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Iron deposits turn blue shrimp gills to orange

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Abstract :

In the grow-out ponds of the blue shrimp (Litopenaeus stylirostris) farms of New Caledonia, animals with orange gills (OG) have been observed over the last ten years, with up to 70% of the shrimp in a given pond being affected. During the processing and marketing of the product, this coloring leads to reduced quality and selling prices, resulting in lower income for the producers concerned. Individual observations and transfer experiments led us to conclude that gill coloration intensity varies according to the intermolt stages, ranging from white in the postmolt stage to a deep orange in the premolt stage, which then disappeared after molting. Histological, biochemical studies and a semi-quantitative analysis by scanning electronic microscopy (SEM + EDX) showed that the coloration is due to layers of iron which settle on the gill tissue surface in a heterogeneous way. Because Cr and Co showed an increase in their concentrations on the whole orange gills but not at their surface, it is possible that elements were mobilized and transported (translocated) from the exoskeleton to the tissue. Animals kept out of contact with sediment show a decrease in OG intensity, suggesting a link with the sediment biogeochemistry. In grow-out ponds, orange gills first appeared in shrimp populations between 10 and 13 weeks after stocking and reached a maximum after 15 weeks. These findings are discussed with a view to identifying the environmental processes that lead to metal accumulation on gills.

Highlights

► Iron oxide accumulates on and colors in orange the gills of the shrimp in the grow-out aquaculture ponds. ► Gill coloration intensity increases during the intermolt period and disappears after molting. ► As well as iron, there was also accumulation of aluminum, cobalt and chrome. ► Orange gills appeared on cultured shrimp 10 to 13 weeks after stocking in ponds.

Keywords : Shrimp, Gills, Metal, Iron, Aluminum, Cobalt, Chrome, Molting cycle

1. Introduction

New Caledonia's lagoon is subject to a high degree of metal contamination pressure (Grenz et al., 2013). Mining activities are the island's primary economic resource and open-cast mining (as well as deforestation) increases the metal discharge into the lagoon waters, thereby contaminating the coastal environment. For 15 years, increasing attention has been given to assessing the impact of metals on marine organisms (e.g. Hédouin et al., 2009; Metian et al., 2008; Briand et al., 2014; Marchand et al., 2016). With regard to crustaceans, a first study aiming to better understand metal and metalloid bioaccumulation in the cultured blue shrimp *Litopenaeus stylirostris* was published 10 years ago (Metian et al., 2010). In parallel, another study investigated the risk for human consumption (Chouvelon et al., 2009). The blue shrimp, which originally came from Latin America and was introduced into New Caledonia in the 1980s, is produced in aquaculture earthen ponds located behind the mangrove forest. Since 2010, the shrimp industry has had to contend with a low-quality product due to the orange coloration on the gills of shrimp produced in a number of farms.

Most aquatic crustaceans primarily use gills for respiration. However, the gill is a multi-functional organ that is also responsible for ion transport, acid base balance and ammonia excretion (Henry et al., 2012). According to Freire et al. (2008), "Crustacean gills constitute the amplified surface of a differentially permeable interface employed for ion and gas exchange between the internal and external media". This exchange area is also subject to environmental contamination. In most crustaceans, the gill is the organ through which a variety of metals is taken up. Patterns of metallic elements occurrence have been reported for several species (Páez-Osuna and Ruiz-Fernandez, 1995; Páez-Osuna and Tron-Mayen, 1996; Pourang et al., 2004). For example, study of the impact of sublethal concentrations of copper and cadmium on the structure and ultrastructure of gills and epipodites shows a profound alteration of the gill structure (Soegianto et al., 1999a,b). Exposure to different levels of a mixture of heavy metals has a far more severe impact than expected on the basis of their individual effect (Frías-Espericueta et al., 2008). Most reports refer to the impact of total metal concentration, without taking the speciation into account, i.e., the ionic, colloidal, and particle forms, (Teien et al., 2008).

Orange gills coloration have already been observed and explained by reduced metal contamination in the hydrothermal deep shrimp *Rimicaris exoculata* (e.g. Zbinden et al., 2004). Lemonnier et al. (2021) recently described pond environmental conditions and ecological processes involved in the metal contamination in shrimp pond in New Caledonia. Results showed high concentrations of reduced iron in pore water at the interface water-sediment (up to 1000 µmol.l^{-1}) during OG periods, close to the values reported for hydrothermal environments (Zbinden et al., 2004). In this study, we aim to describe and explain shrimp gill coloration with regards to metallic deposits and molt cycle, combining field, experimental and laboratory approaches.

2. Materials and methods

2.1. Study site

The farm is located in Teremba bay (TE; 24°44'59.01"S; 165°41'48.93"E) on the west coast of the main island of New Caledonia. This farm is the biggest in New Caledonia, with a total surface of 133 ha, made up of fourteen earthen ponds of about 10 ha each, built behind the mangrove. Ponds were managed using standard techniques and stocked with the blue shrimp *Litopenaeus stylirostris* at densities ranging between 15 and 25 shrimp.m^{-2}. First harvests occurred after about 120 rearing days, when shrimp weight reached 20 g. Shrimps were fed daily with locally produced pellets, including 40% of crude proteins. Water flowing by gravity through all ponds was pumped from the adjacent bay. Daily water exchange may have varied between 5% and 40% of the total pond water volume. Ponds were aerated.

2.2. Sampling and laboratory study

2.2.1. Macroscopic observations

To analyze the relationship to the molt cycle, one hundred shrimps were harvested four times using a castnet at four stations in one pond in March and April 2014. Animals were put directly on ice until they were weighed, sexed, classified for their gill color and their molt stage determined. Six molt stages were defined according to the retraction of the epithelium within setae of the antennal scale

(Chan et al., 1988; Robertson et al., 1987). Shrimps were classified as A and B for the early and late postmolt stages respectively, C for intermolt and D0, D1, and D2 for premolt stages.

2.2.2. Light microscopy

The lateral borders of the cephalothorax of several specimens were rapidly dissected immediately after sampling. The complete gill tissues and the branchiostegites were removed for observation under the light microscope (Leica MZ8, x 50).

2.2.3. Histochemical analysis

Dissected gills were placed in Davidson's fixative for 24h then preserved in 70% alcohol until undergoing histochemical analysis by Perl's method for ferric iron (Perls, 1867). This method reveals the presence of ferric iron in the form of the hydroxide $Fe(OH)_3$. The ferric iron then reacts with a dilute potassium ferrocyanide solution to produce an insoluble blue compound (Prussian blue). Stained slides were examined by light microscopy on a DM3000 Leica (at a total magnification of 400x and 1000x using a 10x ocular). Analyses were conducted on at least ten animals with pronounced orange-colored gills and five animals with uncolored gills. Histochemical analysis were also conducted on the pleiopods for six animals with orange gills (OG). A grading score was arbitrarily defined, based on the surface of gills stained compared to control sections.

2.2.4. Chemical analysis

Twenty shrimps (20 g) without OG (white gills) and 20 with pronounced orange-colored gills were sampled in two ponds. Each animal was rinsed twice with seawater to remove sediment and other particles, weighed and then dissected to separate gills without epipodites from the cephalothorax. All gill samples were individually freeze-dried and weighed. Gills from five animals of each batch were pooled in order to obtain sufficient material for analysis (0.3 to 0.5 g d.w.) and were ground to a powder. Samples were then processed in accordance with the method described in Hédouin et al. (2009) and elements were analyzed using an ICP-OES 730ES VARIAN. The results were expressed in µg per g of dry matter ($\mu g.g^{-1}$ d.w.) for the following elements: Co, Cu, Cr, Fe, Mn, Ni, Pb and Zn.

Certified reference material DORM-3 NRCC (certified / observed) given in µg.g^{-1} dw were used for Cr (1.89 ± 0.17 / 1,87) ; Cu (15.5 ± 0.63 / 14.90) ; Fe (347 ± 20 /336) ; Ni (1.28 ± 0.24 / 1,57) ; Pb (0.395 ± 0.050 / 0.42) and Zn (51.3 ± 3.1 / 54.11).

2.2.5. Electron microscopy observations

Four animals with pronounced orange-colored gills and four with white gills were sampled in the same pond. Gills were dissected, fixed in 3% paraformaldehyde for 2 hours at ambient temperature and kept in PBS until further use. For each gill sample, a tissue section of 0.5 cm was collected and dehydrated in graded ethanol series (75° for 5 min, 95° for 5 min and 100° overnight). The gill pieces were then plunged for 1 min into two consecutive baths of héxamethyldislazane and settled on a cylinder stub with a carbon layer. Images were acquired with a FEI QUANTA 200F at a voltage of 10 KV and a pressure of 10 to 5 Torr (low-vacuum mode, 1300 to 660 Pa). The analyses were carried out in the microscopy laboratory of the University of Montpellier (France). Semi-quantitative micro-analyses were performed with Aztec 2.2SP1 software. The manufacturer provided performance tests and certification of the material once a year. Energy calibration of the EDS system was conducted using the manganese element. Each sample was analyzed on three different areas with five measurements at each location for the following elements: C, O, Na, S, Ca, K, Al, Cu, Fe, Si, Zn, Ni, Mo, Co, Cr, Mn, Ti. Results are expressed in percentage (± SD) of total weight from the 17 elements.

2.3. Animal transfer experiments

2.3.1. In tanks

One hundred and fifty shrimps (21.8 ± 3.0g) were caught in one pond using a castnet and transferred to the laboratory located 80 km from the farm. Animals were individually tagged by injection of colored elastomer (Godin et al., 1996), weighed and determined for their molt stages and gill colors. Animals were dispatched at a density of 15 individuals per tank in eight aerated tanks (300 L of filtered water at 10 µm) and maintained under the same conditions (salinity 35 PSU and 26°C) for 17 days, a period of time considered to be sufficient for the animals to complete a molting cycle (Robertson, 1987). The survey of the concentrations of dissolved iron in the water at the entrance of

the experimental unit showed concentrations below the limit of quantification of 1.21 nmol.l^{-1}. Three tanks were stocked with shrimp with white gills (T_{WG}), three with orange gills (T_{OG}) and two with a mix OG/WG (T_{mix}). During this experiment, water tanks were renewed daily in the morning by 50% with filtered seawater. Animals were fed with local pellets twice a day. Excess food and feces were removed and dead shrimps and molts were counted and removed twice daily. During the experiment, five shrimps per tank were randomly caught at days 4, 8, 11 and 14, examined to determine their molt stage and gill color color, and then put back in the tanks.

2.3.2. In pond cages

Animals (22 g) were caught in one pond characterized by a high proportion of shrimp with orange gills. They were individually tagged by injection of colored elastomer (Godin et al., 1996) according to their gill colors: white gills (WG), light orange gills (LOG), orange gills (OG) and dark orange gills (DOG). Then in April 2014 eighty shrimps, twenty per color category, were placed in two floating cages located in the same pond (1.3 m depth). The aim was to observe the change in gill coloration after two weeks of containment, as the animals were maintained in the same hydrological environment but without direct contact with sediments. Given that a molting cycle lasts about 11 days (Robertson et al., 1987), this duration is considered sufficient for the animals to complete a molting cycle. The cages' rectangular frames (2 m × 2 m) consisted of four polyvinyl chloride pipes (110 mm diameter) connected by PVC elbows and waterproofed with silicone adhesive. The plastic net (Netlon®) of 1 cm mesh size was 4.5 m long, 2.5 m wide and 0.5 m high (Chim et al., 2008). The cages were covered with nylon net. Each cage was anchored to the pond bottom using four vertical metal bars attached by ropes. There was no contact between the bottom of the cages and the pond sediment. A feeding tray was set up in each cage to feed the shrimp. Animals were fed with the same commercial pellets used in the pond. At the end of the experiment, the shrimps were harvested and weighed. The color of the tags and gills were recorded. In addition, fifty-four and fifty shrimps were harvested in the pond at the beginning and at the end of the experiment respectively, in order to determine the color of the gills, assuming that shrimp in the pond were in contact with the sediment.

2.4. *In situ* in pond

Surveys were conducted from September 2014 to June 2015 by the farm's technical staff. Shrimp sampling was carried out every week in four ponds. More than one hundred shrimps were caught quickly using a castnet in different locations in the pond (from 4 to 8), considered as representative of the pond by the farmers. Shrimps were pooled, weighed and examined to analyze the proportion of colored gills in the population.

3. Results

3.1. Observations and analysis of coloration

Macroscopic observation of the shrimps revealed obvious coloration differences between individuals harvested in the same pond. The color of the gills varied from white to dark orange. Four levels of colored gills were distinguished: white gills (WG), light orange gills (LOG), orange gills (OG) and dark orange gills (DOG). Animals in molt stages A, B, C had white gills in more than 90% of cases (Fig. 1). A slight orange coloration appeared at the molt stage D0 in 50% of the animals. This value and the intensity of staining increased until exuviation. All shrimp sampled at the molt stage D1/D2 had colored to strongly colored gills. Light microscopic observations showed that coloration was limited to the gills only (arthrobranchs and pleurobranchs) and was slightly stronger in the anterior part of the branchial cavity (Fig. 2b). The gill chamber did not show any coloration. An example of observations by microscopy after Perls Prussian blue staining on OG and WG are shown in Fig. 2c and 2d. Fine to moderate iron deposition in oxidized form on 80% of the filaments was observed in OG shrimps. In WG individuals, the mineral deposits were scarce or absent. Perl's method revealed also the presence of ferric iron in the form of the hydroxide $Fe(OH)_3$ on a major part of the cuticle surface of the pleiopods. An average of 60% of their surface area showed blue coloration (Appendix A).

Chemical analysis conducted on the whole gills showed that Fe concentration for four pools of five animals was significantly higher (five times higher) in the OG group than in the WG group (Table 1). Values in OG ranged between 1769 and 2415 $\mu g.g^{-1}$ d.w.. These analyses also showed significantly higher mean concentrations in OG compared to WG for cobalt and chrome. The copper level was

significantly lower in OG than in WG. Deposits analyzed by scanning electron microscopy appeared to be less abundant at the base of the gill lamella (Fig. 3).

X-ray micro-analysis and maps (Fig. 3 and 4) of the OG surface revealed the predominance of iron, but also the occurrence of aluminum and lead. The maps showed that the distribution of carbon was similar to that of iron, lead and zinc. The relative rates for most of these elements were similar in the two groups, except for Fe and Al, which were significantly higher (Fig. 5; ANOVA, $P < 0.05$). In OG samples, the Fe content varied from 1.7 to 6.9%, while in white gills it was between 0.1 and 0.3%. From the 17 elements analyzed, the most abundant were, as expected, C with 65.2% and 60.4% and O with 26.3% and 25.2% for the white gill animals and those with OG, respectively (Fig. 5). Among the different metals analyzed, Ni, Cu and Al represented 1.7%, 0.9% and 0.7% respectively of the element weights in the WG group, while in the OG group the relative weights for these elements were 1.5, 0.9% 1.4% and 2.2% respectively. For all other metals, relative weights were less than 1% in the two groups, except for Fe, which was 0.2% and 4.0% in the WG and OG groups respectively.

Histology revealed cuticular colonization by a low to medium bacterial matte on the gill surface (WG and OG not shown). Moderate to strong cuticular colonization by algae, protozoa (Epistylis sp.) and mucus was observed on strongly orange-colored gills. Histological analysis showed an absence of lesions due to the main diseases of shrimp (Taura syndrome virus (TSV), Yellow-head virus, Gill-associated virus (YHV/GAV), White spot syndrome baculovirus (WSBV)) and the absence of Vibriosis and IHHNV (Mermoud et al., 1998; Goarant et al., 2006).

3.2. Animal transfer experiments

3.2.1. In tanks

One week after transfer of shrimp from the pond to tanks containing filtered water, 67% of animals initially selected as OG still had colored gills. Orange gills completely disappeared after two weeks (Table S1). No staining occurred in tanks for the control group without OG. Some molts in tanks showed coloration. Mortality was less than 5% despite high manipulation stress induced by animal transfer from pond to tanks and regular sampling for molt determination. Among 120 shrimps, 68 molts were collected during the experiment, during which animal weight increased on average by 2.2

± 0.3 g. All these findings revealed that the coloration of the gills disappeared after molting in clear water.

3.2.2. In pond cages

The four batches of animals with a single one level of gill coloration at the beginning of the experiment showed different coloration levels after 15 days in cages (Table 2). The general trend revealed a decrease in the proportion of animals with colored and strongly colored gills in cages during the experiment. WG animals placed in the same cages showed coloring of their gills (OG + DOG) in 15% of the cases. DOG animals had gills that remained strongly colored (OG + DOG) or colored in 30% and 25% of cases for cages 1 and 2, respectively. A decrease in coloration intensity was observed in the shrimp population during this experiment. At the end of the experiment, 31% and 34% of animals showed WG or LOG respectively and only 6% had DOG. Mortality mostly affected OG animals irrespective of the cage. During the experiment, the proportions of WG, LOG, OG and DOG did not change in the pond shrimp population (Table 2).

3.3. Surveys of OG proportion in ponds

Figure 6 shows the temporal variations of OG in four ponds stocked at the beginning of the warm season (September 2014). The first OG appeared between 10 and 13 weeks after stocking and reached a maximum around 15 weeks of rearing. This period was followed by a decrease of OG percentage in all ponds until the end of the rearing.

4. Discussion

Gissi et al. (2016) recently reported a lack of high quality data on the effect of metals linked to a lateritic environment on tropical marine and estuarine species. This is particularly true for crustaceans, which can be considered as an ecologically important taxonomic group in tropical environments such as New Caledonia, where Ni, Mn, Co and Cr may show elevated concentrations in coastal waters. In the present study, orange coloration observed in shrimp in aquaculture ponds is due to the accumulation of metals, particularly as a form of iron oxide on the surface of the gills, as shown using

the Perls Prussian blue reaction. A large quantity of iron had already been reported in the gills of *Carcinus maenas* in natural conditions (up to 4000 µg.g^{-1} d.w.) (Martin, 1973). Iron contamination of gills also accounts for this color in *Rimicaris exoculata*, which dominates the megafauna of some of the Mid-Atlantic Ridge hydrothermal vent sites (e.g. Zbinden et al., 2004). In this species, iron oxyhydroxide accumulations in the branchial chamber were observed, covering the inner face of the cuticle and setae (Schmidt et al., 2009) and average concentrations can be as high as 900,460 µg.g^{-1} d.w. for this species (Kádár et al., 2006). Due to the commercial importance of farmed and wild shrimp, metal concentrations in muscle and even in the hepatopancreas and exoskeleton of these species in coastal environments have been extensively studied worldwide (e.g. Carbonell et al., 1998; Guhathakurta and Kaviraj, 2000; Tu et al., 2008). However, few studies have determined metal concentrations in gills from shrimp collected in aquaculture and coastal environments. Table 3 reports the limited data found in the literature. Concentrations recorded in shrimp from hydrothermal ecosystems were well above levels measured from coastal and aquaculture environments. Fe concentrations for shrimp without OG were in the range (80 to 852 µg.g^{-1} d.w) of the values reported in literature. As regards shrimp with OG, the value was similar to that reported for one of the most polluted harbors in the world (Lewtas et al., 2014).

Processes leading to iron accumulation at the surface of the gill chamber but also at the end of the pleiopods have not been described. For aquatic animals, the free metal ion is commonly considered as bio-available (toxic) (e.g. Vuori, 1995). Several studies show that iron speciation, which is sensitive to pH and redox conditions, strongly influences its deposit and accumulation on gills in fish (Teien et al., 2008) and shrimp in hydrothermal systems (Schmidt et al., 2009). This process has been widely described for aquatic animals (1) reared in groundwater, sometimes showing very high concentrations of Fe(II), and (2) living in the vicinity of hydrothermal vents. Iron oxidation of this reduced Fe in the microenvironment immediately adjacent to the gill surface leads to the accumulation of iron oxyhydroxide at the surface of the gills (Teien et al., 2008; Wepener et al., 2001; Schmidt et al., 2009). Moreover, chitin and chitosan are recognized as excellent metal ligands. Shrimp carapaces are even known to be used as an acid mine drainage remediation of effluents for metals including Mn^{2+} and Fe^{2+} (Gamage and Shahidi, 2007), due to their capacity as metal-sorbent biopolymer with a high chitin

content and the presence of calcium carbonate, an acid neutralizing agent (Keteles and Fleeger, 2001; Núñez-Gómeza et al., 2017; Rech et al., 2019). This metal-sorbent capacity could explain the iron accumulation on gills and pleiopods for shrimp reared in ponds.

In our study, elemental X-ray microanalyses and maps of mineral elements suggest an accumulation of aluminum at the surface of the gills, as already reported in fish by Exley et al. (1991). For fish species, gills are described as the principal target organ for this metal and its deposits contribute to osmoregulation and respiration dysfunctioning that can lead to the death of fishes (Poléo et al., 1994). The solubility and speciation of aqueous Al determine its bioavailability. Soluble Al binds to apically and specific groups in the gill lamellar epithelium (Exley et al., 1991). In coastal environments exposed to acid sulfate soils and following oxidation, Al is mobilized from soils, and its solubility increases with decreasing pH, leading to the release of ionic form. Under acidic conditions, Al exposure induces structural abnormalities in the gill for penaeid shrimp juveniles (Russell et al., 2019). Taking into account the above considerations, we hypothesize that acidic conditions in ponds (Lemonnier et al., 2021) could lead to the release of metal ions, particularly Fe and Al and their deposits on the surface of the gills. Because there is a need for contact between shrimp and sediment to develop OG, as shown by the transfer experiment conducted in the present study, acidification of this compartment during rearing could be at the root of this process.

Our study also shows an accumulation of Co and Cr on the whole gills but not at their surface. No data from the literature were found on Co concentration in shrimp gills. By way of comparison with our study, Cr concentrations was reported to be highest for the greentail prawn, *Metapenaeus bennettae*, harvested in Sydney harbor (Lewtas et al., 2014). They were highest in the gill tissue, followed by hepatopancreas, exoskeleton and tail muscle. Metian et al. (2010) reported mean concentrations of 0.27±0.02 and 0.9±0.6 $\mu g.g^{-1}$ d.w. for Co and Cr, respectively, from shrimp cephalothorax farmed in New Caledonia. These values are similar with those reported in white gills. The existence of different pathways is likely to explain differences observed between Fe and Co or Cr. Because Cr and Co did not show an increase in their concentration at the surface of the OG, it is possible that Co and Cr were mobilized and transported (translocated) from the exoskeleton to the tissue (Pourang and Amini, 2001). Laboratory experiments have shown that *L. stylirostris* readily takes up Co and Cr through

exposure to seawater (Metian et al., 2010). The bioaccumulation kinetics of these metals in the whole body was described by a first-order saturation model. This process could explain the increase of their concentrations in the whole organ, especially since Cr decontamination is a slow process (Metian et al., 2010).

Our study shows a significant drop in Cu concentration in gills as a function of gill color. Copper is an essential trace element for biological processes, particularly respiration. The decrease in copper concentration with intensifying OG could be linked to a respiratory dysfunction due to a barrier effect related to iron accumulation. Inhibition of oxygen consumption and decrease in the factor of oxygen have already been shown under sublethal and chronic exposure to metals (Grobler et al., 1989). Changes in circulating haemocyte numbers have been shown for the shrimp *Palaemon elegans* (Lorenzon et al., 2001). However, it was observed that Cu concentration changed in various tissues with the molting cycle (Keteles and Fleeger, 2001; Galindo et al., 2009). This factor should be taken into account in further physiological studies targeting the effect of iron on Cu accumulation in gills. However, a stress effect of metal accumulation on the animals cannot be ruled out at this stage in our work.

Iron accumulation reported by Martin (1973) in the gills of *Carcinus maenas* occurred during stage C3, forming a coating around the branchial lamellae, with a maximum from stage C4 to D3-D4. Similarly, coloration occurred at stage C in our study and increased progressively until stage D2, suggesting progressive accumulation of iron with time. Taking a duration of the molt cycle of around 11 days (Robertson et al., 1987) and using data from Table 1, iron accumulation could be above 150 µg of iron per day and per g d.w. of gills of shrimp in ponds developing orange gills. As observed experimentally, iron coating is rejected during ecdysis along with the integument. Iron accumulation was observed throughout the molting cycle in the hydrothermal vent shrimp *Rimicaris exoculata* (Schmidt et al., 2009). Close correspondence between the color and the molt stages was also shown for this species (Corbari et al., 2008). Molting should also be considered as a mechanism of metal depuration, as reported for Co and Cr in *Litopenaeus stylirostris* (Metian et al., 2010) and for several metals for different crustacean species (e.g. Keteles and Fleeger, 2001).

If we consider that a molting cycle lasts about 11 days (Robertson et al., 1987), the duration between the D0 stage and ecdysis is about 6-7 days. Assuming that all shrimps show coloration from the D0 stage onwards, about 60% of the animals in the same pond should show gill coloration at any one time. This value would explain the rate observed in ponds at the time of the coloration peak. The temporal variability of this percentage between two samplings could be explained by a change of the proportion of animals in the D0 stage – ecdysis over time. Molt synchrony has been already reported in a farmed population (Robertson et al., 1987). The emergence of OG after 11 to 13 weeks occurs following the autotrophy to heterotrophy switch of sediment biogeochemistry (Hochard et al., 2019), when food inputs are at their maximum. Acidification of the pond environment could occur during rearing linked to this change in environmental conditions, inducing the solubilization and accumulation of Fe, Co and Mn, as recently shown at the water-sediment interface in ponds with OG (Lemonnier et al., 2021). Partial harvest after 17 weeks induces a decrease of food inputs into the system and thus a reduction of its eutrophication level. This finding could explain the decrease of the proportion of OG in the population until the last harvest from the ponds.

5. Conclusion

The New Caledonian shrimp farming industry produces between 1500 and 2000 tonnes per year in 19 farms. But New Caledonia is still a small producer, contributing to less than 0.2% of the global harvest. As costs are higher than in other countries, the strategy of this sector is to produce with certification of food quality, consumer safety and environment preservation (Andrier, 2004). Gill coloration issue affects product quality in the processing plant, leading to lower shrimp prices for some farmers. Metal accumulation at the surface of the gills may modify mechanisms of transport, toxicity and bioaccumulation of metal in the shrimp tissues. The absorbed fraction is not considered in this study, given that molting may influence metal concentrations and the distribution between soft tissues and exoskeleton. Metal remaining on the cuticle after exuviation may be bioaccumulated because shrimps ingest their shed carapace after molting. Future studies should be conducted to identify the physiological consequence of iron accumulation at the surface of the gills and on metal bioaccumulation in various organs and, more generally, on the health status of the shrimp.

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Table 1: Metal concentrations in whole gills expressed in µg/g dry weight (n = 4). Chemical analyses were conducted from two pools of 5 gills sampled in two ponds. For each element, different letters indicate a significant difference (ANOVA, $p<0.05$)

	Pronounced Orange Gills	White Gills
	Mean	Mean
Fe	2035±282[a]	383 ± 127[b]
Cu	248±13[a]	319 ± 20 [b]
Zn	84.3±0.6	89.2 ± 1.8
Mn	20.0±4.5	15.7 ± 5.8
Pb	11.25±8.90	5.08 ± 0.47
Ni	1.14±0.23	0.84 ± 0.36
Cr	1.24±0.25[a]	0.71 ± 0.18[b]
Co	0.52±0.04[a]	0.22 ± 0.05[b]

Table 2: Proportion (%) of coloration for each initial group (n = 20 for each initial color) found after two weeks of the pond cage transfer experiment (WG: white gills; LOG: low orange gills; OG: orange gills; DOG: dark orange gills).

		End				
	Initial gills color	WG	LOG	OG	DOG	Dead shrimp (%)
Cage 1	WG	35	30	15	0	20
	LOG	40	40	0	10	10
	OG	15	20	15	0	50
	DOG	35	25	10	20	10
Cage 2	WG	35	35	10	5	15
	LOG	40	40	5	5	10
	OG	35	25	10	0	30
	DOG	10	55	15	10	10
	Mean proportion					
	In both cages	31	34	10	6	19
	In pond at the beginning	46	23	13	18	unknown
	In pond at the end of the experiment	42	26	10	22	unknown

Table 3: Comparative of mean shrimp gill metal concentrations (μg.g^{-1} d.w.) from aquaculture system and coastal environment.

Area	Country	species	Cu	Mn	Ni	Fe	Zn	Cr	Pb	Ref
farm	Mexico	*L.vannamei*	188 to 329	23.0 to 25.8	NA	852	85to 87	-	-	1
lagoon	Mexico	*L. vannamei*	212 to 281	21.5 to **30.6**	1. to 3.2	379	99 to 143	-	-	1
Gulf	Turkey	*P. semiculatus*	253 to 343	-	-	203 to 235	238 to 282	-	17.3 to 26.2	2
Gulf	Turkey	*M. monocerus*	197 to 252	-	-	274 to 332	165 to 212	-	69.9 to 101.2	2
Gulf	Turkey	*P. semiculatus*	141 to 423	-	-	259 to 552	126 to **953**	-	89.5 to **112.3**	3
farm	Australia	*P. monodon*	278	23.8	-	80	140	-	-	4*
Estuary	Australia	*P. merguiensis*	238 to 372	14.6 to 17.3	-	122 to 148	134 to 140	-	-	4*
Bay	Turkey	*P. semiculatus*	665	-	-	438	576	400	-	5
Harbor	Australia	*M. bennettae*	24 to **380**	8.4 to 16	-	175 to **2288**	100 to 106	2.1 to 5.8	4.2 to 15.2	6
Farm	New Caledonia	*L. stylirostris*	248 to 319	15.7 to 20.0	0.84 to 1.1	383 to 2035	83 to 84	0.71 to 1.24	5.1 to 11.3	7

(1) Páez-Osuna and L. Tron-Mayen (1996) ; (2) Kargin et al., 2001; (3) Aytekin et al., 2019; (4) Damano and Denton, 1990 ; (5) Firat et al., 2008; (6) Lewtas et al. (2014); (7) This study

* Assuming a ratio of 6/1 between wet and dry weight.

Data in bold show the highest values for each metal.

Table S1. Results from the pond-tank transfer experiment transfer. Number of shrimp per tank was 15; Molt line indicated the proportion of molt observed during the experiment.

Treatment	T_{WG1}	T_{WG2}	T_{WG3}	T_{OG1}	T_{OG2}	T_{OG3}	T_{mix1}	T_{mix2}
Ratio Male/Female	1.50	0.88	1.50	0.88	2.75	0.25	0.67	1.14
Initial Weight (g)	22.4	22.1	21.0	21.3	21.5	20.9	23.1	22.3
Final Weight (g)	24.1	23.9	23.6	23.7	23.8	23.4	25.4	24.3
Initial OG* (%)	100	100	100	0	0	0	47	53
Final OG (%)$^{+}$	0^{+}	0	0	0	0	0	0^{+}	0
Initial molt stage (%)								
B	7	0	0	0	0	0	0	0
C	40	60	40	0	0	0	13	40
C/D0	7	20	7	0	0	0	27	7
D0	33	20	33	7	0	0	13	0
D0/D1	13	0	0	0	13	7	14	6
D1	0	0	20	40	20	27	0	20
D1/D2	0	0	0	13	27	33	0	0
D2	0	0	0	40	40	33	33	27
Final molt stage (%)								
B	6	6	7	6	6	7	6	6
C	0	0	13	13	7	0	13	7
C/D0	20	0	0	0	7	0	0	40
D0	13	7	33	0	13	0	20	0
D0/D1	13	7	7	0	7	0	7	0
D1	7	46	20	27	0	0	7	7
D1/D2	7	0	7	7	20	0	7	0
D2	27	27	13	47	40	93	33	33
Mortality (%)	7	7	0	0	0	0	7	7
Molt (%)	67	53	73	67	53	40	40	60

***** OG and DOG.

+ Two animals (one per tank) showed a weak coloration classified as LOG.

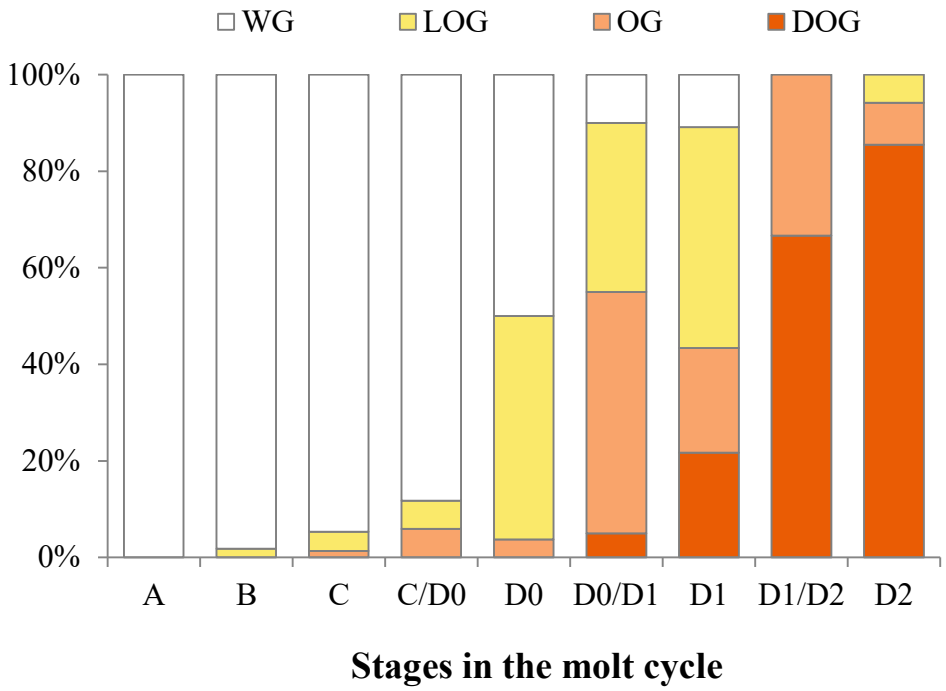


Figure 1. Evolution of the proportion of gill coloration types according to the molt cycle. WG: White gills; LOG: Light orange gills; OG: Orange gills; DOG: Dark orange gills (n = 399).

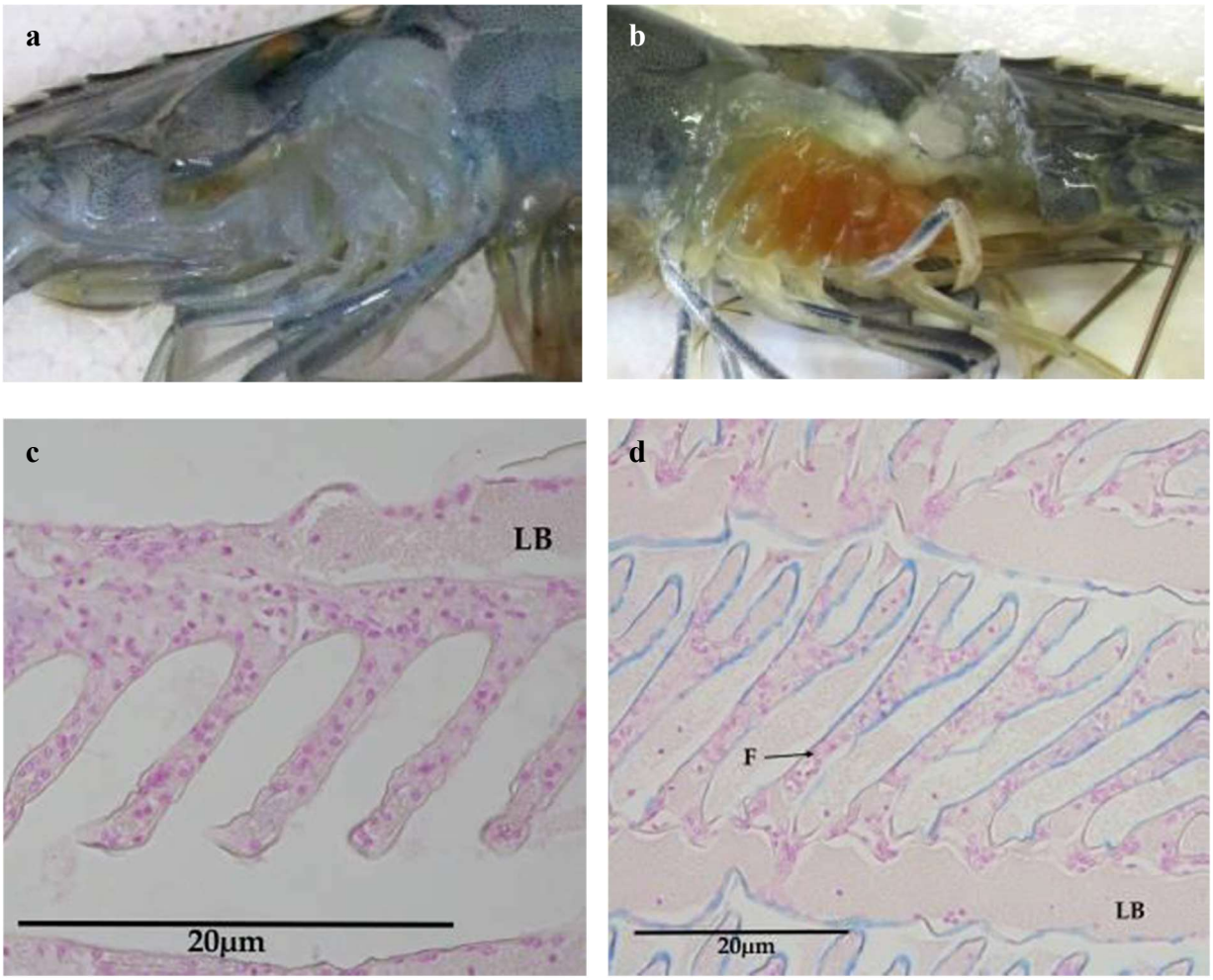


Figure 2: Macroscopic photographs of the cephalotorax of animals (a) without and (b) with dark orange gills (DOG). Histological analysis with Perls Prussian blue staining technique reveals the presence of iron on gill surfaces (blue color). (c) WG and (d) DOG are located on the left and right, respectively. LB: lamella, F: filament.

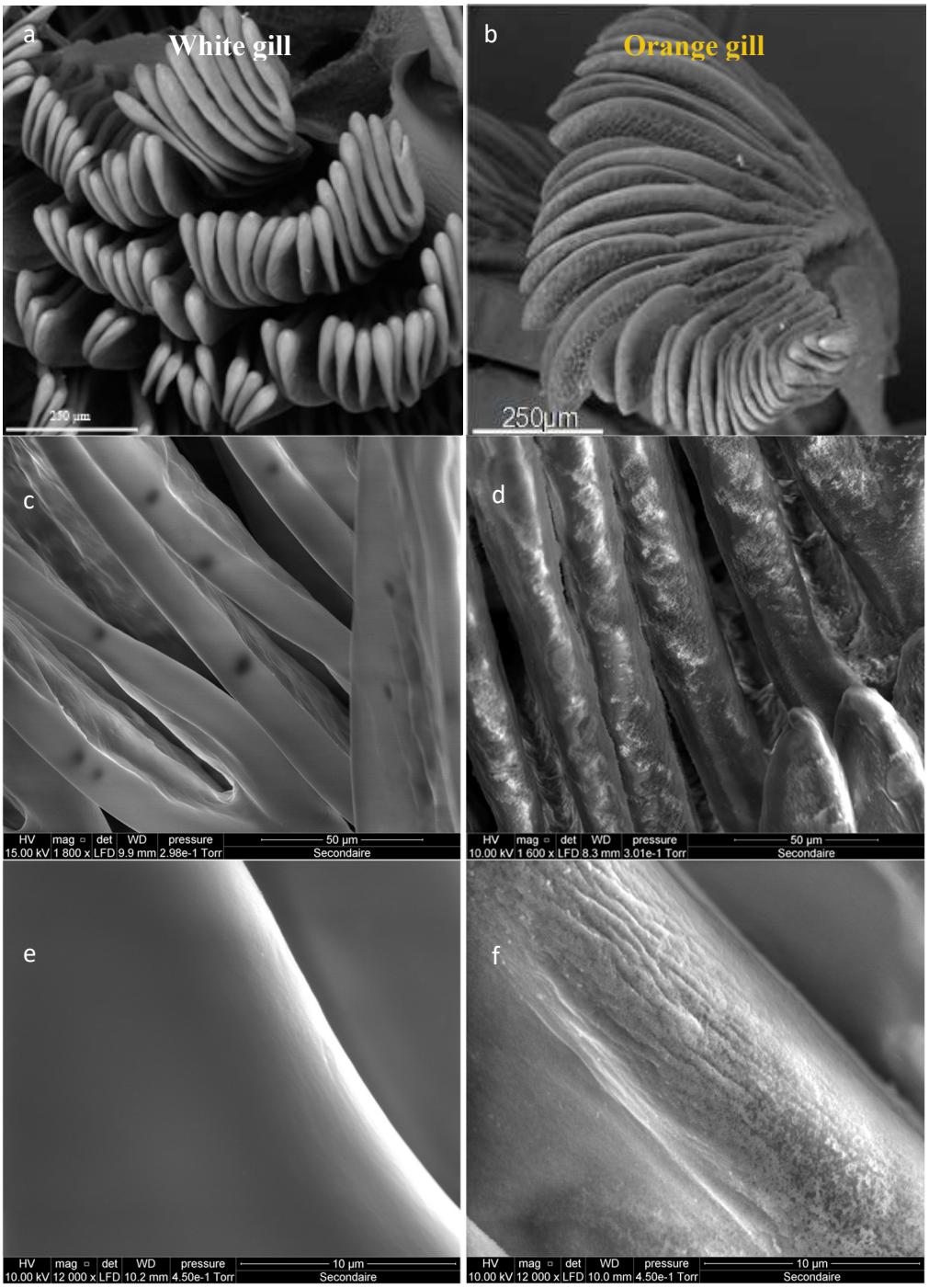


Figure 3: SEM view of gills at different scales. Images on the right (b, d, f) show mineral deposits on dark orange gills. a, c, e: controls (no color change); b, d, f: gills (orange color) with different magnifications of the lateral sides of gill lamellae.

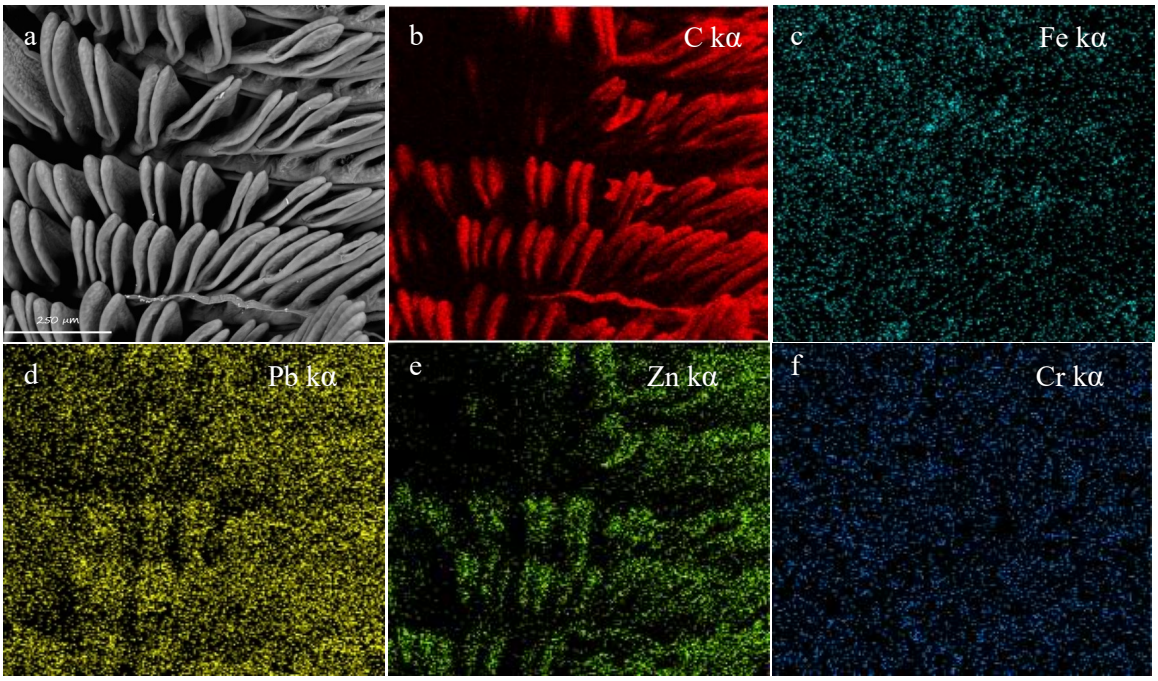


Figure 4: Element X-ray microanalyses and maps of carbon and mineral elements detected at the orange gill surface. (a) SEM image of an orange gill; element maps of carbon (b), iron (c), lead (d), zinc (e) and chrome (f).

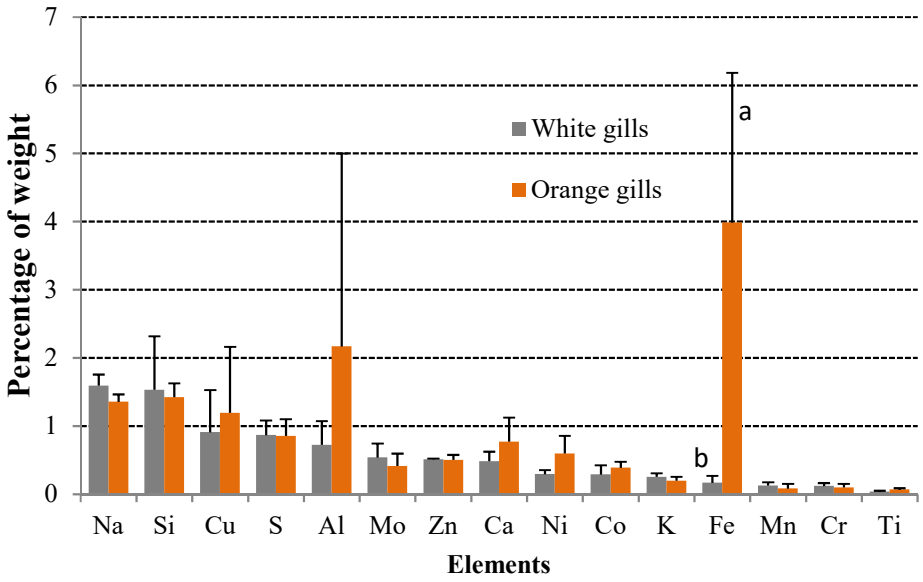


Figure 5: Relative weight (%) of each element analyzed by X-ray microanalysis. Bars (± SD) represent the average of three spectrums for 4 animals with DOG and 4 with WG. Values with different letters are statistically different (ANOVA, $p < 0.05$).

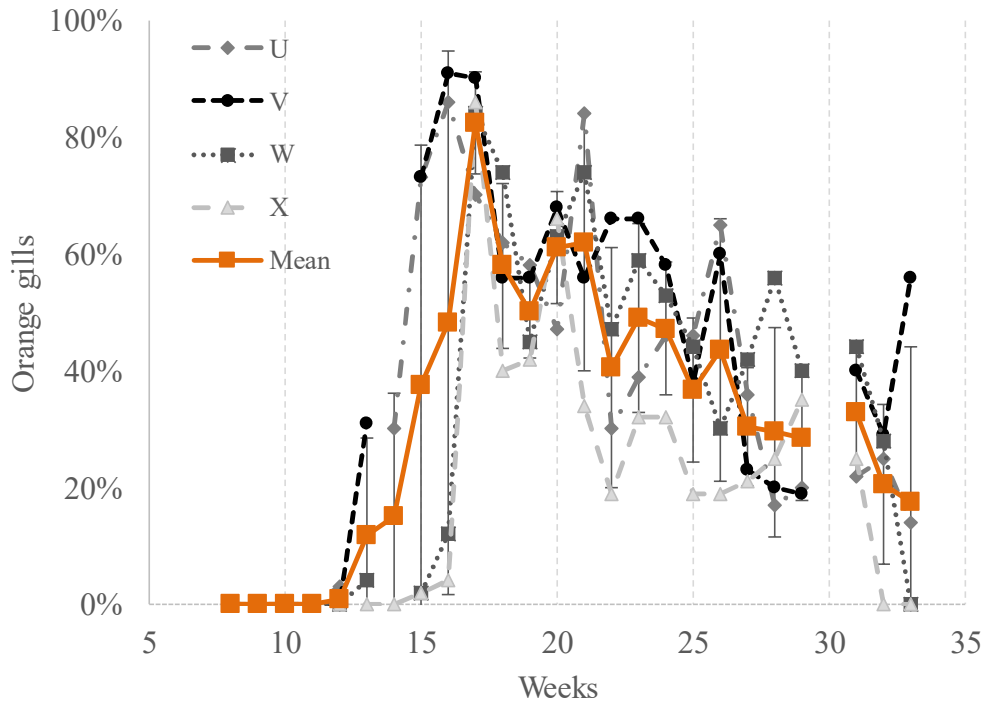
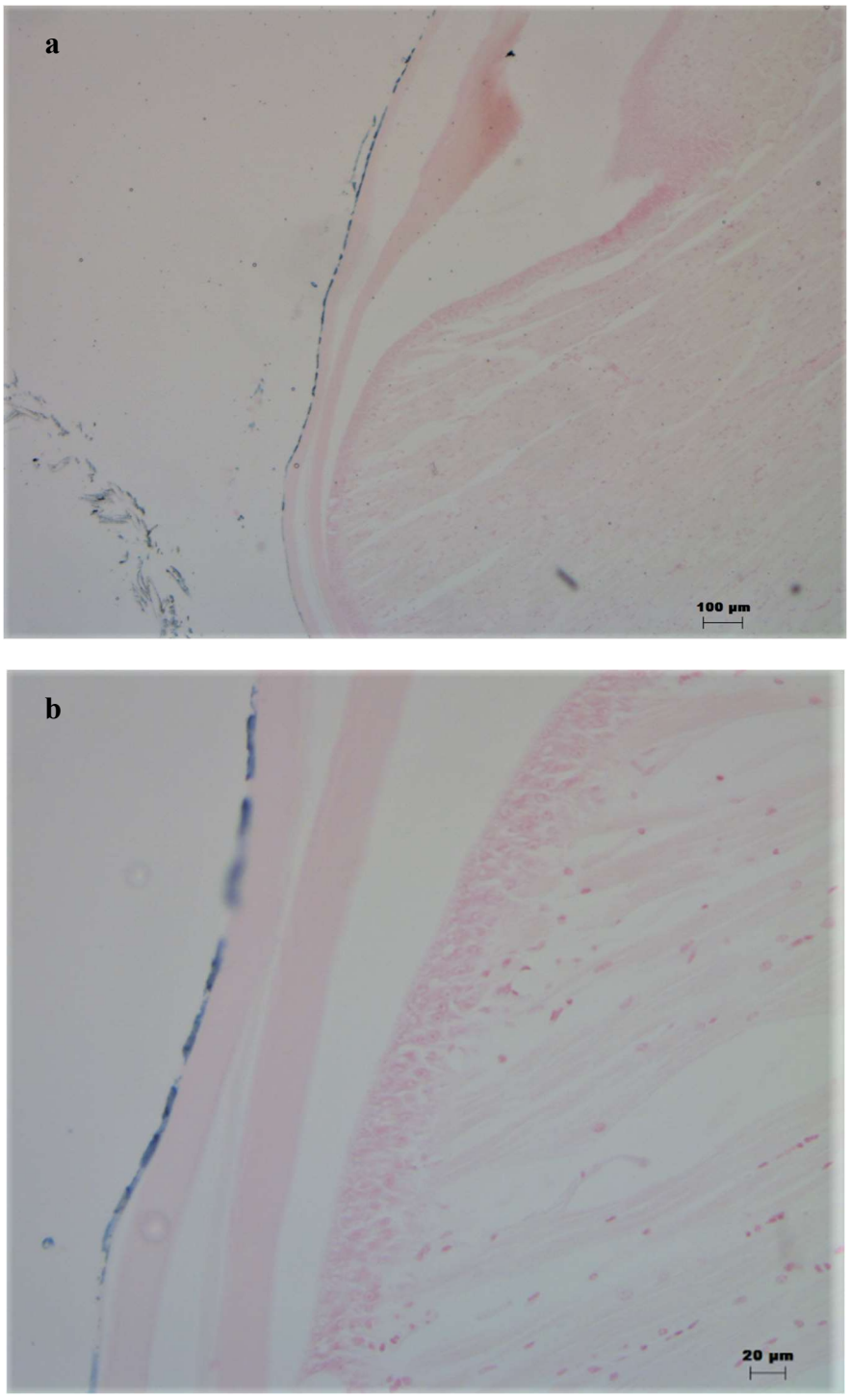


Figure 6: Temporal variations of mean orange gill percentages in the population of four ponds (U, V, W, X), initially stocked in September 2014 (data source: La Sodacal). Only two classes were discriminated by the farmer: with or without coloration.

Appendix A



Example of images of the same pleiopod for a shrimp in a pre-molt stage at two levels of magnification x100 (a) and x400 (b). This histological analysis with Perls Prussian blue staining technique reveals the presence of iron at the surface of the pleiopod (blue color). The cuticle is characterized by separation of the old exoskeleton from the underlying epidermal layer (double layer). From left to the right: outer layer with iron deposit - epidermis - striated skeletal muscle.