtained for the benchmark week (for details see text). Means \pm SEM; n = 11-16; * indicate a significant difference ($p < 0.05$) between treatment groups (permutation test, pairwise post-hoc permutation test with fdr correction)

3.2. Locomotor behaviour

The sample sizes for juvenile crabs tested for the locomotor behaviour test were 14 in each experimental group.

Distance moved

During the benchmark week, crabs moved in average a distance of 425.86 ± 134.34 cm during the 5 min test ($n = 42$). Following exposure, the distance moved was significantly different between groups ($p = 0.009$). Pairwise post-hoc permutation test highlighted differences between FLXVEN5 and the control and FLX5 groups (FLXVEN5-control, $p = 0.012$; FLXVEN5-FLX5, $p = 0.006$) (Fig. 5). Crabs from FLXVEN5 group walked a longer distance than controls or FLX5 group at all experimental time points. Furthermore, the distance moved was significantly different over time ($p = 0.037$). Notwithstanding, pairwise post-hoc permutation test did not show a significant difference over time, but a trend between d3 and d17 ($p = 0.072$). This result is likely to be associated with an increasing intra-group variability in the FLXVEN5 group at d17 and d24 ($d3 = 1.56 \pm 1.22$, $d10 = 1.76 \pm 0.78$, $d17 = 2.25 \pm 1.59$, $d24 = 2.49 \pm 2.69$). Nevertheless, one may notice that crabs from FLXVEN5 showed in average an increasing distance moved over time, while control and FLX5 group exhibited a more stable variability during the entire exposure time.

Velocity

The average velocity of crabs during the benchmark week was 1.42 ± 0.45 cm/s ($n = 42$). The velocity differed significantly between experimental groups ($p = 0.008$). Pairwise post-hoc permutation test demonstrated that FLXVEN5 group had a greater velocity than the control and the FLX5 group (FLXVEN5-control, $p = 0.006$; FLXVEN5-FLX5, $p = 0.006$) (Fig. 6). Crabs exposed to FLXVEN5 walked in average at a greater velocity than control or FLX5 groups. In addition, velocity was significantly different over time ($p = 0.030$). However, pairwise post-hoc permutation test did not show any significant difference between time points, but revealed tendencies between d3 and d17, d24 ($d3$ - $d17$, $p = 0.072$; $d3$ - $d24$, $p = 0.096$). As for the distance moved (see above), this result is linked to the increasing intra-group variability in FLXVEN5 group over time. It may be noticed as well that control and FLX5 groups kept roughly the same variability throughout the entire experimental time course.

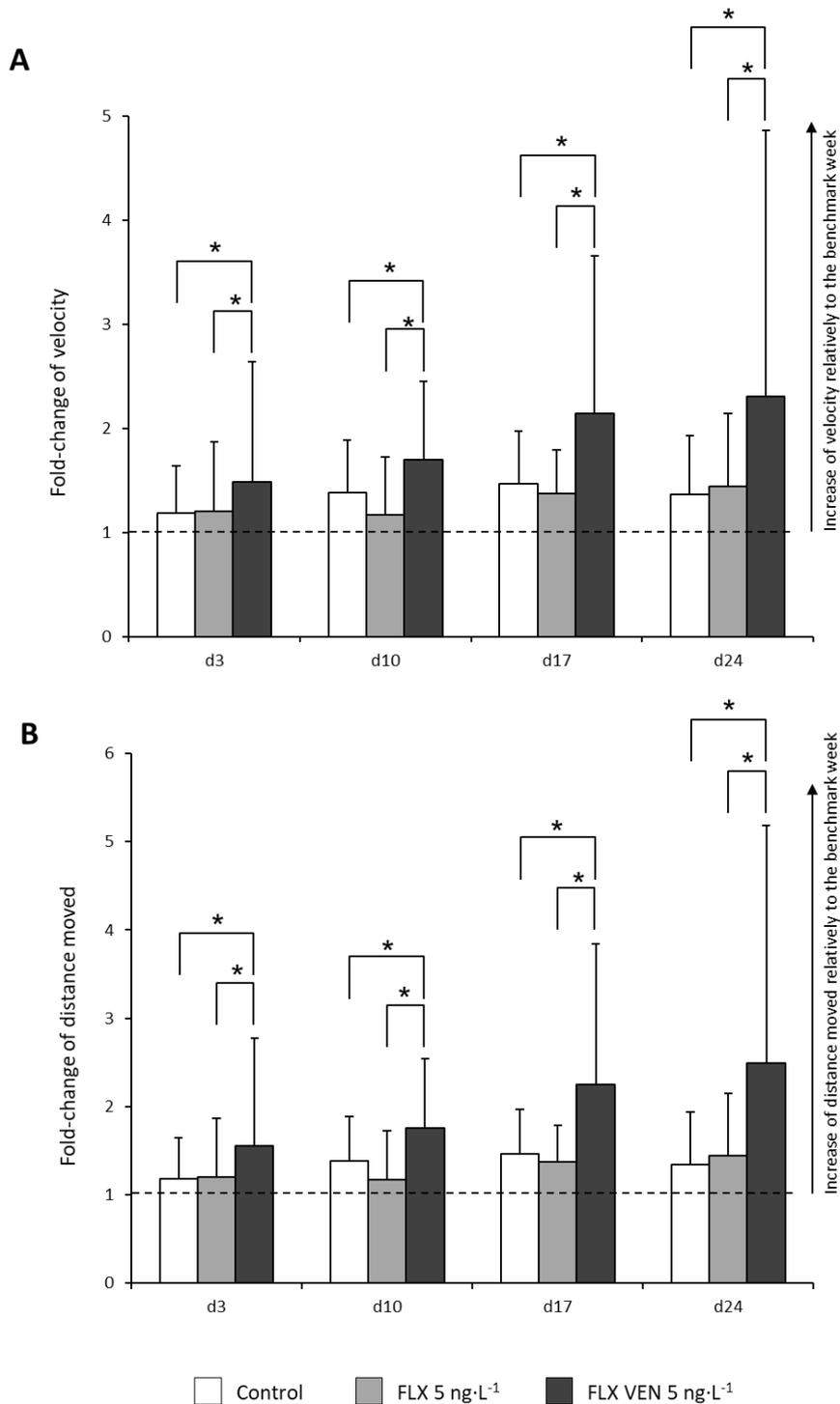


Figure 6. Velocity (A) and distance moved (B), expressed as fold-change, of juvenile crabs during a 5 min openfield test over four time points of exposure to FLX alone at 5 ng·L⁻¹ (FLX5), in mixture with VEN at 5 ng·L⁻¹ each (FLXVEN5), or in control condition. Values are averaged individual data normalised with values obtained from the benchmark week (for details see text). The dotted line represents the baseline corresponding to the benchmark week. Means ± SEM; n = 14; *, significantly different between experimental groups at $p \leq 0.05$; the p-value ($p =$) indicates a trend towards a difference

with the time points labelled with an a (permutation test, pairwise post-hoc permutation test with fdr correction)

Time spent in the periphery

During the benchmark week, crabs spent the majority of their time in the periphery of the openfield arena: 296.30 ± 11.54 s. Following exposure, the time spent in the periphery remained stable between groups ($p = 0.378$) and for any of the time points during the experimental time course ($p = 0.764$). Crabs from all groups spent the vast majority of their time in the periphery of the openfield arena.

Total time spent moving

During the benchmark week, juvenile crabs spent in average 162.86 ± 43.28 s moving in the openfield arena. The total time spent moving was significantly different between groups ($p = 0.036$). Pairwise post-hoc permutation test showed that FLXVEN5 group spent more time moving than controls and FLX5 group (FLXVEN5-control, $p = 0.015$; FLXVEN5-FLX5, $p = 0.015$) (Fig. 7 B), in line with results from the parameters distance moved and velocity. A trend to increase the total time spent moving emerged over time ($p = 0.072$), which followed the same pattern as observed for velocity and distance moved. Moreover, a higher intragroup variability was observed in FLXVEN5 group, whereas in control and FLX5 group it stayed stable.

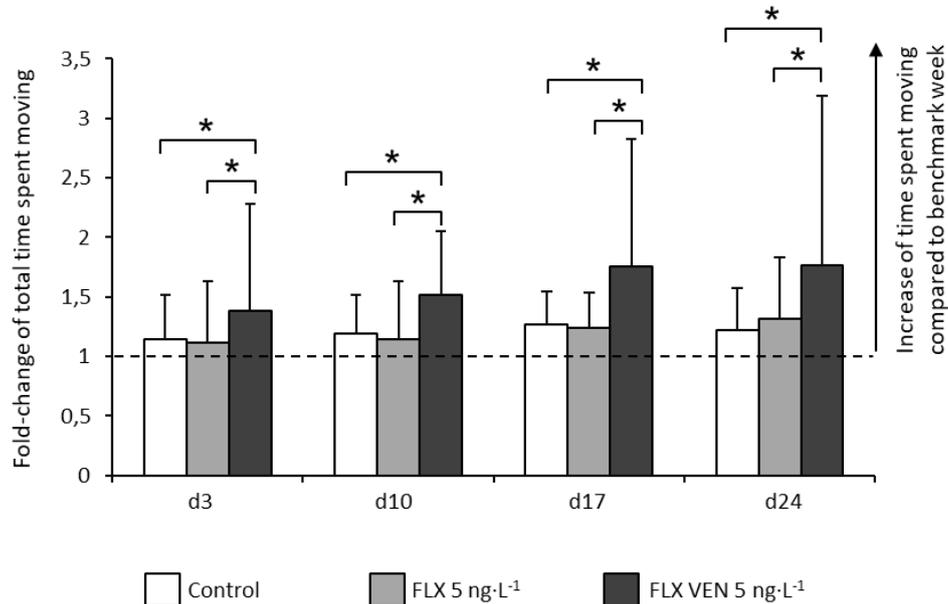


Figure 7. Time spent moving by juvenile crabs during a 5 min openfield test expressed as fold-change over four time points of exposure to FLX alone at $5 \text{ ng}\cdot\text{L}^{-1}$ (FLX5) or in mixture with VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ each (FLXVEN5), or in control condition. Dotted line is representing the baseline of reference data. Values are normalised and expressed as means and SEM. * indicates a significant difference between experimental groups $p \leq 0.05$ (permutation test, pairwise post-hoc permutation test with fdr correction).

In summary, the data suggest effects of antidepressants on colour change, particularly for the FLXVEN5 treatment. The extreme variability and partly opposing responses rendered statistically significant differences impossible. Nevertheless, the pattern of colour change, *i.e.*, brightening of the carapace over time, expressed by the control and FLX groups was not observed in FLXVEN5 group.

By contrast, the antidepressant treatment clearly and significantly affected locomotor behaviour. The FLXVEN5-exposure resulted in a significantly higher velocity, a greater distance moved and more time spent moving as compared to the non-exposed controls or the FLX5-treatment throughout the entire experimental period, *i.e.*, from d3 to d24.

Discussion

Juvenile crabs, which are particularly prone to predation, have to adapt their behaviour in order to survive. They can do this either by changing the colour of their carapace in relation to their environment, or by habitat choice, *i.e.*, by actively moving to an adequate environment the background of which matches their colour pattern. If camouflage is not efficient, they need to flee from their predators. Both, habitat choice and fleeing require locomotor activity, thus providing a link between colour change and locomotion.

Colour change on opposite background

When juvenile shore crabs were placed from the opposite background into an either black or white environment, they were able to adapt their luminance, either becoming darker or whiter over two hours, as previously demonstrated by Stevens et al. (2014a). When white adapted animals were placed on a black background, they became about 20% darker in average, a value consistent with the magnitude of colour change observed in the transparent *Crangon crangon* (Siegenthaler et al., 2018). The amplitude of colour change was, however, extremely variable ranging from half to double of the average colour change. This high inter-individual variability in the capacity of becoming darker or brighter may be explained in part by the highly polymorphic morphological colouration of the cuticle, which overlays the physiological colour change of the chromatophores situated in the epidermis. Notwithstanding, high variability in colour change has been repeatedly reported for various crustacean species, including species with transparent cuticles, and is likely to relate to both, the complexity of the response (Rao, 2001) and numerous external and internal factors that are influencing it, amongst them stress (Detto et al., 2008; Hanumante and Fingerman, 1981b; Hanumante and Fingerman, 1982; Siegenthaler et al., 2018). Moreover, the impression that animals were becoming paler over time could derive from the artificial lighting to which they were continuously adapting (Siegenthaler et al., 2018). Many crustaceans become darker during the day, protecting them with melanin against UV radiation (Fuhrmann et al., 2011). In the experimental environment, UV light was, however, not a factor that would have triggered darkening.

Animals exposed to antidepressants seemed to be affected in their capacity to respond to darker, but not to lighter coloured backgrounds. Dark-adaptation appeared to stabilise towards the end of the experiment at around 20% in the control animals ($19.71\% \pm 0.86$ SEM) while the group treated with FLX at $5 \text{ ng} \cdot \text{L}^{-1}$ seemed to stimulate colour change transiently, indicated by a significant darkening by 27% at d2 ($27.80\% \pm 5.70$ SEM) and a significantly higher normalised colour change at d16 compared to the FLXVEN5 treatment. The latter group, *i.e.*, FLX and VEN at $5 \text{ ng} \cdot \text{L}^{-1}$ each, clearly demonstrated attenuated colour change. Firstly, the animals did not become as pale as those of the two other treatments when adapted to a white background for 21 hours. Secondly, they did not darken as much as the controls and, notably, the FLX treated animals (Tab. 1). Accordingly, the mean colour change in the FLXVEN5 group was only about 15% at all time points (14.78 ± 11.94) and the normalised colour change was mostly lower than that of the benchmark week, *i.e.*, <1 fold colour change. Both, the stimulation in the FLX5 and the attenuation in the FLXVEN5 group led to significant differences between the two treatments.

In a series of experiments Fingerman et al. (1981) as well as Hanumante and Fingerman (1981b, 1983) investigated the role of the monoamine neurotransmitters 5-HT, DA and NE on the antagonistic action of red-pigment dispersion and aggregation as well as on melanin dispersion (Hanumante and Fingerman, 1982; Hanumante and Fingerman, 1982), with the respective neuropeptide hormones being involved in the colour change of *Uca pugilator* and *Macrobrachium ohione* (reviewed in Hanumante and Fingerman, 1982) and other crustaceans. In their studies they also used 5-HT potentiators, such as FLX (Hanumante and Fingerman, 1983b). They found that FLX accelerated the dispersion of pigments in the erythrophores when crabs were transferred from a white to a black background and that it potentiated the indirect action of 5-HT as a neurotransmitter of colour change in *U. pugilator* and *M. ohione*. On the contrary, FLX slowed down the concentration of the pigments when crabs were transferred from a black to a white background. The latter could, however, not be confirmed in the present study. Hanumante and Fingerman (1982) further demonstrated that drugs supposed to reduce norepinephrine levels reduced the dispersion and increased the aggregation of melanin. By using different pharmaceuticals that either inhibit or promote monoamine release, decrease or increase neurotransmitter concentrations, or block their respective receptors, these authors could demonstrate that the monoamines act indirectly on colour change *via* the nervous system, thus triggering the release of pigment dispersing and concentrating neuropeptide hormones. It could also be shown that the different monoamines act differentially on the dispersion or aggregation of the pigments contained in the different types of chromophores: 5-HT elicited pigment dispersion in erythrophores *via* PDH, which could be amplified by the additional administration of FLX (Hanumante and Fingerman 1981). Our data suggest that this was also the case in the *C. maenas* treated with $5 \text{ ng} \cdot \text{L}^{-1}$ waterborne FLX in the present study, as the maximum colour change for all treatments and time points was observed for FLX5 at d2, both as % colour change (27%) and fold change relative to the benchmark value (approximately twofold).

Moreover, the FLX5 group displayed the highest amplitudes of colour change throughout the entire exposure period, being in average 8% above their respective reference value (data not shown).

Other monoamines also intervene in the control of chromatophores and colour change. For instance, DA promotes pigment concentration *via* RPCH in the erythrophores (Hanumante and Fingerman 1983) and NE—or rather octopamine—elicits pigment dispersion in melanophores, which may have interfered with the response to a white environment after dark-adaptation, but may have also affected the adaptation to a white environment before being transferred to a black background. In addition, other neuropeptides, such as the crustacean cardioactive peptide (Nery et al., 1999) or corazonin (Porras et al., 2003), may act on pigment dispersion and aggregation within the chromatophores. How monoamines regulate these neuropeptides in crustaceans, such as *C. maenas*, remains to be elucidated. Nery *et al.* (1999) discovered that crustacean cardioactive peptide acted as a full agonist on pigment dispersion in the erythrophores of the freshwater shrimp *Macrobrachium potiana*. In the crayfish *Procambarus clarki*, Porras *et al.* (2003) demonstrated an antagonistic action of corazonin on erythrophores and leucophores, with corazonin promoting pigment dispersion in leucophores together with a concurrent aggregation of pigments in the erythrophores (Dirksen, 2013). In line with these findings, Alexander et al. (2018) did not observe any activity of corazonin on pigment dispersion in *C. maenas* adapted to a white background, *i.e.*, with maximal aggregation of red pigment, but it might have maintained the leucophore pigment granules fully dispersed, which was not investigated by these authors. Overall, the regulation of colour change in crustaceans consists of a complex system of three to four types of chromatophores in which different neuropeptide hormones elicit either dispersion or aggregation of black/dark brown, red and/or yellow as well as white pigments. The release of these neuropeptides, in turn, is regulated by different monoamines, involving 5-HT, DA and NE/octopamine. As a matter of consequence, when investigating the action of specific monoamines, such as 5-HT, on specific chromatophores, such as erythrophores, for instance, relatively clear responses may emerge, which are coherent with the pharmaceutical action of drugs that act on the respective neurotransmitter levels, such as FLX (Hanumante and Fingerman, 1981a). If, however, integrated quantitative measures of all types of chromatophores are conducted, the responses to compounds that interfere with the monoaminergic regulation of chromatophorotropins and the resulting colour change might emerge less clearly.

It should be further noted that the above mentioned studies used a semi-quantitative chromatophore assay and required high drug levels, *i.e.*, a dose of 20 µg FLX, that was directly injected into the animals in order to obtain significant results. The present study, however, used low environmental levels of antidepressants, which in combination may provoke the concurrent release of different chromatophorotropins. Theoretically, FLX alone should have stimulated the colour change when placing crabs from a white background, on which red pigment and melanin should be fully aggregated, to a black background, which favours the dispersion of the dark pigments. Indeed, the relatively strong colour change in the FLX5 treated animals, notably at d2, conforms to this expectation. The combination

of the two antidepressants FLX and VEN, however, obviously produced the opposite effect. The results rather indicate a significant decrease in the amount of darkening of white-adapted crabs treated with FLXVEN5 when being transferred to a black environment. Actually, this treatment group displayed the lowest overall MGVs when adapted to either black or white environments (tab. 1 and tab.2). As a matter of consequence, the capacity to change colour, either as ΔCC , or % of the absolute MGVs, when transferred from a white to a black environment —to which they perfectly adapted— was reduced, simply because these animals did, in average, not become as pale as the controls or the FLX5 group. Assuming a persistent effect of VEN on NE/octopamine and thus on pigment dispersion in the melanophores, this may have overruled the more dynamic changes due to FLX exposure and attenuated white adaptation.

Locomotor behaviour

Juvenile shore crabs exhibited a stimulation of their locomotor behaviour in presence of FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ each, whereas no effect of FLX alone was observed. Distance moved, velocity and total time spent moving increased significantly through all time points of exposure already beginning at day 3. This shows that a short exposure to very low concentrations of only two antidepressants can have a significant impact on behaviour. Moreover, the increase of locomotion tended to rise over the 24 days of exposure, which may be indicative of an accumulation of the antidepressants (Robert et al., 2017). Our findings are in line with a previous study from Mesquita et al. (2011) that demonstrated an increased distance moved and time spent moving in *C. maenas*. Velocity, however, was not affected in that study after an exposition of 7 days to FLX and the concentrations used in the study of Mesquita et al. (2011) were much higher, *i.e.*, 120 to $750 \text{ }\mu\text{g}\cdot\text{L}^{-1}$, but with adult crabs of reasonable size, *i.e.*, $41.6 \pm 0.97 \text{ mm}$. Similar effects were observed in the shore crab *Hemigrapsus oregonensis*, which displayed a more active behaviour after an exposure to FLX at $30 \text{ ng}\cdot\text{L}^{-1}$ for 7-9 weeks, even in the presence of a predator, making them more prone to predation risk (Peters et al., 2017). In amphipods such as *Echinogammarus marinus* and *Gammarus pulex* exposure to FLX, at 1 - $100 \text{ ng}\cdot\text{L}^{-1}$, significantly increased their velocity after one day or 14 days, respectively (Bossus et al., 2014; De Castro-Català et al., 2017). In aquatic snails, *Urosalpinx cinerea* and *Lithopoma americanum*, exposure to VEN boosted the locomotion, while FLX reduced it after only few hours at high concentrations, $31.3 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ and $345 \text{ }\mu\text{g}\cdot\text{L}^{-1}$, respectively (Fong et al., 2015). On the contrary, other studies showed no effect or opposite effects in various species. Indeed, an acute exposure to FLX at 5 and $25 \text{ mg}\cdot\text{L}^{-1}$ for 15 min of the crab *Pachygrapsus crassipes* did not demonstrate any significant difference in the time spent moving after (Hamilton et al., 2016a), but the time of exposure may have been too short to provoke any behavioural impairment, albeit the very high concentrations. In another study, the freshwater crayfish *Procambarus virginalis* exhibited no alteration of locomotor behaviour when exposed to $1 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ of VEN (Kubec et al., 2019). By contrast, in the rusty crayfish, *Orconectes rusticus*, Tierney et al. (2016) observed a decrease in locomotor activity after an exposure to FLX at 2 and $500 \text{ ng}\cdot\text{L}^{-1}$ for 10 days. In *Gammarus pulex*, the exposure to a similar

range of concentrations, *i.e.*, 10-100 ng·L⁻¹ of waterborne FLX, caused a decrease in swimming activity after 35 min of exposure (De Lange et al., 2006). A study on tadpoles showed that exposition to 0.5 µg·L⁻¹ of FLX or VEN reduced their swimming in response to alarm cues (Barry, 2018). A similar behaviour was observed in the Arabian killifish exposed to FLX at 300 ng·L⁻¹ for 7 days: a reduced swimming in the face of a predator alarm chemical (Barry, 2013). In summary, numerous studies investigated locomotor behaviour after an exposure to either FLX or VEN with very different outcomes in relatively similar experimental settings. Concerning thigmotaxis, no effect, neither with FLX alone, nor in mixture with VEN at 5 ng·L⁻¹ was observed in juvenile shore crabs. Two studies corroborate these results: Mesquita et al. (2011) did not observe any significant effect of FLX on thigmotaxis in *C. maenas*. Thompson et al. (2017) showed that after injection of VEN into zebrafish embryos, the larvae did not behave any different from the controls. Hence, thigmotaxis seems to be not affected by the presence of FLX or VEN at environmentally realistic concentrations.

The altered locomotor behaviour observed in juvenile shore crabs is likely to be linked with the modification of the endogenous concentrations of monoamines such as 5-HT or DA, known to have a role in locomotion in various species. McPhee and Wilkens (1989) showed that injection of 5-HT increased locomotor behaviour in *C. maenas*, whereas DA had no effect. Along the same lines, in the terrestrial snail *Helix lucorum* injection of 5-HT stimulated locomotion and accelerated crawling, while DA decreased locomotion and even prevented it in some animals (Pavlova, 2001). On the contrary, in juvenile lobsters *Homarus americanus*, injection of high doses of 5-HT reduced locomotion by lowering coordination (Peeke et al., 2000). In the crayfish *Procambarus clarkia*, injection of low doses of 5-HT reduced locomotion as well (Tierney and Mangiamele, 2001b). As with colour change and crypsis, the complexity of locomotor responses (Jordan et al., 2007), their hormonal regulation—for instance by crustacean hyperglycaemic hormone—as well as their modulation by the various monoamines appears to result in a high variability of locomotor behaviour. Furthermore, it is unclear to which extent the monoamines act directly on basic motor patterns (Harris-Warrick, 2011), and to which extent they promote the release of neuropeptide hormones, such as crustacean hyperglycemic hormone (CHH), which may relate to increased locomotor activity (Aquiloni et al., 2012; Morris et al., 2010). With respect to the latter, the action of neurohormones from the eyestalk is not straightforward. In an environmental setting, these neurohormones seem to regulate persistent circadian and tidal rhythms of locomotor activity, which increases with darkness and high tide (Strauss and Dirksen, 2010; Williams and Naylor, 1969). Eyestalk extracts, which contain a crude mixture of neuropeptides from the sinus gland appeared to depress locomotor activity, whereas eyestalk ablation increased arrhythmic locomotor activity (Williams et al., 1979). On the other hand, CHH is correlated with increased exercise (Morris et al., 2010) and when injected into the crayfish, *Procambarus clarkii*, the animals spent less time motionless (Aquiloni et al., 2012). It is likely that antidepressants, such as FLX, increase the release of CHH (Robert et al., 2016) and that this may equally contribute to an increased locomotor activity. The complexity of

interacting neuromodulators and neurohormones might even be increased when exogenous substances interfere with the fine-tuned regulation of the animal's physiological state and the locomotion. In the present study, however, the combination of two antidepressants at environmentally realistic concentrations resulted in a rather clear-cut increase of locomotor activity. Whether this was due to the elevated concentration of $10 \text{ ng}\cdot\text{L}^{-1}$ of antidepressants in total, or due to the presence of $5 \text{ ng}\cdot\text{L}^{-1}$ VEN in the mixture cannot be reasoned conclusively.

Crabs that move more may increase their vulnerability in face of predators, as altered locomotion may make them more visible, even if their coloration matches the background. On the other hand, higher velocity may increase their chances to escape from predators. But then, increased locomotion may have an impact on metabolism, as it requires more energy over time. In conjunction with decreased food-intake and predatory behaviour in presence of either FLX or VEN alone or in mixture in different species, as indicated by previous studies (Bisesi et al., 2016, 2014a; Gaworecki and Klaine, 2008; Peters et al., 2017), this could lead to impairment of reproduction or growth, processes which need a great allocation of energetic reserves.

Conclusion

Albeit antidepressants may be detected and quantified at concentrations below levels of toxicity in marine environments, they may still have pernicious effects on sensitive physiological parameters and associated behaviour in non-target organisms. Juvenile crabs exposed to environmentally realistic concentrations of cumulated antidepressants clearly exhibited a different pattern of colour change than controls over the experimental time course showing signs of a reduced capacity to adapt to a white environment. Furthermore, significant effects on locomotor behaviour were demonstrated in presence of the two antidepressants. Indeed, distance moved, velocity as well as time spent moving were increased in crabs exposed to the mixture. These results highlight the potentially sub-lethal effects of low concentrations of antidepressants, which may render juvenile crabs more vulnerable to predators by making them less adapted to their environment or more visible by moving too quickly. Yet, the high variability of these behavioural responses, notably of colour change, makes it difficult to assess negative consequences for survival in crustaceans of the apparently heterogeneous intertidal. It will, therefore, be necessary to scrutinise the sources causing this variability and to identify the mechanisms responsible for the variation in cypsis and/or locomotion.

The mixture of antidepressants showed more pronounced effects on colour change and locomotor behaviour than FLX alone. This confirms that the notoriously low concentrations of single antidepressants in the water bodies are not an argument in favour of their innocuousness, but that rather the cumulated concentrations of several antidepressants and some of their active metabolites need to be considered for estimating the risk emanating from this class of emerging pollutants. Further studies are needed to determine the contribution of each single antidepressant and their interaction in mixtures.

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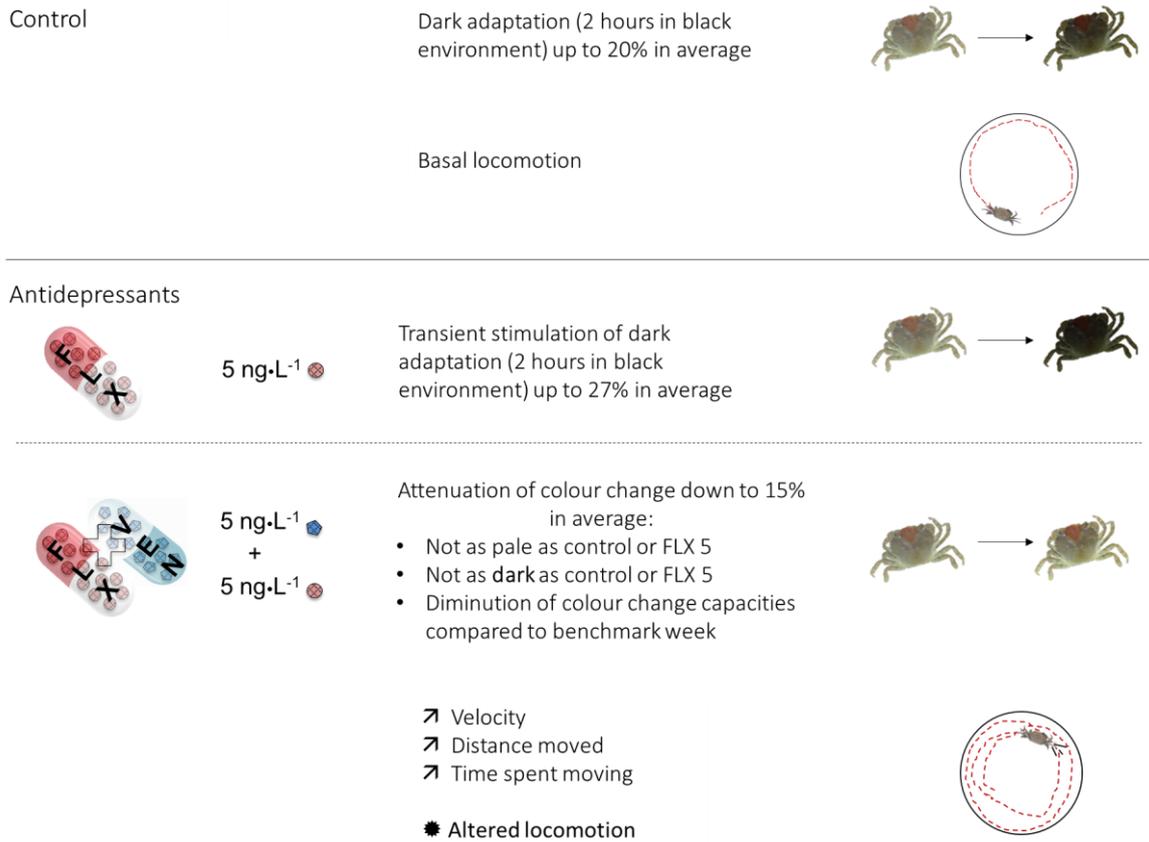


Figure 10. Summary of the major results of the article Effects of environmental antidepressants on colour change and locomotor behaviour in juvenile shore crabs, *Carcinus maenas*.

Article II – Antidepressants modify cryptic behaviour in juvenile cuttlefish at environmentally realistic concentrations

The objective of this study was to analyse the efficiency of cryptic behaviour of juvenile cuttlefish on artificial background designed to elicit two body patterns: uniform and disruptive. Animals were exposed to environmentally realistic concentrations of fluoxetine combined, or not, with venlafaxine during their first month after hatching and their crypsis was monitored weekly.

Antidepressants modify cryptic behaviour in juvenile cuttlefish at environmentally realistic concentrations

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1. Introduction

The common cuttlefish, *Sepia officinalis*, is known to modify its appearance by impressive changes of posture, texture and colour. Indeed, for purposes of communication or camouflage, cuttlefish can display various body patterns on their dorsal side by changing the colour of their mantle as well as their arms and head. In particular, they are able to perform rapid colour change in less than a second depending on the background or situation (Messenger, 2001a).

This rapid colour change in cuttlefish is effected by chromatophores, which are under the control of the nervous system. The chromatophores of cephalopods are specialised cells containing pigments in a sacculus, surrounded by a muscle crown that contracts or relaxes the sacculus (Cloney and Florey, 1968). A contraction pulls apart the sacculus, thus allowing for an expansion and a dispersion of pigments over the surface of the skin, while a relaxation of the muscles will lead to a retraction of the sacculus and a concentration of the pigments. Cuttlefish possess two types of chromatophores: the ‘light’ chromatophores expressing colours ranging from yellow to red and the ‘dark’ chromatophores varying from brown to black (Reiter et al., 2018). The nervous control of chromatophores is influenced by neurotransmitters such as serotonin (5-hydroxytryptamine, 5-HT), glutamate and by various neuropeptides (Loi and Tublitz, 2000; Messenger, 2001a). On the one hand, the rapid relaxation of chromatophores is under the control of 5-HT, while their rapid contraction is brought about by glutamate. On the other hand, a slower and longer lasting contraction is influenced by neuropeptides called FMRFamides (Loi et al., 1996). The chromatophore expansion and contraction are controlled directly by projections from motoneurons in the chromatophore lobes of the brain and, at the highest

level, by the optic lobes (Messenger, 2001a), thereby, related to the perception of the animal's complex environment. As a result, cuttlefish display a rapid and efficient camouflage behaviour.

Because cuttlefish do not benefit of parental care, they need to learn fast on their own how and when to wear an adequate body pattern to hide from potential predators (Dickel et al., 2006). Cuttlefish are already masters of disguise when they hatch from the egg (Hanlon and Messenger, 1988). Even though newly-hatched cuttlefish are able to express complex body patterns, they learn to improve cryptic behaviours while their nervous system is maturing during the first month after birth (Hanlon and Messenger, 1988; Poirier et al., 2005). Cuttlefish can express three main types of chronic body patterns to achieve camouflage: uniform, mottle and disruptive (Hanlon and Messenger, 2018). Uniform patterns, *i.e.*, plain colouration of the whole animal's body, are displayed when the cuttlefish is settling on uniform backgrounds, such as plain sand or rocks. Mottle patterns are characterized by irregular arrangements of small-to-moderate-scale light and dark skin patches. They are displayed on heterogeneous backgrounds with small-to-medium sized light and dark objects, *e.g.*, coarse sand or fine gravel. Disruptive patterns break up the body shape and contours by expressing contrasted large-scale skin components. They may be displayed on heterogeneous habitats, such as coloured pebbles or shells. Mottle and disruptive patterns are often combined to achieve background matching and/or disruptive colouration (Hanlon et al., 2009). Depending on the backgrounds, cuttlefish are able to switch from one pattern to another in milliseconds for dynamic camouflage (Messenger, 2001a).

This high quality camouflage allows the cuttlefish to blend into the environment and stay hidden from predators, but it can also be a strategy to ambush their prey. Thus, any modification of the colour change processes can alter their survival chances. Potential perturbations may derive from a modification of the levels of neurotransmitters involved in the nervous control of chromatophores, such as 5-HT, for instance. Many pollutants detected in marine environments can potentially interfere with the serotonergic system (Mezzelani et al., 2018), but one group of molecules is of particular interest because it is designed to modify 5-HT levels: antidepressants of the class of selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs). Their increasing release in the environment drives from a constant rise of prescriptions (OECD Health Statistics 2019). Consequently, molecules from human excretion or direct disposal of expired pills into water system are found in the aquatic compartments, as wastewater treatment plants lack efficiency to remove these compounds (Bueno et al., 2012). Therefore, residues of antidepressants are continuously detected in concentrations ranging from a few $\text{ng}\cdot\text{L}^{-1}$ to several $\mu\text{g}\cdot\text{L}^{-1}$ in fresh- and marine waters (Gonzalez-Rey et al., 2015; Meador et al., 2016; Metcalfe et al., 2010; Paíga et al., 2016; Schultz et al., 2010). They can be even detected in tap water (Giebułtowiec and Nałęcz-Jawecki, 2014), underlining the persistency of those molecules. Among the various antidepressants, in the present study we focused on two active molecules contained in the most prescribed medicaments and which are, therefore, amongst the most frequently detected ones: fluoxetine (FLX), a SSRI, and venlafaxine (VEN), a SNRI (Fong and Ford,

2014a). FLX was the first SSRI to be commercialised in the 1988 (Pe, 1993) and still is amongst the most prescribed antidepressants. It has thus been extensively studied by ecotoxicologists (Fong and Ford, 2014a; Sehonova et al., 2018), which is why it serves as reference antidepressant in this study. Nearly one decade later, the first SNRIs, with VEN, were commercialised (Zerjav et al., 2009). Both antidepressants are commonly detected in water compartments, but also in various aquatic animals (Fong and Ford, 2014a; Schultz et al., 2010; Silva et al., 2016), even if their concentrations in the water are below detection limits (Klosterhaus et al., 2013; Meador et al., 2016) (Klosterhaus et al., 2013; Meador et al., 2016). Previously, a growing number of studies revealed effects of antidepressants such as FLX and VEN on different behaviours of various marine species: predator and anti-predator behaviour (Bisesi et al., 2014a; Peters et al., 2017), locomotion (Fong et al., 2015; Mesquita et al., 2011), agonistic behaviour (Tierney et al., 2016), photo- and geotaxis behaviour (Guler and Ford, 2010a). Commonly, these studies used much higher concentrations than those detected in water bodies, and they tested single antidepressants only. The environmental exposure scenario is, however, characterised by low $\text{ng}\cdot\text{L}^{-1}$ concentrations of several antidepressants that may, taken together, cumulate to realistic concentrations in the $\mu\text{g}\cdot\text{L}^{-1}$ range (Metcalf et al., 2010). Few studies focused on the effects of environmentally realistic concentration of accumulated antidepressants such as FLX and VEN on different behavioural traits: predator behaviour (Bisesi et al., 2016), predator avoidance (Painter et al., 2009b), reproductive behaviour (Schultz et al., 2011), circadian rhythm (Melvin, 2017). Earlier studies from our laboratory demonstrated impacts of FLX or/and VEN at very low concentrations, *i.e.*, range of 1 to 100 $\text{ng}\cdot\text{L}^{-1}$, on behaviour of cuttlefish: learning and memory processes (Di Poi et al., 2013a), maturation and learning of sand-digging behaviour (Chabenat et al., 2019), predatory behaviour (Chabenat et al., *submitted*), cryptic behaviour and colour change (Bidel et al., 2016a, 2016a; Di Poi et al., 2014).

Juvenile *Sepia officinalis* are developing in coastal areas before starting seasonal offshore migrations. Continuous discharge of various pharmaceutical residues in the vicinity of estuarine outflows may, therefore, influence the physiological and behavioural development of juvenile cuttlefish. Indeed, cuttlefish are known to be highly sensitive to environmental changes with the early-life stages being often more sensitive to pollution (Lacoue-Labarthe et al., 2016). Thus, this study tested the hypothesis that environmentally realistic concentrations of accumulated antidepressants present in the coastal environments affect the colour change of newly-hatched cuttlefish. To test this hypothesis, camouflage response was investigated on artificial backgrounds in *S. officinalis* exposed to two different concentrations of FLX and VEN during the first month after hatching, which is essential for the cuttlefish's development.

2. Material and methods

2.1 Animal rearing and experimental conditions

Clutches of cuttlefish eggs laid by wild animals (*Sepia officinalis*) were collected at various sites in the English Channel off the coast of Luc-sur-mer, France. Eggs of clutches from different females were combined and incubated in four 1000L aquaria with natural seawater at the Centre de Recherches en Environnement Côtier (CREC) of the University of Caen Normandie at Luc-sur-mer, France. The aquaria were supplied by a semi-open flow-through system that renewed 50% of the water body. Water temperature was adjusted to $15\pm 1^\circ\text{C}$ and the photoperiod was set to natural summer conditions (16h light : 8h dark) .

At hatching, individual cuttlefish were immediately isolated in glass beakers (\varnothing 11 cm, height 6 cm), henceforth designated as 'home tank', constituting a semi-static system supplied daily with 250mL of activated charcoal filtered seawater (FSW) at a salinity of 35 PSU. Hatchlings were randomly assigned to either (1) FSW (control), or (2) $5\text{ ng}\cdot\text{L}^{-1}$ FLX (FLX5), or (3) $2.5\text{ ng}\cdot\text{L}^{-1}$ FLX and $2.5\text{ ng}\cdot\text{L}^{-1}$ VEN in combination (FLXVEN2.5) as well as (4) $5\text{ ng}\cdot\text{L}^{-1}$ FLX and $5\text{ ng}\cdot\text{L}^{-1}$ VEN (FLXVEN5) and exposed over a period of 29 days post-hatching (dph). The concentrations of FLX and VEN were renewed together with the daily water changes, which also allowed maintaining appropriate O_2 -levels and low nitrite and nitrate concentrations. Animals were fed daily with one sand shrimp (*Crangon crangon*) until the end of the experiments.

Animal rearing and animal experiments were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments (Protocol approved by the French National Ethical Committee for Animal Experimentation, Agreement number: 035121.02).

2.2 Chemical contamination

FLX and VEN concentrations were chosen to approximate surface water concentrations measured off the coast where the eggs were sampled (Minguez et al. 2016). Final concentrations of either 2.5 or $5\text{ ng}\cdot\text{L}^{-1}$ were obtained from stock solutions of fluoxetine hydrochloride (CAS 56296-78-7, Sigma Aldrich, St. Louis, USA) or venlafaxine hydrochloride (CAS 99300-78-4, Sigma Aldrich) dissolved in FSW. Stock solutions were at $10\text{ }\mu\text{g}\cdot\text{L}^{-1}$ of FLX and VEN, portioned in small aliquots of 0.5, 1 and 2 mL and kept at -80°C until use.

2.3 Behavioural tests

Camouflage responses of the cuttlefish were assessed through the analysis of the body patterns displayed on two different backgrounds: uniform grey or black and white checkerboard. The test apparatus is a custom-made drop-shaped PVC compartment (length 11 cm, height 4 cm, max width 5 cm). Eight independent compartments (static systems) are concentrically arranged to allow the observation of eight individuals, *i.e.*, two animals per exposure condition, simultaneously (Fig. 1). The inside walls and floor of the compartments were either bare medium grey PVC (Fig. 1A), or covered with laminated paper printed with a black and white checkerboard (Fig. 1B) in order to induce a uniform or disruptive pattern response, respectively. The square size of the checkerboard background corresponded to 90% of the cuttlefish dorsal white square (Di Poi et al., 2014). Each cuttlefish passed both behavioural tests at four

different ages: at 7, 14, 21, 28 dph for uniform background, and 8, 15, 22, 29 dph for checkerboard background.

Behavioural tests were conducted in the climate chamber where the cuttlefish were reared and exposed to antidepressants. The tests were performed in a custom-made black tent in order to avoid any external stimuli. A circular LED lamp (Neewer®, Guangdong, China) was positioned 60 cm above the test apparatus. To avoid direct light on cuttlefish, the lamp was placed in an oblique position and the inner surfaces of the tent were lined with white fabrics to diffuse light evenly. All the tests were recorded with a Legria HF R506 Camcorder (Canon, Tokyo, Japan) installed on a tripod adjacent to the lamp.

Each cuttlefish was gently transferred from its home tank into one of the eight compartments previously filled with 50 mL of water from its own home tank to avoid additional stress. For each test session, two cuttlefish per exposure condition (FSW; FLX5; FLXVEN2.5 and FLXVEN5) of exactly the same age were randomly placed in the eight compartments of the experimental apparatus.

The animals were videorecorded during 30 min to evaluate the stability of the body patterns over time.

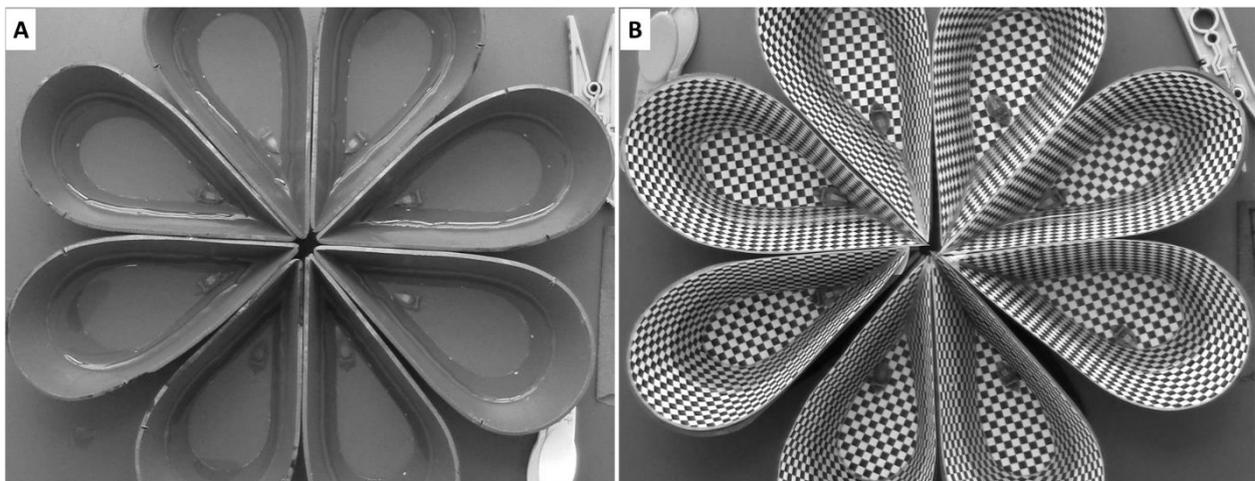


Figure 1. Experimental setup of the behavioural tests with uniform (A) and checkerboard (B) background. Individual and independent drop-shaped PVC compartments were arranged concentrically below the video camera.

2.4 Data analysis

For each video-recording, two still photographs were analysed: one at the beginning of the test, as soon as the cuttlefish was gently transferred into the compartment and stopped to move ($T_0 \pm 1\text{min}$), and one at the end of the test period ($T_{30} \pm 1\text{min}$). Insofar as cuttlefish hatchlings hardly express a true uniform body pattern, the background matching, *i.e.*, the efficiency of camouflage was evaluated by a calculation of a disruptive score on uniform and checkerboard backgrounds. According to Barbosa et al. (2007), juvenile cuttlefish can express up to 11 body components, all which may be more or less expressed. Therefore, each component may be graded from 0 (not expressed) to 3 (strongly expressed). A final grade ranging between 0 (perfect uniform body pattern, *i.e.*, no component expressed) to 33 (perfect

disruptive body pattern, *i.e.*, the 11 components strongly expressed) was assigned to each cuttlefish at T0 (beginning of the test) and at T30 (end of the test, 30 min). The evolution of the calculated disruptive scores according to the developmental stages (from 7 to 28 dph) and the comparison between the exposure conditions (control; FLX5; FLXVEN2.5 and FLXVEN5) were assessed for both behavioural tests (uniform and checkerboard backgrounds). A lower disruptive score on uniform background and a higher disruptive score on checkerboard indicate a more efficient background matching.

All statistical tests were performed using R Studio (Version 1.1.453, RStudio Inc., Boston, USA, <http://www.r-project.org>). As data did not meet the assumptions of normality and homogeneity of variance, we used non-parametric permutation test analyses of data from factorial experiments (function `ezPerm`; “ez” package). The number of iterations was set to 1000. We used mixed within-and-between Ss designs (*i.e.*, repeated factors: time, age; independent factor: experimental groups) to evaluate uniform and disruptive body pattern of juvenile cuttlefish at T0 and T30 between groups and ages. If the null hypothesis was rejected ($p \leq 0.05$), post-hoc permutation tests were performed to highlight any differences between experimental groups and between ages, using the “RVAimemoire” package. We adjusted the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3. Results

Body pattern on uniform background

The disruptive score was significantly different between treatment groups ($p = 0.001$) and over time, *i.e.*, between t0 and t30 min of the test ($p = 0.001$), but there was no difference between ages ($p = 0.479$) (Fig.2). Pairwise comparisons showed a significant difference between each of the exposed groups and the controls (post-hoc permutation test: $p = 0.004$ for each of the treatment groups *vs.* control, respectively). All groups exposed to antidepressants exhibited a better background matching than the control group, *i.e.*, from 7 dph to 28 dph they expressed a lower disruptive score than the control group. Moreover, the score exhibited by the FLXVEN5 group was also different from the two other treatment groups, *i.e.*, FLXVEN2.5 and FLX5 (post-hoc permutation test: FLXVEN5-FLXVEN2.5, $p = 0.027$; FLXVEN5-FLX5, $p = 0.038$). This means that the FLXVEN5 group expressed an even better background matching than the FLXVEN2.5 and FLX5 groups.

In addition, there was a significant interaction between groups and time ($p = 0.016$), *i.e.*, the difference of disruptive score at the beginning and the end of the test was different depending on the group. Pairwise comparisons revealed that no difference of the score over time existed in the control group (post-hoc permutation test: control: control:t0-control:t30, $p = 0.343$), that a slight, but not statistically significant trend towards a difference over time was found in the FLX5 group (post-hoc permutation test: FLX5:t0-FLX5:t30, $p = 0.065$), and a significant difference over the test period existed in both FLXVEN2.5 and FLXVEN5 exposures (post-hoc permutation test: FLXVEN2.5:t0-FLXVEN2.5:t30, $p = 0.004$;

FLXVEN5:t0-FLXVEN5:t30, $p = 0.036$). This means that while the control group exhibits a fairly stable uniform pattern over time, FLXVEN2.5, FLXVEN5 and most probably FLX5 displayed more dynamic evolution of their body patterns. Nevertheless, cuttlefish from those groups improved their body pattern over a 30 min period of time towards a more uniform pattern, resulting in a decreased disruptive score over time.

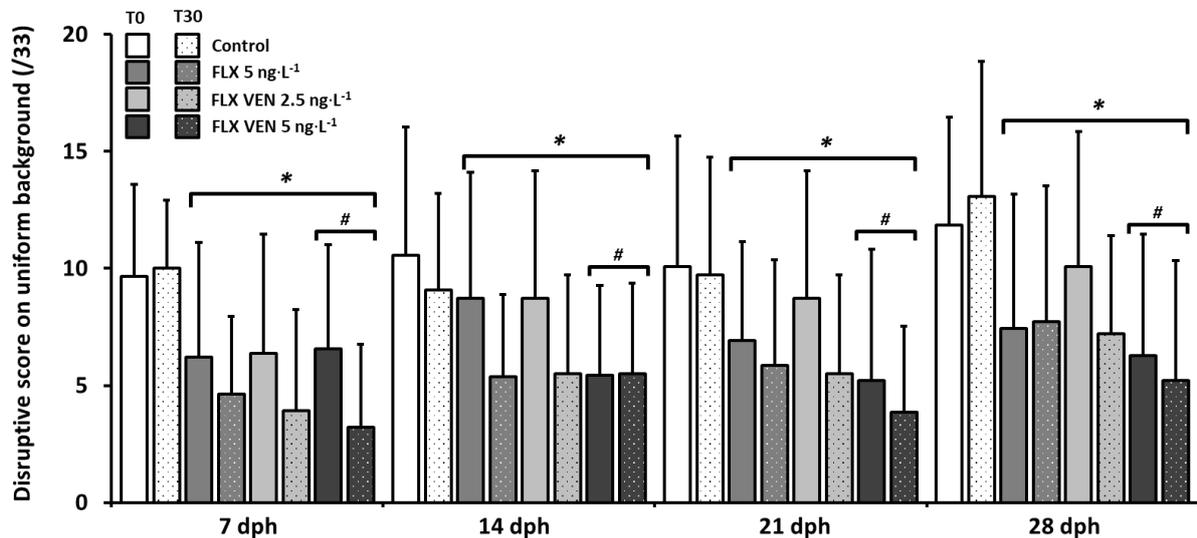


Figure 2. Evolution of disruptive score on a uniform medium-grey background in four treatment groups of juvenile cuttlefish (control, FLX at $5 \text{ ng}\cdot\text{L}^{-1}$, FLX + VEN at $2.5 \text{ ng}\cdot\text{L}^{-1}$ each, and FLX + VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ each), at four different ages (7, 14, 21, 28 days post-hatching [dph]) and over a 30 min test period each (i.e., from T0 to T30). Results are expressed as means \pm SEM of disruptive scores/33, $n=14$. Post-hoc permutation tests with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and age comparisons: * indicates a statistically significant difference between control and all antidepressant exposed groups; # indicates a statistically significant difference between FLXVEN5 and both FLX5 and FLXVEN2.5; $p \leq 0.05$.

Body pattern on checkerboard background (Fig. 3)

The disruptive body pattern was neither significantly different between groups ($p = 0.768$), nor between age ($p = 0.109$), or over the 30 min test period ($p = 0.767$). Overall, juvenile cuttlefish, whether exposed to antidepressants or not, expressed in average the same disruptive score over a 30 min test period during their first month. Nevertheless, it may be noticed that the disruptive score of cuttlefish of 8 dph was generally lower than that of 15, 22 and 29 dph, although no statistically significant difference emerged because of the high intragroup variability ($p = 0.109$). In addition, there was a significant interaction between groups and time ($p = 0.004$) and between ages and time ($p = 0.007$). Yet, pairwise comparisons failed to reveal the statistical differences in both interactions. Again this may be due to the high intragroup variability. However, regarding the means, at 8 dph, cuttlefish from all groups followed the same tendency of a decreasing disruptive score after 30 min (as for the uniform body pattern, see above).

At 15 dph, all cuttlefish exhibited this pattern of decreased disruptive scores after 30 min, except those from FLXVEN2.5 group, which increased their disruptive score. At 22 dph, the control and FLXVEN5 groups slightly decreased their disruptive score after 30 min, but not the FLX5 and FLXVEN2.5 group. Finally, at 29 dph, all cuttlefish, whether exposed or not, displayed an increased disruptive score after 30 min.

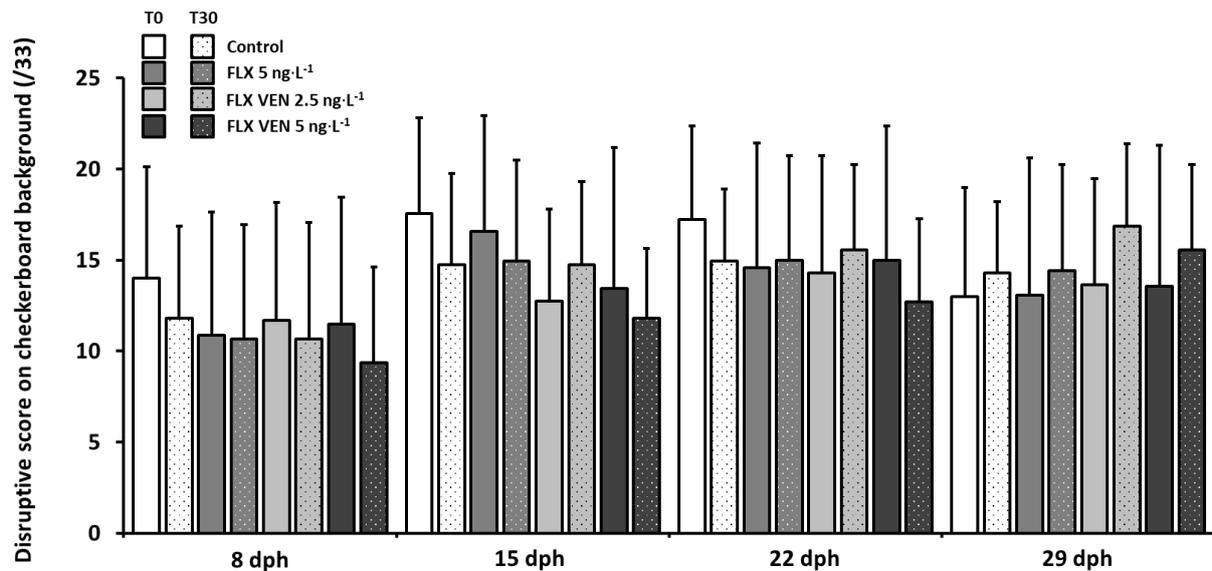


Figure 3. Evolution of disruptive score on checkerboard background in four treatment groups of juvenile cuttlefish (control, FLX at 5 ng·L⁻¹, FLX + VEN at 2.5 ng·L⁻¹ each, and FLX + VEN at 2.5 ng·L⁻¹ each) at four different time points (8, 15, 22, 29 days post-hatching [dph]) and over a 30 min test period each (i.e., from T0 to T30). Results are expressed as means ± SEM of disruptive scores/33, n=16.

Discussion

Like many invertebrate species, cuttlefish hatchlings receive no parental care and must learn on their own how to adapt their behaviours in response to environmental changes or to threats. Notwithstanding, dynamic colour changes are visible on the cuttlefish's body as soon as it hatches, although background matching through chromatic colouration, considered as a primary defence, is still immature. Hatchlings and early juvenile cuttlefish often display an 'inappropriate' body pattern (Hanlon and Messenger, 1988). For instance, they are more likely to express a disruptive colouration when placed on uniform substrates than older animals (Hanlon and Messenger, 1988). Thus, the expression of a uniform pattern for brightness matching on a uniform background seems to be more challenging for hatchling cuttlefish than a disruptive pattern on a contrasted background. Their ability to express a uniform pattern improves in the course of the first two months of life according to their early experience (i.e., rearing environment) (Poirier et al., 2005). The disruptive scores obtained in our control group confirm this difficulty for early juveniles to express a uniform pattern on uniform background, but the lack of improvement with age

may be related to the impoverished rearing conditions as compared to the richer natural environment. Indeed, minimised rearing conditions, which were constrained in our study by the antidepressant exposure, have been shown to slow down the maturation of uniform camouflage abilities in hatchling cuttlefish (Poirier et al., 2005). Our results, therefore, confirm that is useful to evaluate the efficiency of both, uniform and disruptive pattern by the disruptive score, as juvenile cuttlefish did not express uniform body pattern easily.

Cuttlefish exposed to antidepressants at very low environmentally realistic concentrations displayed a less disruptive pattern on a uniform background, which is indicative of a better background matching compared to the control group. This holds particularly true for the highest concentrations of FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ each, *i.e.*, $10 \text{ ng}\cdot\text{L}^{-1}$ of accumulated antidepressants in total. Moreover, cuttlefish exposed to FLX combined to VEN distinctly improved this behaviour over the 30 min test period, and cuttlefish exposed to FLX alone followed this trend, albeit less evidently. On the contrary, the control group displayed a fairly stable, much less dynamic evolution of their body pattern. Similarly, Di Poi et al. (2014) found that two weeks old cuttlefish exposed to FLX at 1 and $100 \text{ ng}\cdot\text{L}^{-1}$ improved their uniform body pattern over a 30 min test period, whereas controls did not. This was, however, not the case in newly-hatched cuttlefish exposed to $1 \text{ ng}\cdot\text{L}^{-1}$ of FLX until two weeks age, which displayed a decreased efficiency to express uniform body patterns (Di Poi et al., 2014). Furthermore, cuttlefish exposed to VEN for 19 days to $100 \text{ ng}\cdot\text{L}^{-1}$ demonstrated a lower quality of the uniform body pattern compared to the controls, while no difference emerged in cuttlefish exposed to $5 \text{ ng VEN}\cdot\text{L}^{-1}$ for 19 days (Bidel et al., 2016). Finally, embryonic exposure at higher concentrations of FLX, *i.e.*, 1 and $10 \mu\text{g}\cdot\text{L}^{-1}$, did not induce any noticeable modification of uniform body pattern. Overall, on a 30 min period, concentrations of FLX had an impact on uniform body pattern, whereas VEN did not. In addition, our results show significant effects of the combination of both FLX and VEN tend towards a better uniform body pattern on medium grey background. In summary, the results of the present and earlier experiments, produced inconclusive responses with respect to the effects of antidepressants on achieving uniform body patterns. Those studies differed slightly in their experimental design and concentrations used, but also in the way of assessing camouflage efficiency. Indeed, Bidel et al. (2016a, 2016b) and Di Poi et al. (2014) used the Mean Gray Value and the Heterogeneity Index from ImageJ[®] to evaluate the efficiency of uniform pattern of cuttlefish despite the fact that juvenile cuttlefish often wear disruptive component and struggle to display a perfect uniform body pattern. Those parameters may be more relevant for adult cuttlefish, which is supposed to master the uniform body pattern, to assess the luminance and brightness against the background while the disruptive score may be more appropriate for young cuttlefish. In addition, there is growing body of evidence pointing to a non-monotonic dose-response for antidepressants with greater effects often at low range concentrations (Fong and Ford, 2014a; Vandenberg et al., 2012) which may explain the variability of response between different studies.

Unlike uniform pattern, disruptive patterns are easily expressed, by hatchlings and juvenile cuttlefish, even if they may not be perfect. This provides them with an efficient camouflage when their environment comprises contrasted components. No distinct effects of antidepressants on the disruptive body pattern of newly-hatched cuttlefish placed in a checkerboard visual environment emerged from the exposures of the present study. Previous studies from Bidet et al. (2016a, 2016b) and Di Poi et al. (2014) are in line with these findings. Indeed, when exposed to either FLX at 1 and 100 ng·L⁻¹ or 1 and 10 µg·L⁻¹, or VEN at 5 and 100 ng·L⁻¹, juvenile cuttlefish did not show any modification of the disruptive pattern on a checkerboard background. Despite the fact that its maturation and its complexity (number of chromatic component expressed) can be modulated by environmental conditions (Poirier et al, 2015), the disruptive pattern seems to be a robust colour pattern, insensitive to waterborne antidepressants, even at the highest concentrations tested or when different antidepressants were combined.

The modification of background matching towards a greater efficiency after an exposure to a SSRI and/or SNRI may be explained by their action on chromatophore organs. Both antidepressants used in this study are designed to modulate 5-HT-levels. In fact, 5-HT is involved in the retraction of chromatophore muscles (Messenger, 2001a). If FLX and/or VEN increase the synaptic concentrations of 5-HT, the exposure to such antidepressants may cause a relaxation of chromatophore's muscles. The relaxation of the muscle crown leads to the coloured pigment concentration thus reducing disruptive colour patterning and bringing forward the uniform coloration by the leucophores. Accordingly, Florey and Kriebel (1969) demonstrated that an application of 5-HT on the skin of *Loligo opalescens* caused a paling due to rapid chromatophore retraction. Loi et al. (1996) obtained similar results with 5-HT, causing a more pronounced retraction than in normal state in an *in-vivo* bioassay with cuttlefish skin. Accordingly, such a direct action on chromatophores may explain the effect of FLX alone as well as in combination with VEN in promoting an improved background matching on uniform substrate due to a retraction of all the chromatophores resulting in a decrease in contrasts between the different skin chromatic components. But, if so, a decrease of the expression of the disruptive pattern should have been observed on the checkerboard background as well, which was not the case, neither for the disrupting score in this study, nor for the heterogeneity index in previous studies (Bidet et al., 2016b, 2016a; Di Poi et al., 2014). Furthermore, both Bidet et al. (2016a, 2016b) and Di Poi et al. (2014) demonstrated that FLX and VEN seemed to influence dopaminergic and noradrenergic system respectively rather than serotonergic system. On the other side, some studies have pointed out the effect of antidepressants on glutamatergic system, in particular by modulating N-methyl-D-aspartate (NMDA) receptors (Musazzi et al., 2013, 2011; Wong et al., 1995). Bidet et al. (2016a) investigated if VEN could modulate binding sites of NMDA-like receptors in brain of cuttlefish. And indeed, an exposure for 20 days post-hatching to either 5 or 100 ng·L⁻¹ of VEN decreased the number of NMDA-like receptor binding sites. Then, VEN may have an impact on chromatophore contraction in neuro-muscular junctions, thus, may enhance a uniform body pattern. Yet, this would mean that this modulation could

exert a similar effect on a disruptive background, but as stated before, no effect could be measured in this condition. Notwithstanding, the modulation of glutamatergic system may have an effect in higher centres, *i.e.*, in brain structures such as optic lobes in cuttlefish. For instance, in *Sepia pharaonis* visual development has been linked with the expression of NMDA receptors in optic lobes (Lee et al., 2013). Previously, exposure to either FLX or VEN has been linked with a decrease in cell proliferation in the optic lobes of juvenile cuttlefish (Bidel et al., 2016b, 2016a). Then, both antidepressants may, putatively, modulate brain development and functions such as visual perception or motor control of chromatic skin components and/or inhibition of the disruptive pattern that cuttlefish hatchling readily express in our experimental conditions. The effects of FLX and VEN on levels of neurotransmitters and structural changes in brain may lead to a different visual information treatment and consequently to modifications in the expression of uniform body pattern.

Juvenile cuttlefish use colour change essentially for camouflage to avoid potential predators, but adult cuttlefish use colour change for signalling both interspecifically, *i.e.*, wearing deimatic displays, and intraspecifically, *i.e.*, wearing intense zebra patterns for male for sexual signalling (Hanlon and Messenger, 1988). The modification of the expression of body pattern in early juveniles may have repercussions further in adult life.

Our study reveals an effect of antidepressants on colour change, specifically when a uniform body pattern is required. The expression of uniform body pattern, immature at hatching (Poirier et al., 2005), seemed to be a more sensitive aspect of cryptic colouration consequently more prone to be modified by the presence of contaminants such as antidepressants. Those results raise one more time questions on the effect of low range concentration of residue of pharmaceuticals on non-target organism with the need to study further those molecules on key behaviours in juveniles.

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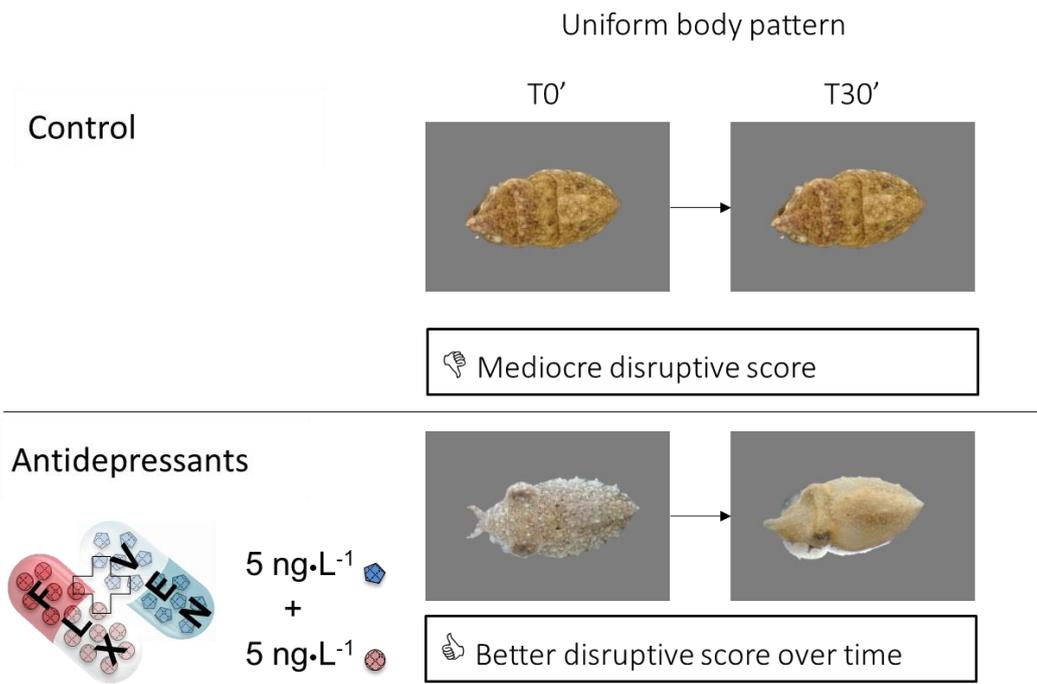


Figure 11. Summary of the main results of the article: Antidepressants modify cryptic behaviour in juvenile cuttlefish at environmentally realistic concentrations

Article III – Hidden in the sand: Alteration of burying behaviour in shore crabs and cuttlefish by antidepressant exposure

This study was designed to evaluate the effects of a sub-chronic exposure to fluoxetine combined, or not, with the venlafaxine on different parameters of the innate burying behaviour in juvenile cuttlefish and crabs. Furthermore, for juvenile cuttlefish that were not buried or only partially buried in the sand, a background matching efficiency was estimated. All parameters were followed weekly in both species.



Hidden in the sand: Alteration of burying behaviour in shore crabs and cuttlefish by antidepressant exposure

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ABSTRACT

Pharmaceuticals such as antidepressants are constantly released into the aquatic environment. Consequently, fluoxetine (FLX) and venlafaxine (VEN), the active molecules of Prozac® and Effexor®, are detected up to several $\mu\text{g.L}^{-1}$ in freshwater and marine coastal waters. Both compounds act on the serotonergic system, which may result in behavioural impairment, especially in juvenile animals presumed to be more susceptible to low concentrations than adults. The objective of this study was to determine whether environmental concentrations of FLX alone or combined with VEN modulate innate burying behaviour in two juvenile marine invertebrates, i.e. *Sepia officinalis* and *Carcinus maenas*. Juvenile cuttlefish were exposed from hatching to 30 days post-hatching to either FLX alone (i.e. 5 ng.L^{-1}) or in mixture with VEN (i.e. either 2.5 ng.L^{-1} or 5 ng.L^{-1} of each antidepressant). Juvenile crabs (< 2 cm carapace width) were exposed for a period of 22 days to 5 ng.L^{-1} of FLX and a mixture of 5 ng.L^{-1} of FLX and VEN each. Several parameters of sand-digging behaviour were analysed weekly in both species. The occurrence of sand-digging behaviour decreased in cuttlefish exposed to a mixture of FLX and VEN at the lowest concentration (2.5 ng.L^{-1} each). Because sand-digging behaviour improved in controls, this decrease was likely to be related to a modification of maturation and/or learning processes. At the mixture of 5 ng.L^{-1} VEN and FLX each, a better body covering was observed in juvenile crabs. In both species, innate behaviour was modified under exposure to mixtures of FLX and VEN at environmentally realistic concentrations. These alterations were observed at an early developmental stage, when animals are particularly prone to predation. Hence, modified maturation of behavioural traits and, putatively, learning processes by exposure to pseudo-persistent antidepressants may affect the survival of these two species in the long term.

1. Introduction

Camouflage is a widespread anti-predator and predator strategy used by many animals to hide through blending into their environment for both, defensive and predator purposes. Among cryptic behaviours, such as body patterning and background matching, burying oneself into the substrate permits to settle and to be concealed, so as to minimize the risk of detection from predators or, otherwise, from prey. Burying is the action of moving into the substratum, the animal being directly in contact with the sediment (Bellwood, 2002). This behaviour results in rapid temporary concealment (Bellwood, 2002). Burying is typical of species living in habitats with moving substrate and requires maintaining a still position (Trueman, 1969). Burying is not to be confused with burrowing, which designates the digging of a cavity into which the

animal may retreat and where it can reside for longer periods of time (Bellwood, 2002). Burying is a common behavioural trait that can be observed in various taxonomic groups, such as flatfish, unshelled cephalopods like the European cuttlefish, *Sepia officinalis*, or crabs like *Carcinus maenas*. The literature describes this behavioural trait as either sand-digging behaviour for cuttlefish (Mather, 1986; Hanlon and Messenger, 2018), or burying behaviour for crabs (Warner, 1977; Bellwood, 2002). Hiding in the sand protects from predators and this concealment is particularly important for the vulnerable juveniles. The same kind of behaviour may also serve to remain unnoticed by the prey, allowing for sudden attacks.

In cuttlefish, burying or sand-digging is described as a fixed behavioural sequence by Mather (1986). It consists of ejecting a series of water jets from their funnel, followed by a lateral wiggling motion of

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the dorsal mantle, so as to cover the animal with sand. Appearing immediately after hatching, sand-digging behaviour is flexible and depends on experience and development (Poirier et al., 2004; Navet et al., 2014). Hence, the juvenile cuttlefish adjust and improve it with age. The sand-digging behaviour may be accompanied by background matching so as to maximise the efficacy of their camouflage (Hanlon and Messenger, 2018). Indeed, cuttlefish frequently remain partially covered by sand and control their skin chromatophores to take the colour, brightness, contrast and granularity of the sand (Hanlon et al., 2009).

The same holds true for crabs, in which the corresponding behaviour is called either burying (Warner, 1977; Bellwood, 2002; McGaw, 2005), or digging behaviour (Cumberlidge and Uglow, 1978). *C. maenas* belongs to the family of Portunidae that typically use their pereopods to enter the substrate in a backward movement (McLay and Osborne, 1985). Once the rear of the body is buried, it is followed either by a body slam (slamming the body against the substrate permitting to entrench themselves deeper by displacing substrate onto the back of the crab) or a sternal push (pushing substrate forwards and towards the side creating a depression in which to settle). Either way, crabs are then pushing further backwards into the substrate to become completely covered. As with cephalopods, numerous crustacean species also adapt their body colours to their surrounding environment, thus reinforcing the burying behaviour by background matching and disruptive colouration (Detto et al., 2008; Stevens et al., 2014a; Duarte et al., 2016). However, this happens less quickly than in cuttlefish and consists of a combination of cuticle pigmentation and chromatophore colouration (Fingerman, 1985; Messenger, 2001; Umbers et al., 2014; Stevens et al., 2014b).

Monamines, especially serotonin (5-hydroxytryptamine, 5-HT), noradrenaline and dopamine, are known to act as neurotransmitters and/or endocrine messengers that regulate many physiological processes in a wide range of animal species (Turlejski, 1996). For instance, they regulate reproduction (Florey and Rathmayer, 1978; Gibbons and Castagna, 1984), growth (Fingerman, 1997), metabolism (Fingerman, 1997; Sarojini et al., 1995) and change of colour (Fingerman, 1997). They are also involved in numerous behaviours in both vertebrates and invertebrates (Weiger, 1997) such as aggressive behaviour (Huber et al., 1997), anxiety-like behaviour (Fossat et al., 2014), locomotor activity and phototactic behaviour (Pavlova, 2001; McPhee and Wilkens, 1989; Perrot-Minnot et al., 2013). Therefore, changes in the concentrations of these neurotransmitters or in the pathways leading to their synthesis, release, action and/or reuptake, may have consequences on various behaviours such as cryptic behaviours, for instance. Potentially, a large panel of environmental pollutants may interfere with serotonergic regulation of behavioural traits. Of particular interest, however, are those pharmaceuticals, which are specifically designed to modulate 5-HT levels (Mezzelani et al., 2018). This is the case, for antidepressants of the families of Selective Serotonine Reuptake Inhibitors (SSRIs) and Serotonin and Norepinephrine Reuptake Inhibitors (SNRIs). These pharmaceuticals are prescribed to treat neurological disorders such as depression and anxious states. Antidepressants are particularly worrying, because of their implications in neurobiological changes that target the serotonergic system (Krishnan and Nestler, 2008). The diversification of antidepressant medication resulted in ever-higher prescription rates, which is why the use of antidepressants is still on the rise worldwide (Nielsen and Göttsche, 2011; OECD Health Statistics 2018). Wastewater treatment plants, however, have limited efficiency in removing these pharmaceutical residues (Bueno et al., 2012). Therefore, SSRIs and SNRIs are continuously discharged into surface waters, leading to a contamination of the aquatic compartments with antidepressants such as fluoxetine (FLX, Prozac®) or venlafaxine (VEN, Effexor®). Residues of both compounds have been detected at low concentrations, from ng.L^{-1} to $\mu\text{g.L}^{-1}$ in freshwater and marine environments (Metcalf et al., 2010; Rúa-Gómez and Püttmann 2012a, 2012b; Bueno et al., 2012; Meador et al., 2016;

Paíga et al., 2016). In 2016, Minguez et al. detected concentrations of VEN up to 1.3 ng.L^{-1} and FLX up to 0.3 ng.L^{-1} in the Northwestern coastal waters of France. It should be noted, however, that, depending on the compound, their respective neurotoxicity might not be well represented by their water concentrations (Ford and Fong, 2016). Especially FLX, that has a half-life greater than 100 days, is readily absorbed to particulate organic matter and strongly accumulates into the biota, even if its residues in the water are below detection limits (Kwon and Armbrust, 2006; Oakes et al., 2010; Meador et al., 2016). Other antidepressants, such as VEN, are more water-soluble and, therefore, appear at higher concentrations in the water bodies (Minguez et al., 2016). Furthermore, although the individual antidepressant concentrations may be in the lower ng.L^{-1} range, the accumulated concentrations often amount to $\mu\text{g.L}^{-1}$ levels (Silva et al., 2015; Sehonova et al., 2018). Accumulated antidepressant concentrations are, however, rarely considered in ecotoxicological studies (Bisesi et al., 2016; Fong and Molnar, 2013; Fong et al., 2015).

Previous studies have shown that FLX and VEN significantly altered camouflage behaviour in cuttlefish, such as background matching (Bidel et al., 2016a, b) and also sand-digging (Di Poi et al., 2014). Thus, the frequency of sand-digging in juvenile *S. officinalis* can be altered at environmental concentrations as low as 1 ng.L^{-1} , but only for the first two weeks of life and without considering any mixture of antidepressants (Di Poi et al., 2014). Similar to cephalopods, digging behaviour in crabs has been identified as a behavioural trait susceptible to environmental pollution. Some studies examined the impact of human pollution on digging behaviour in crabs: Burger et al. (1991) and Zengel et al. (2016), for instance, studied the effects of oil spills on the burying behaviour of fiddler crabs (genus *Uca*), whereas Boere et al. (2011) investigated the consequences of an urban sewage accident on mole crab (genus *Emerita*). No study yet addressed the issue of antidepressants on sand-digging behaviour in crabs, but elevated levels of $120 \mu\text{g.L}^{-1}$ clearly affected locomotion in *C. maenas* (Mesquita et al., 2011) and anxiety-like behaviour in *Pachygrapsus crassipes* (Hamilton et al., 2016).

The present study addresses the question, if similar behavioural changes can be observed with a combination of two representative antidepressants persistently detected in aquatic organisms (Kwon and Armbrust, 2006). The chosen antidepressants have different water solubility, i.e. K_{ow} 4.05 for FLX and K_{ow} 3.20 for VEN (DrugBank; Toxnet). Combining two antidepressants in the exposures represents a more environmentally realistic scenario (Backhaus and Karlsson, 2014; Fong and Ford, 2014). Hence, the aim of the study was to demonstrate that low level mixture concentrations can affect animal behaviour, which is a particularly sensitive endpoint with respect to experimental concentrations in the lower ng -range. Back in 1987, Fluoxetine was the first SSRI antidepressant placed on the market (Das, 2011; Huskamp et al., 2008). Consequently, it was used in many environmental studies examining the effects of antidepressants on aquatic wildlife (Ford and Fong, 2016). It, therefore, served as a kind of reference in the present study, in which it was used alone at the same nominal concentration as the lower concentrated mixture. Somewhat later, the first SNRI antidepressant to be released in 1995 was VEN (Barbui et al., 1999), adding to the already existing environmental antidepressant pollution. The effects of these two quantitatively important and functionally slightly different antidepressants where, therefore, tested in mixture at two different concentrations on a complex behavioural trait, i.e. sand-digging behaviour. Two invertebrate species showing this behaviour, *S. officinalis* and *C. maenas*, the juveniles of which are particularly vulnerable to predators, were used to compare behavioural changes across species. Both animals thrive in the intertidal zone and/or in coastal waters, where they are directly exposed to the discharges carried by the estuarine waters. In addition, the early developmental and juvenile stages are likely to be more sensitive to the low-level pollutant exposure (Lacoue-Labarthe et al., 2016). In this context, the overall objective of the study was to test antidepressants in a more environmentally

relevant scenario in combination with a highly sensitive endpoint shared by different species.

2. Material and methods

2.1. Animal keeping and experimental conditions

All animal experiments were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments (Protocol approved by the French National Ethical Committee for Animal Experimentation, Agreement number: 035121.02).

2.1.1. Juvenile cuttlefish

Eggs of wild cuttlefish (*S. officinalis*, L. 1758) were collected in the English Channel (off the coast of Luc-sur-mer, France; N 49° 19' 43, W 0° 20' 54) and kept at the Centre de Recherches en Environnement Côtier (CREC) of the University of Caen Normandie at Luc-sur-mer, France. Embryos were maintained in carbon-filtered natural seawater (FSW) at a salinity of 35 PSU and 21 ± 1 °C under a natural summer photoperiod (16 h L : 8 h D). After hatching, cuttlefish were individually placed in glass beakers (Ø 11 cm, height 6 cm) supplied with 250 mL of FSW (35 PSU), which was changed once a day. Temperature was maintained at 15 ± 1 °C and photoperiod at 16 h L : 8 h D. Hydrological parameters (T, O₂, NO₂⁻, NO₃⁻) were monitored daily, using a pH and dissolved oxygen meter (HQ30D, Hach company, Loveland, USA), as well as NO₂⁻ and NO₃⁻ tests (Tetra, Melle, Germany). Animals were fed once a day at 9 a.m. with one shrimp (*Crangon crangon*, caught at Luc-sur-mer, and not bigger than the length of the mantle of a juvenile cuttlefish, i.e. < 1 cm) over the entire experiment. Juvenile cuttlefish were exposed over a period of 30 days to either (1) FSW (control), or (2) FSW with 5 ng.L⁻¹ FLX alone (FLX5), or (3) FSW with 2.5 ng.L⁻¹ FLX and 2.5 ng.L⁻¹ VEN in mixture (FLXVEN2.5), as well as (4) 5 ng.L⁻¹ FLX and 5 ng.L⁻¹ VEN in mixture (FLXVEN5). The individuals of each treatment group (n = 14) were submitted to the same sand-digging behavioural test, described in the following, each week over four consecutive weeks. The first month after hatching covers a particularly important period of ontogenetic maturation, during which the cuttlefish learn to camouflage and improve sand-digging behaviour (Lacoue-Labarthe et al., 2016; Poirier et al., 2004).

2.1.2. Juvenile crabs

Juvenile shore crabs (*C. maenas*, L. 1758) of 5–20 mm cephalothorax width were collected on the rocky shore near Yport, France (between N 49° 43' 47, E 0° 16' 33 and N 49° 44' 25, E 0° 18' 47). The intertidal foreshore where the crabs originated from, is composed of chalk and flintstone with several dispatched areas of sand separated by small to large shingles covered, or not, by kelp, green or red algae.

After transfer to the laboratory, animals were separated into different tanks according to their size to avoid cannibalism (density = 15 ± 5 crabs/tank) and acclimated to laboratory conditions over eight weeks until experimental use. The animals were kept in a closed-loop system with filtered and oxygenated artificial seawater (ASW), at a salinity of 35 PSU, 15 ± 1 °C and a 12 h L : 12 h D photoperiod (8 a.m.–8 p.m.). 80% of the water body was renewed once a week with ASW (35 PSU) prepared from dechlorinated tap water using Marine SeaSalt (Tetra, Melle, Germany). Temperature, dissolved oxygen, nitrites and nitrates were controlled daily using a dissolved oxygen meter (HI 9146 HANNA® Instruments, Woonsocket, USA) as well as Visocolor® ECO PF-12 kits (931-241 and 931-244, Macherey-Nagel, Düren, Germany). Animals were fed once a day at 9 a.m. with a cube of roughly 0.5 mm³ of frozen seabass filet (*Dicentrarchus labrax*, obtained from the fish farm 'Aqua-caux', Octeville, France) over the entire experiment.

At the beginning of the experiments (day 0) juvenile crabs were isolated in 250 mL glass beakers (Ø 6 cm, height 10 cm) containing

200 mL ASW (35 PSU) for 7 days, henceforth designated as 'benchmark week'. Each crab was submitted to the burying behavioural test as described below in order to provide reference data for each individual crab against which behavioural changes due to antidepressant exposure were established. Behavioural traits were thus calibrated with a total of 48 crabs prior to the exposures. After one week, i.e. at day 8, juvenile crabs were exposed over a period of 22 days to either (1) ASW (control), or (2) ASW with 5 ng.L⁻¹ of FLX alone (FLX5), or (3) ASW with 5 ng.L⁻¹ of FLX and 5 ng.L⁻¹ of VEN (FLXVEN5). The FLXVEN2.5 was omitted for crabs, as these were relatively older and pre-experiments showed them to be generally less susceptible to antidepressants than cuttlefish. The individuals of each treatment group (n = 16) were submitted to the same sand-digging behavioural test each week at four time points (i.e. after 1; 8; 15; 22 days of exposure). Altogether, this exposure period was considered sufficient to bioconcentrate the antidepressants and provided four measurement points as in cuttlefish.

2.2. Chemical contamination

FLX and VEN concentrations were chosen according to measured surface water concentrations (Minguez et al., 2016; Meador et al., 2016). Final concentrations were prepared from a stock solution of hydrochloride fluoxetine (CAS 56296-78-7, Sigma Aldrich, St. Louis, USA) or venlafaxine (CAS 99300-78-4, Sigma Aldrich), dissolved in distilled water. Stock solutions were at 10 µg.L⁻¹ of FLX and VEN and kept at -80 °C until use. Water was renewed daily, along with a complete change of contaminants to avoid alterations in FLX and VEN concentrations, a decrease of O₂-levels as well as an increase of nitrites and nitrates (NO₂⁻, NO₃⁻) beyond tolerable levels ([NO₂⁻] max = 0.05 mg.L⁻¹, [NO₃⁻] max = 0.15 mg.L⁻¹). Due to the necessity of individual observations (n = 14/16), a semi-static exposure in beakers was employed. Preliminary studies have shown that the actual concentrations did not fall below 75% of nominal concentrations within 24 h, with VEN being more stable than FLX (data not shown).

2.3. Behavioural tests

As the burying, or digging behaviour in crabs and the sand-digging behaviour of cuttlefish examined in this study represent similar types of behaviour, and because the experimental settings for both species were carried out with sandy substrate, the behavioural observations are henceforth referred to as 'sand-digging behaviour' for *S. officinalis* and *C. maenas* alike.

Both, cuttlefish and crabs were randomly placed into individual test arenas. Sand-digging behaviour was recorded during 30 min at 9, 16, 23 and 30 days post-hatching (dph) for cuttlefish and 5 min at 1, 8, 15 and 22 days after the beginning of the experiment for crabs. Distinct test durations were chosen for cuttlefish and crabs, because the crabs bury themselves more quickly than cuttlefish when placed on such sandy substrates.

2.3.1. Sand-digging behaviour test with cuttlefish

Custom-made, drop-shaped PVC apparatuses (length 11 cm, height 4 cm, max width 5 cm) (Fig. 1A) that were saturated with the two contaminants constituted the test arenas. Their bottom was covered with a one cm-thick layer of natural sand (from the sandy beach at Luc-sur-mer). Each compartment was filled with 50 mL of water from each cuttlefish exposure beaker to avoid additional stress. Eight test arenas were arranged concentrically (Fig. 1A) below a circular LED lamp (Neewer®, Guangdong, China). The lamp was adjusted in an oblique position 60 cm above the compartments to avoid direct light on cuttlefish. The camera (Canon Legria HF R506) was installed on a stand adjacent to the lamp. The setup was placed in a custom-made black tent to avoid any external stimuli and surrounded with white fabrics to reflect light. The test began with the gentle transfer of the cuttlefish into the test arena. After the video recording, each animal was returned to

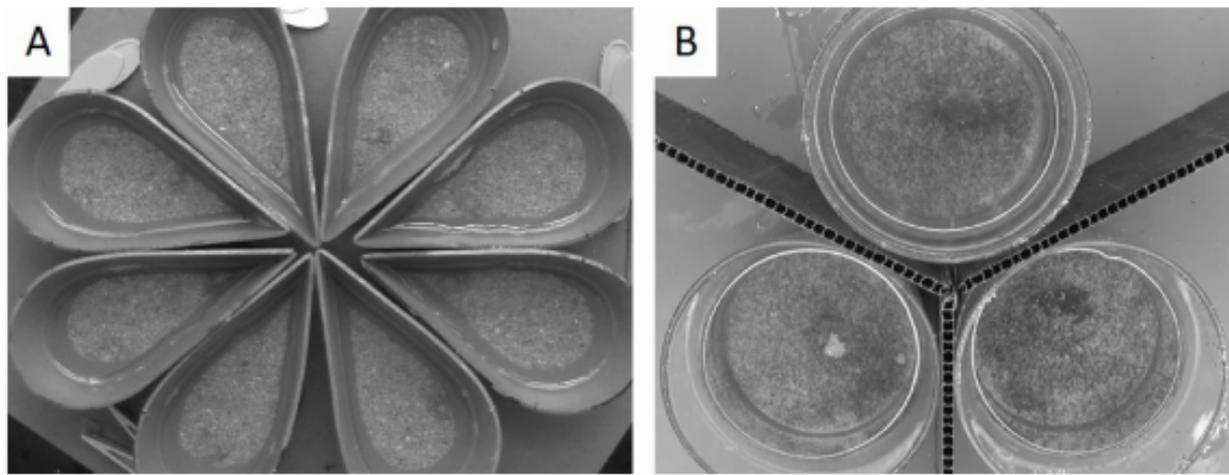


Fig. 1. Setting of sand-digging behaviour showing the individual drop-shaped PVC compartments for cuttlefish (A) and glass containers with the opaque black cardboard for crabs (B).

its exposure beaker.

All tests were conducted in the climate chamber where the cuttlefish were exposed to antidepressants in order to avoid unnecessary handling stress.

2.3.2. Sand-digging behaviour test with crabs

Transparent glass containers (\varnothing 6 cm, height 10 cm, 25 cL, Carrefour, France) (Fig. 1B) constituted the test arenas. They were filled with a two cm-thick layer of mixed white and dark sand (Sansibar Dark and Sansibar White, JBL, Neuhofen, Germany) in order to obtain a medium grey and covered with 150 mL of water from each individual's own exposure beaker to avoid additional stress. The circular LED lamp (Neewer®) was placed 40 cm above the test arena so as to avoid reflection on the water surface. A camera (Sony HDR-CX240E) was installed above the circular lamp to record sand-digging behaviour. Opaque black cardboards were placed between adjacent individual test tanks to avoid mimicry between crabs. The setup was placed in a black tent (Neewer®) to avoid any external stimuli. The test began when crabs were gently placed in their respective transparent glass container. After 5 min of recording, the video was stopped and crabs were returned to their exposure beaker. All tests were conducted in the climate chamber where the crabs were exposed to antidepressants in order to avoid unnecessary handling stress.

2.3.3. Behavioural data analyses

Video-recordings were analysed *a posteriori* and sand-digging behaviours were quantified by manual time measurements as follows. The latency before the first sand-digging behaviour was defined as the time between the placement of the animal into the test arena and the initiation of the sand-digging behaviour. Duration of the sand-digging behaviour sequence corresponds to the period of time from the initiation of sand-digging behaviour until the tested individual stopped moving in the sandy substrate.

Cuttlefish and crabs may perform multiple sand-digging behaviours consecutively, which is why the number of occurrences was counted along the 30 min and 5 min tests, for cuttlefish and crabs, respectively. Periods when cuttlefish and crabs were neither swimming/walking, nor initiating a sand-digging behaviour accounted for the time spent immobile.

Animals were considered buried when the movements underlying sand-digging behaviour were terminated, even if not all parts of the animal's body were concealed. Thus animals that were partially (*i.e.* part of the body visible) as well as completely buried (*i.e.* only the eyes or no feature visible) were considered buried. The body of the animal was contoured manually using the function 'Polygon selections'. The relative surface of the animal's body buried in the sand was quantified

by comparing the total body area of the animal to the visible part of the body after burying, using ImageJ® (Bethesda, Maryland, USA) in order to obtain the percentage of body buried. The closer to 100% the percentage is, the more of the body is covered by sand.

Finally, when cuttlefish were partially buried, or not buried at all, the mean grey level of the animal's visible body part was compared to the mean grey level of sandy background, using ImageJ® in order to obtain the level of homochromy. The closer to 100% the level of homochromy is, the better the background matching.

The percentage of body buried and the level of homochromy were calculated for the first sand-digging behaviour. Using Spearman's rank-order correlation (see section 2.4), the relation between the two strategies, *i.e.* burying and homochromy, was examined. A negative correlation between percentage of body buried and homochromy would indicate that the animals preferably use one of the two strategies. A positive correlation, on the other hand, would signify that both strategies are applied simultaneously.

For the crabs, the first week of test served as benchmark for calibrating the behavioural tests for each animal prior to experimental antidepressant exposure (*i.e.* 'benchmark week'). The results of this benchmarking for the total of all crabs ($n = 48$), subsequently tested in the different treatment groups, are reported for each parameter. All results are expressed relative to these baseline values established during the benchmark week, and which were arbitrarily set to one (note: not all crabs effectuated burying behaviour during benchmarking, which explains the lower numbers of replicates reported in the results section). This benchmarking was not conducted for the cuttlefish, because sand-digging behaviour is continuously improving over the observation period, due to their post-embryonic development.

2.4. Statistical analysis

All statistical tests were performed using R Studio (Version 1.1.453, RStudio Inc., Boston, USA, <http://www.r-project.org>). As data did not meet the assumptions of normality and homogeneity of variance, we used non-parametric permutation test analyses of data from factorial experiments (function `ezPerm`; 'ez' package). The number of iterations was set to 1000. We used mixed within-and-between Ss designs (*i.e.* repeated factor: age; independent factor: experimental groups) to evaluate different behavioural measures: latency before the first sand-digging behaviour, time spent to bury themselves, occurrence of sand-digging behaviours, time spent immobile, percentage of body buried and homochromy with the background (for *S. officinalis* only). If the null hypothesis was rejected ($p \leq 0.05$), post-hoc permutation tests were performed to highlight any differences between experimental groups and between ages, using the 'RVAidememoire' package. We

adjusted the false discovery rate, using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). The correlation between homochromy and percentage of body buried for each group and each age of cuttlefish was evaluated, using Spearman's correlation coefficient (ρ , function cor.test) giving a range between -1 and 1 , with 0 indicating no association (Hollander et al., 2013). The data obtained for crabs were normalised according to results from the benchmark week. All data are reported as medians and interquartile ranges. The number of cuttlefish in each experimental group (Control, FLX5, FLXVEN2.5, FLXVEN5) was always 14 in all tests over the entire exposure period and the number of crabs was always 16 for all experimental groups (Control, FLX5, FLXVEN5). Hence, all statistical results and p-values relate to the same number of replicates for either crabs or cuttlefish and will be not indicated when reporting the statistical probabilities hereafter.

3. Results

3.1. Sand-digging behaviour – cuttlefish

3.1.1. Latency of the first sand-digging behaviour

The latency was not significantly different between groups ($p = 0.730$), but was significantly modified by age ($p = 0.001$; Fig. 2A). Pairwise comparisons showed that it decreased significantly between 9 dph and 30 dph for each group (post-hoc permutation test: $p = 0.024$). This decrease can also be observed in the antidepressant exposed animals, where, by trend, it might be either accelerated (FLX5 and FLXVEN5), or delayed and attenuated (FLXVEN2.5). Importantly, however, the intra-group variability becomes affected by antidepressant exposure, notably in FLXVEN2.5, but also in FLXVEN5 (23 dph and 30 dph) and in FLX5 (23 dph). The high individual variability observed in antidepressant-exposed cuttlefish prohibits any statistically significant difference, as compared to the control. The low 25% and high 75% quartiles, however, underscore that animals may respond to antidepressants, either by increasing, or by decreasing latency.

3.1.2. Time spent to bury

The median time spent to bury themselves, i.e. the duration of the entire sand-digging behaviour sequence was stably situated at 4 s in the control animals across all time points. Antidepressant exposed cuttlefish showed deviations from these control medians, with slight increases in FLX5 and FLXVEN5 at 16 dph as well as a decrease in FLXVEN2.5 at 23 dph. In the exposed groups, we observed that movements of the sand-digging behaviour sequence were more chaotic, thus increasing or decreasing slightly the time to bury themselves. However, these deviations appeared arbitrary, as neither statistically significant differences between groups ($p = 0.272$) and between ages ($p = 0.268$; Fig. 2B), nor clear patterns emerged.

3.1.3. Occurrence of sand-digging behaviours

The occurrence of sand-digging behaviours during the test was significantly different between groups ($p = 0.048$) and between ages ($p = 0.001$; Fig. 2C). Pairwise comparisons showed that cuttlefish from the control group and FLX5 exposed group had a higher occurrence of sand-digging behaviour than cuttlefish exposed to FLXVEN2.5 (post-hoc permutation test: $p = 0.024$ for both comparisons), especially when compared at 30 dph. This accentuated difference was due to an age-dependent increase of digging behaviours in the control group, which could not be observed in the FLXVEN2.5 group. Indeed, the occurrence of sand-digging behaviours was roughly two times higher at 30 dph than at 9 dph, at which about one sand-digging behaviour could be observed during a 30 min test period. This difference was found to be significantly different for pairwise comparisons between ages of 9 and 30 dph (post-hoc permutation test: $p = 0.005$).

3.1.4. Time spent immobile

The time spent immobile was not significantly different between groups ($p = 0.921$), but there was a significant effect of age for all groups ($p < 0.001$). Animals of the youngest group (9 dph) were mobile for more than 5 min, with generally shorter periods of immobilisation than older hatchlings (16, 23 dph). For the 16 and 23 dph of all treatment groups, pairwise comparisons showed an increase of time spent immobile for each group (post-hoc permutation test: $p = 0.006$), with animals remaining immobile for about 28 min of the recording. Subsequently, time spent immobile decreased again at 30 dph, being shorter than in 16 and 23 dph (post-hoc permutation test: $p = 0.008$; $p = 0.015$, respectively; Fig. 2D). This age-dependent increase in mobility in the FLX5 group at 30 dph could, however, not be observed as distinctly as in the other groups. Albeit this difference was not statistically significant (post-hoc permutation test: $p = 0.204$), the 30 dph FLX5 group rather corresponded to the immobility observed at 16 and 23 dph.

3.1.5. Percentage of body buried

The percentage of body buried was not significantly different between groups ($p = 0.877$), but a significant effect of age emerged ($p < 0.001$; Fig. 3A). Pairwise comparisons showed that cuttlefish at 30 dph buried themselves to a greater extent, compared to 9, 16 and 23 dph cuttlefish (post-hoc permutation test: $p = 0.006$, $p = 0.008$, $p = 0.006$, respectively). An effect of antidepressants on the percentage of body buried is difficult to discern, because neither a clear pattern, nor statistically significant differences emerged. Notwithstanding, it may be noted that at 9 dph, the medians are not very different in all groups, whereas they diverged considerably at 16, 23 and 30 dph, although in an inconsistent manner.

3.1.6. Homochromy

The homochromy of unburied body parts of cuttlefish remained stable at levels about 80% or higher, regardless of the group or the age (group: $p = 0.974$; age: $p = 0.210$; Fig. 3B).

A significant correlation between homochromy and percentage of body buried emerged in the control group at 30 dph ($p = 0.040$, $\rho = 0.599$; Table 1), whereas no significant correlation appeared in any other of the exposure groups at any age. The lack of correlation at the earlier time points most likely derives from maturation and a gain in experience for the sand-digging behaviour, which changed over time, whereas homochromy was stable and above 80% from the beginning.

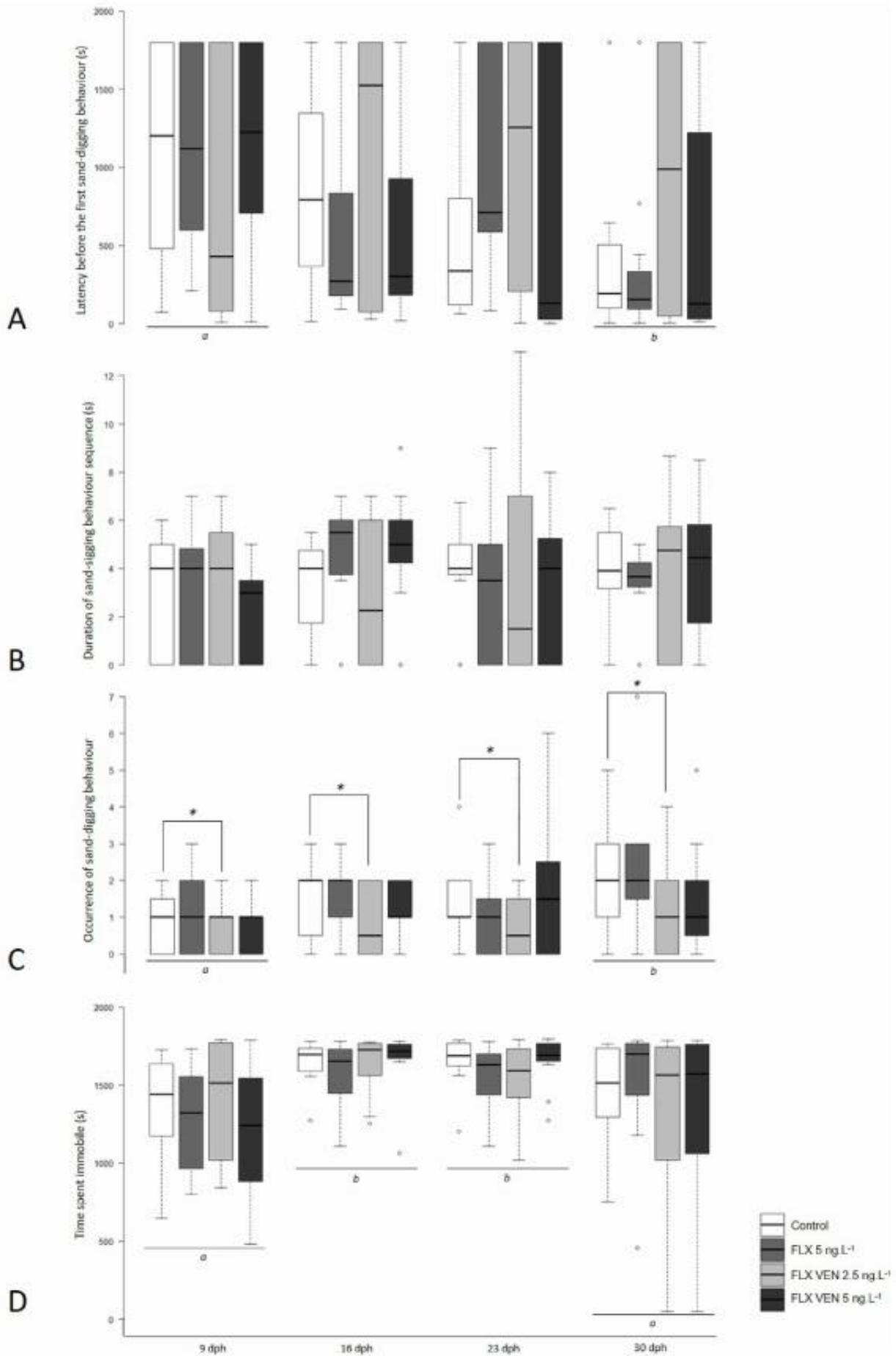
Animals from the FLX5 group had a pattern similar to that of the controls, but the ability to perform both strategies, i.e. to improve sand-digging behaviour, seemed to advance at a slower rate as indicated by the negative Spearman's ρ correlation coefficients from 9 dph to 23 dph (Table 1).

For the combined antidepressant exposures, the pattern was different, without clear improvement with age. In FLXVEN2.5 group, the correlation was positive at each age, but not statistically significant. For the FLXVEN5 group the correlations appear quite variable without any of them being statistically significant (Table 1).

3.2. Sand-digging behaviour – crabs

3.2.1. Latency of the first sand-digging behaviour

The latency during the benchmarking week, when crabs were placed on sand, was 4 ± 2.7 s for those crabs that eventually buried ($n = 40$, i.e. 40 crabs buried over 48 crabs in total). At the beginning (d1) and the end (d22) of the experiment, latency of the first sand-digging behaviour did not deviate from these baseline values whatever the treatment group (Fig. 4A). At d8, median latency increased about 15 s in the controls with respect to the baseline value and about 16.5 and 20 s, in FLX5 and FLXVEN5, respectively, in the exposed animals. Thus, the increase appeared more pronounced in the antidepressant exposed animals, as compared to the controls. Notwithstanding, the



(caption on next page)

Fig. 2. Sand-digging behaviour of four groups of juvenile cuttlefish exposed to fluoxetine (FLX) at 5 ng.L^{-1} , fluoxetine and venlafaxine in mixture (FLXVEN) at 2.5 ng.L^{-1} or 5 ng.L^{-1} each as well as non-exposed controls at four time points (9, 16, 23 and 30 days post-hatching [dph]) of exposure, through: (A) latency of the first sand-digging behaviour, (B) duration of sand-digging sequence (C) occurrence of sand-digging behaviour over a 30-min observation period, (D) time spent immobile during the 30-min test. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and inter-age comparisons: letters a and b demonstrate a significant difference between ages ($p < 0.05$); * indicates a significant difference between groups ($p < 0.05$).

latency of the first sand-digging behaviour was neither statistically different between groups at any of the given time points ($p = 0.698$), nor when comparing the time points of exposure, i.e. d1, d8, d15 and d22 ($p = 0.482$). Hence, the increased latency at d8 most likely represents a deviation without particular significance.

3.2.2. Time spent to bury

The time spent to bury themselves, i.e. duration of the entire sand-digging behaviour sequence during the benchmark week was around $15 \pm 4.1 \text{ s}$ in buried crabs ($n = 40$). A slight increase in controls and both treatment groups became visible at the end of the experiment

(d22), but overall time spent to bury during the experimental exposure was neither statistically different between groups ($p = 0.837$), nor over time ($p = 0.265$; Fig. 4B).

3.2.3. Occurrence of sand-digging behaviours

The occurrence of sand-digging behaviours, during the benchmarking week was 1.0 ± 0.65 , i.e. a large majority of crabs ($n = 40$) buried themselves only once. The buried crabs tended to stay buried until the end of the test. The occurrence of sand-digging behaviour during the test was neither different between groups ($p = 0.206$), nor over time ($p = 0.391$, Fig. 4C). It may be noticed, however, that the

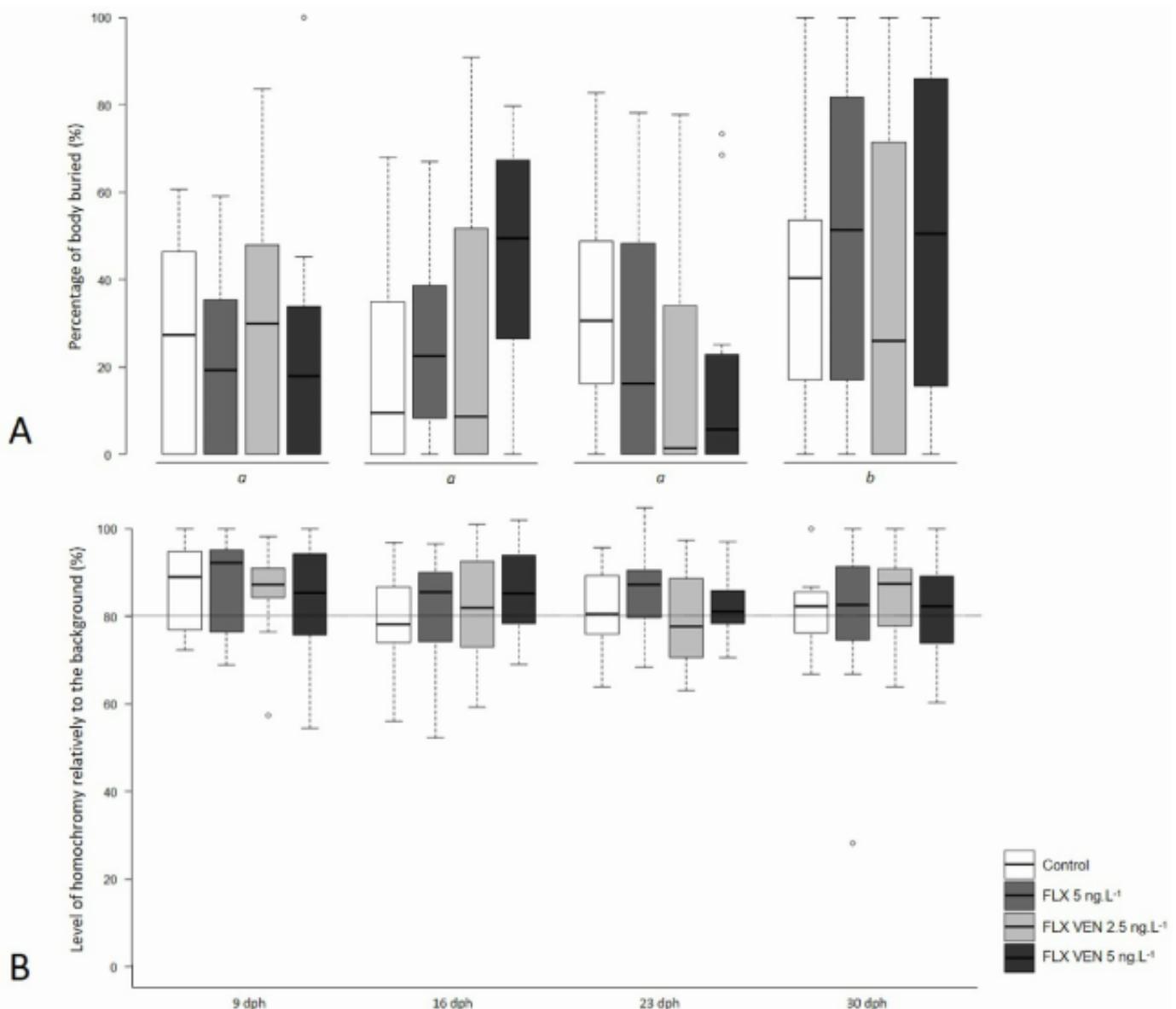


Fig. 3. Efficiency of camouflage on sand of four groups of juvenile cuttlefish exposed to fluoxetine (FLX) at 5 ng.L^{-1} , fluoxetine and venlafaxine in mixture (FLXVEN) at 2.5 ng.L^{-1} or 5 ng.L^{-1} each as well as non-exposed controls at four time points (9, 16, 23 and 30 days post-hatching [dph]) of exposure, through: (A) percentage of bodies surface buried in sand, (B) level of homochromy of unburied body relatively to a sandy background. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers; dotted line represents a level of homochromy equal to 80%. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and age comparisons: letters a and b demonstrate a significant difference between ages at $p < 0.05$.

Table 1

Relation between homochromy and percentage of body buried in juvenile cuttlefish, *Sepia officinalis*. Spearman's correlation coefficient (ρ) was calculated for each treatment group, i.e. *S. officinalis* exposed to fluoxetine at 5 ng.L⁻¹ (FLX5), fluoxetine and venlafaxine in mixture at 2.5 and 5 ng.L⁻¹ (FLXVEN2.5; FLXVEN5) as well as non-exposed animals (Control), at each age (9, 16, 23, 30 days post-hatching [dph]). * indicates a significant correlation at $p < 0.05$

Control	p-value	ρ	FLX5	p-value	ρ
9 dph	0.912	-0.036	9 dph	0.074	-0.534
16 dph	0.469	-0.231	16 dph	0.196	-0.401
23 dph	0.225	0.378	23 dph	0.413	-0.261
30 dph	0.040*	0.599	30 dph	0.079	0.525
FLXVEN2.5	p-value	ρ	FLXVEN5	p-value	ρ
9 dph	0.117	0.477	9 dph	0.231	0.374
16 dph	0.079	0.526	16 dph	0.256	-0.357
23 dph	0.222	0.381	23 dph	0.143	0.449
30 dph	0.327	0.310	30 dph	0.078	0.528

groups exposed to antidepressants, FLX5 and FLXVEN5, shared the highest intra-group variability.

3.2.4. Time spent immobile

The time spent immobile during the benchmark week was 283 ± 68.9 s. The high variability is due to two groups of crabs: (1) crabs that buried themselves quickly tend to stay buried, i.e. did not move until the end of the experiment, and (2) crabs that did not bury themselves quickly or did not bury themselves at all, i.e. did move until the initiation of their first sand-digging behaviour or the end of the test. Overall, the different groups of crabs had the same kind of high intra-group variability, thus the time spent immobile was not significantly different between groups ($p = 0.817$) and time points of exposure ($p = 0.781$; Fig. 4D). Values for all time points and groups oscillated more or less closely around the baseline value.

3.2.5. Percentage of carapace buried

During the benchmark week, crabs ($n = 40$ crabs, i.e. 40 crabs buried of 48 crabs in total) were mostly covered by sand at $74.68 \pm 36.25\%$. Although the medians for all treatment groups and time points were situated close to the baseline levels, the percentage of carapace buried in sandy substrate was significantly different between experimental groups ($p = 0.05$; Fig. 5), but not between the time points of exposure ($p = 0.224$). Pairwise post-hoc permutation tests revealed a significantly higher surface of carapace buried in the FLXVEN5 group, compared to control and FLX5 groups across all time points (post-hoc permutation test: $p = 0.015$ for both comparisons).

4. Discussion

This study presents, for the first time, sub-chronical effects of two different antidepressants, FLX and VEN, in mixtures at environmentally relevant concentrations on the burying behaviour of juvenile invertebrates. The two different species used in this study, *S. officinalis* and *C. maenas*, represent two major groups of marine invertebrates, unshelled molluscs and decapod crustaceans. Both species bury into the substrate, to which they may also adapt their body colouration, in order to avoid predators. Burying is a complex innate behaviour, its main critical parameters being the rapidity with which it occurs as well as its completeness, i.e. the amount of body covered by the substrate. In cuttlefish, epidermal colour change assists the sand-digging camouflage, whereas in crabs the colouration is more fixed; nevertheless they can become darker or brighter through the action of the epidermal chromatophores lying underneath the cuticle. This may modify disruptive colouration, for instance (Detto et al., 2008), but much slower than in cuttlefish. It was, therefore, not considered in this experimental

setting. In addition, cuttlefish are capable of learning, thereby constantly improving these behavioural traits (Poirier et al., 2004). The complex burying behaviour was decomposed into numerous parameters, each of which was often found to change, at least by trend, and, in particular cases, was indeed significantly altered by the exposure to the antidepressants. Even if each single parameter did not reveal highly distinctive modifications of behaviour following antidepressant exposure, at large the observations suggest (1) that environmentally realistic exposures to antidepressants are able to provoke significant behavioural responses in non-target species, (2) that juvenile cuttlefish display more pronounced modifications of burying behaviour than crabs and (3) that a low concentration (2×2.5 ng.L⁻¹, i.e. nominal 5 ng.L⁻¹ of antidepressants in total) may already change behaviour in juveniles of sensitive species. Juveniles with flexible behaviours that can improve during maturation and with experience, i.e. cuttlefish, showed age dependent effects, suggesting that the learning process may be particularly affected by antidepressant exposure.

4.1. Effects of antidepressants on the efficiency of burying behaviour in juvenile cuttlefish

Once they hatch, cuttlefish need to learn quickly how to escape and hide from predators by exhibiting an effective cryptic behaviour composed of background matching, burying into the loose substrate and remaining immobile. In fact, cuttlefish may choose between staying at sight and change the colour of their skin, i.e. perform background matching, or burying themselves as quickly as possible into the substrate. Most likely, both strategies will be combined to improve camouflage, as shown by the positive relation between both parameters in the 30 dph control animals observed in this study (Tab. 1). The antidepressants did not seem to affect the latency before the first sand-digging behaviour occurred. In fact, the antidepressants did not impair sand-digging behaviour as such. They appeared, however, to act on the maturation of this behaviour and, likely, on learning processes, because the juvenile cuttlefish had weekly encounters with the sandy substrate in this study as compared to only two, at hatching and two weeks after, in the study of Di Poi et al. (2014). Indeed, Poirier et al. (2004) demonstrated that early experience of sand influenced subsequent behaviour with sand in a way that sand-digging became more frequent. All cuttlefish, whether contaminated with antidepressants or not, showed a decrease of the latency with age. This confirms that juvenile *S. officinalis* improve sand-digging behaviour with age. In the controls, a clear pattern in the reduction of the latency and in intra-group variability with age emerged. As the reduction of the latency with age in juvenile cuttlefish exposed to FLXVEN2.5 was far less important than in the controls and intra-group variability remained high, one might infer that the antidepressants can decelerate this learning process. In a previous study, Di Poi et al. (2014) showed that, at two weeks of age, latency before the first sand-digging behaviour was significantly higher in cuttlefish exposed to 1 ng.L⁻¹ of FLX than in the control group. Latency was even more increased by a higher FLX concentration of 100 ng.L⁻¹, suggesting that FLX clearly delayed burying and that it is likely to do so in a concentration dependent manner. By trend, the results of the present study are consistent with the increased latency in antidepressant-exposed cuttlefish observed by Di Poi et al. (2014). Latency of sand-digging in cuttlefish may, however, also be lowered by antidepressants. For instance, Di Poi et al. (2014) reported a significantly reduced latency of burying after hatching in *S. officinalis* perinatally exposed to 1 ng.L⁻¹ of FLX. Such reduction in latency might be expected assuming increased serotonin levels, as observed in *Daphnia magna*, for instance (Campos et al., 2016). Indeed, in the other treatment groups, i.e. FLX5 and FLXVEN5, latency tended to decrease, albeit not consistently. Given that burying is part of a complex camouflaging behaviour, in which also background matching is involved, individuals may pursue different strategies, either of which may be affected by the antidepressants, leading to the higher inter-individual variability

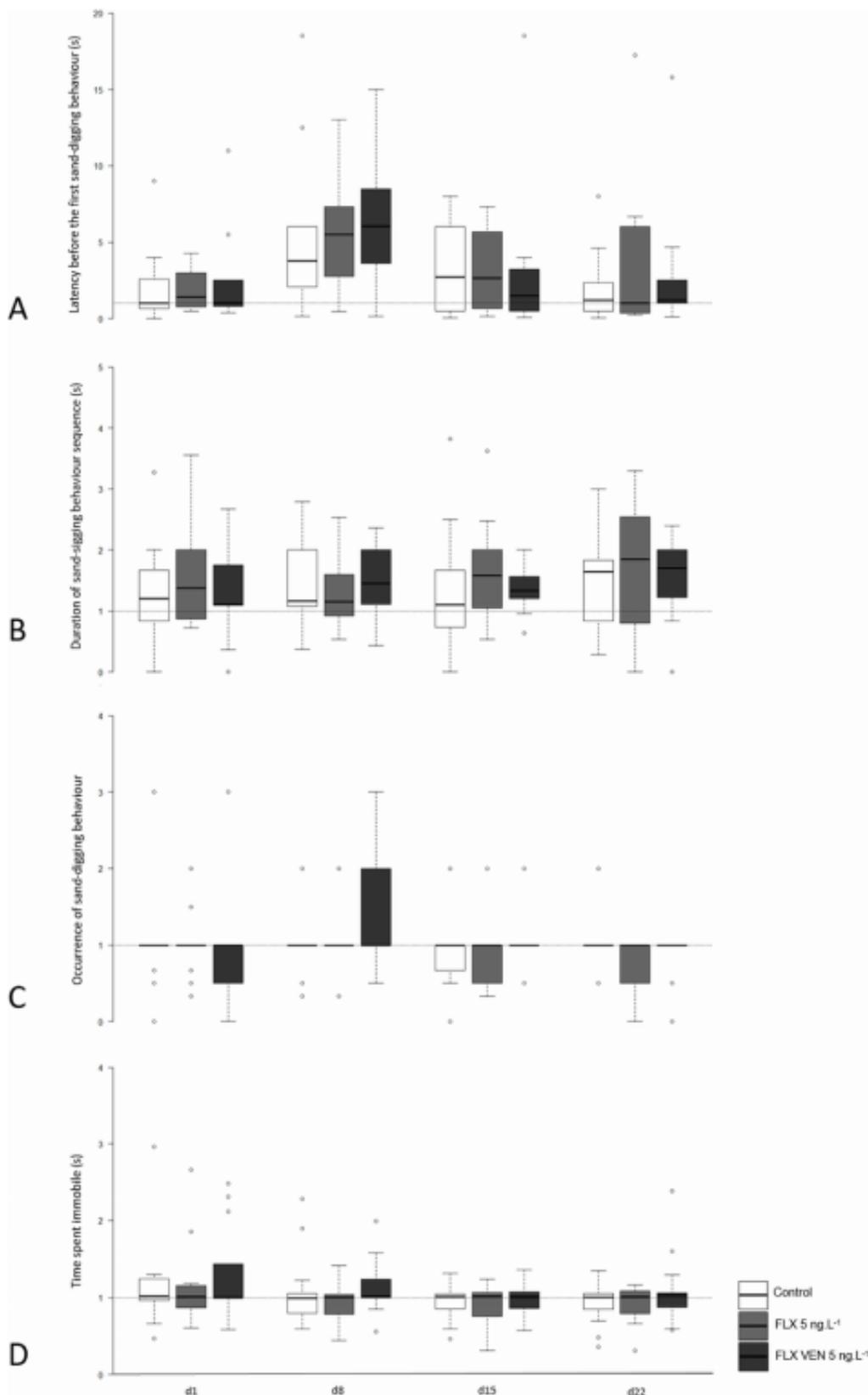


Fig. 4. Sand-digging behaviour of three groups of juvenile crabs exposed to fluoxetine (FLX) at 5 ng.L⁻¹, fluoxetine and venlafaxine in mixture (FLXVEN) at 5 ng.L⁻¹ each as well as non-exposed controls at four time points after 1, 8, 15 and 22 days of exposure, through: (A) latency to first sand-digging behaviour, (B) duration of the sand-digging sequence, (C) occurrence of sand-digging behaviour over the 5-min test, (D) time spent immobile during a 5-min observation period. Data are expressed as fold changes relative to the mean reference value at t0 (benchmark), represented by the dotted line (for details see text). Boxplots showing medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers. Pairwise permutation tests showed no significant difference in either of the measured parameters of the sand-digging behaviour.

observed in the antidepressant exposed animals, as compared to the controls. In this regard, it should be noted, however, that homochromy levels of antidepressant-exposed cuttlefish in this study were high and appeared to be unaffected by the different antidepressants. Notwithstanding, due to slower improvement of sand-digging behaviour in antidepressant exposed juvenile cuttlefish, the interplay between sand-digging behaviour and homochromy was likely to be changed (see below).

Once burying behaviour is initiated, it must be executed quickly and

efficiently. Typically a sequence of the burying behaviour lasted about 5 s or less in the control group of *S. officinalis*. Although no statistical difference between treatment groups emerged, a higher intra-group variability in the antidepressant mixture groups became discernible compared to the control or FLX group. Contrariwise, the intra-group variability of control and FLX group decreased over time, i.e. with age, thus highlighting a maturation and/or learning process. The low concentration mixture tended to prolong the time cuttlefish need to bury themselves, making them more noticeable. Furthermore, we observed

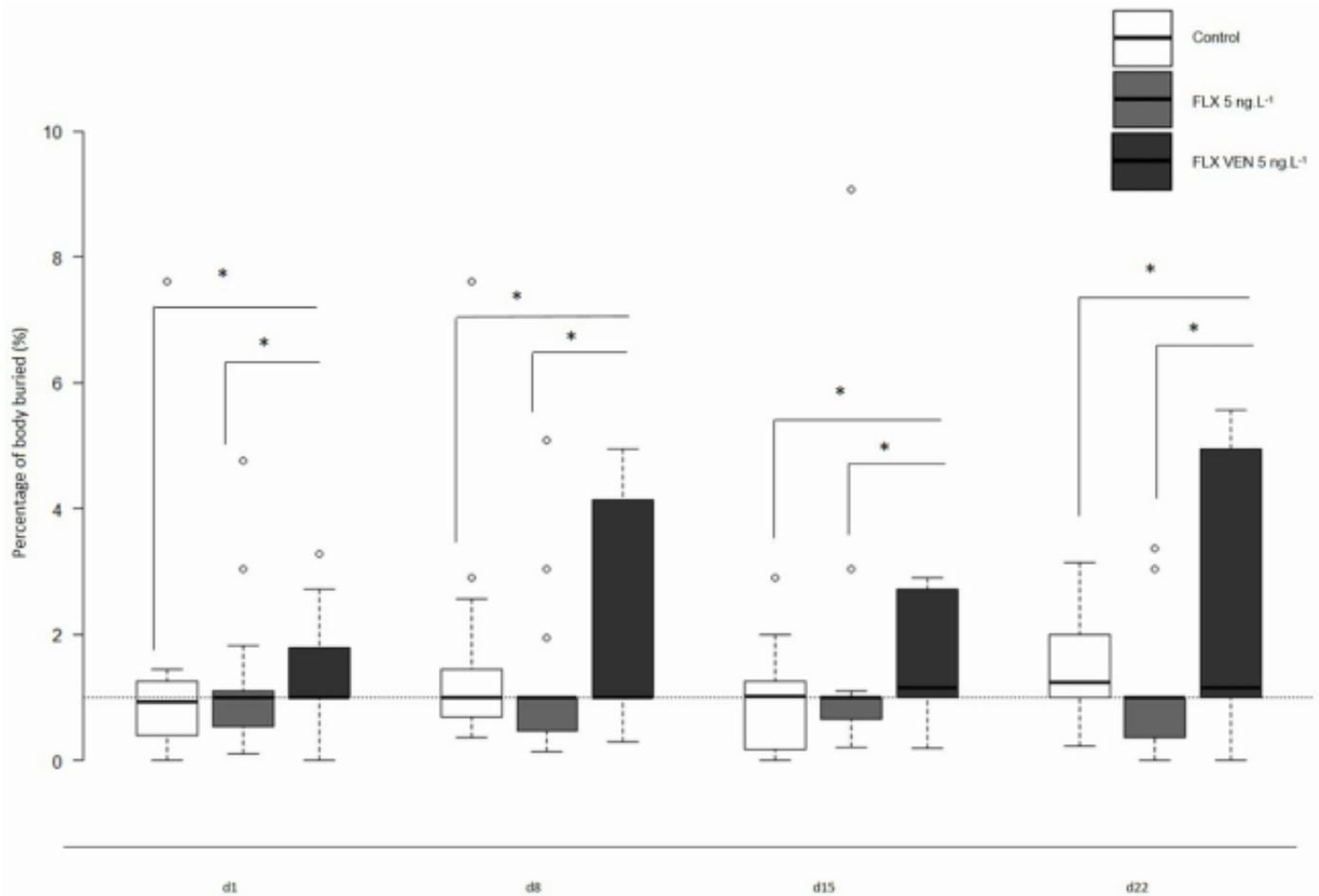


Fig. 5. Percentage of body buried in sand for the three groups of juvenile crabs exposed to fluoxetine (FLX) at 5 ng.L^{-1} , fluoxetine and venlafaxine in mixture (FLXVEN) at 5 ng.L^{-1} each as well as non-exposed controls at four time points after 1, 8, 15 and 22 days of exposure. Data are expressed as fold changes relative to the mean reference value at t_0 (benchmark), represented by the dotted line (for details see text). Boxplot showing medians (horizontal bars), interquartile ranges (boxes) and the highest and lowest values, excluding outliers (whiskers), points represent outliers. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and age comparisons: * indicates a significant difference between groups at $p < 0.05$.

that the sequence of burying behaviour was more chaotic, as indicated by the larger interquartile range and the higher standard deviation, when cuttlefish were contaminated with antidepressants. It is likely that the less precise and more jiggling digging movement of at least some of the individuals would render them more recognisable to the predators.

After being buried, the animals show subtle movements of the mantle while breathing, thus uncovering bits of skin over time. To compensate, cuttlefish may initiate a new sequence of sand-digging behaviour. The decrease in this occurrence of sand-digging behaviours was the most significant parameter affected by antidepressant exposure. According to Di Poi et al. (2014), older cuttlefish are likely to bury more than once during a 30-min test. In our study, however, cuttlefish exposed to a low concentration mixture of FLX and VEN appeared to have a lower occurrence of sand-digging behaviour, regardless of their age. This, once more, suggests that antidepressants exert negative effects on learning processes (Di Poi et al., 2013). Indeed, antidepressants have been pointed out to decrease memory abilities, deterioration or lack of activity in mammals such as rats and humans (El Hage et al., 2004; Eisenreich and Szalda-Petree 2015; Huang et al., 2004; Monleón et al., 2002; Gray and Hughes, 2015; Ulak et al., 2006) as well as in cuttlefish (Di Poi et al., 2013).

An effect of antidepressants on activity, however, could not be recognised, as the time spent immobile during the 30-min test was not clearly affected by any of the treatment groups. As a matter of fact, cuttlefish of each group more or less followed the same pattern: animals were more active at the beginning, i.e. 9 dph, less active at 16 and 23

dph and again more active at the end, i.e. 30 dph. This pattern may correspond to different steps of maturation during the first month of juvenile development (Navet et al., 2014) and could be explained by different kinetics of action of FLX and VEN in the nervous system of cuttlefish. Although these changes appear to be related to age dependent effects, it is important to notice that the two mixture-exposed groups once more share the highest intra-group variability, which points to potential modifications of this particular behavioural trait in certain individuals exposed to antidepressants. Indeed, Fong et al. (2015) found that VEN seems to boost locomotion, while FLX was reducing it in two marine snails, the oyster drill (*Urosalpinx cinerea*) and the starsnail (*Lithopoma americanum*) (see also discussion below on opposite effects of antidepressants in crustaceans). The two antidepressants VEN and FLX differ in the blocking of neurotransmitter uptake and have different effects on neurotransmitters level in the cuttlefish brain (Di Poi et al., 2014; Bidel et al., 2016a). Therefore, they may have different physiological effects and kinetics in cuttlefish, resulting in different modifications of activity and immobility patterns at different time points of the one-month maturation period. This, however, remains mostly speculative and requires testing VEN alone.

4.2. Effects of antidepressants on efficiency of camouflage on sandy substrate in juvenile cuttlefish

Similar to latency before burying and rapidity of the burying sequence, body covering improved with age in the controls and the treatment groups. The antidepressants did not affect the percentage of

body buried at any age in the treatment groups, neither when applied singly, nor in mixtures of different concentrations. These findings confirm earlier observations by Di Poi et al. (2014) with 1 ng.L^{-1} FLX and 100 ng.L^{-1} FLX, suggesting that this parameter is insensitive to antidepressant exposure. The level of homochromy on the natural sandy substrate used in this study was neither affected by the antidepressants in any of the treatment groups, nor at any age. All groups exhibited high homochromy levels of about 80% or more during the entire month after hatching. Similarly, Di Poi et al. (2014) reported that cuttlefish exposed to 1 ng.L^{-1} FLX and 100 ng.L^{-1} FLX did not show significant differences in homochromy from the respective controls. In summary, antidepressants do not seem to impair colour change and camouflage quality against a sandy background. Nevertheless, cuttlefish in the different exposure groups may experience changes in their overall camouflage strategies on sandy substrate. As has been shown in desert arthropods and reptiles (Cloudsley-Thompson, 1991), cuttlefish can express a high homochromy and/or a high percentage of body buried to conceal into their environment. After a three weeks maturation period, juvenile 30 dph cuttlefish from control group were perfectly able to apply both strategies simultaneously ($p = 0.040$, $\rho = 0.599$; Table 1). This ability appeared to be affected in all groups exposed to antidepressants. Because the maturation of sand-digging behaviour was somewhat delayed in the antidepressant exposed animals, the juvenile cuttlefish probably relied primarily on good background matching without burying themselves, *a fortiori*, entirely. Once more, antidepressants appear to have an effect on learning processes related to the maturation of camouflage behaviour, especially on the rapidity of learning. Di Poi et al. 2013 also found that learning was impaired by FLX at an environmental concentration of 1 ng.L^{-1} , but with a different learning paradigm related to predation behaviour. These results suggest that antidepressant exposure may induce broad learning impairments in cuttlefish, as already shown in other species (El Hage et al., 2004; Ulak et al., 2006).

4.3. Effects of FLX and VEN on efficiency of burying behaviour in juvenile shore crabs

Generally, it can be observed that *C. maenas* spend most of their time (76%) during daytime and ebb hidden under rocks or buried in sand (McPhee and Wilkens, 1989). Neither FLX alone, nor in mixture with VEN had any effect on the latency before the first sand-digging behaviour. The duration of the entire sand-digging behaviour sequence seemed to be fixed across all crabs and groups at about 15 s or less. This parameter also remained unaffected by antidepressant exposure. The occurrence of the sand-digging behaviour was neither impacted by the presence of FLX alone, nor in mixture with VEN, but the combined antidepressant treatment groups displayed the highest intra-group variability, a phenomenon also observed with cuttlefish. This variability may, in part, derive from a presumed heterogeneity in age and size structure of the groups composed for the experiments, which could not be controlled as tightly as in cuttlefish. Because antidepressant exposure also increased individual variability in cuttlefish, which were homogenous with respect to age, it is, however, more likely that the FLXVEN treatments provoked this increase in intra-group variability. Either, some individuals may have indeed changed their sand-digging behaviour in response to antidepressant exposure, whereas others did not react to the antidepressants, or the antidepressants elicit opposite effects depending on the individuals. The latter interpretation might be supported by the observation of either decreased or increased latency of burying behaviour in juvenile cuttlefish following antidepressant exposure. Whether this increased variability is related to the mixtures or derives solely from the introduction of VEN into the treatment cannot be assessed conclusively.

Conflicting results to antidepressant exposure have also been observed for mobility. In the present study, crabs exposed to mixtures of FLX and VEN did not show any difference in time spent immobile, even

after 22 days of exposure. In earlier studies, the effects of FLX on behavioural traits such as locomotion, have been investigated with comparable or higher concentrations in different crustacean species. Results differed between species and studies. Following an acute FLX exposure to 5 and 25 mg.L^{-1} for 15 min, Hamilton et al. (2016) did not find any difference in time spent moving in the shore crab, *P. crassipes*. It might be questioned if, despite the quite high concentrations, time was sufficient for uptake and metabolism of the antidepressant in that study. Indeed, it has been shown that FLX requires several hours to days to be accumulated in neural tissues of crustaceans after being injected or administered by oral gavage (Robert et al., 2017). In addition, FLX may require to be metabolised into the equally or, putatively, more active nor-FLX, as suggested by the delayed effects of FLX in *C. maenas* on glycaemia or cardio-respiratory activities observed by Robert et al. (2019, 2016). Accordingly, Mesquita et al. (2011) demonstrated an increase of time spent moving in *C. maenas* at a concentration $120 \mu\text{g.L}^{-1}$ of FLX over 7 days of exposure. This study, however, used a circular arena, rendering it impossible for the animal to find a corner to retreat in (Hamilton et al., 2016). Contrariwise, Tierney et al. (2016) observed a decrease in locomotor activity in the rusty crayfish (*Orconectes rusticus*) after 10 days of exposure at 2 and 500 ng.L^{-1} FLX. These findings may support the idea of antidepressants inducing opposite effects on the same behavioural parameter, i.e. provoking either excitatory or calming/tranquilising effects, depending on the concentration and conditions of exposure. Converse effects of antidepressants are also suggested by the study of De Lange et al. (2006), who showed a decrease in locomotor activity in *Gammarus pulex* after 30 min to 1.5 h of exposure with $10\text{--}100 \text{ ng.L}^{-1}$, whereas the activity of *G. pulex* at higher concentrations ($1 \mu\text{g.L}^{-1}$ to 1 mg.L^{-1}) was similar to the control. To be sure, more data are necessary to confirm whether such opposed effects constitute a general phenomenon of antidepressant exposure, if they are due to different experimental settings (e.g. as proposed by Hamilton et al., 2016), or if they simply are experimental artefacts. Furthermore, it may be noted that most of the concentrations in the studies cited and which affected locomotor behaviour were comparatively higher than the environmentally realistic concentrations used in this study. Moreover, locomotor behaviour is a relatively simple behavioural trait, as compared to the more complex burying behaviour composed of numerous parameters as analysed herein.

4.4. Effects of antidepressants on efficiency of camouflage on sandy substrate in juvenile crabs

When observing crabs burying themselves into the substrate in nature, it can be noticed that they gradually disappear whilst attentively observing their environment. In doing so, part of the anterior carapace remains visible for some time until only the eyes protrude from the surface, before the animal may retreat completely into the substrate. This behavioural sequence was advanced in juvenile crabs exposed to the mixture of FLX and VEN, which exhibited a significantly greater body covering than the FLX5 group or the control group. This may be explained by an enhanced negative phototactic behaviour, either resulting from the mixture exposure, or the VEN contained in these exposures. Earlier studies, using FLX at similar concentrations, reported increased positive phototactic behaviour in a cladoceran species that displays positive phototaxis, i.e. *Daphnia magna* (Storz and Paul, 1998), or in an amphipod species with an usually negative phototaxis, i.e. *Echinogammarus marinus*, (Rivetti et al., 2016; Guler and Ford, 2010). MacPhee and Wilkens (1989) have shown that crabs injected with low doses of serotonin (10^{-6} M) spend less time hidden, thus having an increase of positive phototactic behaviour and time spent moving. Here we observed no effect on locomotion and an opposite effect on phototactic behaviour, i.e. the naturally negative phototactic behaviour was enhanced.

4.5. Differences in burying behaviour between juvenile cuttlefish and shore crabs

Few parameters have been modified by antidepressant exposure in the juveniles of either of the two species. Overall, however, the results point towards an influence on the burying behaviour of juvenile cuttlefish and crabs, which appeared to be slightly, but significantly modified by the mixture of FLX and VEN at concentration as low as 5 ng.L^{-1} in total for cuttlefish and 10 ng.L^{-1} in total for crabs. These modifications were more pronounced in cuttlefish and affected different behavioural components, as compared to crabs. Given the high intra-group variation, indicative of individually varying responses to the antidepressants, the statistically significant differences that emerged are even the more pertinent.

Generally, in our experiments crabs were quicker to engage their first sand-digging behaviour than cuttlefish. To some extent, this may be due the fact they do not exhibit very rapid colour changes, as do cuttlefish. For cuttlefish, this implies different strategies and decisions to be taken in which strategy to engage first. Therefore, in cuttlefish, burying is likely to be a more complex behavioural programme than in crabs. In the latter, burying behaviour might be a more innate and immediate behaviour than is the case for cuttlefish. Indeed, if crabs do not exhibit cuticle colouration that matches with the background (grey sand in this case), even remaining motionless on the substrate could be dangerous, especially if the animal happens to find itself on a substrate to which it has not adapted initially. Hence, the presumably higher behavioural complexity may explain why *S. officinalis* revealed a higher sensitivity to antidepressant exposure than crabs. However, both species are capable of relatively simple forms of learning such as habituation or conditioning (Alves et al., 2007; Orlosk et al., 2011). Although in both cases juveniles were employed in the behavioural tests, the development of both species is quite different. Consequently, age structure and developmental stages in both species differed considerably from one another. Cuttlefish were exposed immediately after hatching, providing a cohort of experimental specimens that was homogeneous in age and development. More importantly, however, cuttlefish learn to improve their behaviour during the first month of maturation and some of the modifications could be explained by an age-dependent effect. Indeed, the hatchlings undergo fundamental changes in their mode of life and this period is characterised by intensive learning and memorization (Navet et al., 2014). In addition, colouration and body patterning have been shown to be subject to learning in *S. officinalis* (Hough et al. 2016). The juvenile *C. maenas* used in this study (< 20 mm carapace width), on the other hand, comprised specimens of different age that were relatively advanced in their development. Chances are that the crabs had already passed the learning period when they were exposed. In cuttlefish, however, it appeared to be precisely the learning process that seemed to be particularly affected by antidepressants.

Besides considerations of age and developmental stage, the actual exposure and uptake may differ between the two species. Cuttlefish are unshelled molluscs, the skin of which may be more penetrable by organic compounds than the mostly rigid cuticle of crustaceans. Although, gills generally provide an enormous epithelial surface and, therefore, must be considered the main entry route for these pharmaceuticals, it is conceivable that the more permeable body surface of cuttlefish may have contributed to an additional uptake of FLX and/or VEN. Furthermore, metabolism and excretion may differ in both species, leading to different body concentrations of the antidepressants and their metabolites. Notably, the digestive gland of cuttlefish is not yet fully developed during the first month post-hatching (Lacoue-Labarthe et al., 2016), a circumstance that might account for reduced metabolic efficiency in maturing juvenile *S. officinalis*. Despite the speculative nature of these considerations, the comparative character of this study underscores the importance of studying responses in different representatives of major phylogenetic groups.

5. Conclusion

Our study confirms that two antidepressants, which can be detected at low concentrations in coastal waters, do not have direct lethal effects at these environmental concentrations. They can, however, impair behavioural features in different marine species. In cuttlefish, the occurrence of sand-digging behaviour is modified and in crabs, the percentage of body buried appears to improve with time. In each species, it was rather the accumulated antidepressants that affected particular features of the sand-digging behavioural sequence. This could mean that FLX, because of its different repartition in the water or its different (neuro-)physiological activity is affecting behaviour to a lesser extent than VEN, which, in the tested mixtures, might be the more relevant compound. It could also mean that the environmentally realistic mixture exposures are having more pronounced effects than single antidepressant exposures. Further studies are needed to elucidate the activity and underlying mechanisms for single antidepressants as well as their combined action.

Devoid of parental care, juvenile cuttlefish and crabs have to face various types of predators without any help. As a defensive behaviour, hiding and blending into their environment by burying and homochromy or disruptive colouration has to be highly efficient early in life. Environmentally realistic concentrations of only two of the numerous antidepressants discharged into surface waters altered at least one feature of the burying behavioural sequence in each of the species investigated. In particular, evidence suggests that acquiring experience was particularly affected. This is underscored by the pronounced alterations in *S. officinalis* hatchlings, which were exposed to the antidepressants during their maturation period. In crabs, this maturation period was either passed or learning does not have the same importance for burying behaviour as in cuttlefish. As a matter of consequence, the antidepressants had lesser effects on *C. maenas*. In summary, this study suggests that behavioural traits that are flexible and improve with experience are likely to be modified by antidepressant exposure at low environmental concentrations during periods when innate behaviour is improved by learning. Given the complexity of behaviour and its ecological relevance, conclusions about the innocuousness of these pharmaceutical residues in the aquatic environments have to be drawn with caution.

Declaration of competing interest

The authors have nothing to declare.

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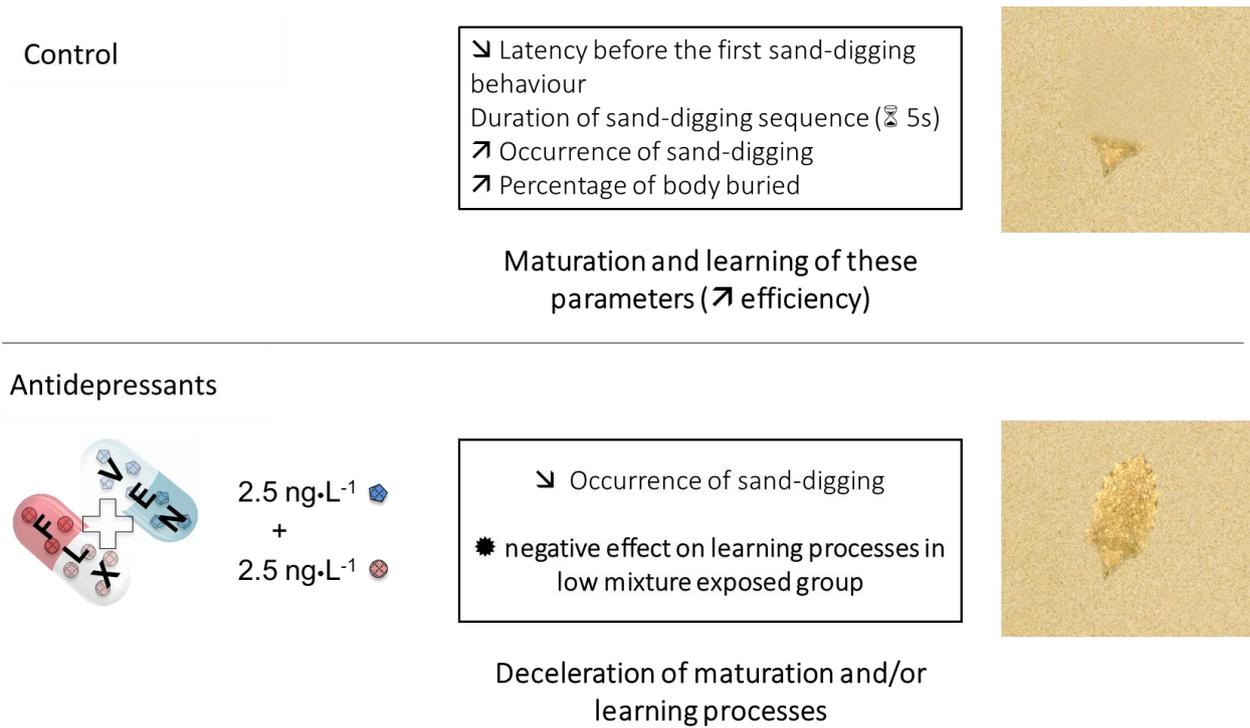


Figure 12. Summary of the results regarding the cuttlefish of the article: Hidden in the sand: Alteration of burying behaviour in shore crabs and cuttlefish by antidepressant exposure

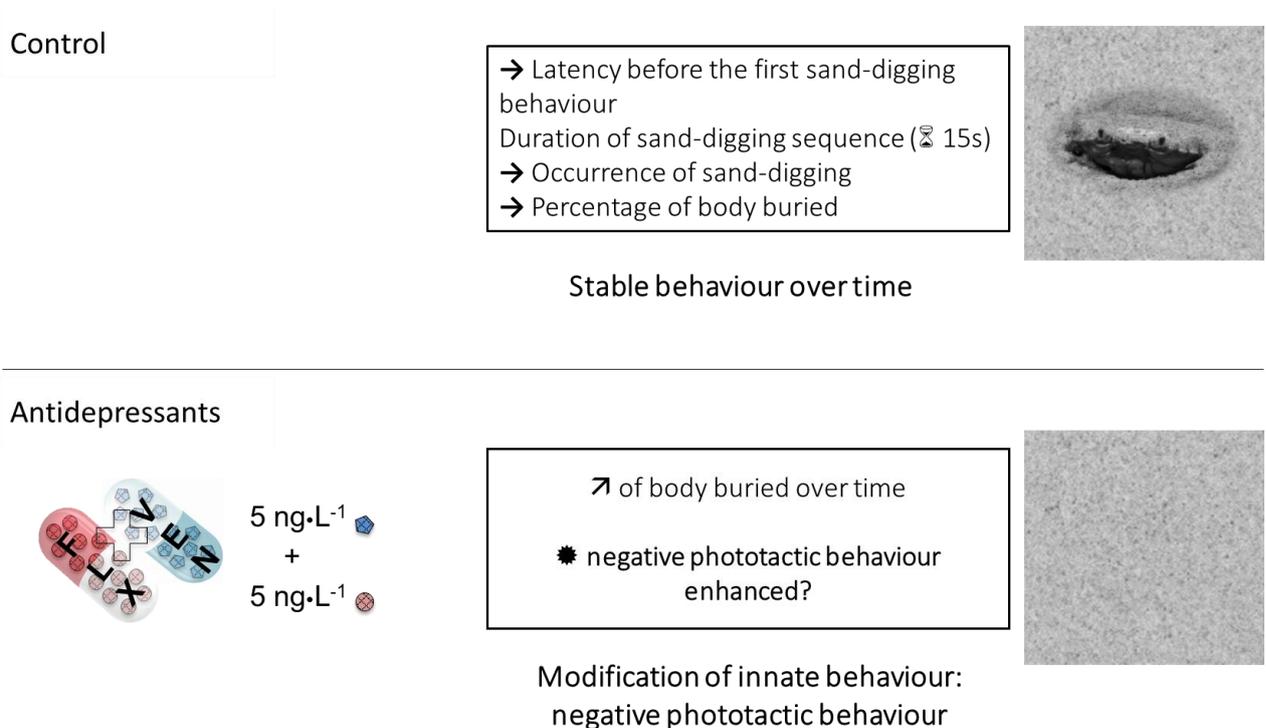


Figure 13. Summary of the results regarding the crabs of the article: Hidden in the sand: Alteration of burying behaviour in shore crabs and cuttlefish by antidepressant exposure

Article IV – Alteration of predatory behaviour and growth in juvenile cuttlefish by fluoxetine and venlafaxine

The aim of the study was to examine if the antidepressants fluoxetine and venlafaxine could affect the predatory behaviour of newly-hatched cuttlefish, which may have repercussions later on growth. The predatory behaviour was followed during the first five days after hatching and growth was monitored at three time points, *i.e.*, 1, 7 and 28 days.

Alteration of predatory behaviour and growth in juvenile cuttlefish by fluoxetine and venlafaxine

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Keywords: antidepressant; monoamines; cephalopod; *Sepia officinalis*; food intake; development

Highlights

- Very low $\text{ng}\cdot\text{L}^{-1}$ concentrations of antidepressants impaired predatory behaviour
- Cumulated fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each decreased feeding motivation
- Cumulated fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each decreased successful prey capture
- Cumulated fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each decreased growth

Abstract

Antidepressants in coastal waters may affect ontogeny of predatory behaviour in cuttlefish, which may, as a result, affect growth of newly-hatched cuttlefish. We investigated the effects of two of the most prescribed antidepressants, fluoxetine (FLX) and venlafaxine (VEN) at environmentally realistic concentrations on the predatory behaviour of hatchlings of *Sepia officinalis*. Newly-hatched cuttlefish were exposed from 1 hour (*i.e.*, day 1) to 5 days after hatching to either FLX alone ($5 \text{ ng}\cdot\text{L}^{-1}$) or combined with VEN ($2.5 \text{ ng}\cdot\text{L}^{-1}$ or $5 \text{ ng}\cdot\text{L}^{-1}$ each) to simulate a more environmentally realistic exposure scenario. Their predatory behaviour was analysed through several parameters: prey detection, feeding motivation and the success in catching the prey. All parameters improved in control animals over the first five days. The combination of FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ each altered the predatory behaviour of the hatchlings by increasing the latency before attacking the prey, *i.e.*, reducing feeding motivation, and also by reducing the number of successful attacks. Consequently, it tended to reduce food intake and affected growth significantly at 28 days post-hatching. Exposures to either FLX at $5 \text{ ng}\cdot\text{L}^{-1}$ or FLX and VEN in mixture at $2.5 \text{ ng}\cdot\text{L}^{-1}$ each tended to have similar effects, even though they were not statistically significant. It is likely that the antidepressants affect maturation of the predatory behaviour and/or learning processes

associated to the development of this behaviour. The slightest delay in maturation processes may have detrimental consequences for growth and population fitness.

1. Introduction

Cuttlefish are active predators capable of catching diverse types of prey (Hanlon and Messenger, 2018), such as small crustaceans and molluscs, but also fish. *Sepia officinalis* becomes an efficient predator over time by maturation of its nervous system and by learning whilst still in the egg and, notably, beginning with hatching (Darmaillacq et al., 2008; Dickel et al., 1997). Indeed, hatchlings do not benefit from parental care because the parents die before their offspring hatch. Juvenile cuttlefish, therefore, have to forage independently (Hanlon and Messenger, 2018). To ensure their survival, they have to learn quickly how to hunt efficiently, as newly-hatched cuttlefish can subsist without feeding only for a few days by relying on the remainders of their internal yolk reserve (Dickel et al., 1997). To avoid any nutrient deficiency, they usually begin to hunt before their yolk is entirely depleted (Wells, 1958). The critical nature of these first days post hatching is underscored by their cuttlebone, which becomes positively buoyant if juveniles do not eat by the fifth day after hatching. As a matter of consequence, the juveniles become unable to hunt and they quickly perish (Boletzky, 1975; Boucau-Camou & Roper, 1995; O'Brien et al., 2017). Furthermore, newly-hatched cuttlefish will have to grow quickly to become efficient and less endangered predators and growth is positively correlated to food intake. Reduced food intake and growth during initial life stage may persist at later life stage and throughout lifetime (Koueta and Boucaud-Camou, 1999).

Juvenile cuttlefish have to adapt their hunting method depending on the prey type (Hanlon and Messenger, 2018). With a crab in sight, cuttlefish will apprehend their prey by jumping from behind with partially open arms and without using their tentacles (Duval et al., 1984). On the contrary, *S. officinalis* will attack a shrimp by ejecting the two tentacles equipped with large suckers, to capture the prey and bring it to the arms and mouth (Messenger, 1977). It appears that juvenile cuttlefish have a preference for shrimps as first prey after hatching over crabs or young fish (Darmaillacq et al., 2006; Guibé et al., 2012). To accomplish this hunting technique, newly-hatched cuttlefish need to (1) detect their prey, (2) assess the distance between themselves and their prey, (3) and eject their tentacles in the right direction and fast enough for successful seizure. These technical parameters need to be improved by maturing and learning over the first few hours to days after hatching.

Behaviour in many species is regulated by the nervous system and influenced by many neurotransmitters. Amongst them, serotonin (5-HT), norepinephrine (NE) and dopamine (DA) are monoamines known to be involved in several behaviours (Weiger, 1997), such as locomotor activity (Pavlova, 2001), aggressive behaviour (Huber et al., 1997), as well as predatory behaviour and food intake (He et al., 2018; Palovcik et al., 1982; Wang et al., 2002). Consequently, any change in the levels of these neurotransmitters may result in modifications of diverse behaviours and bear the possibility of

cognitive impairments during development. Therefore, chemical compounds released into the environment that interfere with the serotonergic, as well as the noradrenergic and dopaminergic system of non-target organisms could disturb behavioural traits in aquatic organisms, such as cuttlefish. A group of pharmaceuticals, the antidepressants of the family of selective serotonin reuptake inhibitors (SSRI) and serotonin-norepinephrine reuptake inhibitors (SNRI), could be of particular concern, as they interact with the pre-synaptic membrane transporters that help to regulate neurotransmitter levels in the synaptic cleft. Because of their continuous release into the aquatic environments worldwide *via* waste water effluents, these pharmaceuticals may be considered pseudo-persistent micropollutants (Bueno et al., 2012; Nielsen and Gøtzsche, 2011; OECD Health Statistics, 2018). Indeed, antidepressants are persistently detected and quantified at low $\text{ng}\cdot\text{L}^{-1}$ concentrations in surface waters (Bueno et al., 2012; Meador et al., 2016; Metcalfe et al., 2010; Paíga et al., 2016; Rúa-Gómez and Püttmann, 2012c, 2012a). Our study combines two of the most prescribed antidepressants, Prozac[®] and Effexor[®], and their respective active molecules fluoxetine (FLX) and venlafaxine (VEN) (Fong and Ford, 2014a). The antidepressants FLX and VEN have been found at the lower $\text{ng}\cdot\text{L}^{-1}$ range typically detected in aquatic compartments (Kwon and Armbrust, 2006), *i.e.*, up to 0.3 and 1.3 $\text{ng}\cdot\text{L}^{-1}$, respectively, in the coastal waters of Northwestern France (Minguez et al., 2016). The frequently reported low $\text{ng}\cdot\text{L}^{-1}$ concentrations of single antidepressants do, therefore, not effectively reflect the true load of these pharmaceuticals, as different antidepressants are released simultaneously into the water bodies and accumulate to higher total concentrations (Klosterhaus et al., 2013; Meador et al., 2016; Richmond et al., 2016). Despite their low toxicity and trace amounts, FLX and VEN released into the aquatic environment may affect particularly sensitive behavioural traits in non-target organisms, such as hatchlings of cuttlefish. Indeed, antidepressants have been shown to induce behavioural impairments in various aquatic species, such as changes in maturing/learning capacities (Chabenat et al., 2019; Di Poi et al., 2013a), camouflage (Bidel et al., 2016a; Di Poi et al., 2014), locomotor activity (Barry, 2013; Guler and Ford, 2010a; Mesquita et al., 2011; Tan et al., 2020) or reproductive behaviour (Campos et al., 2016; Fong, 1998). Predatory behaviour was also affected by the two psychoactive drugs in several aquatic species, such as striped bass (Bisesi et al., 2016, 2014b; Gaworecki and Klaine, 2008), fathead minnow (Stanley et al., 2007; Weinberger and Klaper, 2014), goldfish (Forsatkar et al., 2014; Mennigen et al., 2010) or shore crab (Peters et al., 2017).

Cephalopods have the most developed brain amongst all molluscs allowing for a complex behavioural repertoire (Hanlon and Messenger, 2018). Because of their elevated cognitive capacities (Darmaillacq et al., 2006; Dickel et al., 1997; Mather and Dickel, 2017), which mature during their first life stages, they are particularly sensitive models to study the potential effects of psychotropic substances on animal behaviour. Amongst the various behavioural traits displayed by juvenile cuttlefish, predatory behaviour after hatching is fundamental for growth and survival of the hatchlings. Any modification of foraging that reduces predation success due to pollutants present in the shallow coastal waters where female

cuttlefish lay their eggs could affect population recruitment. Moreover, a fundamentally impaired predatory behaviour following antidepressant exposure may reduce hunting success and, consequently, food intake. Reduced food intake, in turn, is likely to have an impact on other physiological or morphological aspects, such as reduced growth (Koueta and Boucaud-Camou, 1999). Earlier studies showed that embryos of *S. officinalis* do indeed bioaccumulate pollutants such as heavy metals or the antidepressant FLX (Bidel et al., 2016a; Lacoue-Labarthe et al., 2016) and reported effects of antidepressants on early stages of cuttlefish. For instance, Di Poi et al. (2013) demonstrated alterations of cognitive abilities and Bidet et al. (2016a) as well as Di Poi et al. (2014) found that camouflage was affected. Although studies on the effects of single molecules, such as FLX and VEN on predatory behaviour exist (Gaworecki and Klaine, 2008; Mennigen et al., 2010; Stanley et al., 2007), few studies investigated the effects of cumulated antidepressants on this particular behaviour (Bisesi et al., 2016) and no study investigated their effects at very early life stage essential for growth and population survival. Yet, accumulated concentrations of major antidepressants constitute a more realistic environmental scenario than testing single antidepressants. Hence, this study assesses the impact of two antidepressants at cumulated low $\text{ng}\cdot\text{L}^{-1}$ concentrations combining a SSRI (FLX) and a SNRI (VEN) on predatory behaviour and growth —two fundamental ecological parameters— in highly sensitive hatchlings of *S.officinalis*.

2. Material and methods

2.1. Animal rearing and experimental conditions

Eggs of wild cuttlefish (*Sepia officinalis*) were collected in the English Channel (off the coast of Luc-sur-mer, France) at different locations and several times to ensure genetic variability (laid by different females). They were randomly mixed and kept until hatching in filtered natural sea water in an open-loop system under a natural summer photoperiod (16h light:8h dark) in a climate chamber ($15\pm 1^\circ\text{C}$) at the ‘Centre de Recherches en Environnement Côtier’ (CREC) of the University of Caen Normandy at Luc-sur-mer, France. Egg laying days were unknown and spread out over time. Thus, eight hatchlings were collected daily and isolated as explained below until a total of 60 cuttlefish between the 11th and 19th of July 2017 was reached.

At hatching, cuttlefish were transferred immediately to glass beakers (\varnothing 11 cm, height 6 cm; one animal per beaker, henceforth designated ‘home tanks’), assigned randomly to four experimental groups and exposed individually ($n=15$ per group) from their very first hours post-hatching to either (1) carbon-filtered seawater (control), or (2) $5\text{ ng}\cdot\text{L}^{-1}$ FLX alone (FLX5), or (3) $2.5\text{ ng}\cdot\text{L}^{-1}$ FLX and $2.5\text{ ng}\cdot\text{L}^{-1}$ VEN in mixture (FLXVEN2.5) as well as (4) $5\text{ ng}\cdot\text{L}^{-1}$ FLX and $5\text{ ng}\cdot\text{L}^{-1}$ VEN in mixture (FLXVEN5). Cuttlefish were reared over a period of 28 days in a semi-static system supplied with 250 mL carbon-filtered seawater (FSW) to assess the effects of antidepressant exposure on growth. Temperature was maintained at $15\pm 1^\circ\text{C}$ and the photoperiod was set 16h light:8h dark. Constant conditions during the

exposures, *i.e.*, waterborne antidepressant concentrations, salinity of 35 ± 1 PSU, O₂-levels not less than 80%, as well as nitrite and nitrate levels within acceptable levels ($[\text{NO}_2^-]_{\text{max}}=0.05 \text{ mg}\cdot\text{L}^{-1}$, $[\text{NO}_3^-]_{\text{max}}=0.15 \text{ mg}\cdot\text{L}^{-1}$), were maintained by transferring each animal daily to a new home tank filled with renewed FSW and spiked with the respective antidepressant concentrations. Cuttlefish were fed once a day during the predation test with a sand shrimp (*Crangon crangon*). All shrimps were adapted to the size of the cuttlefish to be properly caught and eaten, *i.e.*, a shrimps' length of about 3-6 mm relative to a dorsal mantle length of the juvenile cuttlefish of 1 cm or less. After the predation tests were terminated at day 5, the animals were exposed and fed with one shrimp per day as before over 28 days of exposure to assess the growth of the animals. No mortality was observed during the test duration, *i.e.*, 28 days.

Animal hatchery and animal experiments were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments (Protocol approved by the French National Ethical Committee for Animal Experimentation, Agreement number: 035121.02).

2.2. Chemical contamination

A set of stock solutions at $10 \mu\text{g}\cdot\text{L}^{-1}$ of either FLX hydrochloride (CAS 56296-78-7, Sigma Aldrich, St. Louis, USA) or VEN hydrochloride (CAS 99300-78-4, Sigma Aldrich) was prepared in distilled water and kept at -80°C until use. For daily water renewals of the cuttlefish home tanks, stock solutions were diluted in FSW to the appropriate final concentrations. Preliminary studies confirmed that the actual concentrations after 24 hours were not less than 75% of the nominal concentration. Waterborne FLX and VEN concentrations were chosen to approximate measured surface water concentrations quantified in Northwestern coast of France, *i.e.*, 0.3 and $1.3 \text{ ng}\cdot\text{L}^{-1}$ for FLX and VEN respectively (Minguez et al., 2016). The FLX5 treatment was used as a reference to compare the results of the present study to those of earlier studies, as FLX represents the model antidepressant, which was first SSRI released into the environment and, therefore, has been used in many studies over the last decades to study the effects of this and similar antidepressants on physiological and behavioural responses in aquatic animals.

2.3. Predation test

2.3.1. Experimental setup

Predatory behaviour of each individual cuttlefish was assessed at five time points: from 1 day post-hatching (dph) corresponding to exposure for 1h in contaminated FSW until 5 dph (Fig. 1) using the predation test described in the following. Recordings were conducted at the same hours (between 9 to 11am) to avoid any influence of circadian rhythms on feeding behaviour and to maintain equal exposure durations between the tests, *i.e.*, 24 hours. Each day, cuttlefish were moved within their home tanks (to avoid additional stress) inside a custom-made black tent for predation test recording, in order to avoid any external stimuli. A circular LED lamp (Neewer®, Guangdong, China) served as light source. The lamp was adjusted in an oblique position 60 cm above the tanks with the inner part of the tent being

lined with white fabrics so as to reflect the light in order to avoid direct light on cuttlefish. The camera (Legria HF R506, Canon Camera Co. Inc., Tokyo, Japan) was installed on a stand adjacent to the lamp.

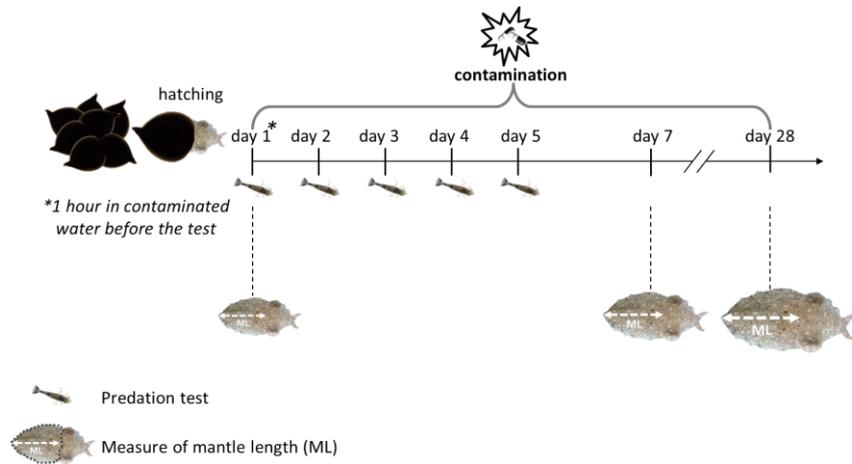


Figure 1. Timeline of the experiments. The day of hatching, the cuttlefish were placed in contaminated water one hour before the first test and the exposure lasted 28 days. Each cuttlefish passed the predation test each day for 5 days. Growth was monitored by measuring mantle length (ML) at three time points, i.e., day 1, 7 and 28 post-hatching.

Four cuttlefish, one from each of the conditions, passed the test simultaneously (Fig. 2). Opaque walls separated the tanks in order to avoid visual inter-individual influence (e.g., mimic) (Fig. 2). The test recording began when a shrimp was inserted in the centre of the home tank with the cuttlefish tending to remain close to the tank's walls. The video recording lasted for 10 min, after which each animal was returned to its experimental exposure zone. If the cuttlefish did not eat the shrimp within the 10 min test period, the shrimp was removed from the tank and excluded from the common batch to avoid any cross contamination or habituation from the shrimp.

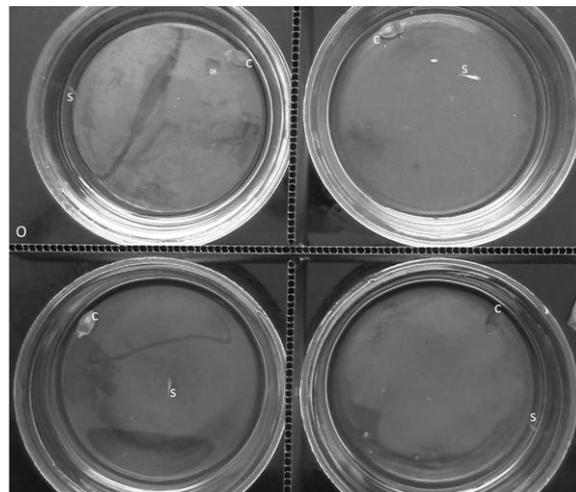


Figure 2. Experimental set-up of the predation test. Cuttlefish passed the test within their home tanks. c: cuttlefish; s: shrimp; O: opaque walls.

2.3.2. Behavioural analyses

Video-recordings were analysed manually *a posteriori*. Predatory behaviour of juvenile cuttlefish was assessed on the basis of (i) whether the prey was actually detected by the cuttlefish, (ii) the cuttlefish showed feeding motivation, *i.e.*, attacked the prey it detected and (iii) feeding behaviour, which was defined as a successful attack and eating the prey.

Prey detection was estimated through six different criteria that were either associated with the presence of the prey, or with the behaviour of cuttlefish. Prey-associated criteria, which accounted for likely detection of the shrimp by the cuttlefish, were assessed as: (i) the shrimp was moving in the beaker, (ii) the shrimp was at a distance less than 3 cm from cuttlefish, (iii) the shrimp was in the cuttlefish's field of vision, which covers a total of 320°. At least two of these prey-associated criteria had to be observed simultaneously in order to accept a prey as detected, even if no obvious behaviour of the cuttlefish towards the shrimp could be observed. Predator-associated criteria that confirmed that shrimp were detected by the cuttlefish were assessed as: (i) the cuttlefish positioned itself towards the prey, (ii) the cuttlefish attempted an attack, but missed the prey (*i.e.*, miscalculated the distance), (iii) the cuttlefish attempted an attack, but the prey escaped. If either of these predator-associated criteria alone, or combined with any other prey- or predator-associated criteria occurred, prey detection could be established with certainty. Eventually, prey detection was expressed as the percentage of one of the following categories relative to the total number of observations: firstly, cuttlefish that successfully detected, attacked and ate the shrimp (detection + success), secondly, cuttlefish that detected the prey but without performing a successful attack (detection + no success), and thirdly, cuttlefish that did not detect the shrimp (no detection).

For *feeding motivation*, two indicators were established:

- (i) The latency before the first attack corresponded to the time between the entry of the shrimp into the home tank and the first time the cuttlefish ejected its tentacles towards the shrimp. If the animals did not attack, the maximum latency of 600 s was assigned.
- (ii) The number of attempts made to catch the prey until the cuttlefish succeeded to seize the shrimp, *i.e.*, accomplishing a successful attack, or until the 10 min test ended (removal of the shrimp).

Feeding behaviour was determined as the number of successful attacks, *i.e.*, the shrimp were attacked and eaten by the cuttlefish within the 10 min of test, for each of treatment groups at each time point.

2.3.3. Food intake

Food intake was determined as the total of shrimps eaten by each cuttlefish over the first five dph, hence the maximum number being five, *i.e.*, one per predation test and time point.

2.4. Growth

Growth was evaluated by measuring the mantle length (ML) of the cuttlefish from the dorsal end to back of the eyes on still pictures (Fig. 1) at 1, 7 and 28 dph using ImageJ[®] (Bethesda, Maryland, USA). Each animal's ML was measured in triplicate, *i.e.*, on three different pictures, for each age and the mean of the three values was taken as its ML.

To guarantee that ML was randomly distributed and the cuttlefish started from the same growth distribution for all treatment groups, ML on day 1 was compared across all groups. This being the case, an analysis across all groups and ages was performed, as described in the statistical analyses, to reveal any difference between treatments and ages.

2.5. Statistical analysis

All statistical tests were performed using R Studio (Version 1.1.453, RStudio Inc., Boston, USA, <http://www.r-project.org>). As data did not meet the assumptions of normality and homogeneity of variance, we used non-parametric permutation test analyses of data from factorial experiments (function `ezPerm`; “ez” package). The number of iterations was set to 1000. We used mixed within-and-between Ss designs (*i.e.*, repeated factors: age; independent factor: experimental groups) to analyse feeding behaviour (*i.e.*, successful attacks), feeding motivation (*i.e.*, latency before the first strike; number of attempts to catch the prey) and growth (*i.e.*, mantle length) at each age. Prey detection was analysed with Friedman test (function `friedman_test`; “coin” package). Additionally, a Kruskal-Wallis test was performed for growth in order to verify the equal distribution of ML among cuttlefish at 1 dph (function `kruskal_test`; “coin” package). For all types of analysis, if the null hypothesis was rejected ($p \leq 0.05$), post-hoc permutation tests were performed to highlight any differences between experimental groups and between ages, using the “RVAidememoire” package (function `pairwise.perm.t.test`). We adjusted the false discovery rate using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

3. Results

3.1. Predation test

Prey detection did neither differ significantly between treatment groups (Friedman test: $p=0.413$), nor between ages (Friedman test: $p=0.698$) (Fig. 2). Each group had a very high detection rate, *i.e.*, more than 87.5% of the animals detected the prey regardless of treatment group or age.

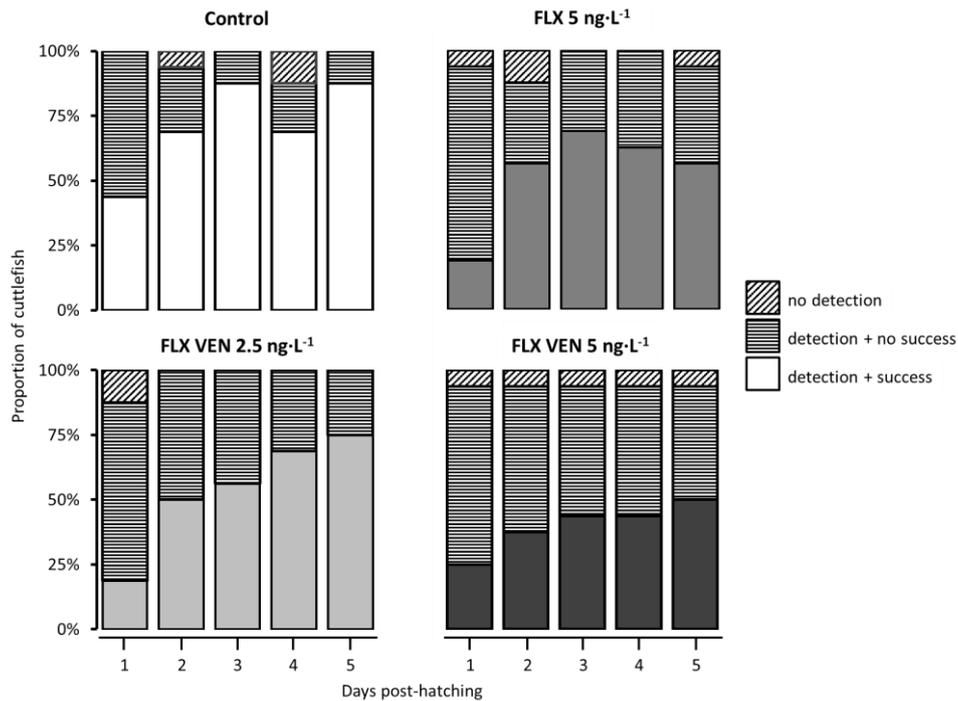


Figure 2. Prey detection during a 10 min predation test by juvenile cuttlefish exposed to four conditions: control (non-exposed group), fluoxetine (FLX) at $5 \text{ ng}\cdot\text{L}^{-1}$, fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$ or $5 \text{ ng}\cdot\text{L}^{-1}$ each. Predation test was conducted at five time points of exposure (1, 2, 3, 4 and 5 days post-hatching). Friedman test showed no significant difference in detection in any group at any age.

Latency before the first attack was significantly different between groups (permutation test across all groups: $p=0.040$). Pairwise comparisons showed that the latency was significantly greater in the FLXVEN5 than in the control group (post-hoc permutation test: $p=0.024$; Fig. 3). Even if no significant difference did clearly emerge between both, the FLX5, the FLXVEN2.5 and the FLXVEN5 group, their latencies tended to be lower than the latter (post-hoc permutation test: FLX5-FLXVEN5, $p=0.072$; FLXVEN2.5-FLXVEN5, $p=0.072$). Similar trends were observed between the control group and either of the FLX5 and FLXVEN2.5 groups (post-hoc permutation test: control-FLX5, $p=0.075$; control-FLXVEN2.5, $p=0.089$).

Importantly, latency evolved with age (permutation test across all groups: $p<0.001$). Indeed, pairwise comparisons showed that latency decreased drastically between the first day after hatching and day 2 (post-hoc permutation test: d1-d2 $p=0.005$), followed by a more moderate decrease between day 2 and day 3 as well as day 4 and day 5, which were, however, statistically non-significant (post-hoc permutation test: $p>0.05$). This pattern is easily observed in the control group (Fig. 3). Within the treatment groups, latency also decreased very rapidly between day 1 and day 2, but developed less steadily in the FLX5 and, notably, in the FLXVEN2.5 groups or even oscillated from day 2 to day 5 in the FLXVEN5 group (Fig. 3). Remarkably, in all treatment groups animals rarely attacked one hour

after hatching, *i.e.*, at day 1, but attacks by hatchlings after 1 hour were more frequent in the control group.

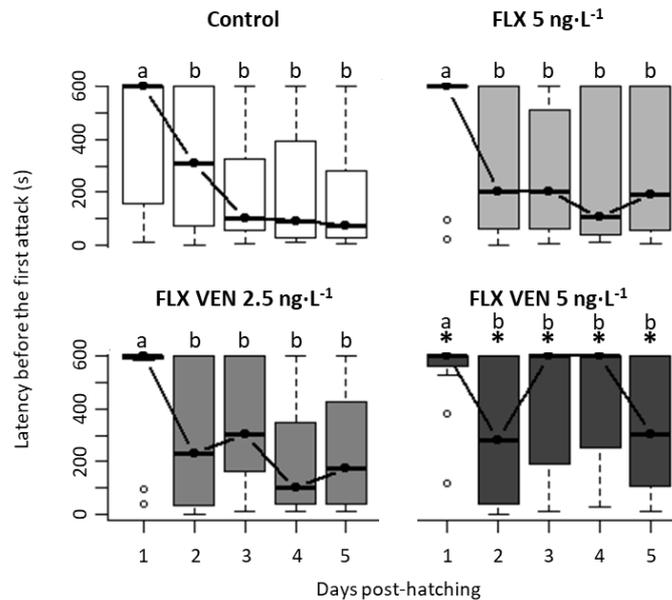


Figure 3. Latency before the first tentacle movement towards the prey, a sand shrimp (*Crangon crangon*), over a 10 min predation test. Newly-hatched cuttlefish were tested at five time points of exposure, *i.e.*, 1, 2, 3, 4 and 5 days post-hatching. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at $5 \text{ ng}\cdot\text{L}^{-1}$, or fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$, or $5 \text{ ng}\cdot\text{L}^{-1}$ each, with non-exposed as controls. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and inter-age comparisons: a and b indicate significant differences between ages within groups ($p < 0.05$); * shows significant differences between the exposed groups and the control group ($p < 0.05$)

The number of attempts made to attack was not significantly different between groups (permutation test across all groups: $p = 0.359$), but it was significantly different between ages (post-hoc permutation test: $p = 0.001$; Fig. 4). Pairwise comparison confirmed that cuttlefish from each group attacked more often at day 5 than during the first 4 dph (post-hoc permutation test: $p = 0.04$). Even if the results did not show any statistical difference, it may be noticed that gradually more and more cuttlefish attacked their prey each day, no matter what the treatment conditions were (post-hoc permutation test: d1-d3, $p = 0.08$; d1-d4, $p = 0.08$). Indeed, the same pattern was seen in successful attacks, with an increasing proportion of cuttlefish attacking their prey each dph (Fig. 5).

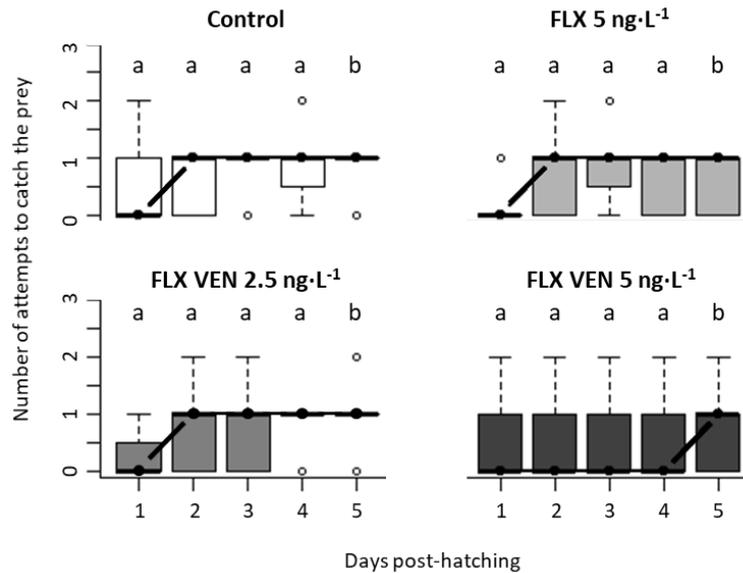


Figure 4. Number of attempts made to catch the prey, a sand shrimp (*Crangon crangon*), over a 10 min predation test. Newly-hatched cuttlefish were tested at five time points of exposure, i.e., 1, 2, 3, 4 and 5 days post-hatching. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at $5 \text{ ng}\cdot\text{L}^{-1}$, or fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$, or $5 \text{ ng}\cdot\text{L}^{-1}$ each, with non-exposed animals as controls. Boxplots show medians (horizontal bars), upper and lower interquartile ranges (boxes) as well as highest and lowest values (whiskers); circles represent outliers. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group and inter-age comparisons: a and b indicate significant differences between ages ($p < 0.05$)

Successful attacks were significantly different between groups (permutation test across all groups: $p = 0.018$). Whereas, the control group showed the highest overall rate of successful attacks (except for d4, Fig. 5), the FLXVEN5 group had the lowest rate of successful attacks (post-hoc permutation test: FLXVEN-control, $p = 0.024$). The FLX5 and FLXVEN2.5 tended to have a lower rate of successful attacks as compared to the control group as well, but these differences did not attain statistical significance (post-hoc permutation test: FLX5-control, $p = 0.084$; FLXVEN2.5-control, $p = 0.084$). The lack of statistical significance may be explained by a high intra-group variability within FLX5 and FLXVEN2.5 groups.

As with the latency, the rate of successful attacks differed between ages (permutation test across all groups: $p < 0.001$). In fact, pairwise comparisons showed an increase in successful attacks with age between d1 and d2, d3, d4, d5 (post-hoc permutation test: d1-d2, $p = 0.015$; d1-d3, d1-d4 and d1-d5, $p = 0.007$, respectively) for each group. Although this statistically significant increase with age was obtained for all groups, the FLXVEN5 treatment stood out as the group with the lowest progressive increase (at d5: 56.25% against 87.5% for control; 62.5% for FLX5; 75% for FLXVEN2.5).

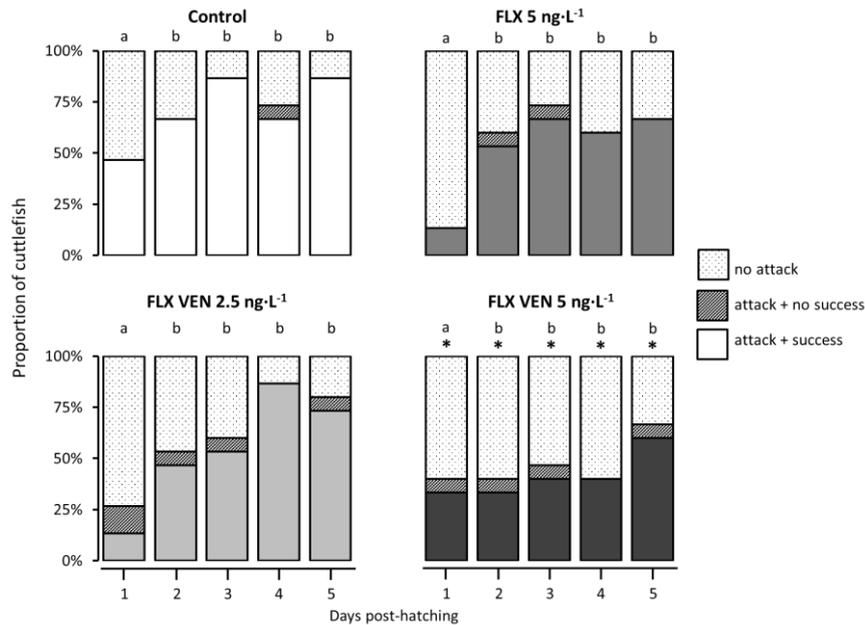


Figure 5. Proportion of cuttlefish which did (i) attack the prey with success, did (ii) attack the prey without success, or did (iii) not attack the prey at all over a 10 min predation test. Four groups of cuttlefish were exposed to either fluoxetine (FLX) at $5 \text{ ng}\cdot\text{L}^{-1}$, or fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$, or $5 \text{ ng}\cdot\text{L}^{-1}$ each, with non-exposed animals as controls. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-groups and inter-age comparisons: a and b indicate significant differences between ages within groups ($p < 0.05$); * shows significant differences between the control group and either of the exposed groups ($p < 0.05$)

The food intake, i.e., number of shrimps eaten throughout the first five dph was not significantly different between groups, even though a trend may be noticed (Kruskal-Wallis test, $p = 0.068$). Cuttlefish from the control group ate in average 3.5 ± 1.1 shrimps during the first five dph, whereas cuttlefish exposed to antidepressants such as FLX5, FLXVEN2.5 and FLXVEN5 groups ate in average 2.6 ± 1.4 , 2.7 ± 1.3 , 2.1 ± 1.8 shrimps respectively.

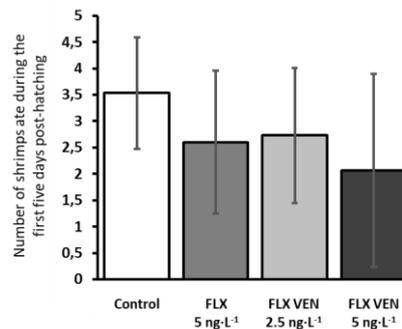


Figure 6. Food intake represented by the number of shrimps ate during the first five days post-hatching by cuttlefish exposed or not (control) to either fluoxetine (FLX) at $5 \text{ ng}\cdot\text{L}^{-1}$, or fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$, or $5 \text{ ng}\cdot\text{L}^{-1}$ each.

3.5. Growth

On the first dph, the MLs of cuttlefish of all groups were statistically equal (Kruskal-Wallis test, $p=0.662$). Indeed, at 1 dph all groups were exhibiting similar mean MLs (\pm SEM): 8.17 ± 0.66 mm for the controls, 8.30 ± 0.86 mm for FLX5, 8.18 ± 0.73 mm for FLXVEN2.5 and 7.88 ± 1.17 mm FLXVEN5. By the end of the experiment, at 28 dph, the mantle length of cuttlefish had significantly increased at each time point, *i.e.*, 1, 7 and 28 dph (permutation test: $p<0.001$; post-hoc permutation test: $p=0.002$ between each time points). There was no obvious difference between the four groups in growth of ML at any age (permutation test: $p=0.217$). Nevertheless, it may be noticed that the mean ML at 28 dph of the FLXVEN5 group appears to be slightly lower than in control group: 13.10 ± 1.44 mm against 13.97 ± 0.82 mm, respectively. Indeed, a post-hoc permutation test between FLXVEN5 and the control group demonstrated a significant difference (post-hoc permutation test: $p=0.032$), highlighting a lower ML.

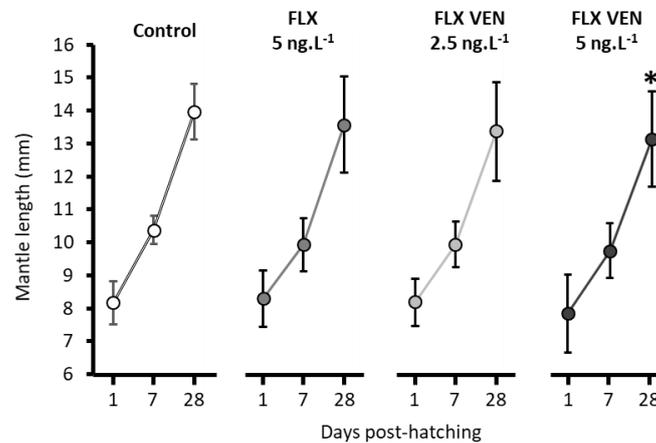


Figure 7. Growth as assessed by mantle length over three consecutive time points (1, 7, 28 days post-hatching) of cuttlefish exposed to fluoxetine (FLX) alone at $5 \text{ ng}\cdot\text{L}^{-1}$, or fluoxetine and venlafaxine in mixture (FLX VEN) at $2.5 \text{ ng}\cdot\text{L}^{-1}$, or $5 \text{ ng}\cdot\text{L}^{-1}$ each, with non-exposed animals as controls. Post-hoc permutation tests adjusted with FDR correction (Benjamini-Hochberg procedure) were performed for inter-group: * shows significant differences between the control group and FLXVEN $5 \text{ ng}\cdot\text{L}^{-1}$ ($p<0.05$)

4. Discussion

The first unconditional step of hunting is to detect the prey. At hatching, cuttlefish are still maturing their visual acuity which begins to develop already at late egg stages (Romagny et al., 2012), enabling them to detect and to gauge their environment (O'Brien et al., 2017). Before attacking, the cuttlefish needs to evaluate the distance to the prey correctly, if the attack shall be successful. Most of the juvenile cuttlefish showed a stable detection of the shrimp from day 1 to day 5, no matter the treatment group. These results suggest that the presence of neither FLX alone, nor the combined exposure with VEN impacted their visual acuity and ability to detect the prey. A previous study from Hedgespeth *et al.* (2014) using the SSRI sertraline showed a decrease of the detection rate after eight days of exposure,

thus, a decrease of feeding rate in juvenile eurasian perch (*Perca fluviatilis*). But this impaired detection rate could only be demonstrated at much higher concentrations of 89 and 300 $\mu\text{g}\cdot\text{L}^{-1}$, whereas at the lowest concentration of 0.12 $\mu\text{g}\cdot\text{L}^{-1}$ no significant decrease in detection was observed. This corroborates our findings that the capacity to detect a potential food source is unlikely to be impacted by antidepressants at low environmental concentrations, as was the case with the more than ten times lower concentration in our experiments with juvenile cuttlefish. Hence, any reduction in feeding activity would necessarily derive from effects other than prey detection.

Maturation of predatory behaviour is essential for newly-hatched cuttlefish since they rely on themselves to satisfy their food requirements. To hunt prey efficiently and successfully, they have to attack their prey fast to avoid possible escapes, once the prey is detected. This necessity for improving their feeding behaviour within the first five days after hatching is corroborated by their high feeding motivation, which is characterised by a progressive decrease in the latency before the first attack as well as an increasing number of attacks over this critical period. The faster and more frequent attacks with time observed in the control group highlight a continuously increasing feeding motivation as well as a sensory-motor maturation that is likely to reflect the neural maturation and learning process (Dickel et al., 2000, 1997; Messenger, 1977). The exposure to the antidepressants FLX and VEN may, therefore, have either delayed the maturation process of the predatory behaviour, the feeding motivation and/or affected learning processes. Indeed, cuttlefish exposed to a combination of FLX and VEN at 5 $\text{ng}\cdot\text{L}^{-1}$ each showed a much greater latency to attack their prey for the first time than the control group during the first five days of exposure. Furthermore, cuttlefish exposed to FLX alone at 5 $\text{ng}\cdot\text{L}^{-1}$ and FLX and VEN at 2.5 $\text{ng}\cdot\text{L}^{-1}$, although not displaying a significantly prolonged latency, did neither show the same clear decrease of latency as observed in the controls. This may be indicative of a similar, but weaker delay in maturation of predatory behaviour, as for the FLX and VEN at 5 $\text{ng}\cdot\text{L}^{-1}$ group. These results are consistent with earlier studies exposing *S. officinalis* to antidepressants. Di Poi *et al.* (2013) demonstrated that FLX was likely to affect memory processing, as perinatal exposure to 1 $\text{ng}\cdot\text{L}^{-1}$ significantly altered acquisition and retention performances in a passive avoidance learning test. In a previous study, we also observed a delay in maturation processes associated with burying behaviour in juvenile cuttlefish when exposed to FLX and VEN at 2.5 $\text{ng}\cdot\text{L}^{-1}$ each (Chabenat et al., 2019). Bidel *et al.* (2015) also investigated the effects of VEN at 5 $\text{ng}\cdot\text{L}^{-1}$ on predatory behaviour and found a tendency towards a greater latency before the first strike compared to the control ($p=0.08$), but the high intragroup variability most likely prevented any statistical significance. Investigations of the brain structures involved in learning, *i.e.*, the vertical lobe and the optic lobes, showed that FLX and VEN induce a decrease in cell proliferation. Bidel et al. (2016a, 2016b) suggest that a decreased cell proliferation may explain the delay in maturation of predatory behaviour in juvenile cuttlefish (Dickel et al., 1997).

As in our experiments the feeding motivation increased with age, the latency before the first attack decreased, whilst the number of attacks generally increased. Despite day-by-day group comparisons did

not turn out statistically significant differences, because of high individual variation in each of the groups, the group exposed to FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ maintained a lower number of attacks over the entire five-day exposure period, suggesting a low feeding motivation in this treatment group. In humans, one of the common side-effects related to SSRIs and SNRIs is the loss of appetite (“Antidepressants - Side effects,” 2018; Capasso and Milano, 2008; Santarsieri and Schwartz, 2015). Furthermore, FLX and VEN are also prescribed for binge-eating disorders and *Bulimia nervosa* because they are considered as appetite suppressants (Carter et al., 2003). In rats, a decrease of food intake is also observed after an exposure with SSRIs or SNRIs (de Oliveira et al., 2004; Jackson et al., 1997; Russ and Ackerman, 1988).

After attacking, which in some cases may need to be repeated several times, the prey has to be seized, and secured by the cuttlefish’s tentacles and arms to be eaten. Over time, newly-hatched cuttlefish become more and more successful in catching their prey and, as a matter of consequence, are able to eat each day. Cuttlefish exposed to FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$ presented the lowest increase of successful attacks over the five days of exposure, meaning that only few cuttlefish of this group have actually eaten every day. This resulted in a lower food intake over the five-day period compared to the control group. As observed with the latency before the first strike, the two other groups exposed to FLX alone at $5 \text{ ng}\cdot\text{L}^{-1}$ or in mixture with VEN at $2.5 \text{ ng}\cdot\text{L}^{-1}$ displayed a similar tendency towards less successful attacks and lower food intake than the control. This suggests that the generally low number of attacks in juvenile cuttlefish exposed to antidepressants is likely to be related to an altered maturation of predatory behaviour, because this behaviour was improved with age in control group. A previous study by Melnyk-Lamont *et al.* (2014) showed that after seven days of exposure to VEN at $1 \text{ }\mu\text{g}\cdot\text{L}^{-1}$, rainbow trout presented a reduced total feed intake per day. Overall, reduced feeding behaviour and food intake in several animal species agree with the known side-effects of SSRIs and SNRIs on human nutrition. Whether they have similar underlying mechanisms remains, however, to be demonstrated. The low improvement of the efficiency of the predatory behaviour may also be related to learning impairments. This and earlier studies of our laboratory consistently point to possible effects of SSRIs and/or SNRIs on learning processes in cuttlefish (Bidel, 2015; Chabenat et al., 2019; Di Poi et al., 2013a).

These effects of SSRI/SNRI antidepressants on behavioural traits in juvenile cuttlefish might be related to changes in the cerebral levels of monoamine neurotransmitters, but differently to what could be expected. In cuttlefish, SSRIs and SNRIs seem to act rather on the DA and NE system than on 5-HT. Especially VEN seems to modulate the NE system Bidel *et al.* (2016a), whereas FLX seems to affect the DA system (Bidel et al., 2016b; Di Poi et al., 2014) of cuttlefish in a yet unknown manner. Indeed, both neurotransmitters have a role in feeding behaviour and both are involved in food intake in fish and mammals (Kulczykowska and Vázquez, 2010 ; He et al., 2018 ; Wang et al., 2002 ; Wellman, 2000) . Although not established in the present study, the modifications of monoamine levels in the brain of cuttlefish resulting from the exposure to environmental concentrations of SSRIs and SNRIs confirmed

by earlier studies could provide an explanation for the lower feeding motivation and feeding success, thus lower food intake of juvenile cuttlefish exposed to antidepressants.

While feeding was reduced during the first days of the cuttlefish life, growth was significantly altered by the presence of antidepressants FLX and VEN at $5 \text{ ng}\cdot\text{L}^{-1}$. A previous study from Di Poi *et al.* (2014) could not demonstrate effects of FLX at 1 or $100 \text{ ng}\cdot\text{L}^{-1}$ on growth in juvenile cuttlefish over two weeks after hatching. Here at 28 dph, *i.e.*, after 4 weeks of exposure, the data indicated an effect of combined FLX and VEN towards reduced growth. Several studies pointed to deleterious effects of FLX on growth in different species. Juvenile guppies, for instance, showed decreased growth after a chronic exposure of 35 days to $30 \text{ ng FLX}\cdot\text{L}^{-1}$ (Pelli and Connaughton, 2015). Tadpoles of *Xenopus laevis* exposed to FLX at $10 \text{ ng}\cdot\text{L}^{-1}$ for a period of 70 days exhibited a reduced growth, probably linked to a decreased food intake (Connors *et al.*, 2009). The amphipod *Hyaella azteca* displayed reduced growth after an exposure to FLX at $33 \mu\text{g}\cdot\text{L}^{-1}$ for 15 days (Péry *et al.*, 2008), while Brooks *et al.* (2003) determined the lowest observed effect concentration for growth to be $5.6 \text{ mg}\cdot\text{kg}^{-1}$ in the same species. In the rotifer *Brachionus koreanus*, growth was reduced after exposure to FLX at 750 to $1000 \mu\text{g}\cdot\text{L}^{-1}$ after only three days of exposure (Byeon *et al.*, 2020). In the polychaete *Capitella teleta*, however, no effect was observed after 18 days of exposure to FLX at 0.001, 0.03, 0.3 or $3.3 \mu\text{g}\cdot\text{g}^{-1}$ dry weight of spiked sediment. Only one study, to the best of our knowledge, demonstrated a stimulation of growth by exposure to FLX on growth: in crayfish, exposure to FLX at $500 \mu\text{g}\cdot\text{L}^{-1}$ enhanced growth, resulting in a greater carapace length of post-moult animals (Tierney *et al.*, 2016). Concerning VEN, no effects on growth could be demonstrated to date. For instance, 0.88 to $80 \mu\text{g}\cdot\text{L}^{-1}$ VEN did not affect growth of fathead minnows (Parrott and Metcalfe, 2017). Similarly, no effect on growth was observed in zebrafish or xenopus embryos after short term exposure to VEN, *i.e.*, 144 hours post-fertilization at $0.3 \mu\text{g}\cdot\text{L}^{-1}$ and 48 hours at $3 \text{ mg}\cdot\text{L}^{-1}$, respectively (Sehonova *et al.*, 2018). Consequently, the literature indicates a greater impact of FLX on growth than VEN. Notwithstanding, quite high concentrations were generally necessary to negatively affect growth by FLX in most of the studies. In our study, a significant decrease of feeding motivation and feeding behaviour in cuttlefish caused by an early exposure to antidepressants at low environmental concentrations, *i.e.*, $5 \text{ ng}\cdot\text{L}^{-1}$ FLX and VEN each, resulted in a trend towards decreased food intake and diminished growth.

5. Conclusion

In view of the reportedly low $\text{ng}\cdot\text{L}^{-1}$ concentrations of single antidepressants in estuarine and coastal waters more distant from wastewater treatment plants or pharmaceutical industries (Birch *et al.*, 2015; Klosterhaus *et al.*, 2013; Meador *et al.*, 2016; Minguéz *et al.*, 2016), it may be questioned if aquatic animals are at risk by the contamination of the water bodies with antidepressants. The increased use and diversity of antidepressants (Nielsen and Gøtzsche, 2011) are likely to result in cumulative concentrations that are higher than those reported for single antidepressants. The combined

concentrations at a cumulated $10 \text{ ng}\cdot\text{L}^{-1}$ of two major antidepressants were able to alter predatory behaviour in hatchlings of cuttlefish by reducing feeding motivation through increased latency before the first attack of the prey and the number of successful attacks. The lack of feeding motivation led to a lower food intake and consequently, lower growth. Exposures to either FLX at $5 \text{ ng}\cdot\text{L}^{-1}$ or FLX and VEN in mixture at $2.5 \text{ ng}\cdot\text{L}^{-1}$ each tended to reduce feeding motivation and successful attacks alike, but to a lesser extent. Importantly, the continuous decrease in latency before the first attack and the increase in the number of attacks observed in the controls suggest that the hatchlings considerably improve predatory behaviour within the first days after hatching. This improvement may derive from neural maturation as well as from experience and learning. One may, therefore, assume that the antidepressant exposure affects these maturation and learning processes. Whichever way the antidepressants affect feeding behaviour, whether they act on the neuronal system or on the levels of neurotransmitters, or both, the significant reduction in feeding behaviour, a trait that is fundamental for survival, shows that the generally very low $\text{ng}\cdot\text{L}^{-1}$ concentrations of antidepressants in the environment give reason for concern. This holds true particularly if sensitive stages of animals with advanced cognitive abilities are exposed to residues of the varying antidepressants in their aquatic environment. Even a minor delay in the maturation and learning processes related to predatory behaviour or a reduced growth can potentially reduce fitness of populations confronted with the accumulated antidepressant pollution of the aquatic environments.

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Authors contribution

A.C performed the experiment and data analyses; A.C, C.B, T.K developed and designed the experiment and data analyses; A.C, C.B, T.K wrote the manuscript.

Notes

The authors declare no competing interest.

Abbreviations

5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; NE, norepinephrine; FLX, fluoxetine; VEN, venlafaxine; SSRI, Selective Serotonin Reuptake Inhibitor; SNRI, Serotonin, Norepinephrine Reuptake Inhibitor

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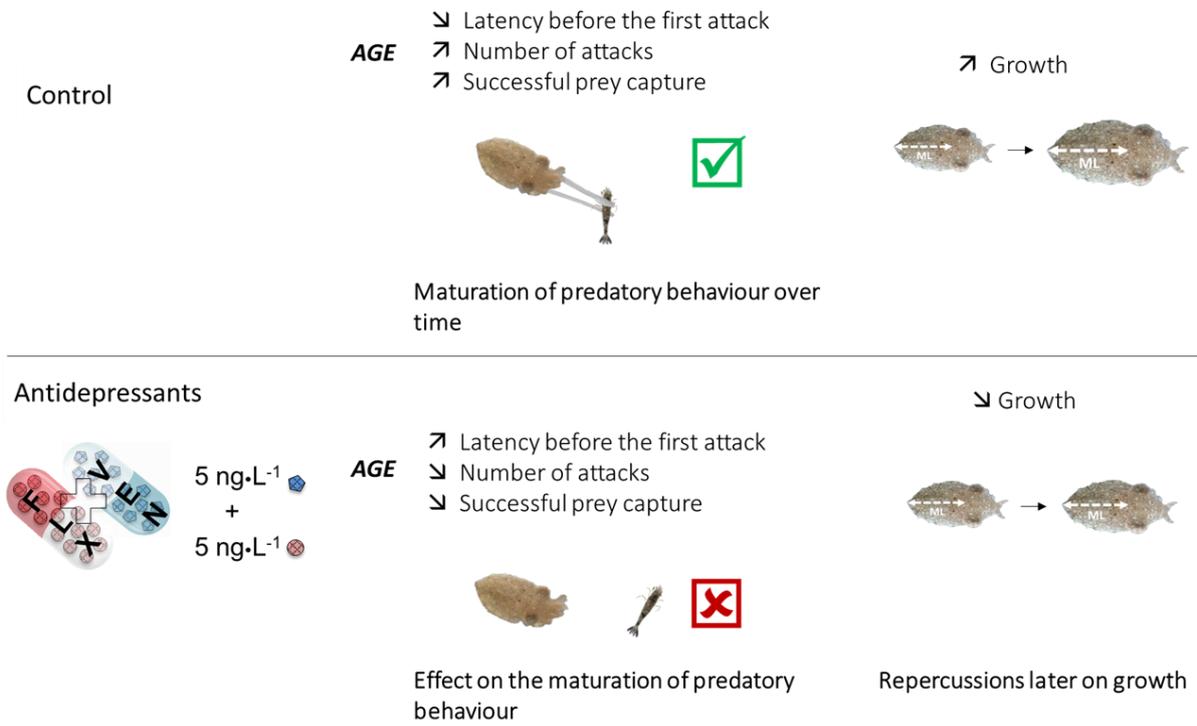


Figure 14. Summary of the results of the article: Alteration of predatory behaviour and growth in juvenile cuttlefish by fluoxetine and venlafaxine

Preliminary results – Antidepressants in the sea: washed away or taken up? Quantifying antidepressants in cuttlefish and crabs

The objective of this study was to determine if antidepressants fluoxetine and venlafaxine could be detected and quantified in (i) juvenile cuttlefish and crabs after a month of exposure and (ii) in adult cuttlefish and crabs collected in natural habitats. For the time being, these results are preliminary in nature. An overview of the chemical analyses was presented in the form of a poster at an international congress at SETAC Toronto 2019.

Antidepressants in the sea: washed away or taken up ?

Quantifying antidepressants in cuttlefish and crabs

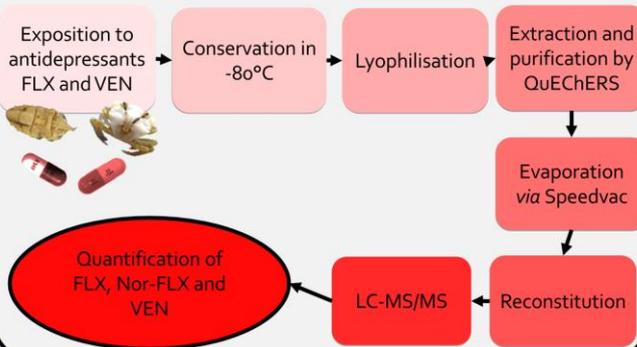
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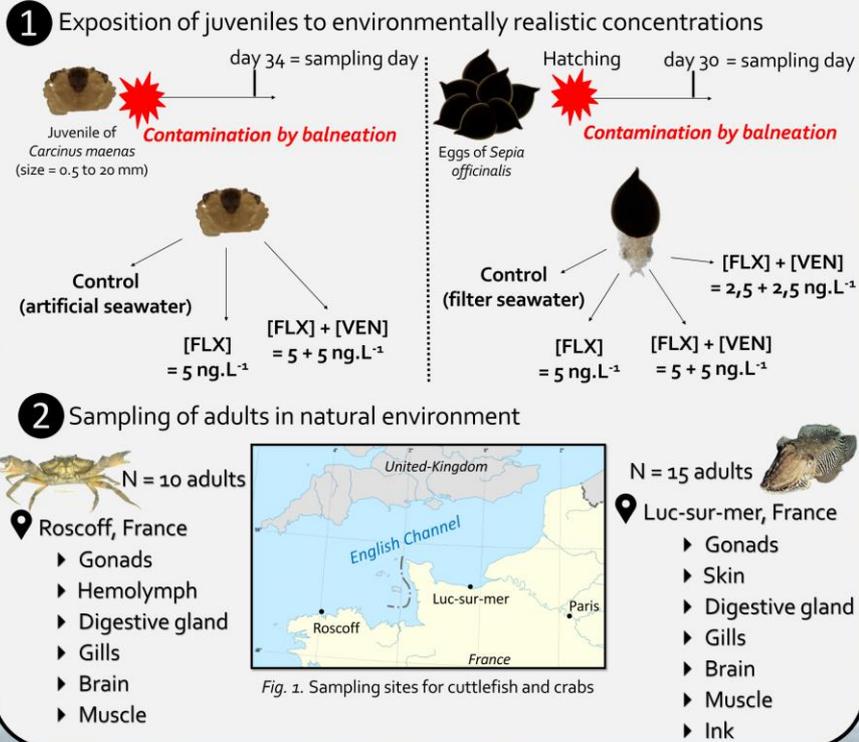
Introduction

- ✓ Prescriptions of **antidepressants** on the rise worldwide
 - ✓ Excretion : contamination of the aquatic compartments
 - ✓ **Fluoxetine (FLX)** and **venlafaxine (VEN)**: detected at low concentrations (from ng.L⁻¹ to µg.L⁻¹)
 - ✓ Toxicity not represented by water concentration
 - ✓ Juvenile **cuttlefish** and **crabs** thrive in the coastal water, close to sources of pharmaceutical pollution
- Development of chemical analysis to detect and quantify both antidepressants in cuttlefish and crabs body

Method



Biological material



Results

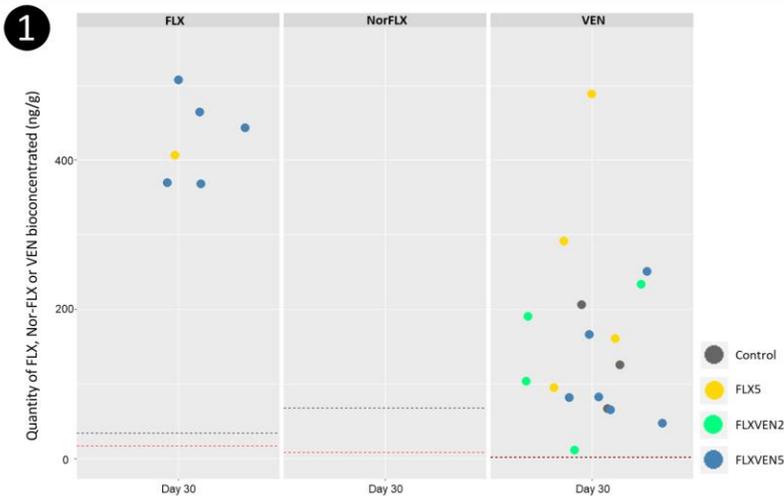


Fig. 2. Quantification of antidepressants bioconcentrated by juvenile cuttlefish depending on treatment after 30 days of exposure by balneation

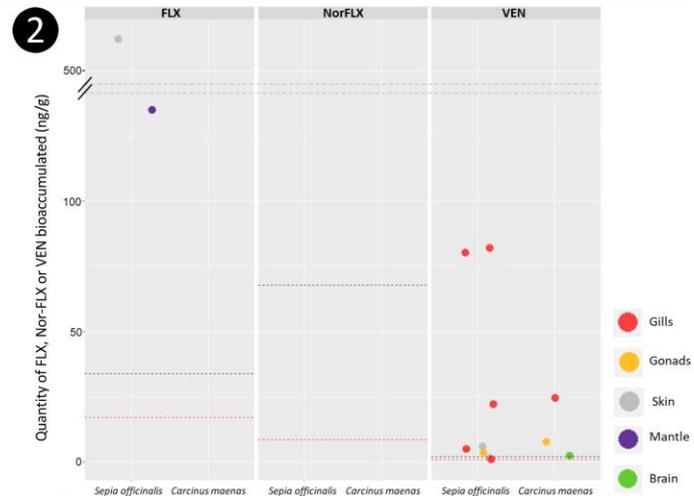


Fig. 3. Quantification of antidepressants bioaccumulated by adult cuttlefish and crabs sampled in their natural environment in different organs

..... **Detection limit** = 3 times higher than background noise signal
 **Quantification limit** = 10 times higher than background noise signal

- Only *Sepia officinalis* bioconcentrated FLX and VEN in their system after 30 days of exposure by balneation, nothing was detected in juvenile crabs after 34 days of exposure
- NorFLX couldn't be quantified nor detected in any of the samples: no metabolism?

- Only *Sepia officinalis* bioaccumulated FLX
- NorFLX couldn't be quantified nor detected in any of the samples: no metabolism?
- Most impacted organs by VEN are gills in cuttlefish, while skin and mantle seem to be the target of FLX

Conclusion & Perspectives

- ✓ Technique permits to detect FLX, VEN and Nor-FLX at **very low level of concentration** (pg/mg)
- ✓ Particular **bioconcentration of VEN** in their system while FLX was less eager to be found, its metabolite **Nor-FLX** wasn't detected at all in any of organisms
- ✓ VEN (and FLX) was detected and quantified in adults cuttlefish and crabs sampled in on the French coast of English Channel



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Discussion & Perspectives

The goal of this doctoral thesis was to examine possible impacts of common antidepressants on cryptic colouration and associated behaviours in juveniles of two invertebrate species, *Carcinus maenas* and *Sepia officinalis*. The Green shore crab and the European common cuttlefish represent two major taxonomic phyla, *i.e.*, crustaceans and molluscs, which together account for more than one third of the marine fauna (Costello et al., 2010). Both species rely on cryptic coloration to hide from predators, notably during their juvenile life stages, but employ different mechanisms of dynamic colour changes with which the antidepressants can potentially interfere. Thus, we asked the question, whether very low, *i.e.*, close to environmental concentrations of more than one antidepressant could have a measurable impact on behaviours and colour change in different marine invertebrates? Indeed, most studies have investigated the impacts of fluoxetine or of venlafaxine at relatively high concentrations of a few $\mu\text{g}\cdot\text{L}^{-1}$ to several hundred or even thousand $\mu\text{g}\cdot\text{L}^{-1}$, whereas antidepressants are mostly detected in the range of the $\text{ng}\cdot\text{L}^{-1}$, at least in the marine environment. Furthermore, single antidepressants have been employed in the majority of studies, but in reality, several antidepressants intended to interfere with serotonergic regulation cumulate to higher concentrations, even if the single compounds may be below the levels of detection. To date, only few studies were conducted with mixtures of molecules. In the marine environment, however, animals are exposed to cumulated amounts of the most frequently prescribed antidepressants discharged into the watercourses that into the estuaries and coastal waters.

Consequences of a waterborne exposure of antidepressants on juvenile cuttlefish

The presence of either fluoxetine alone or combined with venlafaxine at very low concentrations had an impact on cryptic colouration and fundamental behaviours of the juvenile cuttlefish.

First, on a sandy substrate, the effects translated into a lower occurrence of sand-digging behaviour compared to the control group, *i.e.*, a reduced **burying behaviour** (Article III: Chabenat et al., 2019). Indeed, fluoxetine and venlafaxine as low as $2.5 \text{ ng}\cdot\text{L}^{-1}$ caused a modification of this innate behaviour beginning at seven days of exposure until the end of the experiment at 30 days. Because this cryptic behaviour improved in the control group over the first month after hatching, this lower rate in sand-digging behaviour in the antidepressant-exposed cuttlefish could be linked to a delay in maturation and/or learning processes. As a matter of fact, burying behaviour is known to improve progressively over the first weeks after hatching (Poirier et al., 2004). Thus, this innate, yet immature behaviour appears to be sensitive towards the presence of waterborne antidepressants. It may also be speculated that animals are being less anxious over the experimental time course, similarly to human responses to antidepressants. Indeed, previous studies on fish and crustaceans have demonstrated an impact of antidepressants on the anxiety-related behaviours, mostly resulting in a modulation of locomotor activity (Giacomini et al., 2016; Hossain et al., 2020; Simmons et al., 2017). Therefore, a decrease of sand-digging behaviour may be also interpreted as a sign of a reduced anxiety in cuttlefish, because they do not bury themselves or reiterate the sand-digging behaviour.

The cryptic colouration of juvenile cuttlefish, notably the expression of a **uniform body pattern**, was also modified by the presence of fluoxetine and accumulated concentrations of both fluoxetine and venlafaxine (Chabenat et al. *submitted*, article n°II). However, the expression of **disruptive body patterns** did not seem to be affected during the first month after hatching. As for the sand-digging behaviour, Poirier et al. (2005) have demonstrated that the expression of a uniform body pattern is immature at birth and improves during the first weeks after hatching. By and by, newly-hatched cuttlefish will mature their capacity to better display a uniform colouration. Therefore, the presence of contaminants, such as fluoxetine and venlafaxine, even at very low concentrations, is more susceptible to have an impact on improving the expression of a uniform body pattern and sand-digging behaviour than on the expression of a disruptive body pattern. In fact, these two cryptic behaviours as well as the nervous system are still immature in juvenile cuttlefish. This implies ongoing learning processes, while, as suggested by Poirier et al. (2005), the expression of the disruptive pattern might be 'stereotyped' in hatchlings of cuttlefish. Previous studies showed that, during their first weeks of life, newly-hatched cuttlefish were more efficient to wear disruptive components than to display a uniform body pattern (Hanlon and Messenger, 1988; O'Brien et al., 2017). In this respect, our results confirmed previous studies (Bidel et al., 2016a; Di Poi et al., 2014) by showing neither an effect of fluoxetine alone, nor in combination with venlafaxine on disruptive body patterns, no matter the concentrations or the age of cuttlefish.

Newly-hatched cuttlefish exposed to antidepressants also showed a significant alteration of their **predatory behaviour** (Chabenat et al. *submitted*, article n°IV). Indeed, both feeding motivation and successful attacks diminished in cuttlefish exposed to the combination of fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each. As for burying behaviour, it might be assumed that the maturation of predatory behaviour might be affected, thereby reducing the feeding motivation. In fact, controls improved their feeding behaviour rapidly within the five days after hatching, *i.e.*, displayed a progressively shorter latency before the first strike and an increase in successful attacks. Similarly, a previous study exposing cuttlefish to venlafaxine showed a trend towards a higher latency before the first attack (Bidel et al., 2015). Moreover, studies investigating the predatory behaviour in teleosts indicate similar alterations following an exposure to fluoxetine (Forsatkar et al., 2014; Gaworecki and Klaine, 2008; Mennigen et al., 2010; Stanley et al., 2007; Weinberger and Klaper, 2014), venlafaxine (Bisesi et al., 2014a; Melnyk-Lamont et al., 2014) or both antidepressants (Bisesi et al., 2016).

In addition to the effect on feeding behaviour, an effect on growth has been detected. Within the group exposed to both fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$, a slight decrease of food intake during the first days of life significantly impacted later **growth** of cuttlefish at 28 days. Our results add to the concern for the survival of juvenile cuttlefish in the wild, which do not master rapidly how to catch a prey and/or have a lower feeding motivation.

Consequences of a waterborne exposure of antidepressants on juvenile crabs

Juvenile crabs presented rapid behavioural responses to water contaminated with antidepressants.

When placed on sand, juvenile crabs exposed to accumulated concentrations of both fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each exhibited an enhanced **burying behaviour**, *i.e.*, an increase of their percentage of body buried in the sand, starting at day one until the end of the exposure, *i.e.*, over four weeks (Chabenat et al., 2019, article n°III). McPhee and Wilkens (1989) have shown that increased haemolymph levels of 5-HT reduced negative phototactic behaviour by 32% during daylight hours, so that the crabs would be less buried. Assuming that increased burying behaviour corresponds to a negative phototactic behaviour, the accumulated antidepressants rather seem to trigger the opposite effect, as compared to 5-HT alone, leading to more buried animals. In our experiment, this behaviour is not linked to an increase of activity, as all crabs tend to stay buried once they settled in the sand.

Interestingly, on bare ground, as opposed to soft substrate, the **locomotor activity** of the crabs increased after an exposure to a combination of both fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each (Chabenat et al. *submitted*, article n°I). Indeed, juvenile crabs increased their velocity, the distance moved as well as their time spent moving. These parameters were modified over the entire exposure period, from day 3 to day 24 of exposure. Moreover, these effects on the locomotor activity tended to rise continuously across the consecutive time-points of the exposure. These observations are in line with previous results from Mesquita et al. (2011), who showed an increase of distance moved and time spent moving over a 7 days of exposure to fluoxetine in *C. maenas*. However, the concentrations used with larger adult crabs were much higher, *i.e.*, 120 to $750 \mu\text{g}\cdot\text{L}^{-1}$ and beyond concentrations that can be observed in the environment. Other studies demonstrated the same pattern in crabs and other invertebrates (Bossus et al., 2014; De Castro-Català et al., 2017; Peters et al., 2017), whereas a number of studies reported opposite effects, *i.e.*, decreased locomotor activity (De Lange et al., 2006; Fong et al., 2015; Hamilton et al., 2016a; Tierney et al., 2016). As stated earlier, antidepressants may have an impact on anxiety. The experimental setting certainly created a stressful environment, because crabs could hide themselves nowhere. Therefore, they were forced to escape, thus, walking faster on a greater distance and during a greater time searching for a shelter (Kohler et al., 2018). The presence of antidepressants may have enhanced an anxiety-like behaviour in the juvenile crabs, translating into an urge to bury themselves on sandy substrate and resulting in a high locomotor activity on bare ground.

Colour change was also affected by the presence of antidepressants in juvenile crabs, either by fluoxetine at $5 \text{ ng}\cdot\text{L}^{-1}$, or cumulated with venlafaxine. Indeed, exposure to fluoxetine transiently increased the average darkening of the carapace after two days of exposure, but returned to the mean amplitude of colour change that was observed in the controls a week after. In contrast, with accumulated concentrations of both fluoxetine and venlafaxine at $5 \text{ ng}\cdot\text{L}^{-1}$ each, the ability of the crabs to display a paler or darker cuticle diminished. Thus, their colouration was less efficient on the respective

backgrounds. An attenuated colour change combined with an alteration of locomotion may render the cryptic behaviour of juvenile crabs less efficient and may impact their survival in natural environments.

Potential causes and consequences for both species

Low concentrations of both fluoxetine and venlafaxine at $2.5 \text{ ng}\cdot\text{L}^{-1}$ each in cuttlefish and $5 \text{ ng}\cdot\text{L}^{-1}$ in crabs modified their sand-digging behaviour (Chabenat et al., 2019). At first glance, these effects of antidepressants on burying are not necessarily harmful for the animals. On the one hand, juvenile cuttlefish that did not bury themselves still exhibited a good body pattern to match the sandy substrate. On the other hand, juvenile crabs buried better, making them less visible to potential predators. Nonetheless, innate behaviours were modified by the antidepressants in concentrations as low as 2.5 or $5 \text{ ng}\cdot\text{L}^{-1}$. Depending on the environmental conditions, modified behaviours may become inappropriate and may prevent an adequate reaction of the animal to changes of their environment. Notably in cuttlefish, sand-digging is one of the principal strategies to protect from predators at hatching without parental care. Any modification of this behaviour is likely to endanger the juvenile cuttlefish. In addition, if their state of anxiety is lowered they might be less alert to respond properly to a predator appearance. In juvenile shore crabs, an entire body buried might be linked with a negative phototactic behaviour. *A priori*, this is an appropriate innate behaviour of crabs, which helps to protect especially the juveniles from predators. It may, however, also entail to a deficit in oxygen supply/input or to a clogged respiratory channel, if the substrate is not well aerated or sludgy (Bellwood, 2002). Hence, in inappropriate substrates, a prolonged time under the substrate may endanger them as well. In case the crabs stay completely buried without their eyes emerging from the substrate, they might be unaware of the presence of predators when they are about to emerge from the sand.

While on sandy substrate the time spent moving does not seem to be affected by the presence of antidepressants for both cuttlefish and crabs, the locomotor activity of juvenile crabs in an arena with no shelter or substrate was stimulated. If one assumes that fluoxetine and venlafaxine modulate monoamine neurohormonal systems, they could modulate behaviours through multilevel actions on the central and the peripheral nervous system. Central pattern generators, the neuronal circuits that produce rhythmic or stereotyped motor behaviours, such as walking, are well described in arthropods (Ayali and Lange, 2010; Weimann et al., 1991) and monoamines are often cited as neuromodulators of those neuronal circuits, *i.e.*, 5-HT in the production of stereotyped postures in lobster (Kravitz et al., 1984; Kvarda et al., 2012). The monoamine 5-HT could also exert a modulatory action on the proprioceptive feedback in crayfish walking leg (Rossi-Durand, 1993). Thus, such actions could explain the effect of fluoxetine and venlafaxine on locomotion and sand digging, described as a stereotyped behaviour in cuttlefish (Mather, 1986), and crabs (Bellwood, 2002). Probably, a modulation of the serotonergic system by antidepressant residues can also modify many other behaviours regulated by 5-HT.

With respect to camouflage, both species exhibited a modification of their capacity to change colour. The chromatophores of cuttlefish and crabs being controlled either nervously or

neurohormonally, respectively, the results suggest a modulation of the neurotransmitters involved in colour change, such as 5-HT, or glutamate for cuttlefish. Indeed, in cuttlefish, based on the results of Bidel et al. (2016a, 2016b) revealing no impact on the levels of 5-HT, it is rather unlikely that the presence of either fluoxetine or venlafaxine modulated 5-HT levels in the nervous system, thus, causing the better uniform body pattern. Nonetheless, the selectivity of antidepressants has been questioned and increasing evidence points out that SSRI and SNRI are not as selective as supposed. Incidentally, in addition to the serotonergic and noradrenergic systems, the dopaminergic system as well as the glutamatergic system seem to be modulated by SSRIs and SNRIs (Richelson, 2001, 1996; Sánchez and Hyttel, 1999; Shioda et al., 2004). Indeed, Bidel et al. (2016a) showed a diminished number of NMDA-like receptor binding sites in cuttlefish brain after an exposure to venlafaxine. If such a decrease in glutamate receptors would also occur in the peripheral nervous system, it may impact the contraction of chromatophores and prevent the expression of appropriate uniform patterns. Nevertheless, a similar effect should have been observed on a disruptive background, which is not the case. It is, therefore, more likely that effects on the glutamatergic system as well as on the dopaminergic and/or noradrenergic systems may have impacted brain structures. As a matter of fact, Bidel et al. (2016a, 2016b) showed a diminution of cell proliferation in optic lobes following an exposure of either fluoxetine or venlafaxine. Thus, the waterborne exposure to antidepressants is likely to have modulated neurotransmitters levels as well as modified the proper development of brain structures involved in colour change in cuttlefish. On the other hand, in crabs, Fingerman and co-workers have widely investigated the role of monoamines and monoamine potentiators in colour change in crustaceans (Fingerman et al., 1981b, 1981a; Fingerman and Fingerman, 1975; Hanumante and Fingerman, 1983, 1981; Hanumante and Fingerman, 1982). They demonstrated that fluoxetine potentiated the effect of 5-HT, which elicited the dispersion of pigments in erythrocytes (Hanumante and Fingerman, 1983a). Indeed, in our study, this result was observed in crabs exposed solely to fluoxetine. This may signify that level of 5-HT did rise after the exposure to the antidepressant, allowing a high amplitude of colour change. These observations are likely to derive from an action of antidepressants on cAMP (or cGMP) levels involved as second messenger in the signalling cascades of chromatophores upon binding of 5-HT to its receptors (Franzellitti and Fabbri, 2013; Milograna et al., 2016) (Franzellitti and Fabbri, 2013; Milograna et al., 2016). Hanumante and Fingerman (1982) also showed that NE elicited pigment dispersion in melanophores, while DA triggers a pigment concentration in erythrophores. The fact that juvenile crabs exposed to both antidepressants displayed an attenuated colour change may result from a more complex modulation of 5-HT, and perhaps NE and DA as well.

Potentially, the results from this thesis could be worrying, because they suggest that antidepressants at environmentally realistic concentrations could alter behaviour and physiology related to camouflage, which in turn could endanger populations of cuttlefish or shore crabs by diminishing their chance for survival during their first weeks or months of life. In reality, however, it will be difficult

to demonstrate that the subtle changes found in this thesis work translate into population effects. It is also clear that many more questions remain to be answered in order to allow for a solid assessment of the environmental risk associated with SSRI/SNRI-antidepressants. Some leads may be considered: One would be how exactly the antidepressants act on both juvenile invertebrates. Another one would be to know if both species accumulated the antidepressants in their tissues and most importantly, how much and in which?

As for the latter, a major question that has to be answered in order to evaluate the risk of antidepressant compounds is how and to which extent they are taken up, either from the water (bioconcentration), or by food (trophic bioaccumulation). The observed behavioural and colour change responses would need to be related to the actual concentrations present in the animals. Attempts were made within the framework of this thesis to answer these questions by measuring the quantity of fluoxetine and venlafaxine, but also the active metabolite of fluoxetine, *i.e.*, norfluoxetine, in juvenile as well as in adult cuttlefish and crabs (**Preliminary results**). Both antidepressants were found in the whole bodies of juvenile cuttlefish, whereas none were detected in juvenile crabs. Surprisingly, an exposure as long as 34 days did not result in a measurable bioaccumulation of any of the compounds analysed in the whole body of juvenile crabs. A previous study from Robert et al. (2017) revealed the accumulative distribution of fluoxetine and norfluoxetine in the brain, pointing to a potential bioaccumulation of antidepressants in nervous tissues. However, the crabs used by Robert et al. (2017) were bigger and dosed *via* intra-cardiac injection or by oral gavage. Crabs used in this thesis work were less than 2 cm in carapace length and, therefore, their whole body was used for the chemical analyses. Furthermore, concentrations were much higher in the study of Robert et al. (2017). Consequently, the whole body extracts may have ‘diluted’ the molecules of interest concentrated in particular tissues and thus prevented their detection (see also Meador et al. (2016) for discussion). In this context, the technique used to discriminate the presence of both fluoxetine and venlafaxine may have not been sensitive enough. However, adult crabs presented an accumulation of venlafaxine, whereas fluoxetine was not detected. Regarding cuttlefish, both juvenile and adults were contaminated with fluoxetine and venlafaxine. An earlier study of Bidel et al. (2014) suggests that during a waterborne exposure of cuttlefish eggs, fluoxetine passed through the egg membrane and accumulated in the tissues, as it was detected in the embryos. Our results with juvenile cuttlefish showed that both pharmaceuticals could be detected, but also that venlafaxine was present in a few cuttlefish of the control group after 30 days of exposure. The presence of venlafaxine in control animals could derive from an early exposure of eggs collected in nature, or from the shrimps fed to the newly-hatched cuttlefish. These preliminary findings render the issue on the routes of contamination a very important question.

Juvenile cuttlefish and crabs are likely to be exposed through contaminated water *via* their skin or their thin exoskeleton and epidermis below, respectively. Crabs display a rigid exoskeleton whereas cuttlefish possess a large surface of soft skin, which may be more penetrable. Furthermore, the gills

probably present an important entry route for antidepressants. Yet, in crabs it is also protected by the exoskeleton. These morphological differences between both species may explain the lack of detection of fluoxetine and venlafaxine in crabs. Furthermore, having entered the body of either species, the two molecules may be metabolised and excreted differently ensuing a difference in body concentrations. Interestingly, the metabolite of fluoxetine was neither detected in cuttlefish, nor in crabs, which may be interpreted as a lack of metabolisation by both animals. However, an earlier study from Robert et al. (2017) showed the presence of norfluoxetine in crab tissues after injection of fluoxetine, which demonstrated a biotransformation of fluoxetine. In this work, levels of norfluoxetine may have been below the limit of quantification, as the concentration of fluoxetine during the exposure was a lot lower than in Robert et al. (2017) and subjected to the uptake through the epithelia. Furthermore, levels of fluoxetine and norfluoxetine decreased with time (Robert et al., 2017b), which is indicative of detoxification and excretion of the molecules of interest. Concerning cuttlefish, to date no evidence of metabolism has been provided for fluoxetine, or venlafaxine, but the non-detection of norfluoxetine in our experiment may be rather explained by the low concentrations of antidepressants used.

In addition to the waterborne exposure, cuttlefish and crabs may be contaminated as well from food sources. The two active molecules possess different $\log K_{ow}$ (see introduction), thus, present different water solubility, which probably result in different uptake routes. Indeed, based on their chemical properties, fluoxetine which is considered more lipophilic than venlafaxine would be preferentially accumulated in animals. Yet, here we observed a higher accumulation, thus, detection of venlafaxine in both animals (but only in adult crabs) which corroborate previous studies (Maulvault et al., 2018; Moreno-González et al., 2016; Togunde et al., 2012). To examine if the contamination could come from food, it would be interesting to analyse the bioaccumulation of both molecules *via* the ingestion of contaminated prey or food. To this end, we conducted a preliminary study with juvenile crabs using agarose blocks mixed with fresh mussel meat and spiked with either fluoxetine alone or combined with venlafaxine as food. First results showed a bioaccumulation of both fluoxetine and venlafaxine for both conditions (data not shown). The controls, however, were contaminated as well and in the same range of concentrations. We, therefore, had to question whether the mussels used were indeed free of antidepressants and need to repeat the experiments in order to be sure about the levels of contamination. Regarding cuttlefish, it could be envisaged to conduct a series of experiments with shrimps injected with both drugs to analyse if accumulated fluoxetine and venlafaxine come from food and/or waterborne exposure.

Once the active molecules enter the animal, and accumulate, they may act on the serotonergic system in case of SSRIs, and to some degree on the noradrenergic system in case of SNRIs. Generally, the SSRI/SNRI may have effects on the regulation of monoamines like 5-HT, NE or DA in non-target organisms. To which extent this actually is the case and whether the antidepressants have actually modified the levels of monoamines in cuttlefish and/or shore crabs is, however, not established. Yet,

evidence of the evolutionary conservation of reuptake transporters and/or receptors in invertebrates (Caveney et al., 2006), such as molluscs and crustaceans, lead to the hypothesis that levels of 5-HT, as well as NE and DA, were modulated by the waterborne exposure to antidepressants. Indeed, pharmacological studies have demonstrated a modification of behaviour by the use of monoamine receptor agonists and antagonists: 5-HT and DA increased locomotion in *Helix lucorum* (Pavlova, 2001), 3,4-methylenedioxymethamphetamine (MDMA) enhanced prosocial behaviour in *Octopus bimaculoides* (Edsinger and Dölen, 2018) and 5-HT agonists and antagonists modulated photic behaviour in *Gammarus pulex* (Perrot-Minnot et al., 2013). Therefore, in cuttlefish and crabs, any change of behaviour is likely to be explained by a change in monoamine levels. Regarding cuttlefish, Bidet et al. (2016b) investigated the effect of a pre-hatching exposure to fluoxetine at 1-10 $\mu\text{g}\cdot\text{L}^{-1}$ on the levels of monoamines in the brain of newly-hatched animals. Surprisingly, the neurochemical analyses showed no effect on the serotonergic system, but rather on dopaminergic turnover, *i.e.*, the DOPAC/DA ratio (DA and its metabolite, 4-dihydroxyphenylacetic acid). An exposure to fluoxetine at lower concentrations for one month had an impact on DA levels with the group treated with 100 $\text{ng}\cdot\text{L}^{-1}$ having lower concentrations of DA than the group treated with 1 $\text{ng}\cdot\text{L}^{-1}$, but not significantly different than the control. On the other hand, an exposure to venlafaxine at 5-100 $\text{ng}\cdot\text{L}^{-1}$ for 20 days post-hatching induced a decrease in NE levels (Bidet et al., 2016a). Overall, both drugs seem to preferentially influence dopaminergic and noradrenergic systems rather than serotonergic system in young cuttlefish. Regarding crabs, Robert et al. (2019) suggested that fluoxetine was likely to increase 5-HT levels, because an injection of fluoxetine in the μg concentration-range increased glycaemia in *C. maenas*. Yet 5-HT increases glycaemia *via* the stimulation of CHH release, which indirectly proves that fluoxetine had increased 5-HT levels (Robert et al., 2016). Moreover, in crustaceans such as *Daphnia magna*, an exposure to 100 $\text{ng}\cdot\text{L}^{-1}$ fluoxetine increased the levels of 5-HT, while venlafaxine did not, whereas the levels of dopamine and norepinephrine remained unchanged for both antidepressants (Rivetti et al., 2019). Further evidence is provided by a study of Campos et al. (2016), who showed that exogenous fluoxetine strongly increased 5-HT-immunoreactivity in brain neurons. Overall, these results suggest that, in crustaceans, fluoxetine indeed increases 5-HT levels, especially in nervous structures, which may explain the modification of colour change dynamics and behaviour in *C. maenas* in the present thesis.

The rapid effects observed in both cuttlefish and crabs are likely to come from a modification of monoamine levels in nervous structures by the action of antidepressants while the bioaccumulation may have an impact on the longer term. How levels of monoamines are regulated by fluoxetine and venlafaxine in *C. maenas* remains to be elucidated. To better understand the effects of antidepressants on monoamines levels in realistic scenarios, further studies need to be conducted with waterborne exposure at ng-range concentrations and with the use of accumulated antidepressants.

Limits and perspectives

The risk emanating from antidepressants has often been disputed, because of their low environmental concentrations and because effects on aquatic animals could mostly be obtained with concentrations in excess of those found in surface waters. Our studies have shown that environmentally realistic concentrations of antidepressants can have significant effects on behaviours and colour change in juvenile cuttlefish and crabs. In both species and for all parameters studied, the results suggest greater effects with the two antidepressants cumulated, often at the higher concentration of 5 ng·L⁻¹ each, *i.e.*, a total of 10 ng·L⁻¹ of antidepressants. This suggests that the observed effects are concentration dependent and would confirm that it is necessary to consider the cumulated concentrations of these compounds in order to estimate their risk for the biota. However, it cannot be excluded that venlafaxine alone would result in the observed effects, which is why no effect concentrations (NOEC) and low effect concentrations (LOEC) for single antidepressants and their mixtures need to be established for environmental risk assessment. Given that the behavioural traits examined in the framework of this thesis are laborious to measure and time consuming, it is sensible to limit LOEC and NOEC studies of the major antidepressants and their mixtures to the most sensitive parameters, which are measurable within reasonably short time frames, such as predator behaviour or locomotor activity and chromatophore responses. Indeed, in our study, two major parameters present the best sensitivity/practical ratio allowing rapid high-throughput testing: predator behaviour for cuttlefish and locomotor activity for crabs. Both tests offered significant results at short term, *i.e.*, after one or two days of exposure until the end of the experiment, with a reasonable number of replications. As for practicality, they are at low cost and require minimal maintenance. Furthermore, without any intrusive procedure, animals may be used for other behavioural tests to lower the number of animals killed, which is particularly important for cuttlefish (Fiorito et al., 2014). Those sensitive behavioural tests may be used in order to test a great number of psychoactive substances and their combination as well as other types of pollutants.

Early stages of development in animals have always been characterised as sensitive stages more prone to display responses to contaminants (Mohammed, 2013). The possibility to rear both species easily at juvenile stages allows to conduct studies with very low concentrations in order to highlight sublethal effects of antidepressants. Moreover, effects detected at early stages may have repercussions later in life, which is particularly the case for young crabs and cuttlefish that stay in coastal contaminated water during their development. In addition, because we isolated each animal during exposure and testing, we could follow the individual animals throughout the whole experiment. Indeed, each animal was its own replicate, which allowed us to perform paired samples analyses and to use quite high sample sizes. This stands out in behavioural studies, which are often confronted with high intra-group variability. Yet, despite the relatively high number of animals in our studies, we still faced high intra-group variability, which made it difficult to carve out statistically significant results. This underscores

the need to conduct studies with an even greater number of individuals in order to reveal subtle changes between the treatment groups.

A recurrent criticism of the present work was that each substance would have to be tested alone and in mixture at different concentrations in order to understand the contribution of each compound to the observed effects. As mentioned above, for environmental risk assessment this, of course, would be mandatory. Nevertheless, the objective of my thesis was to answer the question whether antidepressants in an environmentally realistic scenario would be able to affect invertebrates, rather than to identify each molecule's effect. In fact, many studies have demonstrated effects of antidepressants on the behaviour of aquatic species, such as fish, crustaceans and molluscs (Bidel et al., 2016b; Fong et al., 2015; Gaworecki and Klaine, 2008; Mesquita et al., 2011; Schonova et al., 2019). Yet, in most cases relatively high concentrations were necessary to observe significant effects of antidepressants. Concentrations in the μg to $\text{mg}\cdot\text{L}^{-1}$ range are, however, rarely observed in the aquatic environments, (Klosterhaus et al., 2013; Ma et al., 2018; Meador et al., 2016; Minguez et al., 2016), especially not when it comes to coastal waters (Minguez et al., 2016; Silva et al., 2012). It has therefore been called into question whether antidepressants pose any environmental risk given their low $\text{ng}\cdot\text{L}^{-1}$ concentrations. The work conducted in this thesis, therefore, intended to elucidate the fundamental question if environmentally realistic antidepressant concentrations can affect aquatic animals. To this end it was considered expedient to focus (i) on very sensitive behavioural parameters, (ii) to expose juvenile animals, which are generally more sensitive to pollutants, and (iii) to cumulate antidepressants to reach higher, but in fact more realistic, concentrations akin to those that can be found in the environment (Evgenidou et al., 2015). In this context, fluoxetine alone was used as a reference compound, so as to link the results to earlier studies. Fluoxetine represents the historically oldest contamination with selective reuptake inhibitor antidepressants, as it was the first SSRI introduced to the market starting in 1987. Consequently, fluoxetine has been studied extensively for more than two decades, while venlafaxine, released few years later back in 1995, has been less studied, but is actually found in higher concentrations in aquatic environment. If future studies focus on the effect of individual antidepressant and the interaction of the combination of a many this would require a large infrastructure, several helping hands, as well as a lot of animals to sacrifice. Using complex behavioural traits and camouflage related endpoints in a comparative physiology approach, *i.e.*, with two species the colour change of which is regulated either neuronally or neuro-hormonally, it was, however, not possible to test several antidepressants alone and in mixtures, as this has been done with toxicity testing by Minguez et al. (2018).

The continuum between behaviour and colour change—or how burying, predation, locomotion and camouflage are linked

Behavioural studies are becoming more and more prevalent in ecotoxicological research, as they permit to point out sublethal effects of pollutants at relatively low concentrations and to correlate the results to direct or indirect ecological effects (Brodin et al., 2014). The behaviours studied in this

research, *i.e.*, burying, predation and locomotion, are interconnected either directly or indirectly with camouflage, including colour change, as well as with growth. Indeed, by examining burying behaviour in both juvenile cuttlefish and crabs, we analysed their capacity to camouflage themselves and to hide from potential predators as well as from prey, especially for cuttlefish for the latter. If the animal is not buried entirely, on a different time scale both species may change the colour of their mantle or body to blend efficiently within their close environment. Our results concerning the juvenile crabs in article I and III are connecting colour changes with burying behaviour: exposed crabs exhibited a lower capacity to change colour while they bury more in the sand. Could this altered colour change overrule enhanced burying behaviour? Would it be associated with an anxiety-like behaviour? Or both? In juvenile cuttlefish, the decreased sand-digging behaviour did not have an impact on the homochromy of their mantle relative to the substrate. But we found that against an artificial medium grey background exposed cuttlefish expressed a more uniform body pattern than controls.

The locomotor activity appears to be essential for survival of juvenile crabs because it allows the animal to go to the next environment with an appropriate background that matches their body colouration to hide from predators for instance. Nonetheless, when background matching is not sufficient and the prey is detected, a proper velocity will help to flee fast and avoid being eaten thereby requiring both camouflage and locomotion. Furthermore, anxiety-like behaviour is often associated with locomotor activity. Thus, it would be interesting to assess the anxiety level either directly by introducing the animals to their predators, *i.e.*, odour, image, shadow, or indirectly by analysing levels of cortisol (Fossat et al., 2015, 2014).

An altered predatory behaviour through a decline of feeding motivation and successful attacks may endanger the cuttlefish by a series of repercussions, *i.e.*, a decrease of food intake leading to an altered development and potentially a modification of innate fundamental behaviours. Indeed, a reduced growth was observed in cuttlefish exposed to antidepressants. This morphological change of the mantle length may be associated to changes of internal structures, such as the brain, which may have repercussions on the maturation of behaviours and related learning processes, but also on visual processes, which can eventually impact the efficiency of colour change and camouflage. A decrease of food intake resulting from an inefficient predatory behaviour may have also an impact on other behaviours, such as locomotor activity. Indeed, if their primary defence, *i.e.*, background matching and/or burying behaviour, is incorrect and the predator or prey seems to detect the animal, the escape or the attack must be done properly, *i.e.*, fast and in the right direction.

From a single modification can come out a waterfall effect on several behaviours and physiological parameters which may endanger the animal.

Conclusion

A few nanograms per litre of only two common antidepressants known to be present in marine environment sufficed to modify innate behaviours and cryptic colouration of two non-target organisms. Although our studies were performed on a sub-chronic level, *i.e.*, one month, the majority of the effects were noticeable already within a few days. This highlights the sensitivity of the behavioural parameters, as well as the vulnerability of early developmental stages of animals. Hence, these behavioural tests constitute a promising approach, especially for micropollutants, which merit to be pursued in the future.

Overall, we feel that there is a need for more behavioural studies in ecotoxicology. Also colour changes, which, as we demonstrated, may be significantly impacted by short and long-term exposures to very low concentrations of antidepressants could be given more attention. To better understand the underlying causes of modified behaviours and colour changes, however, the analyses of monoamines in nervous structures and haemolymph as well as the bioaccumulation of the molecules of interest must be realised.

Despite their low concentrations, notably in marine environments, our results prove that the fluoxetine and the venlafaxine are environmentally active pharmaceutical compounds and are able to induce subtle changes on fundamental behaviours and cryptic colouration in both cuttlefish and crabs. Furthermore, considering the great amount of chemicals and, especially, residues of medication, which end up in the marine environment, it appears necessary to work on 'pollutant-cocktails' in order to simulate as closely as possible environmental realistic scenarios.

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Alteration of camouflage and behaviour in two marine invertebrates, *Sepia officinalis* and *Carcinus maenas*, by antidepressant medication

Juveniles of the common cuttlefish, *Sepia officinalis*, and the green shore crab, *Carcinus maenas*, develop themselves in the intertidal zone and coastal waters impacted by continental pollution such as pharmaceutical residues. This research focused on the effects two antidepressants, the fluoxetine and the venlafaxine, on the camouflage and behaviour of cuttlefish and shore crabs. Both molecules are worrying because they are designed to act on serotonergic system and are commonly detected in aquatic environments. Thus, to approach realistic scenario of exposure fluoxetine was either combined or not with venlafaxine. The results show significant effects of antidepressants at environmentally realistic concentrations, especially the combination of fluoxetine and venlafaxine, on sensitive endpoints such as burying behaviour, colour change and background matching, locomotor activity in crabs and predatory behaviour in cuttlefish. Furthermore, the exposure to antidepressants at early development stage seems to alter maturation and/or learning processes in cuttlefish. Overall, these studies demonstrated the need to investigate further with low range concentrations on key behaviours of non-target species.

Modification du camouflage et du comportement chez deux invertébrés marins, *Sepia officinalis* et *Carcinus maenas*, par des antidépresseurs

Les juvéniles de la seiche commune, *Sepia officinalis*, et du crabe vert, *Carcinus maenas*, se développent au niveau de la zone intertidale et des eaux côtières affectées par la pollution continentale telle que les résidus pharmaceutiques. Cette recherche se concentre sur les effets de deux antidépresseurs, la fluoxétine et la venlafaxine, sur le camouflage et le comportement de la seiche et du crabe vert. Ces deux molécules sont particulièrement inquiétantes car elles sont conçues pour agir sur le système sérotoninergique et sont communément détectées dans les environnements aquatiques. Ainsi, pour se rapprocher de scénarii d'exposition réalistes, la fluoxétine a été associée ou non à la venlafaxine. Les résultats montrent des effets significatifs à des concentrations environnementalement réalistes, d'autant plus avec le mélange des deux antidépresseurs, sur des paramètres sensibles tels que le comportement d'ensemblement, le changement de couleur et la coloration cryptique chez nos deux espèces, ainsi que l'activité locomotrice chez le crabe et le comportement prédateur chez la seiche. En outre, l'exposition de stades de développement précoces aux antidépresseurs semble modifier la maturation et les processus d'apprentissage chez la seiche. Enfin, ces résultats ont démontré la nécessité de mener davantage d'études avec de faibles concentrations sur les comportements clés d'espèces non-cibles.