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Khalid Hussain Rind

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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**MARBEC - Centre pour la Biodiversité Marine, l'Exploitation et la Conservation
Écologie Évolutive et Adaptation**

**Physiology of Threespined Sticklebacks (*Gasterosteus
aculeatus*) of the Camargue at different salinities.**

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Le 30/11/2018

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Table of Contents

Abstract	Erreur ! Signet non défini.
Résumé.....	Erreur ! Signet non défini.
List of Abbreviations	XI
Résumé en Français	XII
1 Chapter 1: General considerations and aims of this study.....	1
1.1 Research objectives	4
2 Chapter 2: Introduction.....	6
2.1 The Camargue area.....	7
2.2 Salinity and precipitation conditions in the Camargue area.....	11
2.3 The threespined stickleback (<i>Gasterosteus aculeatus</i>)	12
2.3.1 The threespined stickleback in Europe	15
2.3.2 Salinity tolerance in the Order Gasterosteiformes threespined Stickleback.....	17
2.3.3 Stickleback morphotypes	20
2.3.4 Plasticity and colonization of new environments	24
2.3.5 Salinity as a barrier to colonization	25
2.4 Ion transporters.....	26
2.4.1 Na ⁺ /K ⁺ -ATPase (NKA).....	27
2.4.2 Na ⁺ /K ⁺ /2Cl ⁻ co-transporter (NKCC)	28
2.4.3 Cystic fibrosis transmembrane conductance regulator (CFTR).....	29
2.5 Major osmoregulatory organs in fish	29
2.5.1 The gills, main site of fish osmoregulation.....	29
2.5.1.1 SW-acclimated fish, NaCl transport.....	31
2.5.1.2 FW-acclimated fish, NaCl transport.....	31
2.5.2 Kidney.....	33
2.5.2.1 Ion secretion in SW-acclimated fish	35
2.5.2.2 Ion reabsorption in SW-acclimated fish.....	36
2.5.2.3 Ion reabsorption in FW-acclimated fish.....	36
2.5.3 Intestine.....	38
2.5.3.1 Structure of the teleost intestine	38
2.5.3.2 Role of the intestine in SW-acclimated fish osmoregulation.....	40

2.5.3.3	Role of the intestine in FW-acclimated fish (Intestinal salt reabsorption).....	42
2.6	Osmegulation in Sticklebacks	43
3	Chapter 3: Materials and Methods.....	44
3.1	Stickleback sampling and rearing.	45
3.2	Experimental design and salinity acclimation.....	48
3.3	Medium and blood osmotic pressure determination	50
3.4	Histology and immunohistochemistry	53
3.5	Electron microscopy.....	57
3.5.1	Scanning Electron Microscopy (SEM)	58
3.5.2	Transmission Electron Microscopy (TEM)	58
3.6	Branchial Na ⁺ /K ⁺ -ATPase activity.....	59
3.7	Oxygen consumption rate.....	59
3.8	Molecular study.....	61
3.8.1	RNA extraction	61
3.8.2	qPCR data analyses.....	62
3.9	Morphometry.....	63
3.10	Statistical Analysis	64
4	Chapter 4: Results.....	65
4.1	Blood Osmotic Pressure	66
4.2	Stickleback morphometric.....	66
4.2.1	Gill ionocytes Morphology	66
4.2.2	Gill rakes	68
4.2.3	Body Morphology.....	70
4.3	Immunolocalization of gill NKA	71
4.4	Immunolocalization of Kidney NKA.....	72
4.5	Immunolocalization of Intestine NKA.....	74
4.6	Relative gill NKA gene expression	75
4.7	Gill ionocytes ultrastructure	77
4.8	Oxygen consumption rate.....	77
4.9	Branchial Na ⁺ /K ⁺ -ATPase activity.....	82
5	Chapter 5: Discussion, conclusion and perspectives	83
5.1	General physiology of osmoregulation	84

5.2	Stickleback Morphometric	86
5.3	Functional Analysis of the main osmoregulatory organs.....	90
5.4	Conclusion.....	95
5.5	Perspectives.....	98
5.5.1	Molecular to ontogenetic analyses.....	98
5.5.2	Morphometry and genetic analyses	100
5.5.3	Macro-evolution.....	101
5.5.4	Micro-evolution	102
5.5.5	Ecotoxicological analysis.....	103
6	References	106
7	Conference Presentations	129
8	Publications	129

List of Figures

Figure 2-1 Evolutionary stages of the Rhône delta since 7200 yr before present time (from L'Homer et al., 1981).....	8
Figure 2-2. Current map of the Camargue with different lagoons along Rhône delta.....	9
Figure 2-3. Living Planet Indices of fish from the Vaccarès, Camargue (Galewski, tour du valat, 2008).	10
Figure 2-4. A Salinity variation among lagoons of the Camargue from 1991 to 2017, B. Precipitation conditions in different lagoons of the Camargue (SNPN, 2017).....	11
Figure 2-5. Gasterosteiformes schematic representation, adapted and modified from Kawahara et al., 2008. (Classifications follow Nelson, 2006).....	12
Figure 2-6. Global distribution of the threespined Stickleback (indicated by the shaded areas) (Wootton, 1984).....	13
Figure 2-7. A) drawings of some of the early developmental stages: 1. Hatching; 2. Hatched embryo; 3. 1-2 days after hatching; 4. 16 days after hatching; 5. 22 days after hatching, young fish possesses all typical characters. From Swarup (1958), B) Female and male threespined stickleback, C) sticklebacks with different lateral plates (Barrett, 2009;). D) Morphological features of threespined stickleback: Jaw length (JL), Head height (HH), Body height (BH), Pelvic girdle length (PGL), Anal fin length (AFL), Caudal peduncle length (CPL), Dorsal fin length (DFL), Second dorsal spine length (DFL2), First dorsal spine length (DFL1), Head length (HL), Standard length (SL), and Total length (TL).	14
Figure 2-8. Phylogenetic relationship of European threespined Stickleback population with different clades. (1. Barents and White Seas, 2. Baltic Sea, 3. North Sea, 4. Atlantic Ocean, 5. Mediterranean Sea, and 6. Black Sea and their drainages) (Figure adapted from Mäkinen et al., 2006).	16
Figure 2-9. Salinity tolerance of Gasterosteiformes at different taxonomic levels, in box Box colours, stenohaline marine (black), stenohaline freshwater (white) or euryhaline (red) species (Kültz, 2015).	18
Figure 2-10. Morphological differences of gill rakers between two ecotypes based on A) Limnetic and B) Benthic axis (Schluter D, 1993).	19
Figure 2-11. Morphotypes of threespined stickleback, lateral plate phenotypes adapted from Bell, (2001) shown include: low (A), (B); intermediate-low (C), (D); partial (E); intermediate-partial (F); and complete (G). (Bell, 2001).	22
Figure 2-12. Number of lateral plates in European's stickleback populations, also from Mediterranean islands (Lucek and Seehausen, 2015).	24
Figure 2-13. Process of osmoregulation; a. in seawater and, b. in freshwater fish.....	26
Figure 2-14. Transmission electron micrographs of gill filaments from killifish in SW (A) and killifish transferred from SW to FW after 30 days (B). Note transformation of the apical region of MRCs (arrows) from a smooth, concave crypt that is recessed below the pavement cells (PVCs) (A) to a convex surface studded with microvilli that extend above the surrounding PVCs (B). Also note that an accessory cell (AC) is not associated with the MRC from FW-acclimated killifish (B)	

and that the distinct, whorl-like microridges on the surfaces of PVCs do not change with salinity (A). (Katoh and Kaneko, 2003).	31
Figure 2-15. Mechanism of NaCl secretion (A) and uptake (B) in the gill of teleosts (Evans, 2008).	33
Figure 2-16. Mechanism of ion secretion and ion uptake in proximal segment I (A) and proximal II (B). Mechanism of ion transporter in distal segment (C) of teleosts (Marshall and Grosell, 2006).	37
Figure 2-17. The general layout of the vertebrate intestine. (A) A cutaway plan from a section of intestine displaying the gross anatomy including mucosa, submucosa, muscle layers and serosa. (B) A close-up of the elaborate foldings of the mucosa which faces the contents of the lumen. (C) A section through a villus displaying the absorptive epithelial cells with associated capillaries and d a close-up of an epithelial cell showing the microvilli of the apical membrane, intercellular spaces and various organelles (Randall <i>et al.</i> , 2001).	39
Figure 2-18. Epithelial ion transport pathways generating the driving forces for water absorption by the marine teleost intestine (Whittamore, 2012).	42
Figure 3-1. Installed fishing site on the Vaccarès lagoon with fixed hoop nets (A), Collection of trapped fish (B), and separation of sticklebacks (C) in order to separate live threespined sticklebacks.	45
Figure 3-2. Camargue with different mesohaline/brackish water sampling sites.	46
Figure 3-3. Camargue with different freshwater sampling sites.	46
Figure 3-4. The “capéchade” fixed hoop net used during sampling from Camargue, the arrows indicate the direction of fish displacement (Bouchereau <i>et al.</i> , 1990).	47
Figure 3-5. Experimental design for relative mra expression of gill NKA, NKCC1, CFTR, and V-ATPase, gill NKA immunolabeling, gill SEM and TEM (Fumemorte), oxygen consumption rate (Vaccarès, Matreau, and Versadou), Kidney TEM (Vaccarès), immunohistochemistry of gill, kidney and anterior and posterior intestines (Vaccarès), and branchial NKA activity (Marteau and Versadou).	49
Figure 3-6. The advance micro-osmometer is the standard system for the determination of the total osmolality in aqueous solutions using the freezing point depression. It necessitates 20 µl of samples.	51
Figure 3-7. Typical cooling curves of water and aqueous solutions.	52
Figure 3-8. Technical steps that are necessary for histological sample preparation (Lignot, 2017).	53
Figure 3-9. Schematic diagram of the immunolabeling technique.	57
Figure 3-10. TEM and SEM for the observation of gill and kidney morphology.	58
Figure 3-11. Experimental setup used for the measurement of oxygen consumption rate.	60
Figure 3-12. Step by step morphometric procedure of stickleback for the analysis of different landmarks.	64
Figure 4-1. Blood osmotic pressure (in mOsm kg ⁻¹) from FW, BW and SW acclimated fish (n = 8, 9 and 11 respectively). Different letters indicate statistical differences.	66
Figure 4-2. Freshwater and seawater SEM and semi thin gill structure with ionocytes, (A&C FW gills, B&D SW gills), FI, filament ionocytes; LI, Lamellae ionocytes; BV, blood vessels.	67

Figure 4-3. SEM of gill with ionocytes: (A) a fish maintained in FW with gill ionocytes, and, (B) a fish transferred to SW with ionocytes. (C) ionocyte morphological measurements. Measurements were obtained from 12 fish maintained in FW and 12 fish transferred to SW. Means \pm SE. BV, blood vessel; GF, gill filament; GL, gill lamellae; FI, filament ionocyte; LI, lamellar ionocyte. 68

Figure 4-4. Gill rake length and density for FW and SW sticklebacks of the Camargue area. 69

Figure 4-5. Body morphology of sticklebacks sampled from FW and mesohaline (SW) habitats, (A); ratios of different ecological significant traits of freshwater and seawater sticklebacks. (B); first and second principle components of morphological variance between FW and SW sticklebacks. 70

Figure 4-6. Number of plates among sticklebacks of camargue. 71

Figure 4-7. Microscopic studies of the gills, (A & B) classical staining of FW & SW gills, (C) freshwater gill ionocytes expressed on both gill filament and as well as on gill lamellae, (D) seawater gill ionocytes expressed only along the gill filament. 72

Figure 4-8. NKA Immunolocalization of the kidney, A, B, C, and D Freshwater fish, E, F, G, and H seawater acclimated fish. CT: collecting duct; T Proximal and/or distal tubule. 73

Figure 4-9. Kidney tubule morphology of sticklebacks collected from the Camargue aquatic habitats. A) Tubule diameter and epithelial thickness of nephrons tubules of freshwater and seawater acclimated fish, B) histological section of a tubule epithelium with the morphological measurements. 74

Figure 4-10. NKA Immunolabeling of the NKA in the intestine, A) anterior intestine and B) posterior intestine of a freshwater stickleback. C) anterior intestine and D) posterior intestine of a seawater-acclimated stickleback. GC. Goblet cells, L. lumen. 75

Figure 4-11. Relative mRNA gene expression for the NKA α 1 and NKA isoforms α 1a and α 1b from fish maintained in FW (n = 8) or acclimated to BW (n = 8) or SW (n = 8). Different letters indicate statistical differences (P < 0.05). Means \pm SEM. 76

Figure 4-12. Relative mRNA expression of different co-transporters in three salinity conditions, freshwater (FW), brackish water (BW) and seawater (SW), (A), relative mRNA expression of CFTR, (B), relative mRNA expression of V-ATPase, and (C), relative mRNA expression of NKCC1. 76

Figure 4-13. Scanning and transmission electron microscopy observations of gill ionocytes of FW and SW fish. Framed a, b, c, d: FW fish; framed e, f: SW fish. AC, apical crypt; DSI, dome-shape ionocyte; GF, gill filament; GL, gill lamellae; HLI, honeycomb-like ionocyte; IC, ionocyte; M, mitochondria; PVC, pavement cell; TVS, tubule-vesicular system. Scale bar: 10 μ m (a), 5 μ m (b, c), 2 μ m (d), 20 μ m (e), 5 μ m (f). 78

Figure 4-14. Scanning electronic microscopy of Gills of freshwater sticklebacks and seawater acclimated sticklebacks. 79

Figure 4-15. Oxygen consumption rate for sticklebacks sampled from the FW Versadou canal and the SW Marteau lagoon and 24 and 72 hours after transfer from SW to FW and from FW to SW, respectively (A). Different temperatures were used: 21, 23 and 25°C; (B) Oxygen consumption rates for sticklebacks sampled from the mesohaline Vaccarès lagoon maintained in SW or FW and 24 and 72 hours after transfer to FW and SW, respectively. 80

Figure 4-16. Oxygen consumption rate for sticklebacks sampled from the mesohaline Vaccarès lagoon maintained in SW or FW and abruptly transferred up to 5 hours to either FW or SW (A).

Oxygen consumption rate for sticklebacks from the Vaccarès lagoon maintained in SW or FW and transferred to FW and SW, respectively for 3 and 6 weeks (B).	81
Figure 4-17. Branchial Na ⁺ /K ⁺ -ATPase activity for fish sampled from the SW Marteau lagoon and the FW Versadou canal and 24 and 72 hours after transfer from SW to FW and from FW to SW.....	82
Figure 5-1 Graphical representation of the results obtained in this study.....	95
Figure 5-2 Distribution of threespined sticklebacks with the main three lineages according to Paepke (2001) and Bell and Foster (1994). Figure adapted from Mäkinen et al., (2008).....	102
Figure 5-3 Phylogenetic relationships among sticklebacks population from three habitats (i.e. Rivers, lakes and estuaries) of Schleswig-Holstein, Germany. Figure adapted from Reusch <i>et al.</i> , 2001.....	103

List of Tables

Table 2-1. Classification of threespined Stickleback (<i>Gasterosteus aculeatus</i>).....	13
Table 2-2. Number of lateral plates in threespined stickleback across its distribution range.....	21
Table 3-1. Different sampling sites with habitats characteristics of Camargue wetland complex.	48
Table 3-2. The number of tissue sampled and condition of their preservation for the different experiments *SEI buffer: 300 mM sucrose, 20 mM Na ₂ EDTA, 100 mM imidazole, pH 7.3.	50
Table 3-3. Trichrome classical histology, different steps for classical staining of tissues.	54
Table 3-4. Different steps of Immunohistochemistry.	56
Table 3-5. List of antibodies used in this research project.	56
Table 3-6. Primer sequence used to amplify the stickleback NKA α 1 subunit and the isoform α 1a, α 1b, CFTR, NKCC1 and V-ATPase.....	61

List of Abbreviations

AC	Apical cell
AFL	Anal fin length
JL	Jaw length
BW	Brackish water
CD	Collecting duct
CFTR	Cystic fibrosis transmembrane conductance regulator
PGL	Pelvic girdle length
DFL	Dorsal fin length
DNA	Deoxyribonucleic acid
DSI	Dome-shape ionocyte
DT	Distal tubule
ESU	Evolutionary significant units
FBW	Fresh body weight
FW	Freshwater
GF	Gill filament
GFR	Glomerular filtration rate
GL	Gill lamellae
HH	Head height
HL	Head length
HLI	Honeycomb-like ionocyte
JL	Jaw length
MMR	Maximum metabolic rate
MRC	Mitochondria-rich cell
NaCl	Sodium chloride
NKA	Na ⁺ /K ⁺ -ATPase
NKCC	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter
PCA	Principal Component Analysis
PGL	Pelvic girdle length
PT	Proximal tubule
PVC	Pavement cell
RR	Respiration rate
SEM	Scanning electronic microscope
SL	Standard length
SW	Seawater

Résumé en Français

I) Objectifs de cette étude

Ces dernières années, la perte de biodiversité a été considérée comme une crise mondiale, importante menaçant éventuellement les habitats écologiques (U.N., 1992). En conséquence, les populations locales subissent des impacts rapides et décroissants, bouleversant les systèmes écosystémiques essentiels et affectant également des écosystèmes particuliers (Garner *et al.*, 2005; Hooper *et al.*, 2005; Helfman, 2007). En outre, il ne s'agit pas simplement du lien entre les espèces et les écosystèmes locaux, mais aussi d'une question d'existence globale des espèces elles-mêmes (Luck *et al.*, 2003). En conséquence, la conservation de populations locales et distinctes est une étape essentielle pour la conservation de la biodiversité (Luck *et al.*, 2003).

Populations périphériques et centrales : cas de l'épinoche à trois épines

Les études comparatives de la niche écologique de populations centrales et périphériques de différentes espèces montrent que les populations en limite d'aire de répartition présentent dans bien des cas, non seulement une originalité écologique par rapport aux populations centrales mais aussi que leur niche écologique est très souvent plus spécialisée que celle des populations centrales. Les effets combinés de l'isolement et de la dérive génétique peuvent aussi amener ces populations périphériques d'espèces très étendues à être plus en péril que les populations centrales. Cela est dû au fait que la faible variation génétique doit diminuer leur potentiel d'adaptation continue. Par conséquent, la connaissance de la diversité génétique et de la structure de la population est inestimable pour une politique et une gestion durable en conservation. Cependant, les décideurs et les gestionnaires ont généralement besoin d'une approche plus pratique pour appliquer les données génétiques à la biologie de la conservation. L'un de ces exemples consiste à prévoir et à classer le potentiel évolutif de différentes populations sauvages en évaluant leur contribution à la diversité mondiale. Ainsi un des articles fondateurs est celui de Foster *et al.* (2003) dans lequel les auteurs ont examiné la situation de l'épinoche à 3 épines aux limites sud de son aire de répartition, en mettant en évidence le cas des populations espagnoles. En effet, ce poisson Téléostéen subi une tendance vers le nord d'extinctions locales dans la péninsule ibérique (Reimchen, 1984; Foster, Baker et Bell, 2003; Araguas *et al.*, 2012). Cette espèce qui est présente dans l'hémisphère nord en Europe, Amérique du Nord et en Asie de l'eau de mer à l'eau douce a subi différentes histoires évolutives au cours de l'Holocène qui ont façonné ses différentes populations avec des morphotypes et des écotypes caractéristiques (ex : nombre variable de plaques latérales, populations d'eau douce ou d'eau de mer, épinoches benthiques ou pélagiques). Une telle diversité évolutive en peu de temps est à l'origine aussi des nombreux travaux consacrés à l'épinoche qui est devenu une espèce modèle en biologie. Son génome est connu et les différences fonctionnelles existant entre les écotypes (populations d'eau de mer et celles d'eau douce) sont maintenant de plus en plus étudiées (Hohenlohe *et al.*, 2010; DeFaveri *et al.*, 2011; Taugbøl *et al.*, 2014). Plus rares cependant, sont les travaux portant sur les populations d'épinoches septentrionales de Méditerranée situées en limite sud de répartition.

Cependant, grâce notamment aux travaux menés sur les épinoches de Camargue (Crivelli et Britton, 1987), il a pu être mis en évidence que ces populations Méditerranéennes présentent une histoire évolutive bien différente par rapport aux populations côtières le long des côtes Européennes (Atlantique, Mer du Nord, Baltique) et continentales d'eau douce. En effet, la population 'marine' de Camargue correspond à un type ancestral avec peu de plaques latérales

(morphotype *leiurus*) et n'a probablement que très peu évolué, le delta du Rhône n'ayant pris sa forme actuelle que depuis ces dernières 7500 années. De plus, l'épinoche présente en Camargue diffère des populations du nord de l'Europe par des traits d'histoire de vie particuliers : espérance de vie plus courte (1 à 2 ans), très fort taux de croissance, maturité sexuelle précoce et période de reproduction très étalée (mai à novembre), ce qui traduit un métabolisme élevé.

Etudier ces populations périphériques en limite sud de répartition est donc un élément très important pour identifier des particularités écologiques, caractériser leur niche écologique et pour affiner les efforts en conservation de la biodiversité.

La Camargue

Dans la zone deltaïque du Rhône, la Camargue correspond à est un haut lieu protégé de la diversité écologique et une réserve de biosphère depuis 1977. Une grande partie de ce delta est protégé avec la réserve naturelle nationale de Camargue (RNNC) située entre le Rhône et le petit bras du Rhône et la petite Camargue (Camargue Gardoise) située à l'ouest du petit bras du Rhône. La RNNC possède ainsi une richesse biologique exceptionnelle et couvre 13 000 ha entre l'étang de Vaccarès mésohalin au nord, les eaux saumâtres eurryhalines au niveau de la partie sud de la Camargue et la mer Méditerranée. L'ensemble de la Camargue est composé d'étangs d'eau douce et de canaux peu profonds avec une végétation abondante, des canaux de drainage d'eau douce avec une végétation submergée, des lagunes mésohaline (eau saumâtre) et euryhaline (saumâtre à hypersaline) toute l'année (Crivelli et Britton, 1987).

La Camargue est également la plus grande région de production de riz en France. La riziculture est réalisée par immersion et pour cela, environ 400 millions de m³ d'eau d'irrigation sont pompés directement depuis le Rhône. L'eau de drainage des rizières est partiellement renvoyée au Rhône par pompage, alors qu'un peu plus de 100 millions de m³ d'eau sont acheminés dans l'étang du Vaccarès, la plus grande étendue d'eau de Camargue. Il est à noter qu'en raison de leur position de réceptacle des eaux de drainage des exploitations rizicoles et des eaux de ruissellement des autres agrosystèmes (maraichage), ces étangs camarguais, s'ils se trouvent au sein de la réserve naturelle, ils sont néanmoins soumis à des contaminations chroniques qui apparaissent tous les ans à cause des pesticides utilisés en riziculture et auxquelles s'ajoutent les polluants transportés par le Rhône. Il s'agit donc d'un milieu particulièrement vulnérable et contraignant car soumis à des contraintes environnementales particulières (ex : pollutions chroniques, modifications rapides de salinités des différents milieux aquatiques) rencontrées par les espèces aquatiques soit lors de migrations actives entre les différentes masses d'eau, soit lors de migrations passives dues aux interventions de régulation des niveaux d'eau (gestion des flux d'eau par l'homme).

Objectifs de l'étude

Par conséquent, ce projet vise à identifier les principales différences morphologiques et dans les performances écophysiologiques d'individus issus des différents milieux aquatiques de Camargue. Le but est d'identifier Des niveaux potentiellement différents d'expression phénotypique et génique pour des protéines clefs pourraient-ils ainsi indiquer des différences entre les peuplements issus de différents habitats aquatiques de Camargue (canaux de drainage, étangs méso et euryhalins) ? Les épinoches de Camargue peuvent-elles être considérées comme provenant de populations différentes ? Ces questions apparaissent d'autant plus importantes que ces épinoches vivant dans les zones humides de Camargue présentent une composante importante de la biodiversité aquatique de cet écosystème et qu'elles constituent une des populations périphériques méridionales parmi les plus importantes en Méditerranée.

L'objectif de ce projet est donc de comprendre les effets de différentes salinités environnementales sur l'adaptabilité des populations d'épinoches à 3 épines.

- Évaluer les différents traits morphologiques des épinoches prélevées dans un environnement présentant des salinités contrastées : canaux d'eau douce, lagunes mésohaline et euryhalines.
- Mesurer les réponses physiologiques aux changements brusques de salinité en utilisant le taux de consommation d'oxygène et l'activité enzymatique de la pompe à sodium (Na^+/K^+ -ATPase = NKA) des branchies comme paramètre osmorégulateur essentiel.
- Étudier les capacités d'acclimatation de l'épinoche de Camargue en considérant des paramètres clés tels que l'osmolalité sanguine, la morphologie des ionocytes, les expressions géniques de la sous-unité $\alpha 1$ de la NKA et des isoformes $\alpha 1a$ et $\alpha 1b$, en plus de l'expression des gènes codants pour le CFTR, la V-ATPase, et le NKCC1.

II) Principaux résultats obtenus

La pression osmotique sanguine montre une différence significative entre les poissons acclimatés aux différentes salinités (ED: 5 ‰ &, 147 mOsm kg^{-1} ; ES: 15 ‰ &, 457 mOsm kg^{-1} ; EM: 30 ‰ &, 911 mOsm kg^{-1}) a été observée dans les pressions sanguines osmotiques.

La morphologie des ionocytes branchiaux a été identifiée par technique d'immunohistochimie en microscopie optique (immunofluorescence indirecte) en utilisant le marquage de la Na^+/K^+ -ATPase. Ainsi, les ionocytes ont été localisés le long des filaments branchiaux et des lamelles chez les poissons maintenus en ED et ES. Dans les poissons en ED, les ionocytes situés sur les filaments étaient nettement allongés sur leur hauteur, et ceux situés le long des lamelles semblaient systématiquement plus aplatis et plus larges. Dans les poissons maintenus en EM, les ionocytes de forme ronde, n'étaient situés que le long des filaments des branchies dans l'épithélium interlamellaire des branchies. De plus, la densité des ionocytes sur une longueur de 100 μm de filament branchial était significativement plus élevée dans les branchies des poissons ED (26 ± 7) par rapport aux poissons acclimatés à l'EM.

Pour les branchies, seule la première branchie de l'arc branchial gauche a été considérée. Les longueurs des branchiospines ont été mesurées de la base à la pointe de ces structures. Une différence significative a été observée au niveau de la morphologie de ces branchies entre des animaux maintenus en ED, ES et EM. La longueur et la densité des branchiospines sont significativement plus élevées chez les épinoches prélevées à partir des canaux ED que chez les poissons pêchés dans les lagunes (ES / EM). Aucune différence statistique dans le nombre de plaques latérales entre les différents habitats n'a pu être identifiée. De plus, aucune différence statistique significative n'a été observée au niveau de la morphologie corporelle entre les épinoches échantillonnées en 2016 et 2017 (Fumemorte, Vaccarès, Marteau et Versadou) et celles échantillonnées depuis 1984 (Malagroy, La Capelière, Fournelet, Baisse Salee et Giraud).

Expression relative de l'ARNm de NKA

Les niveaux de transcription du gène de référence *uba52* n'ont pas changé entre les salinités. Pour $\text{NKA}\alpha 1a$ et $\text{NKA}\alpha 1b$, les expressions géniques relatives des ARNm dans les branchies étaient significativement différentes en fonction de la salinité testée. Cependant, pour $\text{NKA}\alpha 1a$, les expressions géniques relatives des ARNm dans les branchies ne sont pas différentes en raison de la salinité. L'expression relative de l'ARNm de $\text{NKA}\alpha 1a$ est plus élevée dans les branchies des poissons acclimatés à l'ED que dans les poissons ES et l'expression de $\text{NKA}\alpha 1b$ est significativement plus élevée dans les branchies des poissons acclimatés à l'EM que dans les deux

autres salinités. Pour l'expression de la CFTR et de la V-ATPase, aucune différence statistiquement significative entre les différentes conditions de salinité n'a pu être mise en évidence. Par contre, le NKCC1 est surexprimé en condition EM.

Ultrastructure des ionocytes branchiaux

Les observations au microscope électronique à balayage et à transmission ont révélé différentes morphologies cellulaires le long de la surface des branchies de ces poissons. Chez les épinoches maintenues en ED, deux types d'ionocytes ont été observés: les cellules dont la surface apicale et qui est donc au contact avec le milieu extérieur, est en forme de dôme avec des petits éléments sphériques (digitations) recouvrant la surface et les ionocytes dont la surface apicale présente une structure en nid d'abeilles. Tous ces types de cellules ont été confirmés en tant qu'ionocytes en raison de leurs structures internes remplies de nombreuses mitochondries et d'un système tubulo-vésiculaire dense, constitué d'une membrane basale profondément invaginée. Chez les poissons acclimatés à l'EM, seuls les ionocytes possédant une grande crypte apicale ont été observés le long des filaments branchiaux à la base des lamelles branchiales. Ces cellules sont organisées en complexes multicellulaires. Aucun ionocyte n'a pu être observé le long des lamelles branchiales.

Taux de consommation d'oxygène

Pour des épinoches récoltées dans la lagune du «Marteau» (EM), le canal «Versadou» (ED) et la lagune méso-linéaire du Vaccarés (ES), les taux de consommation d'oxygène ont été mesurés à différentes températures (21, 23 et 25°C). Ces niveaux de consommation étaient significativement différents avec une consommation d'oxygène plus élevée à température élevée. Les épinoches prélevées dans la lagune mésohaline de Vaccarés (ES) et conservées initialement en EM ont présenté une réduction significative du taux de consommation d'oxygène immédiatement et jusqu'à 1 heure après le transfert brutal en ED. Une acclimatation plus longue en EM ou ED jusqu'à 3 et 6 semaines n'a pas induit de changement dans le taux de consommation d'oxygène par rapport aux poissons gardés inversement en ED et EM.

Activité branchiale de la pompe à sodium Na^+/K^+ -ATPase

Après transfert direct de EM à ED, l'activité branchiale de la Na^+/K^+ -ATPase des épinoches prélevées dans la lagune EM du «Marteau» a augmenté de 28% et de 40% dans les 24 et 48 heures suivant le transfert, respectivement. Cependant, lorsque les épinoches prélevées dans le canal ED du Versadou sont transférées de ED à EM, l'activité de Na^+/K^+ -ATPase des branchies est significativement réduite de 36% en 24 heures. Par conséquent, il y a une variation de 1,65 fois au niveau de l'activité Na^+/K^+ -ATPase des branchies lorsque les poissons sont transférés brusquement à une salinité différente. De plus, l'activité enzymatique pour les poissons acclimatés ou transférés en EM ou ED semble similaire, bien que ces poissons aient été prélevés à partir de deux habitats contrastés.

III) Discussions

Les modifications de l'osmolalité sanguine sont souvent utilisées comme indicateur des réponses d'acclimatation à la salinité, dans la mesure où la tolérance à la salinité et les fonctions osmorégulatrices sont étroitement liées (Varsamos *et al.*, 2005). Ainsi, la population d'épinoches à trois épines de Camargue s'acclimate rapidement à diverses conditions de salinité. En outre, la pression osmotique du sang mesurée chez ces poissons maintenus à des salinités différentes était toujours proche de 290 mOsm Kg⁻¹, bien que ces valeurs soient significativement plus faibles chez les poissons d'eau douce que chez les poissons acclimatés à des salinités plus élevées. Cependant, les osmolalités plasmatiques mesurées pour des épinoches issues de l'eau douce (ED : rivière Beke) et d'une eau saumâtre (ES : estuaire de Warnow) (nord-est de l'Allemagne) et maintenues en ES (10‰) ou transférées en eau de mer (EM) (35‰) (Schaarschmidt *et al.*, 1999) sont plus élevés (environ 335 et 380 mOsm Kg⁻¹ respectivement) que celles obtenues pour la population méditerranéenne de Camargue. Une telle différence pourrait être due à une fonctionnalité spécifique des mécanismes d'osmorégulation entre ces groupes phylogéographiques différents de mer Méditerranée et de mer du Nord (Mäkinen *et al.*, 2008).

La mesure du taux de consommation d'oxygène est un indicateur indirect du métabolisme chez les poissons (Cech, 1990). De plus, pour des épinoches capturées en milieu marin ou en milieu d'eau douce (population non migratrice) (Bonsall et West Creeks ; Colombie-Britannique, Canada), une différence significative du taux métabolique maximal (MMR « Maximal Metabolic Rate ») a été observée mais aucune différence n'a pu être détectée par rapport au taux métabolique de base (BMR ou SMR « standard Metabolic Rate) (Dalziel *et al.*, 2012). Enfin, suite à des épisodes hypo- ou hyperosmotiques (notre étude sur les épinoches de Camargue), des changements brusques de salinité n'induisent pas de changements significatifs, sauf immédiatement après le transfert. Ceci est cohérent avec des résultats antérieurs sur des épinoches monomorphes de rivière (peu de plaques latérales), des épinoches marines (complètement recouvertes de plaques) et des épinoches polymorphes collectées dans un lac d'eau saumâtre en Norvège : absence d'effets à court terme des variations de salinité sur la consommation d'oxygène et aucun effet sur le SMR (Grøtan *et al.*, 2012). Les taux de respiration peuvent varier cependant, selon les saisons (Meakins, 1975) et il pourrait également y avoir des différences liées à la température notamment, dans la régulation osmotique entre les populations (Schaarschmidt *et al.*, 1999).

Les épinoches prélevées à partir de différents habitats aquatiques de Camargue présentent peu de plaques avec un minimum de 2 et un maximum de 6 plaques latérales. Aucune différence statistique n'a pu être identifiée dans le nombre de plaque latérale entre les différents d'individus issus des différents habitats et possédant des régimes de salinité différents. De plus, aucune différence statistique significative n'a pu être observée en terme de morphologie externe entre les épinoches échantillonnées en 2016 et 2017 (lagunes Vaccarès et Marteau; canaux Fumemorte et Versadou) et celles échantillonnées depuis 1984 (lagunes de Malagroy, La Capelière, Fournelet, Baisse Salée et Giraud). De nombreux agents sélectifs potentiels ont été proposés pour expliquer les changements évolutifs morphologiques parallèles au sein des populations d'épinoche (Voje *et al.*, 2013; Spence *et al.*, 2013; Smith *et al.*, 2014; MacColl et Aucott, 2014). Pour celles de Camargue vivant dans le delta du Rhône, dans des habitats contrastés d'eau de mer, d'eau saumâtre ou d'eau douce, des morphologies corporelles très homologues ont été trouvées et avec aucun effet de salinité sur le nombre de plaques latérales. Ceci est cohérent avec ce qui a déjà été observé pour d'autres épinoches d'eau douce et côtières de la région bioclimatique méditerranéenne, toutes sont faiblement recouvertes de plaques (Münzing, 1963).

Seule la morphologie des branchiospines a révélé une différence entre les épinoches collectées dans les canaux d'eau douce (longues branchiospines) et celles recueillies dans les différentes lagunes mésohaline et euryhaline (courtes branchiospines). Cette divergence peut refléter un mode de vie plus pélagique des épinoches mésohalines (Bell et Foster, 1994). Nos résultats correspondent aux résultats précédents, à savoir ceux de Gross et Anderson (1984). Berner *et al.* (2010) ont également trouvé des structures phénotypiques divergentes dans les populations d'épinoches d'eau douce et marines lorsque au niveau des branchiospines. Par conséquent, la morphologie de ces structures impliquées dans l'alimentation des individus semble déclencher des changements adaptatifs dans des environnements divergents (Mathews *et al.*, 2010) et peut être libre d'évoluer sans liens étroits avec la colonisation de l'eau douce (Larson, 1974). En effet, la longueur de ces structures a déjà été identifiée comme une caractéristique clef permettant des changements de position trophique dans des habitats particuliers (Mathews *et al.*, 2010). Les épinoches sont généralement considérées comme des généralistes trophiques avec des variations morphologiques au niveau des branchies selon un axe benthos - limnos (Schluter et Nagel, 1995; Bell et Andrews, 1997; Willacker *et al.*, 2010); le morphotype limnétique se nourrissant dans les habitats pélagiques (ex : algues flottantes) possède un nombre important de branchiospines, alors que le morphotype benthique présente lui, des longueurs de branchiospines plus courtes (Wootton, 1984; Williams et Delbeek, 1989).

Analyse morpho-fonctionnelle des principaux organes osmorégulateurs

Quatre types distincts d'ionocytes ont été décrits fonctionnellement dans la membrane du sac vitellin des embryons de tilapia (Hiroi *et al.*, 2008; Hiroi et McCormick, 2012) et jusqu'à cinq types ont été rapportés à la surface de la peau et des branchies du poisson zèbre (*Danio rerio*), poisson sténohalin d'eau douce (Dymowska *et al.*, 2012; Guh *et al.*, 2015). De plus, une des cellules observées par en microscopie électronique à balayage a révélé une structure en nid d'abeilles similaire à celle observée le long des lamelles branchiales des épinoches prélevées dans les milieux ED de Camargue ou acclimatés en ED. La présence d'ionocytes similaires chez le poisson zèbre et l'épinoche ED pourrait indiquer qu'il s'agit d'un type cellulaire spécialisé plutôt qu'un ionocyte en stade transitoire. Le second ionocyte de type ED (ionocyte en forme de dôme) présent dans les épinoches de Camargue a été décrit chez diverses autres espèces de Téléostéens vivant dans en ED (Hwang *et al.*, 2011). La transformation de la taille des ionocytes et de la morphologie apicale (passage du type ED plat en EM concave ou EM convexe) peut se produire très rapidement (Choi *et al.*, 2011) et peut entraîner un changement fonctionnel radical de l'absorption de solutés à la sécrétion de sels (Lin, 2013). Ce remodelage pourrait également être dû à un changement de type cellulaire impliquant la prolifération cellulaire et l'apoptose. Dans les épinoches de Camargue, la densité de ces ionocytes ne change pas entre les poissons ED et les poissons acclimatés à l'EM, bien que leur position diffère le long du filament branchial et des lamelles. On considère que ce remodelage des branchies présente un faible coût énergétique, avec une prolifération cellulaire et une apoptose réduite. Cela pourrait également expliquer l'absence de coût métabolique à court terme observé chez les épinoches transférées dans différents environnements de salinité, quel que soit leur environnement d'origine (Grøtan *et al.*, 2012).

Dans les épinoches de Camargue, les niveaux de transcription du gène de référence uba52 n'ont pas changé entre les salinités. Pour NKA α 1, les expressions géniques relatives des ARNm dans les branchies étaient significativement différentes en fonction de la salinité testée. Cependant, pour NKA α 1a, les expressions géniques relatives des ARNm dans les branchies ne sont pas différentes

en raison de la salinité. L'expression relative de l'ARNm NKA α 1 était plus élevée dans les branchies des poissons acclimatés à l'EM que dans les poissons en ED, et l'expression de NKA α 1b était significativement plus élevée dans les branchies des poissons acclimatés à l'EM que dans les deux autres salinités. L'expression génique de la NKA a également été étudiée dans les épinoches transférées de l'EM à l'EM, de l'EM à l'ED, de l'ED à l'ED et de l'ED à l'EM (Taugbøl *et al.*, 2014). Les populations marines et d'eau douce continentale ont pu s'acclimater à ces transferts brusques de salinité avec un profil d'expression de la NKA qui apparaît hautement plastique, l'expression étant augmentée 48 heures et 7 jours après le transfert pour le groupe EM-EM et ED-EM, respectivement. Pour les épinoches de Camargue, le transfert de salinité n'a pas modifié l'expression de la NKA α 1a, bien que l'expression de l'isoforme de la NKA α 1b ait augmenté de manière significative chez les poissons acclimatés à l'EM. Récemment, l'expression des isoformes NKA α 1 a suscité beaucoup d'attention avec une expression inverse de ces isoformes chez les salmonidés en transition entre les environnements ED et EM (Madsen *et al.*, 2009; McCormick *et al.*, 2009). Cependant, la nomenclature actuelle prête à confusion avec des désignations d'isoformes utilisées pour les salmonidés et chez les autres Téléostéens qui sont différentes (Blondeau-Bidet *et al.*, 2016; Wong *et al.*, 2016).

Les épinoches prélevées dans la lagune EM du « Marteau », après transfert direct de l'EM à l'ED, ont montré que l'activité de Na⁺/K⁺-ATPase branchiale augmente de 28% et de 40% dans les 24 et 48 heures suivant le transfert, respectivement. Cependant, lorsque les épinoches prélevées dans le canal ED du « Versadou » sont transférées de l'ED à l'EM, l'activité de Na⁺/K⁺-ATPase des branchies est significativement réduite de 36% en 24 heures. Par conséquent, il y a un changement de 1,65 fois au niveau de l'activité de la Na⁺/K⁺-ATPase des branchies lorsque les poissons sont brusquement transférés dans des conditions de salinité différentes. De plus, l'activité enzymatique pour les poissons acclimatés ou transférés en EM ou en ED semble similaire, bien que ces poissons aient été prélevés dans deux habitats contrastés. Si le coût lié aux changements dans les mécanismes d'osmorégulation est élevé, un choc de salinité aurait dû avoir un impact sur le métabolisme global. Cette plasticité dans les mécanismes d'osmorégulation est observée au niveau cellulaire et moléculaire dans les épinoches de Camargue avec un remodelage des ionocytes des branchies (Rind *et al.*, 2017), un changement de l'activité de la NKA des branchies et une expression génique supérieure de l'isoforme NKA α 1a branchiale par rapport à l'isoforme NKA α 1b ; ces 2 isoformes étant impliquées respectivement dans l'absorption d'ions dans les ionocytes de type FW et dans la sécrétion ionique dans les ionocytes de type SW dans le bar européen (*Dicentrarchus labrax*) (Blondeau-Bidet *et al.* 2016). Ces résultats correspondent à ceux déjà publiés (Jurss *et al.*, 1982; Judd, 2012). Cependant, une activité accrue de NKA chez les branchies a également été documentée lorsque les épinoches d'ED sont transférées en EM (Schaarschmidt *et al.*, 1999). Par conséquent, cette activité accrue de NKA en milieu hyper-osmotique pourrait expliquer l'écart observé entre les résultats obtenus dans notre étude et ceux de Schaarschmidt *et al.* (1999).

Pour les épinoches conservées dans les différentes conditions de salinité, aucune différence n'a pu être détectée dans l'expression relative de l'ARNm pour le CFTR des branchies. Le CFTR est légèrement plus exprimé en ES ou en EM (conditions environnementales hyper-osmotiques) par rapport à la condition ED (condition environnementale hypo-osmotique). Dans l'ensemble, le gène cible (CFTR) présente un profil hautement plastique. Nos résultats concordent avec ceux de Taugbøl *et al.* (2014), puisque dans cette étude, l'expression du CFTR ne variait pas entre des épinoches marines témoins et des poissons acclimatés à long terme à l'ED.

Il est généralement admis que le rôle principal du CFTR est lié à l'excrétion d'ions chez les poissons marins puisque ce transporteur est impliqué dans le transport d'ions chlorure Cl^- (McCormick *et al.*, 2003). Après acclimatation de l'ED à l'EM, une augmentation de l'expression de CFTR a été rapportée chez les anguilles (*Anguilla Anguilla*; Wilson *et al.*, 2004), le saumon atlantique (*Salmo salar*; Singer *et al.*, 2002). Au contraire, McCairns et Bernatchez (2010) ont signalé une expression plus élevée de CFTR dans les épinoches ED élevées en laboratoire. En outre, en ce qui concerne l'acclimatation à long terme pendant plus de 3 semaines, l'expression de CFTR est non modifiée. Pour le CFTR, des résultats très contrastés ont été rapportés chez les poissons. Ainsi, par exemple, le tilapia ED du Mozambique (*Oreochromis mossambicus*) n'exprime pas cette protéine (Hiroi *et al.*, 2005), elle est peu exprimée chez le killifish (*Fundulus heteroclitus*; Marshall *et al.*, 2002), alors qu'il est présent chez le bar rayé (*Morone saxatilis*) mais aucun changement d'expression n'a pu être mis en évidence suite à des changements de salinité (Madsen *et al.*, 2007). Avec des salinités environnementales contrastées, le CFTR peut aussi avoir des localisations intracellulaires différentes : localisation basolatérale pour des poissons en ED et apicale en EM (Marshall *et al.*, 2002). Cela pourrait également être le cas des épinoches de Camargue.

Les poissons euryhalins sont plus plastiques, sur acclimatation à différentes salinités. Ils peuvent notamment remodeler leurs ionocytes branchiaux afin de maintenir l'homéostasie hydrominérale. Dans le cas des épinoches de Camargue, après acclimatation à l'EM, au moins deux semaines le NKCC1 des branchies est surexprimé. Cependant, nous n'avons pas trouvé de différence significative entre l'expression en ED et en ES de NKCC1. Le NKCC est un membre de la famille des cotransporteurs chlorure-cation et le NKCC1 est connu sous le nom d'isoforme sécrétoire largement distribué chez les vertébrés (Lytle *et al.*, 1995; Gagnon *et al.*, 2002). Une augmentation de l'expression de l'ARNm du NKCC des branchies a déjà été observé chez différentes espèces, indiquant une sécrétion de Cl^- lors de l'acclimatation à l'EM comme par exemple, chez le loup rayé (*Morone saxatilis*; Tipsmark *et al.*, 2004), le killifish (*Fundulus heteroclitus*; Scott *et al.*, 2004), l'anguille européenne (*Anguilla Anguilla*; Cutler et Cramb, 2002), la truite brune (*Salmo trutta*; Tipsmark *et al.*, 2002) et le tilapia (*Oreochromis mossambicus*; Wu *et al.*, 2003). Récemment, il a été rapporté que l'abondance de la NKCC (expression des protéines) au niveau des branchies augmente aussi chez les épinoches transférées en EM (35 ppt) pendant une semaine (Divino *et al.*, 2016).

Pour les épinoches de Camargue, il n'a pas été possible de visualiser de manière significative l'expression la V-ATPase dans nos conditions expérimentales de salinité (ED, ES et ES). Chez certains poissons comme le chabot de Longhorn (*Myoxocephalus octodecemspinus*) et l'anguille européenne (*Anguilla anguilla*; Harvey, 2009), la V-ATPase est impliquée dans l'absorption indirecte de sodium à travers la membrane apicale. Dans les branchies, la V-ATPase est présente sur les membranes apicales des cellules pavimenteuses et dans les cellules riches en Na^+/K^+ -ATPase (ionocytes). Il s'agit d'un transporteur actif important pour à la fois l'absorption de sodium et aussi pour l'excrétion d'hydrogène (H^+). La co-localisation de la V-ATPase avec le Na^+ épithélial a été identifié au niveau de la membrane apicale des cellules pavimenteuses situées au niveau du 'bord d'attaque' des branchies du tilapia (*Oreochromis mossambicus*) (Wilson *et al.*, 2000). Chez la truite arc-en-ciel (*Oncorhynchus mykiss*), la V-ATPase est exprimée dans les cellules pavimenteuses et les ionocytes (Wilson *et al.*, 2000). Les preuves du rôle possible de la V-ATPase dans l'absorption des ions chez les Téléostéens comprennent la chute au niveau de l'absorption du sodium par la bafilomycine (inhibiteur spécifique de l'enzyme) chez le tilapia (*O. mossambicus*) et la carpe (*Cyprinus carpio*) (Fenwick *et al.*, 1999) en EM et la diminution de l'activité et localisation de la V-ATPase branchiale (Lin et Randall, 1993; Lin *et al.*, 1994).

IV) Conclusion et perspectives

Le delta du Rhône est le seul delta de France et le second par l'importance de sa superficie en Méditerranée. Il possède des milieux rarement rencontrés ailleurs sur une telle étendue (steppes salées, étangs, mares temporaires, lagunes, marais, etc), jouant un rôle de refuge pour de nombreuses espèces rares de plantes et d'animaux. Ce delta comprend la zone située entre les deux bras du Rhône et la 'petite Camargue' ou Camargue gardoise qui correspond à la partie située à l'ouest du Petit-Rhône. Il s'agit aujourd'hui d'un espace protégé avec une gestion de ses ressources naturelles et la valorisation de son patrimoine qui ont été confiées à la Réserve Naturelle Nationale de Camargue (RNNC) (la plus vieille réserve nationale française) et au Syndicat mixte Camargue gardoise. Si ces sites sont protégés, des risques de pollution existent, néanmoins. En effet, depuis 2011 des analyses d'eau effectuées au sein de la Réserve Naturelle Nationale de Camargue (RNNC) montrent que les canaux de drainage transportent de plus en plus de pesticides provenant de la riziculture avec un transfert de ces polluants vers les étangs et lagunes de Camargue. La petite Camargue est potentiellement moins impactée par ces pesticides du fait de surfaces agricoles moins importantes qu'autour de la RNNC. Ce site pourrait donc servir de milieu témoin par rapport à un biomonitoring de l'ensemble de la zone deltaïque du Rhône. Pour une évaluation de l'état écologique des masses d'eau telle qu'imposée par la Directive Cadre sur l'Eau (DCE ; 2000/60/CE), l'épinoche pourrait être utilisée comme espèce sentinelle de l'environnement puisque cette espèce est déjà utilisée notamment en France pour des tels monitoring des masses d'eau continentales.

Une autre perspective est aussi d'analyser plus finement la diversité des populations de Méditerranée avec notamment une analyse phylogéographique des populations présentes dans les milieux côtiers. Les analyses récentes de phylogénie moléculaire (phylogénies mitochondriales et nucléaires) démontrent clairement des imprécisions quant aux dates de divergences pour les populations européennes du Sud, du Nord et de la façade Atlantique Est (Fang *et al.*, 2018). Il en est de même pour les populations méditerranéennes qui selon les auteurs auraient différemment colonisé les différents bassins de Méditerranée et de la mer Noire et avec également des différences entre les populations insulaires du bassin méditerranéen ouest et les populations continentales et côtières (Lucek & Seehausen, 2015 ; Sanz *et al.*, 2015 ; Mäkinen *et al.*, 2006 ; 2008, ; Lescak *et al.*, 2017 ; Vila *et al.*, 2017 ; Fang *et al.*, 2018).

Il s'agit également de mieux connaître la mise en place de la fonction d'osmorégulation au cours du développement afin d'identifier à quelle période de l'année des changements rapides de salinité

Cette étude est une des rares décrivant les épinoches de Camargue en prenant en compte notamment les capacités physiologiques d'adaptation. Une telle analyse pourrait ainsi préfigurer d'autres analyses évolutives et phylogénétiques. L'épinoche le long du littoral Méditerranéen pourrait aussi être utilisée dans des programmes de surveillance en écotoxicologie. Ainsi, l'ambition générale est de développer une synergie autour de l'épinoche méditerranéenne qui pourrait devenir un modèle de choix, notamment à l'Université de Montpellier. Cette espèce est l'équivalent euryhaline du poisson zèbre en termes de taille et de ses installations de reproduction et de reproduction en captivité (par exemple: fécondation *in vitro* contrôlée). De plus, son génome est connu et publié. Il convient également de noter que les variations prévisibles et imprévisibles

des principaux paramètres environnementaux tels que la salinité et la température doivent augmenter en fréquence et en intensité en raison du réchauffement planétaire actuel. Par conséquent, cette intensification des pressions environnementales constitue une contrainte supplémentaire majeure pour les individus présentant des caractéristiques adaptatives spécifiques et sont déjà à la limite de leurs capacités physiologiques. L'épinoche de la Méditerranée pourrait ainsi être utilisée non seulement en écotoxicologie et en biologie de conservation, mais aussi en écologie fonctionnelle et évolutive.

1 Chapter 1: General considerations and aims of this study

Physiology of Threespined Sticklebacks (*Gasterosteus aculeatus*) of the Camargue at different salinities.

In recent years, the loss in biodiversity has been assumed as a global crisis, eventually threatening ecological significant habitats (U.N, 1992). As a result, local populations are facing rapid and falling impacts, upsetting essential ecosystem systems and also affecting whole particular ecosystems (Garner *et al.*, 2005; Hooper *et al.*, 2005; Helfman, 2007). Besides, it is not simply a connection among local species and ecosystem but also a matter of overall existence of species themselves (Luck *et al.*, 2003). Accordingly, conservation of local distinct populations is a vital step for the conservation of the biodiversity (Luck *et al.*, 2003). Furthermore, in Ecology, central and peripheral populations are key components to be considered. Peripheral populations are essential populations in terms of species biogeography, evolution and conservation (Scudder, 1989; Lesica and Allendorf, 1995; Latta, 2003). Peripheral populations, in natural conditions continue to exist under different environmental conditions and are distinct from core population. Therefore, these peripheral populations may represent particular genetic and phenotypic adjustments to their harsh environments (Yakimowski and Eckert, 2007). Because of small and fragmented size, numerous peripheral populations have low recolonization potential (Lesica and Allendorf, 1995; Channell and Lomolino, 2000; Wisely *et al.*, 2004). Ecologically marginalized populations, in addition, often experience low gene flow and encounter high level of genetic drift, prompting further dissimilarity from central populations (Jones *et al.*, 2001; Lammi *et al.*, 2001; Johannesson and André, 2006). Due to contrasting ecological conditions associated with geographical factors (i.e.: latitude), population may likewise show divergent responses, which, thus, can differently influence life history attributes (i.e.: size and age at reproductive maturation, growth rate, or fertility) (Stearns and Koella, 1986; Berrigan and Koella, 1994; Power and McKinley, 1997; Munch *et al.*, 2003; Heibo *et al.*, 2005; Slaughter *et al.*, 2008). Such life history trait variations have been reported in several taxa including plants (Yakimowski and Eckert, 2007), invertebrates (Lee and Bell, 1999; Lardies *et al.*, 2004) and vertebrates such as fish, reptiles and mammals (Kyle and Strobeck, 2002; Wilson and Cooke, 2004; Kynard, 1997; Yamahira and Conover, 2002). Genetic drift, isolation and low gene flow are the typical threats affecting peripheral population and can contribute to evolutionary divergence between core and peripheral populations (Leppig and White, 2006; Haak *et al.*, 2010). As a result, speciation processes that are most likely occurring in these peripheral populations make them Evolutionary Significant Units (ESU) (Lesica and Allendorf, 1995). Also, these ecologically marginalized populations of wide ranging species are expected to develop unique set of genetic characteristics (Nielsen *et al.*,

2001). Thus, populations at the edge of the species distribution range must be integral parts of the conservation efforts for global biodiversity (Lammi *et al.*, 2001; Johannesson and André, 2006).

Peripheral populations may similarly display distinct developmental rates linked with the length of the developing season (in warmer temperatures) and can also differ according to the latitude (Slaughter *et al.*, 2004). Such differences in growth rate may provide evidence for counter-gradient variation (Conover, 1990). Such growth differences occur when both genetic effect and environmental influences opposes each other across an environmental gradient (Conover and Schultz, 1995). According to the counter-gradient theory, species in higher latitudes have higher growth rates with shorter growing seasons as compared to species with lower latitudes (Conover and Present, 1990; Yamahira and Conover, 2002). Such growth variations have already been reported in a number of freshwater and seawater fish such as Striped Bass (*Morone saxatilis*), Mummichog (*Fundulus heteroclitus*), American Shad (*Alosa sapidissima*) (Conover, 1990), Lake Sturgeon (*Acipenser fulvescens*) (Power and McKinley, 1997), and Atlantic Cod (*Gadus morhua*) (Marcil *et al.*, 2006). Pumpkinseed (*Lepomis gibbosus*), and Guppy (*Poecilia reticulata*) exhibit co-gradient variation, for which genetic and environment effects on phenotype are well aligned along the gradient (Conover *et al.*, 2009). Such variations in growth rates with genetic and phenotypic differences between core and peripheral populations further enhance the conservation value of these peripheral populations.

Also, peripheral populations can live in transition zones where different ecological communities coincide (also known as ecotones). Such environments have received less attention during the last few decades in biodiversity research but they recently received much more attentions in the context of biodiversity conservation (Kark and van Rensburg, 2006). These zones maintain and shape distribution and abundance of organisms (Spector, 2002; Kark and van Rensburg, 2006). Species abundance and richness tend also to peak in these ecotonal zones (with, also, some exceptions). Additionally, in some cases, the species living in an ecotone display genotypes with unique morphological characteristics. These transitional zones can be terrestrial or aquatic, could be large scaled (McIntyre *et al.*, 2008) to local scaled transitions (Boyden *et al.*, 1981). The coastal zone may be visualized at the global scale as an ecotone. For example, Ketchum (1972) describes this

zone as transition between land and sea "where production, consumption, and exchange processes occur at high rates of intensity."

1.1 Research objectives

Peripheral populations in marginal environments and especially in an ecotone, experience extreme selection regimes. Therefore, local selection pressure can strongly influence such peripheral populations. Such ecosystems must deserve careful management of their genetic resources (García-Ramos & Kirkpatrick, 1997). Additionally, notable life-history traits may influence the genetic structure of a species (Lesica & Allendorf, 1995), whereas, a species with poor dispersal capacity is supposed to be more influenced by peripheral location than long-range dispersers (Bohonak, 1999). The populations at the edge of the species distribution limit, overall, have more extreme genetic setups as compared to core / central populations (Johannesson and André, 2006).

Physiological adjustments, on the other hand, have important implications for adaptation in order to counter different environmental salinities. Euryhaline fish for example can survive across wide range of salinity spectrum. Euryhaline capacities facilitate fish to expand from native marine environment to freshwater habitats (Lee & Bell, 1999; Schultz & McCormick, 2012). Subsequently, FW tolerance must rely on enhanced and rapid adjustment capacities to local conditions, potentially through specific co-adapted freshwater osmoregulatory gene complexes (DeFaveri *et al.*, 2011; Jones *et al.*, 2012b).

The three-spined stickleback has a well-documented history of transition from seawater to freshwater habitats, and is an ideal fish to study osmoregulatory physiology. The mechanisms of osmoregulatory adjustments have been studied in various teleosts such as eels, clupeids, salmonids, killifish, tilapia, etc (reviewed by Evans *et al.* 2005; McCormick *et al.*, 2013a) and also in sticklebacks, although limited physiological data are available on this species (De Ruiter and Bonga, 1985; DeFaveri and Merilä, 2014). In addition, most of the work on sticklebacks focused on adaptation to freshwater of northern populations in order to better understand the drivers for rapid evolution (Hohenlohe *et al.*, 2010; DeFaveri *et al.*, 2011; Taugbøl *et al.*, 2014). But no studies are available considering southern populations, notably those of the Mediterranean Sea, which

constitutes the southern limit of the species distribution range in the Western Palearctic (Crivelli & Britton, 1987). Furthermore, in the southern parts of the Mediterranean, stickleback populations have steeply declined. For example, in the Iberian Peninsula, the species is now considered to be endangered after the occurrence of multiple local extinction events (Reimchen, 1984; Foster, Baker & Bell, 2003; Araguas *et al.*, 2012).

The Camargue, the deltaic area of the Rhone river, is an ecological hotspot of diversity and has been a Biosphere Reserve since 1977. It has an exceptional biological richness and covers 13 000 ha between the northern mesohaline, brackish water Vaccarès lagoon and the Mediterranean Sea. It is made up of freshwater ponds and shallow water canals with abundant vegetation, freshwater drainage canals with submerged vegetation, mesohaline (brackish water) and euryhaline (brackish to hypersaline) lagoons flooded throughout the year (Crivelli & Britton, 1987).

This project aims to identify different phenotypic variants and genes that could underlie key physiological differences in terms of performance using the stickleback population living at the southern limit of their distribution, i.e. in the Camargue wetlands. Therefore, the aim of this project is to understand the effects of different environmental salinities on population adaptability of threespined stickleback.

- To evaluate different morphological traits from stickleback sampled from environment with contrasted salinities: freshwater canals, mesohaline lagoons.
- To measure the physiological responses to abrupt salinity changes using oxygen consumption rate and gill NKA activity as a key osmoregulatory parameter.
- To investigate the acclimatory responses of Camargue stickleback by considering key parameters such as blood osmolality, ionocytes morphology, gene and protein expression of $\alpha 1$ NKA, $\alpha 1a$ and $\alpha 1b$ particular isoforms plus CFTR, V-ATPase, NKCC1.

2 Chapter 2: Introduction

Physiology of Threespined Sticklebacks (*Gasterosteus aculeatus*) of the Camargue at different salinities.

2.1 The Camargue area

The deltaic area of the Rhône River along the French Mediterranean coast, also known as the Camargue area. It forms a triangle bordered by the two river branches: Petit Rhône and Grand Rhône. The Camargue is comprised of approximately 145,000 hectares and consists of more or less large interconnected lagoons (“étangs”). The largest lagoon is the “étang de Vaccarès” (Weber and Hoffmann, 1970; Fig. 2.1.).

The evolution of Mediterranean deltas has been driven by the rates of postglacial sea-level rise. Even if the chronology and amplitude of the Holocene sea-level variations differ according to different authors, one commonly considered characteristic is the decrease in the rate of the sea level rise that started 7000 years ago (Labeyrie *et al.*, 1976; L'Homer *et al.*, 1981; Aloisi, 2001; Vella *et al.*, 2005). This phenomenon helps to understand the formation of large deltas (Stanley and Warne, 1994). According to Arnaud-Fassetta (1998), the Rhône delta is primarily controlled by changes in sea level and variation in sediment supply. Along the Mediterranean coast, the Rhône delta began to aggrade and prograde about 6000 years ago because of sedimentary fluxes, climate and anthropogenic forces (Rey *et al.*, 2009).

Coastal wetlands are the most remarkable, but also threatened habitats in the Mediterranean region (Grillas *et al.*, 2004). Under the European Union's Habitat Directive (Natura code 3170, 92/43/CEE, 21 May 1992), wetlands are considered as priority habitats. A wide variety of threats make these habitats endangered including habitat destruction, salinization and hydrological alternation due to climate change as well as anthropogenic activities. Both the hydroperiod and salinity conditions are considered as key factors influencing the aquatic species inhabiting these wetland habitats (Spencer *et al.*, 1999; Bilton *et al.*, 2001; Grillet *et al.*, 2002; Nielsen *et al.*, 2003; Brock *et al.*, 2005). In the Camargue wetland complex, the salinity and hydrology conditions vary considerably because of rainfall and sea level fluctuations. Any drastic modification of the salinity conditions can therefore change the biodiversity for these mesohaline wetlands (Lake *et al.*, 2000; Bauder, 2005; Brock *et al.*, 2005; Pyke, 2005).

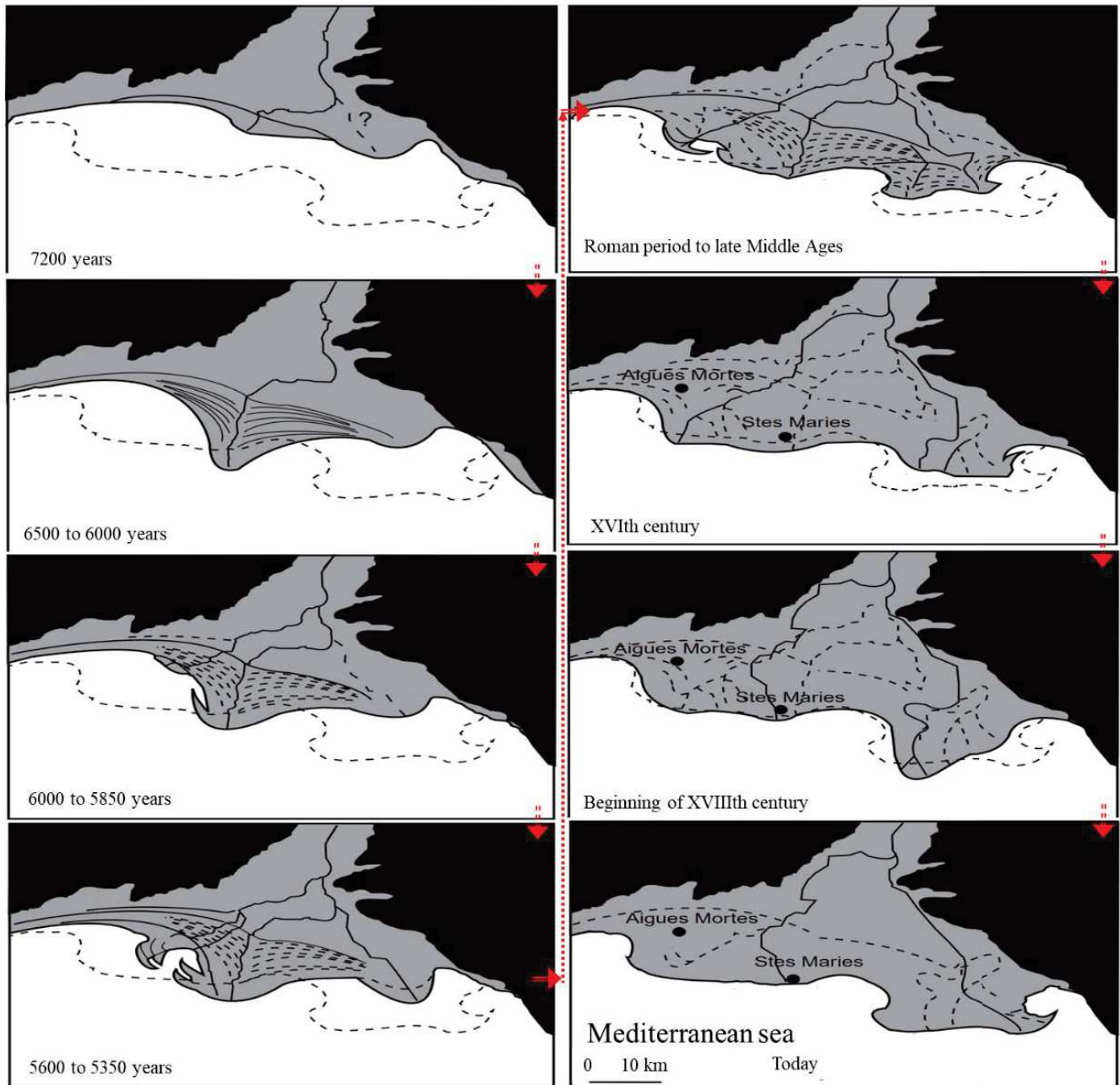


Figure 2-1 Evolutionary stages of the Rhône delta since 7200 yr before present time (from L'Homer *et al.*, 1981).

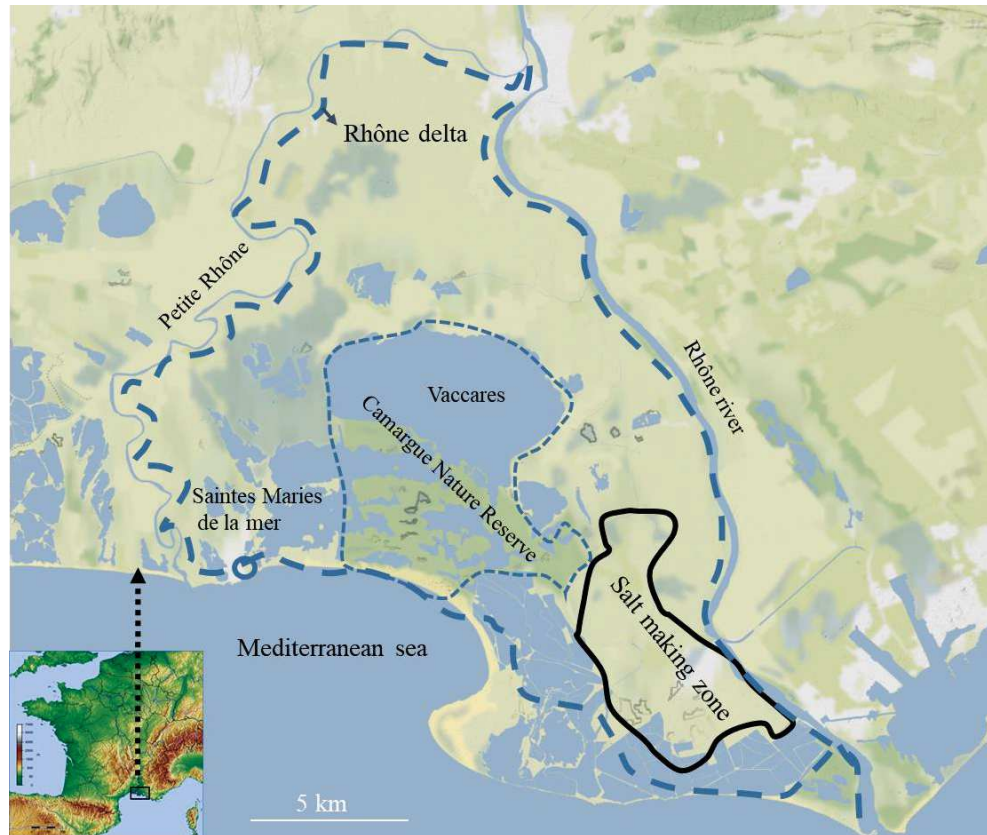


Figure 2-2. Current map of the Camargue with different lagoons along Rhône delta.

Today, the Camargue area is composed of a number of protected wetlands that are highly valuable for biodiversity conservation. It is a site of global conservation concern and a natural reserve since 1975 (Mathevet, 2004). Since 1977, it has been recognized as World Biosphere Reserve in the UNESCO “Man and the Biosphere” program and has been declared as a Ramsar Convention site since 1986. The Camargue is the largest riverine delta in Western Europe, (Fig. 2.2), with a unique biological diversity, and home to exceptional breeds of horse and bull, with also more than 400 species of birds including Pink Flamingoes. The bulls of the Camargue are smaller than other breeds in Europe. Unlike bulls, horses are unique and are considered one of the oldest breed in the world. In France, the Camargue is the only place where pink Flamingoes nest, the population of Flamingo reaches up to 20 000 individuals gathered into flocks where they build their nest. Most of the surface of the Camargue is covered with marshy and irrigated fields and ponds.

However, these wetlands are very close to rural agricultural areas used primarily for livestock and intensive rice (55%) and wheat (30%) cultivation The Camargue is the only place in France with

such a large scale rice production. These rice fields are key environmental features for ground water recharge as well as for providing freshwater to wetlands in order to control soil salinity in flooded fields. But, the use of different pesticides is a potential threat for this transitional environment (Comoretto *et al.*, 2008). Since 2011, the areas used as rice fields have reduced considerably occupying 21 000 ha in 2011 but only 14 000 ha in 2015. As a result, soil fertility decreases with also an increased in salt concentration.

Recently, a decline in the Living Planet Index has been reported from the mid 80ies until 1993, a in the Camargue mostly due to climate conditions. The biodiversity of Camargue especially native species were particularly affected by these prolonged period of freezing with food resources that were almost inaccessible. The fish population of the main lagoon of the Camargue, the ‘étang du Vaccarès’ decreased significantly from 1988 to 1993. Exceptional flood of the Rhône delta followed wet winters (1994-1997), and as a result, significant rises in the water level occurred in the Camargue delta inducing a significant drop in salinity. Such uncommon situation benefited to the fish diversity in the Camargue lagoons. This explains the rapid increase in the global diversity that followed during these years (Fig. 2.3). This situation has persisted over the years, mainly because of a large spill of agricultural wastewater. However, a more accurate analysis shows that the exotic species in the Camargue, responded very strongly to this change in the environment.

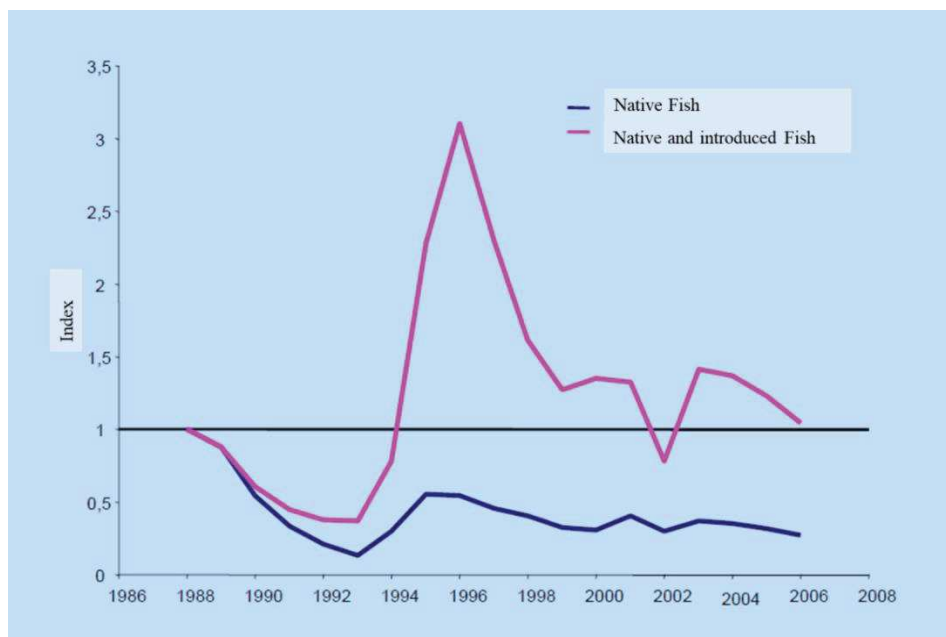


Figure 2-3. Living Planet Indices of fish from the Vaccarès, Camargue (Galewski, tour du valat, 2008).

2.2 Salinity and precipitation conditions in the Camargue area

In the lagoons of the Camargue area, the salinity fluctuates throughout the year. The Vaccarès lagoon, the main lagoon of the Camargue area, presents salinity conditions that are more or less brackish/mesohaline. On the other hand, in the Impériaux (Mesohaline seawater) and Lion / Dame (Mesohaline / hypersaline) lagoons, the salinity presents wide yearly fluctuations (Fig. 2.4A). The highest precipitations occur in the months of September to December, and are minimal in summer (June, July and August) (Fig. 2.4B).

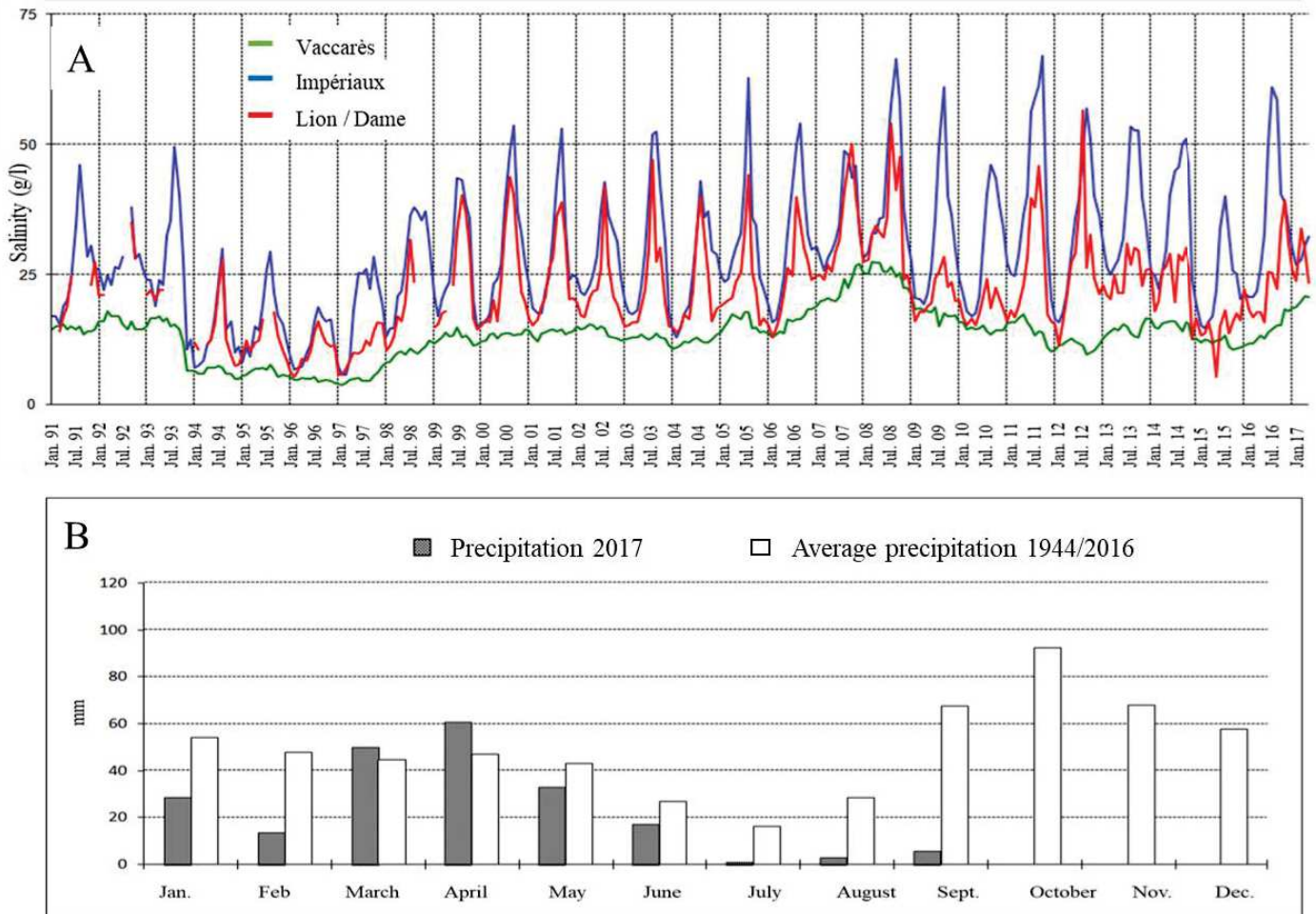


Figure 2-4. A Salinity variation among lagoons of the Camargue from 1991 to 2017, B. Precipitation conditions in different lagoons of the Camargue (SNPN, 2017).

2.3 The threespined stickleback (*Gasterosteus aculeatus*)

The sticklebacks and their close relatives (e.g. Pipefish, seahorse) have unique morphological features with, for instances, armored bodies with a number of lateral plates. They present different morphotypes and are grouped in the most diversified order: the Gasterosteiformes (Svensson, 1988).

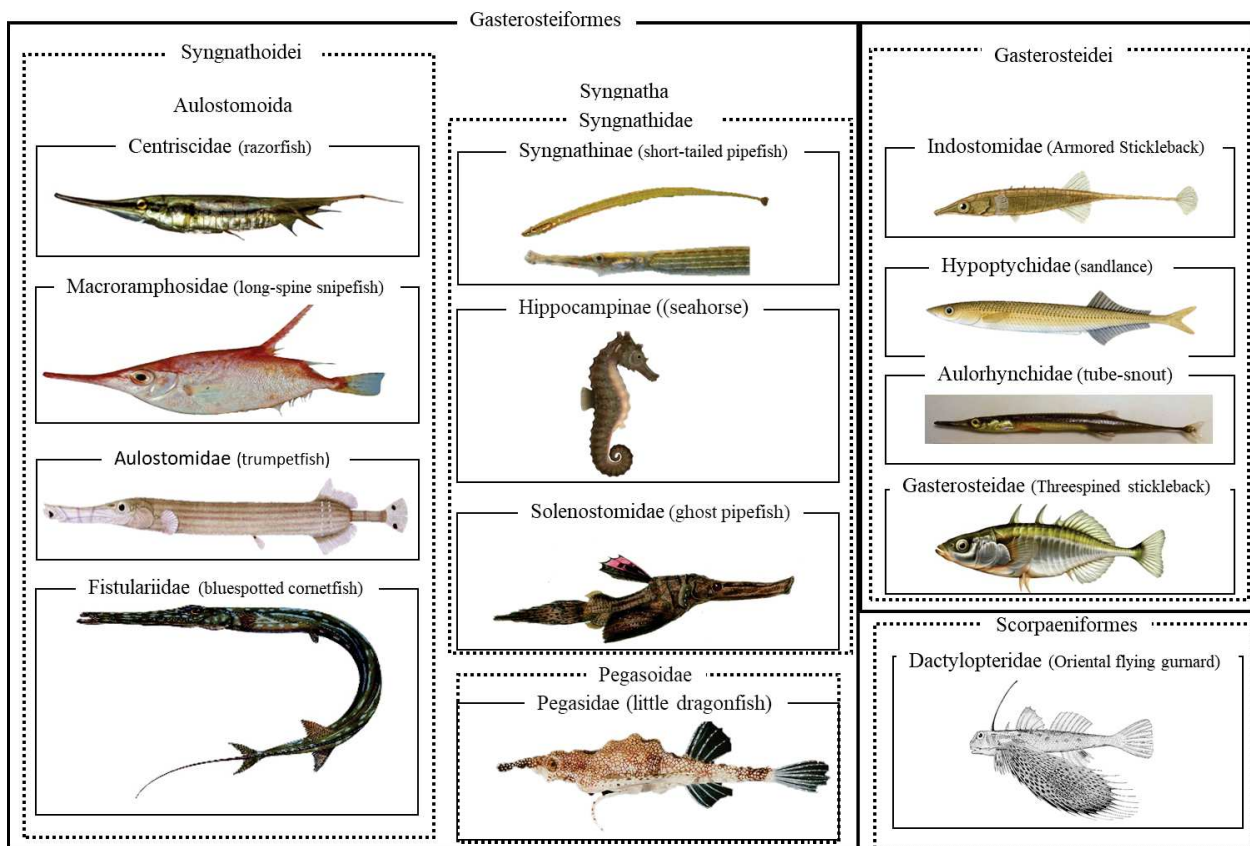


Figure 2-5. Gasterosteiformes schematic representation, adapted and modified from Kawahara *et al.*, 2008. (Classifications follow Nelson, 2006).

Members of this order display various shapes, sizes and modes of reproduction. For example, for members of the gasterosteid and aulorhynchid families, males build a nest using a special glue substance produced by the kidney called spiggin (Wootton, 1976) and females lay eggs in these built nests (Wootton, 1976; Wilson and Laurent, 2002).

Currently, Gasterosteiformes are classified in two suborders Gasterosteoidae and Syngnathoidae with 278 species (Nelson, 2006). Members of the gasterosteoids are mostly distributed within the northern hemisphere (Fig. 2.6) and are found in seawater, brackish water and freshwater habitats, while, the members of syngnathoids found in temperate and tropical live in coastal shallow marine habitats (Nelson, 2006). Additionally, Members of the gasterosteoids also includes threespined sticklebacks (*Gasterosteus aculeatus*), have rapidly adapted and adjusted freshwater habitats with interesting phenotypic diversity variety (Bell and Foster, 1994).

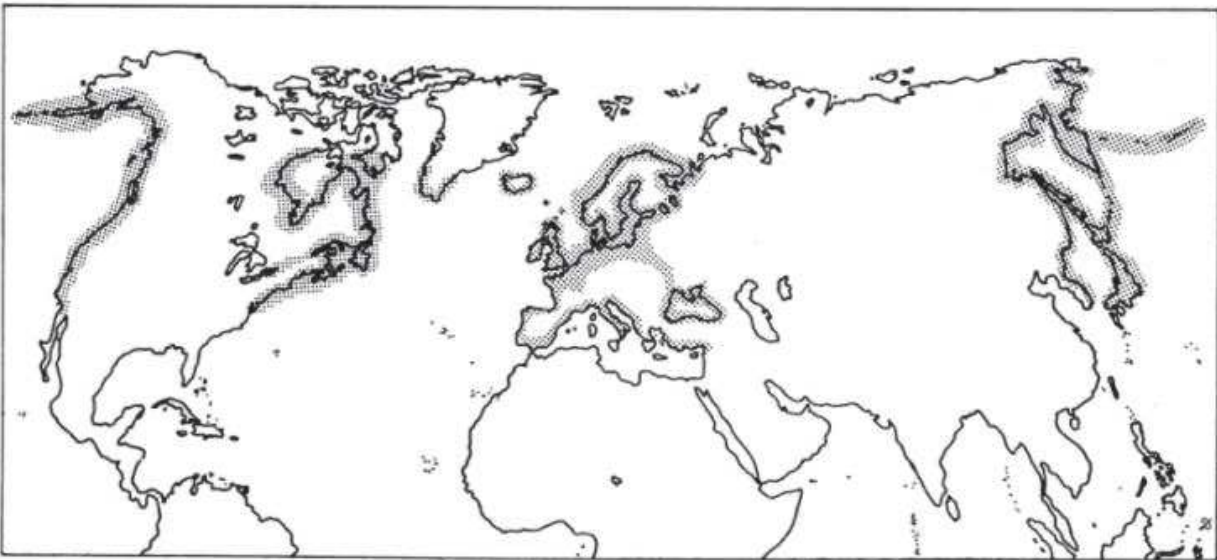


Figure 2-6. Global distribution of the threespined Stickleback (indicated by the shaded areas) (Wootton, 1984).

Table 2-1. Classification of threespined Stickleback (*Gasterosteus aculeatus*).

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Gasterosteiformes
Family	Gasterosteidae
Genus	<i>Gasterosteus</i>
Species	<i>G. aculeatus</i>

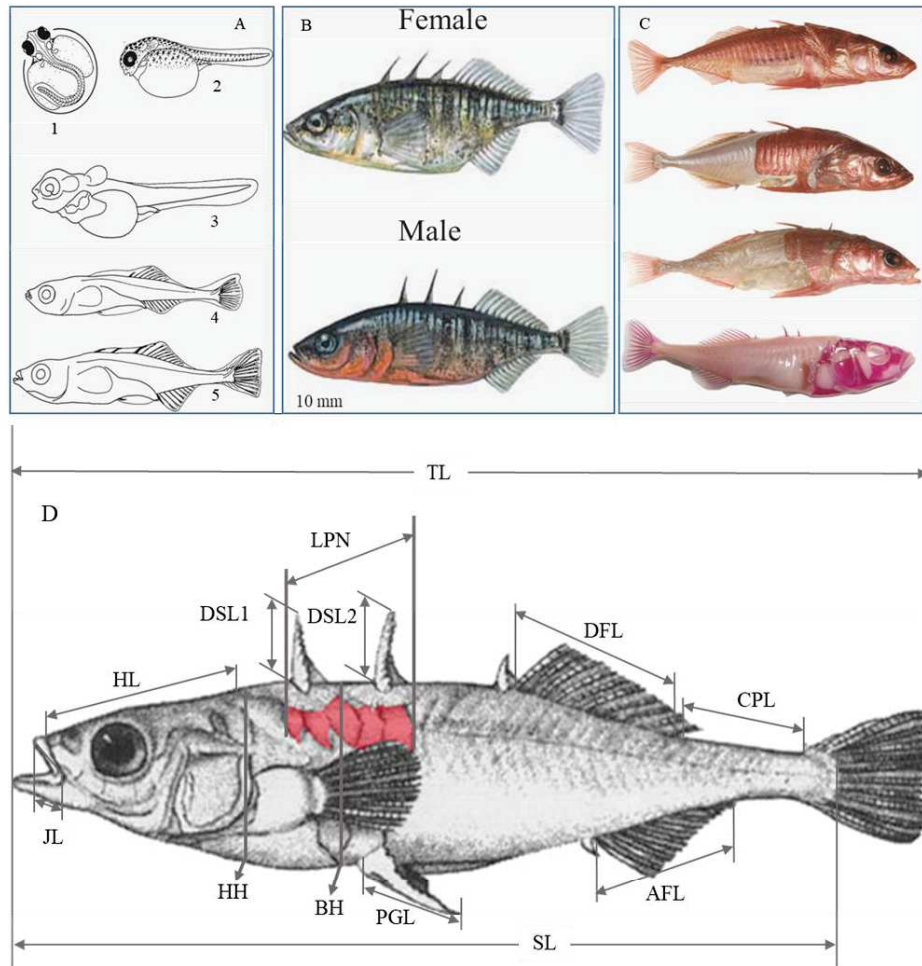


Figure 2-7. A) drawings of some of the early developmental stages: 1. Hatching; 2. Hatched embryo; 3. 1-2 days after hatching; 4. 16 days after hatching; 5. 22 days after hatching, young fish possesses all typical characters. From Swarup (1958), B) Female and male threespined stickleback, C) sticklebacks with different lateral plates (Barrett, 2010). D) Morphological features of threespined stickleback: Jaw length (JL), Head height (HH), Body height (BH), Pelvic girdle length (PGL), Anal fin length (AFL), Caudal peduncle length (CPL), Dorsal fin length (DFL), Second dorsal spine length (DFL2), First dorsal spine length (DFL1), Head length (HL), Standard length (SL), and Total length (TL).

Most recently, the whole genome database of the threespined stickleback has been determined and likelihood of research on the threespined stickleback is expected to be increased (Kingsley *et al.*, 2004).

2.3.1 The threespined stickleback in Europe

Genetic composition of fish fauna in Europe has been affected by Pleistocene climate fluctuations. After glacial retreats and formation of new habitats, species recolonized continental waters during warmer periods from southern refugial zones. Consequently, freshwater fish populations have diverged into many allopatric mitochondrial DNA (mtDNA) lineages (Englbrecht *et al.*, 2000). For instance, typical Scandinavian fish fauna have western, southern and eastern mtDNA lineages which indicate colonization through different refugial routes (Koskinen *et al.*, 2000). The last glaciation process also affected the genetic composition and distribution pattern of the European threespined stickleback. It is generally assumed that marine environment acted as a refugial zone during postglaciation, and potential freshwater habitats colonized by the migratory sticklebacks or by individuals (Münzing, 1963; Bell & Foster, 1994; McPhail, 1993). Also the marine ancestry of freshwater sticklebacks has been revealed by microsatellite and allozyme studies (Reusch *et al.*, 2001).

In Europe, along the distribution range of sticklebacks, from Bay of Biscay to Baltic Sea up to the Barren Sea and the Black sea, several populations share common characteristics with distinct life histories (Paepke, 2001). Coastal/mesohaline populations gather together at shallow coastal areas for spawning while others migrate to riverine waters for reproduction. Both populations co-exist along the marine distribution range. On the other hand, freshwater sticklebacks forming resident population in rivers and lakes, concentrate in Scandinavian coastal areas, the British Isles, Western Europe, and the black sea up to the Baltic drainages (Fig. 2.8). Interestingly, only the extant Mediterranean coastal populations exist as isolated populations from the rest of Europe (Paepke, 2001).

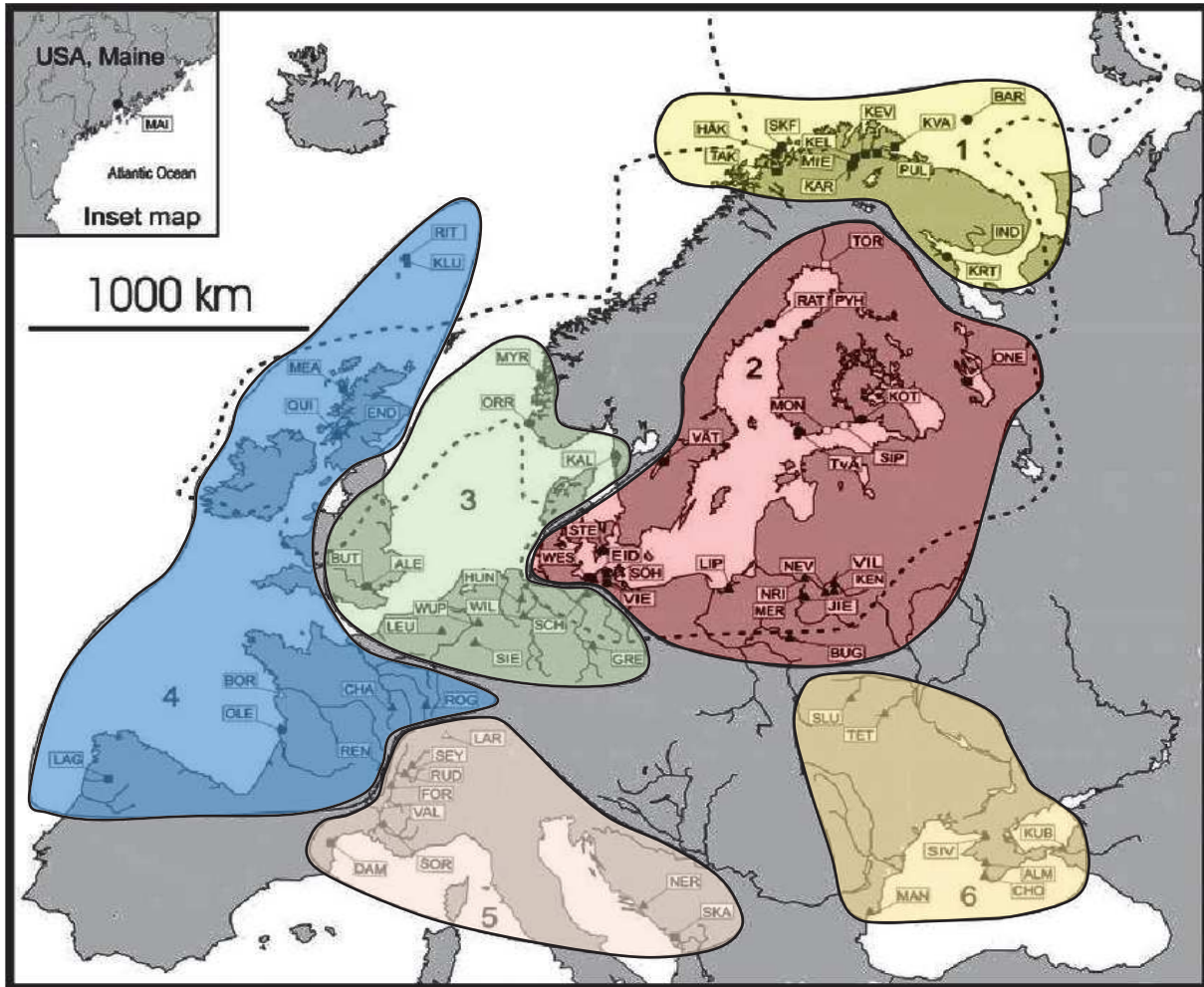


Figure 2-8. Phylogenetic relationship of European threespined Stickleback population with different clades. (1. Barents and White Seas, 2. Baltic Sea, 3. North Sea, 4. Atlantic Ocean, 5. Mediterranean Sea, and 6. Black Sea and their drainages) (Figure adapted from Mäkinen *et al.*, 2006).

The distribution of freshwater threespined stickleback populations within the Mediterranean region is thought to originate from distinct glacial relicts, but their expansion to northern Europe, in contrast, was not issued from this region. On the contrary, northern European freshwater stickleback populations are believed to originate from Atlantic marine populations through multiple recolonization events (Mäkinen *et al.*, 2006). Recently, Swiss researchers, did a comprehensive study on sticklebacks from Western Mediterranean islands, based on mitochondrial sequences (Lucek and Seehausen, 2015). They suggest that sticklebacks from Western Mediterranean islands are genetically distinct from the mainland populations with distinct

genetic lineages. Interestingly, southern French sticklebacks are closely linked with the stickleback population of the Mallorca cluster. Based on microsatellite data (Lucek and Seehausen, 2015), the Southern European threespined stickleback populations are clustered together in a distance-based tree. Even if they are in the same cluster, the Southern populations are genetically distinct even between geographically adjacent populations (Mäkinen *et al.*, 2008; Araguas *et al.*, 2012; DeFaveri *et al.*, 2011). This could be due to limited gene flow, a shrinkage in population size because of a loss of habitats and the introduction of invasive species (Clavero *et al.*, 2009; Araguas *et al.*, 2012).

2.3.2 Salinity tolerance in the Order Gasterosteiformes threespined Stickleback

Salinity is greatly contributing in defining habitat characteristics. In general, salinity in a particular type of habitat represents a key abiotic parameter that governs activity and distribution of fish species (Willmer *et al.*, 2009). In addition, salinity influences the biochemical processes at the cellular level. Climate change, habitat degradation, anthropogenic activities, frequency and severity of salinity changes can eventually exceed the coping ability of many fish species. Such gradual climate-induced salinity changes are further anticipated to increase, resulting in mortality of aquatic organisms in marine coastal ecosystems (Huang *et al.*, 2014).

Particular habitats with different environmental salinities are mostly inhabited by euryhaline fish having a wide range of salinity tolerance. They can alter their adaptive strategy from hyper-osmoregulation in freshwater and diluted seawater to hypo-osmoregulation in seawater and hypersaline environments.

Across the different fish orders, physiological key phenotypic traits of euryhalinity are distributed in a mosaic pattern (Nelson, 2006; Schultz and McCormick, 2012). It appears from a phylogenetic perspective, that the most primitive fish orders (15 orders) are entirely and exclusively marine fish with the exception of lampreys (Nelson, 2006). Twenty orders are moderately advanced and are freshwater inhabitants, except for the Albuliformes, Anguilliformes, Saccopharyngiformes and Clupeiformes. The remaining 27 orders are again mostly marine fish except for the Percopsiformes, Atheriniformes, Cyprinodontiformes, Synbranchiformes and Ceratodontiformes (Kültz, 2015). Therefore, most fish orders contain euryhaline species (Nelson, 2006; Schultz and

McCormick, 2012). Besides, at lower taxonomic levels, mosaic-like pattern of euryhalinity is also observed. For example, the Gasterosteiformes order in a single family (Gasterosteidae) have marine stenohaline, freshwater stenohaline and euryhaline species (Fig. 2.9). At the species level, the threespine stickleback present several derived characters and offers different physiological phenotypes of euryhalinity due to mosaic evolution (Gould, 1977).

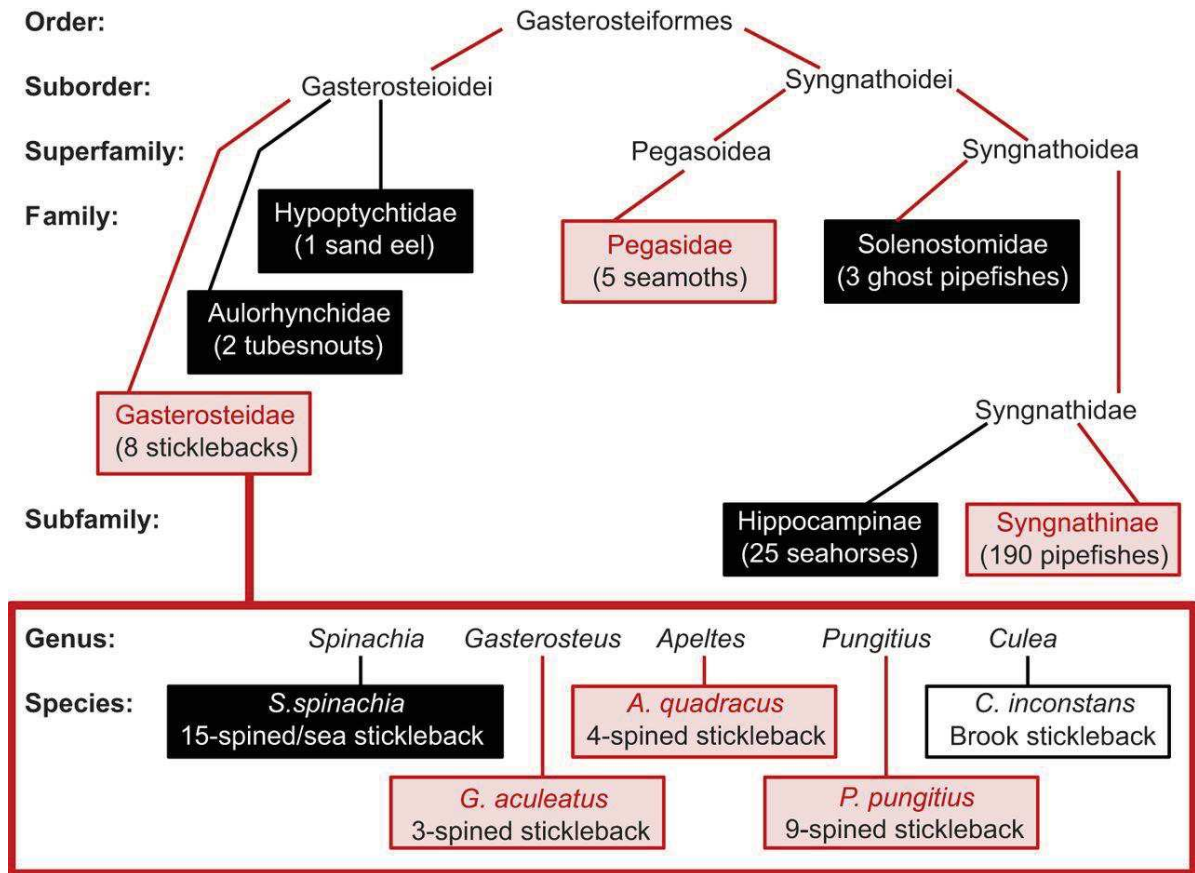


Figure 2-9. Salinity tolerance of Gasterosteiformes at different taxonomic levels, in box Box colours, stenohaline marine (black), stenohaline freshwater (white) or euryhaline (red) species (Kültz, 2015).

In local ecological conditions, different populations often reflect differences in behavior. In similar environmental conditions, populations should possess similar behavioral traits but they should predictably differ from those in other habitats (Foster, 1999; Foster & Endler, 1999). Such ecotypic variances are the product of evolutionary responses to differences in the environment (Endler, 1977, 1986; Foster, 1999; Lee & Bell, 1999; Robinson & Schlüter, 2000). Therefore, parallel

adaptations can offer remarkable insights into the routes that generate biodiversity (Endler, 1986; Foster *et al.*, 1998; Foster & Endler, 1999; Schlüter, 2000).

The threespined stickleback adaptive radiations offer one of the best opportunity to explore patterns of population diversification in response to different environmental conditions because this fish independently and repeatedly adapted to freshwater habitats (Wootton, 1976; Bell & Foster, 1994; McPhail, 1993). Variations in the benthic-limnetic ecotypes in sticklebacks that describe ecological axes of population are also well studied (Fig. 2.10).

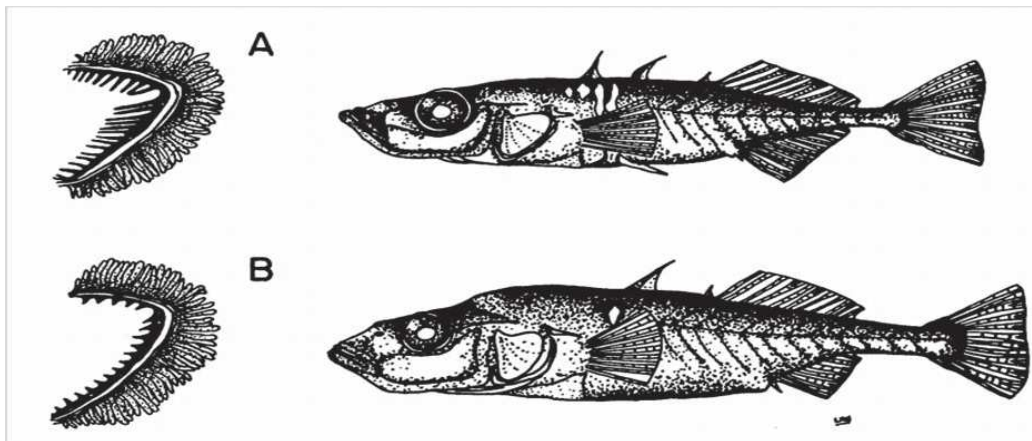


Figure 2-10. Morphological differences of gill rakers between two ecotypes based on A) Limnetic and B) Benthic axis (Schluter D, 1993).

Benthic sticklebacks have deep-bodied forms and live in shallow lakes. They are specialized for feeding on benthic invertebrates while the limnetic ecotype with slender forms is adapted for feeding on plankton (Lavin & McPhail, 1985, Walker, 1997). These benthic-limnetic variations are continuous between the two extremes. Also, trophic divergence is a common pattern of differentiation among populations within a single species and among closely related species (McPhail, 1993; Robinson and Wilson, 1994; Bell and Andrews, 1997). Such differences represent species boundaries (Foster *et al.*, 1998) and have strong implications in the speciation of numerous taxa of post-glacial freshwater fish such as Arctic char (*Salvelinus alpinus*; Malmquist *et al.*, 1992; Adams *et al.*, 1998; Jonsson & Skúlason, 2000; Jonsson & Jonsson, 2001; Guiguer *et al.*, 2002; McCarthy *et al.*, 2004), pumpkinseed sunfish (*Lepomis gibbosus*; Mittelbach *et al.*, 1992; Robinson & Wilson, 1996; Jastrebski & Robinson, 2004), and numerous other groups (Schluter & McPhail,

1992; Robinson & Wilson, 1994; Bernatchez *et al.*, 1996; Logan *et al.*, 2000; Grey, 2001; Ostbye *et al.*, 2006; McPhail 1993; Hindar 1994; Bernatchez *et al.*, 1996).

These local adaptation patterns allow the efficient use of the available food resources. As a result, in some groups, two or more specialized ecotypes can exist with distinct differences in body morphology and feeding behavior. Trophic polymorphism in freshwater fish are exceptionally common (Robinson & Wilson, 1994). The benthic ecotype feeding predominantly on macroinvertebrates are associated with shallow littoral habitats and limnetic ecotype, suspended in the limnetic zones of lakes, primarily feeding on zooplankton (Robinson & Wilson, 1994; Uchii *et al.*, 2007).

Numerous ecological significant traits influence efficiency of foraging behavior of fish in different habitats because these significant traits are strongly related to diet, habitat use and foraging behaviour (Schluter & McPhail, 1992; McPhail, 1993; Robinson & Wilson, 1994). Fish of the benthic ecotype have deeper heads and bodies, fewer gill rakers, more robust pharyngeal teeth and smaller eyes. In lakes, they live in the shallow zones (Scheffer, 1998). In such habitats, the deep body of the benthic ecotype provides mechanical advantages, and thus increases foraging efficiency (Werner, 1977; Ehlinger & Wilson, 1988; Walker, 1997; Svanbäck & Eklöv, 2003). The limnetic ecotypes present morphological significant traits that are well suited for feeding on planktonic preys. The numerous, fine and elongated gill rakers of the limnetic ecotype probably increase the feeding efficiency on small planktons (Magnuson & Heitz, 1971; Gibson, 1988; Gerking, 1994; Langeland & Nøst, 1995; Wright, O'Brien & Luecke, 1983; Drenner *et al.*, 1987; Gerking, 1994; Fig. 2.10).

2.3.3 Stickleback morphotypes

It is believed that phenotypic differences between biological species to evolve take thousands to millions generations (Futuyma, 1998). For instance, for the *Drosophila*, it takes 200,000 years for sympatric speciation completion. For allopatric speciation, it takes about 2.7 million years (Coyne and Orr, 1997). Rapid parallel evolution is also a common process (Thompson, 1998; Hendry & Kinnison, 1999) that support rapid speciation (Howard & Berlocher, 1998; Seehausen & van Alphen, 1999). Phenotypic adaptation through natural selection is now considered as a cornerstone

of evolutionary theory (Thompson, 1917) but our understanding concerning the ecological mechanisms driving adaptive evolutionary changes is still remarkably poor (Schluter, 2001; MacColl, 2011). The genetic and phenotypic divergence and the limits imposed by gene flow to stabilize selection or geographic variations are considered as key question in evolutionary biology (Schluter, 2001; Coyne and Orr, 2004; Benkman and Parchman, 2013).

The threespined stickleback post-glaciation populations exhibit morphological differences that are comparable to what is observed at the species level or even within families (Bell, 1976, 1988; Bell & Foster, 1994; Nelson, 1993). In the threespine stickleback, the phenotypic transition occurs frequently and rapidly in some groups and, infrequently and slowly among others (Bell *et al.*, 1993; Reimchen, 1994). In post-glacial freshwater habitats, phenotypic transition occurred within 2,500-11,000 generations (Bell *et al.*, 1993; McPhail, 1993). Among the different populations of the threespine stickleback, the evolution of major phenotypic differences offers noticeable opportunities to study processes and patterns of evolution at the species level (Bell, 2001).

Table 2-2. Number of lateral plates in threespined stickleback across its distribution range.

Country	Area	Habitat/Salinity	Average lateral plate no.	References
Norway	Lake Glitredammen	FW	11.8	Hansson <i>et al.</i> , 2016
		SW	11.5	
Belgium	Oudenburg+Doel	SW	18	Raeymaekers, 2004
	Hevelee & Herzele	FW	<5	
	Yerseke+Lozen	BW	11	
Canada	Fairy Lake	FW	9.83	Bergstorm, 2002
	Tlell Pond	FW	12.18	
Alaska	Matanuska-Sustina Borough	Wild Sticklebacks	33.2	Cresko <i>et al.</i> , 2004
	Bear Paw lake	FW	3.9	
	Boot lake	FW	5.2	
	Whale lake	FW	2.3	
France	Camargue	Mesohaline	5 ♂ 4 ♀	Crivelli and Brritton, 1987

Across its distribution range, threespine stickleback displays morphological and physiological tractable differences and it is the vertebrate model to understand phenotypic adaptations in relation

to local environmental conditions (Wootton 1984, 2009; Bell *et al.*, 1993; Peichel *et al.*, 2001; Schluter *et al.*, 2010). It is a small teleost fish distributed across northern hemisphere).

They are either marine, anadromous, or freshwater populations (Reimchen, 1994; Wootton, 2009). Instead of having typical scales like other fish, sticklebacks lack scales, but possess bony lateral plates and dorsal spines (Wootton, 1976). On the basis of these lateral plates, three morphs have been described type: (1) the *trachurus* or complete morphotype with complete rows of lateral plates; (2) *semiarmata* or partially plated morphotype; (3) *leiurus* or low-plated morphotype (Hagen and Gilbertson, 1973; Wootton, 1976). In 1983, a fourth morph of stickleback proposed by Ziuganov that is lieura-with-keel (low morph with keel). This fourth morph has already been identified in various European stickleback population (Banbura & Bakker, 1995; Fig. 2.11). These three morphotypes can be clearly and easily defined. The majority of the freshwater populations belong to low-plated morphotype, whereas, the marine and anadromous populations tend to display complete or partial morphs (Moodie and Reimchen, 1976; Bell *et al.*, 1993). These morphological differences in terms of species evolutionary history have also been considered (Bell, 2009; Wootton, 2009; Schluter *et al.*, 2010).

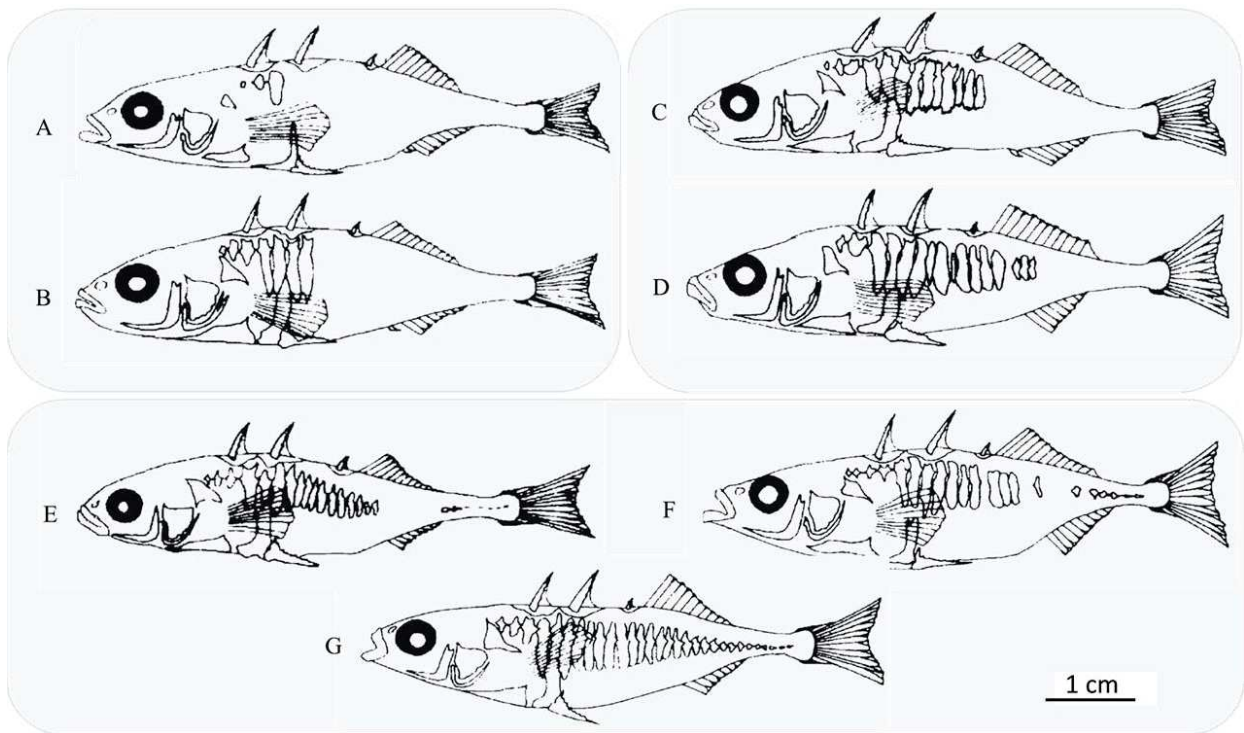


Figure 2-11. Morphotypes of threespined stickleback, lateral plate phenotypes adapted from Bell, (2001) shown include: low (A), (B); intermediate-low (C), (D); partial (E); intermediate-partial (F); and complete (G). (Bell, 2001).

The marine form is phenotypically and genetically uniform and ancestral while the freshwater form derive from anadromous by repeatedly invasion of freshwater habitats (Bell 1976; Withler and McPhail 1985). Reduction in the plate and spine number predominantly reflects genetic changes after colonization of freshwater habitats (Münzing, 1959; Lindsey, 1962; Hagen and Gilbertson, 1973). The genetic basis of armor reduction is well described, and evidences suggest that the same allele (Eda in case of lateral plates) over a wide geographic range, triggers independent evolution of lateral plate reduction (Colosimo *et al.*, 2004, 2005; Cresko *et al.*, 2004; Shapiro *et al.*, 2004; Coyle *et al.*, 2007; Chan *et al.*, 2010). Several hypotheses have already been proposed; among them two consider the nature and intensity of predation (Hagen and Gilbertson, 1973; Reimchen, 1980; Bell *et al.*, 1993) and the bioavailability of dissolved calcium (Spence *et al.*, 2012).

Bony plates, pelvic and dorsal spines, function as defensive elements against predators (Hoogland *et al.* 1957; Reimchen, 1983, 1994). When attacked, sticklebacks with robust plates and spines are more likely to survive (Bañbura *et al.*, 1989; Reimchen, 1992, 2000). In the absence of predatory pressure, however, a tradeoff between swimming ability and number of lateral plates results in the reduction of body armor pressure (Taylor and McPhail, 1986; Reimchen, 2000; Barrett *et al.*, 2008). Most frequently encountered variable is the lateral plate reduction among populations followed by spine length reduction, while, complete loss of lateral reduction is rare (Klepaker and Østbye 2008). Pelvic structures and spines in the absence of predatory fish, make sticklebacks more at risk to aquatic insect predators, for example dragonfly nymphs. (Reimchen, 1980).

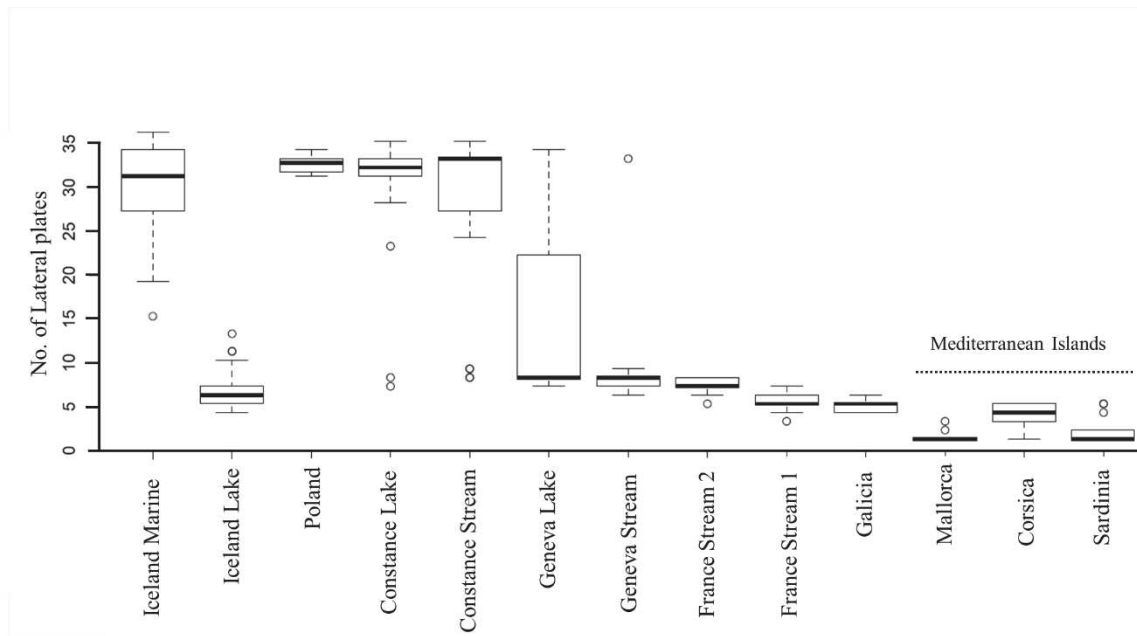


Figure 2-12. Number of lateral plates in European's stickleback populations, also from Mediterranean islands (Lucek and Seehausen, 2015).

2.3.4 Plasticity and colonization of new environments

Fish species that reside in distinct habitats and their differentiated traits that might confer adaptive value in their native environment. After last glaciation, the initial colonists had to tolerate the alterations imposed by the new environment, either through tolerance due to genetic variation or by adaptive plasticity. Though significant amount of research focused on genetic differences linked with divergent natural selection and local ecological adaptations, phenotypic plasticity may have also played key role in colonization and successive adaptations to new habitats. Yet, the role of adaptive plasticity remains unclear (Mery & Kawecki, 2004; Ghalambor *et al.*, 2007, 2015). In non-native environment, plasticity facilitates natural selection and adaptive evolution (Hinton & Nowlan, 1987; Ghalambor *et al.*, 2007), but also adaptive plasticity slows the adaptive evolution by weakening the intensity of directional selection (Wright, 1931; Anderson, 1995; Ancel, 2000). Phenotypic plasticity plays a vital role in colonization of novel environment, in addition to this, also influencing how evolution proceeds after the initial colonization (Ghalambor *et al.*, 2015; Schneider & Meyer, 2017). Additionally, it can be subjected to evolutionary processed after

colonization, resulting in a diversity of procedures and patterns (Pigliucci *et al.*, 2006; Crispo, 2007).

2.3.5 Salinity as a barrier to colonization

Freshwater and seawater fish differ greatly with different major ion concentrations. Therefore, when a fish is moving in a water body with a different environmental salinity, a change in the osmotic gradient of the body fluids and intracellular spaces can occur (Hill *et al.*, 2008; Bradley, 2009). Teleost fish maintain a relatively stable body fluid osmolarity, and face a large osmotic gradient between their blood and the external environment (Hill *et al.*, 2008; Bradley, 2009; Fig. 2.13). For instance, teleosts in diluted seawater and freshwater (hypo-osmotic environments) maintain an internal osmolality of approximately 250-350 mOsm.kg⁻¹. They gain water by osmosis and lose ions by diffusion (Evans *et al.*, 2005; Hill *et al.*, 2008; Hwang *et al.*, 2011). In hyper-osmotic environment, teleosts maintain their blood osmolality at approximately 300-500 mOsm.kg⁻¹. They tend to lose water by osmosis and gain ions by diffusion (Evans *et al.* 2005; Hill *et al.*, 2008; Evans, 2011a, b). Plasma solute concentration is normally raised in euryhaline fish transferred from a hypo-osmotic environment to hyper-osmotic salinities, but it may decrease near levels found in similar species in freshwater over time (Allen *et al.*, 2014). Typically, abrupt transfer to SW results in an immediate increase in plasma osmolality, sodium, and chloride concentrations as well as cortisol levels in some euryhaline teleosts (Mackie *et al.*, 2005; Allen *et al.*, 2014). Therefore, blood osmolality is maintained almost constant or is only slightly variable. It corresponds to approximately 1/3 that of SW, irrespective of whether the fish are in FW or SW (Evans *et al.*, 2005).

A set of physiological and morphological changes in osmoregulatory organs enables teleost fish to maintain this internal homeostasis at different environmental salinities (Marshall and Grosell, 2006; Allen *et al.*, 2009). Gill epithelium, kidney tubules, and the intestinal epithelium are the main osmoregulatory organs responsible for ion uptake/ion excretion (Varsamos *et al.*, 2005; Evans and Claiborne, 2009).

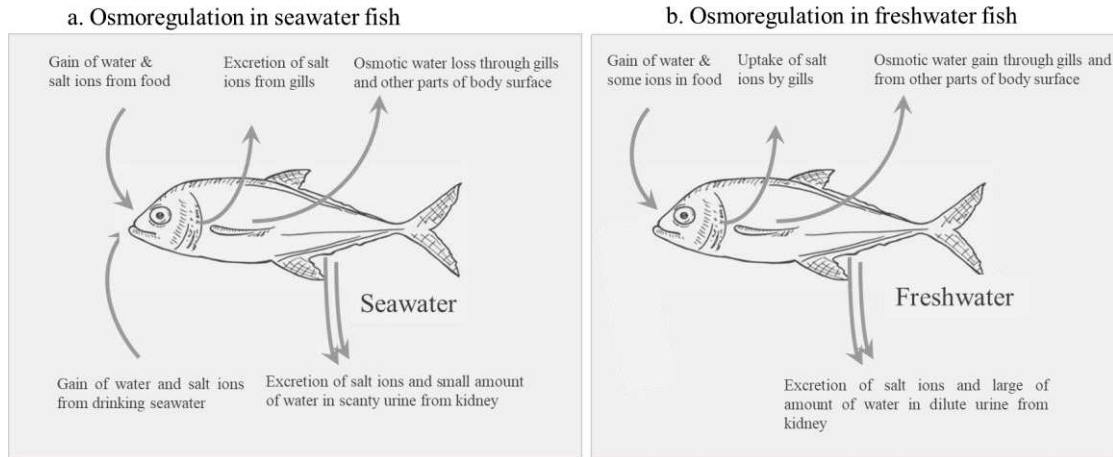


Figure 2-13. Process of osmoregulation; a. in seawater and, b. in freshwater fish.

In freshwater / hypo-osmotic environments, fish produce diluted urine to offset diffusive water gain (Hill *et al.*, 2008; Hwang, 2011), and actively transport ions back into blood plasma (Hwang *et al.*, 2011; Dymowska *et al.*, 2012). As osmoregulation is costly, this process requires energy to actively pump chloride ions (Cl^-), sodium ions (Na^+) and calcium (Ca^{2+}) from the freshwater external environment and back into the blood and into the external environment (Evans & Claiborne, 2006; Hill *et al.*, 2008; Hwang 2011; Hwang *et al.*, 2011; Dymowska *et al.*, 2012).

In seawater / hyper-osmotic environments, fish are unable to produce urine and energy is required to actively secrete ions back to their environment (Evans *et al.*, 2005; Hill *et al.*, 2008; Evans 2011a, b). Cl^- ions are secreted from the blood into the outer environment, while the flow of Na^+ is carried out via paracellular pathway with passive diffusion down the concentration gradients established by the gill epithelium (Evans & Claiborne 2006).

2.4 Ion transporters

The currently accepted model for active NaCl secretion by ionocytes in seawater-acclimated teleosts consists primarily of the cooperative action of 3 major ion-transport proteins: Na^+/K^+ -ATPase (NKA), $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransporter (NKCC), and Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall, 2011). In the model, a basolaterally-

located NKA transports 3 Na⁺ outward in exchange for 2 K⁺, creating low intracellular Na⁺ and a highly negative charge within the cell; the Na⁺ gradient is used to transport Na⁺, K⁺ and 2 Cl⁻ into the cell through a basolateral NKCC1; Cl⁻ then leaves the cells down an electrical gradient through an apical CFTR; Na⁺ is transported back outside the cells via NKA, and then leaves through a paracellular pathway between ionocytes and adjacent smaller cells known as accessory cells (Hiroi and McCormick, 2012).

2.4.1 Na⁺/K⁺-ATPase (NKA)

The sodium pump Na⁺/K⁺-ATPase (NKA), regulates cellular homeostasis and is present in all animal cells (Skou and Esmann, 1992). It is a membrane-spanning enzyme responsible for the active transport of Na⁺ out of and K⁺ into animal cells. NKA is crucial not only for sustaining intracellular homeostasis but also it provides the driving force for many transporting systems including fish gills (Hwang and Lee, 2007). This enzyme consists of three subunits; α , β and γ (Blanco and Mercer, 1998). The α -subunit is responsible for the catalytic and ion regulatory capacity of the enzyme and the β -subunit is associated with protein maturation and anchoring of the enzyme complex in the membranes (Blanco and Mercer, 1998). The γ -subunit has not been reported in teleosts, however, in mammals it appears to modulate affinity of the NKA enzyme for Na⁺, K⁺ and ATP (Therien and Blostein, 2000; Hirose *et al.*, 2003). In teleosts gills, NKA is composed of a catalytic α (α 1-4 isoforms) and an accompanying glycosylated β (β 1-3 isoforms) subunits (Hwang and Lee, 2007). It is expected that euryhaline and anadromous fish have the capacity to adapt to different environmental salinities by modulating NKA function and switching different isoforms of NKA α -subunit (Richards *et al.*, 2003; Mackie *et al.*, 2005; Bystriansky *et al.*, 2006; Nilsen *et al.*, 2007; Li *et al.*, 2014).

Two different isoforms of NKA α -subunit (α -1a and α -1b) has been found in some teleost gills. Their functions are ion uptake in FW and ion secretion in SW fish, respectively. They are differently expressed with salinity changes (Richards *et al.*, 2003; Nilsen *et al.*, 2007). The relationship between ion pumping capacity and activity is likely complex, as tissue NKA activity is a sum of the activity of several isoforms, which are known to vary with salinity (Richards *et al.*, 2003), species (Bystriansky *et al.*, 2006) and tissue type (Cutler *et al.*, 2000; Allen, 2009).

Recently, comprehensive research has been conducted on molecular characterization and expression of NKA isoform ($\alpha 1$) in European seabass with short terms and long term salinity exposure by Blondeau-Bidet and her colleagues (Blondeau-Bidet *et al.*, 2016), interestingly, NKA $\alpha 1a$ displayed close homology (95%) with threespined stickleback. The mRNA expression of NKA $\alpha 1b$ higher in seawater than in freshwater of European seabass by 4.4fold (Blondeau-Bidet *et al.*, 2016),

Most euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Marshall, 2002; Evans *et al.*, 2005). Increases in NKA activity upon salinity challenge are attributed to the precedence or accompaniment of increased NKA α -subunit mRNA abundance, protein amounts, or both and modulation of the hydrolytic rate of this enzyme. A salinity challenge necessitates activation of gill NKA as well as Ionocyte remodeling which is crucial for a fish acclimation.

2.4.2 $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC)

NKCC is key co-transporter to maintain cell volume homeostasis and is widely distributed among many different species including fish (Lytle *et al.*, 1995; Cutler and Cramb, 2002; Lorin-Nebel *et al.*, 2006). NKCC1, a secretory isoform, and NKCC2, the absorptive isoform, have already been identified, located at the basolateral and apical sides of epithelial transporting cells, respectively (Marshall, 2002; Hwang and Lee, 2007). NKCC1 is the most widely distributed isoform and is expressed in a wide range of tissue types whereas NKCC2 appears to be expressed in the kidney (Lytle *et al.*, 1995). Because of prominent expression of NKCC1 in the basolateral membrane-bound protein within the ionocytes of SW-acclimated fish and its association with the operation of chloride secreting epithelia, it is considered to be the secretory isoform in SW fish gills. The NKCC2 may be able to contribute to sodium uptake in some dilute media (Marshall and Grossel, 2006). In mammals, NKCC1 and NKCC2 are each encoded by a single gene. In the European eel, however, NKCC1 is known to be expressed as two distinct genes, with NKCC1a found in most tissues and NKCC1b primarily expressed in the brain (Cutler and Cramb, 2001, 2002; Mackie *et al.*, 2005).

2.4.3 Cystic fibrosis transmembrane conductance regulator (CFTR)

CFTR is probably involved in bicarbonate fluxes and acid-base balance (Marshall and Singer, 2002), but its main function is in osmotic regulation, particularly chloride regulation (Marshall *et al.*, 1999). Cl^- secretion by the apical membrane of chloride cells uses an anion channel with characteristics resembling those of the CFTR that is highly expressed in gill ionocytes (Marshall *et al.*, 1995). However, CFTR may be differently located according to the species: for example in *Fundulus heteroclitus*, ionocytes express a diffuse CFTR in FW (Marshall *et al.*, 2002), but in other species such as *Oreochromis mossambicus*, CFTR is not expressed in these cells in FW (Hiroi *et al.*, 2005); in *Morone saxatilis* there is no change in the localization and expression of the CFTR according to salinity (Madsen *et al.*, 2007). CFTR is present in the apical region of the ionocytes in SW-acclimated killifish (*Fundulus heteroclitus*) but also in pavement cells of freshwater killifish (Marshall *et al.*, 2002; Mc Cormick *et al.*, 2003). Two different isoforms of CFTR were found in the gills of salmon, which are independently expressed (Chen *et al.*, 2001).

2.5 Major osmoregulatory organs in fish

2.5.1 The gills, main site of fish osmoregulation.

Gill epithelium is characterized by the presence of four major cell types: (1) pavement cells (PVCs), (2) ionocytes (chloride cells, mitochondria-rich cells or MRCs), (3) mucous cells and (4) accessory cells (Laurent *et al.*, 1985; Tang *et al.*, 2011). More than 90% of the gill surface epithelium and usually all lamellar surfaces are characterized by PVCs (Laurent, 1984; Wilson and Laurent, 2002). Although these cells, especially on the lamellae, were thought to be the site of trans-epithelial gas transfer, some evidences suggested they might have the roles in ion and acid–base regulation (Evans *et al.*, 2005). The ion exchanges are affected in specialized cells, designated MRCs in the gill epithelium, or more generally ion-transporting cells or ionocytes (Fig. 2.14). These cells also named chloride cells due to their role in chloride excretion in hyperosmotic environments or ion absorption hypo-osmotic environments (Smith, 1930). In teleosts, the localisation and size of chloride cells can vary considerably between species (Evans *et al.*, 2005; Marshall and Grosell, 2006; Allen *et al.*, 2009). In many species, the number of ionocytes in the

gills, their shape and expression level of ion transport proteins involved in salt secretion can be adjusted (McCormick *et al.*, 2009).

In SW, the apical membrane is cup shaped, and the main chloride cell is juxtaposed and interwoven with an accessory cell containing fewer mitochondria (Marshall and Grosell, 2006). Chloride cells are also associated to PVCs, and to accessory cells delineating junctions that can be tight or leaky according to the salinity conditions (Zadunaisky, 1984; Wilson and Laurent, 2002).

The apical surface of the chloride cell is highly plastic, typically with an apical crypt in seawater and changes with salinity from “deep-hole” openings in SW to “shallow-hole” openings in ion-rich FW; in ion-poor FW, the apical surface usually is thrown into microvilli, likely to increase surface area for transport (Marshall and Grosell, 2006).

For SW adapted fish, chloride cells are large cells, abundantly located primarily on gill filaments (Pisam *et al.*, 1988; Uchida *et al.*, 1996). Whereas, for FW adapted fish, chloride cells are often smaller, located on both the filament and lamellae (Hwang and Lee 2007; Laurent *et al.* 2006), and decrease in abundance on the lamellae in (Pisam *et al.*, 1988; Uchida *et al.*, 1996; Allen, 2009). For fish that travel between FW and SW, the appropriate chloride cell function and morphology must be coordinated with the salinity of the environment. Anadromous fish enter SW at a particular life history stage, and the timing of entry is often predictable. Changes in the osmoregulatory mechanisms of anadromous fish are necessarily linked to developmental and environmental cues at the time of juvenile migration (Zydlewski and McCormick, 2001).

Thus, a suite of morphological and functional changes occurs in the osmoregulatory structures enabling constant ion and osmotic homeostasis as fish move from hypo- to hyper-osmotic salinities or reversely (Marshall and Grosell, 2006; He *et al.*, 2009; McCormick *et al.*, 2009).

Ion movements are mediated by different enzymes, primarily Na^+ , K^+ -ATPase (NKA), and ion transporters and ion channels, corresponding to transmembrane proteins located in the basolateral or apical membranes of the chloride cells (Lin and Randall, 1995; Marshall, 2002; Marshall and Singer, 2002; Hirose *et al.*, 2003).

The gills are considered as the major organ responsible for balancing ion movement because of the numerous ionocytes located along the gill filaments and lamellae and their enzymatic equipment, primarily the NKA (Evans *et al.*, 2005; Hwang and Lee, 2007). Hence, the key transporters associated with the NaCl transport process are thought to be NKA, the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$

co-transporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Evans *et al.*, 2005; Varsamos *et al.*, 2005; Hwang and Lee, 2007).

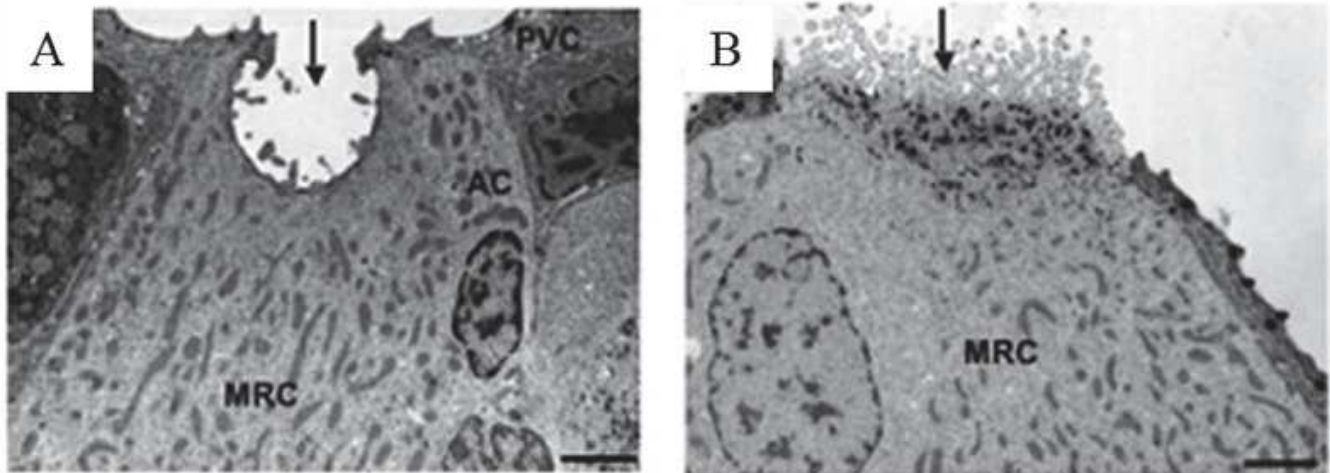


Figure 2-14. Transmission electron micrographs of gill filaments from killifish in SW (A) and killifish transferred from SW to FW after 30 days (B). Note transformation of the apical region of MRCs (arrows) from a smooth, concave crypt that is recessed below the pavement cells (PVCs) (A) to a convex surface studded with microvilli that extend above the surrounding PVCs (B). Also note that an accessory cell (AC) is not associated with the MRC from FW-acclimated killifish (B) and that the distinct, whorl-like microridges on the surfaces of PVCs do not change with salinity (A). (Kato and Kaneko, 2003).

2.5.1.1 SW-acclimated fish, NaCl transport

NaCl secretion by teleost gills is accomplished by secondary active transport of chloride and passive transport of Na^+ across the basolateral cell membrane via NKCC1. Thus, Cl^- enters via the NKCC1 driven by the Na^+ gradient. The driving force for the active transport is NKA, which maintains intracellular Na^+ at low levels and intracellular K^+ at high levels. Cl^- accumulates above its electrochemical equilibrium intracellularly. Chloride channels in the apical membrane allow Cl^- to be secreted followed by passive exit of Na^+ . The Na^+ is secreted passively down its electrochemical gradient through the lateral intercellular spaces and leaky junctions between chloride cells and accessory cells (Evans *et al.*, 2005; Whittamore, 2012). Since both NKA and NKCC carry K^+ into the cell, it is thermodynamically necessary to recycle K^+ out via conductive pathways.

2.5.1.2 FW-acclimated fish, NaCl transport

Smith (1930) and Krogh (1937) using early isotopic experiments proposed that in fish gills, the secretion of acids (H^+ and/or NH_4^+) is linked to the trans-epithelial absorption of Na^+ while the secretion of bases (HCO_3^- and/or OH^-) is independently linked to the trans-epithelial absorption of Cl^- . Since then, these Na^+/Cl^- linked acid/base transport mechanisms have been examined, and the results are still being debated among different species and various external water conditions to the present (Evans *et al.*, 2005; Hwang and Lee, 2007). As it was mentioned above, NKCC2 probably contributes to Na^+ uptake in some dilute media. To take up Na^+ by this means requires a source of extracellular K^+ and sufficient NaCl in the boundary layer to allow Na^+ uptake down its concentration gradient. Hiroi *et al.*, (2005) reported NKCC2 immunofluorescence in the apical region of the chloride cells of tilapia gill membranes when the animals were adapted to FW (Fig. 2.15B). So far, at least two models have been proposed for the apical transport of Na^+ in fish gill cells: (1) an apical V-type of H^+ -ATPase (VHA) electrically linked with Na^+ absorption via the epithelial Na^+ channel (ENaC) and (2) an electroneutral exchange of Na^+ and H^+ via an apical Na^+/H^+ exchanger (NHE) (Hwang and Lee, 2007). Additional link between H^+ extrusion and Cl^- uptake has been proposed. Extrusion of H^+ across the apical membrane via H^+ -ATPase appears to fuel Cl^- uptake by allowing for cytosolic accumulation of HCO_3^- from CO_2 hydration. This occurs because inhibition of H^+ -ATPase activity results in reduced Cl^- uptake in some fish species, at least at low Cl^- concentrations of medium. Such removal of external HCO_3^- would aid HCO_3^- extrusion and would thus favor Cl^- uptake via Cl^-/HCO_3^- exchange if afforded anion channels in the basolateral membrane to exit into the interstitial fluid (Marshall and Grosell, 2006). As well as the linkage between Cl^- absorption with the secretion of HCO_3^- via apical anion exchangers, there are two other possible candidates for the apical Cl^- uptake in fish gill cells are NKCC and Thiazide-sensitive Na^+/Cl^- co-transporter (NCC). Thus, NKA found in chloride cells serves as the driving force for ion absorption at the expense of ATP in FW-acclimated fish, in order to facilitate of ionic and osmotic gradients necessary for ion uptake and general homeostasis in related tissues (Marshall, 2002; Sardella and Kultz, 2009). The possibility of the involvement of the electrogenic Na^+/HCO_3^- cotransporter in the basolateral Na^+ exit of gill chloride cells have been recently raised (Evans *et al.*, 2005).

There are 2 candidate channels for the Cl^- exit: (Evans, 2010), 1) CFTR-like channel of the FW-acclimated fish gill, which is expressed in the basolateral membranes. However, it is expressed in the apical membrane for SW-acclimated fish. On the other hand, another isoform of CFTR, a

candidate for basolateral Cl^- exit based on its basolateral localization in pavement cells (Marshall, 2002; Marshall and Grosell, 2006).

2) Chloride channel (ClC) in fish. For instance, in tilapia, ClC-3 and -5 were cloned and found to be expressed in various organs including gills, but they were suggested to function as intracellular Cl^- channels based on an in vitro functional analysis (Miyazaki *et al.*, 2002).

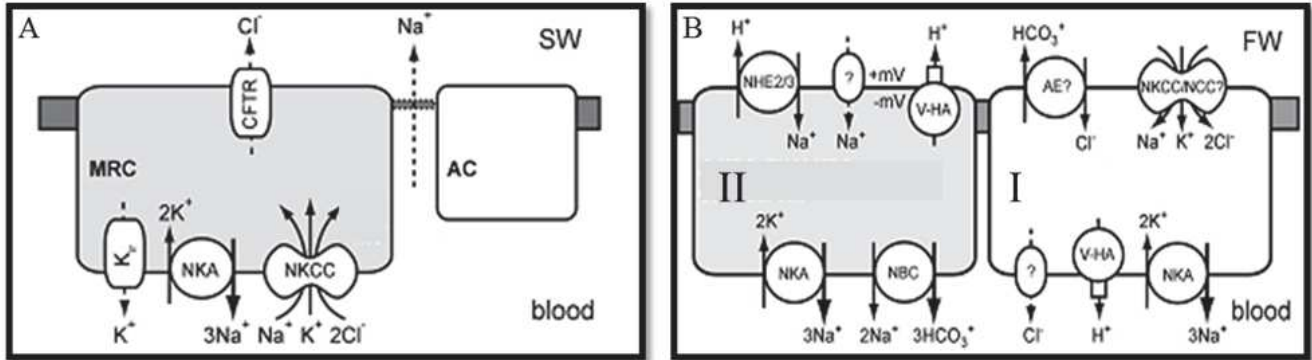


Figure 2-15. Mechanism of NaCl secretion (A) and uptake (B) in the gill of teleosts (Evans, 2008).

2.5.2 Kidney

The nephrons, the functional unit of the vertebrate kidney, are packed together and act for renal osmoregulation. A nephron consists of a renal corpuscle (a Bowman's capsule and a glomerulus), a proximal tubule (PT), and a distal tubule (DT), followed by a collecting duct (CD). Based on the presence of the glomeruli in the nephron, teleost kidney can be broadly divided into three categories: 1) numerous glomeruli (FW and euryhaline species); 2) glomeruli are present but greatly reduced (euryhaline and marine species); 3) glomeruli are completely absent (aglomerular kidneys) (most marine species) (Marshall and Grosell, 2006). The number and size of glomeruli as well as the differentiation of functional segments of teleost nephrons are reduced compared to the mammalian type, and vary according to the evolutionary origin and habitat. Glomerular filtration rate (GFR) varies between species (Beyenbach, 2004; Engelund and Madsen, 2011). GFR is typically 7–10 folds higher in FW than in SW, reflecting the contrasting demands in the two habitats (McDonald *et al.*, 2006). This illustrates that fish kidneys may undergo a major switch from being filtratory in FW to being predominantly secretory in SW (Engelund and Madsen, 2011). The secreted tubular fluid is approximately iso-osmotic to the blood and similar in

composition, dominated by Na^+ and Cl^- , with much smaller concentrations of Mg^{2+} and SO_4^{2-} . Urine is primarily produced by secretion of fluid into the proximal tubule, followed by reabsorption of ions and water in the distal tubule (if present) and bladder (Marshall and Grosell, 2006). In the late proximal tubule and bladder, where the urine is collected and modified, water is reabsorbed following secondary active transport of Na^+ and Cl^- , which passively concentrate Mg^{2+} and SO_4^{2-} , thus, helping reduced renal water losses. The final urine is similar to the rectal fluid in composition, with low concentrations of monovalent ions (Na^+ and Cl^-), but rich in Mg^{2+} and SO_4^{2-} (140 and 80 mM, respectively). It is typically iso-osmotic to slightly hypo-osmotic to the blood plasma. Although the end result of tubular secretion is the net excretion of Mg^{2+} and SO_4^{2-} , the actual rate of divalent ion excretion by the renal system is low compared with the intestine (Marshall and Grosell 2006; Wittamore, 2012).

At the cellular level, the osmoregulatory function of the kidney is based on the presence of NKA immunopositive cells (ionocytes or mitochondria-rich cells) in the different tubules containing the ion transporting enzyme NKA on the basolateral membranes. Associated to different ion and water channels, NKA is the key component in renal solute transport and water homeostasis (Eid and Brandli, 2001; McDonald, 2006; McDonald *et al.*, 2002). The abundance of NKA immune positive cells in the different tubules affects osmoregulatory capacity, which controls the ability of fish to tolerate salinity and its fluctuations (Nebel *et al.*, 2005). The function of the proximal segment(s) of the kidney tubule is somewhat controversial. One primary function is secretion of Mg^{2+} and SO_4^{2-} especially in marine and SW-acclimated species. In FW-kidneys it could play an additional role in excretion of excess water (Beyenbach, 2004).

In some species, there is evidence that the proximal segments of both FW and SW fish are responsible for absorption of Na^+ and Cl^- as well as glucose and other key osmolytes (Beyenbach, 2004). This mechanism is built using the basolateral NKA (Katoh *et al.*, 2008). The apical Na^+ entry pathway into proximal cells is yet unclear but may involve Na^+/H^+ -exchange (Braun and Dantzer, 1997; Ivanis *et al.*, 2008) and Na^+ -glucose cotransporters (SGLT1) (Fig. 2.16B). Like in mammals, the absorptive-type NKCC is absent in fish in this segment (Katoh *et al.*, 2008). A basolateral secretory-type NKCC1 has been observed at least in the killifish (*Fundulus heteroclitus*) proximal segments (Katoh *et al.*, 2008), suggesting a pathway for NaCl secretion which may occur in conjunction with Mg^{2+} secretion (Beyenbach, 2004).

Secreted NaCl is expected to be reabsorbed in the distal and collecting segments. In SW fish, the kidney recycles salt to compensate NaCl secretion from the gills, this process occurs in the proximal I and proximal II segments and in different teleosts (Engelund and Madsen, 2011).

Distal tubules and collecting ducts together make up a variable percentage of the whole nephrons in different fish species (Katoh *et al.*, 2008) and the primary activity here is reabsorption of NaCl in both FW and SW. This is favored by extensive expression of basolateral NKA, apical NKCC2 and NCC cotransporter, and basolateral kidney-specific Cl⁻ channels (Miyazaki *et al.*, 2002; Katoh *et al.*, 2008; Kato *et al.*, 2011; Fig. 2.16C).

Accordingly, the distal segment is absent in the majority of truly marine species. In FW fish, it has been proposed that the distal segments have low water permeability in order to minimize water reabsorption and to promote the formation of hypotonic urine (McDonald *et al.*, 2006). Upon acclimation to SW, the fractional reabsorption of water increases along the nephron by increasing tubular water permeability, as seen in mammalian collecting tubules. Reabsorption of NaCl may promote the osmotic removal of water, thereby creating isotonic urine primarily consisting of MgSO₄ and other unwanted osmolytes (Beyenbach, 2004; Engelund and Madsen, 2011).

2.5.2.1 Ion secretion in SW-acclimated fish

Although Mg²⁺ and SO₄²⁻ concentrations in tubular secretions are elevated far above plasma concentrations and, thus, may contribute to fluid secretion, it appears that the majority of proximal tubule fluid secretion is driven by Na⁺ and Cl⁻ transport in the early proximal tubule. Tubular secretion of Na⁺ and Cl⁻ occurs as secondary active transcellular Cl⁻ transport with electrically coupled paracellular Na⁺ transport. Electrochemical gradients for K⁺ and Na⁺ allow Cl⁻ entry across the basolateral membrane presumably via NKCC and results in cytosolic Cl⁻ concentrations above equilibrium. Apical Cl⁻ secretion is presumably via anion channels (Fig. 2.16A).

Passive entry of Mg²⁺ across the basolateral membrane is thought to occur via Mg²⁺ channels, whereas the active exit of Mg²⁺ across the apical membrane occurs either via Mg²⁺:Na⁺ exchange ultimately driven by NKA or via Mg²⁺/H⁺ exchange driven by H⁺ pumps present in the apical membrane (Fig. 2.16A).

The majority of renal SO₄²⁻ excretion in teleosts with glomerular renal tubules is the result of active tubular secretion. Tubular SO₄²⁻ secretion is facilitated by DIDS-sensitive electroneutral

anion exchange mechanisms in both the basolateral and the apical membrane and by intracellular carbonic anhydrase. Basolateral import of SO_4^{2-} occurs via exchange with OH^- , while apical extrusion into the tubular lumen occurs in exchange for uptake of HCO_3^- or Cl^- (Marshall and Grosell, 2006).

2.5.2.2 Ion reabsorption in SW-acclimated fish

The NaCl reabsorption occurs in the late proximal tubule and in the urinary bladder. The driving force for solute and fluid reabsorption is provided indirectly by the basolateral NKA that creates a favorable gradient for Na^+ entry from the tubular lumen across the apical membrane and drives Na^+ -glucose, Na^+ -amino acid cotransport and Na^+/H^+ exchange. Tubular reabsorption of Cl^- occurs via an electroneutral process also driven by the electrochemical Na^+ -gradient and is suggested to occur via $\text{Cl}^-/\text{HCO}_3^-$ exchange coupled with the Na^+/H^+ exchange process. The urinary bladder of SW fish contributes to NaCl and water absorption, thereby concentrating divalent ions in the urine and minimizing water loss. NKA is localized to the basolateral surfaces of urinary epithelial cells. The urinary bladder in SW fish participates in NaCl and water reabsorption with increasing bivalent ions and minimizing water loss (Fig. 2.16B). The caudal neurosecretory system (urophysis) and its peptides (urotensin I and II) may control transport and contractility of the urinary bladder.

2.5.2.3 Ion reabsorption in FW-acclimated fish

While some reabsorption of electrolytes may occur in the late proximal tubules in marine teleosts, the majority of the monovalent ions are reabsorbed in the water-impermeable distal tubules of non-mammalian vertebrates. In addition; the urinary bladder contributes to conservation of monovalent ions. The mechanisms of reabsorption of monovalent ions in the distal tubules differ from those in the late proximal tubules. In the early distal tubule, there is a lumen positive potential that is dependent on the presence of both Na^+ and Cl^- in the lumen, it involves basolateral NKA and apical NKCC in NaCl reabsorption (Fig. 2.16C). The apical NKCC co-transporter allows for cellular Cl^- accumulation above the thermodynamic equilibrium and Cl^- exit across the basolateral membrane via $\text{K}^+:\text{Cl}^-$ co-transporters or conductive Cl^- channels. The Na^+ and Cl^- absorption via NKCC may rely partly on cycling of K^+ across the apical membrane via apical K^+ -channels. Apical

Na⁺: H⁺ exchange occurs across the apical membrane and may contribute to Na⁺ absorption in amphibian, mammalian, and possibly teleost nephrons, and paracellular Na⁺ transport driven by the lumen positive potential may also contribute to Na⁺ reabsorption (Marshall & Grosell, 2006). With regard to the Ca²⁺ uptake mechanism of fish chloride cells, Flik *et al.*, (1995) proposed a model similar to that for Ca²⁺ reabsorption in mammalian kidneys. In that model, following entry of Ca²⁺ through epithelial Ca²⁺ channels (ECaC) in the apical membranes of cells, Ca²⁺ bound to calbindin diffuses to the basolateral membrane. At the basolateral membrane, Ca²⁺ is extruded via an ATP-dependent plasma membrane Ca²⁺-ATPase (PMCA) and a Na⁺/Ca²⁺ exchanger (NCX). In this way, there is net trans-epithelial Ca²⁺ absorption (Hwang and Lee, 2007).

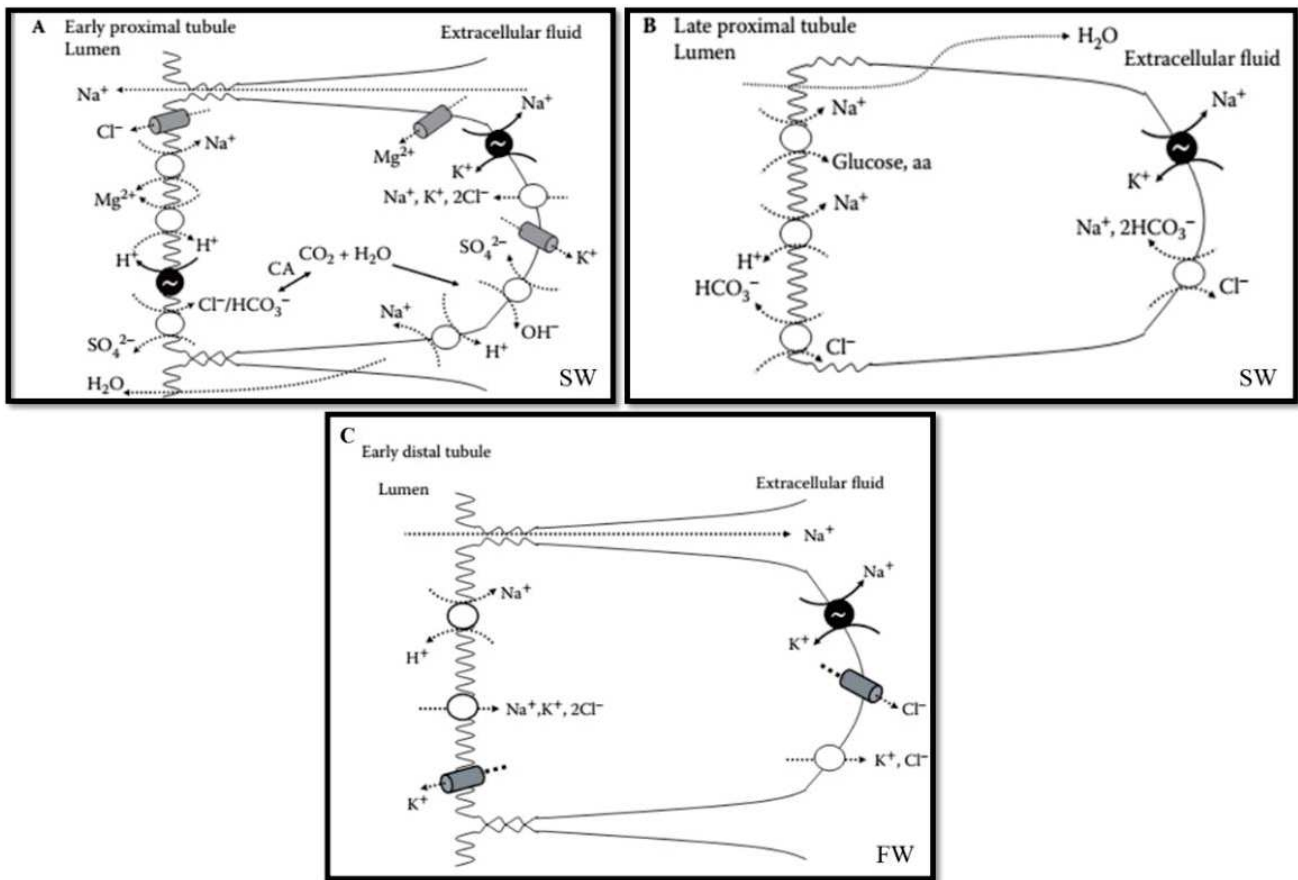


Figure 2-16. Mechanism of ion secretion and ion uptake in proximal segment I (A) and proximal II (B). Mechanism of ion transporter in distal segment (C) of teleosts (Marshall and Grosell, 2006).

2.5.3 Intestine

The primary function of the intestine is the completion of the digestive processes that started in the stomach and the absorption of nutrients. Its functions include digestion, nutriment absorption, hormone secretion, immune protection, and water and salt transfers for hydro-mineral homeostasis. It regulates energy and material exchanges between the environment and the internal medium. It provides a large surface area and is capable of performing both secretory and absorptive functions (Whittamore, 2012). Its structure is variable according to the species, the individual health, the nature of the diet, salinity conditions and developmental stage (Giffard-Mena *et al.*, 2007; Whittamore, 2012). In lampreys, chondrosteans, chondrichtheans and dipnoids, which all have short intestine, the mucosa forms a spiral, valve whereas in teleost fish, the surface area is increased by lengthening the intestine through convolutions (Wilson and Castro, 2010). Dabrowski *et al.*, (1986) found that feeding in SW and FW drastically changes the ionic balance in the euryhaline fish intestine, even though salt intake that accompanies drinking in SW fish largely surpassed dietary intake (Shehadeh and Gordon, 1969; Dabrowski *et al.*, 1986). The acute nature of dietary salt intake may in itself induce an osmoregulatory challenge for the gastrointestinal tract (Taylor and Grosell, 2006).

2.5.3.1 Structure of the teleost intestine

The various constitutive layers and overall structure of the teleost intestine are essentially similar to the mammalian small intestine. It consists of the mucosa, submucosa, muscle layers and serosa (Wilson and Castro, 2010; Fig. 2.17). The mucosa collectively refers to the surface mucus layer, columnar epithelia and lamina propria. A layer of mucus is maintained along the mucosal surface of the intestine, secreted from goblet cells which are found interspersed among the epithelial cells (Fig. 2.17C). As the primary site of contact with luminal contents, the mucus layer has numerous roles including chemical and physical protection, supporting CaCO₃ precipitation and formation of ionic gradients for the benefit of ion and fluid transport. The underlying cell layer constitutes a simple, columnar epithelium possessing characteristic brush border microvilli atop columnar cells connected at their apical poles by tight junctions. Below the columnar epithelial cells is a layer of connective tissue, the lamina propria, containing blood capillaries. This helps support the overlying epithelium and binds it to an additional layer of connective tissue, sometimes referred to as the

submucosa (Wilson and Castro 2010; Wittamore, 2012). The mucosa forms numerous simple folds, and in some cases, more complex secondary and tertiary folds that vastly increase surface area (Wilson and Castro, 2010).

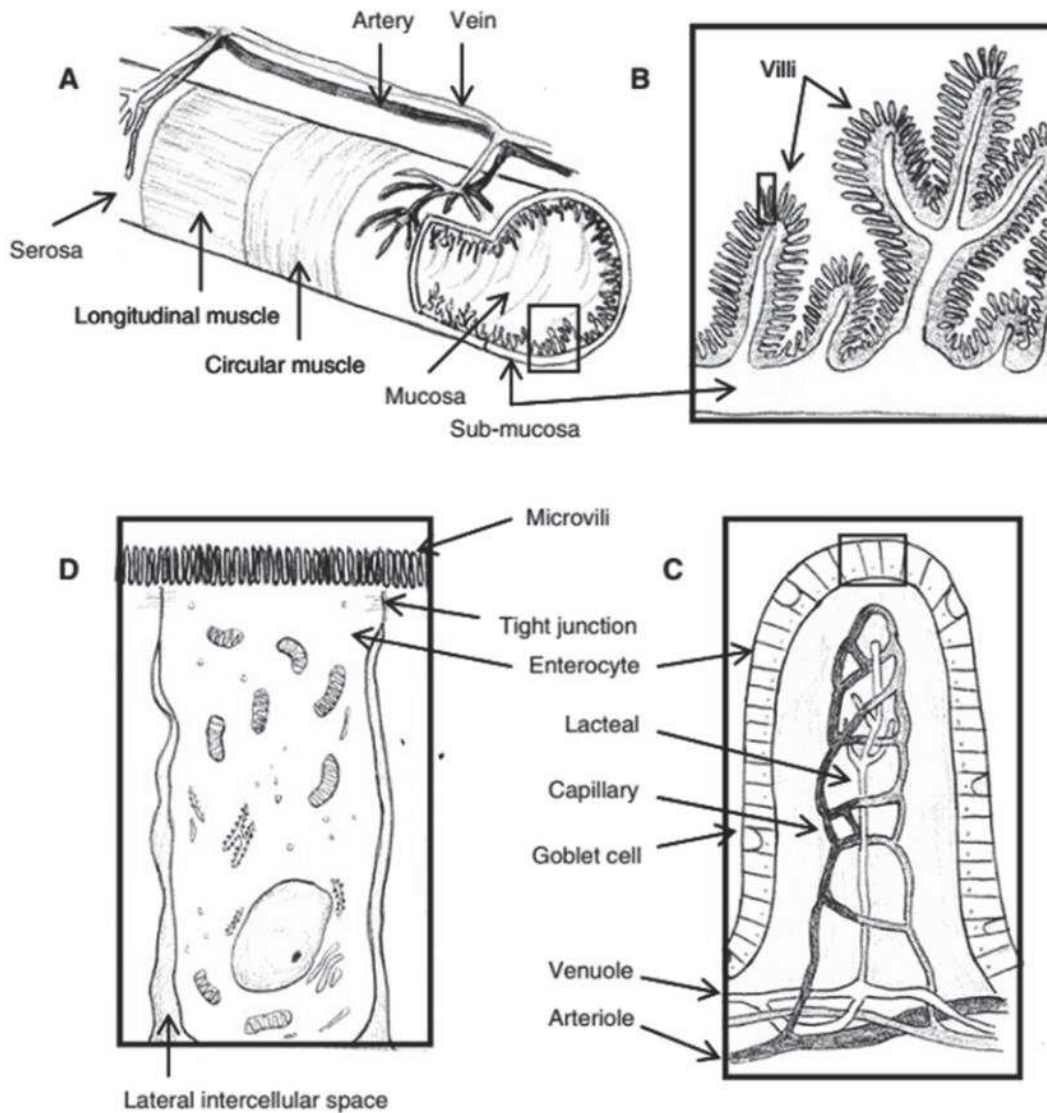


Figure 2-17. The general layout of the vertebrate intestine. (A) A cutaway plan from a section of intestine displaying the gross anatomy including mucosa, submucosa, muscle layers and serosa. (B) A close-up of the elaborate foldings of the mucosa which faces the contents of the lumen. (C) A section through a villus displaying the absorptive epithelial cells with associated capillaries and a close-up of an epithelial cell showing the microvilli of the apical membrane, intercellular spaces and various organelles (Randall *et al.*, 2001).

2.5.3.2 Role of the intestine in SW-acclimated fish osmoregulation

The intestine is involved in osmoregulation, notably for water absorption in SW following active ion uptake in order to avoid dehydration. Ingested SW passes through the gastrointestinal tract and ions must be differentially absorbed across the intestinal epithelium to facilitate water absorption. The intestine has an established role in osmoregulation, and its ability to effectively absorb fluid is crucial to compensating for water losses to the hyperosmotic environment (Marshall and Grosell, 2006; Giffard-Mena *et al.*, 2007). The osmolality of intestinal fluids is further reduced to approximately 400 mOsm in the anterior intestine and as low as 300 to 360 mOsm in the rectal fluids. The main osmolytes of intestinal fluids are not Na^+ and Cl^- , as in SW but, rather, Mg^{2+} , SO_4^{2-} and HCO_3^- . The unique chemistry of the intestinal fluids is the product of combined absorptive and secretory processes as well as the differential permeability of the intestinal epithelium. It should be noted that although the overall function of the marine teleost intestine clearly is to absorb Cl^- and fluids, this epithelium switches, if strongly stimulated, to Cl^- and fluid secretion (Marshall & Grosell, 2006). Between 39 and 85% of imbibed fluid can be absorbed along the length of the intestine, and over the course of the entire gastrointestinal tract almost all the NaCl (up to 99%) is absorbed and excreted (Marshall and Grosell, 2006). Of the potential direct influences, levels of Cl^- appear to have a dominant role in modulating drinking behaviour. For example, increased medium Cl^- stimulates the drinking reflex (Giffard-Mena *et al.*, 2007; Whittamore, 2012). The exact mechanism of water absorption remains unknown and may involve both transcellular and paracellular pathways. However, several co-transporters, including K^+ : Cl^- and Na^+ : glucose transporters, have been demonstrated to transport water and may contribute to transcellular water movement in the marine teleost intestine.

Regardless of the mechanism, in the intestine, water absorption across the marine teleost intestine is tightly linked to the absorption of Na^+ and Cl^- . The Na^+ absorption is ultimately fueled by the basolateral NKA, which extrudes 3Na^+ across the basolateral membrane in exchange for 2K^+ (Marshall and Grosell, 2006). The activity of this enzyme thus maintains low intracellular Na^+ concentration. The apical electrochemical Na^+ gradient energizes the absorption of both Cl^- and K^+ by two parallel co-transport systems: Na^+ : Cl^- (NC) and Na^+ : K^+ : 2Cl^- (NKCC) co-transporters (Fig. 2.18). Removal of Cl^- from the intestinal lumen blocks Na^+ absorption (consistent with NKCC operation), whereas limited Cl^- absorption persists even in the absence of Na^+ ,

demonstrating an alternative Cl^- uptake pathway. However, considering the relatively low K^+ concentration in sea water and intestinal fluids, NKCC by itself cannot explain the often substantial excess net Cl^- absorption. Apical $\text{Cl}^-/\text{HCO}_3^-$ exchange (AE) has been also demonstrated to play a role in Cl^- absorption and accounts for 30 to 70% of net Cl^- uptake (Marshall and Grosell, 2006; Fig. 2.18). The apical $\text{Cl}^-/\text{HCO}_3^-$ exchange performs active transport of not only Cl^- but also HCO_3^- resulting in high luminal HCO_3^- concentrations and highly alkaline intestinal fluids (Marshall and Grosell, 2006).

Endogenous metabolic CO_2 provides cellular HCO_3^- via carbonic anhydrase for the apical anion exchange process, with the resulting H^+ being extruded across the basolateral membrane via an NHE-like transporter. The H^+ extrusion across the basolateral membrane is critical for apical HCO_3^- secretion and ultimately relies on the activity of the basolateral NKA (Grosell, 2006). Exchange of a metabolic waste product (CO_2), which exerts limited osmotic pressure, in exchange for an electrolyte provides an osmotic driving force for cellular water uptake. Basolateral import of HCO_3^- from extracellular fluids appears to also contribute to luminal HCO_3^- secretion and may occur via $\text{Na}^+:\text{HCO}_3^-$ co-transport (NBC) (Grosell, 2006). Bicarbonate secretion also seems to play a role in calcium homeostasis, inhibiting intestinal Ca^{2+} absorption by precipitating CaCO_3 , which is subsequently excreted (Wilson *et al.*, 2002; Wilson and Grosell, 2003). This carbonate precipitation in itself also promotes water absorption, lowering osmolality by removing Ca^{2+} and CO_3^{2-} from solution (Wilson *et al.*, 2002; Marshall and Grosell, 2006). High Ca^{2+} concentrations alone (Wilson *et al.*, 2002) and elevated ambient salinity (Walsh *et al.*, 1991; Taylor and Grosell, 2006) have been shown to stimulate HCO_3^- secretion, which is not surprising considering the role of intestinal anion exchange in Cl^- and water absorption (Grosell *et al.*, 2005; Grosell, 2006).

Regardless of the route of apical Cl^- entry into the epithelial cells, Cl^- transport across the basolateral membrane appears to occur via Cl^- channels and $\text{K}^+:\text{Cl}^-$ co-transporters (Marshall and Grosell, 2006). Compared to Na^+ and Cl^- , the divalent ions (Ca^{2+} , Mg^{2+} and SO_4^{2-}) are poorly absorbed along the intestine (Whittamore, 2012). The Ca^{2+} precipitation (CaCO_3) rate is related to imbibed water. Precipitation of Ca^{2+} (and to a lesser extent Mg^{2+}) removes their osmotic influence and thus helps maintain a favorable osmotic. The reduction in luminal fluids osmolality will be then occurred due to CaCO_3 formation in which Ca^{2+} and CO_3^{2-} are eliminated. Divalent ions (Mg^{2+} and SO_4^{2-}) are inactively accumulated in the digestive tract due to highly reabsorption of Na^+ and

Cl⁻. Their secretion is therefore facilitated by high accumulation of these bivalent ions (Taylor and Grosell, 2006).

2.5.3.3 Role of the intestine in FW-acclimated fish (Intestinal salt reabsorption)

While osmotic dehydration presents a physiological challenge to marine teleosts, FW species instead experience osmotic water gain. In addition to the diffusive water gain across the gills, water ingested with food must be compensated by renal elimination. Secretion of ions is associated with the production of digestive fluids in the stomach and intestine and will thus present a potential disruption of salt balance. However, while some of the secreted ions are recovered by salt reabsorption; ingested food may provide a substantial contribution to ion uptake.

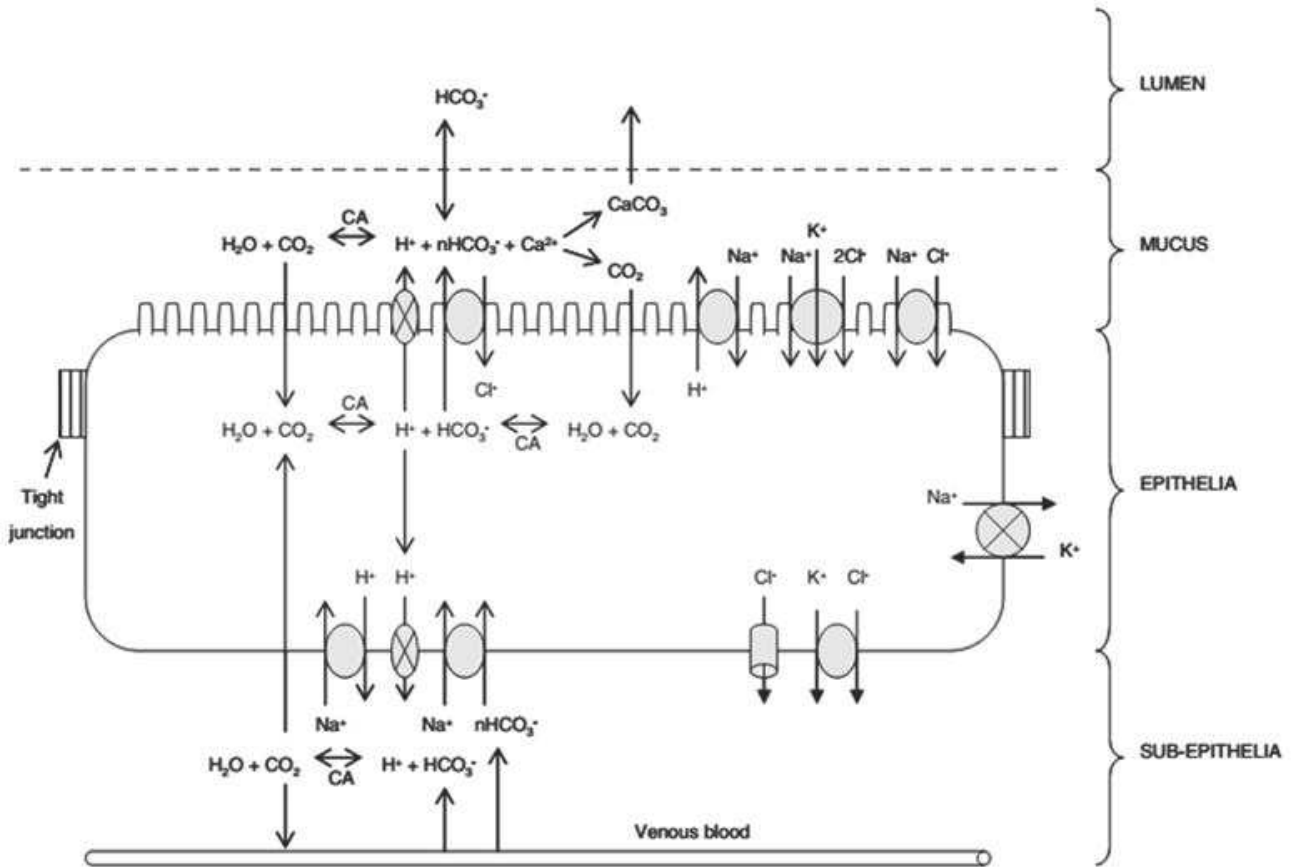


Figure 2-18. Epithelial ion transport pathways generating the driving forces for water absorption by the marine teleost intestine (Whittamore, 2012).

2.6 Osmoregulation in Sticklebacks

Throughout its distribution within the northern hemisphere, stickleback adapted to a wide variety of habitats. In these freshwater habitats, it is expected that they could have lost their osmoregulatory capacities in different salinity conditions. However, recent research studies suggest that they still retained their osmoregulatory capacities enabling them to osmoregulate at different salinities (Grøtan *et al.*, 2012).

In 1958, Baggerman performed the first experiments on salinity preference in sticklebacks in order to explain the effects of light/dark ratio, reproductive maturity, age and interaction of these traits on salinity preference. Barrett *et al.*, (2008) performed experiments on ectodysplasin (*eda*) gene. They found no association of the low-armor *eda* allele with freshwater preference. However, low-plated fish prefer to stay in freshwater environments. Later, Schaarschidt *et al.*, (1999) checked combined effects of salinity and temperature on the enzymatic activity. More recently, McCairns and Bernatchez, (2010) studied adaptive divergence between FW and SW sticklebacks. Shimada *et al.*, (2010) studied different genes with known physiological functions in FW and SW sticklebacks. Jones *et al.*, (2012a, 2012b) explored FW and SW morphotype and ecotypes and adaptive evolution and divergence. Taugbøl *et al.*, (2014) checked the expression of osmoregulatory genes in FW and SW sticklebacks. Wang *et al.*, (2014) studied gene expression responses of stickleback to salinity. Divino *et al.*, (2016) checked salinity tolerance in FW sticklebacks. Kusakabe *et al.*, (2017) studied genetic basis for salinity tolerance of stickleback ecotypes. Finally, Gibbons *et al.*, (2018) studied the effects of both salinity and temperature acclimation on the gills of sticklebacks and NKA enzymatic activity.

However, no physiological studies considered Mediterranean threespined stickleback populations in terms of their osmoregulatory capacity and their capacity to acclimate to different salinities

3 Chapter 3: Materials and Methods

Physiology of Threespined Sticklebacks (*Gasterosteus aculeatus*) of the Camargue at different salinities.

3.1 Stickleback sampling and rearing.

In the Camargue region, a protected area located within the Rhône river delta in Southern France, sub-adult three-spined sticklebacks (*Gasterosteus aculeatus*) were fished using fixed hoop nets of decreasing mesh size from 13 to 6 mm at the end of the hoop net ('capéchade') from different water bodies (Table. 3.1; Fig. 3.2, 3.3; Bouchereau *et al.*, 1990). This framework (Fig. 3.4) includes a "paradière" acting as a barrier and towards tower which fish are directed and trapped in the 'verveux' (fyke net). For the physiological study, fish from 4 different areas were considered. One sampling site corresponds to a freshwater canal ('Versadou') running from the Rhône river and loading freshwater into a brackish/seawater lagoon ('La Contesse' lagoon). A second freshwater body is located near main the Vaccarès brackish water lagoon.



Figure 3-1. Installed fishing site on the Vaccarès lagoon with fixed hoop nets (A), Collection of trapped fish (B), and separation of sticklebacks (C) in order to separate live threespined sticklebacks.

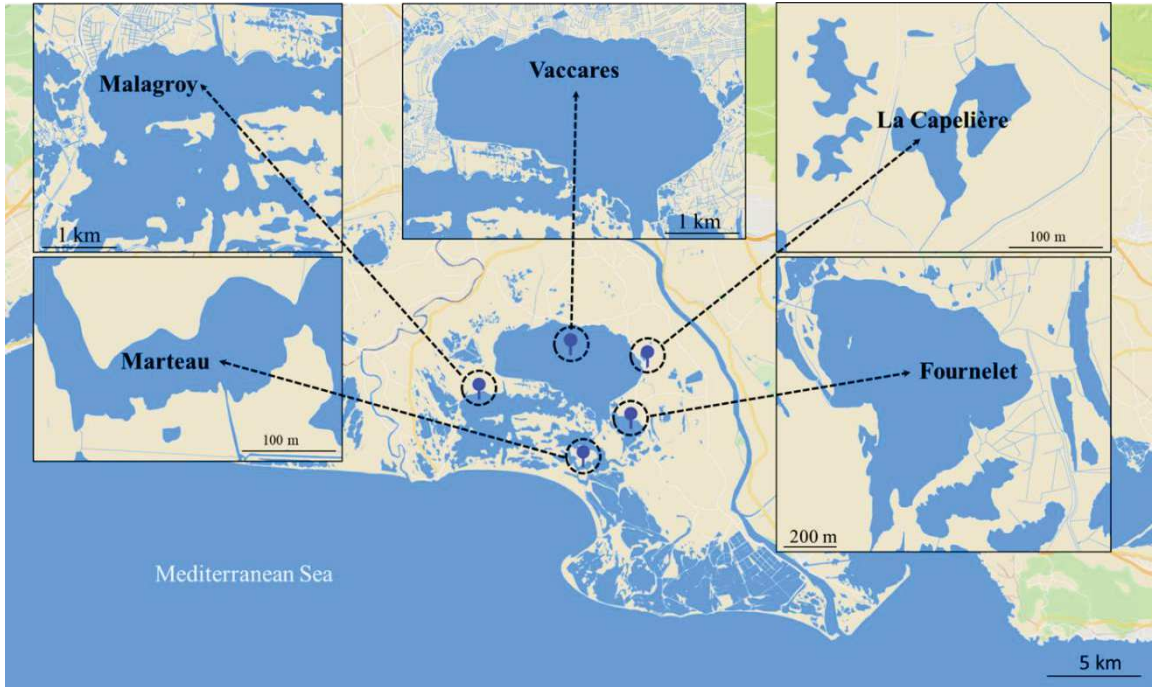


Figure 3-2. Camargue with different mesohaline/brackish water sampling sites.



Figure 3-3. Camargue with different freshwater sampling sites.

The other two sites correspond to a brackish/seawater lagoon ('Marteau' lagoon) and a brackish (BW) lagoon ('Vaccarès' lagoon). Sampling was carried out in summer 2015, December 2016 and February 2017. Captured fish were then directly transported to the laboratory facility at the University of Montpellier, France using separate large oxygenated containers filled with water from the different sampling sites. Fish were then kept at room temperature and under natural photoperiod in separate tanks containing 40-100L of recirculated and filtered water (12 fish per tank with an approximate 1/1 sex ratio). Fish from the BW 'Vaccarès' lagoon were kept at 15‰, 30‰ or 5‰; those from the SW 'Marteau' lagoon were kept at 30‰ and those from the FW 'Versadou' canal and from Fumemorte at 5‰. Fish were fed 3 times a day with chironomid larvae (EXOMARC; Rind *et al.*, 2017), excluding the last day before experimentation. Fish behavior, salinity and temperature levels were checked daily.

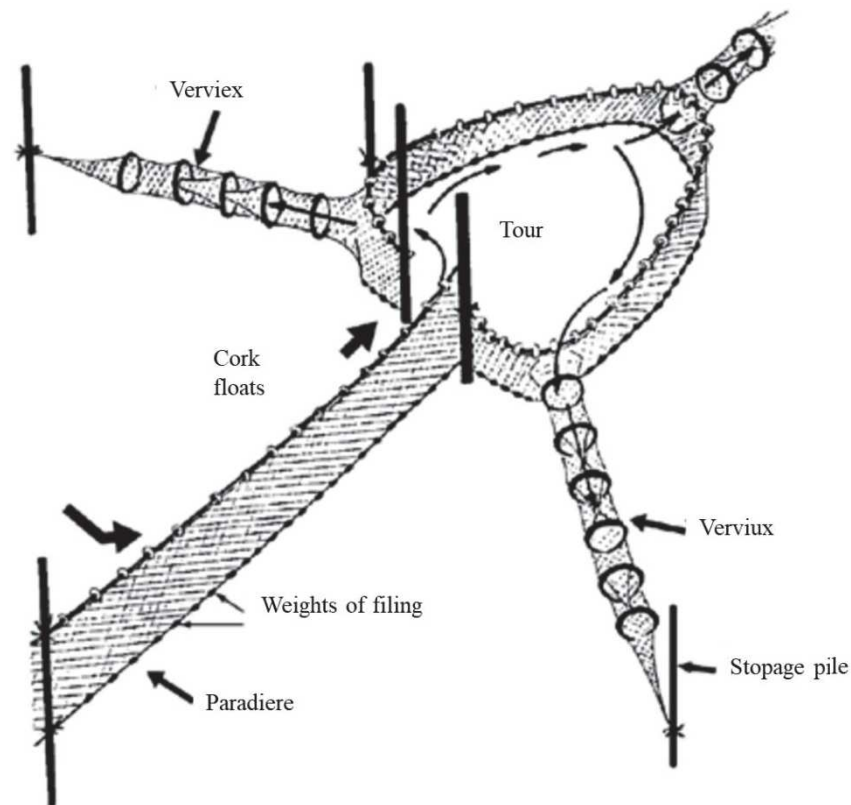


Figure 3-4. The “capéchade” fixed hoop net used during sampling from Camargue, the arrows indicate the direction of fish displacement (Bouchereau *et al.*, 1990).

After a 3-week acclimation period, fish were then either directly used experiments or transferred from BW or SW to FW or from BW or FW to SW. Fish were either kept alive for the respiratory measurements or sacrificed with an overdose of anesthetic (2-phenoxy-ethanol, 500 mg.l⁻¹), and then dissected on ice. Gills were either directly frozen in liquid nitrogen or then kept at -80°C for NKA activity measurements, branchial and kidney NKA gene expression. For other experiments such as light microscopy of gill, kidney, intestine, and scanning electron microscopy (SEM)), whole fish transferred to Bouin's solution for 48 hours (10 fish per condition). For electron microscopy of gills and kidneys, dissected gills were transferred to in mixture of 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 h. Osmotic pressures of the blood extracted from the heart of anesthetized fish were measured with a nano-osmometer (Clifton Osmometer, Clifton Technical physics, Hartford, NY, USA) from eight to ten animals.

Table 3-1. Different sampling sites with habitats characteristics of Camargue wetland complex.

Habitat characteristics	Location names	GPS positions	
Mesohaline	Malagroy	43°30'41.6"N	4°29'16.6"E
	Vaccarès	43°32'16.0"N	4°37'54.0"E
	La Capelière	43°32'26.0"N	4°38'33.9"E
	Marteau	43°27'36.4"N	4°34'58.3"E
	Fournelet	43°29'37.7"N	4°37'24.9"E
Freshwater	Fumemorte	43°31'43.2"N	4°42'10.9"E
	Baisse salée	43°29'15.0"N	4°39'02.7"E
	Giraud	43°29'28.8"N	4°41'42.1"E
	Versadou	43°27'15.2"N	4°35'22.6"E

3.2 Experimental design and salinity acclimation

In order to investigate the osmoregulation capacity and salinity tolerance of stickleback, fish captured from Fumemorte transferred from control FW to BW and SW, after 2 to 3 weeks of acclimation, at least ten fish per condition used for gill relative gene expression of NKA, NKCC1, CFTR and V-ATPase, gills were also used for immunohistochemistry of NKA, scanning electronic microscopy (SEM) and transmission electronic microscopy (TEM). Whereas, fish from Vaccarès

mesohaline brackish water lagoon after 3 to 6 weeks of acclimation, abrupt salinity shocks from 1 hour to 5 hours, 24 hours to 72 hours, we checked bioenergetic cost by measuring oxygen rate of abrupt salinity shocks. The fish with long term acclimation (e.g. 3-6 weeks), their gills and kidney dissected for immunofluorescence of NKA and kidney for TEM experiments.

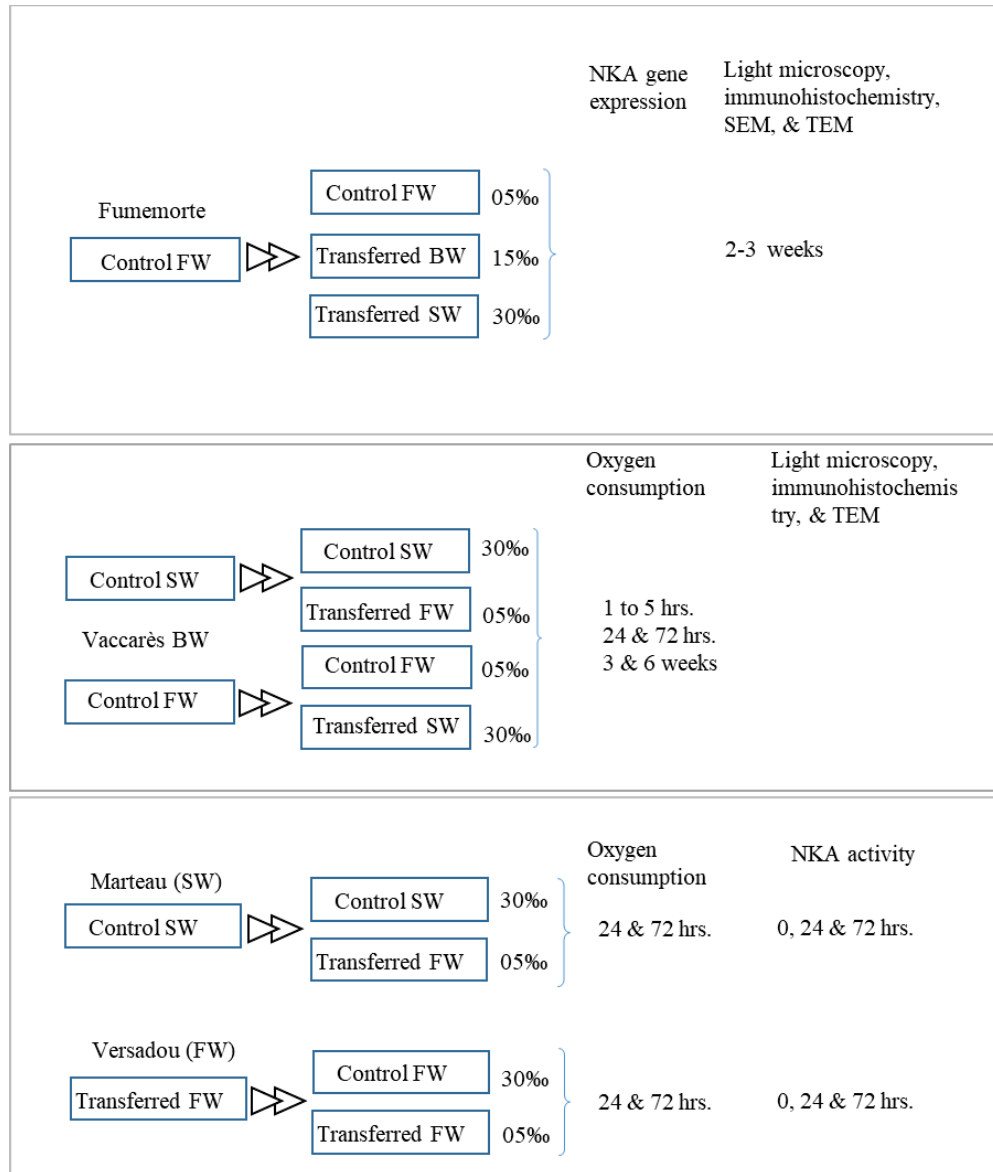


Figure 3-5. Experimental design for relative mra expression of gill NKA, NKCC1, CFTR, and V-ATPase, gill NKA immunolabeling, gill SEM and TEM (Fumemorte), oxygen consumption rate (Vaccarès, Marteau, and Versadou), Kidney TEM (Vaccarès), immunohistochemistry of gill, kidney and anterior and posterior intestines (Vaccarès), and branchial NKA activity (Marteau and Versadou).

Sticklebacks collected from the Marteau SW lagoon is close to the Mediterranean Sea and the Versadou canal (freshwater canal from the Rhône River) were exposed to abrupt salinity changes and oxygen consumption rates were measured 24 to 72 hours after salinity transfer. For branchial NKA activity, at least 10 individuals were used for controls, SW/FW and FW/SW transfers. Fish collected from both the Marteau lagoon and the Versadou canal were also measured before transfer and 24 and 72 hours after transfer (Fig. 3.5).

Table 3-2. The number of tissue sampled and condition of their preservation for the different experiments *SEI buffer: 300 mM sucrose, 20 mM Na₂EDTA, 100 mM imidazole, pH 7.3.

Experiment	Desired tissue	Minimum samples per salinity condition	Condition of samples preservation
Osmotic pressure	Blood plasma	10	at -80°C
Histology Immunohistochemistry	Gill	10	Samples were fixed by Bouin's fixative solution for 48 hours. They were then kept in 70% alcohol
	Kidney	10	
	Anterior intestine	10	
	Posterior intestine	10	
Scanning Electron microscopy (SEM)	Gill	10	samples were fixed by Bouin's fixative solution for 48 hours and were then kept in 70% alcohol
Electron microscopy (TEM)	Gill	10	First fixation: Glutaraldehyde 2.5%+ 0.1M sodium cacodylate for 24h Post-fixation: a mixture (1:1, v/v) of 1% osmium tetroxide + 0.1 M sodium cacodylate buffer for 2h at 4 °C.
		10	
Oxygen consumption rate	Live individuals	10	Live individuals were considered in order to measure oxygen consumption rate.
Branchial Na⁺/K⁺-ATPase activity	Gill	10	Samples were put in the SEI* buffer. They then kept at -08 °C
		10	
Sequencing Gene expression	Gill	10	Samples were put in the RNAlater (Qiagen, Germany). They then kept at -80 °C.

3.3 Medium and blood osmotic pressure determination

Fish were sacrificed with an overdose of anesthetic (2-phenoxy-ethanol, 500 mg L⁻¹) and dissected in order to collect blood samples using a 1-mL syringe coated with heparin (Li-heparin, Sigma-Aldrich, France).. Osmotic pressures of the blood extracted from the heart of anesthetized fish

were measured with advance micro-osmometer (Advanced® Model 3320 Micro Osmometer; Fig. 3.6) from eight to ten animals. The fusion temperature (FT) value obtained with the micro-osmometer was transformed to mOsm kg⁻¹.



Figure 3-6. The advance micro-osmometer is the standard system for the determination of the total osmolality in aqueous solutions using the freezing point depression. It necessitates 20 µl of samples.

The freezing point method allows osmolality determination of an aqueous-based solution (Fig.3.7). When a solute (particles) is dissolved in a solvent (water), the freezing point of that solution is lower than that of the solvent alone. As more solute is added, the freezing point decreases further. By precisely measuring the freezing point of the solution, the osmolality, or concentration, can be determined. Freezing point measurement is non-specific; that is, it will not tell you what the particle is, nor will it tell you the size or shape of the particle. The freezing point depends only on the number of particles in solution.

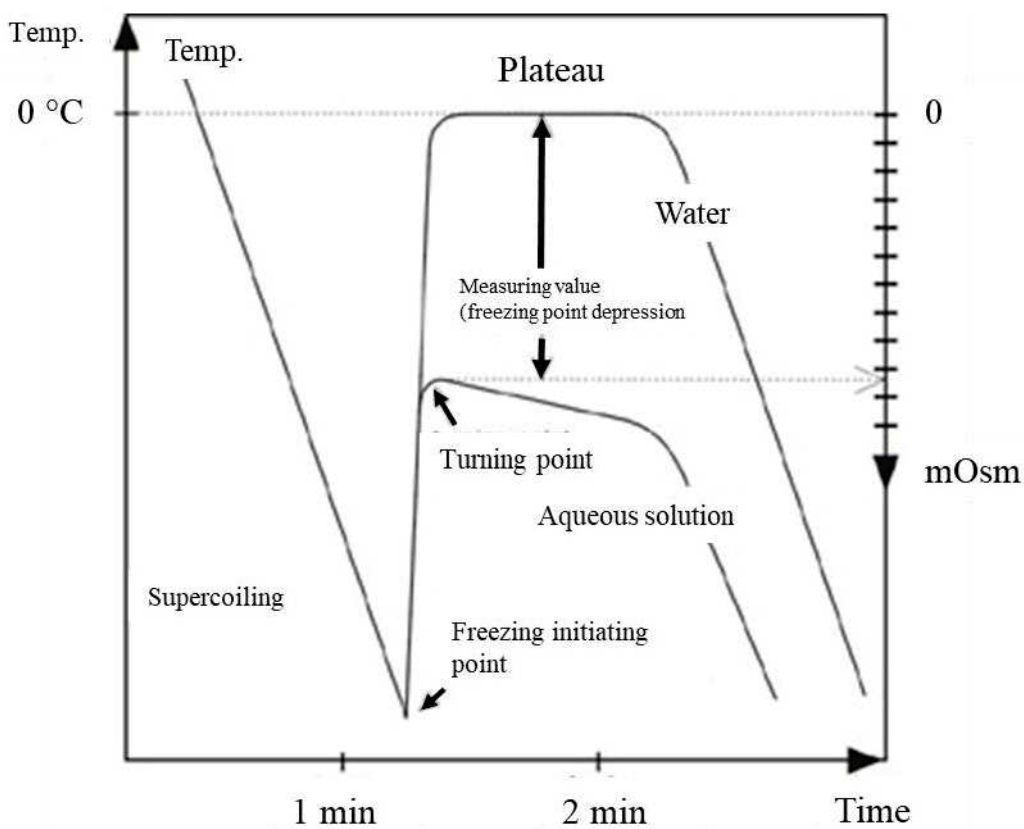


Figure 3-7. Typical cooling curves of water and aqueous solutions.

During the measuring process, the temperature of the sample is measured by a thermistor (a temperature dependent resistor). This is part of the measuring head onto which the tube is placed. The measuring head is attached to guide rods which protect it from accidental damage. At a defined super-cooling the freezing process is started by lowering a needle with ice crystals into the sample tube. The freezing point of the sample is reached. The method of initiating the freezing process is important for the reproducibility of measurements. Dipping a needle with ice crystals into the sample gives more exact results than stirring with a wire which is constantly dipping into the sample. Because of linear correlation between osmolality and freezing point the measurement of freezing point is a determination of osmolality. The results are displayed as mOsm/kg.

3.4 Histology and immunohistochemistry

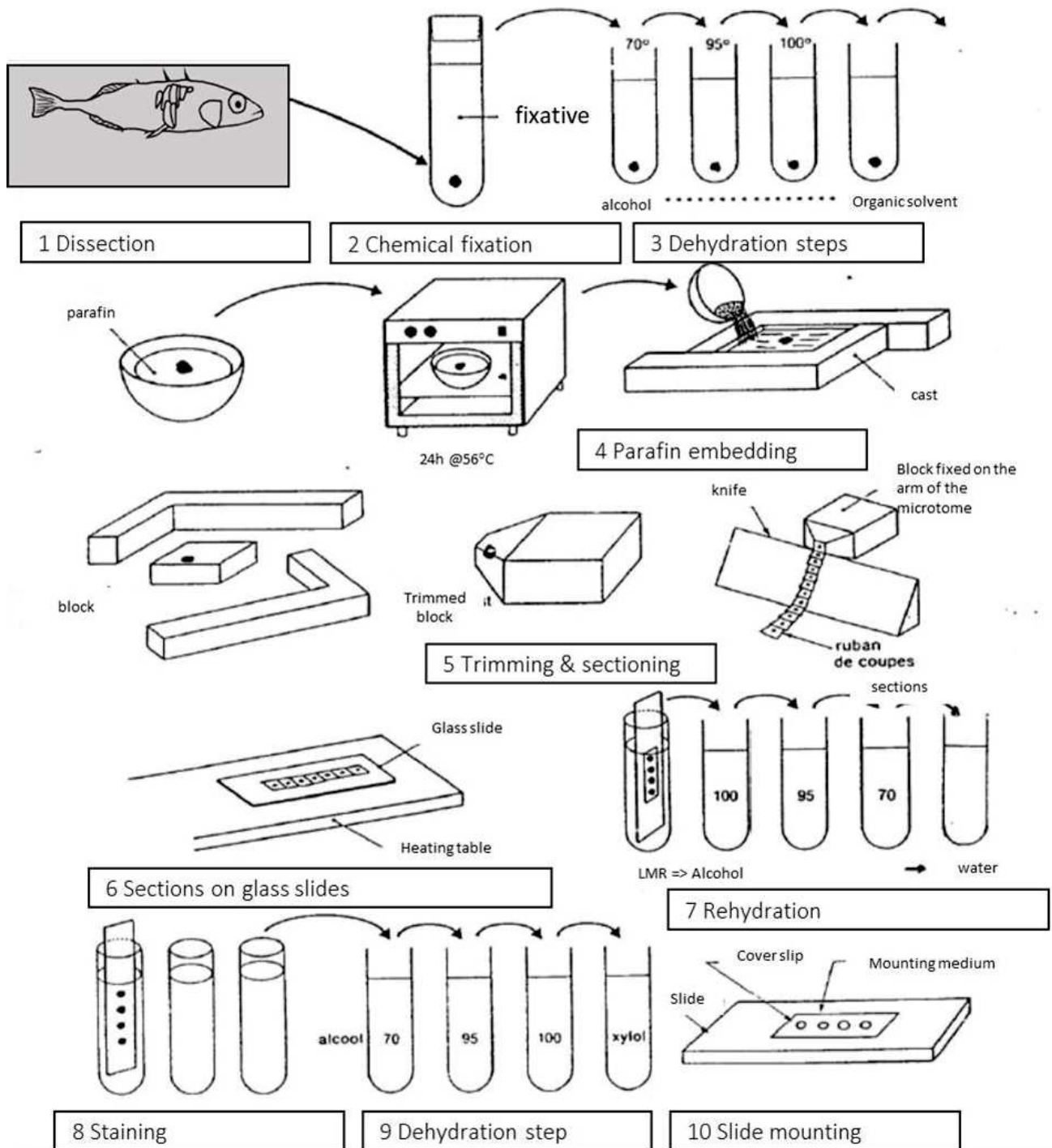


Figure 3-8. Technical steps that are necessary for histological sample preparation (Lignot, 2017).

The morphology of different osmoregulatory organs such as the gills, kidney, anterior and posterior intestine has been studied using trichrome staining and immunohistochemistry. After washing the fixed samples in 70% alcohol, they were dehydrated in an ascending series of ethanol (92%, 100%), overnight in Butanol and processed for embedding in Paraplast X-TRA® (Sigma-Aldrich, P3808). Sections (4 µm) were cut on a Leitz Wetzlar microtome collected on poly-L-lysine glass slides. Dry slides were stained using the Masson's Trichrome Staining Protocol (Martoja and Martoja, 1967; Table 3.3) and selectively staining muscle, collagen fibers, fibrin, and erythrocytes. The general rule in trichrome staining is that the less porous tissues are colored by the smallest dye molecule; whenever a dye of large molecular size is able to penetrate, it will always do so at the expense of the smaller molecule. Others suggest that the tissue is stained first with the acid dye, Biebrich Scarlet, which binds with the acidophilic tissue components. Then, when treated with the phospho acids, the less permeable components retain the red, while the red is pulled out of the collagen. At the same time causing a link with the collagen to bind with the aniline blue.

Table 3-3. Trichrome classical histology, different steps for classical staining of tissues.

Steps	Time	Replicates
Dewaxing with HistoClear (Histological Clearing Agent, Agar, R1345)	10 min	2
Hydration with buthanol	5 min	1
Hydration with 100% alcohol	5 min	1
Hydration with 95% alcohol	5 min	1
Hydration with 70% alcohol	5 min	1
Rinsing with water	10 min	1
Coloration with Hematoxilin	2 min	1
Rinsing with water	5 min	2
Coloration with Fuchine	3 min	1
Rinsing with Acetic acid	-	1
Immersion in Phosphomolibdic acid	5 min	1
Blue Acetic	2 min	1
Rinsing with Acetic acid	-	1
Ethanol 100% alcohol	5 min	2
Buthanol alcohol	5 min	1
HistoClear (Histological Clearing Agent, Agar, R1345)	10 min	2
Slides were mounted in ImmunoHistoMount	-	-

For immunolabeling, several proteins such as NKA has been checked in different osmoregulatory organs (Table 3.5). The sections were then incubated with the primary antibodies in a wet chamber for 2 hours at room temperature (Table 3.4) then diluted in PBS with specific concentration covering the sections. Control sections were incubated in PBS without the primary antibodies. After extensive washing for 3 times of 5 minutes in PBS to remove unbound antibody, the sections were incubated for 1 hour with a secondary antibodies (Table 3.4). Following extensive washing in PBS (3 times for 5 minutes), sections were mounted in 80% glycerine, 20% PBS plus 2% N-propyl-gallate to retard photobleaching (ImmunoHistoMount, Aqueous-based Media, Santa Cruz Bio-technology, USA). Stained and labelled sections were all examined with a Leitz Diaplan microscope equipped with a special filter for fluorescence and associated with a Leica DC 300 F digital camera and its software FW 4000 I (Leica Microsystems, Rueil-Malmaison, France).

Immunofluorescence microscopy is a particularly robust and broadly applicable method generally used to assess both the localization and endogenous expression levels of key proteins such as transmembrane proteins (this study). Using specific fluorophores, the location of bound antibody on histological sections can be visualized. It necessitate to take into consideration the nature of the antigen, the specificity and sensitivity of the primary antibody, the properties of the fluorescent label, the permeabilization and fixation technique of the sample, and fluorescence imaging of the cell. Each protocol requires fine- tuning adjustments depending on the cell type, the antibody, and the antigen used (fig. 3.9).

The basic function of a fluorescence microscope is to irradiate the specimen with a desired and specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. Only the emission light reaches the eye (or detector) so that the resulting fluorescent structures are superimposed with high contrast against a very dark (or black) background. The limits of detection are mostly governed by the darkness of the background and the quality of the antibodies used. In the microscope, the emission light (specific wavelength or defined band of wavelengths often in the ultraviolet, blue or green regions of the visible spectrum) is produced by passing multispectral light from an arc-discharge lamp. Wavelengths passed by the excitation filter reflect from the surface of a dichroic (or dichromatic) mirror, through the microscope objective to reach the specimen with intense light. The emission fluorescent light

gathered by the objective passes back through the dichromatic mirror and is subsequently filtered by a barrier (or emission) filter, which blocks the unwanted excitation wavelengths.

Table 3-4. Different steps of Immunohistochemistry.

Steps	Time	Replicates
Dewaxing with HistoClear (Histological Clearing Agent, Agar, R1345)	5 min	2
Hydration with buthanol	5 min	1
Hydration with 100% alcohol	5 min	1
Hydration with 95% alcohol	5 min	1
Hydration with 90% alcohol	5 min	1
Hydration with 70% alcohol	5 min	1
Hydration with 50% alcohol	5 min	1
Washing with PBS	5 min	1
Solution A (200 ml PBS+ 1.75 g Sodium chlorid3 150 mM+ 40 tween 20 lμ)	10 min	1
Solution B (200 ml PBS+ 10 g dried milk)	20 min	1
Washing with PBS	2 min	3
1 st antibody	2 hours at room temperature	
Washing with PBS	5 min	3
2 nd antibody	1 hour	
Washing with PBS	5 min	3
Slides were mounted in ImmunoHistoMount	-	-

Table 3-5. List of antibodies used in this research project.

Protein localization	Target organ	1 st antibody (μg. ml ⁻¹)	2 nd antibody (μg. ml ⁻¹)
NKA	Gill Kidney Anterior and Posterior	α5 Mouse monoclonal 10	Alexa Fluor 546 goat anti-rabbit 10

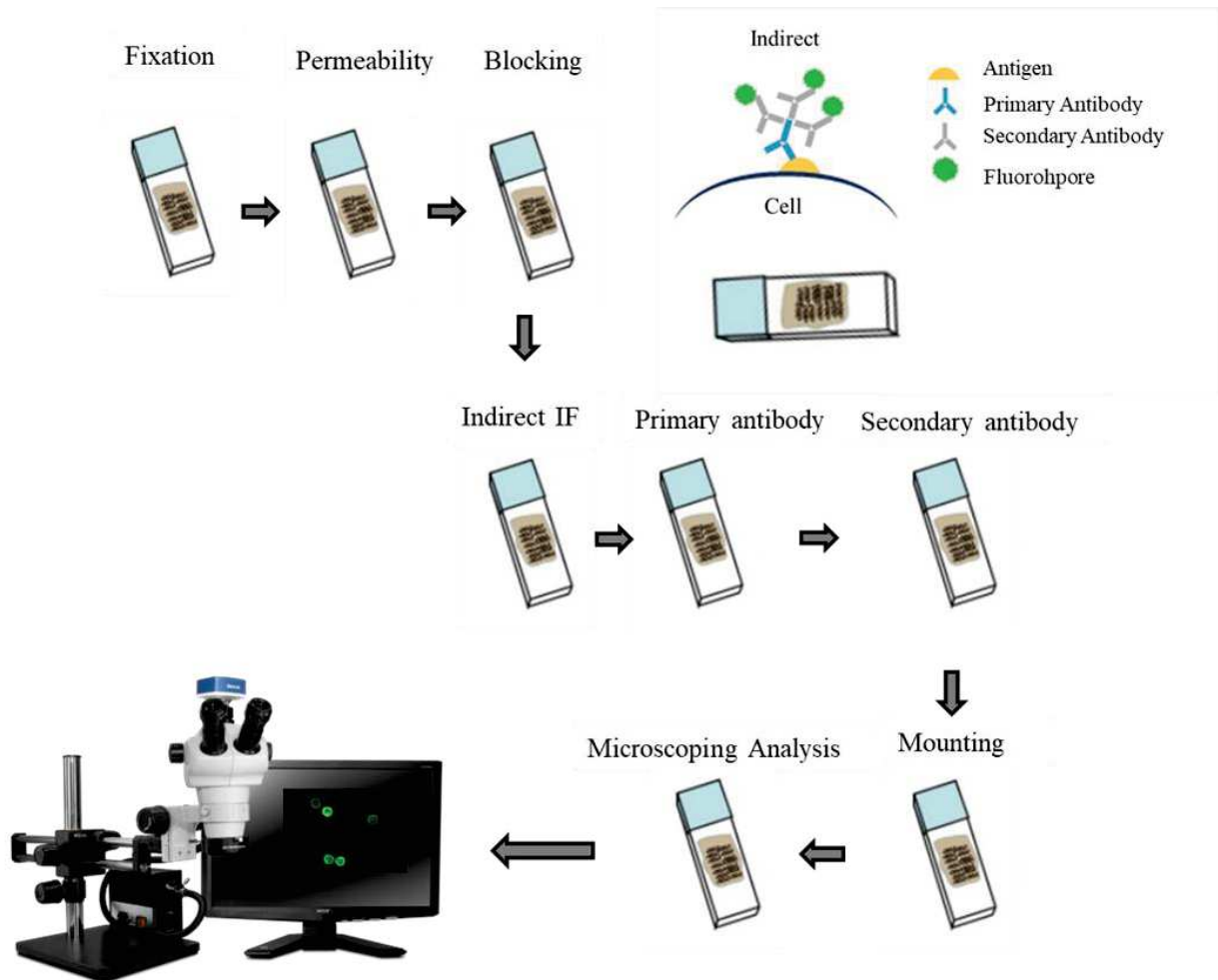


Figure 3-9. Schematic diagram of the immunolabeling technique.

3.5 Electron microscopy

In order to investigate the ultrastructure of the gills two electron microscopic methods (Scanning Electron Microscopy: SEM and Transmission Electron Microscopy: TEM) were used in this study. Electron microscopy can allow the ultrastructural investigation of biological tissues, cells, organelles and molecular elements such as membranes, tissue and cell surfaces. TEMs are the most powerful electron microscopes (resolution 0.3 nanometer). SEMs are generally about 10 times less powerful than TEMs (resolution: about 3 nanometers). However, they produce very sharp, 3D images and biological samples need less preparation.

3.5.1 Scanning Electron Microscopy (SEM)

Gills fixed in Bouin's solution for 48 h (10 fish per condition) and kept in 70% ethanol, were then dehydrated through a graded ethanol series, bathed in hexamethydisilazane (two 1-min cycles) and air-dried. They were subsequently attached to specimen stubs with adhesive carbon tape. The different specimens were then coated with gold for 180 s (~40 mA), using the Edwards Sputter Coater and examined with a FEI Quantum 200 ESEM using a conventional mode (high vacuum) and Thornley-Everhart secondary electron detector.

3.5.2 Transmission Electron Microscopy (TEM)

Dissected gills from five fish in FW and five fish transferred to SW were placed in a mixture (1:1, v/v) of 2% glutaraldehyde in 0.1M sodium cacodylate buffer for 24 h before being processed for transmission electronic microscopy (TEM) using a classical protocol. Post-fixations were performed in a mixture (1:1, v/v) of 1% osmium tetroxide and 0.1 M sodium cacodylate buffer for 2 h at 4°C. Ethanol-dehydrated samples were then embedded in Agar 100 resin (R1031, same formulation as Epon 812). Ultra-thin sections (50–150 nm) were cut on a LKB Bromma 8800 Ultratome 3 or OM2 ultramicrotome and contrasted with uranyl acetate and lead citrate prior to examination on a JEOL 1200 EX transmission electron microscope at 70 kV. Semi-thin sections (0.5–1 μ m) stained with toluidine blue were used for light microscopy studies with a Leitz Diaplan microscope.



Figure 3-10. TEM and SEM for the observation of gill and kidney morphology.

3.6 Branchial Na⁺/K⁺-ATPase activity

From each fish, four gills (from the same side of the fish) were dissected and homogenized in pairs in SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH: 7.4) using a manual potter. The specific, Na⁺- and K⁺-dependent, ouabain-sensitive ATPase activity was measured in these homogenates using 1.4 mM of ouabain as described in detail by Metz *et al.*, (2003). Aliquots (5 µl in triplicate) of homogenate (protein content of 1 mg·ml⁻¹) were incubated in assay medium for 20 min at 25°C. The specific activity was calculated by subtracting the K⁺-independent, ouabain-insensitive ATPase activity from total ATPase activity. ATP hydrolysis was assessed by the amount of inorganic phosphate formed per minute per mg of protein. Sample protein content was estimated using the protocol originally described by Bradford (1976) using a commercially prepared reagent (Sigma-Aldrich, Saint Louis, USA), and bovine serum albumin as standard.

3.7 Oxygen consumption rate

Respiration measurements were conducted in 73 ml transparent metabolic chambers, equipped with an oxygen optode (OXSP5, sensor code SD7-545-214) (Pyro-Science GmbH, Aachen, Germany) previously glued to its inner wall. Optodes were calibrated to 100% (using air-saturated water) and 0% air saturation (using a freshly made 80 mM Na₂SO₃ solution). Each metabolic chamber was equipped with a magnetic stirrer (to ensure correct O₂ mixing in the water column) and partially immersed in a 2 liter tank. This tank contained a water pump connected to the metabolic chamber through a hole on the lid of the chamber also allowing water overflow and was supplied with an aeration system to maintain water fully oxygenated. A structure of 1 mm² mesh was installed at the bottom of the chamber above the magnetic stirrer in order to keep the fish away from the stirrer. Fish were introduced in each of the chambers containing filtered (0.2 µm Whatman) medium at the salinity values at which the animal was acclimated 24h before salinity transfer. After this time, the water of each tank and metabolic chamber was quickly siphoned and replaced with water at the desired salinity.

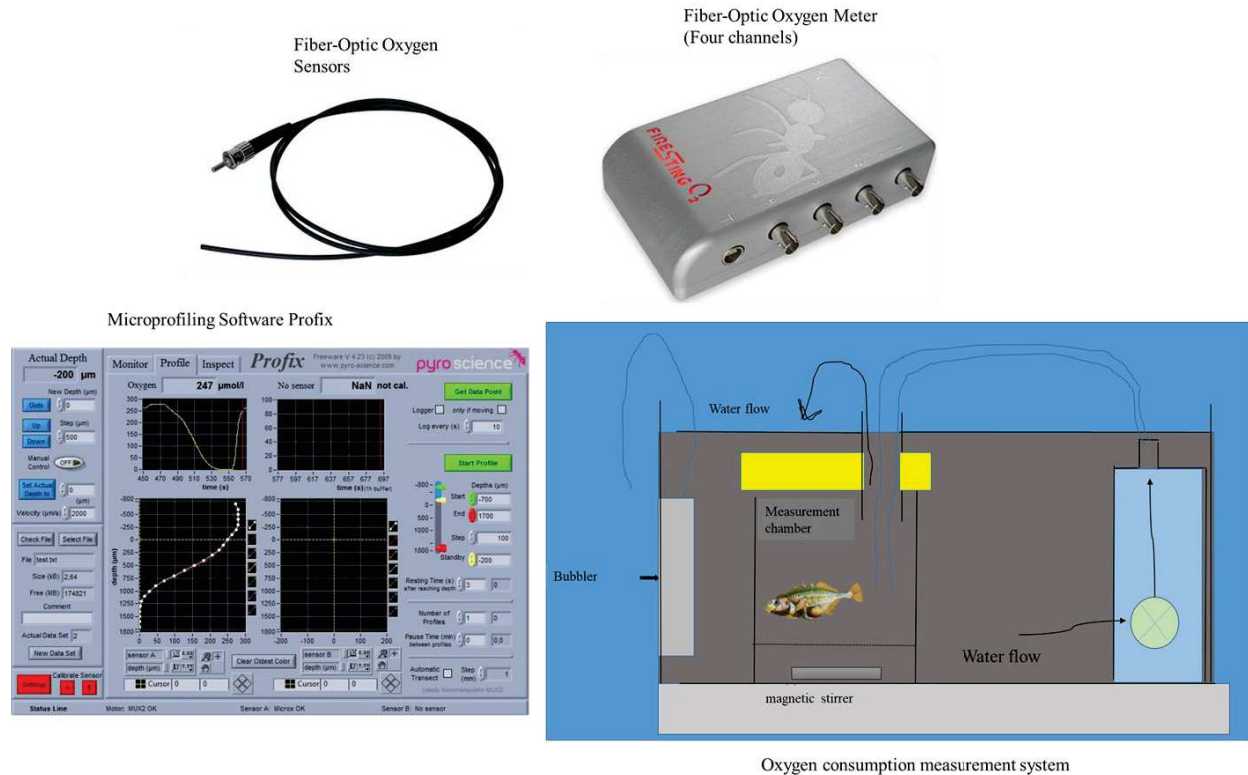


Figure 3-11. Experimental setup used for the measurement of oxygen consumption rate.

The rates of oxygen consumption were measured at different temperatures (21, 23 and 25 °C) and salinities (freshwater 0‰ and seawater 30‰), respectively. Oxygen concentration within each of the chambers was measured at different times before and after water transfer (1 hour before, immediately after transfer, 1, 2, 3, 5, 24, 72 hours after transfer and up to 3 and 6 weeks after salinity transfer) with the water pump and aeration system shut off. Oxygen concentration was measured using a four-channel fiber-optic oxygen meter (FireSting, Pyro-Science GmbH) and recorded through the Pyro Oxygen Logger software. All measurements started in fully oxygenated water (> 98%) and respiration was recorded as a function of declining pO₂ over time. Four parallel measurements were carried out at a time (including a blank) and data were recorded using 5 sec. intervals. Animals in each of the chambers were allowed to breathe for a minimum of 15 minutes at a constant and controlled temperature. After this time, animals were weighted (fresh body weight: FBW) and returned to their medium. Respiration rate (RR) were calculated through linear regression and expressed as mmol mmol O₂ h⁻¹ · g FBW⁻¹.

3.8 Molecular study

3.8.1 RNA extraction

Table 3-6. Primer sequence used to amplify the stickleback NKA $\alpha 1$ subunit and the isoform $\alpha 1a$, $\alpha 1b$, CFTR, NKCC1 and V-ATPase

Gene Symbol	Gene Name	Sequence ID ensembl or genbank	Primer sequence 5' to 3' forward/reverse	Amplicon length (bp)	Annealing temperatu re (°C)	Amplificatio n efficiency
<i>atp1a1</i>	ATPase Na ⁺ /K ⁺ transporting subunit alpha 1	BT027976	CTGCTGGACGACAACCTTGC/ GGATGTTGGCGATGATGAAG	148	60	1.82
<i>atp1a1a</i>	ATPase Na ⁺ /K ⁺ transporting subunit alpha 1 subunit 1a	ENSGAC T0000001 8945	ATGGAGGGAGGAAGAAATC G/CGGTCCCATACTTTCTGTG G	123	60	1.81
<i>atp1a1b</i>	ATPase Na ⁺ /K ⁺ transporting subunit alpha 1 subunit 1b	ENSGAC T0000001 8961	GGGAAGAAGAAGGAGAAGG A/AATCTCAGCAGCCTTGGCG	142	60	1.99
<i>uba52</i>	Ubiquitin A-52 residue ribosomal protein fusion product 1	ENSGAC T0000001 0662	CTTGAGGATGGACGGACACT/ GGCAGATCATCTTGTGCGCAG	142	60	1.92
<i>cfr</i>	cystic fibrosis transmembrane conductance regulator	ENSGAC T0000001 1967	CTTCACCGTATCCATCATCC/ GCCTCCAGTAGCTTGAGTTG	120	60	1.78
<i>atp6v1h</i>	ATPase H ⁺ transporting V1 subunit H	ENSGAC T0000002 2675	AGCAAACCGTCCAGTACATC/ GATCACTTCCCTCCATCAGA	222	60	2.02
<i>slc12a2</i>	solute carrier family 12 (sodium/potass ium/chloride transporter)	ENSGAC G0000001 8343	AGAAGAGGTGGAAGGACTG/ TGCTCCATGTGCTCTTCCTT	223	60	1.82

Total RNA was extracted using Trizol[®] reagent according to the manufacturer's instructions. RNA quantity was assessed by measuring the A260/A280 ratio using the NanoDrop[®] ND- 1000 V3300 Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE, USA) and RNA integrity was checked using an Agilent Bioanalyzer 2100 equipped with an RNA Nano Chip (Agilent Technologies, CA, USA). RNA Integrity Numbers (RIN) were greater than 9.5. One microgram of total RNA was treated with DNase I amplification grade (Invitrogen[™], Life Technologies) and the first strand of complementary DNA (cDNA) was generated using oligo (dT) primers (Invitrogen[™]) and the M-MLV reverse transcriptase (Invitrogen[™]), following the manufacturer's guidelines.

An Echo_525 liquid handling system (Labcyte Inc., San Jose, CA, USA), 0.75 μ l of LightCycler-FastStart DNA Master SYBR-Green I[™] Mix (Roche, Mannheim, Germany) was used to dispense 0.015 μ l of each primer (forward and reverse at 0.2 μ l final concentration), 0.22 μ l of ultra-pure water and 0.5 μ l of cDNA into a 384-well reaction plate. Each sample was run in triplicate. The qPCR conditions were as follows: denaturation at 95°C for 10 min, followed by 45 cycles of repeat amplification (95°C, 15 s), hybridization (60 or 62°C according to the primer pair used, 5 s) and elongation (72°C, 10 s), and a final step at 40°C for 30 s. A melting curve program was performed to control the amplification specificity. Ultra-pure water was used as a no-template control in the qPCR. Primers were designed to amplify the stickleback NKA α 1 subunit and the isoforms α 1a and α 1b as well other genes like CFTR, NKCC and V-ATPase. These primers were based on the *G. aculeatus* genome assembly (http://www.ensembl.org/Gasterosteus_aculeatus/Info/Index).

3.8.2 qPCR data analyses

Three different endogenous reference genes were tested: hypoxanthine phosphoribosyl transferase I (hprt 1), L13A ribosomal binding protein (rpl13a) and ubiquitin A-52 residue ribosomal protein fusion product 1 (uba52). The expression stabilities of these reference genes were evaluated using the BestKeeper's algorithm (Pfaffl *et al.*, 2004). The correlation coefficients were 0.822, 0.850 and 0.885 for hprt1, rpl13a and uba52, respectively, and uba52 was used as the reference gene. The expression level was normalized using the expression of the ubiquitin A-52 residue ribosomal protein fusion product 1 (uba52), which was used as reference gene as reported in Hibbeler,

Scharsack & Becker (2008). Relative gene expression was calculated as $\Delta\Delta C_t$ with E as the efficiency of target amplification (Livak & Schmittgen, 2001). Efficiencies were between 1.81 and 1.99. To normalize the data, we used a corrective formula: $R = [E_{\text{target}}^{\Delta C_{\text{target}}(\text{control-sample})}] / E_{\text{ref}}^{\Delta C_{\text{ref}}(\text{control-sample})}$

3.9 Morphometry

Fish from different sites and sampled from 1994 to 2017 were considered. After sampling, fish were directly fixed in 10% formalin and either kept in alcohol or stained with Alizarin Red S. For bone staining, fixed fish were stained overnight with 0.008% Alizarin Red S in 1% potassium hydroxide, destained in water overnight, then lightly cleared in a 0.25% potassium hydroxide, 50% glycerol solution. The number of lateral plates were then counted from 10 fish collected from the different sampling sites. Also, head height, body height, pelvic girdle length, dorsal fin length and gill raker length were measured using ImageJ (image processing program, national institute of health, Bethesda, MD, USA) and the ratio with the fish length or head height (for the gill rakers) were calculated. For gill rakers, only first gill raker from the left branchial arch was considered. Gill raker lengths were measured from the base to the tip of the rakers, counted and breadth measured as well. Digital images of fish viewed laterally and from their dissected gills were acquired on a Leica DM2500.

Fish morphology also analyzed by using landmark based geometric morphometrics. Seventeen landmarks were digitized on the right side of the body using TpsDIG (Rohlf 2005) in order to get the X and Y coordinates. Digitized landmarks, were then transferred to MorphoJ (Klingenberg) which uses principal component analysis (PCA) for shape variances, then PC coefficient scores were transferred to Excel (2013) to visualize the possible variations between FW and BW/SW fish. Also, The NKA immunolabelling of gill histological sections determined the density and size of the ionocytes, their localization along the gill filaments and lamellae. The NKA fluorescent intensity of these cells was also determined for FW and SW fish (10 individuals per condition). All gill sections were photographed at the same magnification (940). Morphometrical measures (density of chloride cells, size/area, position and intensity of ionocytes) were recorded using Image J freeware (<https://imagej.nih.gov/ij/>). For each individual, ionocytes were counted and measured on the second and third gill arches from both left and right sides. For body morphology, jaw length, snout length, head length, head height, body height, pelvic girdle length, anterior dorsal spine,

dorsal fin length, caudal peduncle length, eye circumference, eye diameter, standard length, total length were measured with ImageJ as well, for plate numbers, at least 10 individuals were counted from left side of the each individual fish considered.

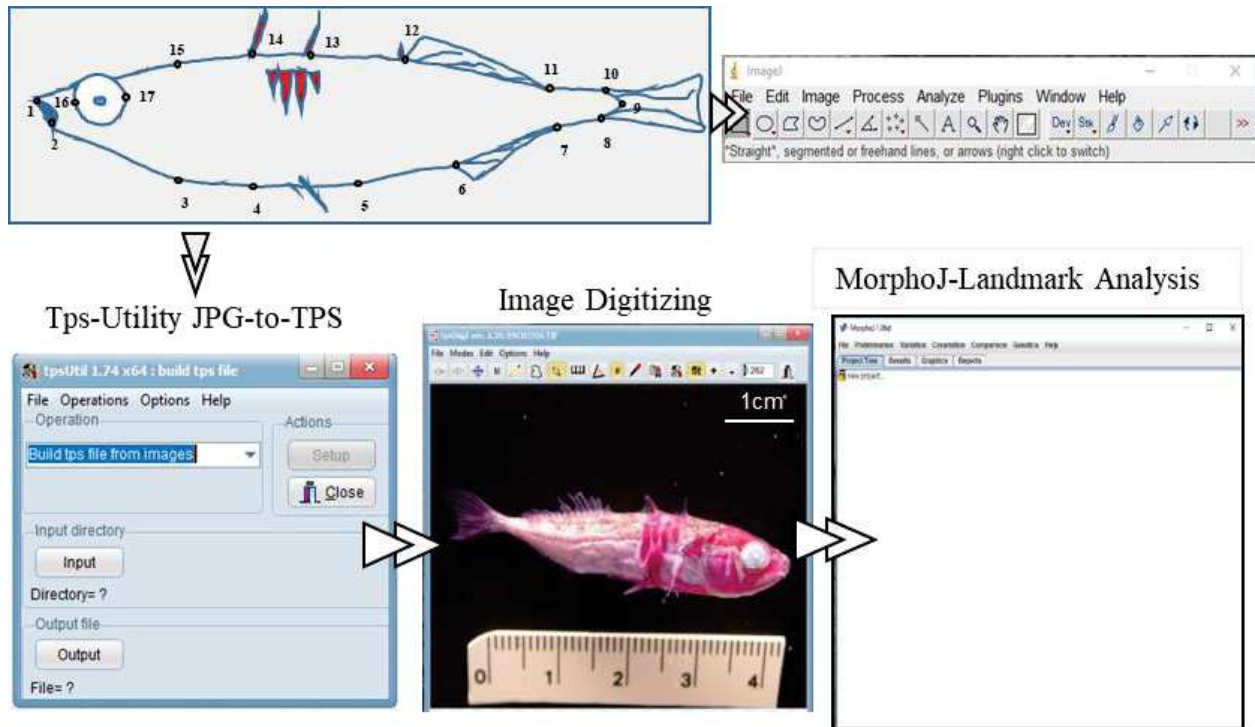


Figure 3-12. Step by step morphometric procedure of stickleback for the analysis of different landmarks.

3.10 Statistical Analysis

Data normality was checked using a Shapiro test. Homoscedasticity was tested by the Bartlett test, and the residual independence was checked with the Durbin-Watson test. Results were evaluated by one-way analysis of variance (ANOVA), followed by Tukey post-hoc test (HSD: honestly significant difference) with a significance level of $p < 0.05$, t tests were performed in order to compare the different ratios, also for ionocyte morphology, t tests were performed in order to compare cell height and cell width. A one-way ANOVA was also used to test gill raker data and ionocyte cell area. All statistical analyses were conducted using the IBM SPSS Statistics 23, and Microsoft Excel (2013). All results are presented as means \pm standard error (S.E.M).

4 Chapter 4: Results

Physiology of Threespined Sticklebacks (*Gasterosteus aculeatus*) of the Camargue at different salinities.

4.1 Blood Osmotic Pressure

Sticklebacks living in the Camargue belong to the low-plated morph, no obvious changes on behaviour, feeding habits, or survival rate (100%) of fish could be detected due to salinity acclimation. A significant difference between fish acclimated to different salinities (FW: 5‰ & 147 mOsm kg⁻¹; BW: 15‰ & 457 mOsm kg⁻¹; SW: 30‰ & 911 mOsm kg⁻¹) was observed in blood osmotic pressures ($F = 74.36$; $P < 0,001$; Fig. 4.1).

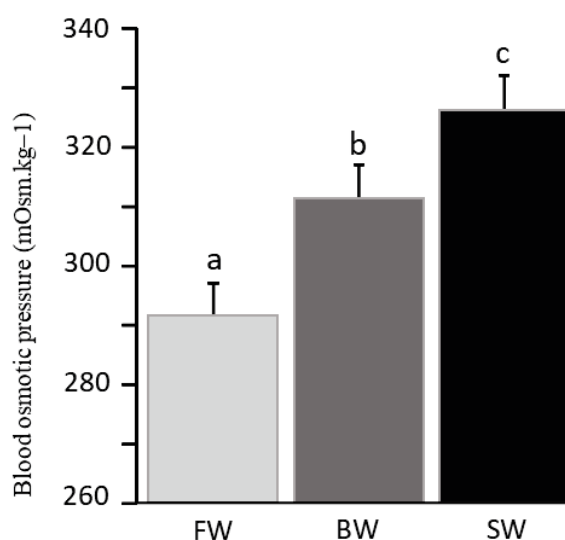


Figure 4-1. Blood osmotic pressure (in mOsm kg⁻¹) from FW, BW and SW acclimated fish (n = 8, 9 and 11 respectively). Different letters indicate statistical differences.

4.2 Stickleback morphometric

4.2.1 Gill ionocytes Morphology

Ionocytes were identified using Na⁺/K⁺-ATPase labelling by immunofluorescence on histological sections and on semi-thin sections (Fig. 4.2; 4.3C). These cells were located along the gill filaments and lamellae in fish maintained in FW and BW, and along the gill filaments in SW-acclimated fish (Fig. 4.3A–C). Drastic differences were observed in gill ionocyte location and size following transfer of the fish from FW to SW. However, fish transferred from FW to BW presented only

mitigated responses. Therefore, comparisons with data from fish transferred to BW were not considered. In fish kept in FW, ionocytes located on the filaments were distinctively elongated from their apical to basal sides ($P < 0,001$), and those located along the lamellae appeared systematically flattened and wider ($P < 0,001$) (Fig. 4.3C). Although different in morphology, these ionocytes did not differ in terms of surface area ($P = 0.51$). In contrast, ionocytes in fish acclimated to SW were round in shape ($P < 0,001$) and were only located along the gill filaments in the interlamellar epithelium of the gills.

Furthermore, ionocyte density within a distance of 100 μm of gill filament was significantly higher in the gills of FW-fish (26 ± 7) compared to SW-acclimated fish (12.5 ± 3.5) ($P < 0.05$). This is due to the presence of lamellar ionocytes in FW-sticklebacks. Ionocytes are therefore twice more numerous along the gills of FW-acclimated fish compared to SW fish. Ionocytes of SW-fish and are also slightly smaller.

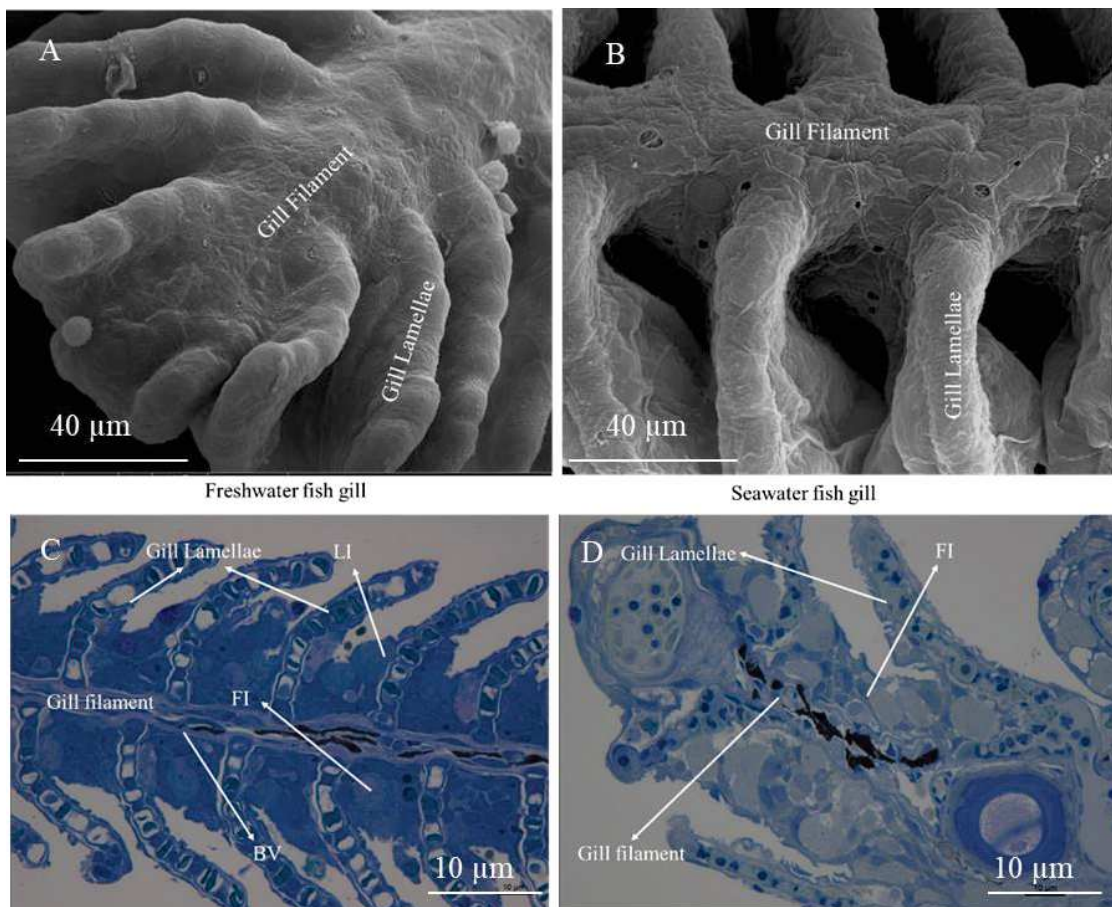


Figure 4-2. Freshwater and seawater SEM and semi thin gill structure with ionocytes, (A&C FW gills, B&D SW gills), FI, filament ionocytes; LI, Lamellae ionocytes; BV, blood vessels.

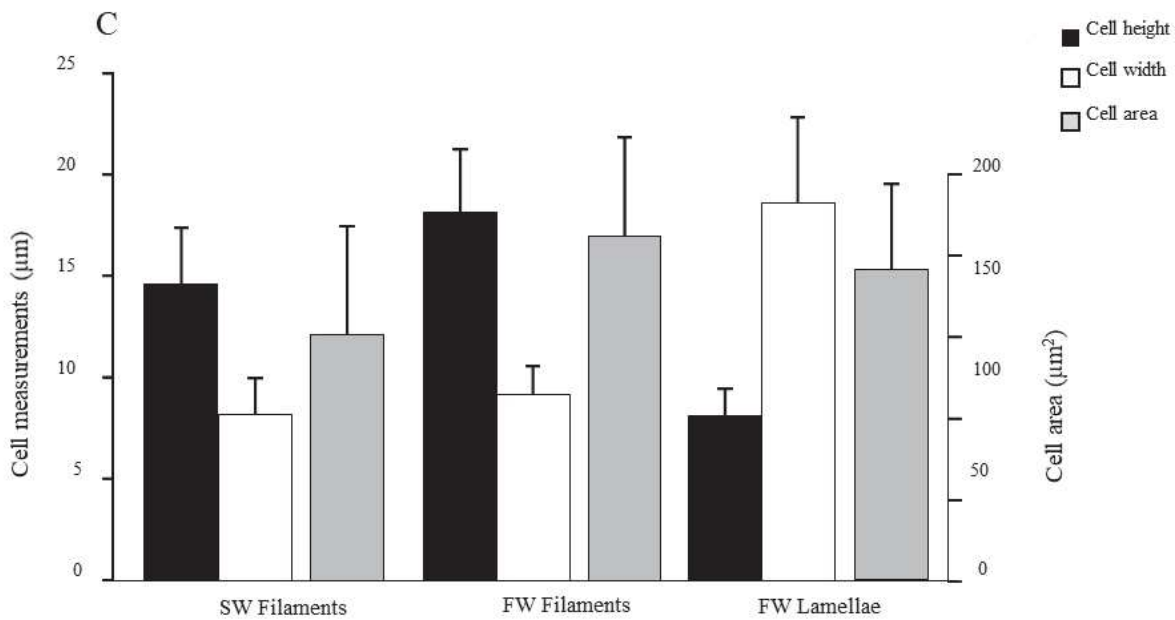
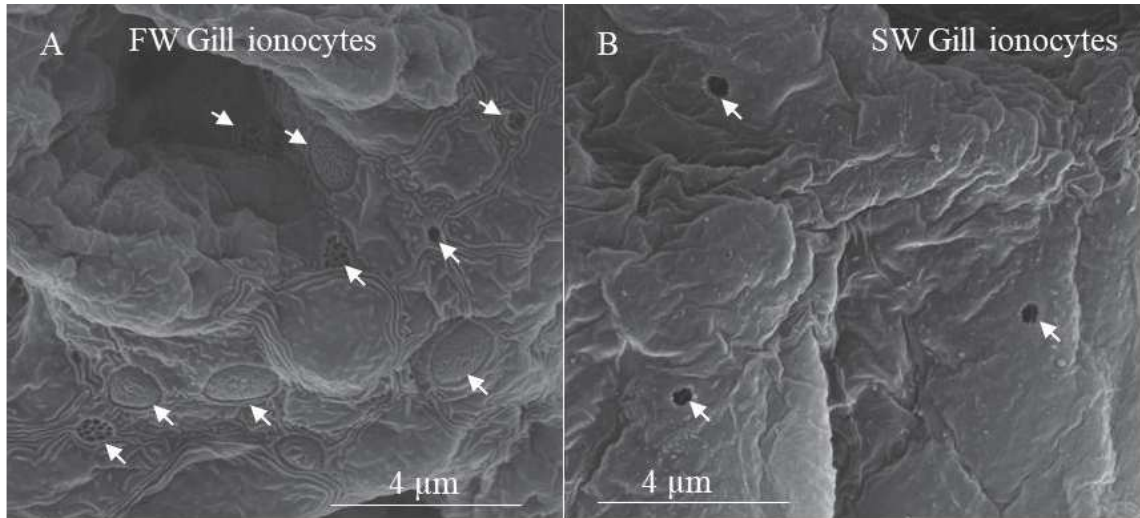


Figure 4-3. SEM of gill with ionocytes: (A) a fish maintained in FW with gill ionocytes, and, (B) a fish transferred to SW with ionocytes. (C) ionocyte morphological measurements. Measurements were obtained from 12 fish maintained in FW and 12 fish transferred to SW. Means \pm SE. BV, blood vessel; GF, gill filament; GL, gill lamellae; FI, filament ionocyte; LI, lamellar ionocyte.

4.2.2 Gill rakes

Stickleback sampled from Camargue. Sampled fish since 1984 and fished fish in 2015, 2016 and 2017. For gill rakers, only first gill raker from the left branchial arch was considered. Gill raker lengths were measured from the base to the tip of the rakers. However, a significant difference is

observed in the morphology of gill rakers between FW and SW/BW sticklebacks. Gill raker length and gill raker density are significantly higher in sticklebacks living in the FW canals compared to the fish living in mesohaline lagoons (SW/BW). (Fig. 3.4).

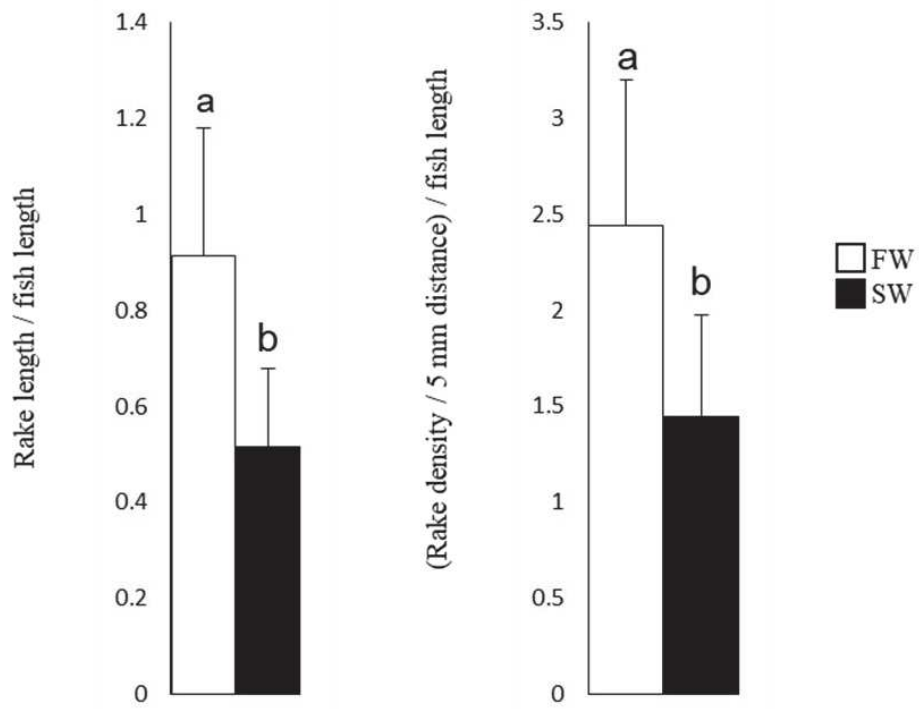
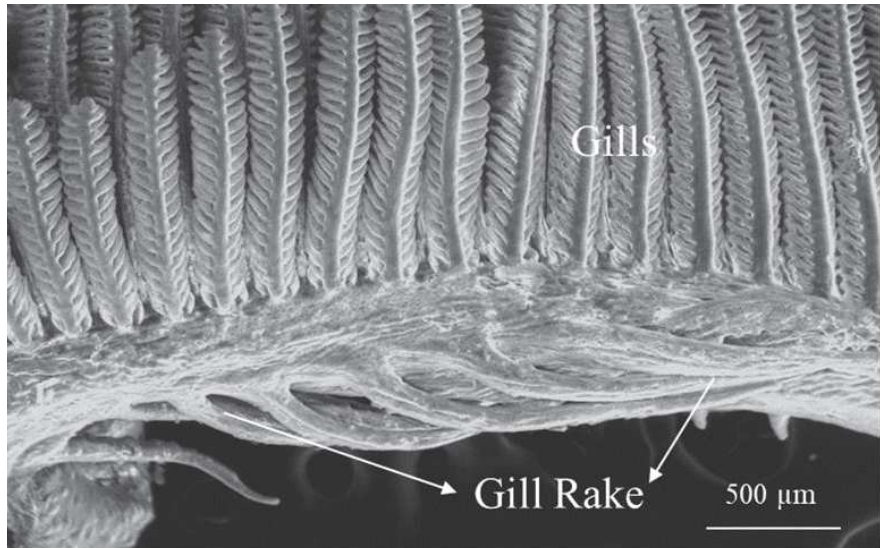


Figure 4-4. Gill rake length and density for FW and SW sticklebacks of the Camargue area.

4.2.3 Body Morphology

Sticklebacks fished since 1984 from different aquatic habitats of the Camargue area presented on average 4.45 ± 0.31 lateral plates, with a minimum of 2 and a maximum of 6 lateral plates observed for some of these fish. No statistical differences in lateral plate number between the different locations and salinities could be identified (Fig. 4.6). Also, no statistical significant differences were observed in terms of body morphology between Sticklebacks sampled in 2016 and 2017 (Fumemorte, Vaccarès, Marteau and Versadou) and those sampled in 1984 (Malagroy, La Capeliere, Fournelet, Baisse Salee, and Giraud; Fig. 4.5A).

The morphogeometric analysis for 17 different morphological traits analyzed for fish from the mesohaline lagoons and freshwater habitats, revealed that 32.937% of the variance is explained by the first component while the second component accounts for only 16.75% of the variance (Fig. 4.5B). The scattered plot for the first and second components indicates limited morphological variations between FW and SW/BW sticklebacks.

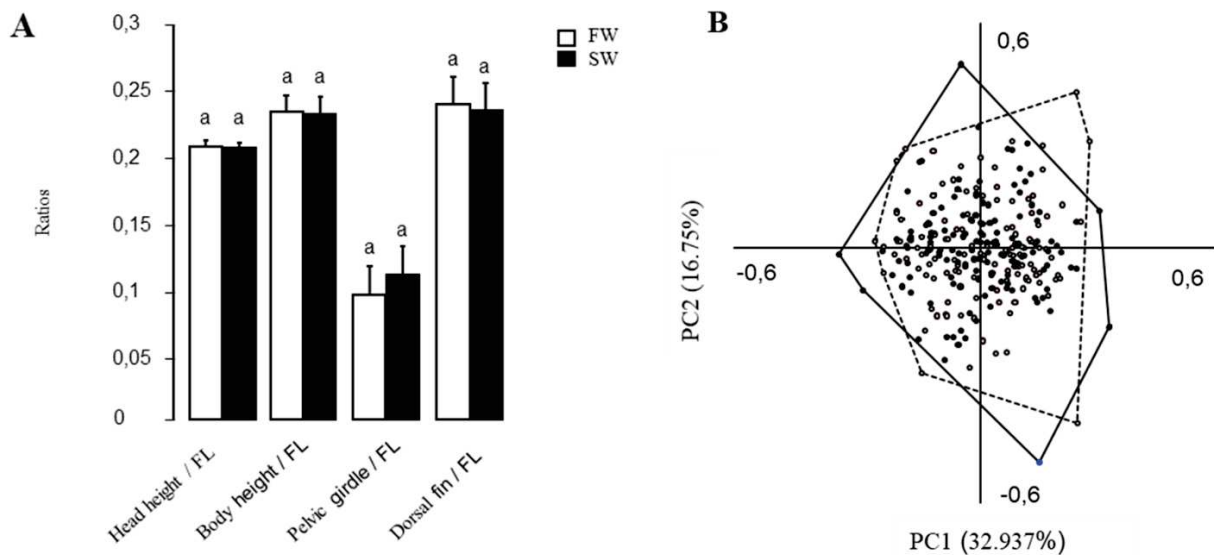


Figure 4-5. Body morphology of sticklebacks sampled from FW and mesohaline (SW) habitats, (A); ratios of different ecological significant traits of freshwater and seawater sticklebacks. (B); first and second principle components of morphological variance between FW and SW sticklebacks.

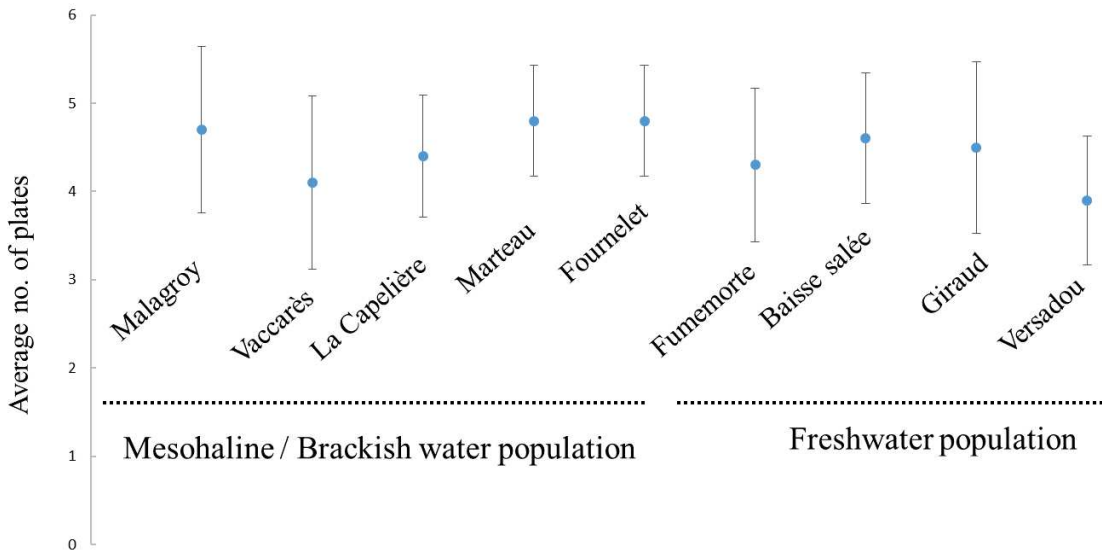


Figure 4-6. Number of plates among sticklebacks of camargue.

4.3 Immunolocalization of gill NKA

Gill ionocytes were identified using NKA immunofluorescence labelling on histological sections. Ionocytes were observed on gill filaments and on gill lamellae (Fig. 4.7D, C). These ionocytes were observed for both freshwater and seawater fish. Interestingly, ionocytes were only expressed on gill lamellae and filaments of freshwater fish (Fig. 4.7A). For seawater acclimated fish, as expected, we could hardly observe any ionocytes on the gill lamellae (Fig. 4.7B).

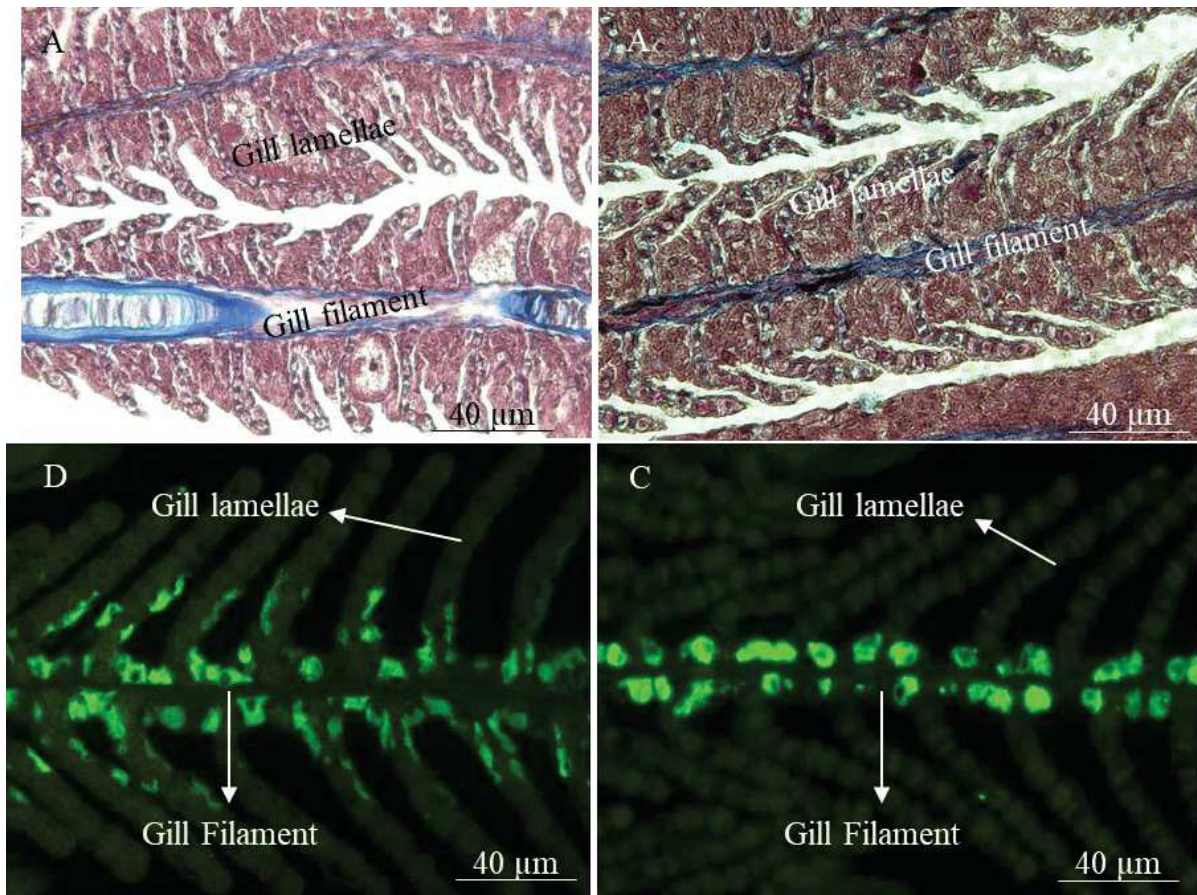


Figure 4-7. Microscopic studies of the gills, (A & B) classical staining of FW & SW gills, (C) freshwater gill ionocytes expressed on both gill filament and as well as on gill lamellae, (D) seawater gill ionocytes expressed only along the gill filament.

4.4 Immunolocalization of Kidney NKA

Histological sections were selected for kidney NKA immunolabeling from sticklebacks kept in freshwater and acclimated to seawater conditions. Previous comparative studies revealed that nephrons are better developed in freshwater adapted fish compared to marine fish (Wendelaar, 1973). In the case of sticklebacks from the Camargue, we did not observe any difference between freshwater- and seawater-acclimated fish. This could suggest a high level of plasticity in local adapted environment (Wendelaar, 1973; Fig. 4.8, 4.9), or the use of an inappropriate tool. Therefore, more work is necessary using Western blots and electron microscopy in order to better identify the NKA protein expression and the morphology of the kidney tubules.

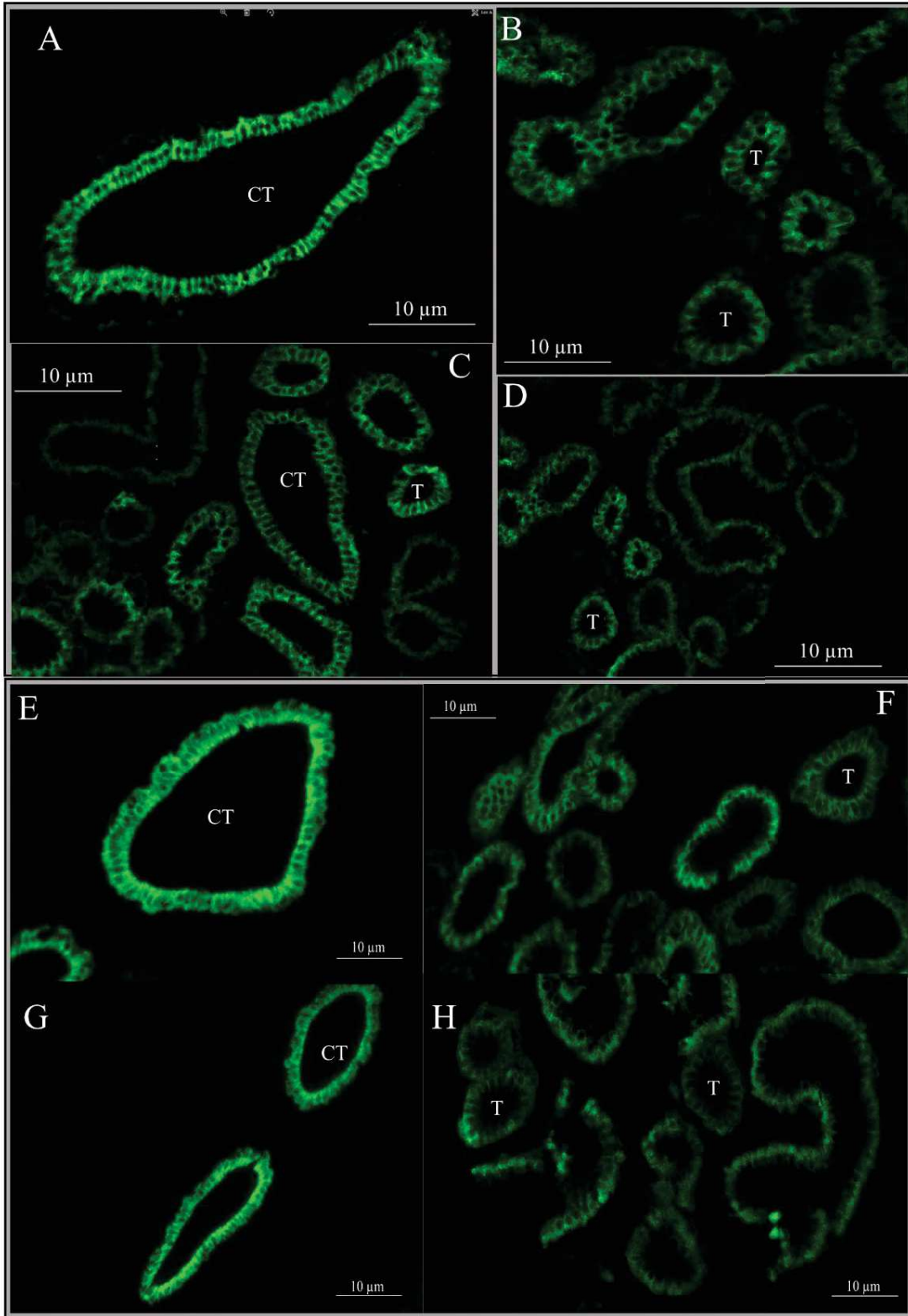


Figure 4-8. NKA Immunolocalization of the kidney, A, B, C, and D Freshwater fish, E, F, G, and H seawater acclimated fish. CT: collecting duct; T Proximal and/or distal tubule.

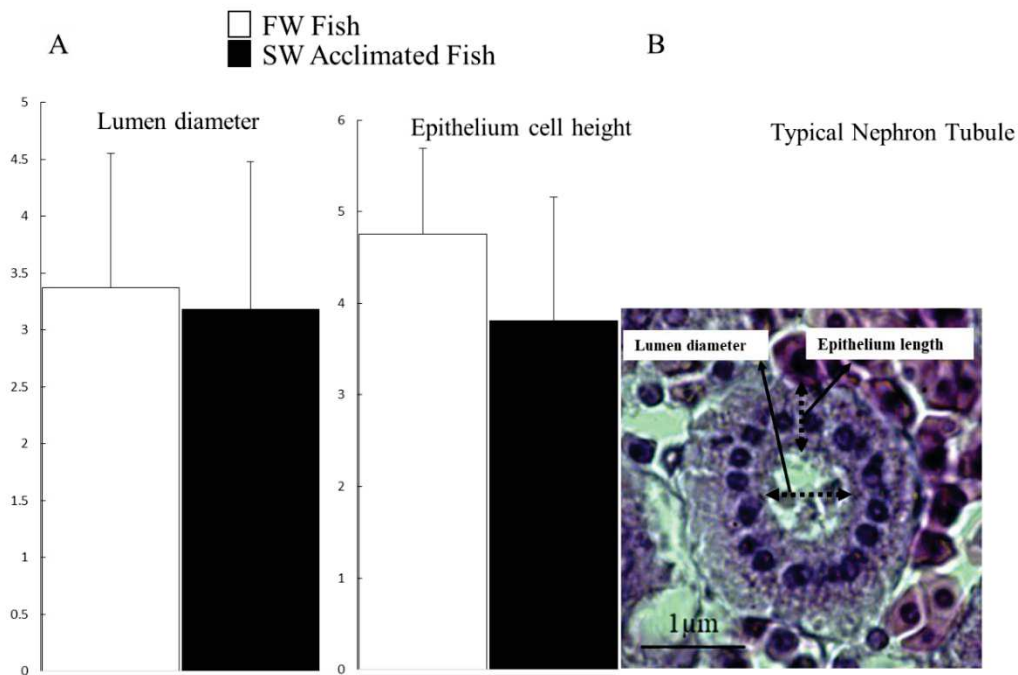


Figure 4-9. Kidney tubule morphology of sticklebacks collected from the Camargue aquatic habitats. A) Tubule diameter and epithelial thickness of nephrons tubules of freshwater and seawater acclimated fish, B) histological section of a tubule epithelium with the morphological measurements.

4.5 Immunolocalization of Intestine NKA

The anterior and posterior intestine of freshwater and seawater acclimated sticklebacks was studied (Fig. 4.10). Strong immunofluorescence was observed along the basolateral membranes of enterocytes of both anterior and posterior intestine of the freshwater and seawater acclimated sticklebacks. However, no significant difference could be observed in the protein expression of NKA between freshwater and seawater acclimated fish from these histological sections.

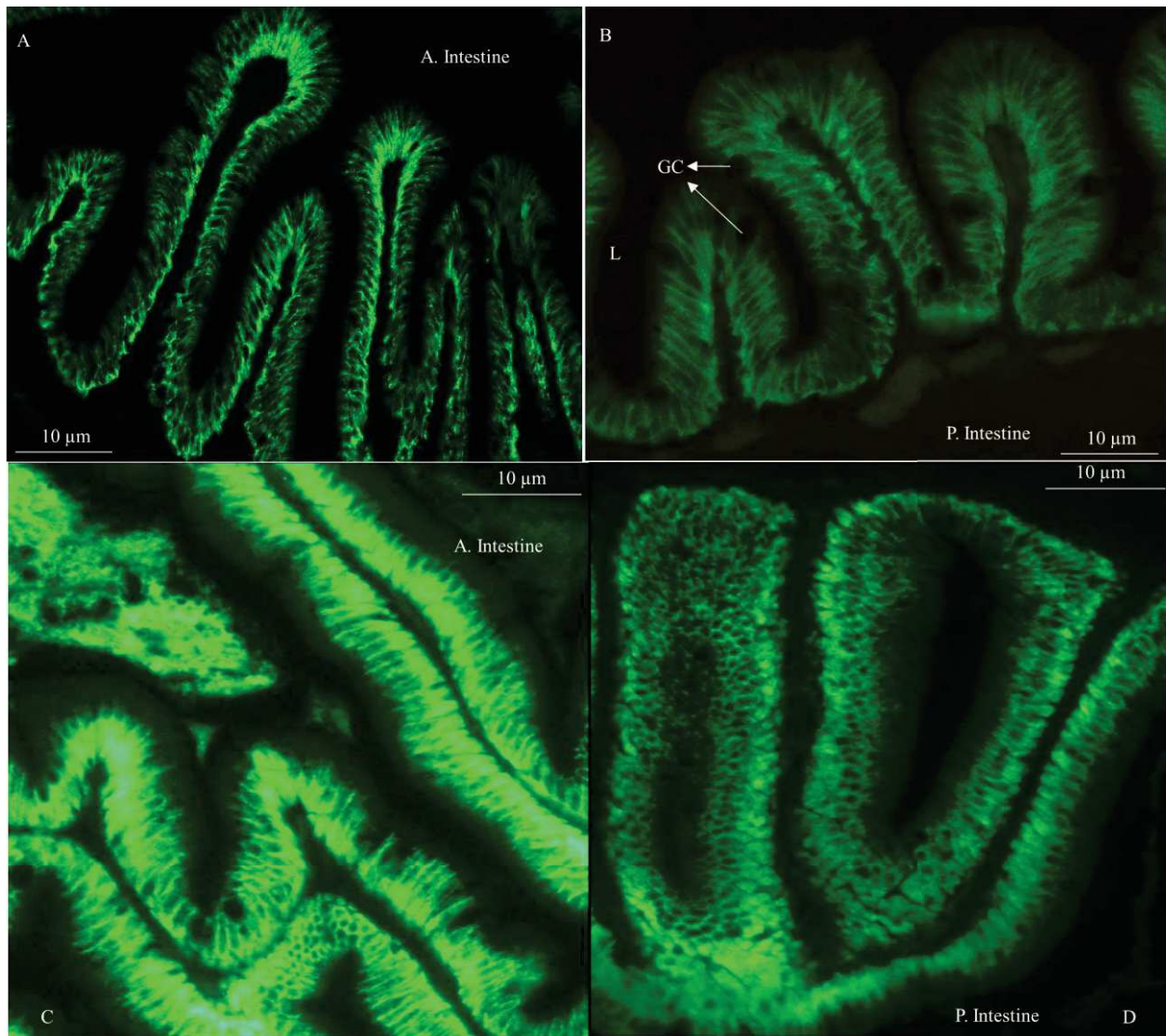


Figure 4-10. NKA Immunolabeling of the NKA in the intestine, A) anterior intestine and B) posterior intestine of a freshwater stickleback. C) anterior intestine and D) posterior intestine of a seawater-acclimated stickleback. GC. Goblet cells, L. lumen.

4.6 Relative gill NKA gene expression

Transcript levels of the reference gene *uba52* did not change between salinities ($P = 0.27$). For $NKA\alpha1a$ and $NKA\alpha1b$, mRNA relative gene expressions in the gills were significantly different depending on the salinity tested ($P < 0.01$ respectively) (Fig. 3.11). However, for $NKA\alpha1\alpha$, mRNA relative gene expressions in the gills are not different due to salinity ($P = 0.09$). Relative $NKA\alpha1a$ mRNA expression was higher in the gills of SW-acclimated fish compared to BW fish and expression of $NKA\alpha1b$ was significantly higher in the gills of SW-acclimated fish compared to

the other two salinities (Fig. 4.11). Whereas, for CFTR and V-ATPase expression, we didn't find any statistically significant differences among different salinity conditions (Fig. 4.12A, B). On the other hand, the NKCC1 over expressed in seawater condition (Fig. 4.12C).

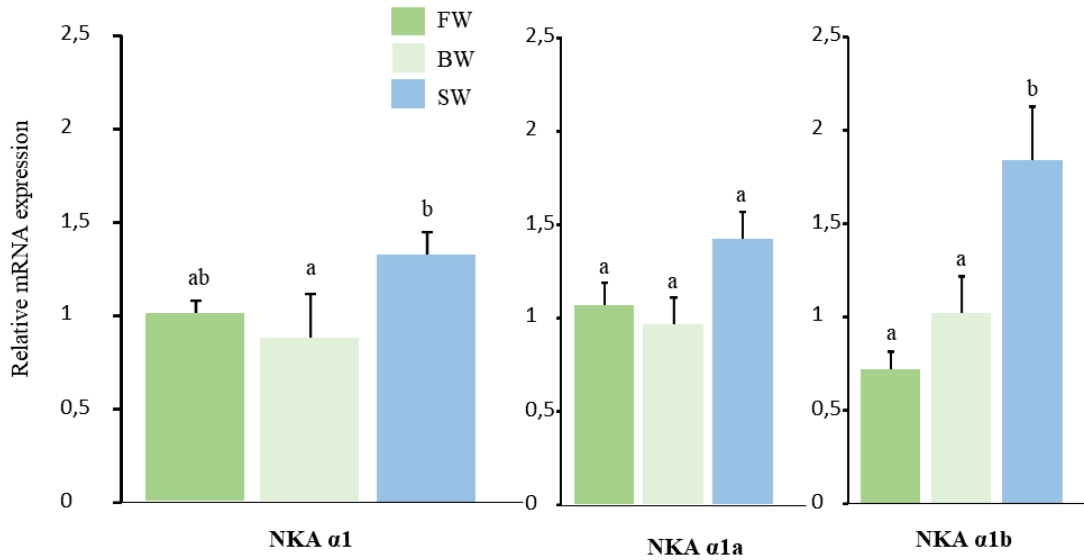


Figure 4-11. Relative mRNA gene expression for the NKA $\alpha 1$ and NKA isoforms $\alpha 1a$ and $\alpha 1b$ from fish maintained in FW (n = 8) or acclimated to BW (n = 8) or SW (n = 8). Different letters indicate statistical differences (P < 0.05). Means \pm SEM.

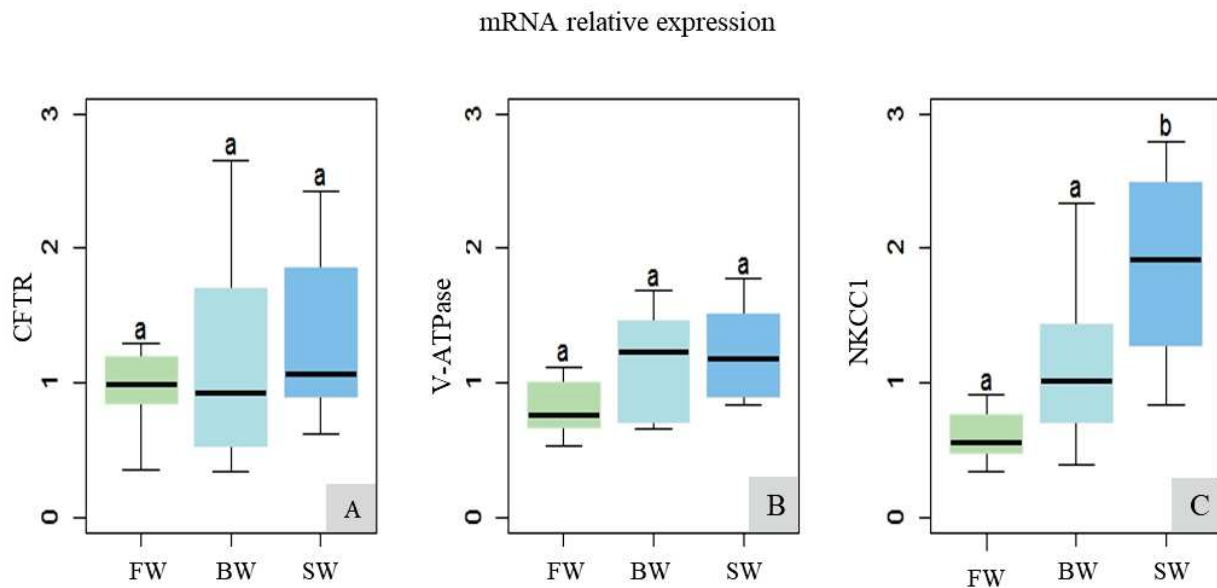


Figure 4-12. Relative mRNA expression of different co-transporters in three salinity conditions, freshwater (FW), brackish water (BW) and seawater (SW), (A), relative mRNA expression of CFTR, (B), relative mRNA expression of V-ATPase, and (C), relative mRNA expression of NKCC1.

4.7 Gill ionocytes ultrastructure

Only fish maintained in FW and those acclimated to SW were considered. Scanning and transmission electron microscopy observations revealed different cell morphologies along the gill surface of these fish (Fig. 4.13). Pavement cells and ionocytes were identified at the two tested salinities but with different morphologies for the ionocytes. In fish maintained in FW, two types of ionocytes were observed: the dome-shaped cells with some spherical elements covering the surface, and ionocytes with a honeycomb-like structure (Fig. 4.13a–c). Occasionally, a different cell type is observed that could be transitional with a reorganization of the apical spherical elements and a deepening of the surface (Fig. 4.13c). All these cell types were confirmed as ionocytes due to their internal structures filled with numerous mitochondria and a dense tubule-vesicular system, consisting of deeply invaginated basal membrane (Fig. 4.13d). In fish acclimated to SW, only ionocytes with a large apical crypt were observed along the gill filaments at the base of the gill lamellae, and these cells were organized as multicellular complexes. No ionocytes could be observed along the gill lamellae.

4.8 Oxygen consumption rate

Sticklebacks collected from the SW ‘Marteau’ lagoon, FW ‘Versadou’ canal and ‘Vacarres’ mesohaline lagoon and kept either in SW or FW presented similar oxygen consumption rates (Fig. 4.15). Also, salinity transfer from SW to FW or FW to SW did not induce any change in the rate of oxygen consumption when recorded after 24 and 72 hours after transfer (Fig. 4.15A, B). However, when measured at different temperatures (21, 23 and 25 °C), oxygen consumption rates were significantly different with higher oxygen consumption at higher temperature (Fig. 4.15B). Sticklebacks collected from the ‘Vacarres’ mesohaline lagoon kept initially in SW presented a significant reduction in oxygen consumption rate immediately and up to 1 hours after abrupt transfer to FW (Fig. 4.16A). However, fish collected from the same environment and kept in FW prior to SW transfer did not modified their oxygen consumption rate during this 6-hour course experiment.

A longer acclimation to SW or FW up to 3 and 6 weeks did not induce any change in oxygen consumption rate compared to fish kept in FW and SW (Fig. 4.16B).

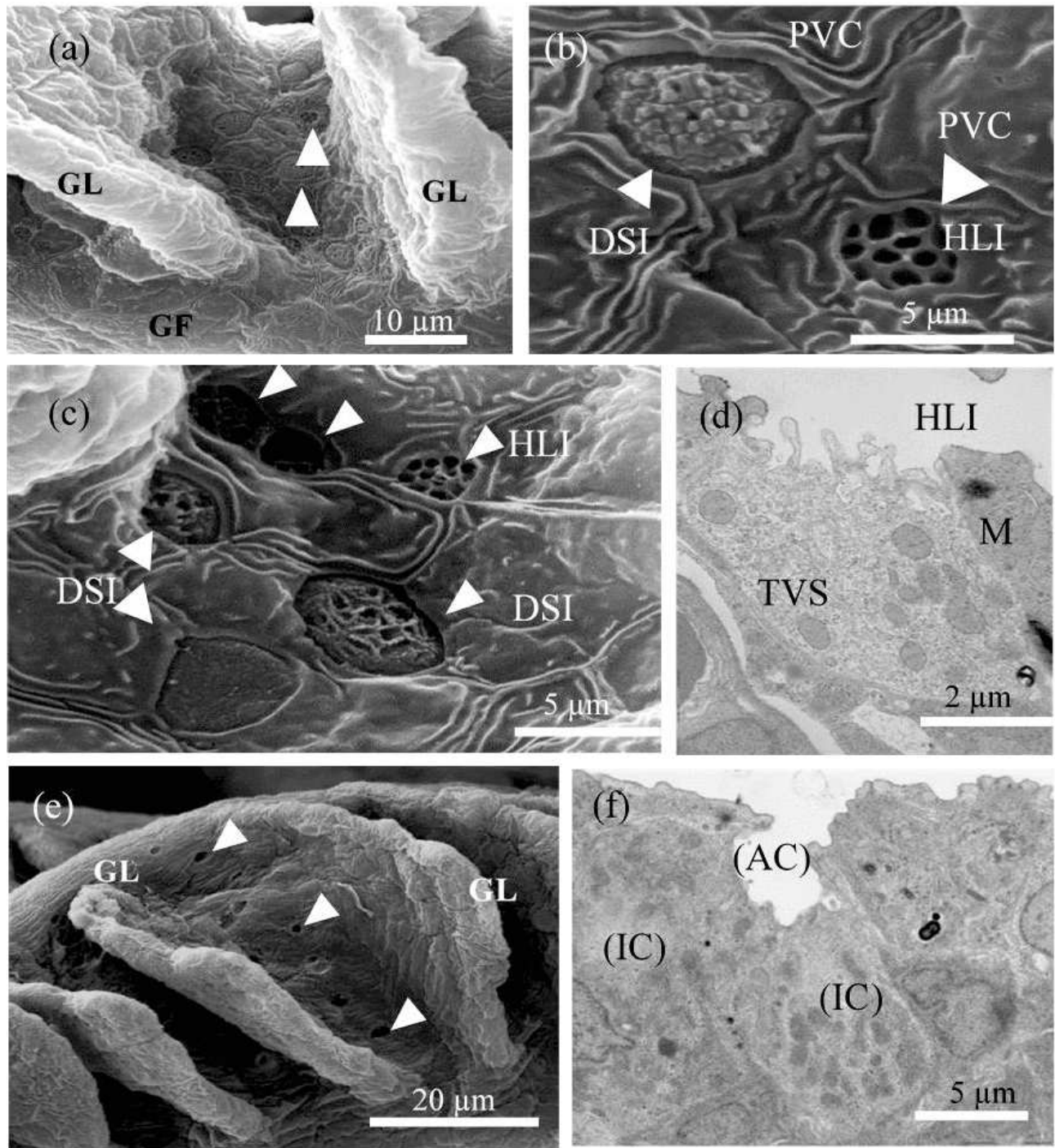


Figure 4-13. Scanning and transmission electron microscopy observations of gill ionocytes of FW and SW fish. Framed a, b, c, d: FW fish; framed e, f: SW fish. AC, apical crypt; DSI, dome-shape ionocyte; GF, gill filament; GL, gill lamellae; HLI, honeycomb-like ionocyte; IC, ionocyte; M, mitochondria; PVC, pavement cell; TVS, tubule-vesicular system. Scale bar: 10 μm (a), 5 μm (b, c), 2 μm (d), 20 μm (e), 5 μm (f).

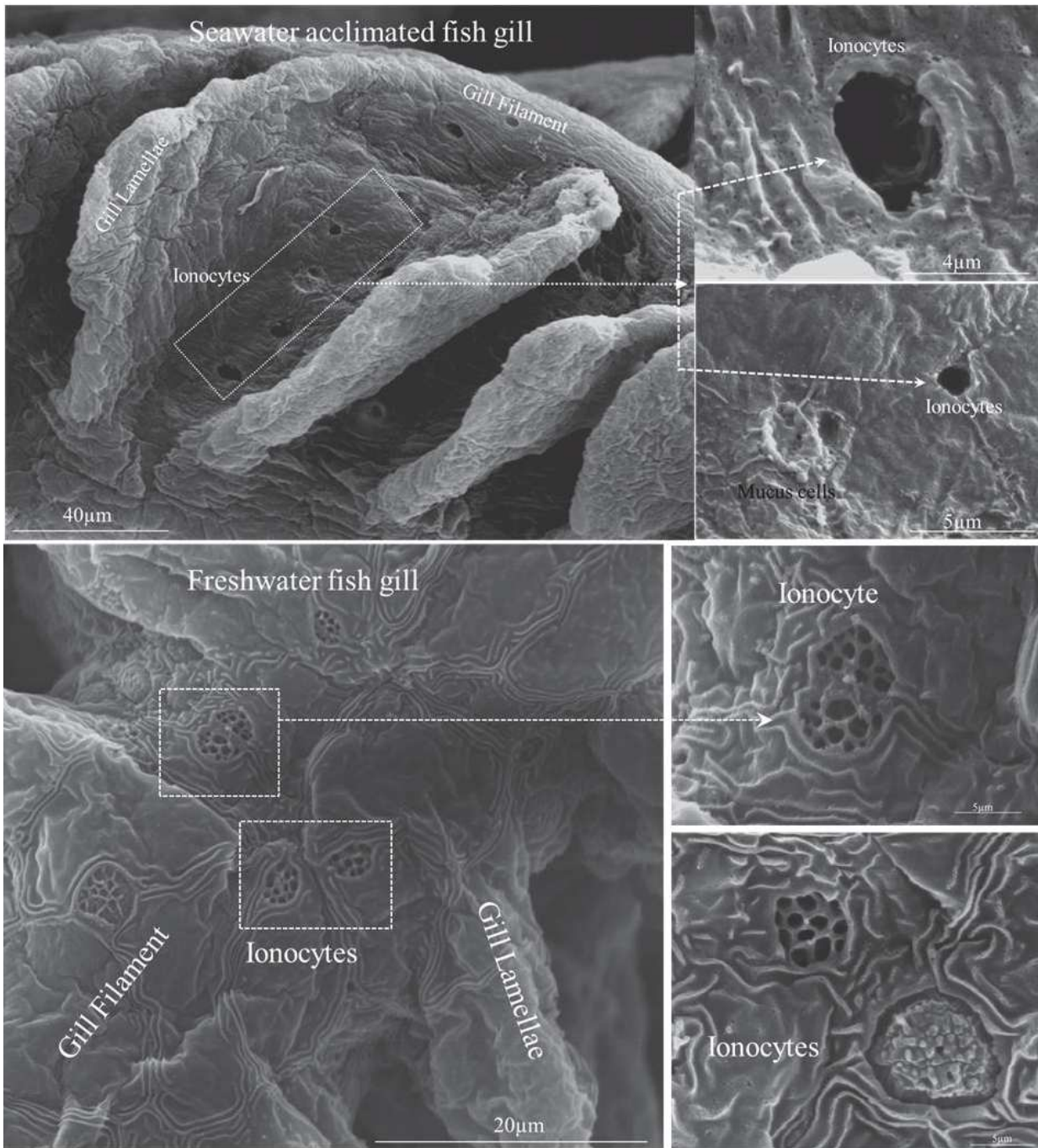


Figure 4-14. Scanning electronic microscopy of Gills of freshwater sticklebacks and seawater acclimated sticklebacks.

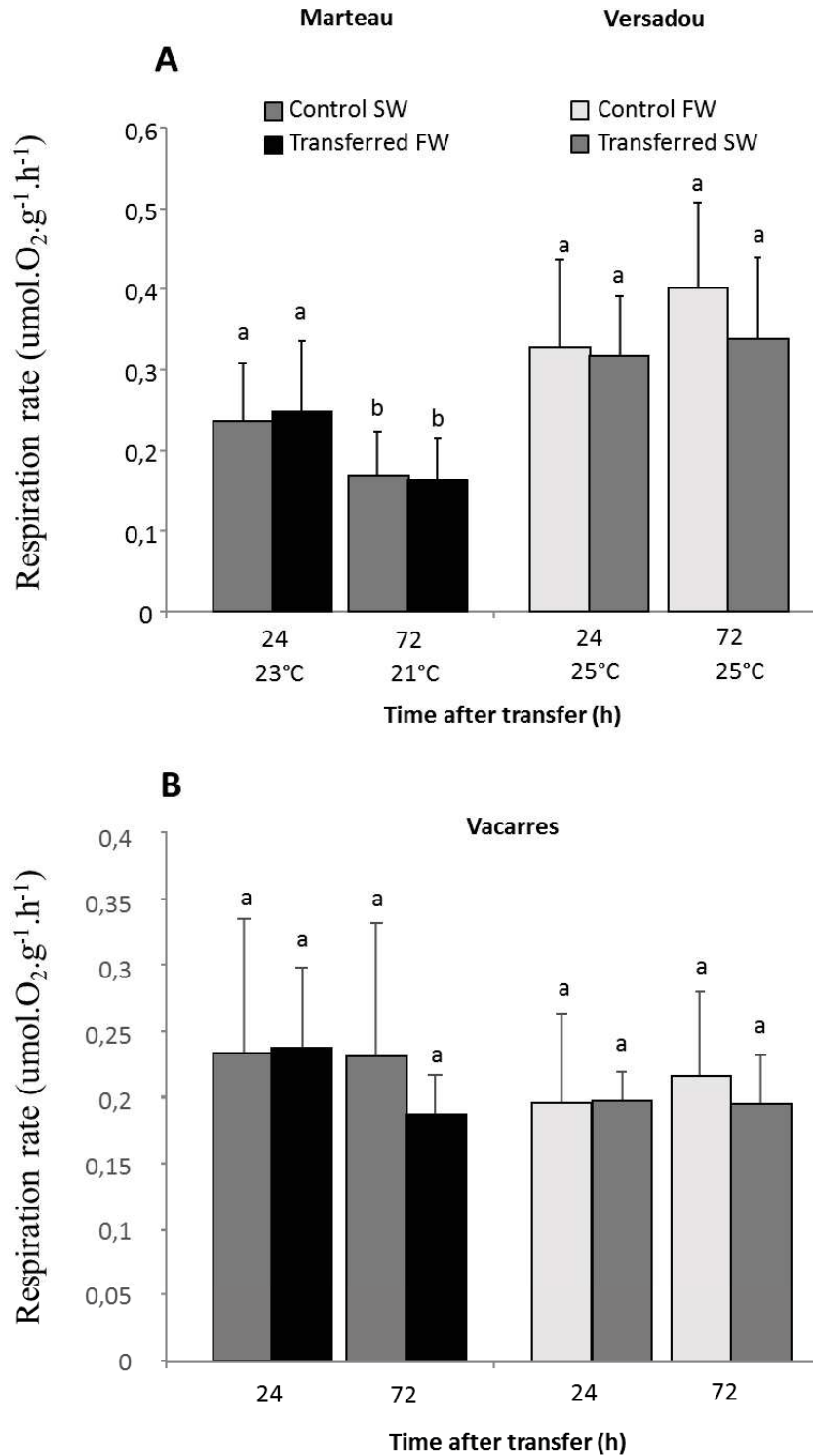


Figure 4-15. Oxygen consumption rate for sticklebacks sampled from the FW Versadou canal and the SW Marteau lagoon and 24 and 72 hours after transfer from SW to FW and from FW to SW, respectively (A). Different temperatures were used: 21, 23 and 25°C; (B) Oxygen consumption rates for sticklebacks sampled from the mesohaline Vaccarès lagoon maintained in SW or FW and 24 and 72 hours after transfer to FW and SW, respectively.

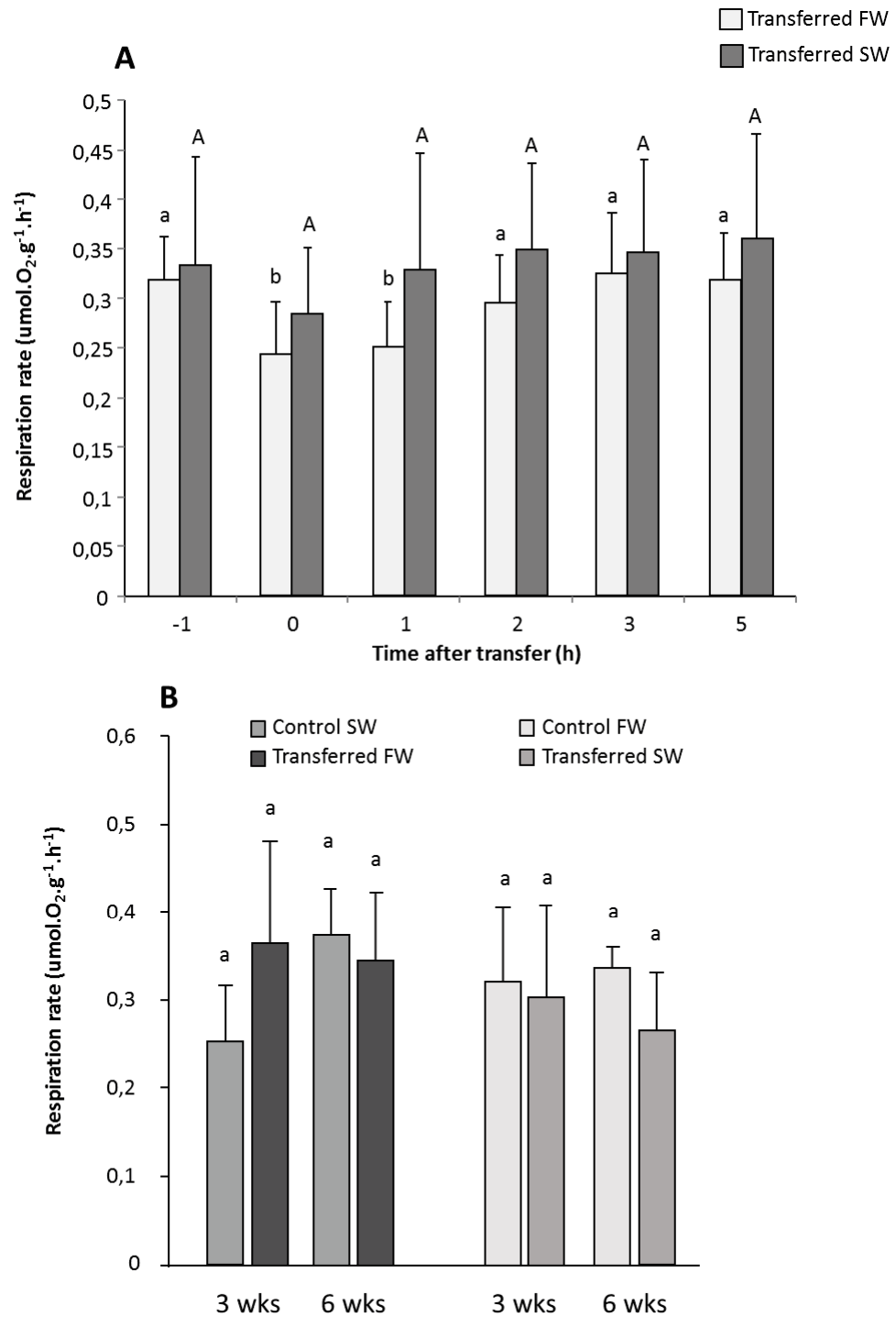


Figure 4-16. Oxygen consumption rate for sticklebacks sampled from the mesohaline Vaccarès lagoon maintained in SW or FW and abruptly transferred up to 5 hours to either FW or SW (A). Oxygen consumption rate for sticklebacks from the Vaccarès lagoon maintained in SW or FW and transferred to FW and SW, respectively for 3 and 6 weeks (B).

4.9 Branchial Na⁺/K⁺-ATPase activity

Following direct transfer from SW to FW, branchial Na⁺/K⁺-ATPase activity of sticklebacks sampled from the SW 'Marteau' lagoon significantly increased by 28% and 40% within 24 and 48 hours post transfer, respectively (Fig. 4.17). However, when sticklebacks sampled from the FW 'Versadou' canal are transferred from FW to SW, gill Na⁺/K⁺-ATPase activity is significantly decreased by 36% within 24 hours. Therefore, there is a 1.65 fold change in gill Na⁺/K⁺-ATPase activity when fish are abruptly transferred to a different salinity. Furthermore, the enzyme activity for fish acclimated or transferred to SW or FW appeared similar although these fish were sampled from two contrasted habitats.

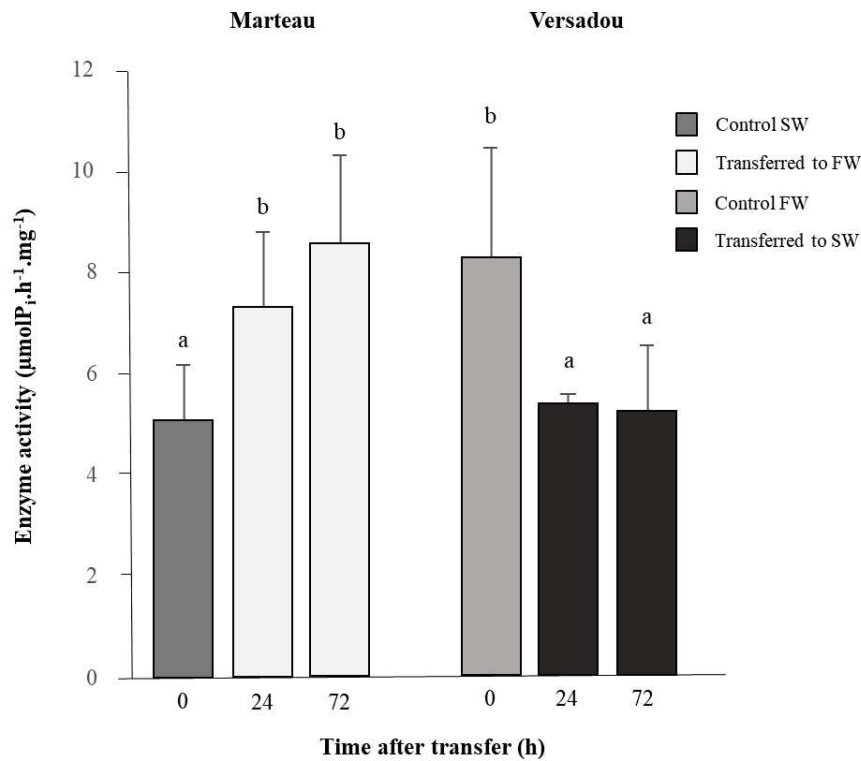


Figure 4-17. Branchial Na⁺/K⁺-ATPase activity for fish sampled from the SW Marteau lagoon and the FW Versadou canal and 24 and 72 hours after transfer from SW to FW and from FW to SW.

5 Chapter 5: Discussion, conclusion and perspectives

Physiology of Threespined Sticklebacks (*Gasterosteus aculeatus*) of the Camargue at different salinities.

5.1 General physiology of osmoregulation

It is generally accepted that osmoregulation is a costly function that must be fueled to allow homeostasis of osmotic and ionic variables in internal body fluid. Nevertheless, changes in blood osmolality are often used as an indicator of salinity acclimatory responses since both, salinity tolerance and osmoregulatory functions are closely linked to each other (Varsamos *et al.*, 2005).

The three-spined stickleback population of the Camargue area rapidly acclimates to various salinity conditions. Also, the osmotic pressure of the blood measured in these fish kept at different salinities was always close to 290 mOsm Kg⁻¹, although, these values were significantly lower in freshwater (FW) fish compared to seawater (SW) acclimated fish. However, plasma osmolality measured in FW and brackish water (BW) sticklebacks from the Beke river and Warnow estuary (northeastern Germany) maintained in BW (10‰) and transferred to SW (35‰) (Schaarschmidt *et al.*, 1999), were higher (circa 335 and 380 mOsm Kg⁻¹ respectively) than the values obtained for the Mediterranean population of the Camargue region. Such a difference could be due to specific functionality of the osmoregulatory machinery between the Mediterranean Sea and North Sea phylogeographic groups (Mäkinen *et al.*, 2008).

Most fish species living in their native freshwater or marine environments are stenohaline and are not able to handle non-native salinities. However, for euryhaline fish such as sticklebacks, the osmoregulatory machinery is adapted to a wide range of salinities. These fish can sustain salinity variations of their environment and move or migrate to non-native salinities (Moyle and Cech, 1996). For instance, the expression of genes related to the osmoregulation is considered to be reduced in native environments because of the selection for reduced energy expenditure (Aykanat *et al.*, 2011). This physiological plasticity facilitates acclimation and the colonization of novel environments, leading ultimately to adaptive evolution (Watt, 1985; Schulte, 2001; Dalziel *et al.*, 2009; Komoroske *et al.*, 2016).

Sticklebacks from the FW canal ‘Versadou’ and the mesohaline ‘Vaccarès’ and ‘Marteau’ lagoons and kept either in FW or SW displayed similar oxygen consumption rates. Also, salinity transfer from FW to SW or SW to FW did not induce any change in the rate of oxygen consumption when recorded after 24 and 72 hours after transfer. Whereas, Sticklebacks collected from the mesohaline

‘Vaccarès’ lagoon and kept in SW presented a significant reduction in oxygen consumption rate immediately and up to 1 hour after abrupt transfer to FW. However, fish collected from the same environment and kept in FW prior to SW transfer did not modified their oxygen consumption rate during this 6-hour course experiment. A longer acclimation to SW or FW up to 3 and 6 weeks did not induce any change in oxygen consumption rate compared to fish kept in FW and SW.

The induced cost of osmoregulation due to changes in salinity are often reflected by changes in the oxygen consumption rate (Sangiao-Alvarellos *et al.*, 2003; Gracia-Lopez *et al.*, 2006). The measurement of oxygen consumption rate, therefore, is an indirect indicator of metabolism in fish (Cech, 1990), and numerous authors have used respiratory methods to assess the standard metabolic rate (SMR), the active / maximum metabolic rate (MMR), the scope for activity, the osmoregulatory cost in fish and, their swimming capacity (Morgan and Iwama 1998; da Silva Rocha *et al.*, 2005; Tsuzuki *et al.*, 2008). For example, resident freshwater sticklebacks have a reduced capacity for prolonged swimming when compared to the anadromous, marine ancestors also collected from the Salmon River (a tributary of the Fraser River, near Fort Langley, British Columbia, Canada) (Taylor and McPhail, 1986). Also, in a polymorphic stickleback population from the freshwater river Sheldt (near Ghent, Belgium), the fully-plated *trachurus* morph have, under similar environmental conditions, higher SMR, MMR and scope for activity compared to the low-plated fish (Tudorache *et al.*, 2007). Furthermore, for wild-caught marine and non-migratory resident freshwater sticklebacks from Bonsall and West Creeks (British Columbia, Canada), a significant difference in MMR and scope for activity was observed, although no difference in SMR could be detected (Dalziel *et al.*, 2012). Finally, when hypo- or hyperosmotic challenges are performed, as seen in this study with sticklebacks of the Camargue area, abrupt changes do not induce significant changes except for SW-acclimated sticklebacks immediately after transfer to FW (within 1 hour after transfer). This is consistent with previous results on monomorphic river-resident sticklebacks (low-plated), marine sticklebacks (completely plated) and polymorphic brackish water sticklebacks collected from a brackish water lake in Norway, indicating no short-term effect of salinity changes on oxygen consumption and no effect on SMR (Grøtan *et al.*, 2012).

Therefore, when comparing the physiological performances of wild caught monomorphic of polymorphic sticklebacks living either in the same habitats or from different saline environments,

SMR may not be sufficient. (Kitano *et al.*, 2010). Respiration rates may vary among seasons (Meakins, 1975), and there might also be temperature related differences in osmotic regulation between populations (Schaarschmidt *et al.* 1999). This indicates that populations adapted to different salinities do differ in their osmoregulatory ability, but this ability probably only occurs under certain conditions such as at very high and very low temperatures in contrasting salinities. Investigating the effects of various additional environmental conditions will clearly be useful.

5.2 Stickleback Morphometric

Sticklebacks have repeatedly colonized freshwater habitats because thousands of new streams and lakes formed during glacial retreats (Bell and Foster, 1994; Schluter, 2000). Therefore, postglacial freshwater stickleback populations share a common suite of adaptation throughout the northern hemisphere such as armor reduction (Bell and Foster, 1994; Colosimo *et al.*, 2005). They also differ significantly in morphology due to divergent selective pressures that they experience in contrasting freshwater habitats (Bell, 1982; Reimchen *et al.*, 1985; Baumgartner, 1992; Spoljaric and Reimchen, 2007). For example, divergent natural selection for the exploitation of benthic vs. limnetic habitats and associated food items is one of the most common diversifying mechanisms affecting resident freshwater sticklebacks (Walker, 1997; Spoljaric and Reimchen, 2007). Sticklebacks also inhabit a large number of coastal brackish water systems throughout the northern hemisphere and present therefore an extremely wide haloniche (Wootton, 1976; Bell and Foster 1994).

Sticklebacks from different aquatic habitats of the Camargue area are low plated with a minimum of 2 and a maximum of 6 lateral plates. No statistical difference could be identified in lateral plate number between the different locations and salinities. Also, no statistical significant differences were observed in terms of body morphology between sticklebacks sampled in 2016 and 2017 (Vaccarès and Marteau lagoons; Fumemorte and Versadou canals) and those sampled since 1993 (Malagroy, La Capelière, Fournelet, Baisse Salée, and Giraud lagoons).

The morphogeometric analysis using 17 landmarks of different morphological traits from fish sampled in the mesohaline lagoons and freshwater habitats, revealed that 32.937% of the variance is explained by the first component while the second component accounts for 16.75% of the variance. The scattered plot for the first and second components indicates limited morphological

variations between sticklebacks from the mesohaline and freshwater habitats. However, a significant difference is observed for the morphology of gill rakers between sticklebacks sampled from these two different habitats. Gill raker length observed significantly higher in sticklebacks living in the FW drainage canals compared to the fish living in the mesohaline lagoons.

The threespined stickleback is a well-known fish for having undergone adaptive radiation following the end of the last glaciation and is well adapted to the freshwater environment (Bell and Foster, 1994). Freshwater populations are less armored and are smaller compared to the marine ancestral populations. These typical morphological traits are interpreted as a result of parallel genetic adaptations to a novel environment (Foster, Baker and Bell, 2004; Colosimo *et al.*, 2005). Many potential selective agents have been proposed to explain the morphological parallel evolutionary changes across stickleback populations (Voje *et al.*, 2013; Spence *et al.*, 2013; MacColl and Aucott, 2014). For instance, it has been suggested that fitness advantages could be related to differences in predator regimes or intensity between different habitats (Reimchen, 1983, 2000). For sticklebacks of the Camargue area living within the Rhône river delta, in seawater, brackish or freshwater habitats, and very homologous body morphologies were observed with, also, no salinity effect on the number of lateral plates. This is consistent with what has already been observed for other freshwater and coastal sticklebacks of the Mediterranean bioclimatic region that are all low-plated (Münzing, 1963). Lateral plate number is genetically determined with a high heritability across all the different morphs even if substantial within-morph variation exists (Hansson *et al.*, 2016). Usually, freshwater fish have 12 plates or less, low plated sticklebacks have less than three plates (Bell, 2001), while marine sticklebacks are predominantly fully plated with more than 30 plates (Bell and Foster, 1994). Also, sticklebacks of the Camargue area present specific life history traits compared to northern populations including a shorter life expectancy (1 to 2 years), a very high growth rate, early sexual maturity, and a very long reproduction period (May to November) reflecting a high metabolism (Crivelli and Britton, 1987). A summer pause in somatic growth has also been detected in sticklebacks living in intermittent Mediterranean streams (Clavero *et al.*, 2009). This highlights harsh environmental conditions for these fish with an annual life cycle compared to those of the Camargue wetland lagoons.

Although fish collected from contrasted saline environments, sticklebacks of the Camargue area may have still the possibility to migrate locally between these different coastal habitats, especially between the mesohaline Vaccarès lagoon (the largest and deepest lagoon of the Camargue area

with, thus, limited salinity variations) and the southern seawater/hypersaline lagoons that are closer to the Mediterranean Sea. Even freshwater drainage canals that are physically isolated from these lagoons can potentially be flooded with brackish water and seawater, notably during severe storm events. Furthermore, the marine regression at the end of the Pliocene period and the anthropogenic use of these lagoons induced recent and regular reshaping of the Camargue region (Rey *et al.*, 2009).

Therefore, these environmental factors could have limited genetic adaptation for these coastal sticklebacks. On a larger scale, it would be necessary to determine whether these coastal sticklebacks have a similar morphology when compared to other southern coastal populations and landlocked Mediterranean sticklebacks. It would be also necessary to test whether the extreme environmental conditions for peripheral southern populations could explain the low number of lateral plates.

Only gill raker morphology revealed a difference between sticklebacks collected from the freshwater canals (large gill rakers) and those collected from the different mesohaline and euryhaline lagoons (short gill rakers). This divergence may reflect a more pelagic lifestyle of the mesohaline sticklebacks (Bell and Foster 1994). Our results fit with previous results i.e. those from Gross and Anderson (1984). Berner *et al.*, (2010) also found divergent phenotypic structures in freshwater and marine stickleback populations when considering gill raker length. Consequently, gill raker morphology seems to trigger adaptive changes in divergent environments (Mathews *et al.*, 2010), and may be free to evolve without strong ties with freshwater colonization (Larson, 1974). Sticklebacks are typically considered as trophic generalists with morphological variations of the gill rakers along the benthic-limnetic axis (Schluter and Nagel, 1995; Bell and Andrews 1997; Willacker *et al.*, 2010); the limnetic morphs foraging in pelagic habitats and having extended gill rakers, while, the benthic morphs have shorter gill rakers (Wootton, 1984; Williams and Delbeek, 1989). Finally, gill raker length has been identified as a key trait permitting shifts in trophic position in particular habitats (Mathews *et al.*, 2010). Therefore, it would be necessary to evaluate food preferences and type of prey to determine whether adaptive niche shifts occurred within the different habitats in the Camargue area (Bolnick *et al.*, 2009). This would then indicate that this coastal population primarily evolves according to their feeding preferences.

Understanding the physiological acclimatory responses to salinity changes is crucial in order to better characterize the ecology of a euryhaline species. In fish, ionocytes are key cells involved in

ion transport. In sticklebacks, these ionocytes were located along the gill filaments and lamellae in fish maintained in FW and along the gill filaments in SW-acclimated fish. Drastic differences were observed in gill Ionocyte location and size following transfer of the fish from FW to SW. However, fish transferred from FW to BW presented only mitigated responses. Therefore, comparisons with data from fish transferred to BW were not considered. In fish kept in FW, ionocytes located on the filaments were distinctively elongated from their apical to basal sides and those located along the lamellae appeared systematically flattened and wider. Although different in morphology, these ionocytes did not differ in terms of surface area. In contrast, ionocytes in fish acclimated to SW were round in shape and were only located along the gill filaments in the interlamellar epithelium of the gills. Furthermore, ionocyte density within a distance of 100 μm of gill filament was significantly higher in the gills of FW fish compared to SW-acclimated fish. This is due to the presence of lamellar ionocytes in FW sticklebacks. Ionocytes are therefore twice more numerous along the gills of FW acclimated fish compared to SW fish. Ionocytes of SW fish are also slightly smaller.

The finely tuned osmoregulatory machinery of fish relies on ionocytes that show high plasticity, both morphologically and in their ion transport functions. The stickleback kept in FW and acclimated to SW, scanning and transmission electron microscopy observations revealed different cell morphologies along the gill surface of these fish. The ionocytes were identified at the two tested salinities (FW and SW) but with different morphologies for the ionocytes. The stickleback kept in FW, there are two different ionocytes were observed, the dome-shaped structures with sphere-shaped elements covering the ionocytes surface, second ones are structures with honeycomb like structures. Occasionally, a different cell type is observed that could be transitional with a re-organisation of the apical spherical elements and a deepening of the surface. All these cell types were confirmed as ionocytes due to their internal structures filled with numerous mitochondria and a dense tubule-vesicular system, consisting of deeply invaginated basal membrane. Whereas, the stickleback acclimated to SW, only one type of ionocytes with large apical crypt were observed along the gill filaments at the base of the gill lamellae, and these cells were organised as multicellular complexes. No ionocytes were observed along the gill lamellae of SW acclimated sticklebacks.

Four distinct types of ionocytes have been functionally described in the yolk-sac membrane of tilapia embryos (Hiroi *et al.*, 2008; Hiroi and McCormick, 2012) and up to five types have been

reported at the surface of the skin and gills of the stenohaline FW zebrafish (*Danio rerio*) (Dymowska *et al.*, 2012; Guh *et al.*, 2015). Furthermore, one of these cells observed through SEM revealed a similar honeycomb structure to that observed along the gill lamellae of the FW sticklebacks of the Camargue region. The presence of a similar type of ionocyte in these two species in FW could indicate that this cell morphotype is a specialized cell type rather than a transitional stage. The second FW type ionocyte with a dome-shaped ionocyte described in sticklebacks of the Camargue region has been described in various other Teleost species living in FW (Hwang *et al.*, 2011). The transformation of the ionocyte size and apical morphology (from flat, concave or convex FW types to SW pit type) can occur very rapidly (Choi *et al.*, 2011) and can involve a functional change from salt uptake to salt secretion (Hwang and Lin, 2013). This remodeling could also be due to a cell switch involving cell proliferation and apoptosis. In sticklebacks of the Camargue region, the density of these ionocytes does not change between FW-fish and SW-acclimated fish, although their position differs along the gill filament and lamellae. It is hypothesized that this gill remodeling takes place at a low energetic cost, with reduced cell proliferation and apoptosis. This could also explain the lack of short-term metabolic cost that has been observed in sticklebacks transferred to different salinity environments, irrespective of their environment of origin (Grøtan *et al.*, 2012).

5.3 Functional Analysis of the main osmoregulatory organs

Euryhaline fish are equipped with complex osmoregulatory mechanisms to counter osmotic stress and to maintain hydro-mineral homeostasis. In fish, ionocytes are involved in ion transport with an exterior apical surface and a basal side close to blood vessels. These ionocytes are located in the yolk sac membrane of embryos, at the surface of the skin in larvae, and along gill filaments and lamellae in juveniles and adults (Hiroi *et al.*, 2008; Dymowska *et al.*, 2012; Hiroi and McCormick, 2012; Guh *et al.*, 2015). The internal structure of these ionocytes are rich in mitochondria with a tubulo-vesicular system composed of basal membrane invaginations containing the sodium pump, Na^+/K^+ -ATPase, or NKA (Evans and Claiborne, 2009; Dymowska *et al.*, 2012).

In sticklebacks from the Camargue area, the transcript levels of the reference gene *uba52* did not change between salinities. For NKA α 1, mRNA relative gene expressions in the gills were

significantly different depending on the salinity tested. However, for NKA α 1a, mRNA relative gene expressions in the gills are not different due to salinity. Relative NKA α 1 mRNA expression was higher in the gills of SW acclimated fish compared to BW fish and expression of NKA α 1b was significantly higher in the gills of SW acclimated fish compared to the other two salinities.

The NKA enzyme located along the heavily folded basolateral membrane forming the tubulovesicular system of the SW and FW type ionocytes plays a critical role in energizing branchial ion transport (McCormick, 1995). A lower gill NKA activity has already been recorded for FW sticklebacks compared to fish transferred to SW (Schaarschmidt *et al.*, 1999). Gene expression of NKA was also studied in SW-to-SW, SW-to-FW, FW-to-FW and FW-to-SW transferred sticklebacks (Taugbøl *et al.*, 2014). Both marine and land-locked FW populations were able to acclimate to these abrupt salinity transfers with a highly plastic NKA expression profile, the expression being increased 48 h and 7 days post-transfer for the SW-to-SW group and FW-to-SW group, respectively. For sticklebacks of the Camargue area, salinity transfer did not modify the NKA α 1a expression although the expression of NKA α 1b isoform significantly increased in SW acclimated fish. Recently, Expression of the NKA α 1 isoforms has received much attention with reciprocal expression of these isoforms in salmonids that were transitioning between FW and SW environments (Madsen *et al.*, 2009; McCormick *et al.*, 2009). However, the current nomenclature is confusing with isoform designations used for Salmonids and other Teleosts that are different (Blondeau-Bidet *et al.*, 2016; Wong *et al.*, 2016).

Within non-salmonid teleosts, Tipsmark *et al.* (2011) showed that Mozambique tilapia undergo NKA α 1a and NKA α 1b switching upon salinity changes. In seabass, NKA α 1a appeared as the predominant isoform in the gills, kidney and posterior intestine with branchial NKA α 1a and α 1b expression that only changed significantly after long-term transfer (2.5 years) from SW to FW (Blondeau-Bidet *et al.*, 2016). Branchial NKA α 1a expression is higher in FW-acclimated sea-bass compared to SW acclimated fish but with the opposite pattern for NKA α 1b. However, no salinity-induced switch was observed between the NKA α 1a and NKA α 1b isoforms in the medaka (*Oryzias latipes*), although the NKA α 1b isoform was more expressed in SW acclimated versus FW acclimated medaka (Bollinger *et al.*, 2016). This is consistent, therefore, with the results obtained for sticklebacks of the Camargue area.

The physiological mechanisms involved in order to maintain ion and water homeostasis are coordinated between the gills, the kidney and the gut (Eddy and Handy, 2012). Also, changes in

salinity and the coordinated physiological response are often reflected by different oxygen consumption rates (Morgan *et al.*, 1997; Gracia-Lopez *et al.*, 2006). This measurement can be used as an indicator of fish metabolism (Cech, 1990) and to assess the osmoregulatory cost (Morgan and Iwama, 1998; da Silva Rocha *et al.*, 2005; Tsuzuki *et al.*, 2008). This cost can impact growth rate (Woo and Kelly, 1995, Boeuf and Payan, 2001; Imsland *et al.*, 2003) with also a minimal osmoregulatory energy demand in an environment that is natural for a particular species, indicating, therefore some fitness-related advantages of being adapted to local environmental salinities (Morgan and Iwama, 1991).

For sticklebacks sampled from the SW ‘Marteau’ lagoon, direct transfer from SW to FW induced, a significant increase of the branchial Na^+/K^+ -ATPase activity by 28% and 40% within 24 and 48 hours post transfer, respectively. When sticklebacks sampled from the FW ‘Versadou’ canal are transferred from FW to SW, gill Na^+/K^+ -ATPase activity is significantly decreased by 36% within 24 hours. Therefore, there is a 1.65 fold change in gill Na^+/K^+ -ATPase activity when fish are abruptly transferred to different salinity conditions. Furthermore, the enzyme activity for fish acclimated or transferred to SW or FW appeared similar although these fish were sampled from two contrasted habitats. If the cost of having a plastic osmoregulatory machinery is high, it was expected that a salinity shock would have impacted the overall metabolism. This plasticity is also observed at the cellular and molecular level in sticklebacks of the Camargue area. There is a remodeling of the gill ionocytes, a switch in gill NKA activity and a higher gene expression of the branchial NKA $\alpha 1a$ isoform compared to the NKA $\alpha 1b$ isoform, these isoforms being involved in ion uptake in the FW-type ionocyte and in ion secretion in the SW-type ionocyte, respectively. This is similar to what has been observed in the European seabass, *Dicentrarchus labrax* (Blondeau-Bidet *et al.*, 2016). These results fit with those already published (Jurss *et al.*, 1982; Judd, 2012). However, an increased gill NKA activity has also been documented when FW sticklebacks are transferred to seawater (Schaarschmidt *et al.*, 1999). In their experiment, fish were transferred to seawater at a higher salinity level (35‰) compared to the ones tested in this study (30‰). As already observed in fish (e.g.: the European seabass and the marbled spinefoot, *Siganus rivulatus*), NKA activity over a large salinity gradient typically presents a U shape curve with maximal activity levels at extreme salinities (Lasserre *et al.* 1971; Saoud *et al.*, 2007). Therefore, this increased NKA activity in the hypersaline condition could explain the discrepancy observed between the results obtained in this study and those from Schaarschmidt *et al.* (1999).

In the ionocytes, the sodium channel, Na⁺/K⁺-ATPase, actively pumps intracellular Na⁺ for extracellular K⁺, thereby driving the Na⁺/K⁺/2Cl⁻ cotransporters (NKCC) to move Cl⁻ from blood plasma into the cell, whereas Cl⁻ ions exit the cell through cystic fibrosis transmembrane conductance (CFTR) embedded in the apical membrane. Na⁺ secretion is thought to occur also passively through leaky tight junctions and between the accessory cells and the ionocytes (Marshall and Grosell 2006; Hwang and Lee 2007). However, for sticklebacks kept at different salinity conditions, no difference could be detected in relative mRNA expression for gill CFTR although it is slightly more expressed in BW or SW conditions compared to the FW condition. Our results fit with those from Taugbøl *et al.*, (2014), since CFTR expression in this study did not vary over a long-term salinity acclimation in SW.

It is generally believed that the main role for CFTR is related to ion excretion in marine fish since this transporter is involved in Cl⁻ ion transport (McCormick *et al.*, 2003). Following acclimation from FW to SW, an increase in the expression of CFTR has been reported in eels (*Anguilla Anguilla*; Wilson *et al.*, 2004), Atlantic salmon (*Salmo salar*; Singer *et al.*, 2002). On the contrary, McCairns and Bernatchez (2010) reported higher expression of CFTR in laboratory-reared FW sticklebacks. Further, when looking at long-term salinity acclimation for more than three weeks, the expression of CFTR tends to decline in SW. For CFTR, much surprising results have been reported across the fish taxa, for example, CFTR in FW Mozambique tilapia (*Oreochromis mossambicus*) fail to express (Hiroi *et al.*, 2005), diffuse in killifish (*Fundulus heteroclitus*; Marshall *et al.*, 2002), no change in the expression in striped bass (*Morone saxatilis*; Madsen *et al.*, 2007), this shows role of CFTR in osmoregulation is quite, overall, a complicated involvement of CFTR in FW osmoregulation. Another possibility is a cellular difference in the localization of CFTR expression in contrasting environmental salinities with a basolateral expression in FW-acclimated fish and an apical localization in SW-acclimated fish (Marshall *et al.*, 2002). This could also be the case for sticklebacks.

Fish gill ionocytes are often structurally or functionally, influenced by contrasting salinities (Evans, 2008). Euryhaline fish are more plastic than stenohaline fish, upon acclimation to different salinities, and can remodel their branchial ionocytes in order to maintain their hydro-mineral homeostasis. For sticklebacks of the Camargue area collected from a BW habitat, a two-week acclimation in SW induced an over expression of NKCC1 in the gills. However, we did not find any difference in NKCC1 expression between FW and BW fish. NKCC is a member of chloride-

cation cotransporter family and NKCC1 is known as the secretory isoform widely distributed in vertebrate species (Lytle *et al.*, 1995; Gagnon *et al.*, 2002). It has been reported an increased in the mRNA expression of gill NKCC which indicates Cl⁻ secretion upon acclimation to seawater in striped seabass (*Morone saxatilis*; Tipsmark *et al.*, 2004), killifish (*Fundulus heteroclitus*; Scott *et al.*, 2004), and European eel (*Anguilla Anguilla*; Cutler and Cramb, 2002), brown trout (*Salmo trutta*; Tipsmark *et al.*, 2002), tilapia (*Oreochromis mossambicus*; Wu *et al.*, 2003). Recently, it has been reported that branchial NKCC abundance is also increased in sticklebacks challenged in SW (35 ppt) for 1 week (Divino *et al.*, 2016).

Recently, it has been suggested that the V-ATPase facilitates Na⁺ uptake across gill epithelium in FW fish generating an electrical gradient to drive sodium uptake through an apical sodium channel (Piermarini and Evans, 2001). However, in Sticklebacks, the V-ATPase is not differentially expressed depending on our experimental salinity conditions (FW, BW, and SW). In fish such as the Longhorn sculpin (*Myoxocephalus octodecemspinosus*) and the European eel (*Anguilla Anguilla*), the V-ATPase is indirectly involved in the uptake of sodium across the apical membrane (Harvey, 2009). The V-ATPase is located on the apical membrane of pavement cells located at the leading edge of the gills in tilapia (*Oreochromis mossambicus*) (Wilson *et al.*, 2000). In rainbow trout (*Oncorhynchus mykiss*), the V-ATPase is expressed in pavement cells and Na⁺/K⁺-ATPase-rich cells (Wilson *et al.*, 2000). This transporter is used for sodium uptake but also for hydrogen excretion. Evidence for the possible role of V-ATPase in ion uptake in FW teleosts includes experiments using bafilomycin (an inhibitor to V-ATPase) that limit sodium take-up in tilapia (*O. mossambicus*) and carp (*Cyprinus carpio*) (Fenwick *et al.*, 1999). Also, when FW rainbow trouts are acclimated to SW, a decrease in V-ATPase activity and localization is observed in the gills (Lin and Randall, 1993; Lin *et al.*, 1994).

It has been hypothesized that V-ATPase in the gills directly pump protons into the environment with also a possible role in acid-base balance. Some studies focussing on isolated mitochondria-rich pavement cells from the gills of the rainbow trout revealed that these cells are rich in V-ATPase (Galvez *et al.*, 2002) and play an active role in sodium uptake and acid extrusion (Galvez *et al.*, 2002; Reid *et al.*, 2003). Studies on branchial V-ATPase in a true marine teleost have yet to be published, but the role of this transporter in acid–base regulation of seawater teleosts is assumed to be minimal (Claiborne *et al.*, 1999).

A schematic representation of the results obtained in this study is illustrated in Figure 5.1.

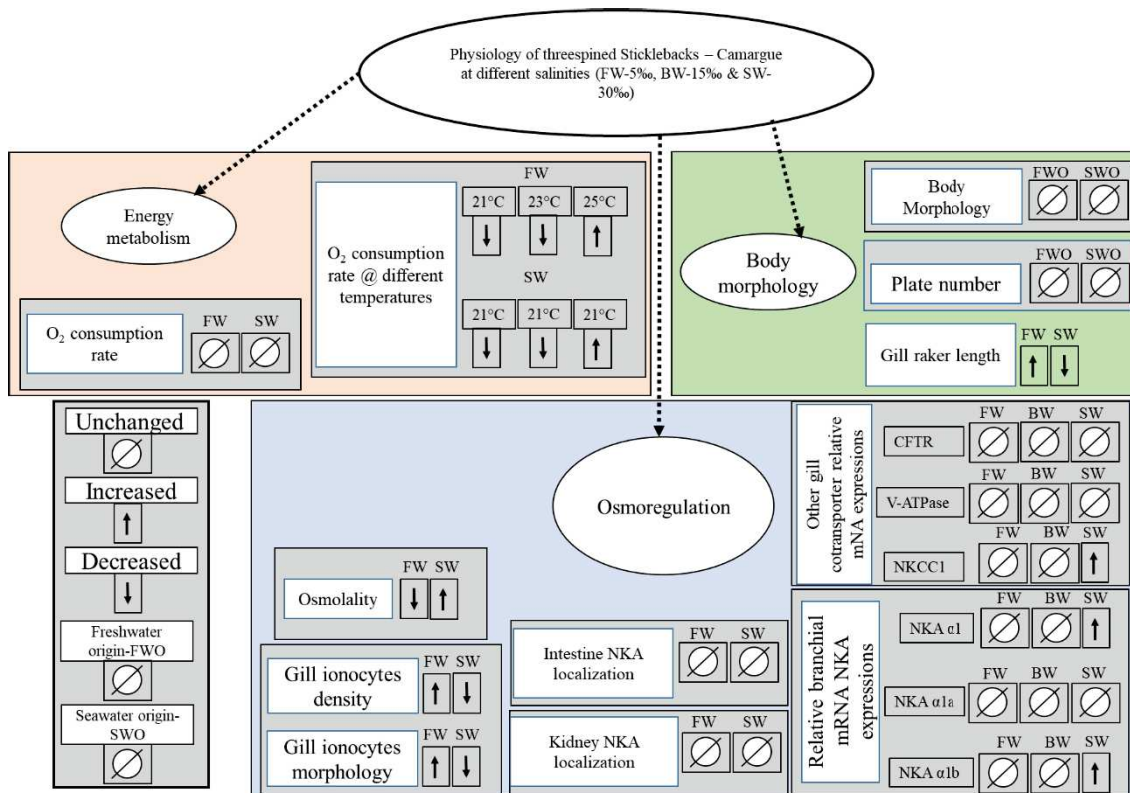


Figure 5-1 Graphical representation of the results obtained in this study

5.4 Conclusion

Sub-adult sticklebacks living in the Camargue area can rapidly acclimate to different salinities (freshwater, brackish water or seawater), with a significant difference observed in blood osmotic pressure between fish acclimated to different salinities (FW: 5‰, 147 mOsm kg⁻¹; BW: 15‰, 457 mOsm kg⁻¹; SW: 30‰, 911 mOsm kg⁻¹).

Sticklebacks fished from the “Marteau” SW lagoon, the FW canal “Versadou” and from the mesohaline lagoon-like “Vaccarès”, unveiled similar oxygen consumption rates kept in either FW or SW. For salinity tolerance, fish were transferred from FW to SW or SW to FW and oxygen consumption measurements recorded after 24 hours and 72 hours, but no change in oxygen consumption rate was observed. Therefore, sticklebacks can move from SW or mesohaline lagoons to FW canals without spending energy to fuel the physiological adjustments. Only sticklebacks from the ‘Vaccarès’ mesohaline lagoon kept initially in SW presented a significant reduction in

oxygen consumption rate immediately and up to 1 hour after abrupt transfer to FW. However, fish collected from the same environment and kept in FW prior to SW transfer did not modified their oxygen consumption rate during a 6-hour course experiment. Also, a longer acclimation up to 3 and 6 weeks to SW or FW did not induce any change in oxygen consumption rate compared to fish kept in FW and SW.

At the gill level, some changes could be observed. For example, gill Na^+/K^+ -ATPase activity of sticklebacks sampled from the SW 'Marteau' lagoon significantly increased by 28% and 40% within 24 and 48 hours post transfer from SW to FW, respectively. When sticklebacks sampled from the FW 'Versadou' canal are transferred from FW to SW, gill Na^+/K^+ -ATPase activity is significantly decreased by 36% within 24 hours. Therefore, there is a 1.65 fold change in gill Na^+/K^+ -ATPase activity when fish are abruptly transferred to a different salinity. Furthermore, the enzyme activity for fish acclimated or transferred to SW or FW appeared similar although these fish were sampled from two contrasted habitats.

Therefore, and as expected, salinity changes induced different protein expression levels within the gills. These changes also induced different blood osmolality values. However, the energy balance do not seem affected. Temperature, however, strongly affects the fish oxygen consumption rates, when measured at different temperatures (21 to 25 °C).

For general body morphology, the stickleback of the Camargue appeared uniform. No statistical significant difference could be observed in terms of body morphology between Sticklebacks sampled in 2016 and 2017 (Fumemorte, Vaccarès, Marteau and Versadou) and those sampled in 1984 (Malagroy, La Capelière, Fournelet, Baisse Salée, and Giraud). In terms of lateral plate number, Sticklebacks fished from different aquatic habitats of the Camargue area presented on average 4.45 ± 0.31 lateral plates, with a minimum of 2 and a maximum of 6 lateral plates observed (no statistical difference in lateral plate number between the different locations of various salinities). Scattered plots obtained from seventeen different morphological traits (digitized with TPsDig) for sticklebacks fished from mesohaline lagoons and freshwater canals indicate very limited morphological variations between these sticklebacks. The morphometric analysis revealed only 32.937% of the variance by the first component while the second component accounts for 16.75% of the variance.

However, a significant difference was observed in the morphology of gill rakers between FW and SW/mesohaline sticklebacks. Stickleback living in the FW habitats their gill raker length significantly higher compared to the fish living in mesohaline lagoons (SW/BW) while gill raker lengths were measured from the base to the tip of the rakers. Only first gill raker from the left brachial arch was considered.

On the semi-thin gill histological sections, branchial ionocytes were identified using Na⁺/K⁺-ATPase labelling by immunofluorescence. In the gills of the FW and BW, the ionocytes were observed on gill filament and gill lamellae, in SW gills along the filaments. Gill ionocytes size and morphology, significant differences were noticed following fish transfer from FW to SW. In FW fish, the gill filament ionocytes, from their apical to basal sides, were observed distinctively elongated, and gill lamellae ionocytes were appeared systematically flattened and wider. While in terms of surface area, these ionocytes did not differ despite of having morphological differences. In contrast, fish acclimated to SW, the ionocytes were round in shape, and located only along the gill filament and in the interlamellar epithelium. Moreover, in FW fish, ionocyte density of filament was significantly higher in the gills compared to SW acclimated fish (within a distance of 100 µm). This is due to the presence of lamellar ionocytes in FW sticklebacks. Ionocytes are therefore twice more numerous along the gills of FW acclimated fish compared to SW fish. Ionocytes of SW fish and are also slightly smaller.

Moreover, Scanning and transmission electron microscopy observations revealed different cell morphologies along the gill surface of these fish. Ionocytes and pavement cells were identified at the two tested salinities but with different morphologies for the ionocytes. In fish maintained in FW, two types of ionocytes were observed: the dome-shaped cells with some spherical elements covering the surface, and ionocytes with a honeycomb like structure. Occasionally, a different cell type is observed that could be transitional with a reorganization of the apical spherical elements and a deepening of the surface. All these cell types were confirmed as ionocytes due to their internal structures filled with numerous mitochondria and a dense tubule-vesicular system, consisting of deeply invaginated basal membrane. In fish acclimated to SW, only ionocytes with a large apical crypt were observed along the gill filaments at the base of the gill lamellae, and these cells were organized as multicellular complexes. No ionocytes could be observed along the gill lamellae.

Whereas, the Relative gill NKA gene expression For NKA α 1a and NKA α 1b in the gills were significantly different depending on the salinity tested. However, for NKA α 1a, mRNA relative gene expressions in the gills are not different due to salinity. Relative NKA α 1 mRNA expression was higher in the gills of SW acclimated fish compared to BW fish and expression of NKA α 1b was significantly higher in the gills of SW acclimated fish compared to the FW and BW. Whereas, for CFTR and V-ATPase expression, we didn't find any significant differences regardless of salinities. On the other hand, NKCC1 was significantly higher in SW acclimated sticklebacks.

The sticklebacks kept in freshwater and acclimated to seawater conditions, histological sections were selected for kidney NKA immunolabeling of kidney tubules, while kidney is main site of many physiological functions aimed to understand the ion transport in contrasting salinities. To best of our knowledge, it is our first study on peripheral stickleback population of the Camargue lagoons. Whereas, preliminary studies on the gut, the anterior and posterior intestine of freshwater and seawater acclimated sticklebacks was studied. Strong immunofluorescence was observed along the basolateral membranes of enterocytes of both anterior and posterior intestine of the freshwater and seawater acclimated sticklebacks. No significant differences were observed in the expression of NKA between freshwater and seawater acclimated fish. Yet again, we did not observe any differences between freshwater and seawater acclimated fish, this could suggest high level of plasticity in local adapted environment. All the results obtained during this research study are summarized in fig. 5.1.

5.5 Perspectives

5.5.1 Molecular to ontogenetic analyses

Future studies should investigate the branchial gene expression of different ion transporters (NKCC, V-ATPase, NHE, CFTR, etc) at different salinities and temperatures with a special focus on some isoforms. The gene expression of these ion transporters should be extended to the kidney because of the multiple roles of this key organ during and after the breeding seasons. It should be interesting also to functionally characterize the ionocytes in the gills and the switching mechanisms of these ionocytes when the fish are transferred to different salinities and temperatures. One hypothesis is that these cells can rapidly (within minutes / hours) modify their shape in order to physiologically switch from hypo- to hyper-osmoregulation and viceversa. Then, cell proliferation

could occur. However, this response could be a non-specific response not necessarily linked to a salinity challenge. For example, the number of gill ionocytes in European seabass *Dicentrarchus labrax* juveniles kept in SW is increased 1h after a confinement stress and is still elevated 7h after (Lignot *et al.*, comm. Pers.). This is accompanied by an increased gene and protein NKA expression. Therefore, a more complete analysis focusing on abrupt salinity transfers, and over a short period of time (within a few hours after transfer) is of special interest in order to better decipher, at the cellular level, between the non-specific stress response and the physiological adjustments. In addition, these adjustments that do not appear to affect the energetic metabolism of the fish, could, nevertheless, specifically affect the gills. A focus on the oxygen consumption of isolated gills directly transferred to different salinities could bring new insights.

Embryos and newly-hatched juveniles do not have well-developed gills. Therefore, it would be interesting to explore osmoregulation mechanisms in contrasting salinities and temperatures on these early stages, with a focus on the ontogenetic development of the osmoregulatory sites. Technically, this is facilitated because fertilized eggs can be easily obtained by an *in vitro* fertilization protocol, similar to that described by Barber and Arnott (2000). Also, a developmental table is already available (Swarup, 1958).

In adults, the use of the spiggin produced by the kidney of the males during nest building is also of special interest. Once eggs (1.2 -1.7 mm in diameter) are fertilized, intense parental care is carried out by the male. It involves nest maintenance and fanning of the eggs to ensure oxygen supply, even at night. The nests are built from vegetation, sand, pebbles and other debris, this material adhering together with the spiggin, an androgen-induced glue protein synthesised by the kidney (Jakobsson *et al.*, 1999). During this period, the glandular transformation of the kidney that only produces spiggin, impairs the osmoregulatory function of this organ, notably due to a reduced glomerular filtration rate (De Ruiter *et al.*, 1985). The loss of the water excreting capacity of the kidney was considered to be partially counterbalanced by the secretion of mucus and, more importantly, by enhanced release of intestinal fluid. Recently; it has been confirmed that the kidney function in terms of water handling is altered in mature males (Madsen *et al.*, 2015). However, these authors could not detect a modification in the trans-epithelial water permeability or salt-secretory activity in the intestine. Salt-absorptive activity in the intestine may, however, be down-regulated during male maturation. Also, the removal of nitrogenous waste products could be taken

by the gills (De Ruiter *et al.*, 1985). A complete study taking into account these 3 organs, cellular and paracellular transport mechanisms, notably in the posterior intestine when the intestine is transformed into a water secretory organ during male maturation would be of special interest.

5.5.2 Morphometry and genetic analyses

The study only focused on sticklebacks from the Camargue area in terms of body morphometry (lateral plate number and gill raker length and density). It would be interesting also to consider other populations of sticklebacks such as those from the French Alps and most importantly, those from the Mediterranean basin (continental and insular land-locked FW populations; offshore marine population, coastal populations). This is emphasized by the recent work by Fang *et al.*, 2018, indicating that all their samples collected from southern Europe formed a monophyletic group composed of 3 clades: a French clade (along the Rhône river, which started to diverge from the rest of the European populations between ca. 42600-23400 years), an Iberian clade (9 populations) and a Mediterranean clade. This Mediterranean clade started to diverge from the Iberian clade between 38.8 and 21.1 kya. It is comprised of freshwater populations of the Balearic Sea, southern Greece, southwest Turkey and the Adriatic (2 separate groups) and Black Sea drainages.

When comparing populations coming from different geographical or ecological origins, it is of first importance to identify the genetic basis underlying local heterogeneity of the results. The markers generally used in population genetics give neutral indications of historical origin (mtDNA sequences) or recent migrations and isolations (microsatellites). They are not directly linked with the investigated character (life history traits, capacities, characters.). However, there is a high probability that populations belonging to distinct phylogeographic lineages or local differentiated groups also developed different characters explaining a part of the observed differences. Because several levels of differentiation are already known for the European *G. aculeatus*, this information can be used to eventually produce a more detailed one.

5.5.3 Macro-evolution

European *G. aculeatus* has already been genetically analysed, sometimes in comparison with the American distribution for the same species, using mainly 2 types of markers giving 2 different pictures: mtDNA sequences and nuclear microsatellites. Mäkinen *et al.*, (2008) conducted a mitochondrial DNA analysis by sequencing both Cytochrome b and Control Region zones. Three main subgroups were detected (Fig. 5.2). Europe is occupied by a big central cluster covering most of the continent. A second group is located at the north of the Black Sea. The third group includes few disparate locations along the European Atlantic coast and one location in the United States (Maine). Microsatellite markers were considerably developed by Peichel *et al.* (2001). This founding publication proposed 288 loci mapped on the 26 chromosomes of the species, permitting an easy use of physically unlinked markers. Most of the following surveys used part of these loci. For instance, Mäkinen *et al.* (2006) proposed with the use of these loci, a phylogeographic structure of the European three-spined sticklebacks that is visibly more complex than the mtDNA one. It is composed of 6 subgroups, including all possible habitat types (coastal, migratory, lake resident, river resident populations). Figure 2.8 shows that the whole West-Mediterranean populations, including the Balkans are clustered into group 5. Because mtDNA and nuclear markers are mainly in agreement in several surveys, but provide different point of view of geographic structure, both markers should be used. Mitochondrial markers are considered to give an historical description of the structure while microsatellites inform on recent migrations and isolations. More or less linked with one of the 3 main groups, smaller but significant units have been demonstrated in the Rhône River (Sorgues tributary), and in the Balkans (Stella, Neretva and Skadar Rivers) (from Mäkinen *et al.*, 2008).

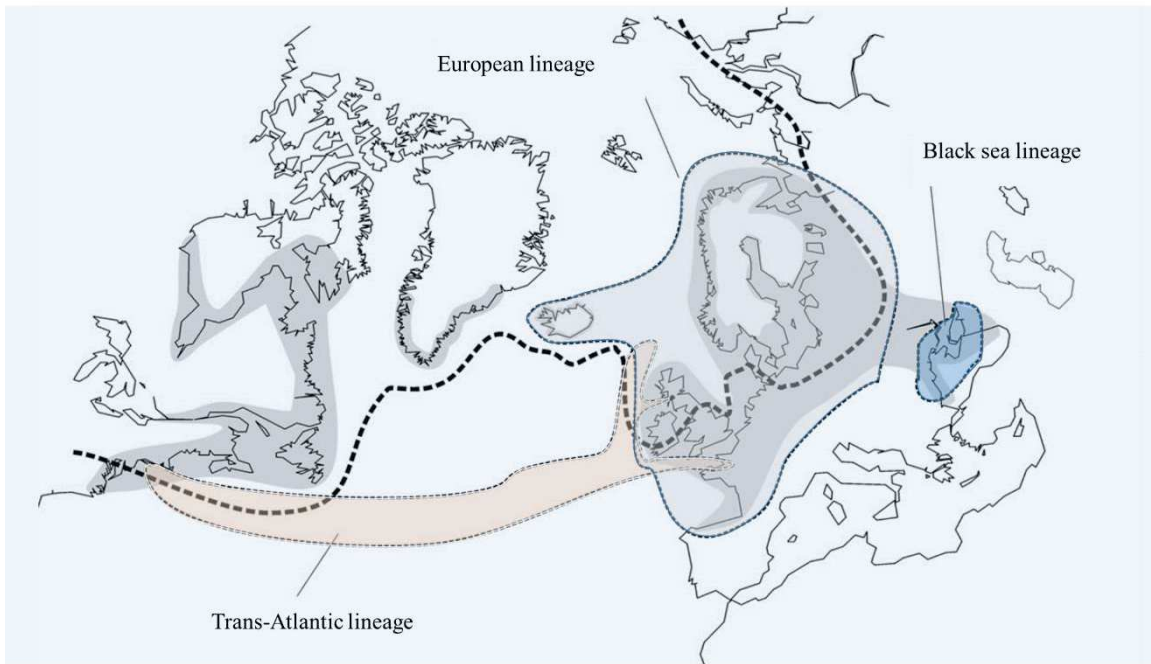


Figure 5-2 Distribution of threespined sticklebacks with the main three lineages according to Paepke (2001) and Bell and Foster (1994). Figure adapted from Mäkinen *et al.*, (2008).

5.5.4 Micro-evolution

The genetic mapping of the populations can be understood in two ways. It is necessary first to situate the sampled localities in the European mtDNA and microsatellite phylogeographic which constitutes "macro-groups". However, several publications have shown that, inside a given macro-group, very detailed micro-groups can be described, frequently linked to habitat types. That is the second level of differentiation constituted by "micro-groups". Very informative structures are sometimes observed between close populations living in different habitats: In Germany, in Schleswig-Holstein region near the Baltic Sea, Reusch *et al.*, (2001) have described a clear link gathering stickleback inhabiting rivers, or inhabiting lakes or estuaries (Figure 5.3). A similar result has been obtained by Raeymaekers *et al.*, (2005) indicating that local micro-evolution can provide the same design of differentiation. Both studies, and several others, highlighted the two alternate hypotheses: (i) a common founding lineage which differentiates lately according to the lake, river or coastal ecology or, (ii) differentiated founders choosing the best ecosystem for them. A first objective would be to assign each investigated sample to a comprehensive macro- and

micro-groups, but there is the possibility, in favourable cases, to describe a new local structure allowing ad hoc hypotheses of origin and of local evolution of *G. aculeatus*.

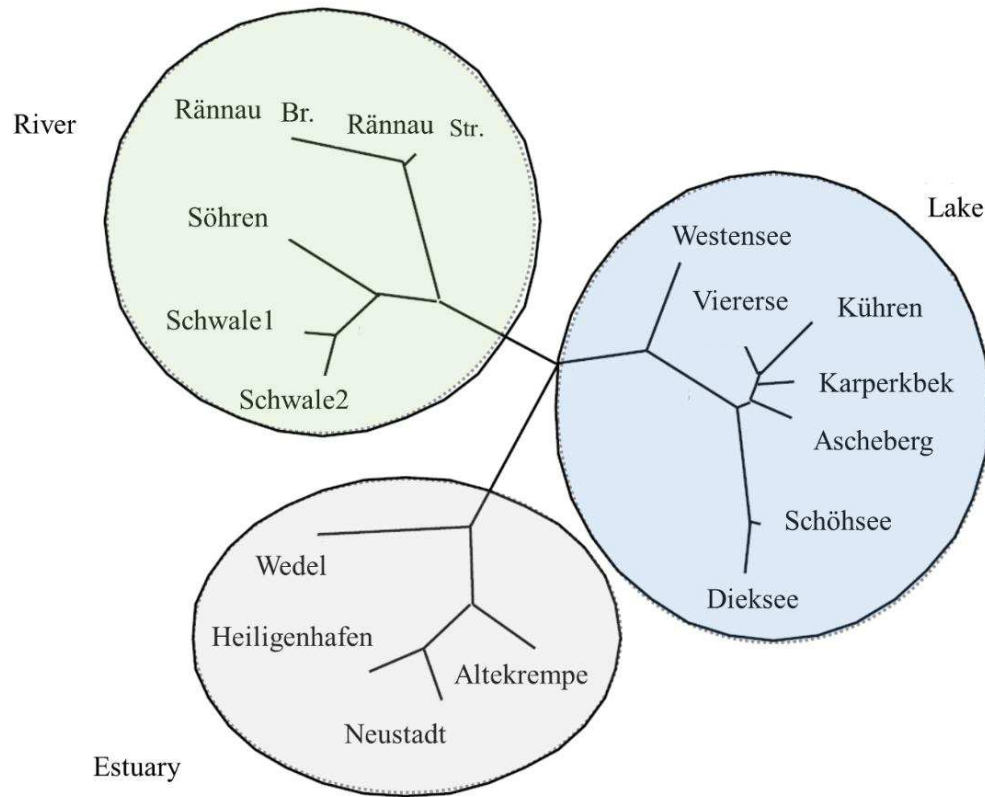


Figure 5-3 Phylogenetic relationships among sticklebacks population from three habitats (i.e. Rivers, lakes and estuaries) of Schleswig-Holstein, Germany. Figure adapted from Reusch *et al.*, 2001.

5.5.5 Ecotoxicological analysis

The Camargue wetlands are surrounded by large surface areas where rice and wheat farming are carried out intensively. Also, the water pumped from the Rhone River that is used for agricultural purposes, represents a total amount of 300-400 million m³ of water per year. This water contains numerous dissolved elements and pollutants that are added to the Camargue aquatic environment every year. Agricultural and rice growing activities also release numerous pollutants into the Camargue lagoons. Therefore, it would be interesting to explore the eco-toxicological effects of these pesticides on sticklebacks of the Camargue area living in different water bodies (freshwater

drainage canals, brackish water and euryhaline lagoons) and to assess the physiological effects of these pesticides using reared sticklebacks kept in laboratory conditions (embryos, juveniles and adults).

The ecological status of the main lagoon (Étang de Vaccarès) was considered “mediocre” in 2009. Since 2011, the annual water quality analyses commissioned by the NNRC have clearly revealed that the freshwater drainage canals and lagoons of the Camargue area are regularly polluted with pesticides, with a direct transfer of these products to the different ponds and lagoons of the Camargue. A total of 13 illegal molecules have been regularly detected in the aquatic environment along with other products that are used for rice growing activities and are either authorized or banned, at concentrations substantially higher than those indicated in the Environmental Quality Standards, especially in the freshwater drainage canals. Through the trophic network, these molecules contaminate fish and fish-eating bird species. Some of these detected pollutants are reprotoxic and/or endocrine disruptors. They can profoundly change the biology and physiology of local species. The three main substances found in high concentrations in the water bodies of the Camargue area are the glyphosate, the bentazone and the penoxsulam. Other herbicides specific to rice cultivation are also measured in the Camargue at higher concentrations than the current standards: oxadiazon from May to September (13 times the SEQ-Eau value), flufenacet (twice the Predicted No Effect Concentration, PNEC value), dimethenamide (3 times the EQS value), 2,4-MCPA in June and the insecticide tebufenozide in September (twice the PNEC). However, these pollutants are not considered reprotoxic and / or endocrine disruptors. Analysis of the latest data collected in 2016 for the two main irrigation canals in the Camargue area again revealed worrying concentrations of anthropogenic chemical contaminants, with an increase in pesticide concentrations in both channels over the preceding years. The local origin of most of the pesticides highlighted in 2016 is clearly related to rice growing.

Sticklebacks are considered as a promising species for biomonitoring quality of the aquatic environment (Sanchez *et al.*, 2007; Roussel *et al.*, 2007). This is due to its wide distribution with a limited home range (Pottinger *et al.*, 2002). It is also a valuable model organism for detecting both androgen- and oestrogen-induced endocrine disruption (Hahlbeck *et al.*, 2004a, b; Hogan *et al.*, 2008; Björkblom *et al.*, 2009). Therefore, sticklebacks are good sentinel fish species and are

used as an effluent-monitoring species in Canada, the United States) and in Europe (Grove *et al.*, 2009). In addition to this, their low economic and recreational values make them easily available. For example, the production of vitellogenin within the gonads and spiggin within the kidneys (glycoprotein serving as glue for the construction of the nest by the males), are recognized as excellent biomarkers of endocrine disruption. Spiggin production may be altered in males during nest construction due to exposure to estrogenic compounds or triggered in females through the effect of androgenic compounds.

Currently, no study in France describes Mediterranean sticklebacks in terms of evolutionary and phylogenetic analyses or in terms of adaptation capacities, or even in monitoring programs in ecotoxicology, despite the highly interesting characteristic of these populations. Indeed, their presence at the southern limit of the species distribution subjects them to strong environmental constraints. The high levels of pollutants identified in the Camargue aquatic environment over the last 6 years demonstrate the urgency to take this pollution into account and carry out an in-depth study of the effects of reprotoxic and endocrine disruptors. This is particularly important in view of the fact that the concentration peaks of these pollutants are observed at a crucial moment of the reproduction period of these fish.

Therefore, the general ambition is to develop a synergy around the Mediterranean stickleback that could become a model of choice, especially at the University of Montpellier. This species is the euryhaline equivalent of zebrafish both in terms of its size and its breeding and reproduction facilities in captivity (e.g.: controlled in vitro fertilization). In addition, its genome is known and published. It should also be noted that predictable and unpredictable variations of key environmental parameters such as salinity and temperature must be increasing in frequency and intensity due to current global warming. Therefore, this intensification of environmental pressures presents a major additional constraint for individuals with specific adaptive characteristics and are already at the limits of their physiological capacities. The Mediterranean stickleback could thus be used not only in ecotoxicology and conservation Biology but also in functional and evolutionary ecology.

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7 Conference Presentations

European Society for Comparative Physiology and Biochemistry, ESCPB. 2016, Barcelona, Spain.
Rind, K., Beyrend, D., Blondeau- Bidet, E., Charmantier, G., Cucchi, P., & Lignot, J. H. * Effects of different salinities on the osmoregulatory capacity of Mediterranean sticklebacks. *Poster Presentation.*

Society of Experimental Biology Annual Meeting, 2017, Gothenburg, Sweden.
Rind, K., Rodriguez-Barrucg, Q., Nicolas, D., **Lignot J.H.*** Are Mediterranean three-spined sticklebacks (*Gasterosteus aculeatus* L.) of the Camargue wetlands with contrasted salinity conditions morphologically and physiologically different? *Oral presentation.*

3eme Colloque d'Ecophysiologie Animale, CEPA 2017, Strasbourg, France.
Rind, K., Rodriguez-Barrucg, Q., Nicolas, D., Lignot J.H. Are Mediterranean three-spined sticklebacks (*Gasterosteus aculeatus* L.) of the Camargue wetlands with contrasted salinity conditions morphologically and physiologically different? *Oral presentation.*

Society of Integrative and Comparative Biology. 2018 San Francisco, California, USA.
Rind, K., Rodriguez-Barrucg, Q., Nicolas, D., **Lignot J.H.** Physiological analysis of the Mediterranean three-spined stickleback living in the Camargue area with different saline conditions? *Oral presentation.*

8 Publications

Rind, K., Beyrend, D., Blondeau- Bidet, E., Charmantier, G., Cucchi, P., & Lignot, J. H. (2017). Effects of different salinities on the osmoregulatory capacity of Mediterranean sticklebacks living in freshwater. *Journal of Zoology*, 303(4), 270-280.

Rind, K., rodriguez-Barucg, Q, Nicolas, D., Cucchi, P., Lignot, J.H. (2018). Morphological and physiological traits of Mediterranean sticklebacks living in the Camargue wetland (Rhône river delta). "Submitted for publication in "Physiological and biochemical zoology".

Abstract / Résumé

Threespined sticklebacks (*Gasterosteus aculeatus*) fished from different areas of the Camargue were studied and challenged to various salinity conditions. Blood osmotic pressures for fish kept in FW (5‰), BW (15‰) and SW (30‰) were different (147, 457 and 911 mOsm kg⁻¹, respectively). However, oxygen consumption results revealed similar values for fish collected from the seawater Marteau lagoon (SW), the freshwater Versadou canal (FW) and the mesohaline/brackish water Vaccarès lagoon (BW). Therefore, sticklebacks of the Camargue can rapidly acclimate to different salinity conditions and move freely among these different habitats with limited energy expenditure. For sticklebacks from the Vaccarès lagoon, a significant reduction in O₂ consumption rate occurred immediately after transfer to FW and up to 1h after transfer. The branchial Na⁺/K⁺-ATPase (NKA) activity of sticklebacks collected from the SW Marteau lagoon directly transferred from SW to FW, revealed a significant increase 24 h and 48 h after transfer (28% and 40%, respectively). However, gill NKA activity was significantly decreased by 36% within 24 h following immediate transfer to SW. Body morphology of sticklebacks was also studied but limited morphological variations could be observed for fish collected from different habitats. The average lateral plate number is 4.45±0.31. However, a significant morphological difference is observed for gill rakers. NKA labeling on histological sections revealed different ionocyte morphologies. In the gills of FW sticklebacks, ionocytes were observed along the gill filaments as well as the gill lamellae. Ionocytes were only identified along the gill filaments for SW fish. For SW fish, the ionocytes were round in shape and slightly smaller than the ionocytes of the FW fish. The ionocytes of the FW fish along the gill filaments were elongated but ionocytes along the gill lamellae were flattened and wider. Ionocytes were also twice more numerous for FW fish compared to SW fish. Also, two types of ionocyte were identified in FW fish: cells with an apical dome shape and ionocytes with a honeycomb-like structure. All these cell types were confirmed as ionocytes due to their internal structures filled with numerous mitochondria and a dense tubulo-vesicular system, with deeply invaginated basal membrane. In SW-acclimated fish, only ionocytes with a large apical crypt were observed along the gill filaments at the base of the gill lamellae. No ionocytes could be observed along the gill lamellae. Relative NKA $\alpha 1$ mRNA expression was higher in the gills of SW acclimated fish compared to BW fish and NKA $\alpha 1b$ was significantly overexpressed in the gills of SW acclimated fish compared to the FW and BW fish. However, for NKA $\alpha 1a$, mRNA relative gene expressions in the gills are not different due to salinity. Whereas, for CFTR and V-ATPase expression, we did not find any significant difference. On the other hand, NKCC1 was significantly higher in SW acclimated sticklebacks. For sticklebacks kept in FW and for those acclimated to SW, analyses of histological sections from the kidney and NKA immunolabeling of the kidney proximal tubules revealed no difference. For the intestine, a strong immunofluorescence was observed along the basolateral membranes of the enterocytes for both the anterior and posterior intestine of FW and SW-acclimated sticklebacks but, again, we did not observe any difference due to salinity conditions. These results suggest a high level of plasticity for these organs.

Altogether, these results indicate that sticklebacks of the Camargue area are morphologically and physiologically homogenous and have strong euryhaline capacities. Some differences were identified however for the gill rakers between fish collected from the FW and mesohaline / euryhaline habitats most probably due to different feeding regimes. Therefore, these fish populations may not mix even if the different Camargue aquatic environments are interconnected.

Les épinoches à 3 épines (*Gasterosteus aculeatus*), pêchées dans différentes régions de Camargue, ont été étudiées et soumises à différentes salinités. La pression osmotique du sang de poissons gardés en eau douce (FW : 5 ppm), en eau saumâtre (BW : 15 ppm) et en eau de mer (SW : 30ppm) était différente (147, 457 et 911 mOsmkg⁻¹, respectivement). Cependant, les résultats de consommation d'oxygène montrent des valeurs similaires pour les poissons capturés dans la l'étang du Marteau (SW), le canal du Versadou (FW) et l'étang mésohalin (eau saumâtre) du Vaccarès (BW). Ainsi, ces épinoches peuvent rapidement s'acclimater à différentes salinités et se déplacer librement entre ces différents habitats avec des dépenses énergétiques limitées. Pour les épinoches du Vaccarès, le taux de consommation d'O₂ diminue immédiatement après le transfert en FW jusqu'à 1 heure après transfert. L'activité branchiale Na⁺/K⁺-ATPase (NKA) des épinoches collectées dans l'étang du Marteau et directement transférées en FW a révélé une augmentation significative 24h et 48h après le transfert (28% et 40%, respectivement). Cependant, l'activité NKA des branchies est réduite de manière significative de 36%, 24h suivant le transfert en SW. La morphologie corporelle des épinoches a également été étudiée mais des variations morphologiques limitées ont pu être observées. Le nombre moyen de plaques latérales est de 4,45 ± 0,31. Cependant, une différence morphologique significative est observée pour les branchiospines. Le marquage NKA a révélé des ionocytes avec différentes morphologies. Ces cellules sont également deux fois plus nombreuses chez les poissons FW que chez les poissons SW. Deux types d'ionocytes ont été identifiés chez les poissons FW: les cellules avec un dôme apical et les ionocytes avec une structure en nid d'abeille. Ces cellules sont bien des ionocytes du fait de leurs structures internes (présence de nombreuses mitochondries et d'un système tubulo-vésiculaire dense avec une membrane basale profondément invaginée). Chez les poissons acclimatés en SW, seuls des ionocytes avec une grande crypte apicale ont été observés le long des filaments branchiaux à la base des lamelles branchiales. Aucun ionocyte n'a pu être observé le long des lamelles branchiales. L'expression relative de l'ARNm de NKA $\alpha 1$ est apparue plus élevée dans les branchies des poissons acclimatés en SW que chez les poissons en BW. L'expression de la NKA $\alpha 1b$ est significativement surexprimée dans les branchies des poissons SW par rapport aux poissons BW et FW. Cependant, pour la NKA $\alpha 1a$, les expressions relatives dans les branchies ne sont pas différentes selon la salinité. Pour l'expression du CFTR et de la V-ATPase, nous n'avons trouvé aucune différence significative. Par contre, NKCC1 est significativement plus élevé chez les épinoches acclimatées en SW. Les analyses histologiques du rein et le marquage de la NKA au niveau des tubules proximaux du rein n'ont révélé aucune différence pour les poissons en FW et en SW. Pour l'intestin, un fort marquage a été observé le long des membranes basolatérales des entérocytes. Cependant, là encore, nous n'avons observé aucune différence due aux conditions de salinité. Ces résultats suggèrent un haut niveau de plasticité pour ces organes.

Globalement, ces résultats indiquent que les épinoches de Camargue sont homogènes au niveau morphologique et physiologique et possèdent de fortes capacités euryhalines. Cependant, des différences ont été identifiées au niveau des branchiospines entre les poissons capturés dans les habitats FW et les habitats BW/SW, probablement en raison de régimes alimentaires différents. Par conséquent, ces populations peuvent ne pas se mélanger même si les différents environnements aquatiques de Camargue sont interconnectés.