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Impacts of the salinity on the physiology, the immunity and the hematology of the striped catfish (Pangasianodon hypophthalmus)

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Impacts of the salinity on the physiology, the immunity and the hematology of the striped catfish (*Pangasianodon hypophthalmus*)

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Nicolas Boileau

Abstract of the master thesis

The impacts of the salinity on the physiology, the immunity and the hematology of the striped catfish (*Pangasianodon hypophthalmus*) affected by a rise of the salt concentration and a prolonged exposure on the response to the bacterial challenge with *Edwardsiella ictaluri*.

The future fish supply is threatened by several anthropogenic factors, deeply compromising the daily protein intake of an important part of the global population. In order to deal with this problematic, several countries developed their aquaculture sectors in order to meet the global fish demand and to become an essential trading partner.

Currently, Vietnamese aquaculture development in the Mekong Delta is one of the best success stories. Prime Minister plan entirely reviews the sector by 2020 and by 2030 in order to become more competitive for the aquaculture products exportations specially for the striped catfish *Pangasianodon hypophthalmus*. However, the dam constructions on the Mekong river and the sea level rise due to the climate change strongly compromise the objectives achievement by increasing the salinity in the area. These saline intrusions mainly occur during the dry season and disrupt the aquaculture sector. Even if it enables to tolerate up to 15 ppt of salinity, striped catfish is highly affected by the saline episodes and tends to be more sensitive to *Edwardsiella ictaluri* infection.

In order to investigate the effect of the salinity on the modulation of the physiology, the immunity and the hematology of this fish species, the experiment was conducted in two phases: a salinity rise which was followed by a two-week exposure at the reached salinity. At the end of each phase, a bacterial challenge was carried out in order to test the cumulative effect of stressors during saline stress.

Results of the physiological parameters show that the osmolality and the ion concentrations (Na^+ and Cl^-) immediately detected the saline stress and that it was not affected by the bacterial challenge. The rise of the osmotic pressure of the plasma induced first a disruption of the hematological parameters (drop of the erythrocyte concentration and hematocrit ratio, increase of the hemoglobin concentration, disturbance of the mean corpuscular volume and growth of the mean corpuscular hemoglobin concentration and the mean corpuscular hemoglobin). However, the long exposure makes the acclimation of these parameters possible by reducing the variability between the different saline treatments. The immune response to these cumulative stressors was firstly observed as a normal modulation during infection of the peroxidase activity and a decrease of its activity fourteen days after the salinity rise. The absence of modulation of several parameters between the salinity rise and the two-week exposure potentially indicates that the slow speed during the first phase was better adapted for the stress response, that acclimation under defined conditions might be possible and that the bacterial challenges were not conclusive.

Studying the influences of environmental conditions on the epigenetic mechanisms and on the stress resistance in earlier life stages of striped catfish might turn out to be interesting in the future, providing the selection program of the PANGAGEN project with additional support.

Résumé du mémoire

Les impacts d'une augmentation de la salinité et d'une exposition prolongée sur la physiologie, l'immunité et l'hématologie du pangasius (*Pangasianodon hypophthalmus*).

L'apport futur en poisson est fortement menacé par plusieurs facteurs anthropogéniques. Dans le but de trouver une solution, des pays ont fait le pari de développer leur aquaculture pour pouvoir répondre à l'augmentation de la demande mondiale en poisson et ainsi devenir un partenaire économique essentiel. Actuellement, le développement de ce secteur au Vietnam est un des meilleurs exemples. Les plans économiques nationaux ont prévu la revue complète du secteur d'ici 2020 et 2030 pour devenir plus compétitif, plus spécialement pour le pangasius *Pangasionodon hypophthalmus*.

Cependant, la construction de barrages sur le Mékong et la montée du niveau des océans à cause du changement climatique compromettent fortement la réalisation des objectifs économiques du pays en induisant l'augmentation de la salinité dans le delta. Les intrusions salines se produisent durant la saison sèche et perturbent totalement les populations piscicoles des fermes aquacoles. Même si le pangasius peut supporter des salinités atteignant 15 ppt, il semble être fortement affecté par ses épisodes et devenir plus sensible à des infections provoquées par *Edwardsiella ictaluri* infection.

Dans le but d'investiguer les effets de la salinité sur la modulation de la physiologie, immunité et hématologie de cette espèce, une expérience a été réalisée en deux temps : une augmentation de salinité et son maintient durant deux semaines. À la fin de chacune de ces deux phases, les poissons ont été soumis à un challenge bactérien dans le but de tester l'effet cumulatif de ces deux stress.

Les résultats montrent que les paramètres physiologiques détectent immédiatement le stress salin et s'y adaptent mais ne modulent pas leur comportement en cas d'infection. L'augmentation de la pression osmotique du plasma provoque dans un premier temps la perturbation des paramètres hématologiques (augmentation de la concentration en érythrocyte et en hémoglobine et de l'hématocrite, perturbation des caractéristiques des cellules sanguines). Cependant, après une période d'acclimatation à la salinité, l'adaptation de ces paramètres semble possible et la réduction de la variabilité entre les différents traitements salins en est la meilleure preuve. La réponse immunitaire à ces deux stress cumulatifs a d'abord induit une modulation normale de l'activité de la peroxydase en cas d'infection mais également le déclin de son activité deux semaines plus tard. L'absence de la modulation de plusieurs autres de ces paramètres indique que la lente augmentation de salinité durant la première phase était mieux adaptée pour les poissons, que l'acclimatation à l'eau salée devrait être possible sous certaines conditions et que les challenges bactériens n'étaient pas concluants.

Au vu de ces résultats, étudier l'influence des conditions environnementales sur les mécanismes épigénétiques durant les premiers stades de vie du pangasius pourrait être intéressant dans le futur et apporter un soutient supplémentaire au programme de sélection du projet PANGAGEN.

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1. Introduction

1.1 Global state of fish supply

The human population prediction reaching 10 billion people by 2050 (United Nations 2017), the future food supply is going to be more and more complicated in a context of global climate change, especially for the fish one (Béné et al. 2015; Worm and Branch 2012). During the last fifty years, the global average consumption of fish per person exploded, increasing from 9.9kg in 1960s to 20 kg in 2014. In 2013, 17% of the global population depended on the fish for their only intake of animal protein while industrialized countries augmented their fish consumption is higher in the (26.8 kg per capita) than in the developing ones (18.8 kg per capita).

In the last decades, the fish supply was ensured by the marine capture reaching 81.5 million tons in 2014 (FAO 2016). But a closer data analysis reveals that the situation is not sustainable in the long term. In several regions, the captures have dropped by one-third between 2007 and 2014 and, at the same time, the total capture for the 25-major species fell by 2.7%. This overall decrease is mainly due to the previous unreasonable exploitation and it is currently reinforced by the increase of the fishing effort. The consequences on the fish population health result in a dramatic shift of the maturation age/size and a reduction of the population size, the overall degradation of the stock state (in 2013, 58.1% of the global stocks already achieved a fully exploited state and 31.4% were biologically unsustainably exploited) and the disruption of the entire marine ecosystem by the collection of the lower trophic levels (Hsieh et al. 2010; King 2007; FAO 2016). In other words, continuing the exploitation the fish resources in the same way will probably lead to a premature extinction of it even without the climate change impacts.

In order to ensure the growing demand and to develop their economy (FAO 2016), some countries as Vietnam have taken up the challenge to develop their aquaculture sector by adding new technologies and scientific knowledge (Worm and Branch 2012). This industry is the equivalent of agriculture in aquatic environment (marine, brackish water and freshwater) including animals and plants. Farming aquatic organisms is the enhancement of their production by stocking, feeding and protecting them from predators (Edwards and Demaine 1998). Offering the main solution for fish production, the aquaculture currently accounts for 44.1% of the total production and it will be soon exceeding the fisheries production (Figure 1). In 2014, this sector provided 73.8 million tons for a first-sale value of US$ 160.2 billion (FAO 2016). The freshwater and brackish water aquaculture represents around 75% of the global production,

more than 35% being ensured by carp, tilapia, catfish, … (FAO 2018b, 2016; Ottinger, Clauss, and Kuenzer 2016)

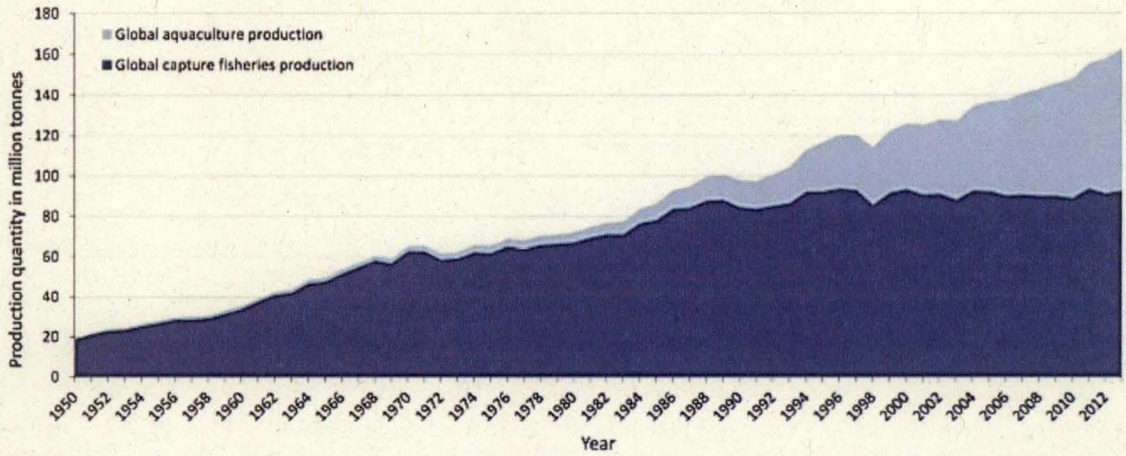

Figure 1 : Global capture fisheries and aquaculture production (millions of tonnes) between 1950 and 2014 (Ottinger, Clauss, and Kuenzer 2016)

Although the life cycle of some species are not yet completely knew(FAO 2018d, [a] 2018, [c] 2018), the aquaculture provides 580 species including 362 finfishes, 104 mollusks, 62 crustaceans, 37 aquatic plants, 9 invertebrates and 6 species of frogs and reptiles(FAO 2016). The economic importance of this sector enables some country to be more competitive in the world fish market which is mainly dominated by the Asian countries providing nearly 90% of total aquaculture production. Distantly followed by Indonesia, India, Vietnam, Philippines and Bangladesh, China provides about 58.8 million tons of aquaculture products which represents nearly 60% of the world's production (Figure 2).(Ottinger, Clauss, and Kuenzer 2016; FAO 2016)

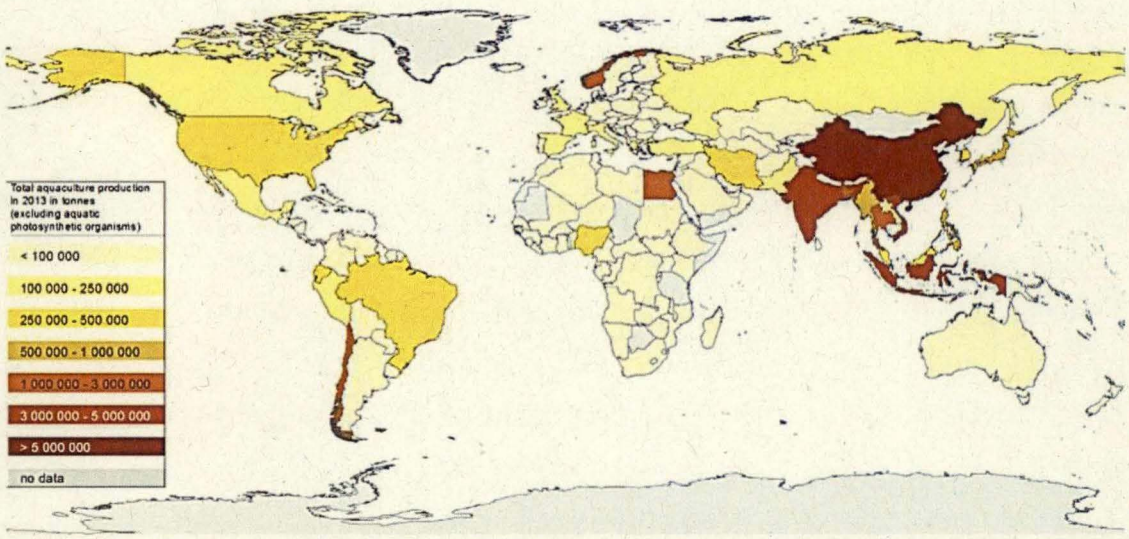

Figure 2 : Map of the total aquaculture production (2013) in tonnes (excluding aquatic photosynthesis organisms) (Ottinger, Clauss, and Kuenzer 2016)

1.2 Fish production in Vietnam

In Vietnam, the average individual fish supply ranged from 30 kilograms to 60 kilograms per year, with an individual daily intake of fish protein from 6 grams to 10 grams. Fish contributes to more than 20% to animal protein supply of the population (FAO 2016). Besides the huge national fish consumption, Vietnam is an interesting study case through its fast-economic development related to the aquaculture for the world market. In 1995, Vietnam became a member of the Association of Southeast Asian Nations (ASEAN)("ASEAN" 2018). With the ASEAN help, the Vietnamese government developed its economy and induced social progress. The evolution of the national fish production makes the increase of the national exports possible. The first evidence of this economic improvement is the signing of the Bilateral Trade Agreement with the United States of America in 2001 which gave to Vietnam a place in the world market. This signature was followed by different Free Trade Agreements (FTA) with China, Japan, Korea, India, Australia and New Zealand. In 2006, Vietnam entered for the first time in the top 10 of fish exporters with a US$ 2.4 billion worth of production (FAO 2006). One year later, the country joined the World Trade Organization becoming an active actor in establishing of international rules for trade. The Vietnamese economy opening still continued in 2015 and 2016 with Trans-Pacific Partnership (TPP) agreement and different FTA with the European Union and the Eurasian Economic Union(H. T. K. Nguyen et al. 2017). In 2017, the national production increased threefold and the total value of the exportation reached a value of US$ 8.3 billion (VASEP 2018a). Since the early 2000s, the agriculture and rural sector were modernized and industrialized. The high correlation between aquaculture production and the Gross Domestic Product (GDP) indicated that this sector provided food, jobs and stable incomes for the population and largely participated to the national economic development (McCoy et al. 2010). Since 2007, fish aquaculture has surpassed for the first-time wild capturing. (Figure 3)

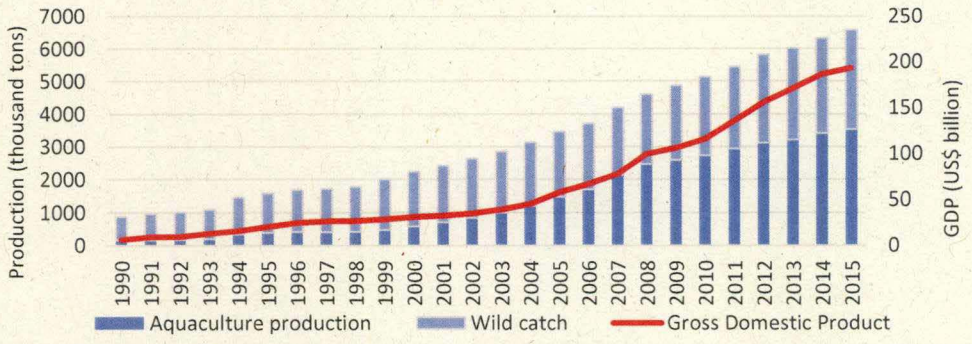

Figure 3: Evolution of wild catch and aquaculture production and gross domestic product of Vietnam (General statistics office of Viet Nam 2016; The World Bank 2018)

This incredible success story will probably continue as indicates the average annual growth between 2004 and 2014 of 12.6% which was higher than China's one (12.2%) (FAO 2016). According to a ministerial decision of Augustus 2013, the aquaculture and capture sectors will be entirely reviewed by 2020 and by 2030 (The Prime Minister 2013a). By 2020, total fishery products have to reach 7 million tons, 65% coming from the aquaculture sector and 35% from wild capture. To achieve the production of 4.5 million tons, 1.2 million hectares will be dedicated. The Master Plan also includes infrastructure expansion, qualitative strain selection and sustainable use of energy, transportation and land use (The Prime Minister 2013a). Projections for 2030 plan 9 million tons of fish including 70% coming from aquaculture while reducing the greenhouse gas emission by 8% and sustainably adapting to climate change.(The Prime Minister 2013b; NDC Partnership 2017)

1.3 Pangasius production

In Vietnam, the pangasius production represents 32.4% of the total aquaculture production and 17.2% of the national fish production (VASEP 2018a). From less than 400 000 tons in 2004, the production reached 1.25 million tons of Pangasius in 2017 using 5 227 hectares and returning US$1.78 billion which is 4.3% higher than the production in 2016 (Figure 4) ("Vietnam Association of Seafood Exporters and Producers" 2018; Ragnar 2017; VASEP 2018a). Between 2005 when the production exploded and 2014, Vietnamese Pangasius achieved 80% of the international food market for this species. Seen as a good substitute to "white fish", Pangasius was exported all around the world: China (23%), US (19%), EU (11%), ASEAN countries (8%), Brazil (6%) and Mexico (6%) being the principal importing countries.

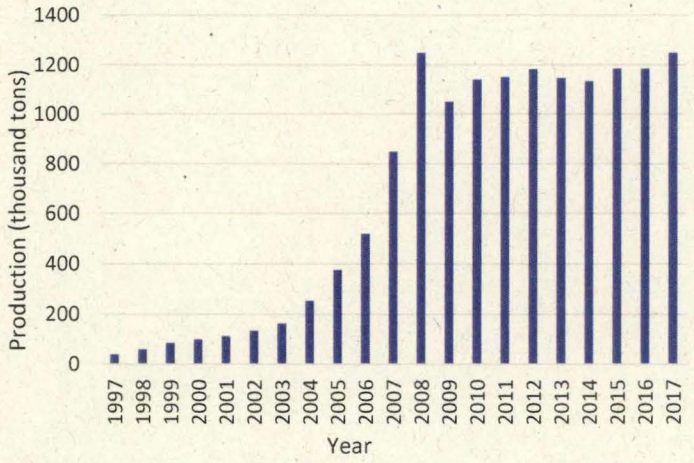

Figure 4: Evolution of the Pangasius production in Vietnam from 1997 to 2017 (Ragnar 2017; VASEP 2018a)

The biggest part of this production comes from the 100 pangasius factories located in the Mekong Delta which represents about 75% of the national Pangasius production(VASEP 2018b). The striped catfish (*Pangasianodon hypophthalmus*) species production covers around 95% of this pangasius production (T. P. Nguyen 2013). In this region, the striped catfish farming provides opportunities to rural people and in particular for women. Due to this important activity, the population density in the Delta is the most important one of the country with 434 people per km² (20% of Vietnam's population) (General Statistics Office Of Vietnam 2016). The Master Plan objectives for 2020 predict the production growth of the Pangasius from 1.8 to 2 million tons, the allocation of 650 000 tons to the exportation and the farming area increase to 10 000 hectares(The Prime Minister 2013a; FAO 2014).

However, even if the Vietnam has become an economic power in the fish product sector, the current production only achieves 78% of the planned one for 2020. The causes of this delay are, for example, the inadequate management skills (for example during saline intrusion) and improper methods of production (Ngoc et al. 2018; H. T. K. Nguyen et al. 2017), and variable environmental conditions inherent in the production site.

1.4 Study site

1.4.1 Mekong Delta

Due to extensive water area and favorable environmental conditions, the Mekong river delta is the most productive area of Vietnam in terms of aquaculture. The 4880 km-long River crosses five countries (*Figure 5*). Originating in the Chinese Himalayas, it successively goes through

Figure 5: Location of the Mekong Delta in Vietnam(Unknown 2018)

Myanmar, Laos, Thailand before flowing in the South China Sea. The total Mekong river basin drains 795 000 km^2 depositing huge amount of sediments at its mouth and brings important freshwater volume. This river is the 12^{th} longest one with the 8^{th} largest flow with an annual discharge of around 475 km^3. The Mekong Delta is located at the river mouth astride Cambodia and the southwest part of Vietnam and covers 40 576 km^2. The Vietnamese part represents approximately 79% of the total Mekong delta. In this work, "Mekong delta" is used to define the Vietnamese region. (Mekong River Commission 2011)·(AQUASTAT survey 2011; Mekong River Commission 2009)

The major characteristic of this type of environment is the average low-elevation, only 0-4 meters above mean sea level. The outer delta plain located nearest to the South China Sea is affected by tides, waves and currents. In order to protect themselves, local populations build ridges and dunes near the coast explaining the slight higher elevation of this region and the lower elevation of the inner plains. (Mekong River Commission 2011)

1.4.2 Environmental conditions

Climate in this subtropical region is dominated by the Southwest Monsoon between May and early October with an average annual rainfall of 1600-1800 mm and up to 2750mm on Ca Mau peninsula, the southernmost region of the delta (Mekong River Commission 2011; Hung 2017; Mikhailov and Arakelyants 2010). Temperature ranges from 25°C to 30°C and maximum precipitation takes place between July and September also corresponding to the flood period of the Mekong river. During this rainy period, water flow of the river can reach 40 000 m^3 per second (Le et al. 2007). In contrast, the dry Northeast monsoon occurring from November and March induce the decrease of rainfalls and of the water flow of the Mekong River flow (2 100 m^3 per second) (Mikhailov and Arakelyants 2010). The alternation of these natural factors influences the water level and the saline concentration of the water in the delta (Figure 6).

Near the coast, the water salinity is higher than the one of inland bends. It induces salinity gradient from 0 ppt in inland plains to 32-33 ppt at the sea interface(Mikhailov and Arakelyants 2010). But the saline front varies according to the season and freshwater supply. Given that freshwater flow decreases in dry season, the saline front is not repelled anymore and progresses into the delta. Salinity in some places increases and freshwater areas become salty during the dry season. All these complex physicochemical properties are influencing the productiveness of the Delta region. Unfortunately, this balance is threatened by different anthropogenic stressors. (Thi Ngoc Trieu and Thanh Phong 2014)

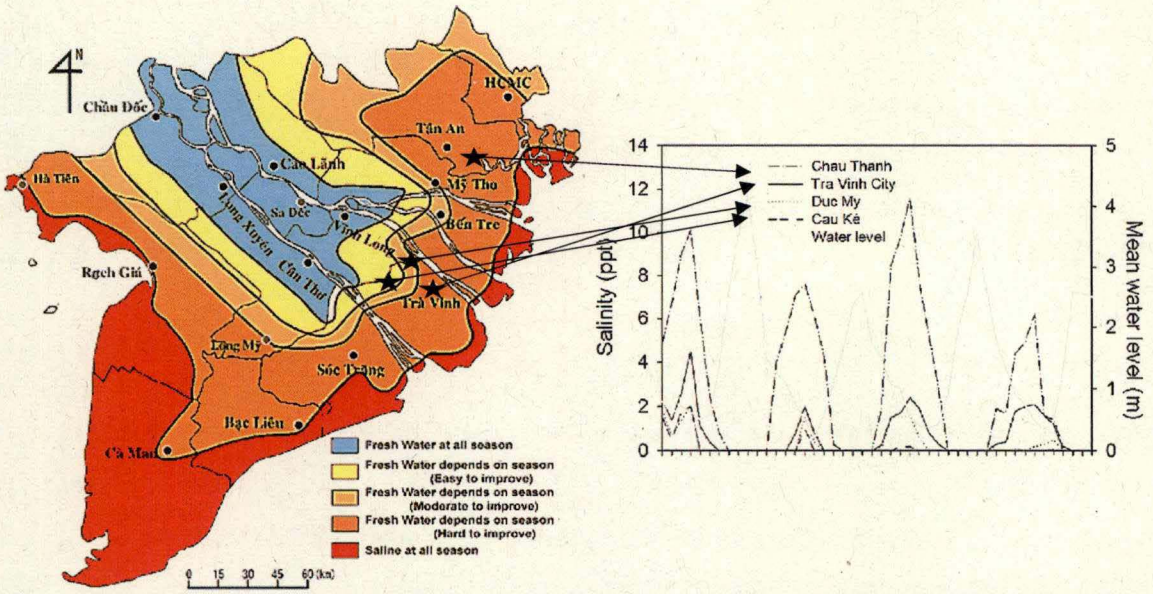

Figure 6: Status of saline intrusion, seasonal salinity (ppt) and water level (m) changes in Mekong delta from January 2011 to October 2014. ("Flood in the Mekong Delta - Nam Kỳ Lục Tỉnh" n.d.; Mélodie Schmitz, Baekelandt, et al. 2017)

1.4.3 Anthropogenic stressors in the delta

One of the major anthropogenic stressors in the delta is the climate change and its impacts on oceans. According to the IPCC report, 90% of energy stored in the climate system accumulated between 1971 and 2010 is stored in the oceans, resulting a temperature increase from 0.09 to 0.13 °C per decade. This energy accumulation induces the thermal expansion of the water mass which contributes between 28% and 41% to the annual sea level rise. This phenomenon is reinforced by the melting of the continental glaciers and the ice sheet, their contribution ranging from 50% to 70% (Stocker et al. 2013). From 1901 to 2010, the global sea level rose from 0.17 m to 0.21 m and the local previsions for sea level near the Mekong Delta add ±0.6 m to the current sea level while. Because of its important coastal zone proportions and its 3 260 km long coastline, Vietnam is one of the most threatened countries in the world (Kreft, Eckstein, and Melchior 2017).

Moreover, climate change also impacts dry and rainy season flows. Dry season discharge would decrease by 2% while total water flow during the rainy season would increase by 5% in the next three decades (Fulton et al. 2018). Minimum monthly flow in the Mekong Delta would drop by 26 and 29% between 2070 and 2100 (Wassmann et al. 2004). In other words, climate change previsions indicate a clear trend about rainfall and sea level which could lead to important threats in the Mekong Delta. (Thi Ngoc Trieu and Thanh Phong 2014)

The second menace is the construction of dams on the river. Mekong River crosses five countries: China, Laos, Myanmar, Thailand, Cambodia and Vietnam. They have all an important energy demand in order to carry on their economic development or to improve living

standards of their population. Due to its important flow, the Mekong river is the perfect location to build hydroelectric dams. Currently, 7 hydropower dams are constructed, 25 are planned and 11 are under construction on the Mekong mainstream. In addition, numerous irrigation and water supply systems and multipurpose constructions are present on the entire Mekong basin (WLE Creater Mekong 2016).

The consequences of these artificial constructions are numerous : sediments do not reach the delta plain, accentuating the shoreline erosion which rises from 4.9 ±6.9 meters a year; the river is fragmented, preventing the migration of potamodromous species like *Pangasianodon hypophthalmus,* the nutrient supply is reduced as the freshwater inflow in the Delta.(X. Li et al. 2017; Mikhailov and Arakelyants 2010)

Because of the climate change, the low elevation, the sea level rise and the freshwater supply decrease, the Mekong Delta's coastline regions will face severe damages. The major consequence is a rise of marine water intrusion (Wassmann et al. 2004). In 2010 (Figure 7A). Usually, the saline intrusions penetrate 55-62 km in the Tien river and 55-60 km in the Hau river during the dry season (April). The salinity reaches high concentration (superior or equal to 12 ppt) in 29% of the area while 11% reaches medium salinity (from 5ppt to 12 ppt) and 13% get to low salt concentration (from 1ppt to 4ppt). In other words, more than half of the area is impacted by salt.

Nonetheless, the prevision for 2100 would plan a graver situation (Figure 7B). The salinity intrusion would shift from 78km to 80km in Hau and Tien rivers and 63% of the delta would be impacted by salinity. Currently, the salinity affects 1.8 million hectares of lands in the Delta during the dry season and salty water enters until 90 km inland. (Thi Ngoc Trieu and Thanh Phong 2014; Mekong River Commission 2010). Saline water in freshwater environment greatly affects physiology of aquatic organisms and lowered crop efficiency in the region, leading to higher rates of mortality or to a lower productivity (Wassmann et al. 2004; Thi Ngoc Trieu and Thanh Phong 2014; Kotera et al. 2008; Pedersen et al. 2014).

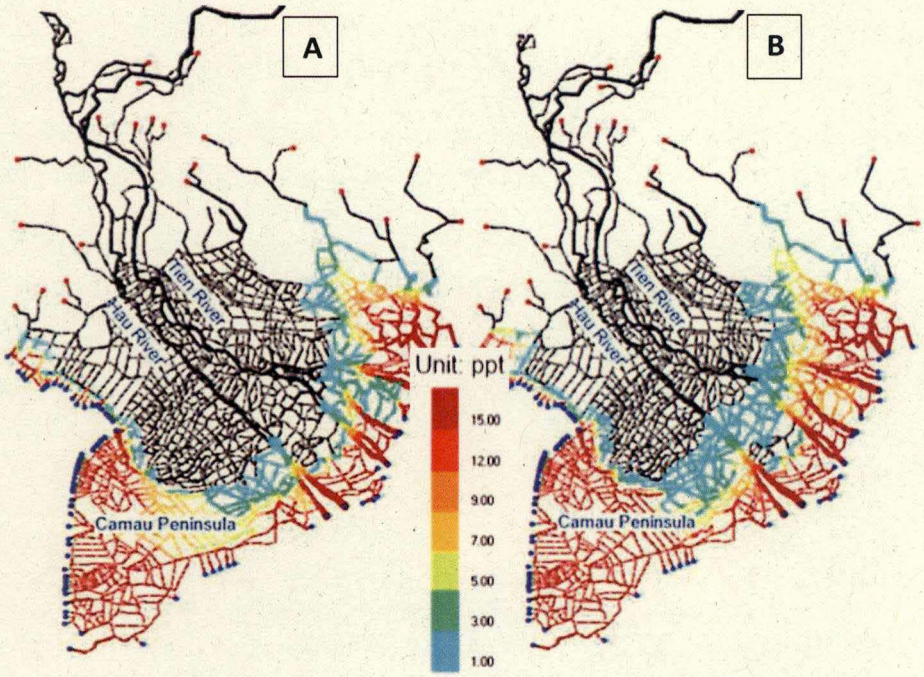

Figure 7: Results of marine intrusions in the Mekong Delta. A: 2010; B: 2100 prevision. This future scenario was planned with sea level rise by 0.73cm and decreasing flow by 29%. (Thi Ngoc Trieu and Thanh Phong 2014)

1.5 Pangasianodon hypophthalmus

Due to its extensive use in the Mekong Delta and its apparent low resistance to saline intrusion, striped catfish (*Pangasianodon hypophthalmus)* is an interesting model in order to better understand the effects of salinity on physiology. Improving the knowledge about this species will enable to secure this sector of aquaculture in the delta region.

1.5.1 Overall description

The striped catfish (*Pangasianodon hypophthalmus* (Sauvage 1878)) is part of the teleost and more precisely of the family of Pangasiidae and the Pangasianodon genus (FishBase Consortium 2016; ITIS 2017). It is characterized by large full-grown adults (20 cm to about 3m). On the head, adults exhibit two paires of barbels and two pairs of nostrils of the same dimensions. Below the operculum, 7-11 branchiostegal rays support the gill membrane. The fins include an adipose one and pelvic ones with 8. (Roberts and Vidthayanon 1991)

Furthermore, the small truncated head pointing in lateral view is formed of the terminal mouth and large eyes. The upper jaw is covered with teeth and entirely protected by the lower jaw when the mouth is closed. In order to be able to equilibrate itself during rapid rise, this species possesses a unique and elaborately vascularized swim bladder with efficient aerial exchanges. Other minor characteristics are also present like the more or less well-joined palatine plates and

vomer bone, low number of vertebrae of the abdominal part of the body, unequal gill rakers on the gill arches, ... In ponds, length of striped catfish is about 80 cm with a weight of 6-7 kg. In the juvenile stage, well-defined stripes are located mid-laterally and abdominally. These are separated at the level of the pectoral fin origin. Other darker stripes are also visible in the middle of the anal fin at this stage but they are lost at the adult stage, which are uniformly grey with greenish and silvery sides. (Roberts and Vidthayanon 1991; FAO Fisheries & Aquaculture 2018) rays (Figure 8)

Figure 8 : Pangasianodon hypophthalmus *(BIOMIN 2015)*

1.5.2 Overall farming methods

At the beginning of the striped catfish production, wild individuals were largely captured in order to provide broods. Because of the fall of the wild population, farmers have entirely replaced the wild capture by stocking of hatchery-produced seed. In captivity, females and males reach sexual maturity respectively at three and two years.

After 22-24 hours of incubation, eggs hatch and larvae feeding are entirely dependent on yolk sac absorption during its first 24 hours. Just before the total yolk sac absorption, larvae are stocked in tanks with a density between 400 and 500 individuals per m^2 in order to avoid cannibalism. In addition to the natural feeds (*Moina sp.*) available in the freshwater, farmers give an emulsion of boiled egg yolk and soybean during the first two weeks, which is progressively replaced by commercial pellets after this period. After 4 weeks, fries are transferred and stocked in a second nursery pond with a lower density of 150-200 individuals per m^2 until they reach the fingerling stage with a body weight of about 14 to 20 grams. The transition from larval stage to fry stage records the lower survival rate with 40-50% while for fry to fingerling transition, the survival rate is 60-70% (Figure 9). The harvest takes place when

fish reaches a body weight around 900 grams(FAO Fisheries & Aquaculture 2018; T. A. Nguyen et al. 2013; Sang et al. 2009). In the Mekong delta, the life cycle of the striped catfish is carried out by different farming systems in different locations independently operating.

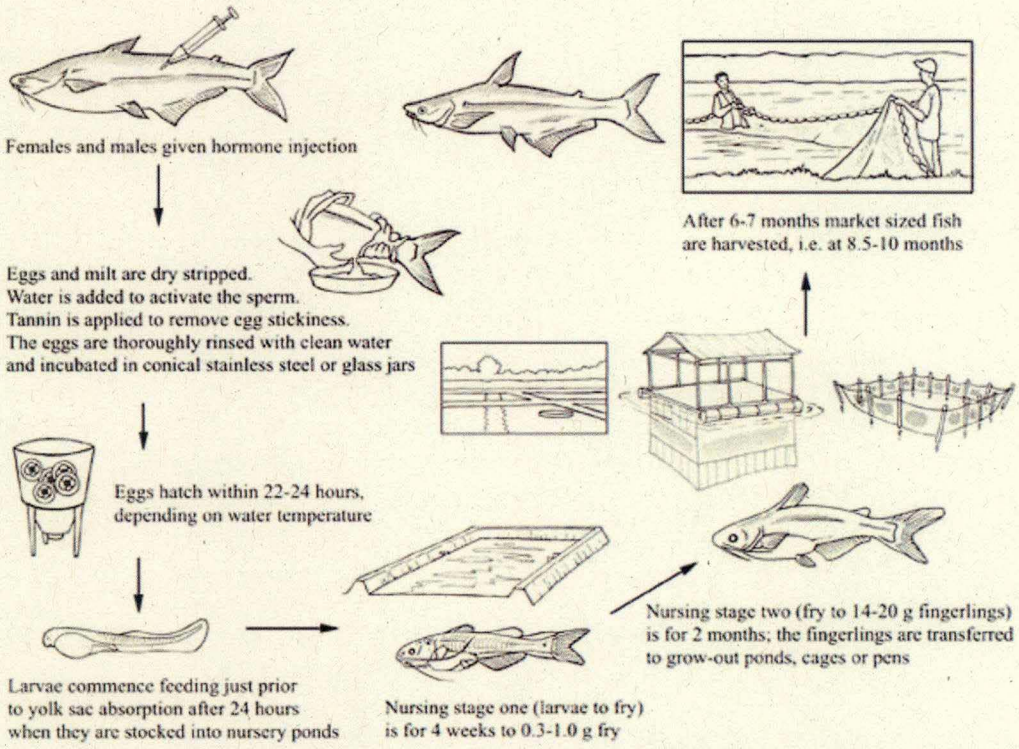

Figure 9 : life and production cycles of P. hypophthalmus*(FAO Fisheries & Aquaculture 2018)*

Specialized hatchery production is located in the upper part of the delta (Figure 10). Their main roles are to manage the brood stock in order to provide gametes, ensure fecundation and hatchery, and to supply nursery and grow out sectors with individuals. First one carries out the larvae development in fry ponds during 1-1.5 months before a transfer to nursery ponds. The stay of fry in these earthen tanks last the same amount of time. After this development period, fingerlings are allocated to the grow out sector that stock them in on-stream and on-canal ponds during 5 to 6 months until the harvest size is reached (De Silva and Nguyen 2011).

When fish are located in the river, farmers use net cages and net pens. First ones are mainly used along Hau and Tien rivers, the two major tributaries of the Mekong river in the delta. With size range from 50 to 1600 m^3, their yields fall between 100 and 120 kg per m^3. Net pen has lower production with a total yield ranging between 30 and 35 (in certain regions 50) kg per m^3. The farming system of striped catfish is able to produce on average between 200 and 400 tons per hectare. Ponds are from 4.5 to 5 meters deep and are connected with the Mekong river in order to ensure a regular water exchange. (T. P. Nguyen 2013; FAO Fisheries & Aquaculture 2018)

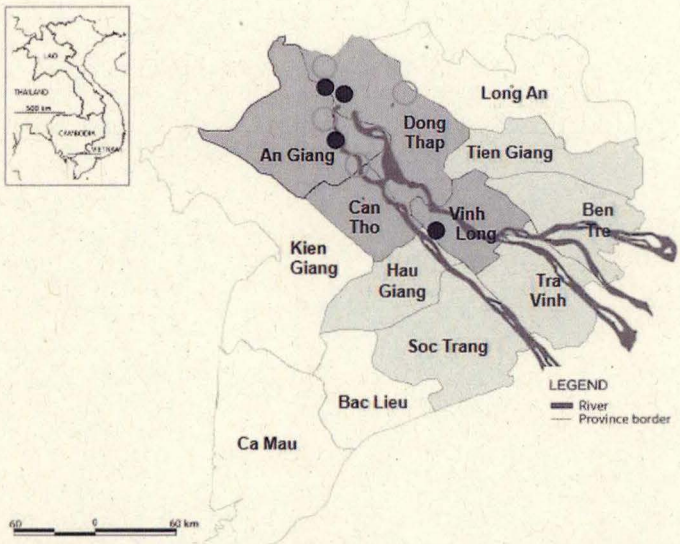

Figure 10: Distribution of striped catfish farming in the Mekong Delta. Dark grey province=main catfish culture provinces, light grey=newly developed catfish provinces, dark point=main hatchery locations, grey circles=main nursery locations.

1.5.3 Ecology of the species

The natural distribution of this freshwater species is the Mekong basin and Chao Phraya river but striped catfish was also introduced in other river basins for fish farming in southern Asia. Currently, pangasius farming also occurs in Bangladesh, China, India, Malaysia and Myanmar (FishBase Consortium 2018; FAO Fisheries & Aquaculture 2018).

In its natural environment, this potamodromous species migrates extensively between upstream where refuge and spawning habitats are located and downstream where feeding and nursery habitats are located (Van Zalinge et al. 2002). Migrations are closely linked to the monsoon season : the descent takes place during the dry season while the return occurs in rainy season.(FishBase Consortium 2016; FAO Fisheries & Aquaculture 2018) Unfortunately, since 2011, wild striped catfish is on the extinction list because of the adults overfishing for farming and of the dams which stop the migration (Vidthayanon, C. and Hogan, Z. 2013). *P. hypophthalmus* usually supports a pH range from 6.5 to 7.5 and a temperature range from 22°C to 26°C(FAO Fisheries & Aquaculture 2018). Given that the temperature of the Mekong river is ranged from 23 ±2.9°C to 29 ±1.5°C and the pH is from 7.3 to 8.4, the Mekong Delta is a perfect place for the striped catfish production (S. Li, Lu, and Bush 2013). Furthermore, striped catfish supports low dissolved oxygen concentration between 0.05 and 0.10 mg/L and highly polluted water (nitrite: LC50 96h = 1.95 mM (Lefevre, Jensen, Huong, et al. 2011)). It also tolerates up to 15 ppt of salt even if the growth is affected from 13 ppt (Kumar et al. 2017). These characteristics enable the high-density production at the adult stage in the Mekong Delta.

1.5.4 Physiology

Striped catfish possesses different physiological features making them well adapted to particular characteristics of the Mekong Delta environment but also vulnerable if any changes happen.

Gills are the first important anatomical organs that enable striped catfish survival. Modern fish possesses well-developed gills with a complex anatomy due to its multifunctionality. Located on the pharynx, they are supported by arches that radiate laterally from the internal base. These bony pieces are covered by richly vascularized connective tissues forming the interbranchial septum. These filaments freely move in the branchial cavity and are protected by an operculum.

First major function of gills is to ensure the respiratory exchanges. Because of the high temperature, concentration of dissolved oxygen in the Mekong river water is very low resulting in recurrent hypoxia episodes. To survive in these conditions, *P. hypophthalmus* had developed an efficient, active and facultative air-breathing. Sometimes, it surfaces and gulps air before turning back to be submerged again. However, unlike other air-breathing fish species, striped catfish possesses well-developed gills that have a high capacity for dissolved oxygen uptake. These other species have reduced gills in order to avoid branchial oxygen loss in hypoxic water. This specific characteristic of striped catfish could be due to its migratory natural behavior and its normal temporary residence in the Delta during these round trips (Damsgaard et al. 2015; Lefevre, Huong, Ha, et al. 2011; Lefevre, Huong, Wang, et al. 2011). Nevertheless, opercula are equipped with a membranous flap along the edge that seems to diminish the contact surface between water and gills when they are closed (Lefevre, Huong, Wang, et al. 2011). Through these adaptations, this species is able to survive at severe hypoxia episodes which only 6kPa of dissolved oxygen in the surrounding water. But oxygen levels in Vietnamese ponds can sometimes reach 5kPa. In this condition, fish must allocate a large amount of time and energy on air-breathing (Lefevre, Huong, Ha, et al. 2011). Well-developed gills are thus useful in hypoxic water but because of their high surface, they become problematic for osmoregulation in saline conditions.

The second function of gills is to ensure osmoregulation of Na^+ and Cl^- ions the kidney activity. In teleost fish, plasma osmotic regulation depends on the ion exchanges adjustment. In this class, 95% of the species possesses a low capacity to maintain their osmotic homeostasis and are called stenohaline. The species of the last five percent, such as the striped catfish, tolerate

range of salinity concentration and are named euryhaline (Kumar et al. 2017). In both cases, they continually adjust their plasma ion concentrations and their water uptake.

In freshwater, ion levels are lower than in the fish plasma. This hyperosmotic condition induces the passive excretion of ions and gain of water and forces fish to continually eliminate large amount of water and to actively uptake ion across gills. In contrast, the plasma of seawater fish is hypoosmotic in higher saline concentration. Therefore, they must respond to the passive loss of water and the gain of ions by the ingestion of seawater and excretion of small volumes of urine and active excretion of salt across gills (Evans 2008; McCormick 2011). The external epithelium of the gills is composed of different types of cells including ionocytes, a category of chloride cells enabling osmoregulation. These mitochondrion rich cells actively absorb ions from surrounding water using three different uptake mechanisms (Dymowska, Hwang, and Goss 2012). Even if the specific mechanism of ion exchanges is still unknown, the most accepted ones are presented in the following descriptions. (Figure 11)

First one is based on the presence of Na^+/H^+ exchangers (NHE) (Figure 11A). These are passive transporters capturing Na^+ ions from the water coupled with the excretion of H^+ ions. In order to limit passive ions excretion, Na^+/K^+ ATPase's (NKA) located in the basal membrane of ionocytes are continually excreting Na^+ ensure a lower concentration of this ion in the cell relative to the surrounding environment. In the other hand, H^+ excretion is driven by ammonia-conducting Rh proteins (Rhcg1) (Kumai and Perry 2012). These latest transporters are specific for NH_3 and not for NH_4^+ forcing this one to get rid of its proton. This proton would then supply NHE exportation. The expression of mRNA and/or protein of these exchangers is increased by acclimation to freshwater. Furthermore, NHE family of mammals also includes nine isoforms. NHE2 and NHE3 were detected in numerous fish species (Yan et al. 2007), (Choe et al. 2005, 3), (Bradshaw, Kumai, and Perry 2011) and are also highly distributed in kidney, ensuring the recapture of Na^+ and Cl^- and the excretion of high quantity of a diluted urine(Kumai and Perry 2012). In addition to NHE, evidence for the presence of Na^+/Cl^- cotransporter (NCC) are more frequent (Figure 11B). This passive transporter captures Cl^- and Na^+ from the water. It would be located in the apical membrane of ionocytes and be able to modify the membrane's electric potential. In addition, basolateral NKA and Cl^- channels keep the concentration of all these ions at a very low level and could contribute to both Na^+ and Cl^- absorption in the cells and to the limitation of the passive excretion of these ions. Recruitment of NCCs mainly happens when fish is transferred from freshwater to seawater. (Kumai and Perry 2012)

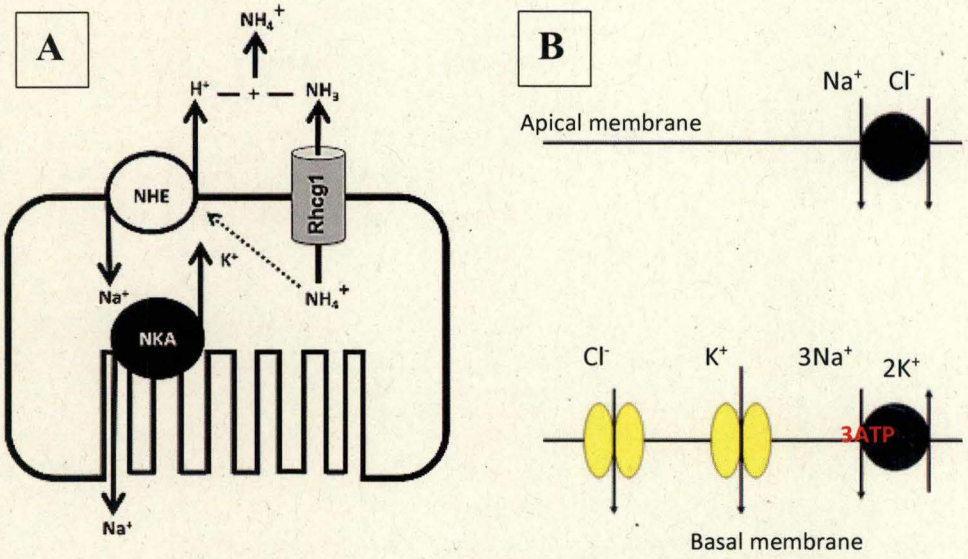

*Figure 11: hypothetical model to explain Sodium uptake in NHE (**A**) and in NCC (**B**) (Kumai and Perry 2012)*

Regulation of osmoregulation when fish are transferred to water with different salt concentration is ensured by endocrine actions ensured by several hormones (Figure 12).

First one is an important corticosteroid, the cortisol secreted by cortical cells of the adrenal gland. This hormone has several physiological roles in metabolism, osmoregulation, growth and immune function but its major function is to promote acclimation. In case of saline stress, plasma levels of cortisol and other catecholamines rise(Schreck et al. 2016). They bind to corticosteroid receptors located in high concentration in the gills, gut and kidney. This link induces mRNA production, protein transcription and enzyme activation. The rise of the transcription and the abundance of transporters (NKA, NKCC and other chloride channels) induce ions release. Moreover, cortisol induces the increase of the number and size of ionocytes. These changes maintain a low level of ions in the plasma if the salt concentration in the surrounding water rises (McCormick 2011; Kumai and Perry 2012). If the salt concentration decreases, cortisol is able by an unknown mechanism to increase the number of NKA inducing the ions uptake. In order to avoid confusion, it could be clarified that NKA possesses two isoforms. The first one is more abundant in freshwater conditions and the other one is more common in seawater. Regulation of these two types is conducted by cortisol, hence its double role in osmoregulation (McCormick 2011). Furthermore, bound with glucocorticoid receptors, cortisol induces gluconeogenesis and the glucose release outside the liver in order to ensure the energy supply of the physiological stress response (Schreck et al. 2016). Second important hormone impacting osmoregulation is the prolactin. This hormone is secreted from specific cells of the pituitary gland. Its major activity is the control of ions release when fish are

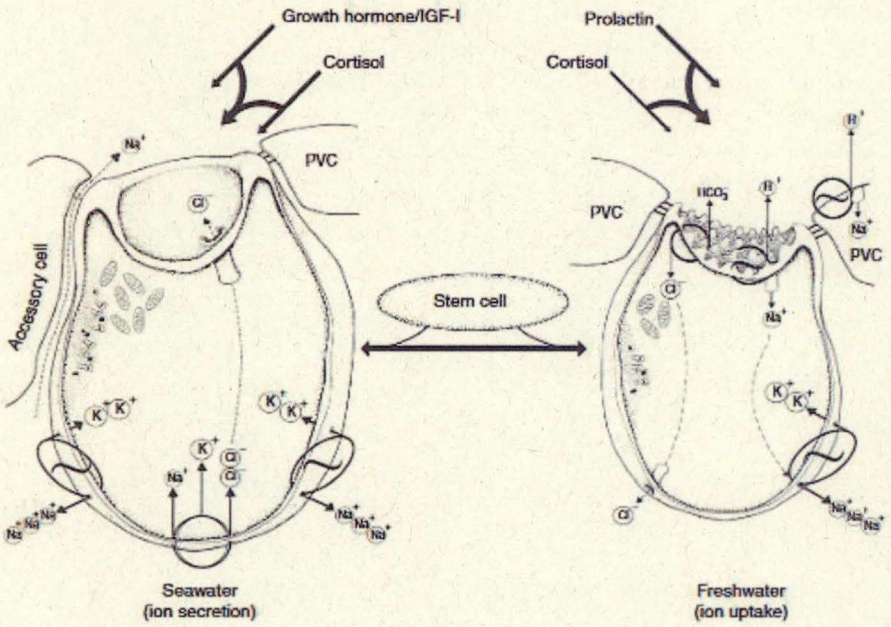

Figure 12 : summary of the morphology, the transport and the hormonal control of gill chloride cells in freshwater and seawater (McCormick 2011)

transferred in hypoosmotic fluids. Even if clear mechanisms are unknown, prolactin seems to inhibit the development of seawater chlorides cells. Resulting consequence is the diminution of permeability of the membrane to ions due to the retention of Na+ and Cl-. Prolactin specific receptors are also located in chloride cells of different organs responsible for the osmoregulation like kidney and intestine. Last hormonal category impacting osmoregulation is growth hormone (GH) which increase the size and the number of mitochondrion-rich cells as well as the density of the NKA and NKCC. Once GH receptors located in liver, gill, gut, and kidney tissues are activated, it stimulates the liver production for insulin-like growth factor-I (IGF-1) that is able to directly increase the activity of NKA. Coupled with cortisol, GH/IGF-1 regulate salt excretion by increasing gill NKA activity. (McCormick 2011)

Nevertheless, other molecules like thyroid hormones (T4, T3) (McCormick 2011) and epinephrine (Kumai and Perry 2012) have an indirect role in osmoregulation.

1.5.5 Immunity system

Due to its migratory adaptions, striped catfish may support low saline concentration up to 15 ppt. However, salt concentration during the saline intrusions in the Delta reaches higher values than that fish may support. During these stressful situations, striped catfish have to allocate its energy to physiological responds which may affect the competence of the immune system(Sopinka et al. 2016). Therefore, coupled with high density ponds which are the perfect place for disease spread, striped catfish are more sensitive to pathogens. The most diagnosed

infectious disease in striped catfish is the Bacillary Necrosis of Pangasianodon caused by *Edwardsiella ictaluri* (Sirimanapong et al. 2014). Described for the first in 1965 (Ewing et al. 1965), the Gram-negative bacterium responsible for this disease induces either an enteric septicemia resulting in a high mortality rate as early as 2 days after the exposure or a chronic form characterized by holes in the head, caused by meningoencephalitis lesions (Crumlish et al. 2014; Ewing et al. 1965). The internal post-mortem symptoms of the infection are the multifocal irregular white lesions on liver, spleen and kidney (Crumlish et al. 2014). In ponds, the disease mainly occurs during the rainy season when the water temperature ranges from 23 to 30°C. (Tu Thanh 2010)

During infectious episodes, a wide variety of immune processes occurs. Vertebrates immune system possesses two types of response. The innate immune system creates a rapid, non-specific reaction to a pathogen while the adaptative system engages the infectious agent with specificity and memory.

One of the immune processes is the activation of the lysozyme activity. Originating in leukocytes, the lysozyme is a mucolytic enzyme of the innate immune system mostly synthesized in the liver (International Union of Biochemistry and Molecular Biology 1961; Saurabh and Sahoo 2008). Its role is the mediating protection against microbial invasion through two main actions. After the action of complement that disrupts the outer cell wall of Gram-negative bacteria, this enzyme induces the hydrolysis of (1→4)-β-linkages between the N-acetylmuramic acid and the N-acetyl-D-glucosamine residues in the peptidoglycan layers (International Union of Biochemistry and Molecular Biology 1961). This action aims to bring down the external defenses of the bacteria and to activate polymorphonuclear leucocytes and macrophages that carry out the phagocytosis.(Saurabh and Sahoo 2008)

The principal cells in charge of the adaptative immune system are the lymphocytes T, in charge of the cell-mediated immunity, and lymphocyte B, the producers of immunoglobulins. These "Y" shaped proteins are also called antibodies and are constituted by two identical heavy chains and two identical chains of glycoproteins. Secreted in the plasma, these molecules contain one or several C-terminal constant domains and one N-terminal variable domain. This last domain participates to the recognition of the pathogen's specific antigens. Once bound to the infectious agent, immunoglobulin C-domain is recognized by immune cells and activates them leading to the neutralization and the destruction of the pathogen in addition to the complement activation.

In bony fish, hematopoiesis and lymphopoiesis of lymphocytes B take place in the upper part of the kidney or in the pronephros. These cells express several transmembrane receptors, each one recognizing a particular antigen on their plasmatic membrane. When an antigen binds to the receptor, B cells produce antigen-specific antibody. In teleost fish, three classes of immunoglobulins have been identified.

Firstly, IgM class is the most ancient and the most prevalent one. This one may either be expressed on the surface of lymphocytes or be secreted in the plasma as an antibody. Teleost serum contains high concentration of IgM but this one is also found in the epithelial mucus and in the intestine. This class has many functions including the activation of the complement, the induction of the phagocytic cell agglutination and the destruction of the pathogens. The following group of immunoglobulins is the IgD class. Secreted form has high affinity for basophils cells inducing antimicrobial and pro-inflammatory factor that activates the B cells. The third class of immunoglobulins is the IgT/Z one, only produced by teleost species. These immunoglobulins possess a varying number of C domains depending on the species and are typical of mucosal tissues like gut, intestine and epithelium. The IgT part is found either as a monomer in the serum or as a tetramer form in the mucous. This molecule is able to pass through the mucosal epithelium due to a specific receptor called fish polymeric immunoglobulin receptor. In addition to these three classes of Ig, teleost fish also processes four IgL isotypes: κ, σ, σ-2 and the λ which seems to be absent of the catfish family. In addition to these isoforms, numerous subtypes of IgL were identified through several species. (Mashoof and Criscitiello 2016). The analysis of the total Ig indicates the intensity of the immune response to an infection.

Under stress conditions, the metabolism rises in order to ensure the energetic demand of the different stimulated metabolic pathways. This overproduction leads to excessive formation of reactive oxygen species (ROS) such as H_2O_2, HO^-, HOCl, … which are dangerous for the organism integrity. In order to protect be protected against destructive effect of these molecules, the antioxidant defenses located in mitochondrion produce and activate oxidase enzymes, such as several kind of peroxidases (Kumari et al. 2015; Blokhina, Virolainen, and Fagerstedt 2003). The activity site of these enzymes is adapted to modify these molecules and to make them inoffensive. However, the production of ROS by the phagocytes is also involved in the immune protection against pathogens. The production of antioxidant enzymes have to be regulated in order decrease in case of infection allowing pathogen eradication process (Mélodie Schmitz, Mandiki, et al. 2016). (Soulié 2017)

Striped catfish osmoregulation and immunity are influenced by a wide range of factors. Even if euryhaline species possess higher acclimation capacity, total immune response depends on the seasons and is modulated by the intensity, the exposure time and the chronicity of the stress. In the Mekong Delta, striped catfish immune system is highly requested. During the rainy season, the high fluctuation in the Mekong River discharge is related to an increase of suspended solids. This phenomenon induces an increase of the particles uptake by gills leading to an inflammatory response. During the dry season, when the salinity level in the Mekong Delta is the highest, the osmolality of the plasma elevates up to 280 mOsm. Moreover, chronical hyperosmotic episodes overstimulate the immune response of striped catfish inducing an immune depression resulting in an increase of the sensitivity to pathogens.

The analysis of these immune system activity factors leads to an overall view of the way striped catfish modulate its response to an infection under saline stress. (M. Schmitz 2017)

1.6 PANGAGEN Project

Striped catfish production in the delta is largely impacted by climate change and dams building in the upper part of the Mekong River. The combination of these two major threats increases the salinity of the Delta water. Even if striped catfish can tolerate saline water, the salinity level during these episodes is too important leading to a stressful situation for the fish. The direct consequence is a reduced total Vietnamese production opening the way for competitors such as China. Targeting this challenge in order to maintain the competitiveness is possible by the creation of "improved" animals through a genetic selection program. These would be more resilient to their environmental changes and more resistant to the pathogens as *Edwardsiella ictaluri,* while keeping a fast growth and a high quality. This study called PANGAGEN project is carried out in a co-partnership by the researchers of Can Tho University (Vietnam), University of Liège and University of Namur (Belgium) and financed by ARES-CCD. Conducted between 2017 and 2021, PANGAGEN project contributes throughout the selection program to sustain the striped catfish value and to supply the quality of the products for the local population.(College of Aquaculture and Fisheries, Vietnam 2017; Farnir and Phuong 2016).

1.7 Master thesis objectives

In parallel to the selection programs of the PANGAGEN project, fundamental studies are conducted to improve knowledge about the effect of the water salinity on striped catfish. The main objective of this master thesis was to investigate the modulation the physiology, the immunity and the hematology of the striped catfish juveniles during a saline stress with or without an infectious episode on. Saline stress was induced during a salinity rise and a followed two-week exposure. At the end of each phase, the bacterial challenge with *Edwardsiella ictaluri* was carried out.

2. Material and method

2.1 Fish and in vivo stress experiment

For each one of the five treatments, five 500L tanks (four occupied by fish and one by the biofilter) were arranged in an independent recirculating system containing in total around 1500L in the wet lab of the College of Aquaculture and Fisheries of the University of Can Tho (Vietnam) (Figure 13). The tanks were firstly cleaned with water and dried completely. To disinfect the system, a second cleaning was carried out with a calcium hypochlorite solution (soaking for 24 hours and then widely rinsed).

Figure 13: treatment tanks disposition

By means of a pump located in the biofilter tank, the water system supplied the four tanks allocated for fish from the top of the tank. To prevent some accidental fish transfer, the water discharge was protected by a drilled tube covered at the top by a net piece. The water came from the bottom of the tank, get through the biofilter and was reinjected in the system. The fish oxygenation was ensured by one air inlet.

The biofilter tank was separated in two compartments by a rigid net surrounded by a nylon fishing net. In the lower part of the biofilter was disposed a nylon net piece of $4m^2$ in order to

filter the biggest particles. The upper part contained around 6Kg of black capsules, one air inlet for the bacteria oxygenation and the water pump (Figure 14). In order to make the development of the biofilter possible, the water circulated in the system for three days before adding the fish.

Figure 14: biofilter tank

The treatment position was randomly assigned (Figure 15).

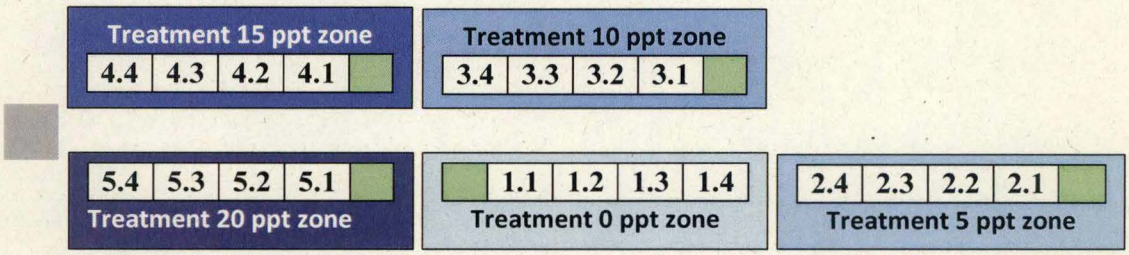

Figure 15: treatments disposition. Legend: in blue: salinity zone (dark blue (high salinity) to light blue (low salinity), in green: biofilter tanks, in grey: air pump.

The fish came from an aquaculture farm and were stored during 2 months before the beginning of the experiment. After the anaesthesia with isoeugenol 50% (10µg/L), they were weighed and randomly distributed in each tank (65 fish/tank). Once in the experimental tank, the fish were daily feed ad libitum twice a day around 10 a.m. and 4.30 p.m. with dry pellets (40% of protein, diameter: 3mm).

The experiment was conducted during 47 days, divided into two steps (Figure 16). In the first one, the salinity was gradually raised each day during 20 days with saline water of around 85-90 part per thousand (ppt). The addition of the saline water took place twice daily directly in the pump of the biofilter in order to reduce the salinity shock for the fish. The first addition of salt occurred in the morning before the feeding. The second addition was conducted in the middle of the afternoon. In order to ensure the exact added quantity of salty water, the salinity (and the water parameters record) was measured before the evening feeding and adjusted.

After the last addition of saline water in the day 20 afternoon, the fish were kept at the reached salinity during 24h without feeding, until the morning of day 22 for the second sampling. Six fish were sampled from each tank: three for the immune parameters and three others for the remaining physiological parameters.

To carry out the bacteria injection in the optimum conditions, this manipulation took place the day following the samplings of the day 20 and 34. For the first bacterial challenge, 10µL of an *Edwardsiella ictaluri* solution (concentration: 10^7 bacteria/ml) were injected to 16 fish (for challenge development, see 2.2 Bacterial culture and survival test). These were isolated in another department of the Aquaculture College to avoid contamination. The third day corresponding to infection peak, 6 infected fish were collected and the mortality was recorded during the next 10 days.

For the second step of the experiment, the salinity was stabilized at the desired salinity (0, 5, 10, 15 and 20 ppt) during two weeks. At the end of this salinity acclimation, 6 fish per tank were collected and 16 other ones were placed in the bacterial challenge system. As previously, 6 infected fish were collected 3 days after the infection and the mortality were recorded during the next 10 days.

During the entire experiment, the feeding quantity allocated to each tank was weighed and the remaining feed in the tank was counted in order to define the feed intake for each tank. Moreover, the mortality was recorded for each tank and the dead fish were weighed. Several water parameters (Appendix 5) were also monitored. The salinity, the pH and the temperature were daily recorded around 4 p.m. during the salinity increase and 2 times a week during the salinity stabilization with a multiparameter probe. The nitrate and the Total Ammonia Nitrogen (TAN) levels were recorded for water samples on days 0, 11, 23, 27 and 34.

2.2 Bacterial culture and survival test

Edwardsiella ictaluri bacteria were collected 1 year ago on infected fish used for previous experiments at 5 ppt and they were stored at -30°C in 500 µL of glycerol and 500µL of broth.

The culture protocol is described in Appendix 1: **Bacterial culture protocol**. At the end of this manipulation, the bacteria count was conducted with the spectrophotometer. A total absorbance equaling a concentration of 10^9 bacteria/ml, the concentrations of 10^8, 10^7 and 10^6 bacteria /ml were reached by dilution with physiological fluid.

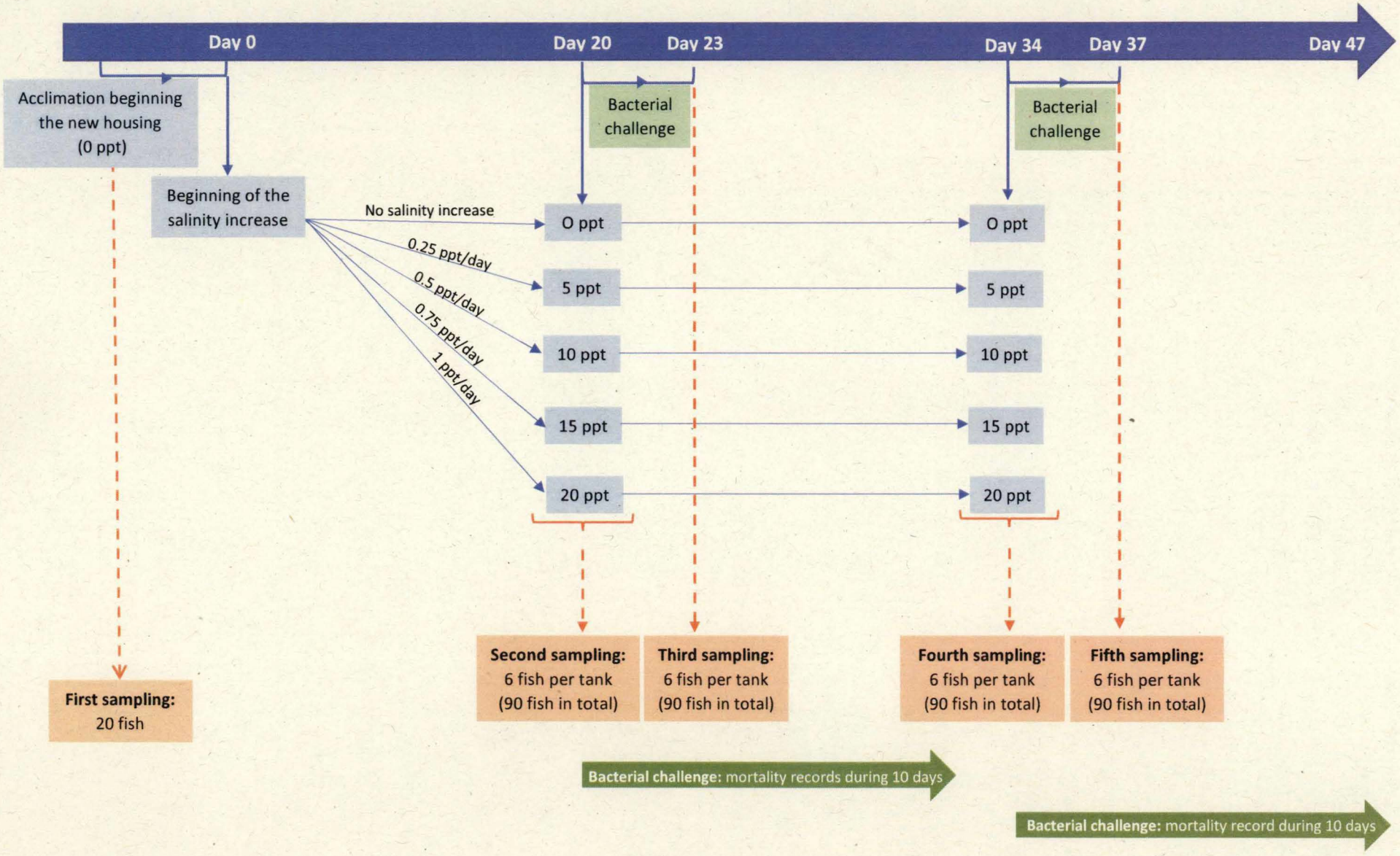

Figure 16: summary of the protocol progress. Legend: blue: relative to the salinity, red: relative to the sampling, green: relative to the bacterial challenge.

The lethal dose at 50% (LD50) was carried out with fish at 0 ppt and at 10 ppt in order to evaluate the bacteria survival rate at high salinity. The fish acclimation at 10 ppt was conducted during 5 days (rise of 2 ppt a day) and kept during 5 days at this salinity before the bacterial injection (100 µL of the corresponding bacteria solutions). For each treatment, 10 fish were challenged after being anesthetized with isoeugenol 50% (10 µg/L).

The results of this challenge test indicated the LD50 at 10^7 CFU for both the 0 ppt and 10 ppt fish.

2.3 Samples collection

For each tank, all the sampled fish were anesthetized in tricaine methanesulfonate MS-222 (100 mg a litre) and weighed. The blood was collected using a sterile heparinized syringe by caudal vein puncture before being euthanized by cervical dislocation within 5min. For the initial status sampling, 10 fish were collected from the storage tank in order to sample plasma, blood, gills, head kidney and liver and 10 other fish were used to collect blood. For the next samplings, 6 fish were randomly selected: 3 for the immune parameters and 3 for the physiological ones. Different tissues were collected.

Blood sampling was used for four analyses: hematocrit ratio, hemoglobin quantification, the proportion of each white blood type and the erythrocyte concentration. It was also used for the plasma production to analyze the lysozyme activity, the peroxidase and the total immunoglobulin, the osmolality and finally the chloride and sodium concentration. For this transformation, the blood was centrifuge at 5000G during 10min at 4°C.

Some gills, the head kidney and the liver were collected and directly fixed in neutral buffer formalin 10% (100ml of 37% formaldehyde solution, 4g of potassium phosphate monobasic, 6.5g of potassium phosphate dibasic 6.5g in 900 ml of distilled water). The rest of the gills was also collected and directly stored in liquid nitrogen to preserve the material for the samplings. They were kept for future histopathology and physiology analyses.

2.4 Physiology parameters protocols

In order to have an overview of the way fish adapt their osmoregulation under stress conditions, the osmolality, the sodium and chloride ions and the glucose concentration in plasma were analyzed in this work.

2.4.1 Osmolality

20 µL of plasma were used to measure the osmolality (mOsm) with a micro-osmometer (The Advanced™ Micro Osmometer Model 3300, Advanced Instrument INC.).

2.4.2 Ion concentrations

2.4.2.1 Chloride concentration

The first step of chloride analysis was the preparation of the buffer (0.99 mL of nitric acid 65%, 10 mL acetic acid 99-100%, 3 drops of Tween 20 was completed with pure water to reach 100 ml and the pH was fixed at 0.89). 20 µL of sample were titrated in the buffer with a chloride analyzer (MK II Chloride Analyzer 926S, Sherwood). The results are expressed in mmol/L.

2.4.2.2 Sodium concentration

Sodium concentration was detected by analyzing 5 µL of plasma in a flame spectrophotometer (Model 420 Clinical Flame Photometer Sherwood). Blank was done with a lithium 1:1000 solution.

2.4.3 Glucose concentration

According to Huggets and Nixon (1957)(Huggett and Nixon 1957), the proteins were removed from the plasma samples by adding 40 µL of perchloric acid 0.33M to 20µL of sample before being mixed and centrifuged during 10min at 3000 rpm and at 25°C. 1.25 µL of the supernatant were diluted in 1.5mL of the reactive solution. This mix was incubated at 38°C during 15min. The final solution was read by spectrophotometry at 436nm and the final concentration was calculated according to the following formula:

$$Glucose\ final\ concentration = \left(\frac{(absorbance - b)}{a}\right) * 100\ (\text{mg/dL})$$

Where a and b are the coefficients of the standard curve.

For more information about the protocol, please consult the Appendix 2.

2.5 Immune parameters protocols

In order to have an overview of the immune changes affecting fish during the stress conditions, the lysozyme and the peroxidase activity, the total immunoglobulin concentration of the plasma were analyzed in this work in addition to the monocytes, lymphocytes and granulocyte proportions.

2.5.1 Lysozymes activity

According to Ellis (1990)(Ellis 1990), Milla et al.(2010)(Milla et al. 2010) and Schmitz and al (2016)(Mélodie Schmitz, Douxfils, et al. 2016) experiments, 10 µL of the plasma sample were placed in the microplate well with 10 µL of buffer (0.05 M of disodium hydrogenophosphate isodecahydrate) and 130 µL of the *Micrococcus luteus* solution (0.6 g/L) in triplicate. The positive control contained 20 µL of buffer and 130 µL of the *Micrococcus luteus* solution and for the negative control was only buffer (150 µL) (in triplicate). The absorbance wells of the microplate were read with the spectrophotometer at 450 nm every 5min within 60min.

The enzymatic activity was calculated according to the following equation:

$$\frac{(OD1 - OD2) * 1000}{time * volume * 0.001} = enzymatic\ activity\ (\frac{unit}{ml})$$

Where: OD1 = initial absorbance

OD2 = final absorbance

1000 = volume conversion (µL ➔ mL)

Time = time of spectrophotometry reading (5 min)

Volume = plasma volume (10 µL)

The 0.001 factor characterises the lysozyme activity: 0.001 unit of absorbance represents 1 unit of lysozyme activity.

2.5.2 Peroxidase activity

For each sample and in triplicates, 5µL of plasma were mixed with 70 µL of Hanks' Balanced Salt Solution (HBSS) no calcium, no magnesium and 25 µL of reactional solution (62.65 mg of Tertramethylbenzidine dihydrochloride and 5.15 µL of H_2O_2 in 10 mL of distilled water). This mix was precisely incubated during 2min at room temperature before adding 25 µL of sulfuric acid (38.88 mL of distilled water and 11.11 mL of H_2SO_4 95-97%) and directly read at 450nm. The final activity was calculated with the following formula:

$$(M - B) * 200 = final\ activity\ (\frac{\mu mol}{ml})$$

Where: M = mean of triplicates

B = blank results

200 = volume conversion (µL ➔ mL)

The quality of the results was validated with the variation coefficient lower than 12%.

2.5.3 Leukocytes proportions

The blood smear was carried out with 10 µL of blood placed on a glass slide, fixed with methanol during 1 minute and dried. The coloration of the lames was carried out following the Chinabut *et al.*(1991) (Chinabut, Limsuwan, and Kitsawat 1991) protocol with Wright and Giemsa stains. For more information about this step, please, consult the Appendix 4.

The counting operation was carried out with an optical microscope at the University of Namur in the Laboratory of Cells and Tissues (LabCeTi). The relative proportion of monocytes, lymphocytes and neutrophils was determined by counting 200 white blood cells per slide and was calculated for each type of white blood cells as following:

$$\frac{number\ of\ one\ type\ of\ leukocytes}{200} * 100 = relative\ proportion\ (\%)$$

2.6 Hematological parameters protocols

The following hematological parameters were analyzed in order to have more information about the global state of fish dealing with saline and bacterial stresses. These parameters were: hematocrit ratio, hemoglobin concentration, erythrocyte concentration and several mean corpuscular parameters.

2.6.1 Hematocrit ratio

For each sample, the blood was centrifuged in microhematocrit tubes during 3 min at 12 000 rpm. The ratio between the sedimented erythrocyte height and the total height of the blood sample in the microtube was finally multiplied by 100 in order to obtain the hematocrit ratio (%).

2.6.2 Hemoglobin concentration

According to Oser (1965)(Oser 1965) and for each sample, 10µL of blood were added to a cuvette containing 2.5mL of Drabkin chemicals (protocol in the Appendix 3) and read at 540nm with the spectrophotometer at 20°C.

The final hemoglobin concentration was calculated with the following formulas:

$$(1) \quad (0.019 + 37.74 * a) * 0.621 = A$$
$$(2) \quad (A * 1.6125) = Hemoglobin\ (\frac{g}{dL})$$

Where: a = absorbance

A = hemoglobin concentration ($\frac{mmol}{L}$)

2.6.3 Erythrocyte density

5 µL of blood were colored in 995 µL of Natt-Herrick solution (3.88g of NaCl, 2.5g of Na_2SO_4, 2.91g of $Na_2HPO_4.12H_2O$, 0.25g of KH_2PO_4, 7.5mL of Formalin 37% and 0.1g of methyl violet in 1L of distilled water and pH adjusted to 7.3). 10 µL of this mix were placed in a counting-chamber Neubauer. The erythrocytes were counted with microscope on 5 squares (the 4 corners squares and the central square of the counting grid (4*4 squares) on the two parts of the counting chamber. The number of the erythrocyte by mm^3 was determined with the formula bellow:

$$\frac{a * 200}{0.02} = erythrocyte\ density\ (\frac{cells}{mm^3})$$

Where: a = total number of erythrocytes counted

0.02 = volume of counting chamber

200 = dilution factor

2.6.4 Mean corpuscular parameters:

The following indicators were calculated according to the previous hematological parameters.

- Mean corpuscular volume (MCV) = $\frac{Hematocrit\ rate\ (\%)*10}{Erythrocytes\ density\ (\frac{10^6}{mm^3})}$ fL
  which represents the average volume of an erythrocyte,
- Mean corpuscular hemoglobin (MCH) = $\frac{Hemoglobin\ (\frac{g}{dL})*10}{Erythrocytes\ density\ (10^6/mm^3)}$ pg
  which represents the average hemoglobin weight of an erythrocyte.
- Mean corpuscular hemoglobin concentration (MCHC) = $\frac{Hemoglobin\ (\frac{g}{dL})*100}{Hematocrit\ rate\ (\%)}$ g/dL
  which represents the average hemoglobin concentration in an erythrocyte.

2.7 Overall health indicators

Several health indicators were calculated:

- Mean weight gain (g)

$$\frac{Total\ initial\ weight\ of\ fish\ in\ tank}{living\ fish\ number} - \frac{Total\ final\ weight\ of\ fish\ in\ tank}{livinf\ fish\ number}$$

- Cumulated mortality rate (%)

$$\frac{(Dead\ fish\ number\ during\ the\ previous\ days + dead\ fish\ number\ of\ the\ day)}{Total\ fish\ number\ at\ the\ beginning\ of\ the\ experimental\ phase} * 100$$

- Feed efficiency (g/g)

$$\frac{\sum(Total\ feed\ provided\ during\ a\ time\ period/living\ fish\ number)}{Mean\ weight\ gain\ during\ the\ phase}$$

2.8 Statistical analyses

The objectives of the statistical analyses were to test the influence of the saline treatment and o f the time on the physiological, immunological and hematological parameters of the striped ca tfish. This work was carried out with the Rstudio program: R Core Team (2017). R: A languag e and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Several packages were used as:

- ggplot2 (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2009)
- Rmisc: Ryan M. Hope (2013). (Rmisc: Ryan Miscellaneous. R package version 1.5. https://CRAN.R-project.org/package=Rmisc)
- car (J. Fox and S. Weisberg (2011). An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA: Sage. http://socserv.socsci.mcmaster.ca/jfox/Boo ks/Companion)
- fitdistrplus (Marie Laure Delignette-Muller, Christophe Dutang (2015). fitdistrplus: An R Package for Fitting Distributions. Journal of Statistical Software, 64(4), 1-34. http:// www.jstatsoft.org/v64/i04/.)
- lme4 (D. Bates, M. Maechler, B. Bolker, S. Walker (2015). fitting linear mixed-effects models using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067. i01.)
- lsmeans (R. V. Lenth (2016). Least-Squares Means: The R Package lsmeans. Journal of Statistical Software, 69(1), 1-33.doi :10.18637/jss.v069.i01)
- base (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/)

After removing all the outliers following the Cook distance method, the normality of data residuals was tested by the Shapiro test and the normal QQPLot. These observations were followed by the residual homoscedasticity analysis with the graph observation of the residuals in function of fitted value and the Levene test. If the residuals followed a normal distribution and if the homoscedasticity was respected, a linear model with a normal distribution was used to test the different effects. If one of these hypotheses were not respected, the function descdist() helped to find a modification (logarithm, square root, inverse or logistic) in order to reach to normal distribution. If one of those fitted, it was included in the analysis by using a generalized linear model.

Moreover, the random effect induced by the parameter "Tank" was tested and included in the model if its effect was significative.

In the case of a (generalized or not) linear model, a posteriori contrast was applied in order to detect the significative effects of each parameters and their levels when the p value of the comparison was lower than 0.05.

If the data distribution did not correspond to any cited distribution, the Kruskal Wallis non parametric test was applied on the data. The results of this analysis were compared together with a Wilcoxon test on the Time and the Treatment. The obtained p values were finally compared to the mean and the standard deviations of each group.

In order to compare the variability between groups, the coefficient of variation (%) was calculated:

$$\frac{Standard\ deviaton\ of\ the\ group}{Mean\ of\ the\ group} * 100$$

3. Results

3.1 Experiment conduction

After 4 days of acclimation to their new-housing, some fish in all tanks developed a disease which the symptoms were damaged gills, mouth strongly skinned (in some case, the bone of the lower jaw was visible), injuries on the body, skin clarification, white eyes and sometimes profound wounds on the head (Appendix 6). The tank 3.4 was completely lost and as well as 52 fish in total from the other tanks. Given that the use of antibiotics would affect the immune system, a treatment with salt was applied to the tanks and fish. First, a high salinity soak (20 ppt) was applied during 1 h in ± 20 cm of water. After this, the water level was raised in order to decrease the salinity to 2-3 ppt and the fish were kept in this remaining salinity during 3 days. The water was finally renewed twice in order to decrease a maximum the salinity. The system was reconnected and kept in observation during 3 days. In order to avoid feed waste and consequently bad water quality, the fish were not fed during the disease event. Thereby, the total weight of the fish in the tank was recorded a second time after 24h without feed and new fish were added to complete the tanks where it was necessary. After 2 days of acclimation, the fish did not display any symptom of disease, were swimming well and normally ate except for the tank 3.4. It was decided to isolate this tank from the other tanks of the treatment 3 during five additional days and to increase the salinity separately. From the day 14 to 18, four dead fish were found with symptoms of a new disease (distended abdomen, swimming on the back at the surface, bloody flow from the anal orifice) in the treatments 3 and 4. Salinity being around 7 and 10 ppt respectively and the number of dead fish stopping increasing, no salt treatment was provided unlike the first disease event, but the food recipients were changed. Thereafter, none of these symptoms were detected on living and dead fish. The dead fish of this pathological event were not taking into account in the mortality record.

The pH (7.56±0.22), temperature (29.5±0.59°C) and total ammonia nitrogen (0.92±0.55mg/L) records were in the suitable range for *Pangasianodon hypophthalmus* development (Islam et al. 2019; Güroy et al. 2016).

In the following sections, the results will be presented, for each major group of variables, in different phases, related to the different experimental conditions: **phase 1** = the increase of salinity from 0 ppt (Day 0) to the defined one (5, 10, 15 and 20 ppt) (Day 20) ; the **1st bacterial challenge** at the end of the salinity increase (Day 20 – Day 23); **phase 2** = the two-week exposure to the fixed salinity (Day 20 – Day 34); the **2nd bacterial challenge** at the end of the

constant salinity period (Day 34 – Day 37). The means, standard deviations and coefficients of variation of each group are presented in the Appendix 7.

3.2 Feed efficiency, weight gain and mortality rate

The feed efficiency and the weight gain per fish and the final mortality rate per treatment according to the different salinities for the phases 1 and 2 and the bacterial challenges are presented in the Table 1.

Table 1: Mean and standard deviation for the feed efficiency (FE) (g/g) and the weight gain (WG) (g) per fish and the mortality rate (MR) (%) per treatment at the end of the phases 1 and 2 in function of the salinity concentration (ppt)

Salinity conc. (ppt)	0	5	10	15	20
Phase 1 (Day 0 - Day 20)					
FE: mean ± SD (g/g)	1.49(±0.14)a	1.17(±0.15)b	1.46(±0.33)ab	1.35(±0.12)a	3.94(±2.44)c
WG: mean ± SD (g)	8.70(±2.07)a	12.80(±0.70)b	7.87(±1.16)a	9.05(±0.98)a	2.81(±2.41)c
MR: (%)	2.7(±3.4)a	0 (±0.0)a	2.3(±2.7)a	1.2(±0.8)a	79.23(±11.0)c
Phase 2 (Day 20- Day 34)					
FE: mean ± SD (g/g)	2.17(±0.87)a	1.23(±0.51)b	1.09(±0.42)b	0.86(±0.10)b	-
WG: mean ± SD (g)	4.67(±1.37)a	7.99(±2.15)b	7.24(±3.17)ab	8.99(±1.90)b	-
MR: (%)	6.3(±3.1)a	3.7(±3.2)a	5.9(±7.2)a	7.0(±4.6)a	-
1st bacterial challenge (Day 20 – Day 23)					
FE: mean ± SD (g/g)	-	-	-	-	-
WG: mean ± SD (g)	-	-	-	-	-
MR: (%)	5.0(±5.8)ab	0(±0.0)a	0(±0.0)a	7.5(±9.6)b	60.0(±25.8)c
2nd bacterial challenge (Day 34 – Day 37)					
FE: mean ± SD (g/g)	-	-	-	-	-
WG: mean ± SD (g)	-	-	-	-	-
MR: (%)	52.5(±34.0)a	2.5(±5.00)b	10.0(±20.00)b	22.5(±25.00)b	-

At the end of the **phase 1** (Day 20), 79.23(±11.0) % of the fish at 20ppt died and the mortality rate rose from 16ppt. Because the survival fish were insufficient for this saline treatment at the end of the phase 1, the number of fish during the following bacterial challenge differs from those under lower saline treatment (5, 10 and 15 ppt) and in freshwater. Therefore, the variables of the fish submitted at 20 ppt during the bacterial challenge are not exploited for the variable statistical analyses. No dead fish was recorded for the treatment under 5ppt and the mortality rate at 10 and 15 ppt did not differ from the one at 0ppt ($p<0.05$). The other mortality rates slowly increased but never reached 5%. For the **1st bacterial challenge**, no fish died for saline treatment at 5 and 10 ppt but the final rate for the treatment at 20 ppt reached 60.0(±25.8) %. During the **phase 2**, the treatment under 15ppt recorded the highest mortality rates (7.0(±4.6) %) and the rise started around the day 27. **For the 2nd bacterial challenge**, 52.5(±34.0) % of the fish in freshwater died 5 days after the injection. The mortality rate of the fish under 5 ppt was the only one below 5%. Dead fish number was recorded and cumulated for each tank during the two phases of the experiment duration and during 10 days after the *Edwardsiella ictaluri*

inoculation for the challenges test in order to calculate a cumulated mortality rate (%) per treatment (Figure 17).

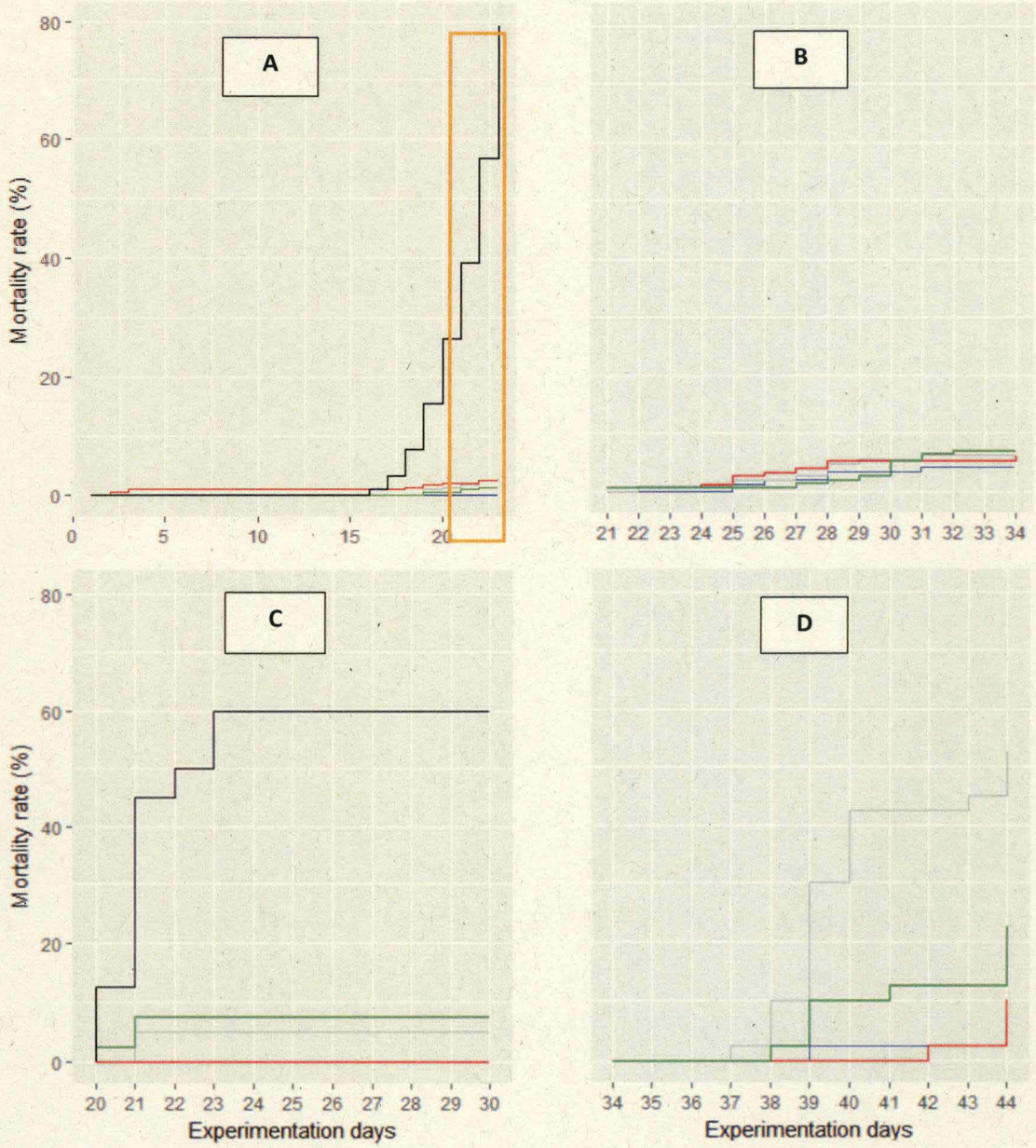

Figure 17: Average mortality rate (%) of the phase 1 (A) (n=65), the phase 2 (B) (n=65-(dead fish during phase1)-(sampled fish)), the 1st bacterial challenge (C) (n=10) and the 2nd bacterial challenge (D) (n=10). Legend: saline treatments at 20ppt (black), 15ppt (green), 10ppt (red), 5ppt (blue) and 0ppt (grey). Framed area (orange): dead fish record during the three following days when took placed the sampling time, the injection for the first bacterial challenge and the biofilter cleaning.

3.3 Physiological parameters

The results of the physiological parameters (osmolality and sodium, chloride and glucose concentration) are presented in the following section. All the significative p values are indicated in brackets and no control treatment differed from each other for all the physiological parameters.

3.3.1 Osmolality

During the **phase 1** (Day 0 - Day 20), the plasma osmolality ranged from 264.10 (±20.73) to 426.33 (±37.95) mOsm according to the **salinity concentration** ($p<0.05$), with a rather low variation coefficient within a same treatment (3.32 to 13.23%). Osmolality increased with salinity and the values were significantly higher ($p<0.05$) in fish at 10, 15 and 20 ppt. When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the plasma osmolality increased in function of the **salinity** ($p<0.05$), ranging from 268.90 (±9.29) to 345.67 (±43.06) mOsm, with a rather low variation coefficient within the same treatment (3.04 to 12.46%). The significative values are observed at 5, 10 and 15 ppt ($p<0.05$). For the **1st bacterial challenge** (Day 20- Day 23), the osmolality significantly increased with the **salinity** ($p<0.0001$) and the significative values are observed at 5, 10 and 15 ppt ($p<0.05$). They ranged from 263.90 (±13.78) to 349.30 (± 43.99) mOsm with rather low variation coefficient within the same treatment from 2.99 to 12.60%. For the **2nd bacterial challenge** (Day 34 – Day 37), the osmolality rose according to the **salinity** ($p<0.05$) ranging from 263.18 (± 9.98) to 330.00 (±19.76) mOsm, with a low variation coefficient within the same treatment (2.64 to 5.99%). The significative values were observed at 5,10 and 15 ppt. (Figure 18)

3.3.2 Sodium concentration

At the end of the **phase 1** (Day 20), the plasma sodium concentration ranged from 103.16 (±17.95) to 186.54 (±26.07) mmol/L according to the **salinity** ($p<0.05$), with a rather high variation coefficient within the same treatment (9.74 to 25.79%). Sodium concentration increased with salinity and the values were significantly higher ($p<0.05$) in fish at 15 and 20ppt. When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the plasma sodium concentration increased with the **salinity** ($p<0.05$), ranging from 100.72 (±14.41) to 162.46 (±14.10) mmol/L, with a rather high variation coefficient within the same treatment (8.68 to 22.37%). The values were significant at 5, 10 and 15 ppt. For the **1st bacterial challenge** (Day 20- Day 23), the plasma sodium concentration rose in function of the **salinity** ($p<0.05$) and the significative values were observed at 5, 10 and 15 ppt ($p<0.05$). They ranged from 106.20

(±15.70) to 155.61 (±14.51) mmol/L with a rather high variation coefficient within the same treatment (9.32 to 20.37%). During the **2nd bacterial challenge** (Day 34 – Day 37), the plasma sodium concentration increased with the **salinity** ($p<0.0001$). They ranged from 96.91 (±10.31) to 161.90 (±14.34) mmol/L with a rather high variation coefficient within the same treatment (8.86 to 13.64%). Values at 5, 10 and 15 ppt were higher than at 0 ppt ($p<0.05$). (Figure 18)

3.3.3 Chloride concentration

During the **phase 1** (Day 0 - Day 20), the plasma chloride concentration ranged from 100.27 (±13.67) to 187.67 (±26.91) mmol/L, according to the **saline treatment** ($p<0.05$) with a rather high variation coefficient within the same treatment (8.12 to 15.07%). Chloride concentration increased with salinity and the values were significantly higher in fish at 5,10 and 15 ppt ($p<0.05$).When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the plasma chloride concentration rose with the **salinity** ($p<0.05$), ranging from 100.87 (±10.59) to 146.42 (±21.46) mmol/L with a rather high variation coefficient within the same treatment (6.48 to 14.66%). The values were significant in fish under saline conditions ($p<0.05$). During the **1st bacterial challenge** (Day 20 – Day 23), the plasma chloride concentration increased in function of the **salinity** ($p<0.05$) and the significant values were observed under saline conditions ($p<0.05$). They range from 95.74 (±13.54) to 147.57 (±23.28) mmol/L with a variation coefficient within the same treatment from 6.48 to 15.78%. For the **2nd bacterial challenge** (Day 34 – Day 37), the plasma chloride concentration increased with the **salinity** ($p<0.05$) ranging from 99.50 (±9.31) to 140.83 (±10.87) mmol/L with a rather low variation coefficient within the same treatment (5.11 to 9.35%). The values for the fish at 5, 10 and 15 ppt were higher than for the fish in freshwater ($p<0.05$). (Figure 18)

3.3.4 Glucose concentration

During the **phase 1** (Day 0 - Day 20), the plasma glucose concentration differs with the **salinity** ($p<0.05$) ranging from 31.21 (±23.62) to 74.46 (±21.96) mg/dL, with a high variation coefficient within the same treatment from 18.69 to 75.70%. However, the values were significant at 20 ppt at which the glucose concentration dropped ($p<0.05$). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the plasma glucose concentration was affected by the **salinity** ($p<0.05$) but remained relatively constant, around 61.20 (±17.33) to 78.39 (±19.46) mg/dL with a high variation coefficient within the same treatment (18.22 to 28.32%) even if the values significantly decrease at 10 ppt ($p<0.05$). For the **1st bacterial challenge** (Day 20 – Day 23), the plasma glucose concentration differed due to the bacterial challenge ($p<0.05$).

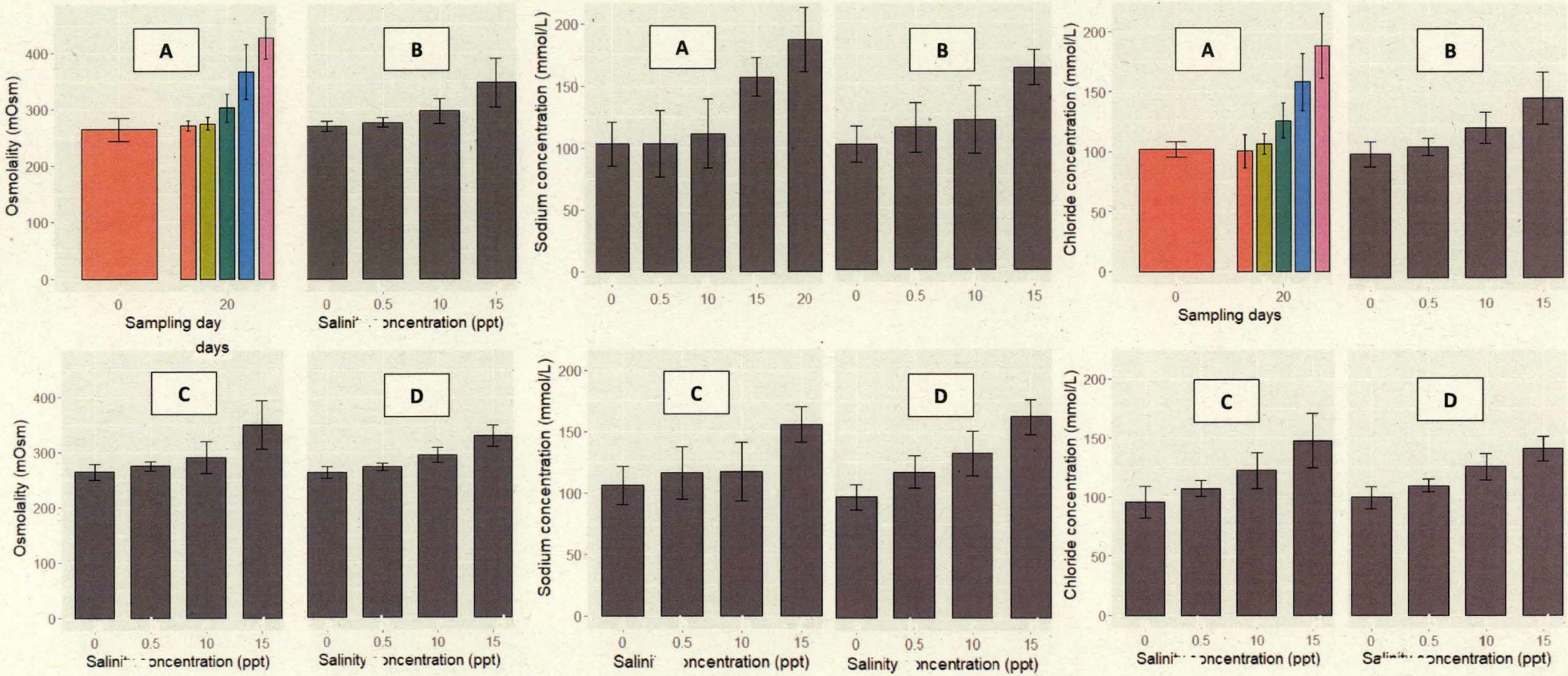

*Figure 18: Means and standard deviations of the osmolality (mOsm), sodium and chloride concentrations (mmol/L) of the striped catfish plasma during the phase 1 (Day 0- Day 20) (A), the phase 2 (Day 20-Day 34) (**B**), the 1st bacterial challenge (Day 20 -Day 23) (C) and the 2nd bacterial challenge (Day 34- Day 37) (**D**) in function of the salinity concentration (ppt). Statistical letters = significant change ($p<0.05$) between the groups within the same graph. Legend: red = 0 ppt, brown = 5 ppt, green = 10 ppt, blue = 15 ppt and violet = 20 ppt.*

For the sane fish, glucose concentration equaled 67.97 (±20.00) mg/dL with variation coefficient of 29.42% and increased when fish were infected equaling 84.24 (±23.60) mg/dL with a coefficient variation of 24.06%.

The graphs of the glucose concentration after the salinity rise, the phase 1 and the 1st bacterial challenges are included in the Figure 19.

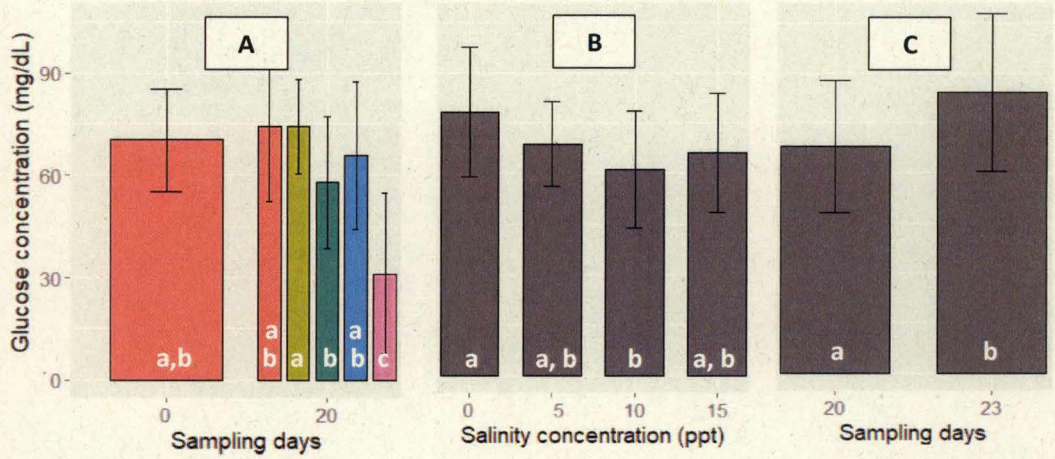

Figure 19: Means and standard deviations of the glucose concentration (mmol/L) of the striped catfish plasma after the phase 1 (Day 0- Day20) (A); the phase 2 (Day 20-Day 34) (B) and the 1st bacterial challenge (Day 20-Day 23) (C) in function of the salinity concentration (ppt) or of the bacterial challenge. Statistical letters = significant change (p<0.05) between the groups within the same graph. Legend: red = 0 ppt, brown = 5 ppt, green = 10 ppt, blue = 15 ppt and violet = 20 ppt.

During the **2nd bacterial challenge** (Day 34 – Day 37), the glucose concentration of the plasma differs in function of the **salinity** ($p<0.05$), the **bacterial challenge** ($p<0.05$) and the **interaction** between these two factors ($p<0.05$). Ranging from 50.67 (±16.32) to 79.16 (±19.90) mg/dL with a high variation coefficient within the same group (12.26 to 34.47%), the results are presented in Table 2.

Table 2: Means and standard deviations of the glucose concentration after the 2nd bacterial challenge in function of the salinity concentration (ppt).

Salinity concentration (ppt)	0	5	10	15
Sane fish (Day 34)				
Mean ± SD (mg/dL)	82.0(±17.0)a	63.6(±7.8)b,c	63.3(±15.5)b,c	66.3(±13.6)a,b
CV (%)	20.76	12.26	24.45	20.59
Challenged fish (Day 37)				
Mean ± SD (mg/dL)	52.3(±15.1)b	79.2(±19.9)a,c	50.7(±16.3)b	67.1(±19.1)a,b
CV (%)	28.86	25.14	32.21	28.51

Legend: Statistical letters = significant change (p<0.05) between the groups. CV= coefficient of variation.

3.4 Immune parameters

The results of the immune parameters (total immunoglobulin, lysozyme and peroxidase activity and leukocytes proportions) are presented in the following section. All the significative p values are indicated in brackets. For the control analysis, no one differed for lysozyme activity and total immunoglobulin concentration but one or several control treatments were significantly different for peroxidase activity and leukocytes proportions. Analyses of these last three parameters were carried out in Belgium.

3.4.1 Total immunoglobulin concentration

During the **phase 1** (Day 0 – Day 20), the plasma total immunoglobulin concentration ranged from 7.91 (±2.88) to 20.12 (±4.15) mg/mL according to the **salinity** ($p<0.05$) with a high variation coefficient within the same treatment (20.62 to 49.09%). Total immunoglobulin concentration increased with salinity up to 10 ppt and then decreased. Values were significantly higher in fish at 10 ppt and 20 ppt($p<0.01$). When the fish were submitted to the **fixed salinity** (Day 20 - Day 34), the plasma total immunoglobulin remained around the same values, ranging between 12.10 (± 6.66) and 18.51 (±7.18) mg/mL according to the **salinity** ($p<0.05$), with high a variation coefficient within the same treatment (37.8 to 55.1%). However, value was significantly different at 5 ppt. Plasma total immunoglobulin concentration of **1st bacterial challenge** (Day 20-Day 23) varied through the **time** ($p<0.05$), **salinity** ($p<0.05$) and **interaction** between these two factors ($p<0.05$) ranging from 6.98(±5.42) to 19.67(±4.29) mg/mL with a huge variation coefficient within a treatment (21.81 to 77.67%). The significant values were observed in freshwater and under saline conditions but also during bacterial challenge ($p<0.05$).

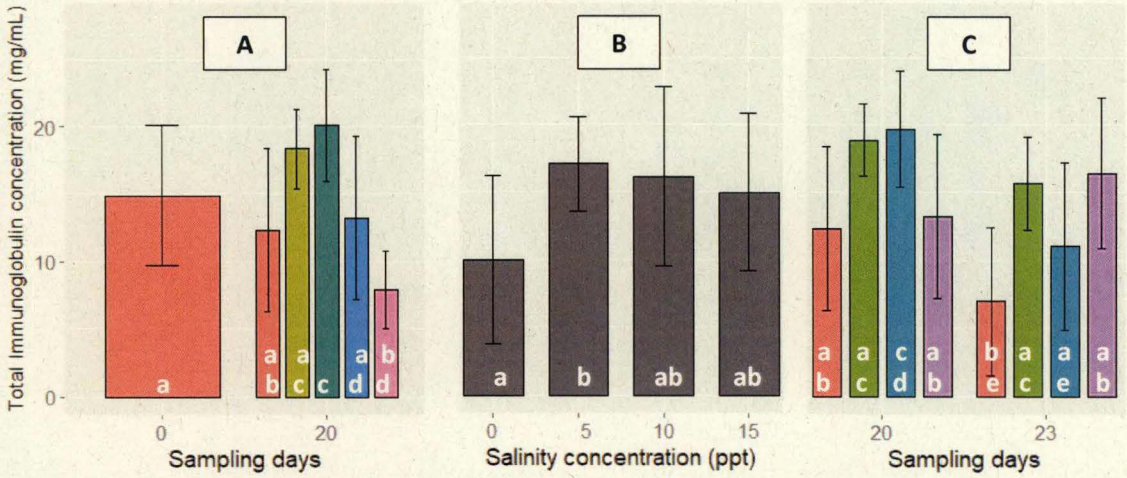

Figure 20: Means and standard deviations of the total immunoglobulin concentration (mg/mL) of the striped catfish plasma during the phase 1 (Day 0 - Day20) (Legend: red =0ppt, brown =5ppt, green =10ppt, blue =15ppt and violet =20ppt.) (A); the phase 2 (Day 20-Day 34) (B) and the 1st bacterial challenge (Day 20 – Day 23) (Legend: red =0ppt, green =5ppt, blue =10ppt, violet =15ppt) (C) in function of the salinity concentration (ppt) or the sampling day. Statistical letters = significant change ($p<0.05$) between the groups within the same graph.

During the **2nd bacterial challenge** (Day 34 – Day 37), the plasma total immunoglobulin concentration of the plasma remained constant, equaling to 15.50 (±7.28) mg/mL with a high variation coefficient of 46.96%. Total immunoglobulin concentration for the phase 1, phase 2 and the 1st bacterial challenge are presented in the Figure 20.

3.4.2 Lysozyme activity

At the end of the **phase 1** (Day 20), or the **phase 2** (Day 20 – Day 34), the plasma lysozyme activity ranged from 145.89 (±43.13) to 231.11 (±67.07) U/mL (phase 2 : 150.48 (±70.81) to 213 (±60.80) U/mL) according to the **salinity** ($p<0.05$) with a rather high variation coefficient within the same treatment (21.06 to 29.56% (phase 2 : 28.43 to 47.06%). Values were significantly lower ($p<0.05$) for fish at 5 ppt relative to fish at 10 ppt. For the **1st bacterial challenge** (Day 20 – Day 23), the plasma lysozyme concentration rose according to the **salinity** and the **bacterial challenge** ($p<0.05$) for the fish at 10 and 15 ppt ($p<0.05$). Values ranged from 169.35 (±74.38) to 238.26 (±76.04) U/mL with a high variation coefficient within the same salinity treatment from 27.46 to 43.92%. The plasma lysozyme activity during the **2nd bacterial challenge** (Day 34 – Day 37) differed because of the **bacterial challenge** ($p<0.05$). For the sane fish, lysozyme activity equaled 189.37 (±83.58) U/mL with a high variation coefficient of 44.14% and increased when fish were infected which equaling 227.08 (±45.49) U/mL with a rather high coefficient variation (20.03%). The graphs of the lysozyme activity of the phase 1, phase 2 and the 2nd bacterial challenges are presented in the Figure 21.

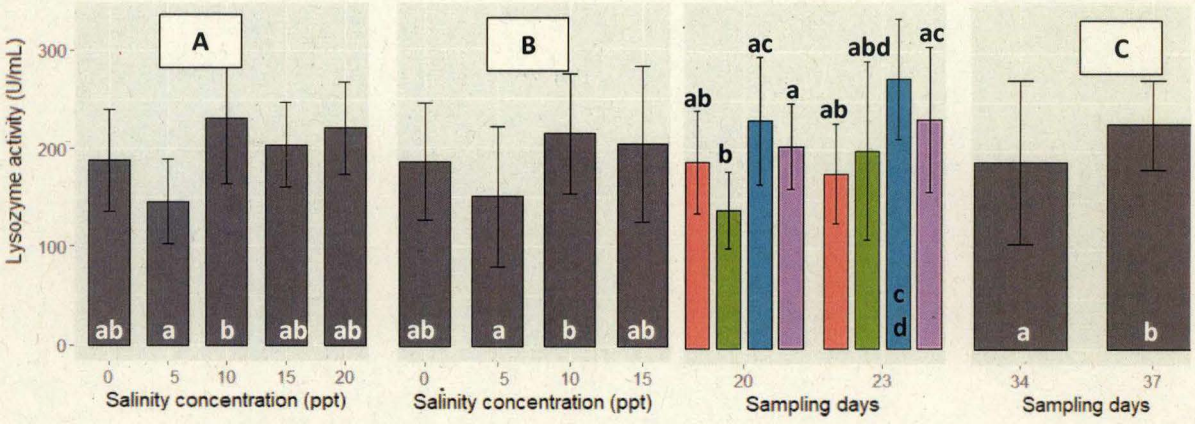

Figure 21: Means and standard deviations of the lysozyme activity (U/mL) of the striped catfish plasma after the phase 1 (Day20) (A), phase 2 (Day 20-Day 34) (B), 1st bacterial challenge (Day 20 – Day 23) (C) and 2st bacterial challenge (Day 34- Day 37) (D) in function of the salinity concentration (ppt) or of the bacterial challenge. Statistical letters = significant change ($p<0.05$) between the groups within the same graph. Legend: red=0ppt, green=5ppt, blue=10ppt, violet =15ppt

3.4.3 Peroxidase activity

During the **phase 1** (Day 0 - Day 20), the plasma peroxidase activity was affected by the **salinity rise** ($p<0.05$). At the initial status, value equaled 55.46 (±18.49) mg/mL with a high variation coefficient (33.35%) and increased until to 82.22 (±34.09) mg/mL with a high variation coefficient (41.46%). The plasma peroxidase activity for **the phase 2** (Day 20 – Day 34) differed due to the **two-week exposure** ($p<0.05$). After the salinity rise, peroxidase activity equaled to 85.59 (±35.81) mg/mL with a variation coefficient of 41.84% and then decreased until 70.99 (±39.30) mg/mL with a coefficient variation of 55.36%. The plasma peroxidase activity for the **1st** and **the 2nd bacterial challenge** (Day 20 – Day 23) differed because of the **bacterial challenge** ($p<0.05$). For sane fish, peroxidase activity equaled to 85.59 (±35.81) (2nd one : 70.99 (±39.30)) mg/mL with a variation coefficient of 41.84 (2nd one: 55.36) % and decreased until to 58.94 (±19.22) (2nd one : 40.38 (±37.46)) mg/mL with a variation coefficient of 32.61 (92.75)% when fish were infected. Graphs of peroxidase activity of phase 2, 1st and 2nd bacterial challenges are presented in the Figure 22.

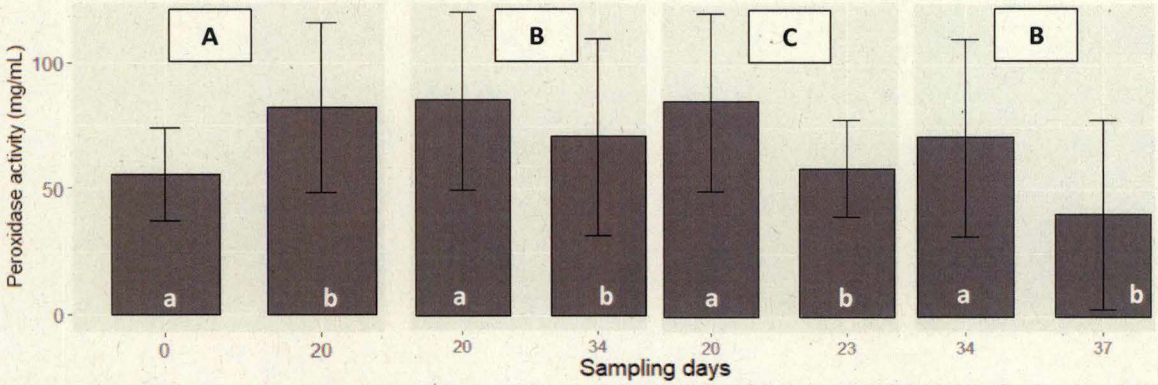

*Figure 22: Means and standard deviations of the peroxidase activity (mg/mL) of the striped catfish plasma for the phase1 (Day 0 – Day 20), phase 2 (Day 20-Day 34) (**A**), 1st bacterial challenge (Day 20- Day 23) (**B**) and 2st bacterial challenge (Day 34-Day 37) (**C**) in function of the two-week exposure or the bacterial challenge. Statistical letters = significant change ($p<0.05$) between the groups within the same graph.*

3.4.4 Leukocytes proportions

3.4.4.1 Granulocyte proportion

At the end of the **phase 1** (Day 20), the blood granulocyte proportion varied according to **salinity** ($p<0.05$) ranging from 2.23 (±1.49) to 8.44 (±5.23) % with a high variation coefficient within the same treatment from 27.75 to 67.09%. Significant values were recorded at 20 ppt ($p<0.05$). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood granulocyte proportion differed due to the **two-week exposure** ($p<0.05$). After the salinity rise, granulocyte proportion equaled to 3.15% (±1.92) with a huge variation coefficient of 60.98% and then increased until reaching 4.45% (±2.00) with a variation coefficient of 44.87% fourteen

days later ($p<0.05$). For the **1st and the 2nd bacterial challenges** (Day 20 – Day 23 and Day 34 – Day 37), the blood granulocyte proportion varied because of the **infection** ($p<0.05$). For the sane fish, it equaled 3.15 (±1.92) % (2nd one: 4.45% (±2.00) %) with a huge variation coefficient of 60.98% (2nd one: 44.86%) and then increased until to reach 6.69 (± 2.56) % (2nd one: 6.84 (±3.72)%) which a variation coefficient of 38.24% (2nd one: 54.52%).

3.4.4.2 Monocyte proportion

At the end of the **phase 1** (Day 20), the blood monocyte proportion dropped before rising according to the **salinity** ($p<0.0001$). Values ranged from 3.47 (±1.73) to 17.73 (±11.50) % with a huge variation coefficient (49.79 to 79.22%). Significant effects were observed at 5, 10 and 20 ppt ($p<0.05$). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood monocyte proportion was affected by the **two-week exposure** ($p<0.0001$) and the **salinity** ($p<0.0001$). Ranging from 3.50 (±1.81) to 17.54 (±5.93) % with a high variation coefficient within a group (33.81 to 85.12%), significative values were lower at 5 and 10 ppt ($p<0.05$) and monocyte proportions were higher after the constant salinity period in each treatment except at 10ppt ($p<0.05$). During the **1st bacterial challenge** (Day 20 – Day 23), the blood monocyte concentration differed because of the **salinity** ($p<0.0001$) and the **infection** ($p<0.0001$). They ranged from 3.50 (±1.81) to 28.24 (±15.44) % with a huge variation coefficient within a group (36.16 to 77.86%). Monocyte concentration decreased with salinity but monocyte proportions were higher after the bacterial challenge for each treatment ($p<0.05$). For the **2nd bacterial challenge** (Day 34 – Day 37), the blood monocyte proportion was influenced by the **infection** ($p<0.0001$), the **salinity** ($p<0.05$) and the **interaction** between these two factors ($p<0.05$). Values ranged from 6.42 (±3.81) to 22.99 (9.74) % with a huge variation coefficient within a group (33.81 to 85.12%). Monocyte proportion for infected fish did not differ from each other and were in the same range as the sane fish in freshwater (see above for more details on Day 20) (Figure 23).

3.4.4.3 Lymphocyte proportion

During the **phase 1** (Day 0 - Day 20), the blood lymphocyte proportion was affected by the **salinity** ($p<0.0001$), ranging from 73.83 (±15.74) to 94.29 (±5.48) % with a rather low variation coefficient within the same treatment (2.43 to 21.32%). The lymphocyte proportion increased before dropping and the values were significant at 5, 10 and 20 ppt ($p<0.05$). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood lymphocytes proportion differed according to the **two-week exposure** ($p<0.0001$) and the **salinity** ($p<0.05$). The lymphocyte

proportion significantly increased at 5 and 10 ppt and decreased after the constant salinity period ($p<0.05$) in each treatment. Values ranged from 77.84 (±6.88) to 94.29 (±2.30) % with a variation coefficient within the same salinity treatment of a day from 2.43 to 12.65%. During the **1st bacterial challenge** (Day 20 – Day 23), the blood lymphocyte proportion varied according to the **salinity** ($p<0.0001$) and the **infection** ($p<0.0001$), ranging from 65.03 (±16.28) to 94.13 (±2.34) % with a rather low variation coefficient within a group (2.49 to 25.04%). Values were significantly lower under saline condition but increased with the disease ($p<0.05$).

For the **2nd bacterial challenge** (Day 34 – Day 37), the blood lymphocyte proportion are influenced by the **infection** ($p<0.0001$) and the **interaction** between this parameter and the salinity ($p<0.05$). Lymphocyte proportion for the sane fish increased until to reach 10ppt and then decreased but when they were infection, it remained constant until 10ppt and then significantly decreased ($p<0.05$). Values ranged from 68.74 (±12.59) to 89.32 (±4.01) % with a variation coefficient within the same salinity treatment of a day from 4.49 to 18.68%.

Graphs of lymphocyte proportion of phase 1, phase 2, 1st and 2nd bacterial challenges are presented in the Figure 23.

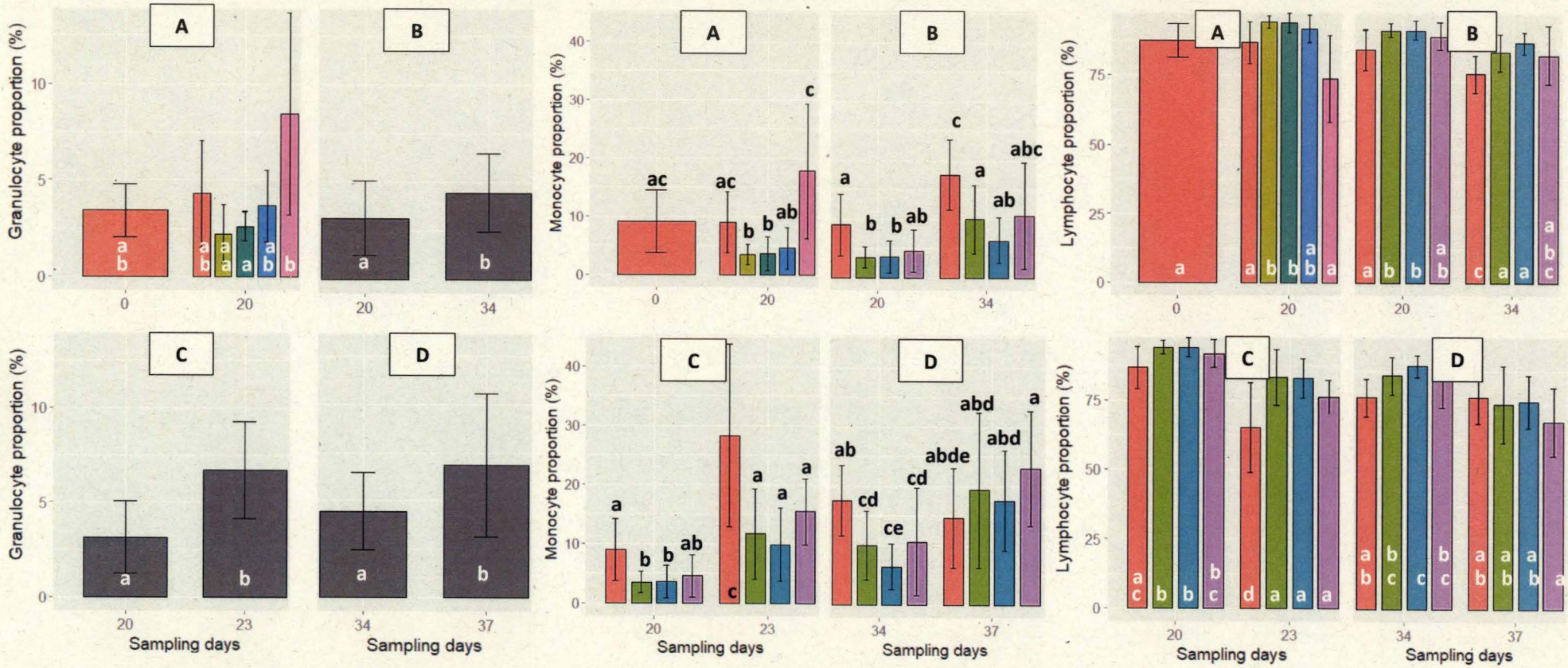

Figure 23: Means and standard deviations of the granulocyte, monocyte and lymphocyte proportions (%) of the striped catfish blood during the phase 1 (Day 0 - Day20) (Legend: red = 0 ppt, brown = 5 ppt, green = 10 ppt, blue = 15 ppt and violet = 20 ppt.) (A); the phase 2 (Day 20-Day 34) (B), the 1st bacterial challenge (Day 20- Day 23) (C) and the 2nd bacterial challenge (Day 34 – Day 37) (D). (Legend: red = 0 ppt, green = 5 ppt, blue = 10 ppt, violet = 15 ppt) (C) in function of the salinity concentration (ppt) and the sampling day. Statistical letters = significant change ($p<0.05$) between the groups within the same graph.

3.5 Hematological parameters

The results of the analyses of the hematological parameters (erythrocyte concentration, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin and the mean corpuscular hemoglobin concentration) are conducted in the following section. All the significative p values are indicated in brackets. For the control analysis, no one differed for erythrocyte concentration but one or several control treatments were significantly different for the other hematological parameters.

3.5.1 Erythrocyte concentration

During the **phase 1** (Day 0 - Day 20), the blood erythrocyte concentration was affected by the **salinity** ($p<0.05$). They ranged from 1.3946 (±0.4319) to 2.9804 (±0.3824) 10^6cells/µL with a variation coefficient within the same saline treatment from 1.46 to 30.97% and decreased with the salt concentration when it reached 15 and 20 ppt ($p<0.05$). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood erythrocytes concentration relatively constant through the **salinity** ($p<0.05$) ranging from 2.113(±0.497) to 2.782 (±0.436) 10^6cells/µL with a rather high variation coefficient (15.68 to 23.50%). Significant effect was recorded between 5 and 15 ppt ($p<0.05$). During the **1st bacterial challenge** (Day 20 – Day 23), the blood erythrocyte concentration was influenced by the **infection** ($p<0.0001$), the **salinity** ($p<0.0001$) and the **interaction** between these two factors ($p<0.05$). For the sane fish, erythrocyte concentration differed between 5 and 15 ppt but when they are infected, it rose in freshwater ($p<0.05$). Values ranged from 2.1458 (±0.4476) to 4.5767 (±0.6785) 10^6cells/µL with a rather high variation coefficient (13.46 to 34.43%). For the **2nd bacterial challenge** (Day 34 – Day 37), the blood erythrocyte concentration of the blood was affected by the infection ($p<0.0001$). The erythrocyte concentration of the sane fish equaling 2.3544 (±0.4453) 10^6cells/µL with a variation coefficient of 18.92% was lower than the one with the bacterial challenge which reaches 3.1178 (±0.6022) 10^6cells/µL with a variation coefficient of 19.31%. (Figure 24)

3.5.2 Hematocrit ratio

During the **phase 1** (Day 0 - Day 20), the blood hematocrit ratio was not affected. The hematocrit ratio decreased with the salinity ($p<0.05$) when it reached at least 10 ppt ($p<0.05$). Values ranged to 48.09 (±7.50) % to 33.04 (±11.6) % with a rather high variation coefficient (4.15 to 34.07%). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood hematocrit ratio differed according to the **two-week exposure** ($p<0.05$). After the salinity rise, values equaling 38.93 (±6.34) % with a variation coefficient of 16.28% was lower than the one

fourteen days later which reached 42.33 (±5.41) % with a variation coefficient of 12.78%. During the **1st bacterial challenge** (Day 20 – Day 23), the blood hematocrit ratio differed in function of the **salinity** ($p<0.05$) for the fish in freshwater and those submitted to 10 and 15ppt ($p<0.05$). They ranged from 34.37 (±8.91) to 42.90 (±5.21) % with a variation coefficient for the same salt concentration from 12.15 to 25.92%. For the **2nd bacterial challenge** (Day 34 – Day 37), the blood hematocrit ratio was influenced by the **infection** ($p<0.05$), the **salinity** ($p<0.05$) and the **interaction** between these two factors ($p<0.05$). Hematocrit ratio was significantly lower at 15ppt with bacterial challenge ($p<0.05$). Values ranged from 32.54 (±6.55) % to 43.55 (±5.95) % with a rather high variation coefficient of a group (5.56 to 20.12%). (Figure 24)

3.5.3 Hemoglobin concentration

During the **phase 1** (Day 0 - Day 20), the blood hemoglobin concentration was affected by the **salinity** ($p<0.001$). It significantly decreased in freshwater before increasing under saline condition ($p<0.05$). Values ranged from 8.25 (±1.56) to 12.62 (±2.70) g/dL with a rather high variation coefficient within a group (6.13 to 21.61%). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood hemoglobin concentration was influenced by the **two-week exposure** ($p=0.0003$) and the **interaction** between this factor and the salinity ($p<0.0001$). Hemoglobin concentration ranged from 8.25 (±1.56) to 13.11 (±1.90) g/dL with a rather high variation coefficient within a group treatment (14.38 to 20.30%). Values increased at the end of the salinity rise but decreased under high salinity concentration fourteen days later. During the **1st bacterial challenge** (Day 20 – Day 23), the blood hemoglobin concentration varied according to the **infection** ($p<0.0001$), the **salinity** ($p<0.0001$) and the **interaction** between these two factors ($p<0.05$). Ranging from 6.80 (±1.69) to 11.45 (±2.15) g/dL with a rather high variation coefficient (6.68 to 24.77%), values increased significantly for the sane fish according to the salinity but strongly decreased when they are infection except at 15 ppt ($p<0.05$). For the **2nd bacterial challenge** (Day 34 – Day 37), the blood hemoglobin concentration was affected by the **salinity** ($p<0.05$) because it slowly decreased among saline conditions ($p<0.05$). Hemoglobin concentration ranged from 9.73 (±2.49) to 12.36 (1.94) g/dL with a rather high variation coefficient (15.62 to 25.60%). (Figure 24)

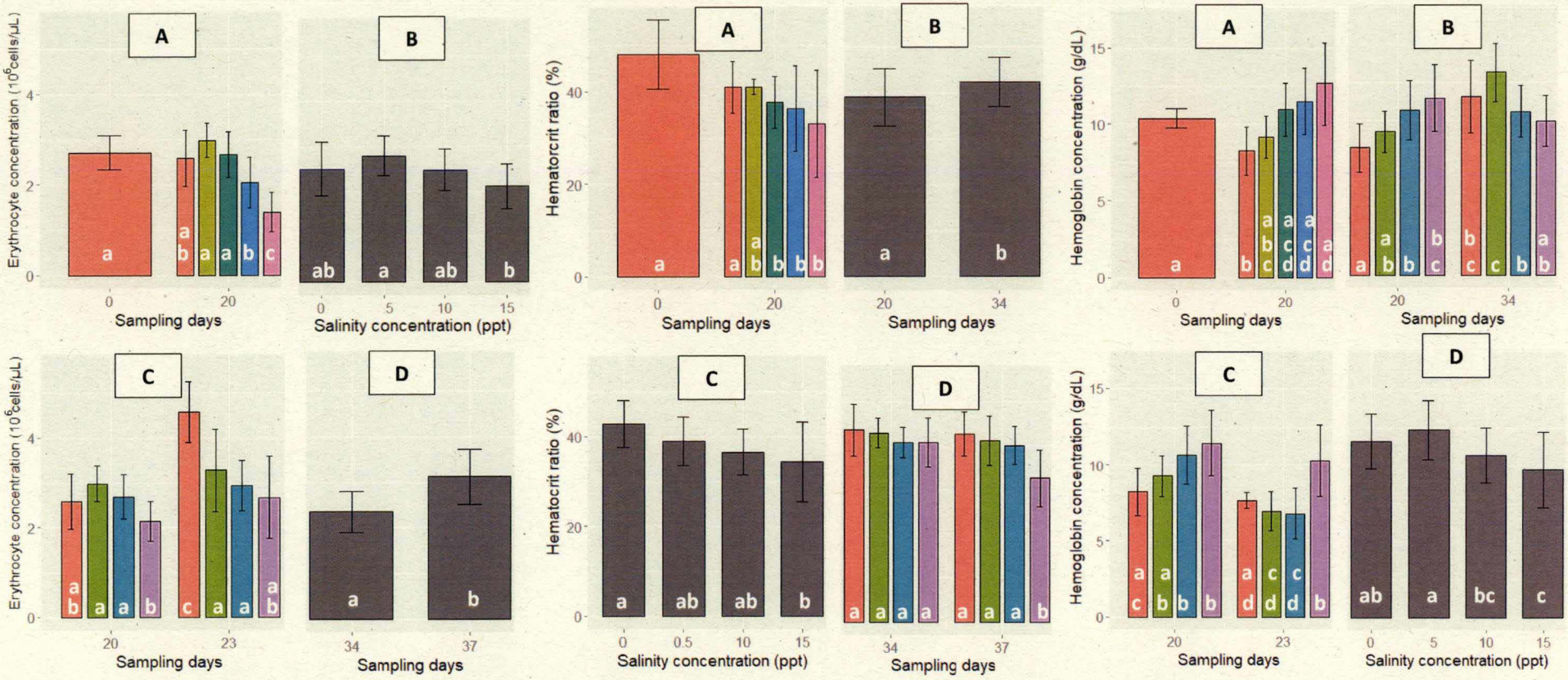

*Figure 24: Means and standard deviations of the erythrocyte concentration (10^6cells/µL), hematocrit ratio (%) and hemoglobin concentration (g/dL) of the striped catfish blood during the phase 1 (Day 0 - Day20) (Legend: red = 0 ppt, brown = 5 ppt, green = 10 ppt, blue = 15 ppt and violet = 20 ppt.) (**A**); the phase 2 (Day 20-Day 34) (**B**), the 1st bacterial challenge (Day 20 – Day 23) (**C**) and the 2nd bacterial challenge (Day 34 – Day 37)(**D**) (Legend: red = 0 ppt, green = 5 ppt, blue = 10 ppt, violet = 15 ppt) (**C**) in function of the salinity concentration (ppt) and the sampling day. Statistical letters = significant change ($p<0.05$) between the groups within the same graph.*

3.5.4 Mean corpuscular volume (MCV)

During the **phase 1** (Day 0 - Day 20), the blood MCV was affected by the **salinity** ($p<0.05$) resulting significative effect between low and high salinities. Dropping before increasing, values ranged from 69.89 (±10.17) to 134.13 (47.93) fL with a rather high variation coefficient within the same treatment (14.56 to 38.58%). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood MCV differed according to the **two-weeks exposure** ($p<0.0001$) and the **salinity** ($p=0.0019$), ranging from 69.89 (±10.17) to 99.12 (±22.42) fL with a high variation coefficient within a group (14.56 to 24.38%). MCV was generally significant fourteen days after the salinity rise with higher values ($p<0.05$). During the **1st bacterial challenge** (Day 20 – Day 23), the blood MCV was influenced by the **infection** ($p<0.0001$), the **salinity** ($p<0.05$) and the **interaction** between these two factors ($p<0.05$). MCV was lower in freshwater when the fish are infected ($p<0.05$) but values ranged from 50.19 (±14.13) to 91.99 (±24.38) g/dL with a rather high variation coefficient (12.55 to 26.93%). For the **2nd bacterial challenge** (Day 34 – Day 37), MCV is affected by the **infection** ($p<0.0001$). For the sane fish, MCV equaling 92.61(±17.58) fL with a variation coefficient of 18.98% is higher than the MCV for the infected fish which reached 63.75 (±20.46) fL with a variation coefficient of 20.46%. (Figure 25)

3.5.5 Mean corpuscular hemoglobin (MCH)

During the **phase 1** (Day 0 - Day 20), the blood MCH increased with the **salinity** ($p<0.05$). Ranging from 15.72 (±2.34) to 51.99 (±6.56) pg with a high variation coefficient within the treatment (12.95 to 41.85%), values were significant at 15 and 20 ppt. When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood MCH is affected by the **two-week exposure** ($p<0.05$), the **salinity** ($p<0.05$) and the **interaction** between these two factors ($p<0.0001$). Values were significantly elevated under higher salinity or fourteen days later ($p<0.05$) and ranged 15.72 (±14.91) to 29.72 (±8.90) pg with a high variation coefficient within a group (14.91 to 29.94%). During the **1st bacterial challenge** (Day 20 – Day 23), the blood MCH differed according to the infection ($p<0.0001$) and the salinity ($p<0.0001$), ranging from 8.54 (±1.30) to 29.72 (±4.47) pg with a high variation coefficient (14.91 to 36.06%). Values generally increased with the salinity ($p<0.05$). For the **2nd bacterial challenge** (Day 34 – Day 37), the blood MCH for the sane fish equaled 24.39 (±4.90) pg with a variation coefficient of 20.10% and decreased until 18.40 (±4.56) pg (variation coefficient :24.77%). (Figure 25)

3.5.6 Mean corpuscular hemoglobin concentration (MCHC)

For the **phase 1** (Day 0 - Day 20), the blood MCHC differed according to the salinity ($p<0.05$) and increased with it ($p<0.05$). Values ranged from 20.58 (±5.05) to 40.46 (±13.44) g/dL with a high variation within the same treatment (15.78 to 33.23%). When the fish were submitted the **fixed salinity** (Day 20 – Day 34), the blood MCHC was influenced by the **salinity** ($p<0.05$) and the **interaction** between this factor and the two-week exposure ($p<0.0001$). MCHC ranged from 18.05 (±3.90) to 31.39 (±7.46) with a high coefficient variation within a group (15.02 to 23.75%). After the salinity rise, the MCHC increased but fourteen days later it decreased according to the salinity. During the **1st bacterial challenge** (Day 20 – Day 23), the blood MCHC was affected by the infection ($p<0.05$) and the salinity ($p<0.0001$) and increased at 10 and 15 ppt. Values varied from 17.10 (±2.11) to 34.15 (±7.60) g/dL with a high coefficient variation (12.32 to 31.39%). However, For the **2nd bacterial challenge** (Day 34 – Day 37), the blood MCHC is not affected. It equals 27.72 (± 5.61) g/dL in average with a coefficient variation of 20.25%. (Figure 25)

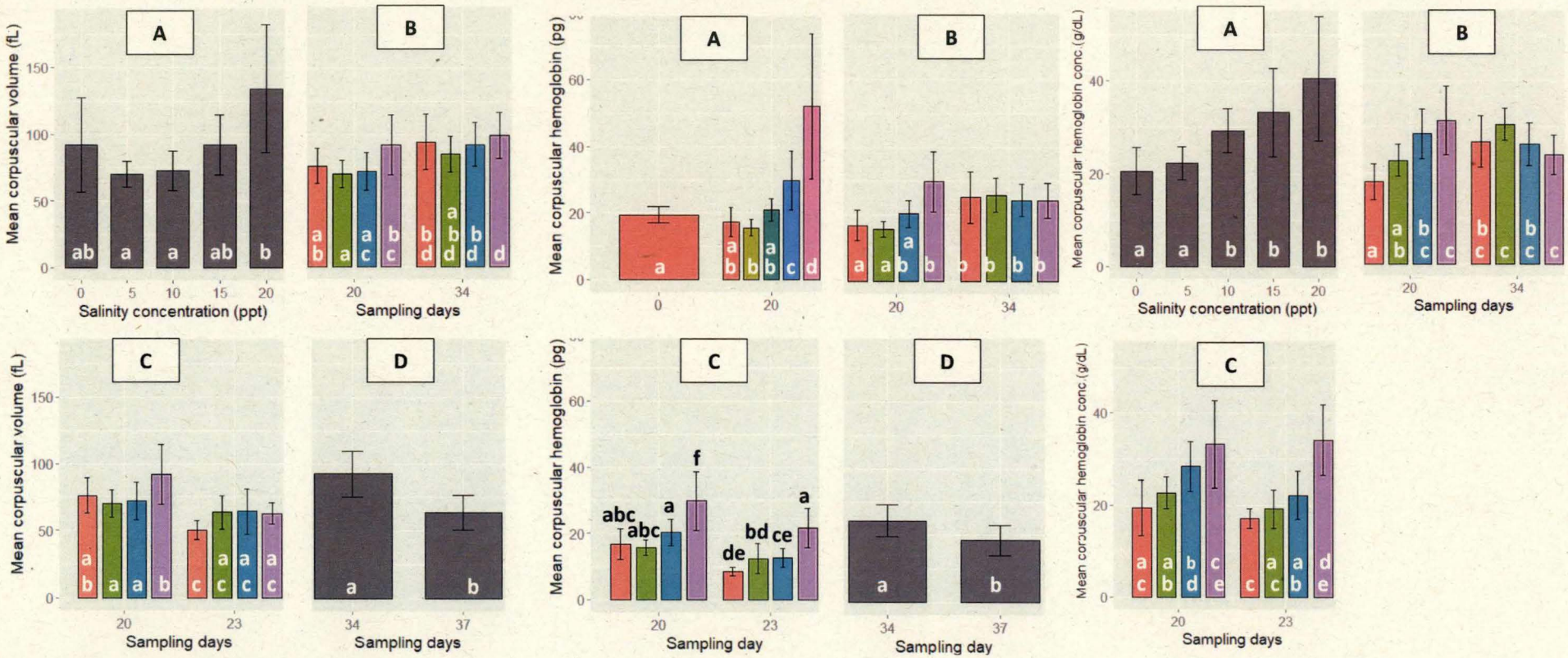

Figure 25: Means and standard deviations of the mean corpuscular volume (fL), mean corpuscular hemoglobin (pg) and the mean corpuscular hemoglobin concentration (g/dL) of the striped catfish blood during the phase 1 (Day 0 - Day20) (Legend: red = 0 ppt, brown = 5 ppt, green = 10 ppt, blue = 15 ppt and violet = 20 ppt.) (A); the phase 2 (Day 20-Day 34) (B), the 1st bacterial challenge (Day 20 – Day 23) (C) and the 2nd bacterial challenge (Day 34 – Day 37) (D) (Legend: red = 0 ppt, green = 5 ppt, blue = 10 ppt, violet = 15 ppt) (C) in function of the salinity concentration (ppt) and the sampling day. Statistical letters = significant change ($p<0.05$) between the groups within the same graph.

4. Discussion

4.1 Overall consideration

Before the results discussion, several experimental facts have to be mentioned.

First of all, the experimental system construction and the disease event before the salinity rise delayed the beginning of the experiment for one month. However, the overall planification and the intensive work during the two following months make to recover that delay in order to finish on time. Consequently, variables analyses took place in Viet Nam and in Belgium during and after the experiment.

Moreover, as seen at the end of the 1st bacterial challenge, the mortality rate of the fish submitted to 0, 5, 10 and 15 ppt did not exceed 5% on average. In addition to this observation and the absence of visible disease symptoms during the autopsy for the majority of sampled fish, the bacterial challenge was not decisive after 3 days and a lot of fish submitted to these salt concentrations were still alive and looked healthy 8 days after the infection. In order to recheck the bacteria virulence, another culture and inoculation test were carried out with the same bacterial strain. After the successful *in vitro* culture, bacteria solution was injected to another set of fish with the same background as the one of those used during the first bacterial test. The results were identical to the previous sampling: no mortality was recorded and only a few fish proportion showed symptoms of weak intensity. Because of the ability of bacteria to induce immune reaction without mortality even with a strong virulence decrease, the same strain was reinoculated for the second bacterial challenge. The comparison with the control groups is still possible and enable to verify if bacterial response ability was impacted by the salinity and/or by the bacterial challenge.

Challenging fish in salty or freshwater with the bacteria strain developed in other saline concentration was not totally appropriate, their survival being deeply compromised. For future researches, this parameter may be taken into account from the bacteria culture step, and comparison of the variability of virulence intensity and ACH50 might be previously conducted. However, the modulation of the immune reaction in case of salinity events with non-biological stressors such as temperature (T. H. P. Nguyen 2015), transportation (Zanuzzo et al. 2019), chemicals (Wang et al. 2019; Stoddard et al. 2019), hypoxia (Abdel-Tawwab et al. 2019),... may also be considered.

Furthermore, the experimental design did not allow for the statistical comparison between the two bacterial challenges because of the time interval between them. For a future experiment, it could be interesting to submit another set of fish to a salinity rise starting 20 days before the end of the two-week exposure in order to carry out the bacterial challenges at the same time with the same bacteria material and under the same environmental conditions. In this case, it could be possible to compare the impact of a salinity rise and an exposure time on the immune response to a bacterial challenge.

Additionally, the statistical comparison between each phase for each parameter had to be done separately from the rest of the data set.

However, this study offers interesting insights about immunological, physiological and hematological responses of the striped catfish after (1) a stress induced by a salinity increase (2) followed by a constant salinity exposure; with or without infection.

4.2 Feed efficiency, weight gain and mortality rate discussion

During the phases 1 and 2, the significant effects detected in the weight gain, feed efficiency and mortality within the same treatment were consistent. Indeed, during the phase 1, fish under 5ppt displayed the best feed efficiency and the lowest mortality rate. In general, the growth performance of the fish under low salinities was better than in the higher ones or in freshwater (P. T. H. Nguyen et al. 2014; Jahan et al. 2019) and that was also confirmed in this study at the end of the salinity rise.

Nevertheless, different results were recorded at the end of the phase 2: the best feed efficiency and weight gain were observed for the fish under 15 ppt even if the mortality rate was the highest one. This was probably explained by the death of the weak fish (probably also the smallest ones) and the adaptation of the remaining ones to the high salinity (T. H. P. Nguyen 2015; Phuc, Mather, and Hurwood 2017), reinforcing the idea that the selection program of more resistant striped catfish strain is possible even during a chronic saline stress. However, the observed shift of the feed efficiency and the weight gain in comparison to the phase 1 ones could also indicate an exhausting risk for the fish and have to be investigated.

The mortality rates recorded during the bacterial challenges are difficult to interpret because of the virulence lost and the increased of the mortality rate in the previous phases just before the challenges.

4.3 Physiological variables

A short comparative summary of the physiological parameters among the different experimental steps is presented in the Table 3.

Table 3: Comparative summary of the results for the physiological variables between different experimental steps for each saline treatment

		Salinity concentration (ppt)				
	Variables	0	5	10	15	20
Phase 1	Osmolality	-	-	↑	↑	↑
	Chloride concentration	-	-	↑	↑	↑
	Glucose concentration	-	-	-	-	↓
Phase 2	Osmolality	Only salinity effect				NR
	Sodium concentration					NR
	Chloride concentration					NR
	Glucose concentration					NR
1st bacterial challenge	Osmolality	Only salinity effect				NR
	Sodium concentration					NR
	Chloride concentration					NR
	Glucose concentration	Only infection effect (↑)				NR
2nd bacterial challenge	Osmolality	Only salinity effect				NR
	Sodium concentration					NR
	Chloride concentration					NR
	Glucose concentration	↓	-	-	-	NR

Legend: status quo (-), decrease (↓) or rise (↑) of the parameter value at the corresponding salinity concentration in function of the experimental step. NR = no record

4.3.1 Osmolality and ion concentrations

When the fish were submitted to different salinities, the same trend was observed throughout the whole experiment: the osmolality as well as the chloride and sodium concentrations increased according to the salinity gradient and did not vary with the constant salinity period or the bacterial challenges, the values being consistent with the information reported previously for the striped catfish and other siluridae species(Lefevre, Jensen, Huong, et al. 2011; T. H. P. Nguyen 2015; Malakpour Kolbadinezhad, Coimbra, and Wilson 2018; Souza-Bastos and Freire 2009; Kumar et al. 2017; M. Schmitz 2017). The constant salinity period during the 1st bacterial challenge and the phase 2 enabled the decrease of the coefficient variation and the emergence of significative differences for the sodium concentration which was not possible at the end of the phase 1 because of the rapid salinity increase. The lower variability recorded for the fish at 20 ppt was probably a consequence of the high mortality rate recorded before the sampling which "removed" the fish with a lower tolerance threshold, inducing a lower interindividual variation among the survivors. In other words, osmoregulation was only affected by the saline treatment. As the salinity stressor induced similar trends in the different variables of osmoregulation tested, the analyses may be limited to the osmolality in future experiments.

4.3.2 Glucose concentration

The secondary stress indicator, namely the glucose concentration, generally increased in case of stress (P. T. H. Nguyen et al. 2014; Jahan et al. 2019; Ahmmed et al. 2017) as observed during the 1st bacterial challenge (Sopinka et al. 2016). However, the plasma glucose concentration remained relatively constant during the phase 1 and for the saline treatment of the phase 2 (compared to the initial status (Day 0)) whereas previous studies indicated its stimulation in presence of salt. However, glucose concentration reduction and a stabilization was already observed for striped catfish under constant salinity concentration (Phuc, Mather, and Hurwood 2017). This effect is probably explained by the slow salinity rise carried out during the first step of this experiment coupled with the high interindividual variability. However, the drop of this variable at 20 ppt is an indication about the complete disruption of the glucose metabolism due to the salinity toxicity at this concentration. All the values were consistent with the information reported previously for the striped catfish or other catfish species (Souza-Bastos and Freire 2009; Sarma et al. 2013). The results of the 2nd bacterial challenge seem to indicate that the fish submitted to 5 ppt were more resistant to the infection and that the bacteria did not survive at 15 ppt (the salt is a common natural antiseptic in aquaculture to threaten microbial diseases (Mélodie Schmitz, Mandiki, et al. 2016; Souza-Bastos and Freire 2009)), leading to the absence of significant effects for these two treatments. At the end of the phase 2, the fish in freshwater appeared significantly more stressed than at the beginning of the experiment, making the interpretation of the following bacterial challenge results for this treatment more complicated. The glucose concentration at 10 ppt significantly decreased. This is probably the consequence of the energetic reserves depletion instead of an immunodepression status under high salinity concentration.

Inefficient stress indicator in case of acute response to specific stressors, glucose concentration interpretations for a chronic stress (constant salinity period) in addition to an acute stress (following bacterial challenge) is difficult to interpret because of its implication in other general metabolic processes (Sopinka et al. 2016). But because of the experimental constraints, other plasmatic stress indicators as cortisol were not possible to analyze. The gills Na^+/K^+ ATPase activity could provide additional information on the stress levels during saline stresses and infections. Unfortunately, the analysis of this parameters is underway and the results are not yet available. Increasing under high saline concentrations and decreasing in case of *Edwardsiella ictaluri* infection (Mélodie Schmitz, Douxfils, et al. 2016; Yuasa et al. 2003).

4.4 Immune parameters

A short comparative summary of the immune variables among the different experimental steps is presented in the Table 4. The analyses of the peroxidase and the total immunoglobulin have been carried out in Belgium. The transport conditions could not be optimal, the results of these two immune markers must be interpreted with caution.

Table 4: Comparative summary of the results for the immune variables between different experimental steps for each saline treatment

	Salinity concentration (ppt)					
	Parameters	0	5	10	15	20
Phase 1	Total immunoglobulin conc.	-	-	↑	-	↓
	Peroxidase activity	Only salinity rise effect (↑)				
	Granulocyte proportion	-	-	-	-	-
	Monocyte proportion	-	↓	↓	-	-
	Lymphocyte proportion	-	↑	↑	-	-
Phase 2	Total immunoglobulin conc.	Only salinity effect				NR
	Lysozyme activity	-	-	-	-	NR
	Peroxidase activity	Only exposure effect (↓)				NR
	Granulocyte proportion	Only exposure effect (↑)				NR
	Monocyte proportion	↑	↑	-	-	NR
	Lymphocyte proportion	↓	↓	↓	-	NR
1st bacterial challenge	Total immunoglobulin conc.	-	-	↓	-	NR
	Lysozyme activity	-	-	-	-	NR
	Peroxidase activity	Only infection effect (↓)				NR
	Granulocyte proportion	Only infection effect (↑)				NR
	Monocyte proportion	↑	↑	↑	↑	NR
	Lymphocyte proportion	↓	↓	↓	↓	NR
2nd bacterial challenge	Total immunoglobulin conc.	No effect				NR
	Lysozyme activity	Only infection effect (↑)				NR
	Peroxidase activity	Only infection effect (↓)				NR
	Granulocyte proportion	Only infection effect (↑)				NR
	Monocyte proportion	-	↑	↑	↑	NR
	Lymphocyte proportion	-	-	↓	↓	NR

Legend: status quo (-), decrease (↓) or rise (↑) of the parameter value at the corresponding salinity concentration in function of the experimental step. NR = no record

For this section, only the proportion for each white blood cell type was carried out and not their absolute concentrations. Its analysis in future experiments will probably bring news information about the strength of the immune reaction. However, leukocyte proportions already enabled the detection of a shift in the immune response.

During the salinity rise, the lymphocyte proportion and total immunoglobulin increase at 10 ppt indicated the presence of an immune stress. At 20 ppt, the decrease of the total immunoglobulin concentration as well as the high variability of the granulocyte and monocyte proportions tend to indicate a disturbance in the immune response and a high variation between individuals. The increase of peroxidase activity indicates an overall oxidative stress and a high variability within

the same saline treatment. The higher lysozyme activity in the plasma in comparison of the fish under 5 ppt suggests an innate immunity stimulation.

Under 5ppt, the absence of significant effects detected in the plasma immune markers and the slight changes in the monocyte and lymphocyte proportions suggest a better overall health status. The absence of significant effects at 15 ppt could indicate an overall inhibition of the immune system or an intermediate status between 10 ppt and 20 ppt treatments. However, this last hypothesis is not consistent at the light of the poor mortality rate recorded in this treatment.

At the end of the two-week exposure, peroxidase activity and lymphocyte proportion dropped markedly, indicating an overall stress reduction and probably an acclimation to the salinity conditions. Lysozyme activity and total Ig were similar to those observed at the end of the salinity rise. In other words, the prolonged exposure of the fish to a saline stressor tended to decrease of the adaptative immunity and the general stress status response but did not regulate the innate immune response.

In both bacterial challenges, peroxidase activity diminished and granulocyte proportion augmented. For the 1st bacterial challenge, lymphocyte and monocyte proportions showed opposite trend in each treatment: when the first one increased, the other one was lowered. This trend was recorded during the second bacterial challenge only for the fish under 10 and 15 ppt. Lysozyme activity did not vary during the 1st bacterial challenge (even if the variability at 5 and 10 ppt rose) while it recorded an overall increase during the 2nd one. Total immunoglobulin was not affected (except at 10 ppt) by the 1st bacterial challenge and remained constant for the second one, probably indicating a better resistance of the fish at 5 ppt and the non-viability of the bacteria at 15 ppt. The immune reaction after the two-week exposure seems to be less impacted by the salinity.

4.4.1 Overall discussion for the immune markers

The immunity stimulation during acute or chronic saline stressors was already described in previous studies for the striped catfish and euryhaline species (Mélodie Schmitz, Douxfils, et al. 2016; Jiang et al. 2008; Mélodie Schmitz, Ziv, et al. 2017).

Under saline stress conditions, the metabolism rises in order to ensure the energetic demand of the different stimulated metabolic pathways. This overproduction leads to excessive formation of reactive oxygen species (ROS). In order to protect the organism against destructive effect of these molecules, the antioxidant defenses, such as the peroxidase production, are enhanced

(Kumari et al. 2015; Blokhina, Virolainen, and Fagerstedt 2003). The slight decrease of the peroxidase activity at the end of the two-week exposure suggests that the fish could be acclimated to the salinity. Following this observation, the prolongation of this experimental step in future study could provide new information about stress acclimation that could be used by the selection program of the PANGAGEN project. Moreover, the production of ROS being involved in the immune protection against pathogens, the production of antioxidant enzymes decreased in case of infection in order to not diminish the pathogen eradication process (Mélodie Schmitz, Mandiki, et al. 2016).

The stimulation of the lysozyme for the fish under high salinity in comparison to those at 5 ppt during the salinity rise indicates the stimulation of the innate immune system. Their maintenance during the two-week salinity exposure coupled with a granulocyte proportion rise indicates the persistence of inflammatory markers under sterile inflammation which could threaten the organism integrity and induce tissue damages (Chen and Nuñez 2010).

4.5 Hematological variables

A short comparative summary of the hematological variables among the different experimental steps is presented in the Table 5.

Table 5: Comparative summary of the results for the hematological variables between different experimental steps for each saline treatment

	Salinity concentration (ppt)					
	Parameters	0	5	10	15	20
Phase 1	Erythrocyte concentration	-	-	-	↓	↓
	Hemoglobin concentration	↓	-	-	-	-
	Hematocrit ratio	-	-	↓	↓	↓
	Mean corpuscular volume	-	-	-	-	-
	Mean corpuscular hemoglobin	-	↓	-	↑	↑
	Mean corpuscular hemoglobin conc.	-	-	↑	↑	↑
Phase 2	Erythrocyte concentration	-	-	-	-	NR
	Hemoglobin concentration	↑	↑	-	-	NR
	Hematocrit ratio	Only exposure effect (↑)				NR
	Mean corpuscular volume	-	↑	↑	-	NR
	Mean corpuscular hemoglobin	↑	↑	-	-	NR
	Mean corpuscular hemoglobin conc.	↑	↑	-	↓	NR
1st bacterial challenge	Erythrocyte concentration	↑	-	-	-	NR
	Hemoglobin concentration	-	↓	↓	-	NR
	Hematocrit ratio	Only salinity effect				NR
	Mean corpuscular volume	↓	-	-	↓	NR
	Mean corpuscular hemoglobin	↓	-	↓	↓	NR
	Mean corpuscular hemoglobin conc.	-	-	-	-	NR
2nd bacterial challenge	Erythrocyte concentration	Only infection effect (↑)				NR
	Hemoglobin concentration	Only salinity effect				NR
	Hematocrit ratio	-	-	-	↓	NR
	Mean corpuscular volume	Only infection effect (↓)				NR
	Mean corpuscular hemoglobin	Only infection effect (↓)				NR
	Mean corpuscular hemoglobin conc.	No effect				NR

Legend: status quo (-), decrease (↓) or rise (↑) of the parameter value at the corresponding salinity concentration in function of the experimental step. NR = no record

Hematological responses against saline stress is strongly dependent on fish species. Some euryhaline species tend to be affected by a salinity-induced osmoregulatory dysfunction leading to an erythrocyte and hemoglobin concentrations and hematocrit ratio drop while freshwater species tend to raise these markers (Burgos-Aceves, Lionetti, and Faggio 2019). Hematological indicators of striped catfish being very sensitive to environmental conditions, some isolated effects could probably not be explained (Phuc, Mather, and Hurwood 2017; Shahjahan et al. 2018). However, it was clearly observed that these indicators are influenced by salinity(Shahjahan et al. 2018) and infection with *Edwardsiella ictaluri* (Abdelhamed et al. 2018; Burgos-Aceves, Lionetti, and Faggio 2019).

At the end of the salinity rise, the erythrocyte concentration dropped when fish were submitted to high salinity (15 ppt) but this effect completely disappeared 14 days later. Even if the effect of the salinity on the erythrocyte concentration depends on the fish species, its duration and its intensity, trends and values range were already observed in previous studies for pangasius (Witeska 2013; Jahan et al. 2019; Phuc, Mather, and Hurwood 2017). During the phase 1, hematocrit ratio rather varied according to the red blood cells concentrations even if the variation in the high salinity treatments (>10 ppt) significantly increased. Furthermore, the mean corpuscular volume (MCV) allowed for the detection of a more homogenous size under 5, 10 and 15 ppt which were lower than under 20 ppt. The blood hemoglobin concentration significantly increased, as expected (Phuc, Mather, and Hurwood 2017), indicating a growth of the mean corpuscular hemoglobin concentration (MCHC). The mean corpuscular hemoglobin (MCH) show a weight gain of hemoglobin under high salinities. In other words, red blood cells under acute and important saline stress are bigger and their hemoglobin concentration are higher in order to balance their disappearance.

At the end of the two-week exposure, the strong variability observed after the salinity rise tended to be reduced: erythrocyte remained constant in concentration as the MCH and the MCV and hemoglobin concentration rose for fish at 5 ppt, indicating a higher MCHC for this last treatment. Hematocrit ratio being a representation of red blood cells volume in the blood, the slight increase of these indicators during the phase 2 while erythrocyte concentration remained constant could only be explained by an overall MCV growth but the strong variability depending on the salinity limits our conclusions (in freshwater and under 20 ppt, variation coefficients were twice as the one 5 ppt).

During bacterial challenges, different effects were observed in the light of the salinity history. After the first salinity rise, fish in freshwater underwent an erythrocyte number gain while hemoglobin concentration and hematocrit did not change, indicating a MCV and a MCH reduction. At 5 ppt, hemoglobin concentration slightly decreased while erythrocyte concentration and hematocrit remained constant. However, this effect was not significant enough to influence MCH or MCHC. The same trend was observed at 10 ppt but, in this case, MCH was impacted and also decreased. At 15 ppt, red blood cells count, hematocrit ratio and hemoglobin concentration seem not to be affected by the infection, even if the variability between the individuals seems to hide some effects that can be detected with the decrease of the MCV and MCH. Hematocrit ratio was never affected by the infection probably due to a trade-off between erythrocyte concentration and MCV. However, the controls groups did not

differ from the fish in freshwater in several cases indicating a strong influence of the environmental conditions.

The bacterial challenge at the end of the two-week exposure was not affected by the salinity except for hematocrit ratio when it reached 15 ppt. The quasi-identical response than for the sane fish tend to indicate the absence of infection consequence. However, the only significative effect was the red blood cells concentration rise which is opposite to normal infection reaction. The release of erythrocyte from hematopoietic organ generally happens when cells are damaged because of environmental factors in order to ensure their overall metabolic activity(Witeska 2013). This effect could be induced by the relocation of the fish under new environmental conditions for the bacterial challenge. The observation of the cells abnormalities in future experiments could be carried out to challenge this hypothesis. MCHC was totally not affected by the salinity or the infection.

4.5.1 Overall discussion for the hematological parameters

Even if blood parameters were completely disrupted during the salinity rise, *Pangasianodon hypophthalmus* was able to acclimate at the salinity even under 15 ppt. This finding is crucial for future ecological studies and the continuity of its fish farming in the Mekong delta. The weakness of these during the 1st and the 2nd bacterial challenges seems to be due to the loss of virulence of the bacteria strain.

4.6 Overall discussion

In order to conclude this discussion, some interesting new findings have to be pointed. The osmotic response is instantly adapted to the salinity rise and the fish did not attempt to modify it even during prolonged exposure. Firstly, it leads to an overall exhaustion of the organism and the trigger of both innate and adaptative immunity response. But the exposition for several days at the same salinity level induces the diminution of the adaptative immunity and the general stress level, the recovery of the feed efficiency and the adaptation of the hematology factors. The absence of clear response of several parameters at the end of the salinity rise suggests that the slow saline concentration rise (maximum 1 ppt a day) and the daily split addition of salty water enabled the fish to have a better adaptation. This fact might to be investigated.

5. Conclusion and perspectives

Stress exposure induces the activation of numerous physiological mechanisms, from gene expression to metabolism changes and immune disturbances. In vertebrates, immune response is highly specialized through complex structures and regulatory systems. Different stressors may induce suppressive or cumulative effects, the overall reaction strongly depending on the intensity, the duration, the stress type and the species. Short term stress may stimulate immune response under certain conditions while chronic stress induces immunodepression and increased sensitivity to infection. Several concomitant stressors highly threat the sustainability of the striped catfish farming in the Mekong delta such as salinity and *Edwardsiella ictaluri* infection.

In this study, an important salinity rise caused an immediate adaptative response to the osmotic pressure of the plasma inducing the disruption of the hematological parameters and, as expected, the triggering of innate and adaptative immune reactions. Nonetheless, the response of some parameters was lower than expected. After a relatively long exposure to salt, the stabilization of the interindividual variability seems to stabilize and several significant effects tend to disappear, giving new insights about stress acclimation. Investigating the influence of a longer exposure period and the salinity rise speed might be carried out in future experiments in order to improve our knowledge on striped catfish survival during saline events.

Moreover, in light of all these results, the bacterial challenges were not conclusive even if the bacterial antigens were recognized by several immune factors. In order to be sure of the link between long exposure to high salinity and immune response, the modulation of immunity under saline stress might be carried out with a non-biological stressor.

Studying the influences of environmental conditions on the epigenetic mechanisms and on the stress resistance in earlier life stages of striped catfish might turn out to be interesting in the future, providing the selection program of the PANGAGEN project with additional support.

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7. Appendices

Appendix 1: Bacterial culture protocol

The stored bacteria were cultured on Trypticase soy agar at room temperature during 2 days. One colony was isolated and put in Brain Heart Infusion Broth for 2 additional days. All the obtained colonies were collected and rinsed with physiological fluid as following described. After the addition of 5 µL of physiological fluid, these bacterial solutions were centrifuged at 7500rpm during 5min at room temperature and a maximum of liquid was removed. This step was carried out three times in a row. After the cleaning, the solid part was diluted in 5 mL of physiologic fluid.

Appendix 2: glucose protocol information

Phosphate buffer 0.6M (pH = 7.5):

- 13.625g of KH_2PO_4,
- 28.36g of Na_2HPO_4,
- 500ml of pure water.

Phosphate buffer 0.1 M (pH = 7.5)

- 100mL of phosphate buffer 0.6 M,
- 500 mL of distilled water.

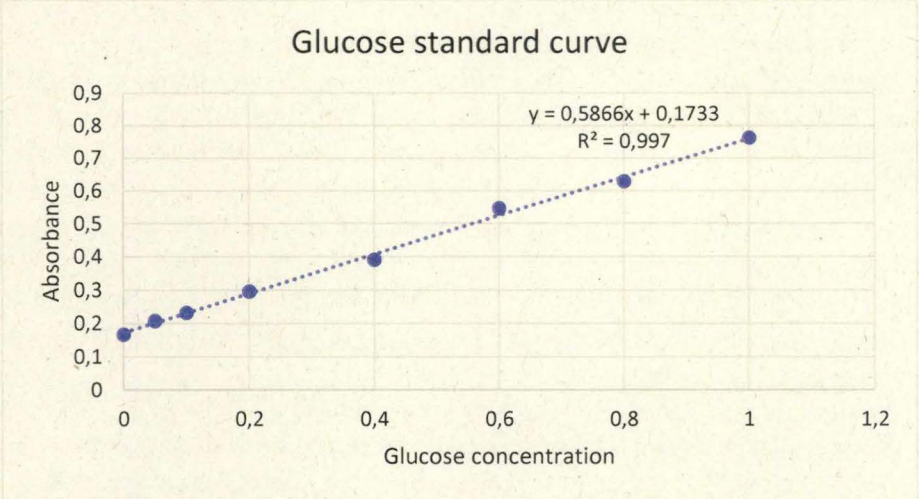
Reactive solution:

- 2000 units of glucose oxidase Type X-S,
- 147 units of peroxidase Type 1 (147U),
- 125mg of 2,2-Azino-di-(3-ethylbenzoline sulfonate (ABTS)
- 500mL of phosphate buffer 0.1M (pH = 7.5)

The standard curve was realized following the concentration in the table below.

Glucose standard curve		
Glucose standard solution (µL)	Pure water (µL)	Final concentration (mg/ml)
0	100	0
5	95	0.05
10	90	0.1
20	80	0.2
40	60	0.4
60	40	0.6
80	20	0.8
100	0	1

The absorbance related to the glucose concentration was used to determine the a and b coefficients (y = a*x +b) of the trend line of the standard curve.



Appendix 3: hemoglobin protocol information

Darbkin chemical protocol	
Solution 1	20g of $K_3Fe(CN)_6$ diluted in 1L of distilled water
Solution 2	75g of $KHCO_3$ and 5g of KCN diluted in 1L of distilled water
10mL of solution 1 and 10 mL of solution 2 were mixed with 980mL of distilled water	

Appendix 4: erythrocyte protocol information

Time	Chemical
10min	Wright's stain
3min	Distilled water adjusted to pH 6.5
35min	Giemsa solution (1ml in 10 ml of distilled water)
Rinsed with water	
10min	Distilled water adjusted to pH 6.2 solution
Rinsed with water and dried	

Appendix 5: water parameters protocols

Nitrate:

2.5g of sulphanilamide were added to 250ml of solution composed of 36ml of HCl 1M and distilled water.

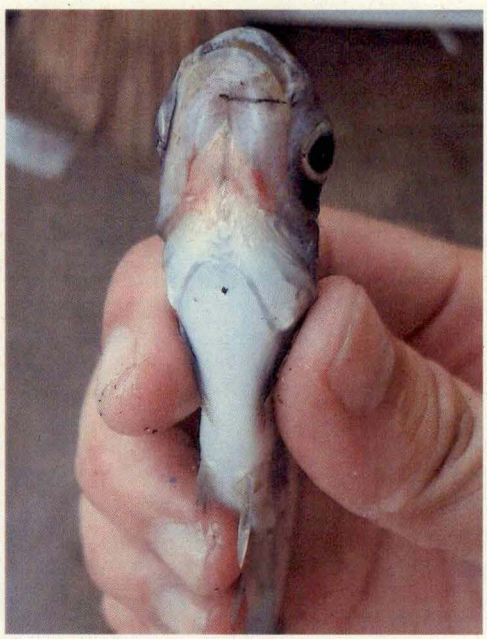
Total ammonium nitrogen

For each water sample (Days 0, 11, 23, 27 and 34), 5ml of water were mixed with 200 µL of solution A (11.1 mL of phenol, 88.9mL of ethanol 96%), 200µL of solution B (0.5g of $Na_2[Fe(CN)_5NO].2H_2O$ in 100mL of distilled water) and 500 µL of solution C (20g of trisodium citrate, 1g NaOH and 25 mL of NaOCl in 100 mL of distilled water). After waiting 1h, the mix was read either at 630 nm for the freshwater samples or at 640 nm for the saline water.

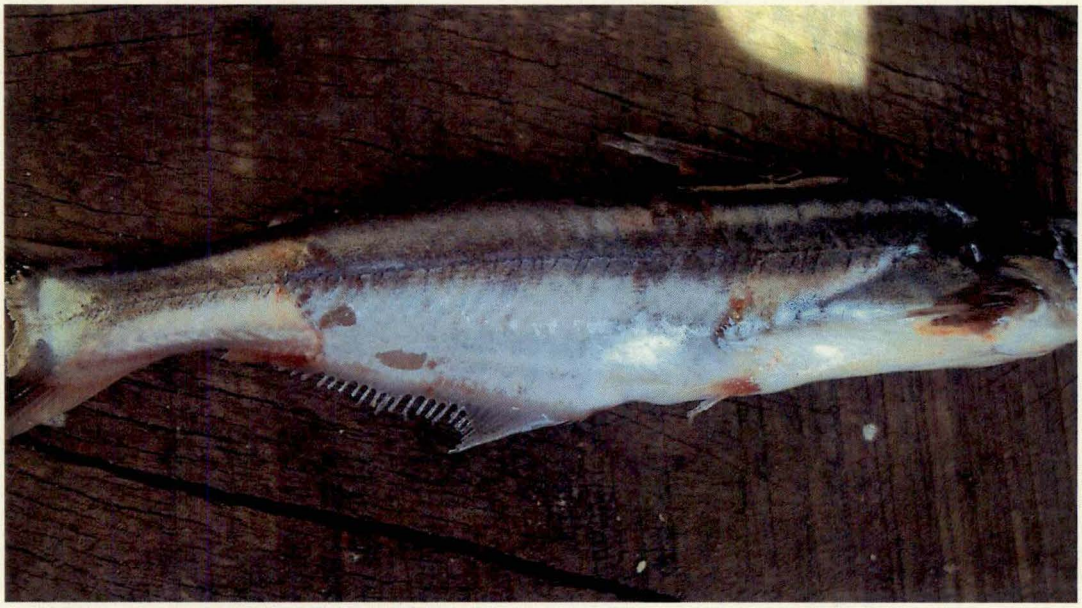
The calibration curve was carried out with 9 mL of tap water and 1mL of standard solution. This solution was prepared with 2mL of the solution 0 (0.2358g of $(NH_4)_2SO_4$ in 100 mL of distilled water) in 98 mL of distilled water. The mix was diluted 1:2, 1:4, 1:8, 1:16. The standard curve was designed with 5mL of the mix, the previous dilution and 5 mL of tap water in which were added 200µL of solution A, 200µL of solution B and 500µL of solution C. The tubes were read with the same wavelength than previously.

Appendix 6: fish symptoms of the pre-experimental disease

Legend: apparent bone of the lower jaw.



Legend: bloody gills.



Legend: injuries on the body and grey color of the skin.

Appendix 7: mean, standard deviation and variation coefficient of the parameters according to the salinity concentration (ppt)and/or the sampling day.

Salinity conc. (ppt)	0	5	10	15	20
Osmolality					
Phase 1					
Day 0					
Mean ± SD (mOsm)	264.10(±20.73)a	-	-	-	-
CV (%)	7.85	-	-	-	-
Day 20					
Mean ± SD (mOsm)	270.4(±9.0)a	273.8(±11.3)a	301.8(±25.0)b	366. 0(±48.4)c	426.3(±38.0)d
CV (%)	3.32	4.13	8.28	13.23	8.90
1st bacterial challenge (Day 20 – Day 23)					
Mean ± SD (mOsm)	263.9(±13.8)a	275.0(±8.2)b	290.6(±29.2)c	349.3 (±44.0)d	-
CV (%)	5.22	2.99	10.03	12.60	-
Phase 2 (Day 20- Day 34)					
Mean ± SD (mOsm)	268.9(±9.3)a	275.9 (±8.4)b	295.7 (±21.8)c	345.7(±43.1)d	-
CV (%)	3.46	3.04	7.36	12.46	-
2nd bacterial challenge (Day 34 – Day 37)					
Mean ± SD (mOsm)	263.2(±9.99)a	273.1(±7.21)b	295.1(±13.3)c	330.0(±17.8)d	-
CV (%)	3.79	2.64	4.50	5.99	-
Sodium concentration					
Phase 1 (Day 0 - Day 20)					
Mean ± SD (mmol/L)	103.2(±18.0)a	103.4(±26.7)a	111.8(±27.6)a	157.1(±15.3)b	186.5(±26.1)c
CV (%)	17.41	25.79	24.66	9.74	13.98
1st bacterial challenge (Day 20 – Day 23)					
Mean ± SD (mmol/L)	106.2(±15.7)a	116.3(±21.3)b	117.2(±23.9)b	155.6(±14.5)c	-
CV (%)	14.79	18.31	20.37	9.32	-
Phase 2 (Day 20- Day 34)					
Mean ± SD (mmol/L)	100.7(±14.4)a	114.2(±19.8)b	120.4(±26.9)b	162.5(±14.1)c	-
CV (%)	3.46	3.04	7.36	12.46	-
2nd bacterial challenge (Day 34 – Day 37)					
Mean ± SD (mmol/L)	96.9(±10.3)a	117.4(±13.2)b	132.7 (±18.1)c	161.9(±14.3)d	-
CV (%)					-
Chloride concentration					
Phase 1 (Day 0 - Day 20)					
Day 0					
Mean ± SD (mmol/L)	101.80(±6.18)a	-	-	-	-
CV (%)	6.07	-	-	-	-
Day 20					
Mean ± SD (mmol/L)	100.3(±13.7)a	106.3(±8.6)b	125.4(±14.6)c	157.6 (±23.8)d	187.7(±26.9)e
CV (%)	17.41	25.79	24.66	9.74	13.98
1st bacterial challenge (Day 20 – Day 23)					
Mean ± SD (mmol/L)	95.7(±13.5)a	107.2(±7.0)b	122.2(±15.4)c	147.6 (±23.3)d	-
CV (%)	14.79	18.31	20.37	9.32	-
Phase 2 (Day 20- Day 34)					
Mean ± SD (mmol/L)	100.9(±10.6)a	106.9(±6.9)b	122.4(±12.4)c	146.4(±21.5)d	-
CV (%)	3.46	3.04	7.36	12.46	-
2nd bacterial challenge (Day 34 – Day 37)					
Mean ± SD (mmol/L)	96.9(±10.3)a	117.4(±13.2)b	132.7(±18.1)c	161.9(±14.3)d	-
CV (%)	10.64	11.23	13.64	8.86	-

Salinity conc. (ppt)	0	5	10	15	20
Glucose concentration					
Phase 1					
Day 0					
Mean ± SD (mg/dL)	70.28(±15.03)ab	-	-	-	-
CV (%)	21.38	-	-	-	-
Day 20					
Mean ± SD (mg/dL)	74.5 (±22.0)ab	74.2(±13.9)a	57.9 (±19.2)b	65.9 (±21.7)ab	31.2(±23.6)c
CV (%)	29.49	18.69	33.16	33.01	75.70
Phase 2 (Day 20- Day 34)					
Mean ± SD (mg/dL)	78.4(±19.5)a	68.7(±12.5)ab	61.2(±17.3)b	66.1(±17.8)ab	-
CV (%)	24.83	18.22	28.32	26.87	-
1st bacterial challenge	Day 20			Day 23	
Mean ± SD (mg/dL)	67.97(±20.0)a			84.24(±23.6)b	
CV (%)	29.42			28.02	
Total immunoglobulin concentration					
Phase 1 (Day 0 – Day 20)					
Mean ± SD (mg/mL)	12.3 (±6.2)a	16.7(±5.95)bc	20.1 (±4.15)b	13.3(±6.03)ac	7.9 (±2.88)a
CV (%)	49.09	35.67	20.62	45.44	36.46
Phase 2 (Day 20- Day 34)					
Mean ± SD (mg/mL	12.1 (±6.66)a	18.5(±7.18)b	17.2(±7.61)ab	14.9 (±5.63)ab	-
CV (%)	24.83	18.22	28.32	26.87	-
1st bacterial challenge					
Sane fish (Day 20)					
Mean ± SD (mg/dL)	12.33(±6.1)ab	16.95(±6.3)ac	19.67(±4.3)cd	13.27(±6.0)ab	-
CV (%)	49.09	36.86	21.81	45.44	-
Challenged fish (Day 23)					
Mean ± SD (mg/dL)	6.98(±5.42)be	15.66(±3.5)ac	11.03(±6.2)ae	16.43(±5.5)ad	-
CV (%)	77.67	22.06	56.06	33.69	-
Lysozyme concentration					
Phase 1 (Day 0 – Day 20)					
Mean ± SD (U/mL)	187.8(±51.98)ab	145.9 (±43.1)a	231.1(±67.1)b	204.0(±43.0)b	220.9(±47.5)b
CV (%)	27.68	29.56	29.02	21.06	21.48
Phase 2 (Day 20 – Day 34)					
Mean ± SD (U/mL)	185.9(±58.8)a,b	150.5(±70.8)a	213.9(±60.8)b	203.3(±79.2)b	-
CV (%)	31.61	47.06	28.43	38.98	-
2nd bacterial challenge	Day 34			Day 37	
Mean ± SD (U/mL)	189.4(±83.58)a			227.1(±45.49)b	
CV (%)	44.14			20.03	
1st bacterial challenge					
Sane fish (Day 20)					
Mean ± SD (U/mL)	187.8(±52.0)ab	139.4(±39.3)b	229.6(±64.5)ac	204.0(±43.0)a	-
CV (%)	27.68	28.22	28.08	21.06	-
Challenged fish (Day 23)					
Mean ± SD (U/mL)	176.5(±50.4)ab	199.3(±90.2)a bd	272.1(±60.6)cd	230.7(±73.4)ac	-
CV (%)	28.56	45.25	22.27	31.79	-
Peroxidase activity					
Phase 2	Day 20			Day 34	
Mean ± SD (mg/mL)	85.59 (±35.81)a			70.99 (±39.30)b	
CV (%)	41.84			55.36	

Salinity conc. (ppt)	0	5	10	15	20
1st bacterial challenge		Day 20		Day 23	
Mean ± SD (mg/mL)		85.59 (±35.81)a		58.94 (±19.22)b	
CV (%)		41.84		32.61	
2nd bacterial challenge		Day 34		Day 37	
Mean ± SD (mg/mL)		70.99(±39.30)a		40.38(±37.46)b	
CV (%)		55.36		92.75	
			Granulocyte proportion		
Phase 1 (Day 0 – Day 20)					
Mean ± SD (%)	4.30 (±2.74)ab	2.23 (±1.49)a	2.62 (±0.73)a	3.65 (±1.84)ab	8.44 (±5.23)b
CV (%)	63.76	67.09	27.75	50.49	61.96
Phase 2		Day 20		Day 34	
Mean ± SD (%)		3.15 (±1.92)a		4.45 (±2.00)b	
CV (%)		60.98		44.87	
1st bacterial challenge		Day 20		Day 23	
Mean ± SD (%)		3.15(±1.92)a		6.69%(±2.56)b	
CV (%)		60.98		38.24	
2nd bacterial challenge		Day 34		Day 37	
Mean ± SD (%)		4.45(±2.00)a		6.84(±3.73)b	
CV (%)		44.86		54.52	
			Monocyte proportion		
Phase 1 (Day 0 – Day 20)					
Mean ± SD (%)	8.95 (±5.26)a,b	3.47 (±1.73)c	3.60 (±2.85)c	4.54 (±3.43)ac	17.73(±11.5)b
CV (%)	58.83	49.79	79.22	77.89	64.88
Phase 2					
Day 20					
Mean ± SD (%)	8.95(±5.26)a	3.50(±1.81)b	3.57(±2.73)b	4.54(±3.53)ab	-
CV (%)	58.93	51.73	76.63	77.86	-
Day 34					
Mean ± SD (%)	17.54(±5.93)c	9.96(±5.75)a	6.42(±3.81)ab	10.60(±9.02)ac	-
CV (%)	33.81	57.73	59.38	85.12	-
1st bacterial challenge					
Sane fish (Day 20)					
Mean ± SD (%)	8.95(±5.26)a,b	3.50(±1.81)c	3.57(±2.73)c	4.54(±3.53)b,c	-
CV (%)	58.93	51.73	76.63	77.86	-
Challenged fish (Day 23)					
Mean ± SD (%)	28.24(±15.44)d	11.68(±7.60)a	9.84(±6.15)a	15.46(±5.59)a	-
CV (%)	54.68	65.10	62.48	36.16	-
2nd bacterial challenge					
Sane fish (Day 34)					
Mean ± SD (%)	17.54(±5.93)a	9.96(±5.75)b,c	6.42(±3.81)b	10.6(±9.02)abc	-
CV (%)	33.81	57.73	59.38	85.12	-
Challenged fish (Day 37)					
Mean ± SD (%)	14.62(±8.43)ab	19.34(±13.1)ac	17.57(±8.53)ac	22.99(±9.74)a	-
CV (%)	57.68	67.64	48.58	42.37	-
			Lymphocyte proportion		
Phase 1					
Day 0					
Mean ± SD (%)	87.48(±6.21)a	-	-	-	-
CV (%)	7.10	-	-	-	-
Day 20					
Mean ± SD (%)	86.75(±7.7)a	94.29(±2.3)c	93.96(±3.5)c	91.82 (±5.0)ac	75.56(±15.7)a
CV (%)	8.89	2.43	3.74	5.48	13.37

Salinity conc. (ppt)	0	5	10	15	20
Erythrocyte concentration					
Phase 1					
Day 0					
Mean ± SD (10^6C/μL)	2.711(±0.377)a	-	-	-	-
CV (%)	19.55	-	-	-	-
Day 20					
Mean ± SD (10^6C/μL)	2.591(±0.622)ab	2.980(±0.38)a	2.689(±0.500)a	2.046(±0.553)b	1.395(±0.43)c
CV (%)	24.01	13.46	18.09	27.02	30.97
Phase 2 (Day 20 – Day 34)					
Mean ±SD (10^6C/μL)	2.497(±0.586)ab	2.782(±0.436)a	2.477(±0.46)ab	2.113(±0.497)b	-
CV (%)	23.45	15.68	18.47	23.50	-
1st bacterial challenge					
Day 20					
Mean ± SD (10^6C/μL)	2.591(±0.622)ab	2.980(±0.382)a	2.689(±0.500)a	2.046(±0.553)b	-
CV (%)	24.01	13.46	18.09	27.02	-
Day 23					
Mean ± SD (10^6C/μL)	4.577(±0.679)c	3.274(±0.922)a	2.939 (±0.561)a	2.670 (±0.92)ab	-
CV (%)	23.20	15.12	13.18	20.20	-
2nd bacterial challenge	Day 34			Day 37	
Mean ± SD (10^6C/μL)	2.3544 (±0.4453)a			3.1178 (±0.6022)b	
CV (%)	18.92			19.31	
Hematocrit ratio					
Phase 1					
Day 0					
Mean ± SD (%)	48.09(±7.50)a	-	-	-	-
CV (%)	15.60	-	-	-	-
Day 20					
Mean ± SD (%)	40.99(±5.59)a	40.99(±1.70)ab	37.65(±5.56)b	36.28 (±9.35)b	33.04(±11.6)b
CV (%)	13.64	4.15	14.77	25.78	34.07
Phase 2	Day 20			Day 34	
Mean ± SD (%)	38.93 (±6.34)a			42.33 (±5.41)b	
CV (%)	16.28			12.78	
1st bacterial challenge (Day 20 – Day 23)					
Mean ± SD (%)	42.90 (±5.21)a	39.05(±5.38)ab	36.51(±5.14)ab	34.37(±8.91)b	-
CV (%)	12.15	13.77	14.07	23.92	-
2nd bacterial challenge (Day 34 – Day 37)					
Day 34					
Mean ± SD (%)	43.55 (±5.95)a	42.95 (±3.30)a	40.66 (±3.48)a	40.41 (±5.65)a	-
CV (%)	13.66	11.82	7.68	13.49	-
Day 37-					
Mean ± SD (%)	42.70 (±5.05)a	41.15 (±5.55)a	40.05 (±4.34)a	32.54 (±6.55)b	-
CV (%)	5.56	10.83	13.88	20.12	-
Hemoglobin concentration					
Phase 1					
Day 0					
Mean ± SD (g/dL)	10.35 (±0.63)a	-	-	-	-
CV (%)	6.13	-	-	-	-
Day 20					
Mean ± SD (g/dL)	8.24(±1.56)bc	9.29(±1.34)acd	10.65(±1.9)ade	11.45 (±2.15)ae	12.62(±2.7)ae
CV (%)	18.86	14.38	15.04	18.82	21.61

Salinity conc. (ppt)	0	5	10	15	20
Phase 2					
Day 20					
Mean ± SD (g/dL)	8.24 (±1.56)a	9.29 (±1.34)ab	10.65 (±1.92)b	11.45(±2.15)bc	-
CV (%)	18.86	14.38	15.04	18.82	-
Day 34					
Mean ± SD (g/dL)	11.54 (±2.34)bc	13.11 (±1.90)c	10.58 (±1.69)c	9.96 (±1.66)bc	-
CV (%)	20.30	14.53	15.96	16.65	-
1st bacterial challenge					
Day 20					
Mean ± SD (g/dL)	8.24 (±1.56)ac	9.29 (±1.34)ab	10.65 (±1.92)b	11.45 (±2.15)b	-
CV (%)	18.86	14.38	15.04	18.82	-
Day 23					
Mean ± SD (g/dL)	7.68 (±0.51)ad	6.96 (±1.30)cd	6.80 (±1.69)cd	10.28 (±2.34)d	-
CV (%)	6.68	18.64	24.77	22.72	-
2nd bacterial challenge (Day 34 – Day 37)					
Mean ± SD (g/dL)	11.60 (±1.81)ab	12.36 (±1.94)a	10.67 (±1.79)ab	9.73 (±2.49)b	-
CV (%)	15.62	15.71	16.78	25.60	-
Mean corpuscular volume					
Phase 1 (Day 0 – Day 20)					
Mean ± SD (fL)	92.01(±35.50)ab	69.89(±10.17)a	72.51(±14.8)a	91.99(±22.4)ab	134.1(±47.9)b
CV (%)	38.58	14.56	20.42	24.38	35.69
Phase 2					
Day 20					
Mean ± SD (fL)	75.95(±13.24)ab	69.89(±10.17)a	72.51(±14.8)ac	91.99(±22.4)bc	-
CV (%)	17.43	14.56	20.42	24.38	-
Day 34					
Mean ± SD (fL)	94.18(±20.68)bd	84.74(±13.6)abd	92.3(±16.64)bd	99.12(±17.64)d	-
CV (%)	21.96	16.09	18.03	17.80	-
1st bacterial challenge					
Day 20					
Mean ± SD (fL)	75.95(±13.24)ab	69.89(±10.17)a	72.51(±14.8)a	91.99(±22.4)b	-
CV (%)	17.43	14.56	20.42	24.38	-
Day 23					
Mean ± SD (fL)	50.19(±7.09)c	63.58(±12.61)ac	63.98(±17.2)ac	62.63(±7.86)ac	-
CV (%)	14.13	19.83	26.93	12.55	-
2nd bacterial challenge	Day 34			Day 37	
Mean ± SD (fL)	92.61(±17.58)a			63.75(±13.04)b	
CV (%)	18.98			20.46	
Mean corpuscular hemoglobin					
Phase 1					
Day 0					
Mean ± SD (pg)	19.37 (±2.51)a	-	-	-	-
CV (%)	12.95	-	-	-	-
Day 20					
Mean ± SD (pg)	16.68 (±4.59)ab	15.72 (±2.34)b	20.19(±3.94)ab	29.72 (±8.90)c	51.99(±6.56)d
CV (%)	27.50	14.91	19.53	29.94	41.85
Phase 2					
Day 20					
Mean ± SD (fL)	16.68 (±4.59)a	15.72 (±2.34)a	20.19(±3.94)ab	29.72 (±8.90)b	-
CV (%)	27.50	14.91	19.53	29.94	-

Salinity conc. (ppt)	0	5	10	15	20
Day 34					
Mean ± SD (fL)	25.14 (±7.59)b	25.79 (±5.09)b	24.27(±4.76)b	24.07 (±5.14)b	-
CV (%)	30.19	19.75	19.61	21.37	-
1st bacterial challenge					
Day 20					
Mean ± SD (fL)	16.68(±4.59)abc	15.72(±2.34)abc	20.19(±3.94)a	29.72 (±8.90)f	-
CV (%)	27.50	14.91	19.53	29.94	-
Day 23					
Mean ± SD (fL)	8.54 (±1.30)de	12.41(±4.47)bd	12.56(±2.84)ce	21.54 (±5.92)a	-
CV (%)	15.19	36.06	22.62	27.48	-
2nd bacterial challenge		Day 34		Day 37	
Mean ± SD (fL)		24.39 (±4.90)a		18.40 (±4.56) b	
CV (%)		20.10		24.77	
Mean corpuscular hemoglobin concentration					
Phase 1 (Day 0 – Day 20)					
Mean ± SD (pg)	20.58 (±5.05)a	22.32 (±3.52)a	29.28(±4.66)b	33.20 (±9.49)b	40.46(±13.4)b
CV (%)	24.52	15.78	15.91	28.57	33.23
Phase 2					
Day 20					
Mean ± SD (g/dL)	18.05(±3.90)a	22.71(±3.41)ab	28.41(±5.44)bc	31.39(±7.46)c	-
CV (%)	21.59	15.02	19.16	23.75	-
Day 34					
Mean ± SD (g/dL)	26.78(±5.55)bc	30.50(±3.47)c	26.15(±4.61)bc	23.97(±4.25)b	-
CV (%)	20.74	11.39	17.64	17.74	-
1st bacterial challenge					
Day 20					
Mean ± SD (g/dL)	18.05(±3.90)ac	22.71(±3.41)ab	28.41(±5.44)bd	31.39(±7.46)ce	-
CV (%)	21.59	15.02	19.16	23.75	-
Day 23					
Mean ± SD (g/dL)	17.10(±2.11)c	19.20(±4.14)ac	22.15(±5.22)ab	34.15(±7.60)de	-
CV (%)	12.32	21.55	23.55	22.26	-

Legend: Statistical letters = significant change ($p<0.05$) between the groups. CV= coefficient of variation.

Abstract of the master thesis

The impacts of the salinity on the physiology, the immunity and the hematology of the striped catfish (*Pangasianodon hypophthalmus*) affected by a rise of the salt concentration and a prolonged exposure on the response to the bacterial challenge with *Edwardsiella ictaluri*.

The future fish supply is threatened by several anthropogenic factors, deeply compromising the daily protein intake of an important part of the global population. In order to deal with this problematic, several countries developed their aquaculture sectors in order to meet the global fish demand and to become an essential trading partner.

Currently, Vietnamese aquaculture development in the Mekong Delta is one of the best success stories. Prime Minister plan entirely reviews the sector by 2020 and by 2030 in order to become more competitive for the aquaculture products exportations specially for the striped catfish *Pangasianodon hypophthalmus*. However, the dam constructions on the Mekong river and the sea level rise due to the climate change strongly compromise the objectives achievement by increasing the salinity in the area. These saline intrusions mainly occur during the dry season and disrupt the aquaculture sector. Even if it enables to tolerate up to 15 ppt of salinity, striped catfish is highly affected by the saline episodes and tends to be more sensitive to *Edwardsiella ictaluri* infection.

In order to investigate the effect of the salinity on the modulation of the physiology, the immunity and the hematology of this fish species, the experiment was conducted in two phases: a salinity rise which was followed by a two-week exposure at the reached salinity. At the end of each phase, a bacterial challenge was carried out in order to test the cumulative effect of stressors during saline stress.

Results of the physiological parameters show that the osmolality and the ion concentrations (Na^+ and Cl^-) immediately detected the saline stress and that it was not affected by the bacterial challenge. The rise of the osmotic pressure of the plasma induced first a disruption of the hematological parameters (drop of the erythrocyte concentration and hematocrit ratio, increase of the hemoglobin concentration, disturbance of the mean corpuscular volume and growth of the mean corpuscular hemoglobin concentration and the mean corpuscular hemoglobin). However, the long exposure makes the acclimation of these parameters possible by reducing the variability between the different saline treatments. The immune response to these cumulative stressors was firstly observed as a normal modulation during infection of the peroxidase activity and a decrease of its activity fourteen days after the salinity rise. The absence of modulation of several parameters between the salinity rise and the two-week exposure potentially indicates that the slow speed during the first phase was better adapted for the stress response, that acclimation under defined conditions might be possible and that the bacterial challenges were not conclusive.

Studying the influences of environmental conditions on the epigenetic mechanisms and on the stress resistance in earlier life stages of striped catfish might turn out to be interesting in the future, providing the selection program of the PANGAGEN project with additional support.