Aquaculture
Article in Press
Acceptation date : 2011-01

http://dx.doi.org/ 10.1016/j.aquaculture.2011.01.008

Calcein staining of calcified structures in pearl oyster Pinctada margaritifera and the effect of food resource level on shell growth

Clémentine Linard[a, c], Yannick Gueguen[b], Jacques Moriceau[a], Claude Soyez[a], Bélinda Hui[a], Aurore Raoux[a], Jean Pierre Cuif[d], Jean-Claude Cochard[a, e], Marcel Le Pennec[c] and Gilles Le Moullac[a,*]

[a] Ifremer, Laboratoire de Domestication de l'Huître Perlière, Centre Océanologique du Pacifique, Tahiti, BP 7004, 98719 Taravao, Tahiti, Polynésie française
[b] Ifremer, Laboratoire de Biotechnologie et Qualité de la Perle, Centre Océanologique du Pacifique, Tahiti, BP 7004, 98719 Taravao, Tahiti, Polynésie française
[c] Laboratoire Biodiversité Terrestre et Marine, UMR CNRS EA4239, Université de la Polynésie Française, BP 6570, 98702 Faa'a, Tahiti, Polynésie française
[d] Laboratoire Interactions et Dynamique des Environnements de Surface, UMR 8148-IDES, Université Paris XI, Orsay, France
[e] Ifremer, Département Environnement Littoral et Ressources Aquacoles, Brest, Technopole de Brest-Iroise, BP 70, 29280 Plouzané, France

*: Corresponding author. Tel.: +689 546006; fax: +689 546099.
20 E-mail address: Gilles.Le.Moullac@ifremer.fr

Abstract:

Marine mollusc shell growth has been widely measured using fluorochrome marking. In order to test the efficiency and reliability of calcein staining on Pinctada margaritifera shells and pearls, the present study examined two administration methods, different concentrations and several immersion times. Immersion in a 150 mg L−1 calcein solution for 12 h to 24 h appeared to be the best method for marking P. margaritifera shells. For pearl marking, injection of a 200 mg L−1 calcein solution into the pearl pouch was the optimal method. Calcein marking was then used to measure the influence of food resource levels on the shell growth. Groups of 23-month-old P. margaritifera were fed at three trophic levels for two months. The two highest food levels tested (6000 cell mL−1 and 15 000 cell mL−1) induced uniform growth between the dorsal and ventral sides of shell, whereas the lowest food level (800 cell mL−1) induced greater growth on the dorsal side. Shell deposits from the ventral side were observed using a scanning electron microscope, revealing that the difference of the trophic level over two months had modified the thickness of the aragonite tablets formed. These results showed that the trophic level is a major factor conditioning P. margaritifera development.

Keywords: Bivalvia; Pearl; Shell; Growth; Scanning electron microscope

ABSTRACT

Marine mollusc shell growth has been widely measured using fluorochrome marking. In order to test the efficiency and reliability of calcein staining on *P. margaritifera* shells and pearls, the present study examined two administration methods, different concentrations and several immersion times.Immersion in a 150 mg.L^{-1} calcein solution for 12 h to 24 h appeared to be the best method for marking *P. margaritifera* shells. For pearl marking, injection of a 200 mg.L^{-1} calcein solution into the pearl pouch was the optimal method. Calcein marking was then used to measure the influence of food resource levels on the shell growth. Groups of 23-month-old *P. margaritifera* were fed at three trophic levels for two months. The two highest food levels tested (6 000 cell.mL^{-1} and 15 000 cell.mL^{-1}) induced uniform growth between dorsal and ventral sides of shell, whereas the lowest food level (800 cell.mL^{-1}) induced greater growth on the dorsal side. Shell deposits from the ventral side were observed using a scanning electron microscope, revealing that the difference of trophic level over two months had modified the thickness of the aragonite tablets formed. These results showed that trophic level is a major factor conditioning *P. margaritifera* development.

Key words: *Bivalvia*, pearl, shell, growth, scanning electron microscope

1 INTRODUCTION

The black-lip oyster, *Pinctada margaritifera cumingi*, Linnaeus 1958 (Class: *Bivalvia*, Order: *Pterioida*, Family: *Pteriidae*), is used for the production of black pearls, and ranks among the most important commercial species in French Polynesia (Tisdell and Poirine 2000). Annual pearl production has increased from about 100 kg to more than 8 000 kg within the last 20 years, with an annual turnover of about US$ 170 million. This activity represents the second most important industry in French Polynesia after tourism. The main production sites are located in the Tuamotu-Gambier archipelago, where they represent the principal source of income for a quarter of local Polynesian families.

French Polynesia has the advantage of possessing natural stocks of these pearl oysters, which produce large numbers of larvae. Pearl oyster spat are commonly collected with lightweight spat collectors made from plastic mesh. Spat collectors are generally deployed for around 2 years and the pearl oysters are harvested when they attain a dorso-ventral shell height of approximately 50-65 mm (Cœroli *et al.* 1984, Pouvreau and Prasil 2001). *Pteriidae* shells grow by the deposition of layers of carbonate crystals and organic matrix. The shell is usually externally covered by a tanned protein sheet, *"the periostracum"*. Within the periostracum, a

layer made up of calcite prisms, "*the prismatic layer*", is commonly described. There is then an internal layer composed of parallel aragonite tablets, *"the nacreous layer"* (Wilbur and Saleuddin 1983). Growth is a variable that depends on both the genetic basis of traits under selection and the impact of the environment (Nasr 1984, Gervis and Sims 1992, Sims 1993, 1994). Genotype-by-environment interactions dictate the final phenotype, and evidence of such interactions has been recently been demonstrated for the *Pinctada* genus (Kvingedal *et al.* 2008, Gu *et al.* 2009). Age, nursery culture methods, predator composition and abundance, and degree of fouling are all factors that can affect shell growth (Gervis and Sims 1992, Taylor *et al.* 1997, Southgate and Beer 2000). The principal environmental factors influencing growth in most polar, temperate and tropical species are food availability, nutritional value of microalgae and temperature (Honkoop et Beukema 1997, Lodeiros *et al.* 1998, Laing 2000, Pouvreau *et al.* 2000a, 2000b, Yukihira *et al.* 1998b, 1999, 2000, Ahn *et al.* 2003). Despite the economical importance of this species, the impact of the environment on shell and pearl growth of *P. margaritifera* remains poorly understood.

In order to measure growth of calcified structures, several techniques have already been used. For example, shell growth has been assessed through shell length, shell height or shell width measurements (Pouvreau *et al.* 2000a, Hwang *et al.* 2007). However, external size quickly becomes a poor indicator of growth and age because growth rate varies among individuals and according to reproductive status (Pouvreau 1999). External measurements may therefore lead to biased growth estimations. Other studies have investigated shell perforation, sawing and emersion as methods to measure *P. margaritifera* shell growth during a known period (Caseiro 1993). However, these methods are likely to disrupt normal growth, as they cause physical damage (Caseiro 1993). Fluorochrome dyes have been used to validate growth checks (Day *et al.* 1995, Hales and Hurley 1991) and to investigate the layer formation pattern (Nakahara, 1961, Rowley and Mc Kinnon 1995, Schöne *et al.* 2003). These chemical stains bind to calcium and are incorporated into newly mineralizing shell, producing an internal growth mark that can thus subsequently be used for the analysis of environmental effects on short term growth rates (Nakahara, 1961, Schöne *et al.* 2003). Calcein is a fluorochrome that is easy to use and to detect and has been widely tested as growth marker for many marine animals, including bivalves, gastropods (Day *et al.* 1995, Rowley and McKinnon 1995, Kaehler and McQuaid 1999, Moran 2000, Eads and Layzer 2002, Moran 2005, Thébault *et al.* 2006, Riascos *et al.* 2007, Lucas *et al.* 2008).

In this study, a protocol using calcein fluorescent marking was developed to measure *P. margaritifera* shell and pearl growth. Calcein staining efficiency was assessed comparing

different administration methods, concentrations and immersion times, and the protocols were then used to investigate shell and pearl growth. Shell growth was described and measured on the ventral and dorsal sides and the influence of food abundance on the shell growth variability was examined and quantified. Furthermore, the influence of the food resources on the structure of the aragonite tablets, which compose the nacreous layer of the shell, was examined using scanning electron microscopy.

2 MATERIAL AND METHODS

2.1 Chemicals

Calcein was purchased from Sigma Aldrich, France. The stain powder was dissolved over 12 h at 24 °C in filtered (0.1 µm) seawater using a magnetic stirrer. The following concentrations were prepared: 50, 100, 150 and 200 $mg.L^{-1}$. Calcein administration techniques, concentrations and immersion periods were tested (Table 1).

2.2 Calcein shell marking

The efficacy of calcein for marking shells was tested on 10-month-old pearl oysters (N=84, shell length average: 35 ± 0.02SD mm) produced in the Ifremer hatchery in Vairao (17°48'S, 149°17'O) Tahiti, Society archipelago, French Polynesia. Shell marking was performed either by injection or immersion. For the injections, two concentrations of calcein (50 and 100 $mg.L^{-1}$) were tested on *P. margaritifera* pearl oysters (N=3/condition). Calcein was introduced using a sterile syringe inserted through the byssus hole into the palleal cavity until the cavity was filled with marker solution. Care was taken to insert the needle without injuring the pearl oyster. Animals were placed between wet towels for 30-40 min before returning them to 25-L tanks in an open seawater circulation system (120 $L.h^{-1}$ water turnover). Seawater temperature was measured and seen to remain around 28 ± 0.3SD °C. For the immersion protocol, three calcein concentrations (50, 100 and 150 $mg.L^{-1}$) were tested in 3-L aquaria. For each concentration, samples of three pearl oysters were taken after 6 h, 12 h and 24 h immersion time. A control batch was also kept under the same conditions but without calcein treatment, to evaluate toxicity of the stain. Calcein immersions were realized in aquaria equipped with an aerated sea water system and protected from light. During immersion, no water changes were made. After immersion and injection trials, the *P. margaritifera* pearl oysters were reared in 25-L tanks. Seawater (27 °C) was renewed 4 times per hour. A diet of 10 000 algal cell mL^{-1}, composed of a 2:1 mix of *Isochrysis galbana* (T-Iso) and *Chaetoceros gracilis* cells

was supplied continuously. Marking success was evaluated two months later. To investigate ventral marking, shells were sawn according to the third section shown in Fig. 1.

2.3 Dynamics of shell growth

To explore shell growth dynamics, fifteen 40-month-old *P. margaritifera* pearl oysters (mean shell length: 122 ± 13.8SD mm) shipped from the Takapoto atoll (14°41'S, 145°14'O, Tuamotu archipelago, French Polynesia) were used. Two marking of pearl oysters were carried out at an interval of two months. The first and the second marking were done in similar conditions by immersion in a 150 $mg.L^{-1}$ calcein solution in 3-L aquaria during 24h. Between the two marking, the pearl oysters were reared in lagoon. To explore shell growth with respect to anterior, ventral, posterior, dorsal and central parts of the shell, samples were sawn eight times, from the umbo to the growing edge, to obtain nine parallel shell pieces (Fig. 1). Anterior and posterior pieces were then sawn three more times, perpendicular to the section axis. Growth of the prismatic and nacreous shell layers was studied by examining 12 prismatic points and 35 nacreous points on each shell (Fig. 1).

2.4 Influence of trophic level on shell growth

In this experiment, 23-month-old pearl oysters (N=60, mean shell length: 85 ± 5.7SD mm) were firstly put into three 500-L seawater tanks (N=20/tank) equipped with an open sea water circulation system (water renewal of 96 $L.h^{-1}$) and acclimatized over two weeks before the staining was performed and the diet treatments started. The *P. margaritifera* pearl oysters were then submitted to a 150 $mg.L^{-1}$ calcein immersion for 24 h. Three level were tested : 800 $cell.mL^{-1}$, 6 000 $cell.mL^{-1}$ or 15 000 $cell.mL^{-1}$ composed of a microalgae mix (*Isochrysis aff. galbana*, and *Chaetoceros aff. gracilis*, v:v) continuously supplied for two months. The mean temperature of seawater during this experiment was 29.4 ± 0.2 SD °C Mortalities were recorded during the experiment. Ten specimens per treatment were collected after the first month of experimentation and ten more, one month later. To investigate ventral and dorsal growth, shells were sawn at the third section shown in Fig. 1.

2.5 Calcein pearl marking

To mark pearls, two grafted adult populations aged 40 months (mean shell length: 122 ± 13.8SD mm) shipped from the Takapoto atoll were used. The first population (N=27) was grafted four months before the staining experiment and the second one (N=22), twenty-four months before the staining experiment. Both injection and immersion methods were tested.

For the injection method, 1 mL of the calcein solution was introduced into the pearl pouch using a sterile syringe. Two concentrations of calcein were tested: 150 and 200 $mg.L^{-1}$. The immersion staining protocol was the same as that described above for shell marking and the same calcein concentrations were tested as with the injection method (150 and 200 $mg.L^{-1}$). Double marking was carried out at a two-month interval for both groups of grafted specimens. Consequently, at the end of experimentation, pearls were aged six months in the first batch and twenty-six months in the second batch. The grafted pearl oysters were reared in 25-L tanks in same conditions described in section 2.2. Marking efficiency and pearl growth were evaluated two months later. Pearls were incorporated into a wax block before being sawn transversally.

2.6 Calcein detection

The shells and the pearls were sawn using a “Swap Top Trim Saw” machine (Inland, Middlesex, United Kingdom), which includes a diamond Trim Saw Blade (Thin Cut) IC-40961. Shell and pearl edges were then polished for 5 s with various grades of water sandpaper sheet. The shell and pearl sections were then examined under a Leitz Dialux 22 compound fluorescence microscope equipped with a I3 filter block and an optical micrometer. To determine calcein marking success on shell and pearls, calcein was recorded in the prismatic and/or nacreous layers using a binary system (1: mark; 0: no mark). Then, to measure shell and pearl growth, increments deposited between the two calcein marks were measured using an optical micrometer (Fig. 2).

2.7 Shell deposit structure

The structure of the shell deposit was observed by scanning electron microscopy (SEM). Pearl oyster shells were sawn at the third section shown in Fig. 1. The ventral side of the shells was isolated, embedded in resin and polished using various grades of diamond paste, down to a final 0.25 µm grade. To reveal microstructural features, polished shell sections were etched for 40 s with a formic acid solution (1 %) mixed with glutaraldehyde (3 %). SEM observations were conducted using a Philips XL30 instrument housed at the Laboratoire Interactions et Dynamique des Environnements de Surface, Orsay, France.

2.8 Statistical analysis

In order to evaluate marking efficiency according to the different administration methods (injection *vs*. immersion), calcein concentration and immersion times, Khi^2 tests or

Fisher tests were realized using XLSTAT software 2007.7 (Bouyer 1996, Scherrer 1984). Nacreous deposits were square root transformed to normalize data distribution and a one-way ANOVA was performed to compare nacreous deposition between the dorsal and ventral side of the shell. Effect of trophic level on shell growth on the ventral and dorsal sides was analysed using a two way ANOVA following by a PLSD Fisher test using Statview version 5.0 software. Square root transformed pearl deposit on 4 and 24 month-old pearls was compared using the Student t test.

3 RESULTS

3.1 Optimization of shell and pearl marking

For both shell and pearl marking experiments, no mortality caused by calcein staining was recorded on the *P. margaritifera* pearl oysters. Shell marking frequency was significantly different according to the administration method. Using the immersion method, 85 % of the shells were marked, whereas only 35 % of the shells were marked after calcein injection into the palleal cavity (Khi^2=17.211; df=1; p<0.0001) (Fig. 3a). Calcein concentration also significantly influenced the shell marking frequency (Fisher test; p=0.012). After immersion in a 150 $mg.L^{-1}$ calcein solution, 100 % of *P. margaritifera* shells were marked. After immersion in 50 and 100 $mg.L^{-1}$, calcein solutions, only 65 % and 89 % of shells were marked, respectively (Fig. 3b). An effect of immersion time was also observed. Shell marking frequencies were significantly higher for 12 h and 24 h than for 6 h immersion (Fisher test; p=0.023) (Fig. 3c).

For pearl marking, the injection of calcein into the pearl pouch showed double the efficiency of the immersion method (88 % *vs.* 43 %) (Khi^2=16.67; df=1; p<0.0001) (Fig. 4a). Furthermore, the concentration of calcein significantly influenced the pearl marking frequency (Khi^2=5.10; df=1; p=0.024), using a 200 $mg.L^{-1}$ calcein concentration delivered by injection, 100 % of the pearls were marked whereas only 75 % of pearls were marked at 150 $mg.L^{-1}$ (Fig. 4b). In addition, pearl age significantly influenced calcein incorporation: 97 % of the 4 month-old pearls were marked, while only 36 % of 24 month-old pearls were marked (Khi^2=6.41; df=1; p=0.011), for all treatments combined.

3.2 Dynamics of shell growth

Shell growth was measured using calcein fluorochrome marks on 15 shells of *P. margaritifera*, each sawn into eight sections (Fig. 1). The thickness measurements of the

prismatic and nacreous deposits at the anterior, ventral and posterior area of the shells were not significantly different (F=1.07; df=1; p=0.3031). After two months of growth, shell deposit rate at the nacreous layer of the dorsal side measured 5.12 ± 4.36SD µm.day^{-1}, whereas shell deposit rate at the nacreous layer for the anterior, ventral and posterior sides were significantly lower (3 ± 2.13SD µm.day^{-1}, 2.88 ± 2.53SD µm.day^{-1}, 2.76 ± 1.92SD µm.day^{-1}, respectively) (Fig. 5). The difference observed for the dorsal part compared to the anterior, ventral and posterior area is significant (F=2.78; df=3; p=0.0458). No calcein mark was detected in the central area of the shell. Hence, shell growth could only be observed at the perimeter of the valves.

3.3 Shell growth is correlated with trophic level

To evaluate the influence of food resources on *P. margaritifera* shell growth, 3 microalgal diets were tested over two months. Shell deposition on the ventral and dorsal sides was significantly higher for the 15 000 cell.mL^{-1} diet than for the 6 000 cell.mL^{-1} one, and significantly higher for the 6 000 cell.mL^{-1} diet than for the 800 cell.mL^{-1} one (Fig. 6) (F=22.14; df=2; p<0.0001).

Differences between ventral and dorsal growth depended on diet (F=10.50; df=1; p<0.0017). For the 800 cell.mL^{-1} diet, dorsal nacreous increment was significantly higher than ventral nacreous increment (p=0.0001) (Fig. 6). The dorsal growth rate was about 6.3 ± 3.0SD µm.day^{-1} whereas ventral growth rate was about 2.6 ± 1.3SD µm.day^{-1}. In contrast, no significant differences between ventral and dorsal sides were detected at 6000 cell.mL^{-1} (p=0.3072) or at 15 000 cell.mL^{-1} (p=0.2042).

3.4 Influence of trophic level on aragonite tablet thickness

The present study demonstrated that shell increment on the ventral side is greater for higher food level than for lower food level. Following the lowest and highest diets (800 cell.mL^{-1} and 15 000 cell.mL^{-1}), shell deposit structure on the ventral side of *P. margaritifera* pearl oyster was observed by SEM. Observations showed that the food level seemed to act on the aragonite tablet thickness of the shell. Observations showed that during the two months of the experiment, the thickness of the aragonite tablets of the shell reduced with time whatever the food level considered. Aragonite tablets deposited just after calcein staining looked thicker than the later aragonite tablets deposited at the end of the experiment (Fig. 7 a, b, c, d).

When a section of identical thickness was compared between shells of pearl oysters from the different diet treatments at the start and end of the experimental period, two

differences were observed in the aragonite tablets. In the initial stage of the experiment, the number of aragonite tablets composing a 10 µm section was lower in oysters from the higher food treatment (N=13) than in those from the lower food treatment (N=17) (Fig. 7 b, c). Indeed, aragonite tablets were thicker in the higher food treatment. At the end of the experiment, the opposite trend was recorded: the number of aragonite tablets in a 10 µm section was higher for the higher food level (N=20 for the lower food level, N=21 for the higher food level) (Fig. 7 a, d). At the end of the experimental period, therefore, aragonite tablets of the shell in oysters fed at the lower food level were thicker than those in shells of oysters fed at the higher food level.

3.5 Pearl growth analyses

Pearl growth rate was evaluated at two points after the graft. Pearl growth rate was significantly higher for 4 month-old pearls than for 24-month-old pearls ($p=0.0002$) (Fig. 8). Pearl growth rates were 1.44 ± 0.04SD µm.day^{-1} for the 4-month-old pearls and $9.21.10^{-2} \pm 0.01$SD µm.day^{-1} for 24-month-old pearls.

4 DISCUSSION

During recent decades, fluorochromes have attracted attention as they can serve to create a fluorescent line in calcified structures. In this study, calcein marking efficiency was investigated on *P. margaritifera* to measure shell and pearl growth and to evaluate the influence of food levels on the growth of these structures. In our study, no mortality due to calcein administration was recorded. Compared with other species, *P. margaritifera*, therefore, presented a good resistance to the calcein marking process (Day *et al.* 1995, Bumguardner and King 1996, Gelsleichter *et al.* 1997, Eads and Layzer 2002). Several administration methods, concentrations and immersion times were tested. The most efficient method for marking shells, was shown to be immersion of pearl oysters in 150 mg.L^{-1} calcein solution for 12 to 24 h. Marking success increased with concentration and immersion time. Similar results have been obtained for other taxa: polychaete worms (*Serpula columbiana*), gastropods (*Concholepas concholepas, Nucella ostrina*), bivalves (*Comptopallium radula, Mesodesma donacium*) and fishes (Wilson *et al.* 1987, Brooks *et al.* 1994, Day *et al.* 1995, Rowley and Mc Kinnon 1995, Kaehler and Mc Quaid 1999, Moran 2000, Eads and Layzer 2002, Iyengar 2002, Thébault *et al.* 2006, Riascos *et al.* 2007). To mark pearls, injection into the pearl pouch clearly gave better results than immersion. Twice as many pearls were marked with the injection technique compared with the immersion method. Our results showed that

all pearls were marked with injected calcein at 200 mg L^{-1} concentration.. Calcein marks have been observed in shells as well as pearls up to one year after marking, demonstrating that marks made in this way have a long-term life span. Riascos *et al.* (2007) noted a seven-month life span in *C. concholepas* and *M. donacium*.

Using calcein marking, we followed the growth rate of three parts of the *P. margaritifera* shell, the peripheral part, the dorsal part and the central part, which were shown to differ. Disruptive shell growth has been evoked for many species (Day *et al.* 1995, Rowley and Mc Kinnon 1995, Kaehler and Mc Quaid 1999). The peripheral part of the shell is composed of anterior, ventral and posterior areas and has active bio-mineralization. Present results showed an active deposition rate for nacreous and prismatic layers, with similar deposition rate in the two. Secondly, the dorsal side of the shell was distinguished by a nacreous deposit rate 1.8 times higher than on the ventral side over the two-month study period. Lastly, the central part of the shell is defined by a fluorochrome-free area. To explain this fluorochrome-free area, two hypotheses can be proposed. Either, 24 h is not sufficient for calcein incorporation into the central area of the shell, or the measurement technique is not adapted to detecting the growth pattern of this area.

Physiological processes are affected by endogenous and exogenous factors. Exogenous factors include physical (temperature), chemical (oxygen, salinity) and biological (food availability) parameters, of which food resources are considered to be one of the most important (Nasr 1984, Sims 1993, Honkoop et Beukema 1997, Yukihira *et al.* 1998b, Laing 2000). Using calcein marking, we measured the impact of trophic level on ventral and dorsal shell growth in *P. margaritifera*. After the two-month experiment, the lowest food level clearly favored growth of the dorsal side of the shell over that of the ventral side. However, when micro-algal concentration was higher, shell growth increased and was equal between dorsal and ventral parts. Consequently, the highest shell deposits on ventral and dorsal sides were recorded at the highest food level. The positive influence of food resources on shell growth has also been demonstrated for mussel *C. meridionalis* and *M. edulis* (Griffiths 1980b, Bayne and Worrall 1980). Winter (1978) evocated an optimum food concentration beyond which growth rate declined. In our experiment, food optimum was not reached since no growth rate decline was observed when microalgae concentration rose. These results are in line with Yukihira *et al.* (1998b), who detected an optimal food concentration between 10 000 cell.mL^{-1} and 20 000 cell.mL^{-1} for *P. margaritifera*. Even though this bivalve is adapted to the oligotrophic lagoon waters of French Polynesian, our results show that *P. margaritifera* can also adapt itself to higher trophic levels.

SEM observations of *P. margaritifera* shells differed according to trophic level simultaneously revealing a temporal evolution of mineralization rate and the influence of trophic level on aragonite tablet thickness. Shells of pearl oysters fed the highest food level had thicker aragonite tablets than those of pearl oysters fed the lowest food level. Consequently, there seems to be a higher shell deposition rate in pearl oysters fed at the highest food level due to a combination of thicker aragonite tablets and an increasing shell deposit rate.

Plasticity and modification in growth expression due to genotype by environment interactions have already been demonstrated for the *Pinctada* genus (Kvingedal *et al.* 2008, Gu *et al.* 2009). Among endogenous parameters known to affect growth, age is presumably the most important. In the present study conducted in Vairao, Tahiti, the pearl growth rate for the 4-month-old pearls over a two month period was 1.44 ± 0.04 µm.day^{-1}, and the pearl growth rate for 24 month-old pearls was $9.21.10^{-2} \pm 0.01$ µm.day^{-1}. The pearl growth rate decreased by about 15 times between 4-month-old pearls and 24-month-old pearls. The influence of pearl age on pearl growth has been already studied in *P. margaritifera* in Takapoto until the 13th month after the grafting process, revealing a growth rate of 3.60 ± 1.60 µm.day^{-1} (Caseiro 1993). Takapoto and Vairao differ in temperature and chlorophyll-a concentration. Temperature in Takapoto ranks between 26.5 °C and 30.5 °C while temperature in Vairao ranks between 25 °C and 29 °C. Buestel and Pouvreau (2000) found a chlorophyll-a concentration of about 0.28 µg.L^{-1} at depths between 8 and 10 m, whereas chlorophyll-a concentration measured in Vairao lagoon in 2000 at a depth of 7 m was about 0.184 µg.L^{-1} (Pers. Comm. Le Moullac). These indications, coupled with the age of the pearls, may contribute to explaining pearl growth differences between those two sites.

Description of the impact of the environment on *P. margaritifera* shell growth is a valuable area of research because of the production challenge. French Polynesia is the top exporter of cultured *P. margaritifera* black pearls in the world. The country is composed of more 115 islands with different environmental conditions. Maximizing growth rate of pearl oysters will reduce the time needed to rear them to operable size for the pearl grafting operation and, therefore, increase farm profitability. Temperature is another environmental factor of prime importance for growth in pearl oysters (Pandya 1976, Honkoop and Beukema 1997, Laing 2000, Yukihira *et al.* 2000, Tomaru *et al.* 2002, Saucedo *et al.* 2004, Gu *et al.* 2009), so we will examine its effects in our future research.

5 Acknowledgements

This study is part of a collaborative project (GDR ADEQUA) supported by the "Service de la perliculture" of French Polynesia. It is also supported by Ifremer and University of French Polynesia. Authors are grateful to Marcel Le Pennec for helpful discussions and assistance. Thanks also to Helen McCombie-Boudry for improving the English in this paper.

6 References

Ahn, I.Y., Surh, J., Park, Y.G., Kwon, H., Choi, K.S., Kang, S.H., Choi, H.J., Kim, K.W., Chung, H., 2003. Growth and seasonal energetics of the Antarctic bivalve *Laternula elliptica* from King George Island, Antarctica. Mar. Ecol. Prog. Ser. 257, 99-110.

Bayne, B.L., Worrall, C.M., 1980. Growth and production of Mussels *Mytilus edulis* from two populations. Mar. Ecol. Prog. Ser. 3, 317-328.

Bouyer, J., 1996. Méthodes statistiques-Médecine-Biologie, ed. INSERM, France.

Brooks, R.C., Heidinger, R.C., Kohler, C.C., 1994. Mass-Marking Otoliths of Larval and Juvenile Walleyes by Immersion in Oxytetracycline, Calcein, or Calcein Blue. N. Am. J. Fish. Manage. 14, 143-150.

Buestel, D., Pouvreau, S., 2000. Particulate matter in Takapoto lagoon waters: potential food for cultivated pearl oysters. Oceanol. Acta. 23, 193-210.

Bumguardner, B.W., King, T.L., 1996. Toxicity of oxytetracycline and calcein to juvenile striped bass. T. Am. Fish. Soc. 125, 143-145.

Caseiro, J., 1993. La Nacre noire de Polynésie: Bio-minéralisation, paramètres de croissance, effets chromatiques dans la coquille et la perle de *Pinctada margaritifera*. Thèse, Université Claude Bernard-Lyon I. 386pp.

Cœroli, M., De Gaillande, D., Landret, J.P., AQUACOP (Coatanea, D.), 1984. Recent innovations in cultivation of molluscs in French Polynesia. Aquaculture. 39, 45-67.

Day, R.W., Williams, M.C., Hawkes, G.P., 1995. A comparison of fluorochromes for marking abalone shells. Mar. Freshwater Res. 46, 599-605.

Eads, C.B., Layzer, J.B., 2002. How to pick your mussels out of a crowd: using fluorescence to mark 10 month-old oyster freshwater mussels. J. N. Am. Benthol. Soc. 21, 476-486.

Gelsleichter, J., Cortes, E., Manire, C.A., Hueter, R.E., Musick, J.A., 1997. Use of calcein as a fluorescent marker for elasmobranch vertebral cartilage. T. Am. Fish. Soc. 126, 862-865.

Gervis, M.H., Sims, N.A., 1992. The biology and culture of pearl oysters (*Bivalvia: Pteriidae*). ICLARM, Metro Manila, Philipines/ODA, London, England.

373 Griffiths, R.J., 1980b. Natural Food Availability and Assimilation in the Bivalve
374 *Choromytilus meridionalis*. Mar. Ecol. Prog. Ser. 3, 151-156.
375 Gu, Z.F., Wang, Q.Y., Fang, J., Ye, N.H., Mao, Y.Z., Shi, Y.H., Wang, Y., Wang, A.M.,
376 2009. Growth of Cultured Pearl Oyster (*Pinctada martensii*) in Li'an Lagoon, Hainan
377 Island, China. J. Shellfish Res. 28, 465-470.
378 Hales, L.S., Hurley, D.H., 1991. Validation of daily increment formation in the otolithsof
379 juvenile silver perch, *Bairdiella chrysoura*. Estuaries. 14, 199-206.
380 Honkoop, P.J.C., Beukema, J.J., 1997. Loss of body mass in winter in three intertidal bivalve
381 species: an experimental and observational study of the interacting effects between water
382 temperature, feeding time and feeding behaviour. J. Exp. Mar. Biol. Ecol. 212, 277-297.
383 Hwang, J.J., Yamakawa, T., Aoki, I., 2007. Growth of wild pearl oysters *Pinctada fucata*,
384 *Pinctada margaritifera* and *Pinctada sugillata* (Bivalvia: Pteriidae) in Taiwan. Fish. Sci.
385 73, 132-141.
386 Iyengar, E.V., 2002. Sneaky snails and wasted worms: kleptoparasitism by *Trichotropis*
387 *cancellata* (Mollusca, Gastropoda) on *Serpula columbiana* (Annelida, Polychaeta). Mar.
388 Ecol. Prog. Ser. 244, 153-162.
389 Kaehler, S., McQuaid, C.D., 1999. Use of the fluorochrome calcein as an in situ growth
390 marker in the brown mussel *Perna Perna*. Mar. Biol. 133, 455-460.
391 Kvingedal, R., Evans, B.S., Taylor, J.J.U., Knauer, J., Jerry, D.R., 2008. Family by
392 environment interactions in shell size of 43-day old silver-lip pearl oyster (*Pinctada*
393 *maxima*), five families reared under different nursery conditions. Aquaculture. 279, 23-28.
394 Laing, I., 2000. Effect of temperature and ration on growth and condition of king scallop
395 (*Pecten maximus*) spat. Aquaculture. 183, 325-334.
396 Lodeiros, C.J., Rengel, J.J., Freites, L., Morales, F., Himmelman, J.H., 1998. Growth and
397 survival of the tropical scallop *Lyropecten (Nodipecten) nodosus* maintained in suspended
398 culture at three depths. Aquaculture. 165, 41-50.
399 Lucas, T., Palmer, P.J., Wang, S., Scoones, R., O'Brien, E., 2008. Marking the shell of the
400 saucer scallop *Amusium balloti* for sea ranching using oxytetracycline, calcein and alizarin
401 red S. J. Shellfish Res. 27, 1183-1188.
402 Moran, A.L., 2000. Calcein as a marker in experimental studies newly-hatched gastropods.
403 Mar. Biol. 137, 893-898.
404 Moran, A.L., 2005. A simple technique for physical marking of larvae of marine bivalves. J.
405 Shellfish Res. 24, 567-571.

406 Nakahara, H., 1961. Determination of growth rates of nacreous layer by the administration of
407 tetracycline. Bull. natl. Pearl Res. Lab. 6: 607-614.
408 Nasr, D.H., 1984. Feeding and growth of the pearl oyster *Pinctada margaritifera*_L. in
409 Dongonab Bay, Red Sea. Hydrobiologia. 110, 241-245.
410 Pandya, J.A., 1976. Influence of temperature on growth ring formation in the pearl oyster,
411 *Pinctada fucata* (Gould) of the Gulf of Kutch. Indian J. Mar. Sci. 5, 249-251.
412 Pirker, J.G,. Schiel, D.R., 1993. Tetracycline as a fluorescent shell-marker in the abalone
413 *Haliotis iris*. Mar. Biol. 116, 81-86.
414 Pouvreau, S., 1999. Etude des mécanismes expliquant la croissance de l'huître perlière
415 *Pinctada margaritifera* au sein de l'écosystème conchylicole du lagon de l'atoll de
416 Takapoto (Polynésie française). Thèse, Ecole Nationale Supérieure d'Agronomie de
417 Rennes. 267pp.
418 Pouvreau, S., Tiapari, J., Gangnery, A., Lagarde, F., Garnier, M., Teissier, H., Haumani, G.,
419 Buestel, D., Bodoy, A., 2000a. Growth of the black-lip oyster, *Pinctada margaritifera*, in
420 suspended culture under hydrobiological conditions of Takapoto lagoon (French
421 Polynesia). Aquaculture. 184, 133-154.
422 Pouvreau S., Gangnery, A., Tiapari, J., Lagarde, F., Garnier, M., Bodoy, A., 2000b.
423 Gametogenic cycle and reproductive effort of the tropical blacklip pearl oyster, *Pinctada*
424 *margaritifera* (Bivalvia: Pteriidae), cultivated in Takapoto atoll (French Polynesia). Aquat.
425 Living Resour. 13, 37-48.
426 Pouvreau, S., Prasil, V., 2001. Growth of the black-lip oyster, *Pinctada margaritifera*, at nine
427 culture sites of French Polynesia: synthesis of several sampling designs conducted between
428 1994 and 1999. Aquat. Living Resour. 14, 155-163.
429 Riascos, J., Guzman, N., Laudien, J., Heilmayer, O., Oliva, M., 2007. Suitability of three
430 stains to mark shells of *Concholepas concholepas* (Gastropoda) and *Mesodesma donacium*
431 (Bivalvia). J. Shellfish Res. 26, 43-49.
432 Rowley, R.J., McKinnon, D.I., 1995. Use of the fluorescent marker calcein in bio-
433 mineralisation studies of brachiopods and other marine organisms. Bulletin de l'institut
434 océanographique, Monaco. 14.
435 Saucedo, P.E., Ocampo, L., Monteforte, M., Bervera, H., 2004. Effect of temperature on
436 oxygen consumption and ammonia excretion in the Calafia mother-of-pearl-oyster
437 *Pinctada mazatlanica* (Hanley, 1856). Aquaculture. 229, 377-387.
438 Scherrer, B., 1984. Biostatistique, ed. Gaëtan Morin, Montréal-Paris.

Schöne, B.R., Tanabe, K., Dettman, D.L., Sato, S., 2003. Environmental controls on shell growth rates and delta O^{-18} of the shallow-marine bivalve mollusk *Phacosoma japonicum* in Japan. Mar. Biol. 142, 473-485.

Sims, N.A., 1993. Size, age and growth of the black-lip pearl oyster, *Pinctada margaritifera* (L.) (Bivalvia ; Pteriidae). J. Shellfish Res.12, 223-228.

Sims, N.A., 1994. Growth of wild and cultured black-lip pearl oysters, *Pinctada margaritifera* (L.) (Pteriidae, Bivalvia), in the Cook-Islands. Aquaculture. 122, 181-191.

Southgate, P.C., Beer, A.C., 2000. Growth of blacklip pearl oyster (*Pinctada margaritifera*) juveniles using different nursery culture techniques. Aquaculture. 187, 97-104.

Taylor, J.J., Southgate, P.C., Rose, R.A., 1997b. Fouling animals and their effect on the growth of silver-lip pearl oysters, *Pinctada maxima* (Jameson) in suspended culture. Aquaculture. 153, 31-40.

Thébault, J., Chauvaud, L., Clavier, J., Fichez, R., Morize, E., 2006. Evidence of a 2-day periodicity of striae formation in the tropical scallop *Comptopallium radula* using calcein marking. Mar. Biol. 149, 257-267.

Tisdell, C.A., Poirine, B., 2000. Socio-economics of pearl culture: Industry changes and comparisons focusing on Australia and French Polynesia. World Aquaculture. 11, 30-37, 58-61.

Tomaru, Y., Kumatabara, Y., Kawabata, Z., Nakano, S., 2002. Effect of water temperature and chlorophyll abundance on shell growth of the Japanese pearl oyster, *Pinctada fucata martensii*, in suspended culture at different depths and sites. Aquac. Res. 33, 109-116.

Yukihira, H., Klumpp, D.W., Lucas, J.S., 1998a. Effects of body size on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima*. Mar. Ecol. Prog. Ser. 170, 119-130.

Yukihira, H., Klumpp, D.W., Lucas, J.S., 1998b. Comparative effects of microalgal species and food concentration on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima* (Bivalvia: Pteriidae). Mar. Ecol. Prog. Ser. 171, 71-84.

Yukihira, H., Klumpp, D.W., Lucas, J.S., 1999. Feeding adaptations of the pearl oysters *Pinctada margaritifera* and *P. maxima*. Mar. Ecol. Prog. Ser. 182, 161-173.

Yukihira, H., Lucas, J.S., Klumpp, D.W., 2000. Comparative effects of temperature on suspension feeding and energy budgets of the pearl oysters *Pinctada margaritifera* and *P. maxima*. Mar. Ecol. Prog. Ser. 195, 179-188.

Wilbur, K.M., Saleuddin, A.S.M., 1983. The Mollusca. (4): Physiology Part 1.

Wilson, C.A., Beckam, D.W., Dean, J.M., 1987. Calcein as a fluorescent marker of otoliths of larval and juvenile fish. T. Am. Fish. Soc. 116, 668-670.

Winter, J.E., 1978. Review on knowledge of suspension-feeding in Lamellibranchiate Bivalves, with special reference to artificial aquaculture systems. Aquaculture. 13, 1-33.

Table 1: Summary of administration techniques, concentrations and immersion times tested for the calcein fluorochrome on *Pinctada margaritifera.*

Experiment	Administration technique	Concentration ($mg.L^{-1}$)	Immersion times (hours)
Shell marking	Immersion	50	6, 12, 24
		100	6, 12, 24
		150	6, 12, 24
	Injection into the palleal cavity	50	/
		100	/
Pearl marking	Immersion	150	24, 43
		200	24, 43
	Injection into the pearl pouch	150	/
		200	/

List of Figures

Figure 7: *P. margaritifera* pearl oyster shell from a low trophic level treatment (800 cell.mL^{-1}) (a, b) and a high trophic level treatment (15 000 cell.mL^{-1}) (c, d) at an interval of two months. b, c: Aragonite tablets deposited immediately after staining. a, d: Aragonite tablets deposited two months later.

Figure 8: Comparison of the pearl deposit rate (µm.day^{-1}) for 4-month-old and 24-month-old pearls. 4-month-old pearls: N=5; 24-month-old pearls: N=4. Bars indicate standard deviation.

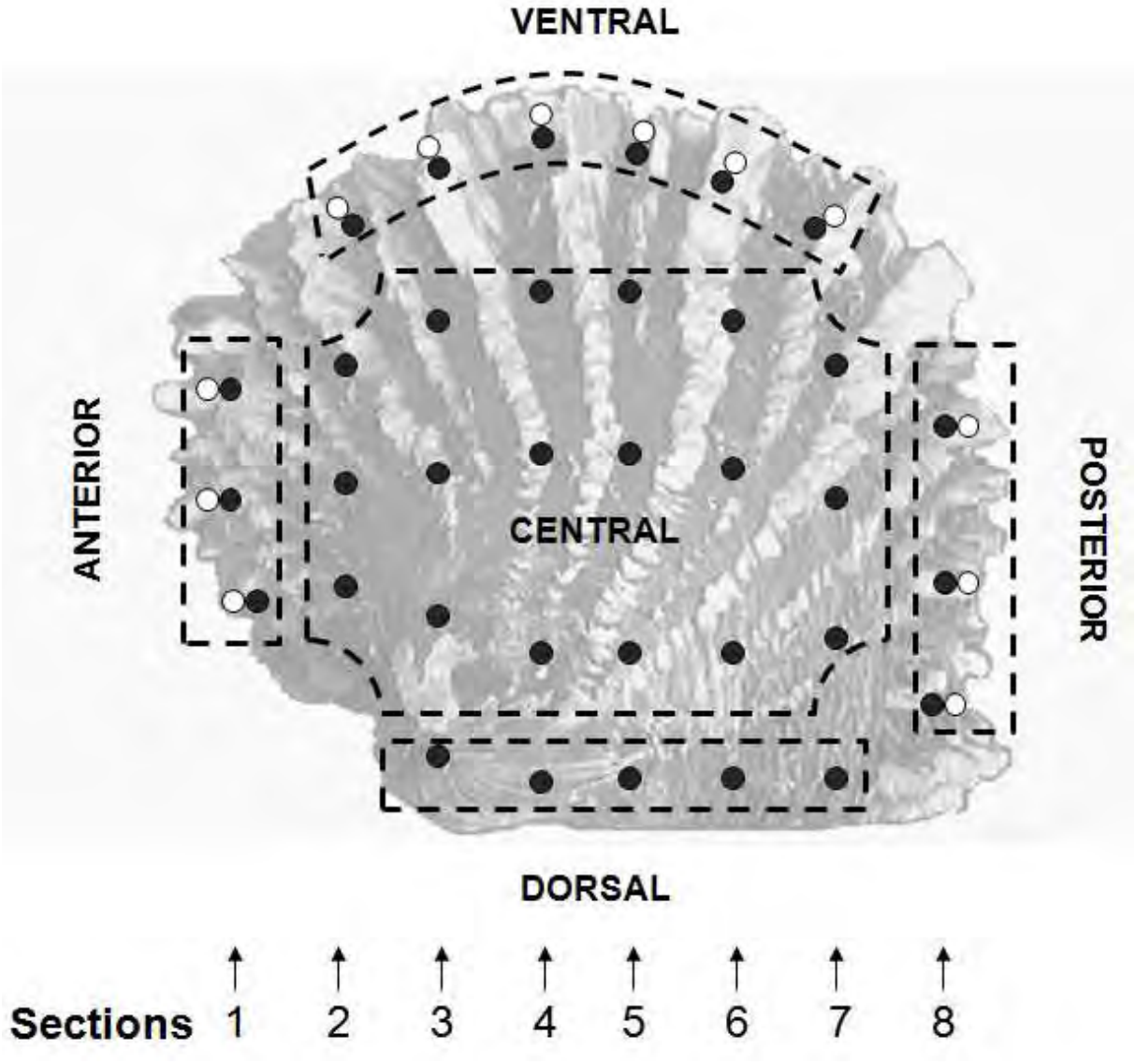

VENTRAL
ANTERIOR
CENTRAL
POSTERIOR
DORSAL
Sections 1 2 3 4 5 6 7 8

Figure 1

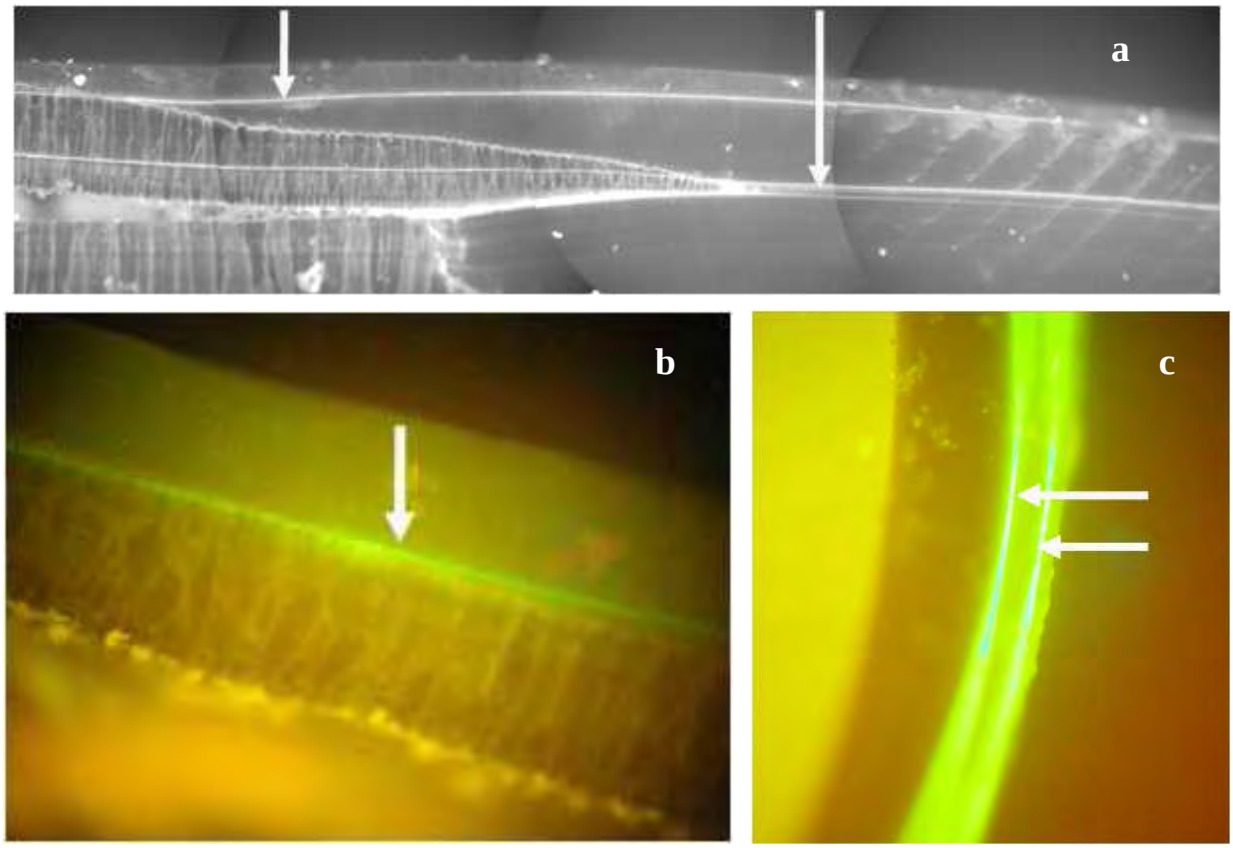


Figure 2

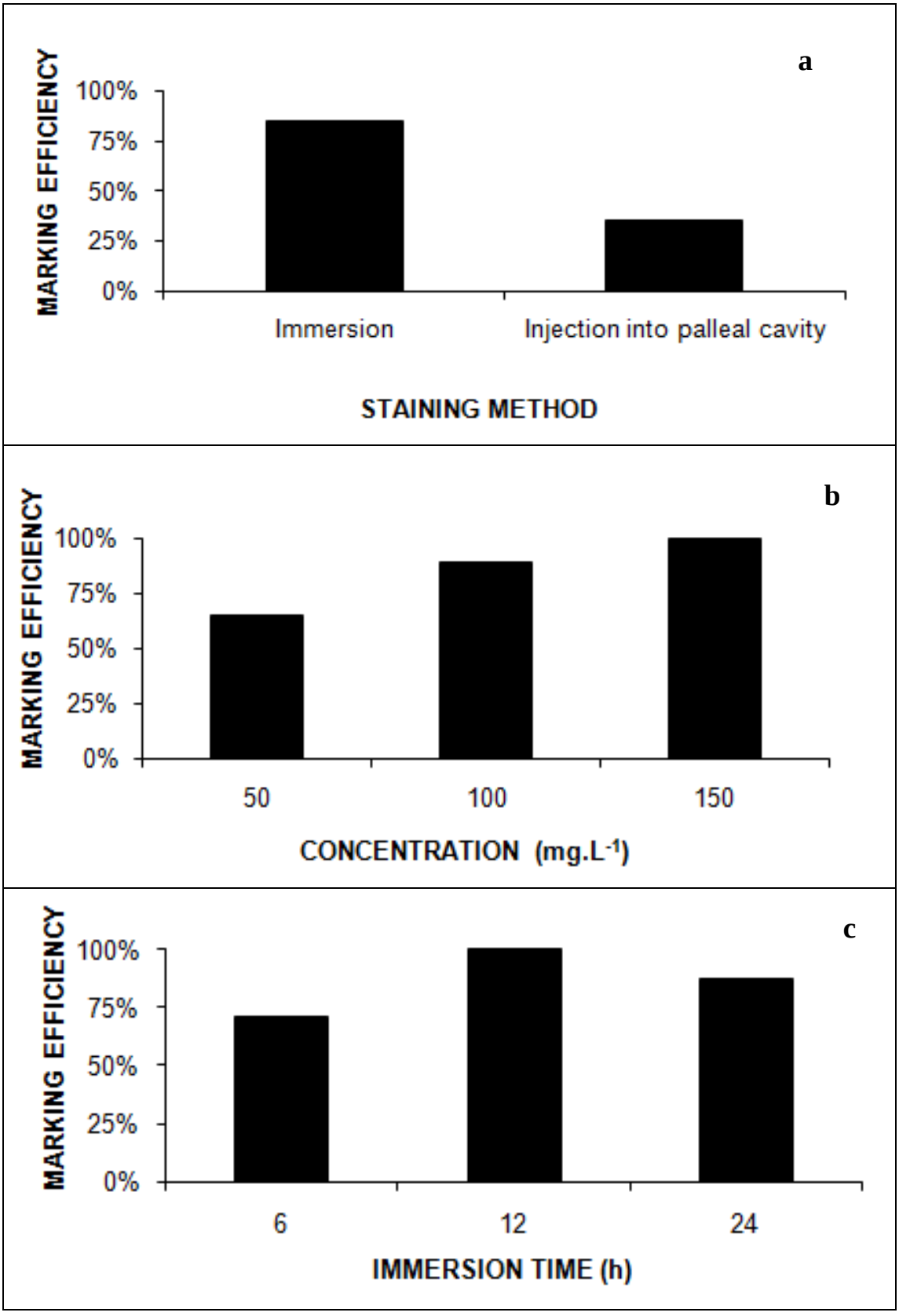
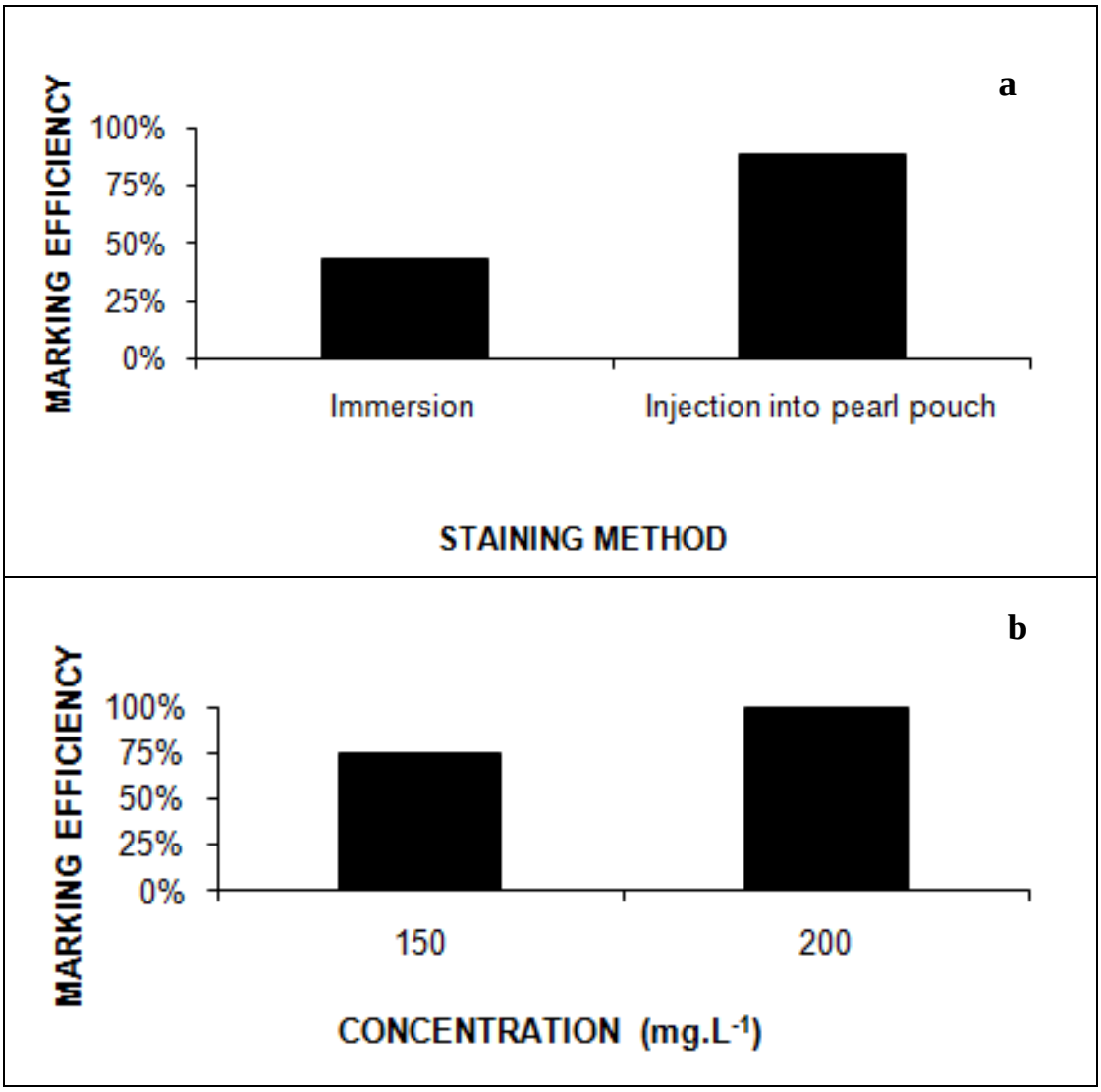

a
MARKING EFFICIENCY
100%
75%
50%
25%
0%
Immersion
Injection into palleal cavity
STAINING METHOD
b
MARKING EFFICIENCY
100%
75%
50%
25%
0%
50
100
150
CONCENTRATION (mg.L-1)
c
MARKING EFFICIENCY
100%
75%
50%
25%
0%
6
12
24
IMMERSION TIME (h)

Figure 3



561

562 Figure 4

563

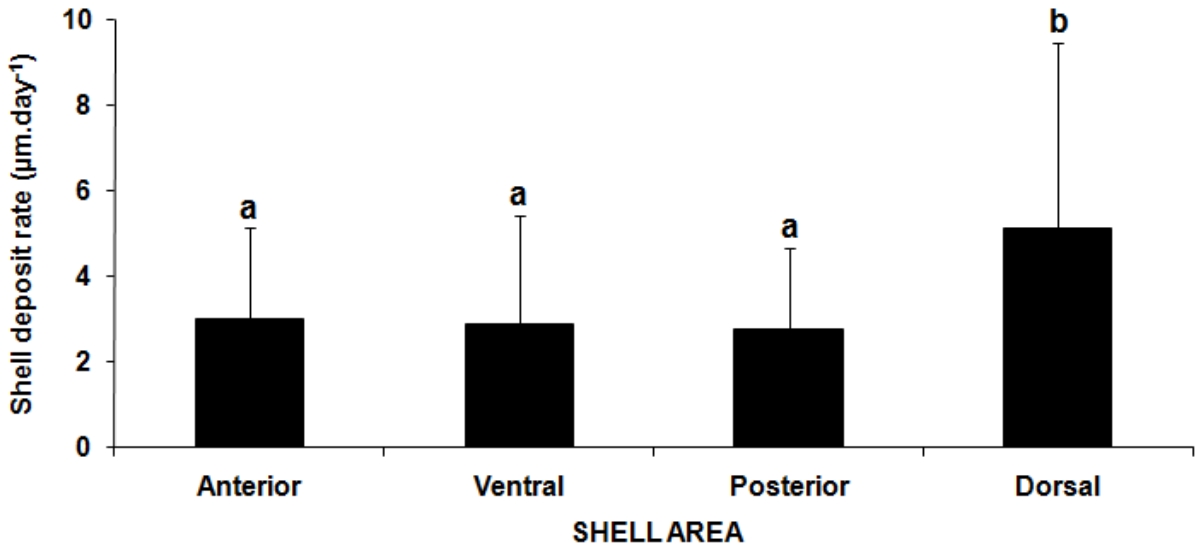

Shell deposit rate (µm.day-1)
10
8
6
4
2
0
a
a
a
b
Anterior
Ventral
Posterior
Dorsal
SHELL AREA

Figure 5

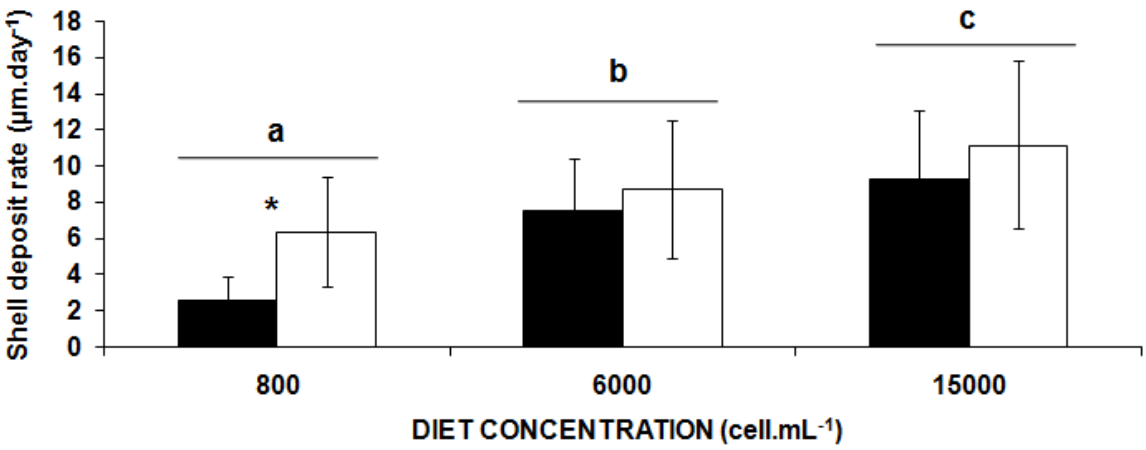

Shell deposit rate (µm.day-1)
18
16
14
12
10
8
6
4
2
0
a
b
c
*
800
6000
15000
DIET CONCENTRATION (cell.mL-1)

Figure 6

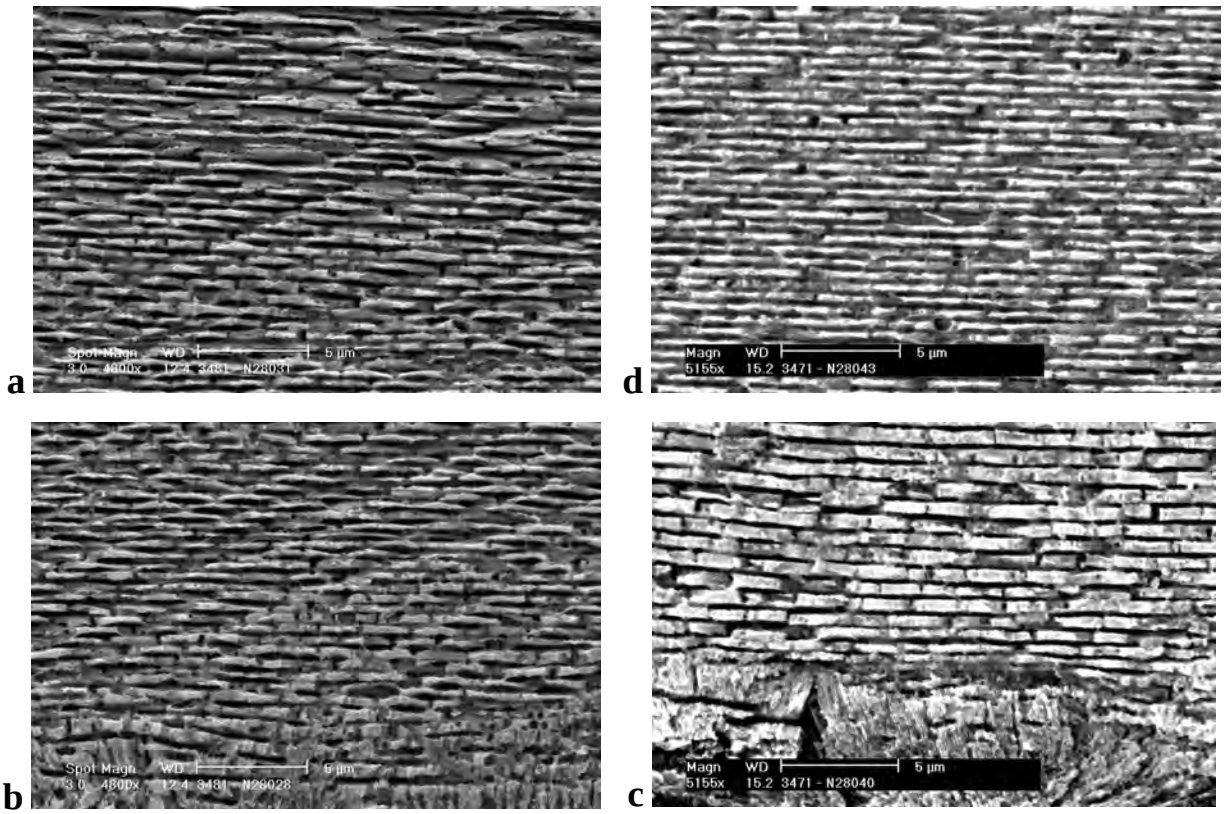

a
d
b
c
Magn WD 5 µm
5155x 15.2 3471 - N28043
Magn WD 5 µm
5155x 15.2 3471 - N28040

Figure 7

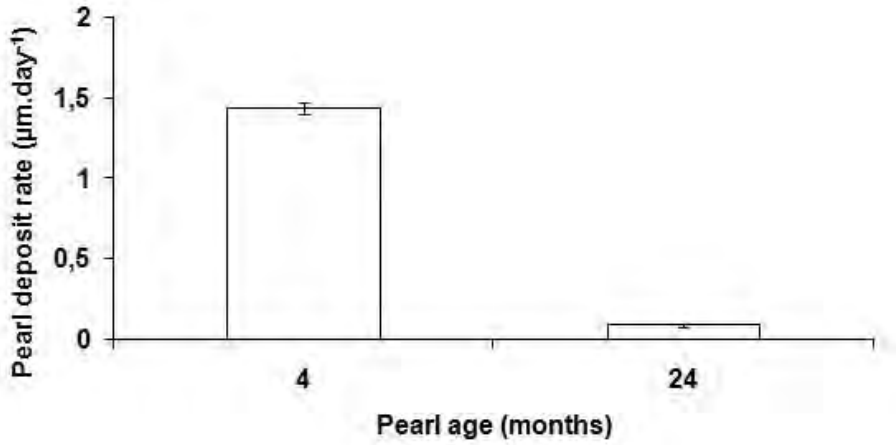


Figure 8

602

603

604

605