

Anesthesia in Pacific oyster, *Crassostrea gigas*

Marc Suquet^a, Goulwen de Kermoysan, Ricardo Gonzalez Araya, Isabelle Queau, Luc Lebrun, Pierrick Le Souchu and Christian Mingant

Ifremer, Station expérimentale Argenton, Département de Physiologie fonctionnelle des organismes marins (PFOM), 11 presqu'île du Vivier, 29840 Argenton, France

Received 16 September 2008; Accepted 6 January 2009

Abstract – A reliable anaesthesia and sampling protocol for Pacific oysters will enable experiments to be conducted without sacrificing animals and will facilitate successive sampling of individuals for gametogenesis studies. As no such techniques were available for *Crassostrea gigas*, the present study aimed to define suitable anaesthetic conditions for use with this species. Three groups of ten oysters (mean weight \pm SD, 32.1 ± 9.0 g) were anaesthetised in 5 L containers. Among different chemicals: benzocaine, eugenol and three different types of magnesium chloride (a laboratory one – Flucka[®] – and two designed for agriculture – DEUSA International[®] and Dead Sea Works[®]) and concentrations tested, one laboratory (concentration: 72 g L^{-1}) and one agricultural (Dead Sea Works[®] 50 g L^{-1}) type of magnesium chloride were the most effective, respectively inducing anaesthesia in $73 \pm 3\%$ and $80 \pm 3\%$ after three hours. Lower oyster weight and a two day period of starving prior to treatment significantly increased the number of anaesthetised animals. Using this protocol, losses of 0 to 10% of oysters were observed one week after anaesthesia. Increasing anaesthesia duration from 3 to 16 h resulted in a significant increase in the number of anaesthetised oysters (from 50 ± 10 to $97 \pm 7\%$) but no increase in mortality ($7 \pm 11\%$). On the other hand, reducing water temperature from 19.5°C to 15.3°C , resulted in a significant decrease in anaesthesia efficacy. A reliable anaesthesia protocol was developed: 100% of Pacific oysters are anaesthetised using $50 \text{ g L}^{-1} \text{MgCl}_2$ Dead Sea Work[®] for a 16 h duration. This long duration facilitates tissue sampling and does not increase the working time needed. This protocol was validated by monthly anaesthesia and gonad sampling during a three month period with the loss of only a single oyster. It can thus be used for routine applications.

Key words: Anaesthesia / Relaxant / Magnesium chloride / Gonad sampling / Pacific oyster / *Crassostrea gigas*

Résumé – Anesthésie de l'huître creuse, *Crassostrea gigas*. Un protocole complet d'anesthésie chez les huîtres creuses devrait faciliter l'échantillonnage successif des mêmes animaux, sans les sacrifier, favorisant notamment l'étude de la gamétogenèse. Cette étude a pour but de définir des conditions fiables d'anesthésie adaptées à l'huître creuse, *Crassostrea gigas*. Trois groupes de 10 huîtres chacun (poids moyen \pm écart-type, $32,1 \pm 9,0$ g) ont été anesthésiés dans des récipients de 5 L. Parmi les différents anesthésiants testés : benzocaïne, eugénol et trois types de chlorure de magnésium différents (un de laboratoire – Flucka[®] et deux agricoles : DEUSA International[®] et Dead Sea Works[®]), les chlorures de magnésium de laboratoire (concentration : 72 g L^{-1}) ou destinés à l'agriculture (Dead Sea Work, 50 g L^{-1}) sont les plus efficaces, anesthésiant respectivement $73 \pm 3\%$ et $80 \pm 3\%$ des huîtres après un traitement de trois heures. Un poids plus faible des huîtres ou une mise à jeun préalable de deux jours améliorent significativement les résultats. Une semaine après l'anesthésie, des pertes comprises entre 0 et 10 % des huîtres sont observées. L'accroissement de la durée du traitement de 3 à 16 h augmente de façon significative le nombre d'huîtres anesthésiées (de 50 ± 10 à $97 \pm 6\%$) sans modifier la mortalité observée ($7 \pm 11\%$). La baisse de la température de l'eau de $19,5$ à $15,3^\circ\text{C}$ diminue significativement l'efficacité de la technique. Ce protocole d'anesthésie s'avère très fiable : 100 % des animaux sont anesthésiés après un bain dans une solution de 50 g L^{-1} de MgCl_2 (Dead Sea Work[®]) durant 16 h. Cette longue durée facilite l'échantillonnage et n'accroît pas la charge de travail. Le protocole a été validé par l'anesthésie et le prélèvement mensuel successif de gonades, réalisés durant une période de trois mois ; à l'exception d'une seule huître, aucune mortalité n'a été constatée. Ce protocole peut être utilisé lors de pratiques aquacoles.

^a Corresponding author: msuquet@ifremer.fr

1 Introduction

Tissue sampling in bivalves usually relies on destructive method: the shell is removed and tissues are sampled. As a consequence, successive sampling cannot be carried out in a single individual and no method is available for example, for individual reproductive cycle monitoring. Sophisticated non invasive techniques have been recently developed using magnetic resonance imaging (MRI): MR imaging can be used in marine bivalves to depict the soft tissue anatomy of Pacific oysters (Pouvreau et al. 2006). However feasible its potential application in aquaculture remains too expensive at present.

Anaesthesia may be used to reduce stress associated with handling and transportation. It also facilitates tissue biopsies and sex determination. Such techniques have been successfully studied in fish species (Mylonas et al. 2005). In molluscs, anaesthesia has mainly been studied in pearl producing species as it induces wide valve opening and thus eases the surgical techniques used in pearl production. Propylene phenoxetol was successfully used in *Pinctada albina*, *Pinctada maxima* and *Pinctada imbricata* (Mills et al. 1997; O'Connor and Lawler 2002; Mamangkey in press). Anaesthesia has been used to facilitate the study of the digestive system, from the mouth to the anus, in pearl oyster *Pinctada fucata martensii* (Handa and Yamamoto 2003).

Anaesthesia has also been studied in aquaculture species grown for food production: magnesium chloride is a suitable non-toxic anaesthetic for sea urchins, *Paracentrotus lividus* (Arafa et al. 2007) and Sydney rock oyster, *Saccostrea glomerata* (Butt et al. 2008). Among 6 anaesthetics, magnesium chloride induces rapid anaesthesia and recovery of flat oysters, *Ostrea edulis*, without mortality (Culloty and Mulcahy 1992).

Biopsy with needles is a means of obtaining tissue samples for sex determination, physiological and genetic studies. Compared with controls, no increase in mortality was recorded after mantle biopsy of freshwater mussels (Berg et al. 1995), and no mortality was observed after gonad sampling of anaesthetised pearl oysters (Acosta Salmon and Southgate 2004).

In Pacific oyster, the only available study on anaesthesia was published by Namba et al. (1995), who recorded shell opening in oysters treated with magnesium chloride. Higher shell opening rates were observed with increasing anaesthesia duration and $MgCl_2$ concentration. However, result variations due to changes in anaesthesia conditions including, for example, the use of different anaesthetics, the effect of oyster weight and temperature or light conditions were not quantified. Subsequent mortality was also insufficiently documented, and gonad sampling after anaesthesia was not studied. Finally, the use of laboratory $MgCl_2$ is expensive for large water volumes.

A practical anaesthesia and sampling protocol would facilitate successive samplings on individuals, for different kinds of descriptive studies such as those on gametogenesis, while avoiding oyster sacrifice. This paper aims at establishing a reliable anaesthesia and sampling protocol for the Pacific oyster by describing the effect of rearing parameters and gonad sampling on the number of oysters anaesthetised and their survival after one week. Preliminary experiments conducted in our laboratory showed that adding microalgae modified neither the number of anaesthetised oysters nor the number that died.

Furthermore, increasing artificial light intensity from dark to 60 lux had also no significant effect on oyster anaesthesia.

2 Methods

Adult Pacific oysters (mean weight \pm SD, 32.1 ± 9.0 g) were transferred from Aber Benoit (North Brittany, France) to the Argenton experimental hatchery. They were maintained in flow-through seawater tanks and fed daily according to Chavez-Villalba et al. (2002). Treatments that are non toxic to humans were used in this study. Unless otherwise stated, the following standard conditions were used for anaesthetic treatment: 3 replicate groups of 10 oysters each were anaesthetised in 5 L containers (water temperature: 19 °C) using 50 g L⁻¹ Dead Sea Work[®] $MgCl_2$ ($MgCl_2$: 46.5–47.5%, $CaCl_2$: 2.0–2.2%, $NaCl$: 0.5–0.8%, KCl : 0.2–0.5%) and water; dilution medium: 3 L freshwater and 2 L seawater to maintain salinity of oyster rearing environment at 35–38‰. Oysters were considered as anaesthetised when, after three successive gentle pressures on valves, shell closure was not observed. The control groups received no anaesthetic. After three hours, the number of anaesthetised oyster was assessed. Oysters were then returned to clean seawater. The oysters were judged to have recovered when they closed their valves by themselves. Survival was monitored for one week after exposure to the anaesthetic treatments. A set of 9 experiments was conducted during this study.

2.1 Chemicals and concentrations

To select the most suitable anaesthetic and its concentration, different chemicals and doses were tested. Three types of magnesium chloride were tested, one intended for laboratory use (Flucka[®], Czech Republic) and two designed for agriculture (DEUSA international[®], Germany and Dead Sea Work[®], Israel). $MgCl_2$ was dissolved in freshwater and seawater volumes, mixed to maintain the salinity of the oyster rearing environment in the anaesthetic solution (Table 1). Benzocaine and eugenol (99%) were dissolved in ethanol (0.1 g benzocaine in 1 ml ethanol and 1 ml eugenol in 9 ml ethanol). To examine the effect of ethanol exposure, three groups of five oysters each were maintained in 5 L containers with 0, 10 and 100 ml L⁻¹ of ethanol for three hours. Oysters were then returned to clean seawater. No mortality was observed after one week in the ethanol test.

$MgCl_2$ Dead Sea Work[®] concentration. The second experiment aimed at determining the effect of $MgCl_2$ Dead Sea Work[®] concentration: oyster groups were submitted to 35, 50 or 72 g L⁻¹ diluted in fresh and seawater solutions, designed to maintain salinity at a constant value (Table 1).

2.2 Starvation

Because filtration could be enhanced in unfed oysters, the effect of starvation before treatment on subsequent anaesthesia success was tested in oysters maintained for 0, 2 and 5 days out of seawater before the experiment.

Table 1. Chemicals, doses and medium used during the 9 experiments (N° refer to experiments 1 and 2).

N°	Chemicals	Concentration (g L ⁻¹), except mentioned unit	Dilution medium		
			Fresh water (L)	Seawater (L)	Ethanol (ml)
1	MgCl ₂ DEUSA®	72	5		
2	Benzocaine	500 mg L ⁻¹		5	25
3	MgCl ₂ DEUSA®	50	3	2	
4	Eugenol	1.5 ml L ⁻¹		5	68
5	Benzocaine	1200 mg L ⁻¹		5	60
6	Eugenol	5 ml L ⁻¹		5	225
7	MgCl ₂ Flucka®	50	3	2	
8	MgCl ₂ Flucka®	72	5		
9	Dead Sea Work®	50	3	2	
10	Dead Sea Work®	35	2.1	2.9	
11	Dead Sea Work®	72	5		
12	Control			5	

2.3 Water temperature

To assess the effect of water temperature, groups of oysters were anaesthetised at 15.3 ± 0.4 °C or 19.5 ± 0.4 °C. Water temperature was maintained using thermo-baths. In addition to non anaesthetised oysters at 19.5 °C, a second control group was used to test the effect of temperature changes (from 19.5 °C to 15.3 °C, maintained at 15.3 °C during three hours and then back to 19.5 °C) on oyster mortality without anaesthetic.

2.4 Anaesthesia duration

To determine the effect of anaesthesia duration, oyster groups were left in the anaesthetic solution for 3 h, 4 h, 6 h, 16 h, 19 h and 22 h. At the end of anaesthesia, the percentage of recovered oysters (presenting closed valves) 30 min after their return to clean seawater was assessed in relation to anaesthesia duration.

2.5 Oyster weight

The effect of oyster weight was assessed by exposing two oyster populations of differing mean weight (32.1 ± 9.0 g and 66.6 ± 16.7 g) to anaesthesia. Two control groups were used for this experiment, one representing each population.

2.6 Gonad sampling

The effect of sampling was observed by sampling gonads (sample volume < 0.05 ml) on the left side of anaesthetised oysters (above the adductor muscle) of the two weight groups (experiment 6), using a 1 ml syringe and a needle (0.6×30 mm, 23 gauge). Survival of the two oyster populations was then monitored compared with two controls (1: non anaesthetised and non sampled oysters, 2: anaesthetised but non sampled oysters).

2.7 Filtration capacity

This experiment aimed to assess filtration capacity during anaesthesia. Three groups of three oysters each were individually transferred to 2 L beakers after 4 h or 16 h anaesthesia treatment or without anaesthesia (control). 10^5 ml⁻¹ *I. affinis. galbana* were added to each beaker. Algae consumption was estimated every 10 min by Coulter measurements (Rico-Villa et al. 2006). In order to avoid limiting conditions, experiments were stopped when oxygen (Bioblock WTW Oxi 340) and algae concentrations were respectively lower than 5.9 mg L⁻¹ and 0.7×10^4 ml⁻¹.

Individual algae concentration (IAC) was estimated as:

$$IAC = (N_1 - N_2)(DW \times T)^{-1}$$

where N_x is algae concentration assessed during sampling x (number ml⁻¹), DW is dry weight (obtained after lyophilisation at -50 °C for 72 h of previously frozen samples and then weighed to the nearest 0.01 g) and T is time duration in minutes between two successive samplings.

2.8 Method validation

The final experiment aimed at validating the protocol established in this work by determining the effect of successive anaesthesia and gonad sampling. Oysters were submitted monthly to 5 h or 16 h anaesthesia and sample were taken from their gonads. This experiment lasted three months (four samplings).

For each experiment, the percentages of anaesthetised oysters were recorded and the percentage of oysters surviving was recorded after one week.

2.9 Statistical analysis

To compare the percentages of oysters anaesthetised between the different treatments, the controls were excluded from statistics – no anaesthetised oysters having been observed

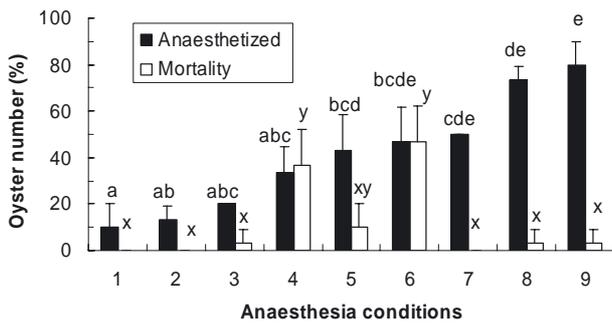


Fig. 1. Effects of anaesthetics and their concentrations on the percentage of anaesthetised oysters and subsequent mortality. 1: MgCl₂ DEUSA® 72 g L⁻¹, 2: Benzocaine 500 mg L⁻¹, 3: MgCl₂ DEUSA® 50 g L⁻¹, 4: Eugenol 1.5 ml L⁻¹, 5: Benzocaine 1200 mg L⁻¹, 6: Eugenol 5 ml L⁻¹, 7: MgCl₂ Flucka® 50 g L⁻¹, 8: MgCl₂ Flucka® 72 g L⁻¹ and 9: MgCl₂ Dead Sea Work® 50 g L⁻¹ (Different letters refer to significantly different results within parameter: *a* to *e* normal font for percentage of anaesthetised oysters, *x* and *y* bold font for mortality, mean ± SD).

in these groups. On the other hand, controls were used in the analysis of mortality, when mortality was recorded seven days after anaesthesia. After angular transformation, the percentages from each of the three replicates of 10 oysters per treatment were compared using one way ANOVA, except for experiment 6 in which a Student-t test was used. When the ANOVA result indicated that differences were significant, a Tukey a posteriori test was used for comparison. When variances were not homogeneous, a Kruskal-Wallis non parametric test was used (experiments No. 3 and 4).

3 Results

Compared to eugenol (1.5 ml L⁻¹), benzocaine (500 mg L⁻¹) and MgCl₂ DEUSA® (50 and 72 g L⁻¹), a significantly higher percentage of anaesthetised oysters ($p < 0.001$) was observed using both the laboratory (72 g L⁻¹) and agricultural Dead Sea Work® (50 g L⁻¹) magnesium chloride (Fig. 1). After one week in clean seawater, a significantly higher ($p < 0.001$) mortality was recorded using eugenol (1.5 and 5 ml L⁻¹) compared with other chemicals, except benzocaine (1200 mg L⁻¹). The percentage of anaesthetised oysters was not significantly different between MgCl₂ concentrations 35 g L⁻¹ (23 ± 6%), 50 g L⁻¹ (47 ± 15%) or 72 g L⁻¹ (43 ± 15%) Dead Sea Work® magnesium chloride. No mortality was observed one week after this second experiment.

Increasing the duration of starvation before anaesthesia from 0 to 2 days resulted in a significantly higher ($p < 0.001$) percentage of anaesthetised oysters. A further increase of starvation duration from 2 to 5 days did not increase this percentage further (Fig. 2). Oyster mortality was not significantly affected by the length of starvation period.

A significantly higher ($p < 0.001$) number of oysters was anaesthetised at 19.5 °C (70 ± 0%) compared with 15.3 °C (47 ± 6%). No mortality was observed in either of the controls (constant 19.5 °C for the 1st control and temperature change

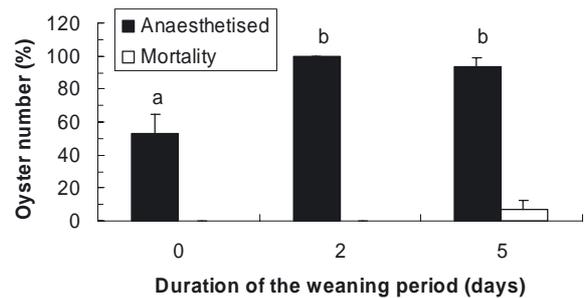


Fig. 2. Effect of preceding starvation duration on the percentage of anaesthetised oysters (Different letters refer to significantly different results; mean ± SD).

from 19.5 to 15.3 and back to 19.5 for the 2nd control) or following the anaesthesia treatments. Increasing anaesthesia duration from 3 to 16 h significantly increased ($p < 0.01$) the percentage of anaesthetised oysters (Fig. 3a). After 16 h duration, close to 100% oysters were anaesthetised. Increasing anaesthesia treatment duration did not significantly change oyster recovery percentage observed after 30 min (Fig. 3b). After 60 min in clean seawater, shell closure was observed in 100% oysters. No significant increase was observed in mortality percentage when increasing anaesthesia duration from 3 to 22 h. A significantly higher ($p < 0.05$) number of oysters (67 ± 21%) in the lower body weight group was anaesthetised compared with the larger group (27 ± 12%). No subsequent mortality was observed. Gonad sampling induced 0 to 10% mortality. However, regardless of oyster mean weight, sampling did not induce significantly higher mortality compared with non sampled oysters.

No algae consumption was observed in oysters maintained during 4 h or 16 h in anaesthetic solution compared with non anaesthetised animals ($p < 0.001$; Fig. 4). Monthly anaesthesia and gonad sampling over a three month period did not appear to increase mortality as only one animal died (5 h anaesthesia treatment). When maintained for 5 and 16 h in 50 g L⁻¹ Dead Sea Work® MgCl₂, 90 to 100% and 100% oysters were anaesthetised, respectively. Shell opening was wider when anaesthesia duration was increased to 16 h, facilitating gonad sampling.

4 Discussion

Among the different chemicals tested, two types of magnesium chloride were selected as the most suitable anaesthetics for Pacific oyster. Magnesium chloride has been used successfully for many invertebrates such as sea urchin, *Paracentrotus lividus* (Arafa et al. 2007), cephalopods (Messenger et al. 1985), scallop, *Pecten fumatus* (Heasman et al. 1995), queen conch, *Strombus gigas* (Acosta Salmon and Davis 2007), pearl oyster, *Pinctada albina* (Norton et al. 1996), flat oyster (Culloty and Mulcahy 1992), but also Pacific oyster (Namba et al. 1995). Invertebrate muscle contractibility depends on interactions between Ca²⁺ and Mg²⁺ ions (Altura et al. 1987). The facilitating effect of Ca²⁺ ions on synaptic transmission is inhibited by Mg²⁺ (Kandel et al. 2000). Because Dead Sea

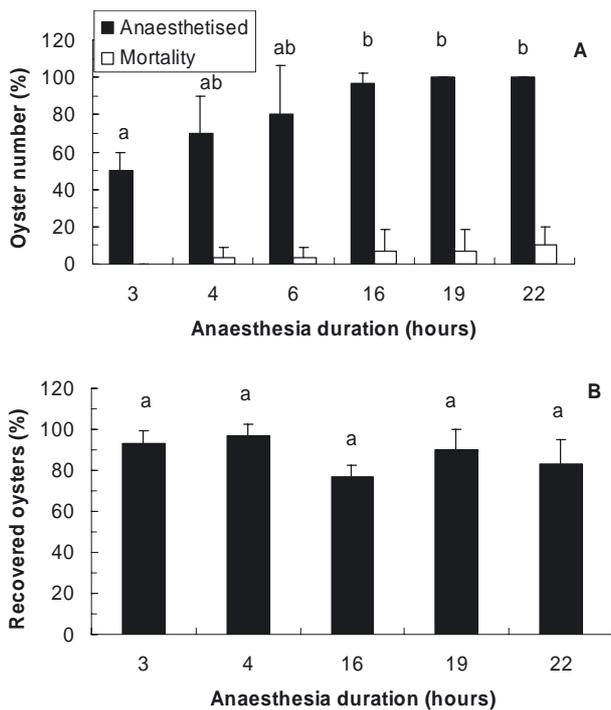


Fig. 3. Effect of anaesthesia duration. A: effect on the percentage of anaesthetised oysters, B: effect on the percentage of recovered oysters after 30 min in clean seawater (Different letters refer to significantly different results; mean \pm SD).

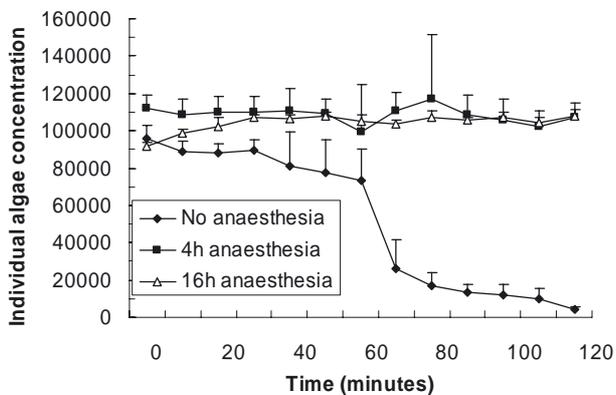


Fig. 4. Algae consumption in oysters. Changes in individual algae concentration in relation with time and oyster anaesthesia (mean \pm SD).

Work[®], MgCl₂ intended for agricultural purposes, is as effective as a laboratory magnesium chloride but 14 times less expensive, we selected this product as the best anaesthetic for Pacific oysters.

Depending on concentration used (500 and 1200 mg L⁻¹), benzocaine induced anaesthesia in 10 to 40% oysters only. Benzocaine is efficient in pearl oysters (1200 mg L⁻¹; Norton et al. 1996), but is not considered as suitable for juveniles of green sea urchin, *Strongylocentrotus droebachiensis* (Hagen 2003). Whatever the concentration used, eugenol, the active ingredient of clove oil, induced mortality in all oysters that opened their shells. On the other hand, eugenol is suitable

for many fish species (Mylonas et al. 2005), juvenile prawns, *Penaeus japonicus* (Hiroko et al. 1974) and octopus, *Octopus minor* (Seol et al. 2007).

As observed in our preliminary experiments, adding micro-algae to the anaesthetic medium did not increase the oyster percentage that opened their shells. This addition was intended to trigger filtration behaviour and thus the effect of magnesium chloride into the oyster. On the other hand, a previous two-day starvation period increased the percentage of anaesthetised oysters, suggesting that anaesthesia could indeed be strengthened by filtration.

In contrast to our work on size in the present study, Norton et al. (1996) found that pearl oyster size (range 9 to 17 cm) had no effect on anaesthesia success. Using propylene phenoxetol, O'Connor and Lawler (2002) found that pearl oyster size had only a small effect on the time they needed to open their valves.

Increasing anaesthetic treatment duration increased the number of anaesthetised Sydney rock oysters (Butt et al. 2008). After 3 h, a maximum of 86% anaesthetised Pacific oysters was observed by Namba et al. (1995). In the present work, 100% oysters were opened after a 16 h anaesthesia treatment and this duration did not increase subsequent oyster mortality. Anaesthetising all the oysters without subsequent mortality is essential for studies describing individual cycles.

In contrast to the present work, MgCl₂-induced anaesthesia in scallop was not affected by temperature (ranging from 12 to 24 °C, Heasman et al. 1995). Namba et al. (1994) found no significant effect of water temperature on Pacific oyster adductor muscle relaxation. However, these authors used lower temperature values (5 and 15 °C) than those used in the present work (15.3 and 19.5 °C).

Monthly anaesthesia and gonad sampling over a three month period did not induce oyster mortality. This method therefore provides a valuable tool for studying individual cycles such as gametogenesis. The effects of successive samplings have never been previously described either in anaesthetised Pacific oyster or in any similar species. Non lethal effects of anaesthesia were not investigated in the present study. A possible effect of repetitive sampling on gonad development cannot be rejected without further study. Although exposure to magnesium chloride did not have long term effects on immunological parameters in Sydney rock oysters (Butt et al. 2008), secondary effects remain possible in Pacific oyster.

In conclusion, a complete and practical protocol for Pacific oyster anaesthesia was established in this study. Pacific oyster can be anaesthetised using 50 g L⁻¹ MgCl₂ Dead Sea Work[®] for a 16 h duration. This long duration improves shell opening in 100% oysters, facilitating tissue sampling. Furthermore, a 16 h anaesthesia treatment does not increase the working time needed: the oysters are transferred to the anaesthetic solution at the end of the afternoon and sampled the following morning. This protocol was validated by four successive anaesthesia treatments and gonad samplings tested on the same animals, with a very low subsequent mortality. It will now be tested on other bivalve species.

Acknowledgements. The authors wish to thank R. Brizard (Ifremer) and M. Jamin (*Cabinet vétérinaire aquacole*) for fruitful discussions

on the protocols. Many thanks to H. McCombie-Boudry for corrections to the English.

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