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Effect of temperature, food availability, and estradiol injection on gametogenesis and gender in the pearl oyster *Pinctada margaritifera*

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Abstract :

The black-lip pearl oyster *Pinctada margaritifera* is a protandrous hermaphrodite species. Its economic value has led to the development of controlled hatchery reproduction techniques, although many aspects remain to be optimized. In order to understand reproductive mechanisms and their controlling factors, two independent experiments were designed to test hypotheses of gametogenesis and sex ratio control by environmental and hormonal factors. In one, pearl oysters were exposed under controlled conditions at different combinations of temperature (24 and 28°C) and food level (10,000 and 40,000 cells mL^{-1}); whereas in the other, pearl oysters were conditioned under natural conditions into the lagoon and subjected to successive 17β-estradiol injections (100 µg per injection). Gametogenesis and sex ratio were assessed by histology for each treatment. In parallel, mRNA expressions of nine marker genes of the sexual pathway (*pmarg-foxl2*, *pmarg-c43476*, *pmarg-c45042*, *pmarg-c19309*, *pmarg-c54338*, *pmarg-vit6*, *pmarg-zglp1*, *pmarg-dmrt*, and *pmarg-fem1-like*) were investigated. Maximum maturation was observed in the treatment combining the highest temperature (28°C) and the highest microalgae concentration (40,000 cells mL^{-1}), where the female sex tended to be maintained. Injection of 17β-estradiol induced a significant increase of undetermined stage proportion 2 weeks after the final injection. These results suggest that gametogenesis and gender in adult pearl oysters can be controlled by environmental factors and estrogens. While there were no significant effects on relative gene expression, the 3-gene-pair expression ratio model of the sexual pathway of *P. margaritifera*, suggest a probable dominance of genetic sex determinism without excluding a mixed sex determination mode (genetic + environmental)

1. Introduction

The black pearl industry, which is the second most important economic resource forFrench Polynesiaafter tourism, is based on the exploitation of the black-lip pearl oyster Pinctadamargaritifera (L.). Breeding of selected donor oyster linesproviding high-performance graft tissue is a way to improve the production of quality pearls (Tayalé et al., 2012; Ky et al., 2013, 2014). Such genetic selection is based oncontrolledpearl oyster reproduction in laboratory,which requires an understanding of the physiological mechanisms involved and the factors controlling them(Gueguen et al., 2013).The artificial reproduction of the pearl oyster P. margaritiferaimpliesbeing able to control gametogenesis and the synchronization of maturity stagesof the breeders, butmostly importantly requires the management of sex ratio for broodstock husbandry, whichappears to be the mainlimitation. Indeed, reproduction of pearl oyster under controlled conditions mainly depends on the availability of females, which unfortunately are not abundant amongyoung farmed batches of this species due to itsprotandrous hermaphroditism (Tranter, '58). This particular reproductive strategy pose a problem of generation time for the renewal of oyster lines in hatcheries because it takes 4-5 years for a significant number of females to produce offspring (Chávez-Villalba et al., 2011).

In the animal kingdom, sex determination can be genetic (GSD); environmental (ESD); or the result of an interaction of both these factors (Valenzuela et al., 2003; Wedekind and Stelkens, 2010). The main factors involved in ESD mechanisms are temperature, food availability and population density (Pradeep et al., 2012). In marine bivalves, only a few studies have covered the influence of these latter parameters on sex ratio. Stenyakina et al.(2010) showed a male-biased sex ratio in fasting mussel *M. charruana*. In Pacific oyster *Crassostreagigas*, an alternative and irregular protandrous hermaphrodite, a male-biased sex ratio was obtained in adults by conditioning at low temperature (8 °C) (Fabioux et al., 2005)whereas in spat of the same species it was obtained by conditioning at high temperature (25°C)(Santerre et al., 2013).

The pearl oyster is a suspension feeder particularly abundant in the Polynesian archipelagos where annual temperatures range from 22 to 31 °C (Zanini and Salvat, 2000; Fournier et al., 2012; Le Moullac et al., 2012). *P.margaritifera* reaches sexual maturity at the end of its first year. Its production of gametes is continuous, asynchronous, and linked to the pearloyster size (Pouvreau et al., 2000). Most studies show that the reproduction cycle in bivalves is generally influenced by environmental factors (Sastry, '79; Bayne and Newell, '83; Gervis and Sims, '92). In temperate areas,Fabioux et al.(2005)showed that the gametogenic cycle of*C.gigas* depends on temperature andfood availability. In tropical area such as French Polynesia,however, there is no clear seasonal cycle because there is little temperature variations. Pearl oysters are reproductively active almost all year round (Pouvreau et al., 2000; Le Moullac et al., 2012), withabundant trophic resources promoting gametogenesis and spawning(Fournier et al., 2012). A recent study in *P.margaritifera* confirmed that gametogenesis is modulated by food level:under-feeding decreases or stops germinal activity preventing any resumption of gametogenesis (Le Moullac et al., 2013). However, a betterunderstanding of the influence of environmental factors on gametogenesis remains essential for controlling this crucial step in the rearing cycle.

Although some debates occur in the scientific community (Scott, 2012, 2013), fluctuations in levels of sex steroidshave been found to be correlated with the sexual maturation cycle in a number of bivalves, thus suggesting that sex steroids may play important stimulatory roles in their reproductive regulation (Matsumoto et al., '97; Osada et al., 2004; Gauthier-Clerc et al., 2006; Ketata et al., 2007).This hypothesis is supported byVaraksina and Varaksin('91) and Varaksina et al. ('92) whoreported that injections of estradiol, progesterone and testosterone stimulated both oogenesis and spermatogenesis in adult Japanese scallop

Mizuhopectenyessoensis. In the clam*Mulinialateralis*, administration of methyltestosterone accelerated sex maturation and resulted in an increase in spawning frequency (Moss, '89),while estradiol injections stimulated vitellogenesis in the oyster *C. gigas*(Li et al., '98; Osada et al., 2003). There is also evidence indicating that the gender of bivalves can be affected by sex steroids.Moss, ('89),showed that methyltestosterone fed to spawned clam *M. lateralis*increased the male/female ratio from 0.8 to 1.6. Mori et al.('69) showed that injection of estradiol into the oyster *C. gigas* at early stages of seasonal maturation induced sex reversal from male to female. In the pearl oyster *P. margaritifera*,no studies have yet been published on the possible effects of sex steroids on gametogenesis or gender determination.

The genetic and/or environmental process that establishes the gender of an organism (Penman and Piferrer, 2008), leads to specific molecular cascades transforming an undifferentiated gonad into a testis or an ovary (Piferrer and Guiguen, 2008). Recently, a gonad transcriptome analysis of *P.margaritifera*, using Illumina-based RNAseq, made it possibleto propose a new model of the reproduction of *P. margaritifera* based on a dual histo-molecular approach(Teaniniuraitemoana et al., 2015); this revealed thatthe regression phase of the gonad could open the time window for adult sex-determination. In addition,after examining expression of nine identified marker genes of the sexual pathway, a model was formed based on the expression ratios of 3 gene pairs. This model makes it possible to predict sexual pathway in this hermaphrodite species. This 3-genes-pair ratio sexual pathway signature concerns the ratios*pmarg-c43476*/*pmarg-fem1-like*, *pmarg-foxl2*/*pmarg-c54338* and *pmarg-foxl2*/*pmarg-fem1-like* and strongly suggeststhe involvement of*pmarg-foxl2* and *pmarg-fem1-like* in sex determination and differentiation in *P. margaritifera*(Teaniniuraitemoana et al., 2015).

In this context, the main objective of the present study was to examine, using two separate experiments,(1) the effect of environmental combinationsof temperature (24 and 28 °C) and trophic level (10,000 and 40,000microalgae cells mL^{-1}) and (2) the effect of injected 17β-estradiol (100 µg) on gender, especially sex ratio, gametogenesis and mRNA level of nine marker genes of the sexual pathway.

2. Materials and Methods

2.1. Biological material

The effect of environmental factors (temperature and trophic level) has been tested on 360three-years-old male and female adult pearl oysterswith an average size of 118 mm ± 8.67. These pearl oysters were transportedfrom Takaroa (Tuamotu Archipelago, French Polynesia) to Vairao lagoon (Tahiti, French Polynesia) where they were acclimated for 3.5 months.

The effect of 17β-estradiol injection was tested on 260two and a half-years-old male pearl oysters with an average size of 65 mm ± 8.13. These pearl oysterswere issued from a multi-familial cross, according to Ky et al.(2013), in the Ifremer hatchery by crossbreedingbroodstock oysters (4 males and 6 females)originated from the Apataki atoll (Tuamotu Archipelago, French Polynesia) in June 2011, and were reared in the Vairao lagoon.

2.2. Initial biopsy and standardization by gonad regression

Before the start (d0) of the two experiments (environmental: Exp1, February to April 2013; and hormonal:Exp2, January to March 2014), all pearl oysters (n = 360for Exp1 and n = 260 for Exp2) were labeled individually and sexed by biopsy. They were then placed in the

lagoon for a minimum of 2 weeksfor recovery. The biopsy was performed using a needle mounted on a piston syringe inserted in the gonadic part of the visceral mass to collect the gametes. Sex was determined by observation undera binoculardissecting microscope.

After the recovery period in the lagoon, pearl oysters were subjected toa period of under-feeding to stop the production ofgonial cells(Le Moullac et al., 2013)and synchronize gametogenesis. For Exp1, oysters were placed randomly in 8tanks (250 L) and then fed for 7 dayson 4,400 cells mL^{-1}microalgae (1/3 *Chaetocerosgacilis* and 2/3 *Isochrysisgalbana*) at a temperature of 28 °C. For Exp 2, oysterswere placed randomly in 26 tanks(30L) and maintained for ten days with continuously renewed sea water at 28 °C and a meanchlorophyll-*a* concentrationof 0.015 µg L^{-1}. This phase ended by the sampling of 40 and 20 individuals in Exp1 and Exp2, respectively, to define the initial state ingametogenesis of population in eachexperiment before treatments.

2.3. Pearl oyster conditioningaccording to temperature and trophic level (Exp1)

Pearl oysters were reared attwo trophic levels (10,000 cells mL^{-1} and 40,000 cells mL^{-1}) and two temperatures (24 °C to 28 °C) for 8 weeks.Four experimental treatments were realized in the laboratory in duplicate (24 °C-10,000 cells mL^{-1}, 24 °C-40,000 cells mL^{-1}, 28 °C-10,000 cells mL^{-1}, 28 °C-40,000 cells mL^{-1}). Oysters (n = 320) were distributed between8 experimental tanks (250 L), with32 males and 8 females per tank. Seawater renewal (100 L h^{-1}) was maintained constant. Homogenization of the environment in the tanks wasachieved by "air-lifts", and photoperiod was maintained at 12:12. The tankswere cleaned once a week. Pearl oysters were fed with a mixed diet composed of 2:1 (v:v) of *Isochrysisgalbana*(T-Iso)and *Chaetocerosgracilis*cells, supplied continuously withBlackstone dosing pumps. Cooled sea water was produced using a plate heat exchanger stocked with cold fresh water, and warmed sea water was produced using a 2,500-watt electric heater. Each rearing tank wassampled automatically every 3 minfor fluorescence and temperature measurements. The fluorometer(???) wasfirst calibrated foralgal concentration (cells mL^{-1}) and the temperature probe was calibrated using a reference electronic thermometer(???).After 30 days (d30), 16 males and 4 females were randomly sampled from each tank. The 160 remaining pearl oysters were sacrificed at the end of the experimentafter 60 days(d60) to assess the final state.

2.4. Pearl oyster conditioning using estradiol injection (Exp2)

17β-estradiol (E_2) was purchased from Sigma-Aldrich (Stenheim, Germany). E_2 was dissolved in 100% ethanol, which was then diluted 1:100 with 1-µm filteredsterilized seawaterto give a 0.5 µg $µL^{-1}$ stock solution. From this solution, 200 µL were injected directly into the gonad of each animal (n = 120). The remaining pearl oyster (n = 120), were injected with 200 µL of a 1:100 mixture of ethanol and sterilized seawater toserve as a control. Three successive injections were performedonce a week at the beginning (day 0, d0), at day 7 (d7) and at day 14 (d14) of the experiment. To minimize stress caused by gonad injections, oysters were anesthetized using $MgCl_2$ following the protocol provided bySuquet et al.(2009). After each injection, the pearl oysters were putback in the lagoon, where they were hung on ropes ingroups of 20 on a submerged long-line at 7 m depth.Two weeks after the last injection (d28), the first half of the pearl oysters was collected. The second half was sacrificed at the end of the experiment,6 weeks after the final injection (d56).

2.5. Histology

For each oyster, gonad tissue was sampled for histological examination using methods described by Fournier et al. (2012).After dissection, each visceral masse was fixed in formalin solution 10% diluted in seawater for 48 h before being transferred to 70% ethanol for 48 h for preservation. Then, the visceral masses were cut along thesagittal plane with a

microtome blade and were dehydrated through a graded ethanol series, embedded in paraffin, sectioned at 3–4 µm on a rotary microtome, stained with Giemsa dye and, finally, mounted on microscope slides.Gonad development stage and sex were then determined under a light microscope(???) and thus samples were classified according to theten different categories of gonadic tissues described previously in Teaniniuraitemoana et al. (2014):male and female at “Early” stage (the gonad is in early gametogenesis), “Intermediate” stage (the gonad is developing), at “Mature” stage (the oyster is ready to spawn), “Regressed” stage (the gonad has stopped generating gametes); “Inversion” (the gonad presents male and female gametes together)and “Undetermined” (the gonad contains no gametes at all).

2.6. Real time PCR

For each oyster, a piece of gonad tissue was sampled, putin RNAlater™ (Qiagen) (50 mg/mL) and then stored at -80 °C for subsequent total RNA extraction.

Total RNAwas extracted using TRIzol® Reagent (Invitrogen) and treated with DNAse I using a DNA-*free*™ Kit (Ambion) following manufacturer’s instructions. RNA concentrations were measured using an ND-100 spectrophotometer (Nanodrop Technologies) at 260 nm, using the conversion factor 1 OD = 40 µg/mL RNA.For each sample, 0.5 µg of total RNA were reverse-transcribed using a Transcriptor First Strand cDNA Kit (Roche) and amplified by real time PCR on a Stratagene MX3000P. The amplification reaction contained 12.5 µL 2X SYBR green qPCRMaster Mix (Stratagene), 10 µLcDNA template, and 2.5 µL ofeach primer (4 µM) in a final volume of 25 µL. Each run included a positive cDNA control and a blank control (water) for each primer pair. Relativegene expression was calculated using two reference genes, *ef1a* and *gapdh1*, using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001), as follows: Relative expression$_{(\text{target gene, sample x})}$ = $2^{\wedge -}(Ct_{\text{target gene, sample x}} - Ct_{\text{reference gene, sample x}})$. PCR efficiency (E) was estimated for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of the cDNA control to ensure that E ranged from 90 to 110%. The 9 primerpairs used for amplificationand described inTeaniniuraitemoana et al.(2015) are listed in Table 1.

2.7. Molecular sexual pathway determination

To determine the molecular sexual pathway of oysters, we used the model proposed byTeaniniuraitemoana et al.(2015) based on the expression ratios of four genes: *pmarg-c43476*/*pmarg-fem1-like*, *pmarg-foxl2*/*pmarg-c54338* and *pmarg-foxl2*/*pmarg-fem1-like*. According to this model, oysters aremolecularly on thefemale pathway when they combine a *pmarg-c43476*/*pmarg-fem1-like* gene ratio ≥ 0.02225 and a *pmarg-foxl2*/*pmarg-c54338* gene ratio ≥ 12.25; or a *pmarg-c43476*/*pmarg-fem1-like* gene ratio ≥ 0.02225, a *pmarg-foxl2*/*pmarg-c54338* gene ratio < 12.25 and a *pmarg-foxl2*/*pmarg-fem1-like* gene ratio ≥ 0.1003. Oystersare on the male pathway when they present a *pmarg-c43476*/*pmarg-fem1-like* gene ratio < 0.02225 or a combining *pmarg-c43476*/*pmarg-fem1-like* gene ratio ≥ 0.02225, a *pmarg-foxl2*/*pmarg-c54338* gene ratio < 12.25 and a *pmarg-foxl2*/*pmarg-fem1-like* gene ratio < 0.1003.

2.8. Statistical analysis

Gametogenesis, sex ratio and sexual pathway were analyzed using Fischer's exact testin XLStat software. The mean of relative gene expressions were compared using the non-parametric Kruskall-Wallis test, and pairwise multiple comparisons were carried out according to the procedure of Wilcoxon using Bonferroni correction inR software (R Development Core Team, 2011). All these statistical analyses were conducted with the threshold of significance $\alpha = 0.05$.

3. Results

3.1. Effects of temperature and trophic levelon gametogenesis and sex ratio

Significant differences in the proportion of reproductive stages were revealed between the different treatments(Fisher's exact test;*pvalue*<0.05)in the two experiments (Fig. 1 and 2). At d0 of experimentation(initial state), there was a significantly higher proportion of regressed gonads,than that expected, with 100% and 80% in Exp1 and Exp2, respectively, probably caused by the initial under-feeding.

In the experiment testing environmental factors(Exp1, Fig. 1), after 60 days of conditioning, a significantly lower proportion(5%) of mature gonadswas obtainedthan expected for pearl oysters conditioned at 24°C and10,000 cells mL^{-1}. For pearl oysters conditioned at 28 °C and 40,000 cells mL^{-1}, a significantly higher proportion(65.7%) of mature gonads was obtainedthan expected. In this latter treatment, a higher proportion of mature pearl oysterthan the expected distribution was also found after only 30 days of conditioning. Regressed gonads wereobserved in all temperature and trophic level combinations. Significantly higher proportions of regressed gonads were found in the treatmentcombining 24 °C and 10,000 cells mL^{-1}after both30 and 60 days of conditioning. Pearl oysters in the regressed stage were also found in the treatment28 °C-40,000 cells mL^{-1}, but in significantly lower numbers than the expected distribution.

In addition, Fisher's exact test revealed significant differences in the proportion of females and animals in inversion(*pvalue*< 0.001; Table 2). Conversely, the proportion of males did not change significantly. At d30, changes in the sex ratio were found only at low trophic level. In the 28 °C-10,000 cells mL^{-1}treatment,three individuals became sexually undifferentiated, plus one female animaldied. At d60significant effects of environmental factors on the sex ratio were observed. At low trophic level and 28 °C, a significant proportion of females (50%)wasundergoing inversion to become male. In pearl oysters fed with a concentration of 40,000 cells mL^{-1}, a significantlysmaller proportion of inversion was observed. At 24 °C, two “female to male” inversions were observed whereas only one was seen at 28 °C.

3.2. Effects of estradiol injection on sex ratio and gametogenesis

In the hormonal experiment (Exp2, Fig.2), after 28 days, there were significantly higher proportions of regressed and undifferentiated gonads for pearl oysters injected with 17β-estradiol. These two stages were not found at all after 56 days in this treatment, where the mature stage then appeared significantly dominant (51%) as in the control treatment (60%). Some pearl oysters in regression were also found in the control group at the end of the experiment (2%) but in significantly lower proportionsthan in the expected distribution.

The effect of 17β-estradiol injection was only tested inmalesin order to evaluatewhether induction of male to female sex-reversal was possible in *P. margaritifera*. Fisher’s exact test (*pvalue*< 0.05) revealed significant differences in the proportion of male pearl oysterscompared with the expected distribution (Table 2). Indeed,for pearl oysters injected with 17β-estradiol and sampled at d28, there was a significant lower proportion (82%) of males, whereas the proportion of undetermined individualsappeared significantly higher (18%)than expected.

3.3. Gene expression analysis reveals male pearl oysters at the onset of female differentiation

Gene expression analysis of nine candidate marker genes (*pmarg-foxl2*, *pmarg-c43476*, *pmarg-c45042*, *pmarg-c19309*, *pmarg-c54338*, *pmarg-vit6*, *pmarg-zglp1*, *pmarg-dmrt* and

pmarg-fem1-like)wasperformed on regressing maleand undeterminedsamples after 60 days of conditioning in Exp1 andafter 28 days in Exp2. Statistical testscarried out on mRNA levels did not show significant effects of environmental factors (Kruskal-Wallis) or 17β-estradiolinjection (Wilcoxon) on gene expression.

However, the use of the 3-gene-pair expression ratio model of the sexual pathway of *P. margaritifera*, proposed inTeaniniuraitemoana et al.(2015), on the present gene expression dataset revealedthat nine male pearl oysters exhibiteda female sexual molecular pathway (i.e. combining a *pmarg-c43476*/*pmarg-fem1-like* gene ratio ≥ 0.02225 and a *pmarg-foxl2*/*pmarg-c54338*gene ratio ≥ 12.25; or combining a *pmarg-c43476*/*pmarg-fem1-like* gene ratio ≥ 0.02225, a *pmarg-foxl2*/*pmarg-c54338* gene ratio < 12.25 and a *pmarg-foxl2*/*pmarg-fem1-like* gene ratio ≥ 0.1003) (Table 3).One of these specimens was found in the treatment 28 °C-10,000 cells mL^{-1} and another in 28 °C-40,000 cells mL^{-1} and three were found in the treatment 24 °C-10,000 cells mL^{-1} in Exp1. In Exp2, we found two individuals among the oysters injected with 17β-estradiol and two others in the control condition. Regarding their proportions, theFisher's exact test revealed significant differences in Exp1 but not in Exp2. Indeed,the proportion of oysters on the female and male pathways was significantly lower in treatments 24 °C-40,000 cells mL^{-1} and 28 °C-40,000 cells mL^{-1},respectively,than the expected proportions(*pvalue*< 0.05; Fisher's exact test).

Comparing gene expression between individuals determined molecularly to be on female and male sexual pathways;Wilcoxon test revealed significant differences for*pmarg-foxl2* and *pmarg-fem1-like* mRNA levels but not for the seven other genes tested (Fig. 3). Indeed, the mRNA level of *pmarg-foxl2* appeared significantly higher (4.6E-05 ±9.7E-05; *pvalue*< 0.05) in oysterson the female sexual pathway than in those on the male sexual pathway, whereas *pmarg-fem1-like* appeared significantly lower (1E-03 ±4.4E-03; *pvalue*< 0.01).

4. Discussion

In this study, we report the effect of different combination of temperature, food quantity and 17β-estradiolon gender determination and dynamics of gametogenesis in adult *P. margaritifera*. For this purpose, we conducted two separated experiments. In the first experiment, oysters were reared in controlled conditions of light, temperature and food availability. In the second experiment, oysters were injected 3 timesat weeklyintervals with 17β-estradiol and rearedinnatural conditions into the lagoon.

4.1. Effect of food availability and temperature

4.1.1. Effect on gender determination

The main objective of our environmental experiment was to test the hypothesis of environmental sex determinism (ESD) in *P. margaritifera*adults.

Numerous vertebrates exhibit this sex determinism mode, mainly consisting in "temperature-dependent sex determination" (TSD)(Pieau et al., '99). For instance, among reptiles, gonadic differentiation is known to bebiased towards femaleat high temperature in most turtle species (Shoemaker and Crews, 2009), but biased towards male in some lizards (Hulin et al., 2009). In marine bivalves, only a few studies have shown the effect of environmental factors on sex ratioas a manner of induction of a male-biased sex ratio(Fabioux et al., 2005; Stenyakina et al., 2010; Santerre et al., 2013). Our results show a significant sex ratio change, determined histologically, for oysters conditioned at high temperature and low trophic level.Indeed in these environmental conditions, 50% of the initial females changed sex after 60 days,

probably due to an energetic deficit. However, when looking at the molecular level using the 3-gene-pair expression ratio model, the proportion of regressed males identified as being on the male sexual pathway was significantly lower than expected in the treatment combining higher temperature conditions (28 °C)with high trophic level (40,000 cells mL^{-1}). It is therefore highly likely that we could induce a sex–reversal from male to femaleusing such conditions.Most previous studies that revealed a change in sex ratio in adult bivalves were conducted with a long conditioning time, generally over 1 year, as in the Pacific oyster *C. gigas* (more males at approximately 8 °C) (Fabioux et al., 2005) or as in the tropical Cortez oyster *C. corteziensis* (more females at 9 °C) (Rodríguez-Jaramillo et al., 2008). Our experiment lasted only two months,which corresponds to two reproductive cycles in *P. margaritifera*,but this duration could be insufficient to observe a significant effect of an environmental factor, especially temperature, on sex ratio.

The energetic deficit, hypothesized above, as source of sex inversion of 50% of the females, could be explainedbythe temperature fixed at 28 °C that would have increased the oxygen consumption and the metabolic rates, increasing the need for energy, as already demonstrated from 24 to 27 °C (Chávez-Villalba et al., 2013). Furthermore, in some molluscs, the production of female gametes is supposed to be more energetically costly than the production of male gametes, estimated at 50% more energy (Russell-Hunter, '79). To achievesex inversion, pearl oysters should initiate gametogenesis to renew a reproductive cycle.Bayne ('76)indicated that gametogenesis only begins when energetic reserves are sufficient. Poorly fed pearl oysters probably do not have enough energy from the diet and reserves at 28 °C to initiate gametogenesis and therefore change sex. At 28 °C and a high trophic level considered as a sufficient input of energy, we found only one female in the course of sexual inversion,suggesting that there areother forcing factors. A recent studyon*P. margaritifera*, has shown that at equal ingestion, females assimilate organic matter of ingested food better than males, and thus require a higher oxygen consumption (Chávez-Villalba et al., 2013). This suggests that when females are subjected to a stress, an increase of their oxygen consumption could exacerbate the energetic deficit. Rearing techniques may be partially responsible and considered as stressful. We could hypothesize that the weekly cleaninggenerateda detrimental chronic stress for maintaining the female sex or/and the manifestation of new females. Some previous work has shown that the proportion of females has decreased for cultured pearl oysters compared with the natural stocks (Thielley, '93). In addition, Lacoste et al.(2014) observed a negative effect of monthly cleaning on female sex on farmed pearl oysters reared in a lagoon. It therefore appeared important to find non-stressful cleaning methods in order to consider only the effect of the environmental factors and promote females in aquaculture rearing.Energetic cost of sex inversion would be also of great interest for further studies to understand and to model sex determinism in *P. margaritifera*.

4.1.2. Effect on gametogenesis

Food availability is known to be an important factor for bivalve development, affecting broodstock energy reserves, fecundity, quality and quantity of eggs, and larval development(Berntsson et al., '97; Utting and Millican, '97; Hendriks et al., 2003; Delgado and Pérez-Camacho, 2005). In *P. margaritifera*, the present study clearly demonstrates, in experimental conditions, that the trophic level is a key parameter in the control of gametogenesis. Low trophic level seemed detrimental to the production of gametes, resulting in a high proportion of oysters in regression and therefore the cessation of gonialcellproduction. Conversely, a high concentration of microalgae promotedgonialcell proliferation and gamete maturation. These results confirm those of a study carried out byLe Moullac et al.(2013)showing that gonadic maturation and gonial proliferation of the pearl oyster is controlled by microalgal concentration. In this previous study, only 11% of individuals showedcomplete gametogenesis at the end of the first month of conditioning,

whereas in our study,under the effect of a higher trophic level (x 2.5), the proportion of oysterswith complete gametogenesisreached60%.The role of temperature in gametogenesis has also been demonstrated in many species of bivalves. Indeed, it was shown thatelevated temperatures in temperate zone (i.e. ~ 19-20 °C) promoted reproductive effort and maturation of germ cells (Mart nez and P rez, 2003; Fabioux et al., 2005). More precisely, it has been shown that the temperature regulates the speed and thus the duration of gametogenesis (Enríquez-Díaz et al., 2009).From a bioenergetic point of view,conditions were met in the present study to provide the maximum energy to oysters, i.e., a temperature of 28 °C (Yukihira et al., 2000) and a trophic level close to saturation (Le Moullac et al., 2013). Under these conditions, pearl oysters realized complete gametogenesis, going to spawning for some male and female individuals (data not shown). This study therefore offers an avenue forconditioning pearl oysters for reproductive maturation,but further research will be required to test for an effect of food quantity and quality on gamete quality and onthe subsequent implicationsfor offspring.

4.2. Effect of 17β-estradiol

The role of estrogens in the hormonal regulation of bivalve reproduction was suggested to be similar to that which they fulfill in vertebrate endocrine systems. Many studies in vertebrates, especiallyteleosts,showed that 17β-estradiol has a significant effect on reproduction, stimulating ovarian development and vitellogenin synthesis (de Vlaming et al., '80; Chang et al., '95; Kang et al., 2002). In this context, we made the first test of the potentialfeminizing effect of 17β-estradiol by directly injecting it into male gonadsof*P. margaritifera*.While no sex reversal from male to female was established during this experiment, a significantly lower proportion of male and a significantly higher proportion of undetermined oysters were found in the 17β-estradiol-injected group after 28 days (i.e. 14 daysafter the third injection). Furthermore, regarding gametogenesis, we also foundsignificantly lower proportions of intermediate and mature gonads. These results strongly suggested that 17β-estradiolhas a negative effect on male gonad development in *P. margaritifera*, as in fishes. For example,Chang et al.('95)observed that E_2 treatment completely suppressed testicular development and spermiation in 2-years-old black porgy*Acanthopagrusschlegeli*. However, in *P. margaritifera*, it seemed that this negative effect disappears 6 weeks after the last injection,giving way to a positive effect on spermatogenesis. Indeed, although at the end of the experiment, a significantly higher proportion of mature gonadswas reported in 17β-estradiol-injected oysters than in the control group, we observedan increase of sperm motility under the microscope for mature oysters of the 17β-estradiol-injected group compared with the control group at this time (data not shown).These results may seem to be contradictory to previous reports indicating the feminizing effect of estradiol in bivalves (Mori et al., '69; Varaksina and Varaksin, '91), but these effects are in accordance with other reports showing that estradiol can stimulate male reproductive activities. Indeed, it has been reported in other marine bivalves that estradiol stimulates spermatogenesis(Varaksina et al., '92; Wang and Croll, 2004), potentiates sperm release and spawning (Wang and Croll, 2003, 2006).If we supposed that 17β-estradiol quantity decreased in the gonad over the experiment, we can suggest that a residual quantity of 17β-estradiol (at the end of the experiment) could have a positive effect on spermatogenesis, whereas a high dose leads to the regression of the male differentiation. Therefore, our results show an effect of 17β-estradiol in reproduction of *P. margaritifera*, but further studies are needed to elucidate its function and mechanism of action.

4.3. Probable dominance of genetic sex determinism in adult *P. margaritifera*.

The application of the model of the sexual pathway of *P. margaritifera*to real-time PCR results revealed that nine regressed male pearl oysters, overall the experiments,were molecularly on the female sexual pathway (i.e. across all conditions whether in the environmental and hormonal experiments). These nine animals represent 10% of the total

analyzed pearl oysters. This ratio is not different to the female ratiofound in population of this average height (Chávez-Villalba et al., 2011), suggesting that whatever an environmental or hormonal treatment applied here, apreviously established genetic control seems to be dominant. In the Pacific oyster *C. gigas*, two genetic models have been proposed for sex determination (Guo et al., '98; Hedrick and Hedgecock, 2010). Whatever the model, the authors assumed that sex in *C. gigas*would be controlled by a single major gene. Herein, we showed that among the nine marker genes only *pmarg-foxl2* and *pmarg-fem1-like* appeared significantly differently expressed between pearl oysters in male and female sexual pathways, indicating that these two genes are involved at the top of the molecular cascades of sex determination in *P. margaritifera*. More recently,Santerre et al.(2013) suggested that *C. gigas*exhibits a mixed sex determination (GSD + TSD), ascan be observed for many fishesand amphibians (Valenzuela et al., 2003). In *P. margaritifera*, we did not exclude a mixed sex determination mode, GSD with ESD, but it appeared that adult sex determinism in this species is governed by more complex mechanisms. Indeed, this study covered only the effect of the exogenous factors temperature and food availability and the endogenous factor estradiol in adult pearl oysters. More studies will be required to better understand sex determination in this species, such as spat sex determination asrecently studied in *C. gigas*(Santerre et al., 2013), an alternative hermaphrodite mollusc, or with regard topopulation density as in *Crepedulafornicata*, a sequential hermaphrodite mollusc(Proestou, 2005).

Furthermore, this study also confirmed the utility and the capacity of the previously determined model based on the expression of three gene pairs to identifyoystersatthe onset of the female pathway.

5. Conclusion

In the present study, we found evidence for the environmental and,for the first time,hormonal control of the reproduction of*P. margaritifera*. Although no sex-reversal from male to female was observed, we suggest that environmental factors, and especially temperature combining with a predominant genetic controlmechanism, are involved in the gender determination of adult pearl oysters. Furthermore, high temperature and high trophic level appeared the best combination for broodstock conditioning in futurebreeding programs aimed at the sustainable development of pearl farming in French Polynesia. However, the sex determinism of *P. margaritifera* remains to be explored,with the two candidate genes *pmarg-foxl2* and *pmarg-fem1-like* as the best starting points. Herein we covered adult sex determinism and it would be of great interest to study sex determinism in spat during first gonadic differentiation, likeSanterre et al.(2013) in the Pacific oyster *C. gigas*.

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Tables

Table 1. Primers used for real time PCR.

Gene names	Genbank Accession numbers	Primer names	Forward vs Reverse	Sequences (5'-3')
pmarg-foxl2	KJ907378	FOXL2_1S	F	TCCGGATCACAGTGTCTCAG
		FOXL2_1AS	R	GACAGCAACGCACAGAATGT
pmarg-c43476	KM874285	C43476_VF_1S	F	ACCCTTACCAATCACCCTGC
		C46476_VF_1AS	R	TAACCAGGCCCGGGACTAT
pmarg-c45042	KM874286	C45042_VF_2S	F	GAGTGGAAGCAAGACAGACCA
		C45042_VF_2AS	R	TCCCAGAGGTTGCCAGAATAC
pmarg-c19309	KM874287	C19309_NFMRF_2S	F	GATGCACTAGACACGCCTGA
		C19309_NFMRF_2AS	R	ACATGGATCTCTGTAACGCGT
pmarg-c54338	KM874288	C54338_NFMRF_1S	F	CGTCTTCTCTAATTCACG
		C54338_NFMRF_1AS	R	CAGTACACACCATGATTGA
pmarg-vit6	KM874283	Vit-6_2S	F	GTCCGCCCAGTAAACAAAGA
		Vit-6_2AS	R	CAATGTTGGTCCAGCTTCCT
pmarg-zglp1	KM874284	ZGLP1_4S	F	AGGCTTCTTTGTCAGACCACA
		ZGLP1_4AS	R	GTGCTGAGAGATGTGATCCCA
pmarg-dmrt	KJ907374	Dmrt_2S	F	AACCTCTGGGTGACAACTGG
		Dmrt_2AS	R	ATCCAGGACCACAGGTTCAG
pmarg-fem1-like	KJ907377	FEM-1_4S	F	ACTCCACAACCAGCTTTCACA
		FEM-1_4AS	R	GTGCCGATGTGACACTGAGA
ef1a	-	EF1a5_S	F	CCACGAGTCCTTACCAGAGG
		EF1a5_AS	R	TGGATCACTTTTGCTGTCTCC
gapdh1	-	GAPDH1_S	F	AGGCTTGATGACCACTGTCC
		GAPDH1_AS	R	AGCCATTCCCGTCAACTTC

Table 2.Number of male, female, as well as undetermined and inversion stages of *Pinctadamargaritifera* in each condition of the two experiments.

Time	Condition	Male	Female	Undetermined	Inversion
Experiment 1 (Exp1)					
d0	-	32	8	0<	0<
d30	24°C – 10,000 cells mL^{-1}	31	7	1	0<
	24°C – 40,000 cells mL^{-1}	32	8	0<	0<
	28°C – 10,000 cells mL^{-1}	29	6	3	0<
	28°C – 40,000 cells mL^{-1}	30	8	0<	0<
d60	24°C – 10,000 cells mL^{-1}	30	5	4	0<
	24°C – 40,000 cellsmL^{-}	28	6	2	2
	28°C – 10,000 cells mL^{-1}	32	2<	2	4>
	28°C – 40,000 cells mL^{-1}	30	7	0<	1
Experiment 2 (Exp2)					
d0	-	19	0	1	0
d28	17β-Estradiol	36<	0	8>	0
	Control	43	0	5	0
d56	17β-Estradiol	39	0	0<	0
	Control	47>	0	0<	0

> indicates a significant higher number and < a significant lower number between conditions ($p < 0.05$, Fisher exact test).

Table 3.Number of pearl oysters *Pinctadamargaritifera* determined in male or female sexual pathway using the predictive 3-gene-pair expression ratio model of the sexual pathway proposed in Teaniniuraitemoana et *al.* (2015).

Condition	Femalepathway	Male pathway	Total
Experiment 1 (Exp1)			
24°C – 10,000 cells mL^{-1}	3	24	27
24°C – 40,000 cells mL^{-1}	0<	13	13
28°C – 10,000 cells mL^{-1}	1	12	13
28°C – 40,000 cells mL^{-1}	1	1<	2
Subtotal	5	50	55
Experiment 2 (Exp2)			
17β-Estradiol	2	20	22
Control	2	11	13
Subtotal	4	31	35
Total	**9**	81	90

> indicates a significant higher number between conditions ($p < 0.05$, Fisher exact test).

Figures

Fig. 1 Frequency of maturity stages observed by histology in the environmental experiment (Exp1). Number of animals (n) sampled at each sampling time and each treatmentare indicated below each histogram. The corresponding sampling times are indicated above histograms with horizontal brackets.> indicates a significant higher number and < a significant lower number between conditions than predicted ($p < 0.05$, Fisher's exact test).24-10: 24 °C-10,000 cells mL^{-1}; 24-40: 24 °C-40,000 cells mL^{-1}; 28-10: 28 °C-10,000 cells mL^{-1}; 28-40: 28 °C-40,000 cells mL^{-1}; d0: the beginning of the experiment; d30: after 30 days of conditioning; and d60: after 60 days of conditioning, the end of the experiment.

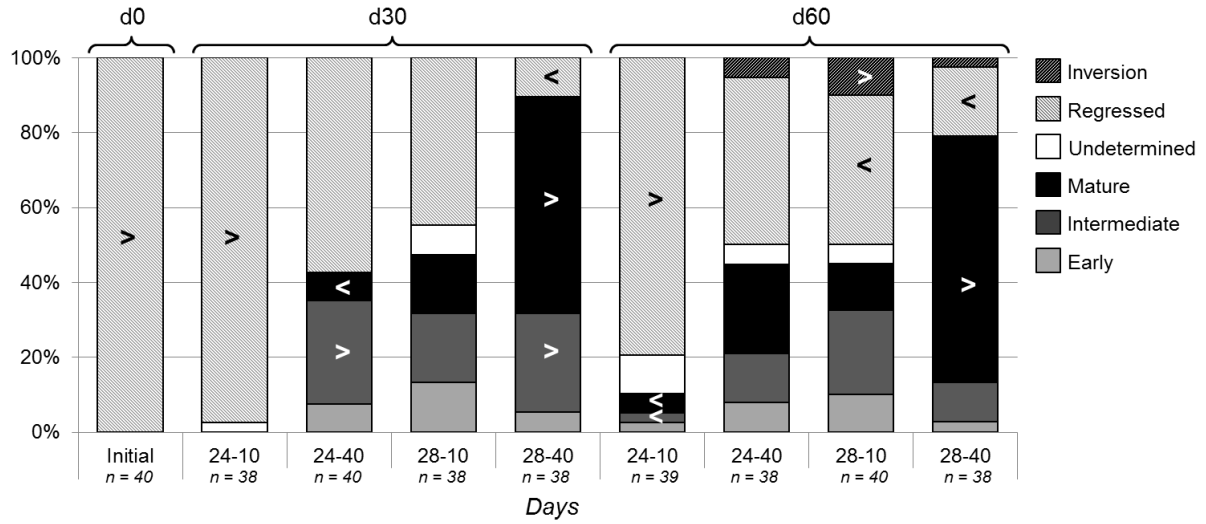

Fig. 2 Frequency of maturity stages observed by histology in the hormonal experiment (Exp2). Number of animals (n) sampled at each sampling time and each treatmentare indicated below each histogram.The corresponding sampling times are indicated above histograms with horizontal brackets. > indicates a significant higher number and < a significant lower number between conditions than expected($p < 0.05$, Fisher's exact test).17β-Estradiol: oysters injected with a 0.5 µg μL^{-1}17β-estradiol solution; Control: oysters injected with a 1:100 mixture of ethanol and sterilized seawater (Control solution);d0: the beginning of the experiment; d28: after 28 days of experiment, two weeks after the final injection; and d56: after56 days of experiment, 6 weeks after the final injection, the end of the experiment.

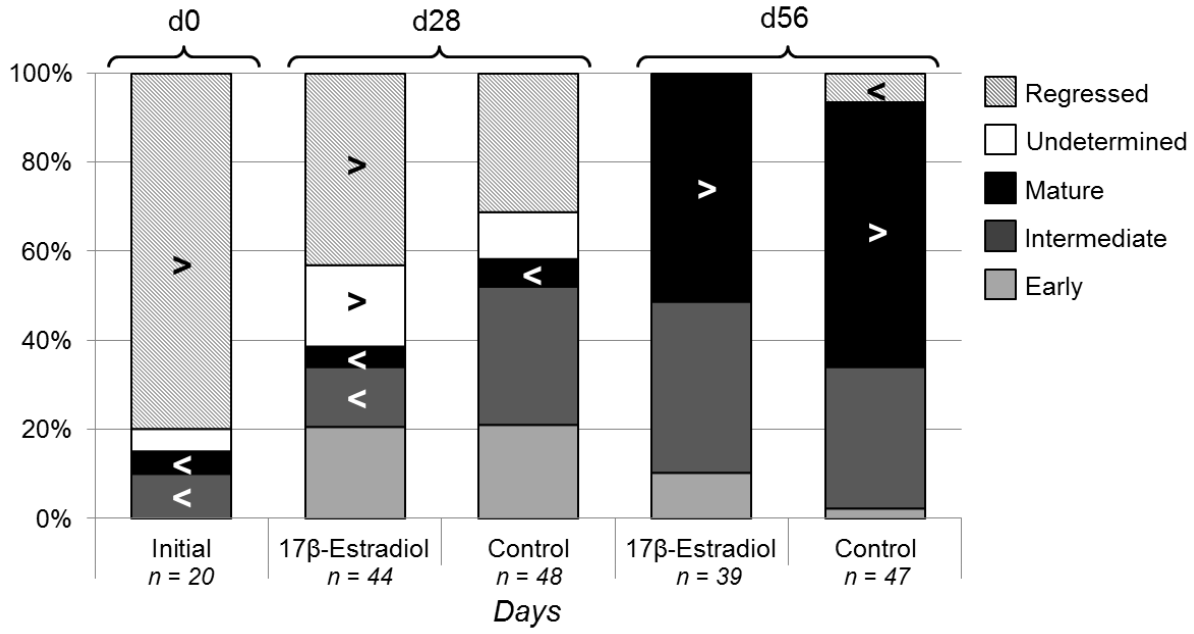

Fig. 3 Relative expression profiles (real time PCR) of *pmarg-foxl2* (A), *pmarg-c43476* (B), *pmarg-c45042* (C), *pmarg-c19309* (D), *pmarg-c54338* (E), *vit-6* (F), *zglp1* (G), *pmarg-dmrt* (H), and *pmarg-fem1-like* (I) in pearl oysters on male and female sexual pathways. * indicates statistically significant differences determined by a Wilcoxon test ($p < 0.05$).