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Feeding the pearl oyster *Pinctada margaritifera* during reproductive Conditioning

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Abstract :

This study aimed to model the food intake of *P. margaritifera* in order to examine the relationship between food level and reproductive activity. The effect of microalgae concentration on ingestion rate and assimilation efficiency was studied over a broad concentration range, using a mixture of Isochrysis galbana and Chaetoceros gracilis. Reproductive effort was assessed using three microalgae concentrations of 0.5, 7 and 18 cell µL-1. Reproductive status was assessed by gonad development index (GDI) – the ratio of the gonad surface to the visceral mass surface – and histological analysis of the gonad based on the presence (continuous or discontinuous) or the absence of gonial cells (GC). Ingestion is a saturating function of seston concentration for bivalves modeled with an adapted Michaelis- Menten function. The maximum ingestion rate of *P. margaritifera* adults was 193.50 106 cell.h-1.g-1 dw and the half saturation coefficient was 15 cell.µL-1. The concentration of 18 cell µL-1, supplied for 45 days induced a significantly higher GDI than the other treatments. GC decreased significantly and even stopped when pearl oysters were under-fed, suggesting that the mitotic process of the germinal stem cells was altered. Differentiation of germinal stem cells, therefore, appears to be controlled by food availability.

Keywords : *Pinctada margaritifera*, food level, ingestion rate, reproduction, gametogenesis.

1. Introduction

The artificial control of reproduction in the pearl oyster *Pinctada margaritifera* would enable a managed supply of spat to be produced for pearl farms throughout French Polynesia. Moreover, it would open the way for selection based on families with desired traits. In the wild, the pearl oyster, *Pinctada margaritifera*, undergoes continuous gametogenesis because

trophic resources and temperature are very stable and favorable throughout the year in its area of distribution (Pouvreau, Gangnery, Tiapari, Lagarde, Garnier & Bodoy 2000). As a consequence of the relative abundance of reproductively mature pearl oysters, hatcheries can rely on wild broodstock for spat production. However, such an approach is not feasible for a selection program based on specifically chosen broodstocks, families or individual genitors. All steps of reproduction would need to be controlled, including reproductive conditioning, which would have to be synchronized in order to have the chosen parents for a desired cross ready to breed at the same time.

As the growth and reproduction of bivalves are controlled by temperature (Janzel & Villalaz 1994; Albentosa, Beiras & Perez-Camacho 1994; Borcherding 1995) and food (Chavez-Villalba, Cochard, Le Pennec, Barret, Enriquez-Diaz & Caceres-Martinez 2003; Delaporte, Soudant, Lambert, Moal, Pouvreau & Samain 2006; Newell, Tettelbach, Gobler & Kimmel 2009), it is necessary to understand the physiological mechanisms of nutrition relating intake to reproductive development. Qualitative requirements in terms of sterols and fatty acids during oogenesis in reared pearl oysters show that the essential polyunsaturated fatty acids could be supplied by a diet composed of 2 algae species: T-*Iso* and *Chaetoceros gracilis* (Vahirua-Lechat, Laure, Le Coz, Bianchini, Bellais & Le Moullac 2008), but the physiological mechanisms controlling gonadal activity must also be understood to achieve optimal conditioning of broodstock in the hatchery.

Most basic knowledge on *P. margaritifera* broodstock conditioning is lacking. Although the quantitative nutritional requirements of pearl oyster reproduction are not known, the basis of the feeding behavior is understood to be driven by temperature and nutrition as in most bivalves (Bayne & Newell, 1983). Qualitative nutritional needs of *P. margaritifera* were studied by Vahirua-Lechat *et al.* (2008), who analysed female gonads in terms of fatty acids and sterols. Their results suggested that *Chaetoceros sp*. and Tahitian *Isochrysis aff. galbana*

(T-*Iso*) supply the main fatty acids and sterols, naturally present in the female gonads. Moreover, Hashimoto, Hyodoh, Hirose, Nishikawa, Katano & Nakano (2008) showed that the growth rate and glycogen content of *P. fucata* were highest when oysters were fed with *C. gracilis* and *Isochrysis*. Retention efficiency of *P. margaritifera* gills reached 98 % for the microalgae *C. gracilis* and T-*Iso*, and the highest CR values for this pearl oyster were observed with this combination of algae (Pouvreau, Jonquières & Buestel 1999). Chlorophyll *a* concentration is a bulk estimator of phytoplankton biomass but provides no information on the nutritional status of the pearl oyster. The range of chlorophyll *a* concentration in Polynesian lagoons such as Takapoto (Northern Tuamotu) is 0.1 to 0.8 µg L^{-1} (Buestel & Pouvreau, 1999; Delesalle, Sakka, Legendre, Pagès, Charpy & Loret 2001). In the laboratory, the transposition of these data into microalgae concentrations around pearl oysters gives an equivalent of 1000 to 8000 cell mL^{-1} of *Isochrysis aff. galbana* (T-*Iso*). On this basis, some preliminary feeding trials were carried out for reproductive conditioning, resulting in the spawning of about 50 % of the broodstock. However, such trials produced a drop in gametogenesis, since gonial cells disappeared for both males and females (Le Moullac, Hui, Vonau, Levy & Cochard 2009). These results indicated that more knowledge was needed in order to find ways of sustaining gametogenesis and gamete maturation in the hatchery.

The present study describes experiments aiming to define the quantitative nutritional requirements of *P. margaritifera* and to describe the link between food and the reproductive parameters of gonadic growth and gametogenesis. The influence of food was assessed (i) to determine the kinetic parameters of feeding: the maximum ingestion rate (Imax), the half saturation coefficient (Xk) and the assimilation efficiency (AE); (ii) to describe the link between food level and reproduction, studied by measuring gonad size; and (iii) to observe the germinal activity by histology, focusing on frequency of gonial cells in the gonad.

Materials and methods

Experimental set up

The pearl oysters were placed in tanks of 500 L volume with controlled flow-through. Seawater was renewed at the rate of 100 L h^{-1} and maintained at 26 °C during all the experiments. The pearl oysters were fed with a mixed diet composed of a 2:1 (v:v) mix of *Isochrysis galbana* (T-Iso) and *Chaetoceros gracilis* cells, supplied continuously with Ismatech rotary piston pumps.

Experimental designs

Experiment 1: Ingestion and assimilation estimations

The pearl oysters used in this experiment measured on average 108 ± 7 mm in height. Four tanks were used to allow simultaneous testing of 2 microalgae concentrations and measure the ingestion rate at the population scale. Each microalgae concentration was tested in two tanks: one with 6 pearl oysters and a control tank without any oysters. Different microalgae levels were tested that were representative of ambient environmental algae concentration around cultured pearl oysters. An automated sampling device recorded algal fluorescence at a rate of 3 measurements per hour. Ingestion rate (I) was calculated by the difference between the control and experimental tanks according to the following equation:

$$Iobs = (\mu alg\ control - \mu alg\ exp) * flowrate / dw$$

Since ingestion is a saturating function of algal concentration, ingestion was modeled by a hyperbolic function adapted from the Michaelis-Menten function:

$$Imod = (Imax \times \mu alg\ exp)/(Xk + \mu alg\ exp)$$

Where,

- Iobs is the observed ingestion rate, Imod is the modeled ingestion rate and Imax is the maximal ingestion rate; all three are expressed in cell.h^{-1}.g^{-1} dw.

- µalg is microalgae concentration in the control tank (control) and in the experimental tank with pearl oysters (exp), expressed in cell.μL^{-1}.

- dw is dry weight, expressed in g.

- flow rate is expressed in L h^{-1}.

- Xk is the half saturation coefficient, expressed in cell.μL^{-1}.

Dry weights were estimated using the length-weight relationship $W = 9.94\ 10^{-6}\ H^{2.78}$, where W is the tissue dry weight and H the shell height (Pouvreau 1999).

Assimilation efficiency (AE) of organic matter (OM) was assessed by analyzing microalgae and biodeposits according to the method of Conover (1966). The pearl oysters were laid out in a collector, in which the deposits were collected on a 10 µm sieve. Biodeposits were centrifuged for 15 min at 4500 t min^{-1}. The supernatant was removed and the pellet washed twice with ammonium formate (37 % in distilled water). The pellet was then put in a pre-weighed aluminum cup to be dried at 60 °C for 48 hours before being burnt at 450 °C for 4 hours. Microalgae OM was obtained by the centrifugation of 5 liters of the microalgae mixture and treatment of the pellet according to the same procedure as for the biodeposits. The absorption efficiency (AE, %) was then calculated according to the equation of Conover (1966):

$$AE = (\%OM_{\mu alg} - \%OM_{biodepot}) / (1 - \%OM_{biodepot}) \times (\%OM_{\mu alg})$$

Experiment 2: Dynamics of reproduction during broodstock conditioning

Pearl oysters (N = 120) of 90 ± 8 mm height were placed in 6 experimental tanks and conditioned for 45 days. The oysters were fed with the mixed diet described in the *Experimental set up* section and supplied continuously at one of three concentrations of microalgae corresponding to a daily supply of 1 %, 2 % or 3 % of oyster dry weight in algal dry weight per day (2:1 mixture of *Isochrysis galbana* (T-*Iso*) and *Chaetoceros gracilis* cells), resulting in microalgae concentrations of 0.5, 7 and 18 cell μL^{-1} around the pearl oysters.

Each food level was tested in duplicate. Initial sampling was of 20 pearl oysters. During the experiment, samples of 10 pearl oysters were taken from each tank (20 per food level) on days 15, 30 and 45. To maintain a constant biomass in the tanks, the sampled pearl oysters were replaced by others that had been adapted to similar experimental conditions.

Experiment 3: Relationship between food level and germinal activity

Forty pearl oysters of 85 ± 6 mm mean height were conditioned for 60 days. The oysters were fed with the mixed diet described in the *Experimental set up* section, supplied continuously at two food levels: 1.5 and 15 cell μL^{-1}. Initial samples of 10 pearl oysters were taken at the start of the experiment, and then two more samples of 10 pearl oysters were taken from each food level treatment on days 30 and 60.

Gonad analysis

Measurement of the reproductive effort

After flesh dissection, the visceral mass (VM) was drained on absorbant paper, weighed and put in 10 % formalin seawater for 72 h before being transferred into 70 % ethanol. VM were cut along the sagittal plane and digital images obtained using a desktop scanner (Fig. 1). The digital pictures were then analysed using ImageJ software. Gonad size was characterized using a gonad development index (GDI), which is equal to the ratio of the gonad surface (G) to the VM area of a sagittal section (GDI = G/VM).

Histology

For histological analysis, the fixed gonads were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned into 3 – 4µm slices on a rotary microtome, stained with Giemsa dye and finally mounted on glass microscope slides. Gametogenesis was analysed by recording the presence or absence of the gonial cells (GC). Normal gametogenesis was characterized by the continuous presence of gonia along the gonadal epithelium. Two situations were described as abnormal: the absence of any germ cells or the presence of clusters of gonia.

Statistics

AE was analysed using arcsine square root AE/100 value. Means were compared between the microalgae concentrations, using one-way ANOVA followed by Fisher's tests. The relationship between food level and reproduction was analyzed by examining changes in the visceral mass using two-way ANOVAs on the arcsine square root-transformed GDI. When a significant effect ($p < 0.05$) was found, a Multiple Range Test (Tukey honest significance difference test: HSD) was used to determine which means were significantly different. In all cases, a significance level (α) of 0.05 was used. Impact of food level on germinal activity frequency was analyzed using Fisher's exact test, which is designed for small samples.

Results

Ingestion rate modeling and assimilation efficiency

Eighteen measurements of ingestion rate were recorded over 24h, from 4 to 45 cell μL^{-1} algae. A double-inverse linearization allowed us to obtain a constants to estimate Imax and Xk, the kinetic parameters of feeding behaviour (Fig. 1). The slope **a** of the equation of the double inverse linearization was $7.55\ 10^{-8}$ and the ordinate of origin **b** was $5.18\ 10^{-9}$. This provided the result of Imax = 1/**b** = $1/ 5.19\ 10^{-9} = 1.93\ 10^{8}$ cell.h^{-1}.g^{-1} dw.

Xk, a trophic parameter, was obtained from the projection on the X-axis of the ingestion rate value corresponding to the half of the maximum ingestion rate (1/2 Imax) of the Y-axis where X_k=**a** x Imax = $7.55\ 10^{-8}$ x 1.93 108 = 15 cell.μL^{-1}.

AE efficiency significantly changed according to the microalgae concentration ($F=4.862$, $p=0.0078$). AE values did not significantly change between 4 and 25 cell μL^{-1} and remained between 64 to 70 %. At the microalgae concentration of 35 cell μL^{-1}, the AE dropped significantly to reach 43.9 % (Fig. 2).

Impact of food level on visceral mass weight and gonad development

The development of the gonad in the visceral mass is shown in Figure 3. The two-way ANOVA showed that food level significantly influenced GDI ($F = 21.292$, $p < 0.0001$), although time did not ($F = 1.498$, $p = 0.226$) (Fig. 4). The food level × time interaction was significant, however ($F = 2.499$, $p = 0.0435$), showing a similar effect as for VM weight: the GDI decreased significantly from the 30^{th} experimental day onwards (Fig. 4) with the lowest diet.

Impact of food level on the germinal activity

The gametogenic process of males appears to have significantly modified germinal activity (Fisher p value = $1.46\ 10^{-7}$), which slowed down (Fig. 5b) or stopped (Fig. 5c) according to the food level (table 1). At the low food level, the degradation of the germinal process increased as the experiment went on. Gonial cells were totally lacking in 89 % of males after 30 days of low food conditioning and 100 % after 60 days. At the high food level, the activity of GC decreased after 30 days of food conditioning and the proportion of males with continuous germinal activity fell to 11 %, whereas germinal activity had completely recovered (Fig. 5a) in 60 % of the males after 60 days of food conditioning.

Discussion

The functioning of bivalves for growth and reproduction is based on a relationship with environmental conditions, including water temperature and food level (Delaporte *et al*. 2006, Pouvreau, Bourles, Lefebvre, Gangnery & Alunno-Bruscia 2006). In depth investigations of pearl oyster reproduction are needed to enhance hatchery performances and to improve our understanding of reproductive behaviour in the wild and its implications for natural populations. The present study clearly demonstrated that algal supplements constitute key parameters that exert active control over the gametogenesis of *P. margaritifera* in laboratory condition. Our experimental approach allowed us to find a suitable food level for conditioning

218 *P. margaritifera* pearl oysters in a hatchery. Using a 2:1 mixture of *Isochrysis galbana* and
219 *Chaetoceros gracilis* cells, we showed that the ingestion rate increases with food density at a
220 decelerating rate until a maximum is reached above which ingestion rate remains constant.
221 AE was maximal until the microalgal concentration of 30 cell μL^{-1} corresponded to twice the
222 half saturation coefficient; it then decreased significantly.
223 Knowing the feeding parameters, it was then useful to study the link between food level and
224 reproduction by testing the impact of food level on gonad growth. To study this relationship,
225 we developed a tool to estimate reproductive effort, based on image analysis of pictures of the
226 gonad within the visceral mass. Other techniques, such as histology (Saucedo, Rodriguez-
227 Jamarillo & Monteforte 2002) or quantitative histology (Enriquez-Diaz, Pouvreau, Chavez-
228 Villalba & Le Pennec 2009), are too complex to use for the estimation of reproductive effort.
229 With this technique, we were able to show the relationship between food level and the
230 reproductive effort in *P. margaritifera.* The quantities of microalgae provided during this
231 conditioning experiment corresponded to 1, 2 and 3 % of oyster dry weight in algal dry
232 weight per day. Expressing this quantity as a percentage allowed us to compare these ratios
233 with those tested on other bivalves in controlled environments (Chavez-Villalba, Barret,
234 Mingant, Cochard & Le Pennec 2002; Delaporte *et al*. 2006). The smallest ratio proposed
235 during our experiments would be insufficient for mollusks from temperate areas as the ratios
236 they require lie in the higher range of 3 to 5 %. Our highest ratio tested (3 %) is at the bottom
237 of this range. Our results revealed the relationship between the food and the reproductive
238 effort for *P. margaritifera.* According to Utting & Millican (1997), 3 % seems to be a ratio
239 sufficient to compensate for the needs of the majority of the mollusc species for growth and
240 reproduction.
241 The effect of environmental conditions on gametogenesis was clearly revealed by observation
242 of the male cell line showing that at low trophic level, spermatogenesis slowed down or

stopped in *P. margaritifera*. This experiment was built with males because first females do not appear before 2 year old and a balanced sex ratio is attained only around 9 year old (Chavez-Villalba, Soyez, Huvet, Gueguen, Lo, Le Moullac 2011). The relationship between food level and gametogenesis occurs also in older females in which ovogenesis stops at low trophic level. (Le Moullac *et al*. 2009). Although the importance of energetic reserves has been remarked in bivalves in general (Chavez-Villalba *et al.* 2003), pearl oysters have no specialized reserve tissue and very little information is available on their energy partitioning. Reproduction in this species appears to be highly dependant on immediate food availability. A similar relationship was found in the echinoderm *Lytechinus variegatus*, in which histological examination of the testicular tubules of starved individuals revealed the presence of spermatocytes, spermatids and some mature spermatozoa, but the absence of a germinal epithelium. When animals were re-fed, the testicular tubules developed thin, discontinuous patches of germinal epithelium 8 and 16 days later and continuous zones of germinal epithelium in the testes 32 days later (Bishop & Watts, 1994). Food availability is known to be an important factor for bivalve development, affecting broodstock energy reserves and gametogenesis, duration of the maturation process, fecundity, quality and quantity of eggs, and larval development (Muranaka & Lannan, 1984, Utting & Millican 1997, Chavez-Villalba *et al*. 2003, Delaporte *et al*. 2006).

The total or partial lack of gonia along the tubular epithelium in our study suggests that the mitotic process of the germinal stem cells (GSC) was altered. In adult Pacific oyster *C. gigas*, the population of germinal cells appears to be renewed annually by proliferation at the beginning of the cycle of reproduction. The GSC differentiation into gonia and multiplication of these gonia represent the beginning of gametogenesis. Intensity and duration of differentiation of GSCs and multiplication of gonia are controlled by mechanisms utilizing environmental parameters such as temperature and photoperiod (Fabioux, Huvet, Le Souchu,

Le Pennec & Pouvreau 2005). The results of the present work showed that differentiation of GSCs is probably under the control of food level. The analysis of this step of gametogenesis is, therefore, a relevant tool to assess the quality of the environment for broodstock conditioning. Similar studies focusing on females relate to the frequency distribution of the oocyte size (Lango-Reynoso, Chavez-Villalba, Cochard & Le Pennec 2000; Bishop & Watts 1994).

From a practical point of view, our work has provided some useful information on the hatchery broodstock conditioning of the pearl oyster *P. margaritifera*.

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365

365 Table 1: Effect of food level on gonial cell line activity after 30 and 60 days of
366 conditioning.

Food level	Conditioning period (days)	GC lacking	GC cluster	Continuous GC
initial	0	10 %	0 %	90 %
1.5 cell μL^{-1}	30	89 %	11 %	0 %
	60	100 %	0 %	0 %
15 cell μL^{-1}	30	56 %	33 %	11 %
	60	10 %	30 %	60 %

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Figure captions

Figure 1: Ingestion by the pearl oyster *P. margaritifera* and modeling of ingestion according to algal concentration.

Figure 2: Assimilation efficiency of the pearl oyster *P. margaritifera* according to algal concentration (means are presented with standard deviation, N = 4) Significant differences between the different experimental groups are shown by different letters (Tukey post hoc test, $P < 0.05$).

Figure 3: Gonad development in the saggital plane of the visceral mass of the pearl oyster *P margaritifera*.

Figure 4: Effect of food level on gonad index of the pearl oyster *P. margaritifera* over 45 days of conditioning at 0.5 (light grey), 7 (grey), 18 (dark grey) cell μL^{-1}, initial (white). (means are presented with standard deviation, N = 20 at day 0, 15 and 30 and N = 40 at day 45). Significant differences between the different experimental groups are shown by different letters (Tukey post hoc test, $P<0.05$).

Figure 5: Histological view of a male gonad showing continuous (a), discontinuous (b) and lacking (c) gonial cell (GC) line. Arrows indicate gonial cells.

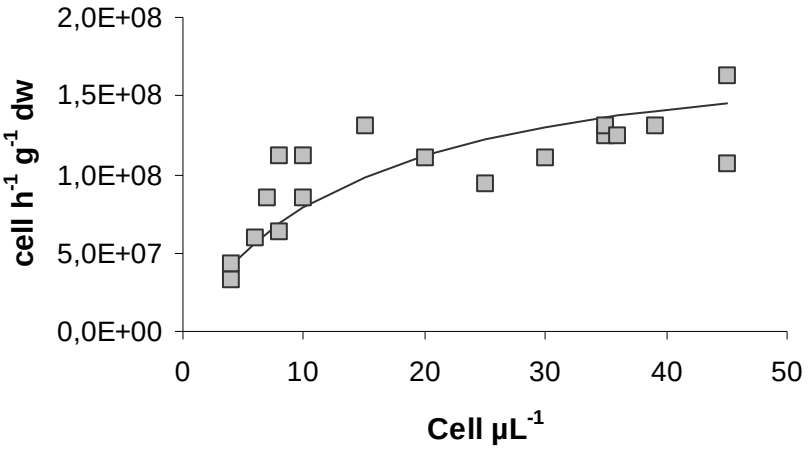


Figure 1

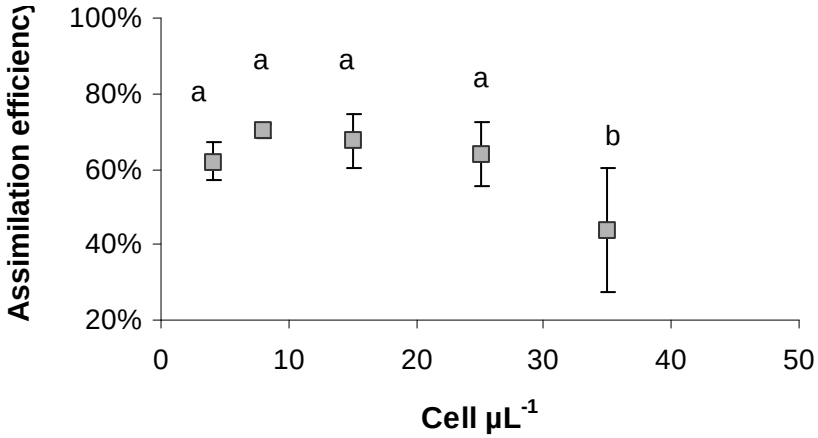


Figure 2

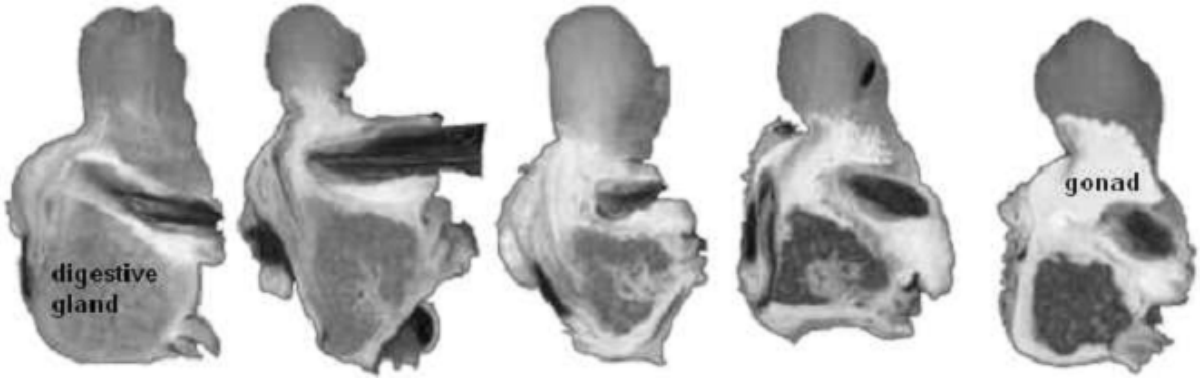


Figure 3

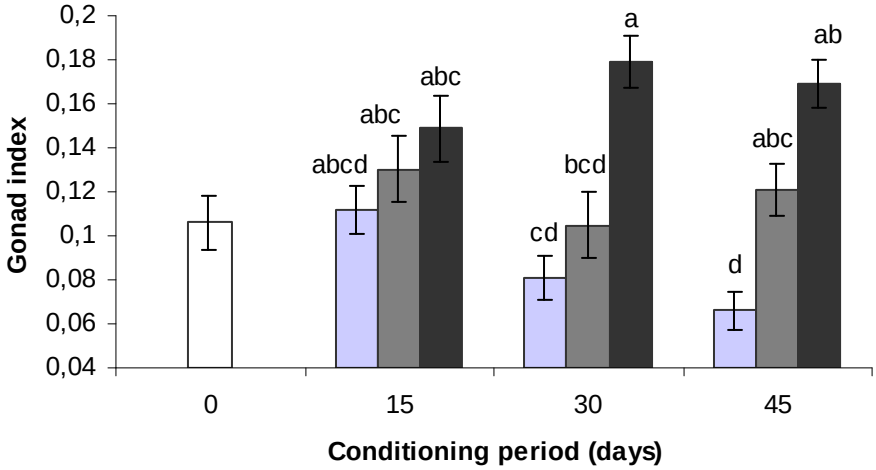
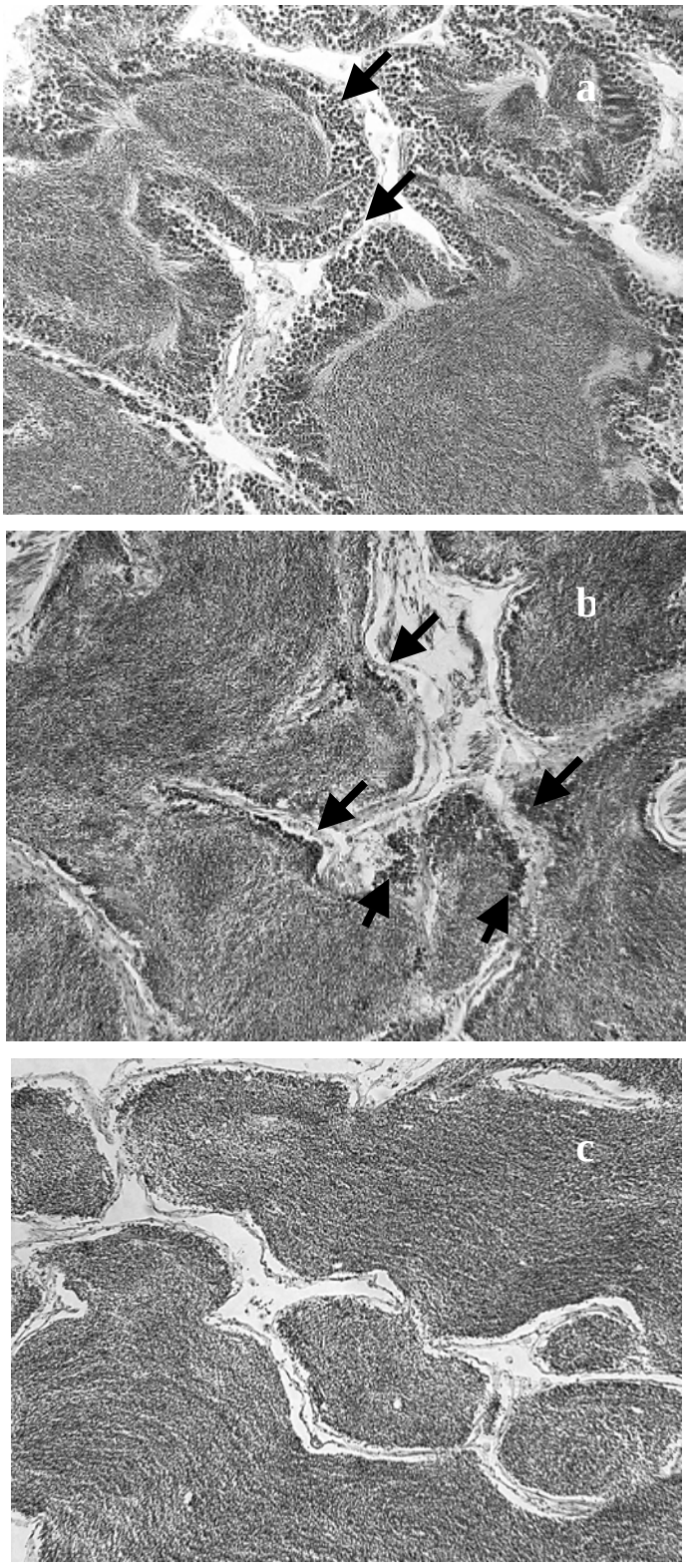


Figure 4



411 Figure 5

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