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ANIMAL SCIENCE

Effects of agricultural-grade magnesium chloride as an anesthetic agent and a gonad sampling technique on white scar oysters (*Crassostrea belcheri*)

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Abstract: The effects of agricultural-grade magnesium chloride ($MgCl_2$) and gonad sampling on white scar oysters (*Crassostrea belcheri*) were evaluated. Oysters were anesthetized with 4 $MgCl_2$ solutions at 4 concentrations (0, 30, 50, or 70 g L^{-1}) for 24 h. The most effective solution was subsequently used to study the effects of gonad sampling using needles of different sizes (20G, 22G, or 24G) on *C. belcheri*. The results revealed no significant differences in the percentage of anesthetized oysters after exposure to 30, 50, or 70 g L^{-1} $MgCl_2$ for 24 h. However, the 50 g L^{-1} $MgCl_2$ solution was most effective at inducing anesthesia in *C. belcheri,* with 86.67±6.67% after 15 h. No significant differences in mortality rates were observed 7 days after anesthesia among the oysters immersed in 30, 50, or 70 g L^{-1} $MgCl_2$. The 20G, 22G, and 24G needles enabled gonad sampling for sex determination, with no mortality in any treatment group after 15 days. Therefore, a cost-effective anesthesia protocol and gonad sampling method was established for *C. belcheri*. These protocols are valuable for *C. belcheri* broodstock management, facilitating effective breeding and other research studies requiring *C. belcheri* survival after tissue sampling.

Key words: anesthetic agent, broodstock management, *C. belcheri*, muscle relaxation, tissue sampling.

INTRODUCTION

Crassostrea belcheri, commonly known as the white scar oyster, is a large oyster that tolerates a wide range of salinities. *C. belcheri* is extensively distributed across Southeast Asian countries, including the Philippines, Vietnam, Malaysia, Indonesia, Myanmar, and Thailand (Angell 1986, Phuwan et al. 2018, Maung et al. 2021, Ninwichian et al. 2021). In Thailand, *C. belcheri* is considered an economically important species that is cultured extensively in near-shore areas, shallow-water bays, and estuaries (Phuwan et al. 2018). *C. belcheri* culture and the culture of other oyster species in Thailand, relies primarily on natural seedlings. However, a decrease in both the quality and quantity of these natural seedlings coupled with a reduction in their growth rates, has been observed, leading to a decrease in oyster production (Choopunth & Tanyaros 2002, Pianpon & Eiadkaew 2023). Oyster production in Thailand decreased from 15,746.97 to 8,294.26 tons from 2020–2022 (Fishery Statistics Group 2022, 2023). Consequently, producing *C. belcheri* from hatcheries is an efficient approach for ensuring an adequate supply of high-quality oyster seedlings. However, one important step to enable efficient *C. belcheri* production from hatcheries before breeding events relies

on determining the sex of the oysters and assessing the maturity of their gonads. Among 2,000 natural *C. belcheri* broodstocks collected from Bandon Bay, Thailand, the observed sex ratio was approximately 70% female and 30% male (P. Ninwichian, unpublished data). In general, techniques for determining oyster sex or assessing the maturity of their gonads use either lethal or nonlethal procedures. For lethal procedures, the oyster shells must be opened and removed; subsequently, the gonad tissues are sampled to determine sex and evaluate gamete quality before stripping. If the gametes are not ready or if the required number of male and female oysters is insufficient to ensure successful fertilization, many oysters can be sacrificed. Therefore, the development of noninvasive methods to determine oyster sex and assess gamete quality while minimizing the loss of valuable oyster broodstocks would be beneficial for both oyster aquaculture and research (Suquet et al. 2009, 2010). In nonlethal procedures, the oyster sex can be distinguished from the release of gametes from mature oysters using the thermal shock technique; however, when an oyster does not release gametes, the sex of the oyster is difficult to determine. Alternative nonlethal methods, such as magnetic resonance imaging (MRI) and nuclear magnetic resonance (NMR), have also been proposed for sex determination in oysters (Davenel et al. 2006).

Anesthesia is another effective noninvasive method for reducing stress and mortality rates and facilitating tissue biopsy and gametogenesis studies in oysters (Butt et al. 2008, Suquet et al. 2010, Puchnick-Legat et al. 2015, Rojas-Figueroa et al. 2023). The selection of appropriate chemical substances for anesthesia is crucial. Chemical substances, namely benzocaine, eugenol, propylene phenoxetol, magnesium sulfate ($MgSO_4$), and magnesium chloride ($MgCl_2$), have been used to anesthetize oysters (Acosta-Salmón et al. 2005, Suquet et al. 2009, Puchnick-Legat et al. 2015, Hintz et al. 2017). Among these, $MgCl_2$ has been identified as the most effective chemical substance for anesthesia in oysters because it is safe, easy to use, and highly effective in inducing muscle relaxation (Acosta-Salmón & Davis 2007, Suquet et al. 2009, Puchnick-Legat et al. 2015). $MgCl_2$ has been used as an anesthetic agent in different oyster species, including the European flat oyster (*Ostrea edulis*) (e.g. Culloty & Mulcahy 1992, Suquet et al. 2010), Sydney rock oyster (*Saccostrea glomerata*) (Butt et al. 2008), Chilean flat oyster (*Ostrea chilensis*) (Alipia et al. 2014), mangrove oyster (*Crassostrea rhizophorae*), mangrove oyster (*Crassostrea gasar*), and Pacific oyster (*Crassostrea gigas*) (e.g. Puchnick-Legat et al. 2015). According to Suquet et al. (2010), the use of laboratory-grade or agricultural-grade $MgCl_2$ did not affect oyster anesthesia rates. In addition, researchers used 23G needles to randomly sample gonads from anesthetized oysters for sex identification (Suquet et al. 2009, 2010, Puchnick-Legat et al. 2015). This method has been found to be effective and safe, with 100% survival rates in several oyster species, including Pacific oysters (Suquet et al. 2009, 2010, Puchnick-Legat et al. 2015) and mangrove oysters (*C. rhizophorae* and *C. gasar*) (Puchnick-Legat et al. 2015). Although anesthesia and gonad sampling methods have been studied in many oyster species, these methods have not been investigated in *C. belcheri*. This species can tolerate a wide range of salinities (12–30 ppt, Tan & Wong 1996), unlike other oyster species commonly studied. These physiological differences and the concentration of $MgCl_2$, are hypothesized to influence muscle relaxation. The objective of this study was to evaluate the effects of agricultural-grade $MgCl_2$ at different concentrations (0, 30, 50, and 70 g L^{-1}) on the anesthesia of *C. belcheri*. Agricultural-grade

$MgCl_2$ from Dead Sea Works® was selected because of its cost-effectiveness, global availability, and potential for hatchery applications. The solution of $MgCl_2$ that was most effective was subsequently used for biopsy to sample the gonads of *C. belcheri* using needles of different sizes (20G, 22G, and 24G). The established protocol could provide a safe and effective anesthesia methodology for *C. belcheri* that can be used for sex determination and broodstock management for efficient breeding programs and also could be applied in other studies in which *C. belcheri* is used as an experimental organism.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were performed according the guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Prince of Songkla University.

Preparation of white scar oysters

A total of 300 adult *C. belcheri* were transferred from Bandon Bay, Kanchanadit, Surat Thani, Thailand to the Prince of Songkla University, Surat Thani campus, Thailand, where the experiment was conducted. Before the experiment was initiated, all the oysters were acclimated in a plastic tank containing 1200 L of aerated 15 ppt water (with the same water salinity as that in Bandon Bay where the oysters were collected) for 3 and 10 days for experiments 1 and 2, respectively. During the acclimation period, the oysters were fed a commercial shellfish diet (Instant Algae Shellfish Diet 1800®, Reed Mariculture, Campbell, CA, USA) at a daily ratio of 3% of the mean dry meat weight twice a day at 8:30 and 16:30. However, all the oysters were left unfed for 24 h before the experiment was started to enhance filtration.

Experimental design

Experiment 1: Effects of $MgCl_2$ concentration and treatment duration on white scar oyster anesthesia

A commercial agricultural reagent containing approximately 47% $MgCl_2$ (Dead Sea Works®, Israel) was used as the chemical substance for oyster anesthesia. After the acclimation period, a total of 120 individual oysters with a mean initial weight of 131.45±16.29 g were randomly distributed into plastic tanks containing $MgCl_2$ at 4 different concentrations, i.e., 0, 30, 50, and 70 g L^{-1}, in triplicate, at a stocking density of 10 oysters per tank (10 L each). The salinity was maintained at 15 ppt for the 0 g L^{-1} $MgCl_2$ group. In the $MgCl_2$ group, $MgCl_2$ was dissolved in freshwater at rates of 30, 50, or 70 g L^{-1}, and after mixing well, the salinities of the waters for oyster rearing were maintained at 19, 32, and 45 ppt, respectively.

To determine anesthesia duration, the oysters in each group were immersed in $MgCl_2$ solutions of different concentrations, and the percentage of anesthetized oysters was checked every 3, 6, 9, 12, 15, 18, and 24 h after immersion in the $MgCl_2$ solutions. Oysters were considered anesthetized when shells did not close after the opening valves were gently touched 3 times (Suquet et al. 2010). Five anesthetized oysters from each tank (n=15) were randomly collected, and their openings were measured using a Vernier caliper. The shell opening rate was calculated as

$$\text{Shell opening rate (\%)} = [\text{Width of shell opening (mm)}/\text{shell length (mm)}] \times 100$$

After the shell opening rate was measured, all anesthetized oysters from each experimental tank were transferred to rearing tanks containing

40 L of $MgCl_2$ free with aeration, at a salinity 15 ppt. The percentage of oysters that recovered after anesthesia in different solutions of $MgCl_2$ was assessed every 30 min until all the oysters recovered. Anesthetized oysters were considered to have recovered when they could close their valves by themselves. Mortality rates were observed during anesthesia and after anesthesia for 7 days. For 7 days, the oysters were fed a commercial shellfish diet twice a day at 8:30 and 16:30 at a daily ratio of 3% of the mean dry meat weight. Water quality parameters were monitored. Temperature was measured with a glass-mercury thermometer (±0.1°C) and ranged from 26 to 28°C. Dissolved oxygen (DO), pH, nitrite, and ammonia levels were measured every other day using water quality test kits (PARA Test, Aquacare, Thailand). The DO concentration ranged from 5.0–7.5 mg L^{-1}, and the pH ranged from 7.5–8.0. Nitrite and ammonia were maintained at levels less than 0.25 and 0.05 mg L^{-1}, respectively. 100% water renewal was carried out every other day.

Experiment 2: Effects of needle size on gonad sampling

After an acclimation period of 10 days, a total of 120 individual oysters with a mean initial weight of 127.70±22.56 g were randomly selected. Fifteen individuals were left as nonanesthetized oysters with no gonad sampling, and 105 individuals were immersed in 50 g L^{-1} $MgCl_2$ for 6 h (the most effective concentration and immersion time in $MgCl_2$ solution that generated at least 60% of the cumulative percentage of anesthetized oysters in experiment 1). After 6 h in the anesthesia bath, a total of 60 anesthetized oysters and 15 nonanesthetized oysters were selected and randomly distributed into 5 experimental groups in triplicate, with 5 individual oysters in each tank. Each experimental group was defined as follows: no anesthesia, anesthesia with no gonad sampling or gonad sampling using sterile 20G (0.9×38 mm), 22G (0.70×38 mm) or 24G (0.55×38 mm) needles.

For gonad sampling, 0.02–0.03 mL of gonad fluid was collected from the left side above the adductor muscle of each individual using a 1 mL disposable syringe fitted with sterile needles of different sizes as previously described. Each gonad sample was subsequently immersed in 0.1 mL of 15 ppt water for 30 min in a 1.5 mL tube. A portion of the immersed gonad sample from each oyster was subsequently observed under a compound microscope at 20× objective magnification, and the sex of the oysters was determined from the gonad samples. After the gonad sampling step, all the oysters were transferred to rearing tanks containing 40 L of $MgCl_2$ free aerated water at 15 ppt. After these processes, the oysters were observed for recovery at 30 min intervals until all the oysters had recovered. The mortality rate in each group was monitored for 15 days. For 15 days, the oysters were fed, and water quality was monitored as described in the previous sections.

Data analysis

The data are presented as the mean±SE. The percentage of anesthetized oysters, anesthesia duration, mortality, and shell opening rates were analyzed by one-way analysis of variance (ANOVA) using SPSS version 22. Duncan's multiple range test was used to determine significant differences between treatments, and the differences were considered statistically significant at $P < 0.05$. Nonanesthetized oysters from the control group were excluded from the statistical analysis for the percentage of anesthetized oysters.

RESULTS

Effect of $MgCl_2$ on *Crassostrea belcheri* anes-

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The effects of agricultural-grade $MgCl_2$ at different concentrations on *C. belcheri* anesthesia are shown in Table I. Anesthesia of *C. belcheri* with $MgCl_2$ at concentrations of 0, 30, 50, and 70 g L^{-1} for 24 h did not affect the mortality rate of *C. belcheri* during the anesthesia period (0.00±0.00%), and the mortality rate 7 days after anesthesia was not significantly different among the experimental groups (0.00±0.00–6.67±6.67%) ($P > 0.05$). In the assessment of anesthesia induction rates in *C. belcheri*, when $MgCl_2$ sulutions of 30, 50, and 70 g L^{-1} were used over a 24-h period, the anesthesia induction rates were 76.67±3.33%, 86.67±6.67%, and 73.33±12.02%, respectively. At 50 g L^{-1}, $MgCl_2$ resulted in the highest anesthesia rate for *C. belcheri*, but no significant difference in the anesthesia rate was observed in this group when compared to the 30 and 70 g L^{-1} $MgCl_2$ groups ($P > 0.05$).

The percentages of anesthetized *C. belcheri* at different time points among the different $MgCl_2$ solutions are shown in Table II. It was found that the 50 g L^{-1} solution yielded greater

Table I. The percentage of *Crassostrea belcheri* anesthetized with agricultural-grade $MgCl_2$ at different concentrations for 24 h and the mortality rate during the anesthesia period and 7 days after anesthesia.

$MgCl_2$ concentration (g L^{-1})	The total cumulative anesthesia (%)	Mortality rate (%)	
		During the anesthesia period	7 days after anesthesia
0	0^{ex}	0.00±0.00	0.00±0.00
30	76.67±3.33	0.00±0.00	0.00±0.00
50	86.67±6.67	0.00±0.00	0.00±0.00
70	73.33±12.02	0.00±0.00	6.67±6.67

The data are presented as the mean ± standard error (n=3). ^{ex}=excluded for statistical analysis.

Table II. The percentage of anesthetized *Crassostrea belcheri* at different time points and with agricultural-grade $MgCl_2$ at different concentrations.

Time period of anesthesia (h)	The cumulative anesthesia (%)		
	$MgCl_2$ concentration (g L^{-1})		
	30	50	70
3	$10.00±10.00^{a, C}$	$3.33±3.33^{a, C}$	$0.00±0.00^{a, D}$
6	$33.33±8.82^{ab, B}$	$60.00±5.77^{a, B}$	$10.00±10.00^{b, CD}$
9	$60.00±5.77^{a, A}$	$63.33±6.67^{a, B}$	$36.67±6.67^{b, BC}$
12	$66.67±6.67^{a, A}$	$83.33±5.77^{a, A}$	$60.00±10.00^{a, AB}$
15	$73.33±3.33^{a, A}$	$86.67±6.67^{a, A}$	$66.67±8.82^{a, A}$
18	$76.67±3.33^{a, A}$	$86.67±6.67^{a, A}$	$73.33±12.02^{a, A}$
24	$76.67±3.33^{a, A}$	$86.67±6.67^{a, A}$	$73.33±12.02^{a, A}$

The data are presented as the mean ± standard error (n=3). Different lowercase or uppercase letters denote statistically significant differences at P < 0.05. Different superscripted lowercase letters indicate significant differences within the same anesthesia period at the different $MgCl_2$ concentrations. Different superscripted uppercase letters indicate significant differences for the same $MgCl_2$ concentration with different anesthesia durations.

cumulative anesthesia at both 6 h (60.00±5.77%) and 9 h (63.33±6.67%) than the 30 g L^{-1} (33.33±8.82% and 60.00±5.77%, respectively) and 70 g L^{-1} (10.00±10.00% and 36.67±6.67%, respectively) solutions. However, no significant difference was observed in cumulative anesthesia rates during the 6- and 9-h periods when 30 and 50 g L^{-1} $MgCl_2$ were used ($P > 0.05$). Conversely, the use of 50 g L^{-1} $MgCl_2$ resulted in a significantly greater cumulative anesthesia rate than the use of 70 g L^{-1} $MgCl_2$ ($P < 0.05$).

Furthermore, at anesthesia periods of 3, 12, 15, 18, and 24 h, the use of $MgCl_2$ at concentrations of either 30, 50, or 70 g L^{-1} resulted in no significant difference in the cumulative anesthesia rate of *C. belcheri* ($P > 0.05$). When *C. belcheri* was anesthetized for 15 h, a $MgCl_2$ concentration of 50 g L^{-1} yielded the highest anesthesia rate of 86.67 ± 6.67%, and increasing the anesthesia time from 15 h to 18 and 24 h did not result in an increase in the cumulative anesthesia rate. In contrast, at $MgCl_2$ concentrations of 30 and 70 g L^{-1}, up to 18 h was required to achieve maximum cumulative anesthesia rates of 76.67±3.33% and 73.33±12.02%, respectively, and increasing the anesthesia time from 18 to 24 h did not result in an increase in the cumulative anesthesia rate (Table II).

Additionally, extending the anesthesia time from 3 (10.00±10.00%) to 6 h (33.33±8.82%) and then to 9 h (60.00±5.77%) with 30 g L^{-1} $MgCl_2$ significantly increased the cumulative anesthesia rate of *C. belcheri* ($P < 0.05$). However, extending the anesthesia time beyond 9 h did not significantly increase the cumulative anesthesia rate of *C. belcheri* ($P > 0.05$). Similarly, extending the anesthesia time from 3 (3.33±3.33%) to 6 h (60.00±5.77%) and then to 12 h (83.33±5.77%) with 50 g L^{-1} $MgCl_2$ significantly increased the cumulative anesthesia rate of *C. belcheri* ($P < 0.05$). Extending the anesthesia time from 3 (0.00±0.00%) to 9 h (36.67±6.67%), and then to 15 h (66.67±8.82%) with 70 g L^{-1} $MgCl_2$ also significantly increased the cumulative anesthesia rate of *C. belcheri* ($P < 0.05$). Overall, extending the anesthesia time from 12 to 24 h with $MgCl_2$ at 30, 50, and 70 g L^{-1} did not significantly increase the cumulative anesthesia rate of *C. belcheri* ($P > 0.05$) (Table II).

Effect of $MgCl_2$ on shell opening and the shell opening rate in *Crassostrea belcheri*

The effects of $MgCl_2$ on shell opening in *C. belcheri* and the percentage of shell opening to body length in *C. belcheri* are shown in Table III. After anesthesia, with 30, 50, and 70 g L^{-1} $MgCl_2$, the shell openings of *C. belcheri* were 2.80±0.30, 3.83±0.84, and 3.33±1.28 mm, respectively, and the percentages of shell opening to body length after anesthesia were 3.30±0.35, 4.36±0.25, and 3.95±0.37%, respectively. Anesthetizing *C. belcheri* with 50 g L^{-1} $MgCl_2$ resulted in the highest shell opening rates, which were significantly

Table III. Shell opening and percentage of shell opening rate of *Crassostrea belcheri* after anesthetization with agricultural-grade $MgCl_2$ at different concentrations.

$MgCl_2$ concentration	Body length	Shell opening	Shell opening rate
(g L^{-1})	(mm)	(mm)	(%)
30	85.03±0.96[a]	2.80±0.30[b]	3.30±0.35[b]
50	88.53±1.99[a]	3.83±0.84[a]	4.36±0.25[a]
70	84.30±1.91[a]	3.33±1.28[ab]	3.95±0.37[ab]

The data are presented as the mean ± standard error (n=15). Different superscripted letters in the same column indicate statistically significant differences at $P < 0.05$.

greater than those obtained with 30 g L^{-1} $MgCl_2$ ($P < 0.05$) but not significantly different from those obtained with 70 g L^{-1} $MgCl_2$ ($P > 0.05$).

Effect of $MgCl_2$ on the recovery of anesthetized *Crassostrea belcheri*

The cumulative percentages of recovered oysters in each period after the oysters were anesthetized with 30, 50, or 70 g L^{-1} $MgCl_2$ are shown in Figure 1. At 30 min, the recovery rates of *C. belcheri* in all the experimental groups remained low, with recovery rates of 8.93±4.49%, 3.33±3.33%, and 7.87±3.96% for 30, 50, and 70 g L^{-1} $MgCl_2$, respectively. At 60 min, the recovery rates of anesthetized *C. belcheri* increased, with recovery rates of 69.64±10.86%, 35.00±7.64%, and 15.28±9.72% for 30, 50, and 70 g L^{-1} $MgCl_2$, respectively. *C. belcheri* anesthetized with 30 g L^{-1} $MgCl_2$ had a significantly greater recovery rate than those anesthetized with 50 or 70 g L^{-1} $MgCl_2$. In contrast, *C. belcheri* anesthetized with 70 g L^{-1} $MgCl_2$ had a lower recovery rate than the other experimental groups, especially at 60, 90, 120, 150, and 210 min. In addition, *C. belcheri* anesthetized with 30 g L^{-1} $MgCl_2$ had a 100% recovery rate at 150 min, whereas those anesthetized with 50 and 70 g L^{-1} $MgCl_2$ reached 100% recovery rates at 240 min.

Gonad sampling of *Crassostrea belcheri* using needles of different sizes

The results of the gonad sampling of *C. belcheri* using needles of 3 different sizes, i.e., 20G, 22G, and 24G after anesthesia with 50 g L^{-1} $MgCl_2$ are shown in Table IV. Needles of 3 different sizes were used to sample *C. belcheri* gonads for sex identification, with a 100% success rate. The *C. belcheri* gonads used in this study were not in the initial stage of development or the spent stage. Therefore, needles of all 3 sizes could be used to collect gonad samples for sex identification in *C. belcheri*. Gamete samples of male and female *C. belcheri* collected using the 3 different needles are shown in Figures 2a-c and 2d-f, respectively. The reproductive cells of both male and female *C. belcheri* collected using the 3 needles presented a milky white appearance, and the morphology of their reproductive cells was similar to that of the reproductive cells of *C. belcheri* that were released into the water

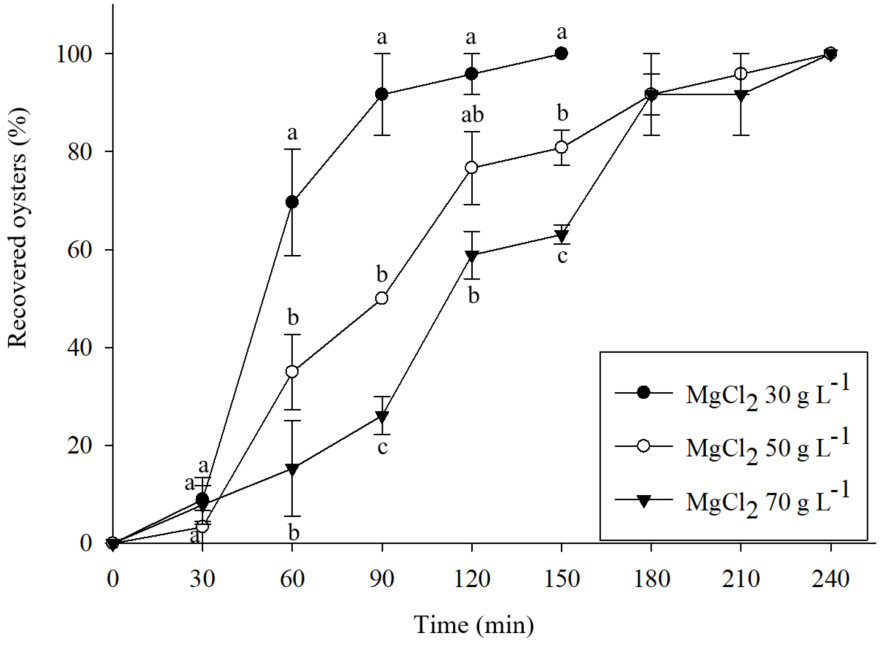

Figure 1. Cumulative percentage of recovered oysters in each period after anesthesia with $MgCl_2$ at different concentrations. Different superscripted letters within the same anesthesia period at different $MgCl_2$ concentrations denote statistically significant differences ($P < 0.05$).

during spawning (data not shown). After 30 min of exposure to 15 ppt water, *C. belcheri* male gametes collected using the 3 needles remained motile. The female gametes remained relatively round without cell rupture. When the mortality rates of all the experimental groups were compared, it was found that the sampling of *C. belcheri* gonads with the needles of 3 different sizes did not affect the mortality rate (0.00±0.00%) after 15 days of gonad sampling ($P > 0.05$).

Recovery of anesthetized *Crassostrea belcheri* after gonad sampling using needles of different sizes

The cumulative percentage of recovered oysters in each period among the groups of anesthetized *C. belcheri* with no gonad sampling and those with gonad sampling with needles of 3 different sizes is shown in Figure 3. The results indicated that the anesthetized *C. belcheri* in all the experimental groups did not recover from anesthesia after 30 min. After 60 min, the percentage of recovered *C. belcheri* in the group sampled using a 24G needle (13.33 ± 13.33%) was greater than that from the other experimental groups (0.00 ± 0.00%) ($P > 0.05$). After 90 min, the *C. belcheri* recovered from anesthesia in all the experimental groups. The percentages of recovered oysters in the group sampled using 20G (40.00 ± 11.55%), 22G (33.33 ± 6.67%) and 24G (40.00 ± 11.55%) needles were greater than those of oysters that did not undergo gonad sampling (6.67 ± 6.67%) ($P > 0.05$). After 120 min, the percentage of recovered oysters in the group sampled using a 24G needle (86.67 ± 13.33%) was greater than that in the other experimental groups ($P > 0.05$). However, after 150 min, the percentages of recovered oysters in the group sampled using 22G (93.33 ± 6.67%) and 24G (93.33 ± 6.67%) needles were greater than in those sampled using a 20G needle (86.67 ± 13.33%) and in those without gonad sampling (73.33 ± 6.67%) ($P > 0.05$). After 180 min, 100% of the oysters sampled with 22G and 24G needles had recovered. Oysters sampled with a 20G needle and those without gonad sampling had 100% recovery after 210 and 240 min, respectively.

DISCUSSION

Assessment of the cumulative percentages of *C. belcheri* anesthetized with 30, 50, and 70 g L^{-1} agricultural-grade $MgCl_2$ for a period of 24 h revealed that the use of 50 g L^{-1} $MgCl_2$ resulted in the highest cumulative percentage of anesthetized *C. belcheri*. However, the percentage

Table IV. Gonad sampling of *Crassostrea belcheri* using needles of different sizes.

Parameter	Sex identification		Mortality rate after 15 days of gonad sampling (%)
	Male	Female	
	(%)	(%)	
Nonanesthetized oyster with no gonad sampling	UI	UI	0.00±0.00
Anesthetized oyster with no gonad sampling	UI	UI	0.00±0.00
Anesthetized oyster sampling with 20G needle size	13.40	86.60	0.00±0.00
Anesthetized oyster sampling with 22G needle size	26.60	73.40	0.00±0.00
Anesthetized oyster sampling with 24G needle size	46.60	53.40	0.00±0.00

The data are presented as the mean ± standard error (n=15). UI=Unidentified.

of *C. belcheri* anesthetized with 50 g L^{-1} $MgCl_2$ was not significantly different from that anesthetized with 30 or 70 g L^{-1} $MgCl_2$. *C. belcheri* anesthetized with $MgCl_2$ at concentrations of 30, 50, and 70 g L^{-1} presented a 100% recovery rate from anesthesia, indicating that $MgCl_2$ is a safe and effective chemical for inducing anesthesia in *C. belcheri*. The results of this study are consistent with the study by Suquet et al. (2009), who reported that a greater percentage of Pacific oysters (*C. gigas*) were anesthetized when 50 g L^{-1} Dead Sea Work® $MgCl_2$ was used than when using 35 or 72 g L^{-1} $MgCl_2$ was used. However, the success rates of anesthetizing oysters with 50, 35 and 72 g L^{-1} $MgCl_2$ were not significantly different. Our study revealed that 50 g L^{-1} $MgCl_2$ was the recommended concentration for use in *C. belcheri* because this concentration provided the highest cumulative percentage of anesthetized *C. belcheri* (86.67±6.67%) after exposure to $MgCl_2$ for 15 h. Additionally, this concentration provided the highest shell opening rate (4.36±0.25%), which facilitates tissue sampling, and it was safe for *C. belcheri* since no mortality was observed during the 7 days after anesthesia. The results obtained in this study and those of previous studies demonstrated that $MgCl_2$ is an effective chemical for anesthetizing invertebrates, including commercial scallops (*Pecten fumatus*) (Heasman et al. 1995), sea urchins (*Paracentrotus lividus*) (Arafa et al. 2007) and oysters, e.g., Sydney rock oysters (*S. glomerata*) (Butt et al. 2008), Pacific oysters (Suquet et al. 2009, Puchnick-Legat et al. 2015), European flat oysters (*O. edulis*) (Suquet

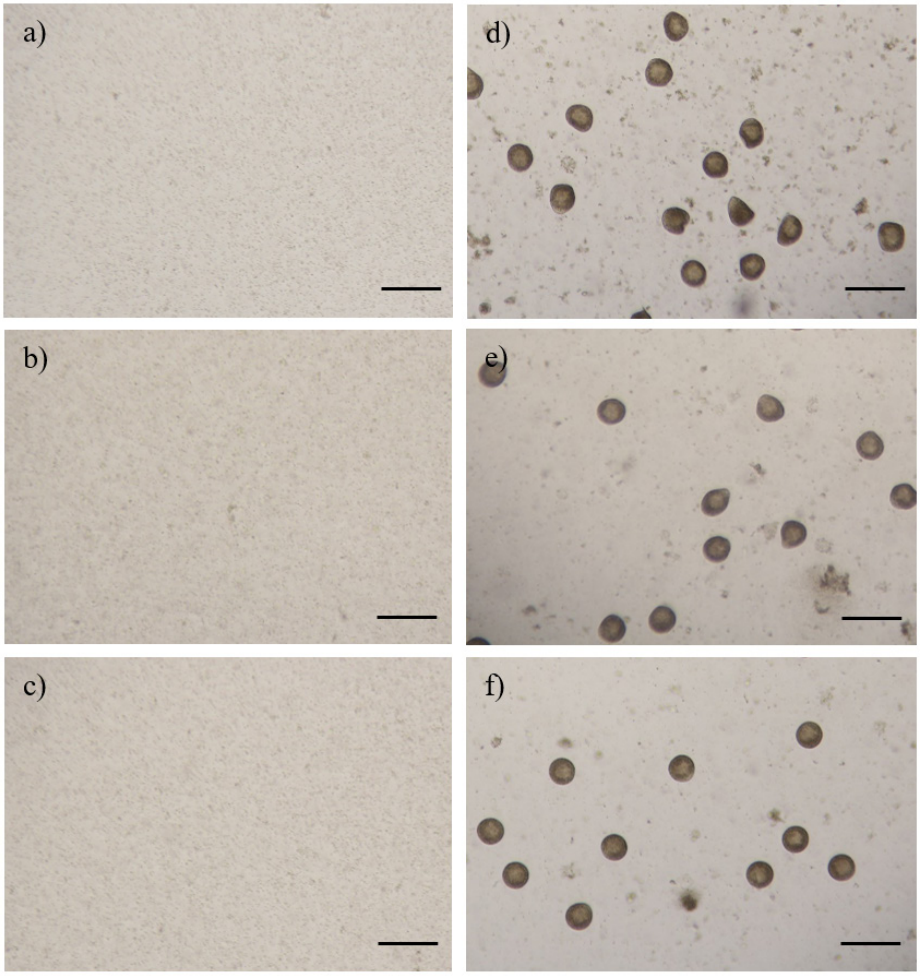

Figure 2. Images of the male gametes of *C. belcheri* sampled using 20G (a), 22G (b), and 24G (c) needles, and images of the female gametes of *C. belcheri* sampled using 20G (d), 22G (e), and 24G (f) needles after 30 min of exposure to 15 ppt water. Images were obtained at 20× magnification; scale bar = 100 µm.

et al. 2010), Chilean flat oysters (*O. chilensis*) (Alipia et al. 2014), mangrove oysters (*C. gasar* and *C. rhizophorae*) (Puchnick-Legat et al. 2015), and green-lipped mussels (*Perna canaliculus*) (Azizan et al. 2021), essentially because $MgCl_2$ is a nontoxic chemical. Additionally, $MgCl_2$ is readily available, convenient to use, cost-effective, and effective in inducing muscle relaxation, as described in many studies (Heasman et al. 1995, Acosta-Salmón & Davis 2007, Butt et al. 2008, Alipia et al. 2014). The ability of $MgCl_2$ to effectively induce anesthesia in invertebrates is related to magnesium ions (Mg^{2+}). In general, the contractibility of invertebrate muscle is influenced by the interaction between calcium ions (Ca^{2+}) and Mg^{2+} ions (Altura et al. 1987, Namba et al. 1995). Mg^{2+} is a calcium channel blocker that suppresses Ca^{2+} entry into the cell. The concentration of Ca^{2+} within the cell remains below the level needed to trigger the release of acetylcholine, causing synaptic transmission failure and leading to muscle relaxation or shell opening in shellfish (Namba et al. 1995, Kandel et al. 2000). Considering the cumulative percentage of *C. belcheri* anesthetized with 50 g L^{-1} $MgCl_2$, the results were similar to those of a previous study in mangrove oysters (*C. gasar*) (Puchnick-Legat et al. 2015). The results of the previous study demonstrated that 86.70±21.70% of *C. gasar* samples were anesthetized after 12 h of exposure to 50 g L^{-1} $MgCl_2$. In contrast, the cumulative percentage of anesthetized *C. belcheri* differed from that of other oyster species, i.e., the Sydney rock oyster, Pacific oyster, and mangrove oyster (*C. rhizophorae*), since these oyster species had 100% anesthesia rates after exposure to 50 g L^{-1} $MgCl_2$ for 6 h (Butt et al. 2008, Puchnick-Legat et al. 2015). In addition, Suquet et al. (2009) reported that 100% of Pacific oysters were anesthetized after being exposed to 50 g L^{-1} $MgCl_2$ for 16 h. Moreover, studies on European flat oysters and Chilean flat oysters revealed that both species experienced 100% anesthesia after being immersed in 50 g L^{-1} $MgCl_2$ for 3 h (Alipia et al. 2014). This study and previous studies demonstrated that the duration required to anesthetize oysters varies across studies. Factors that affect the time required for anesthesia may include species, size, age, duration of fasting prior to anesthesia, temperature, salinity, ability to close the shell for long periods, type of chemical and chemical

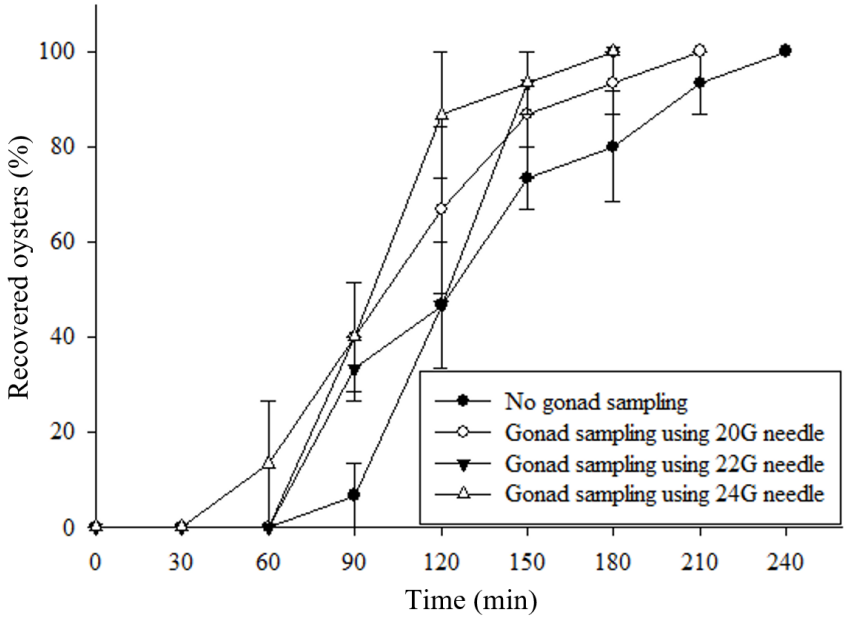

Figure 3. Cumulative percentage of recovered oysters after each period of anesthesia without and with gonad sampling using needles of different sizes.

concentration (Suquet et al. 2009, Vatsos & Angelidis 2012, Puchnick-Legat et al. 2015, Kishore et al. 2022, Rojas-Figueroa et al. 2023).

Furthermore, the results revealed that although no significant difference in the anesthesia rate was observed when the concentration of $MgCl_2$ increased from 50 to 70 g L^{-1}, the 70 g L^{-1} $MgCl_2$ solution resulted in the lowest cumulative percentage of anesthetized *C. belcheri* (73.33±12.02%) compared to the 30 g L^{-1} (76.67±3.33%) and 50 g L^{-1} (86.67±6.67%) $MgCl_2$ groups. These results are consistent with those of the study by Alipia et al. (2014), who reported that a higher concentration of $MgCl_2$ (60 g L^{-1}) resulted in a lower percentage (70%) of anesthetized Chilean flat oysters than the use of lower concentrations (30, 40, and 50 g L^{-1}), which resulted in 96–100% anesthesia. The reason why *C. belcheri* exhibited the lowest percentage of anesthesia at a 70 g L^{-1} $MgCl_2$ concentration may be attributed to the increased salinity of the water due to the higher $MgCl_2$ concentration. The use of 70 g L^{-1} $MgCl_2$ effectively increased the salinity of the anesthetic solution (45 ppt) to levels much greater than the natural salinity that *C. belcheri* inhabits (15 ppt). The sudden increase in salinity may have caused osmotic shock in *C. belcheri*, leading to stress and causing the oysters to close their shells for an extended period to protect themselves from abrupt changes in salinity. As a result, the $MgCl_2$ was less able to penetrate the tissues of *C. belcheri*, leading to a lower anesthesia rate at a concentration of 70 g L^{-1} $MgCl_2$. A similar observation was reported by Alipia et al. (2014) in a study of Chilean flat oysters. The recovery time after anesthesia was shorter for *C. belcheri* anesthetized with 30 g L^{-1} $MgCl_2$ than for those anesthetized with 50 or 70 g L^{-1} $MgCl_2$, possibly due to the lower amount of Mg^{2+} ions. At 30 g L^{-1} $MgCl_2$, Mg^{2+} ions may have inhibited the Ca^{2+} ion process to a less extent than $MgCl_2$ solutions of higher concentrations, enabling faster restoration of synaptic connections compared to that in the 50 and 70 g L^{-1} $MgCl_2$ groups.

Needles of all 3 sizes did not affect gamete cell morphology, and *C. belcheri* had a 100% survival rate 15 days after gonad sampling. The results agree with those of many studies in other oyster species, including blacklip pearl oysters (*Pinctada Margaritifera*) (Acosta-Salmón & Southgate 2004), European flat oysters (Suquet et al. 2010), Pacific oysters, and mangrove oysters (both *C. rhizophorae* and *C. gasar*) (Puchnick-Legat et al. 2015). Thus, needles of all 3 sizes (20G, 22G, and 24G) can be used to sample the reproductive cells of *C. belcheri* broodstocks without causing mortality. Considering the percentage of recovery over time after gonad sampling, the *C. belcheri* sampled using 22G and 24G needles required a shorter period to recover from anesthesia than those sampled using the 20G needle and the control group without gonad sampling. Further investigation is required to determine the reason for the faster recovery rates in *C. belcheri* sampled using smaller needles (22 G and 24 G) than in those sampled using larger needles (20 G) and in the control group with no gonad sampling. Therefore, gonad sampling using 20G, 22G, and 24G needles can be beneficial for the management of *C. belcheri* broodstocks in hatcheries; the number of male and female broodstocks to be used can be determined and sexually mature *C. belcheri* broodstocks can be selected to increase the efficiency of *C. belcheri* breeding.

In conclusion, the concentrations of agricultural-grade $MgCl_2$ (30, 50, and 70 g L^{-1}) used to anesthetize *C. belcheri* for a period of 24 h did not significantly affect the cumulative percentage of anesthetized *C. belcheri*. However, the use of 50 g L^{-1} $MgCl_2$ resulted in the highest cumulative percentage of anesthetized *C. belcheri* (86.67 ± 6.67%) and provided the maximum shell

opening for tissue sampling. The recovery time was shorter for oysters anesthetized with 30 g L^{-1} $MgCl_2$ (150 min) than for those anesthetized with 50 or 70 g L^{-1} $MgCl_2$ (240 min). Additionally, 20G, 22G, and 24G needles were suitable for gonad sampling, and did not affect the survival rate of *C. belcheri*.

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