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A NONLETHAL ANESTHESIA PROTOCOL FOR ACCESSING THE MANTLE CAVITY OF OLYMPIA OYSTERS IN THE LABORATORY OR FIELD

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ABSTRACT An effective method of anesthesia for Olympia oysters (*Ostrea lurida*) would allow for nonlethal sampling of tissues for genetic analysis, biopsy for diseases, assessing reproductive status, and collection of brooding larvae. The use of magnesium sulfate ($MgSO_4$) as an anesthetization method for Olympia oysters was assessed in laboratory trials and field use. Three replicate groups of 10 oysters were exposed to $MgSO_4$ at three concentrations (0, 75, 85, and 100 g/L) in the laboratory to investigate the optimal concentration for anesthetization. Laboratory trials determined that 45 min of treatment with 100 g/L $MgSO_4$ was the most effective. In the field, more than 14,000 oysters were exposed to $MgSO_4$ as an anesthetic to assess reproductive status and validate the procedure. In field trials, the anesthetization method of 45 min air exposure followed by 45 min submersion in 100 g/L $MgSO_4$ was found to have a success rate >80%. No influence of sampling date, location, or reproductive status on anesthetization was detected. Shell height was negatively correlated with anaesthetization success rate, with small oysters more likely to open their shell in response to $MgSO_4$.

KEY WORDS: Olympia oysters, *Ostrea lurida*, anesthetization

INTRODUCTION

In most bivalve molluscs, it is challenging to determine the health and reproductive stage in a nonlethal manner. Bivalves tightly close their two valves inhibiting inspection and sampling without removing the shell and sacrificing the individual. Non-invasive techniques have been developed including the use of magnetic resonance imaging (MRI) which allows for visualization of soft tissue. Pouvreau et al. (2006) successfully used MRI to identify internal structures and assess gonadal evolution in *Crassostrea gigas*. These techniques are expensive, hard to implement in the field, and do not allow for the collection of samples.

Anesthesia has been used to collect tissue samples with oysters, *Pinctada margaritifera* (Acosta-Salmón & Southgate 2004), and tissue biopsies of freshwater mussels, *Actinonaias ligamentina* and *Quadrula quadrula* (Berg et al. 1995), with limited mortality. Anesthetization also allows for repeated sampling of the same individual and reproductive monitoring. Methods exist for anesthetizing various molluscs including conchs (Acosta-Salmón & Davis 2007), mussels (Lellis et al. 2000), scallops (Heasman et al. 1995), and oysters (Culloty & Mulcahy 1992, Butt et al. 2008, Suquet et al. 2009, Suquet et al. 2010, Alipia et al. 2014, Puchnick-Legat et al. 2015), but species have varied responses to different anesthetics.

It would be beneficial to have an anesthesia method for the Olympia oyster *Ostrea lurida* (Carpenter, 1864). The Olympia oyster is the only oyster native to the west coast of North America. Olympia oysters are an economically, culturally, and ecologically important species in the Puget Sound region of northwest Washington State. These native oysters have been part of the diet of local Native American tribes for thousands of years (Blake 2003), were harvested in large quantities by European settlers between the late 1800s and early 1900s (Baker 1995, White et al. 2009, Blake & zu Ermgassen 2015), and continue to be an important species for both restoration and commercial aquaculture (Trimble et al. 2009). Although the wild fishery in Puget Sound ended in the early 1900s, farmed Olympia oysters continue to command high prices in a niche market (Trimble et al. 2009). Ecologically, *O. lurida* are ecosystem engineers that create biogenic habitat (Blake & Bradbury 2012) that increases biodiversity (Pritchard et al. 2015) and provide benthic-pelagic coupling by filtering local waters (Ruesink et al. 2005). Native oyster reefs were depleted and degraded by 1920 in Washington State (White et al. 2009); the decline was attributed to overharvest from fisheries, decreases in water quality, and habitat loss (Kirby 2004, Pritchard et al. 2015, Baker 1995). Over the last 20 years, multiple projects have aimed at restoring *O. lurida* populations (Peter-Contesse & Peabody 2005, Dinnel et al. 2009, Blake & Bradbury 2012).

It is important to have long-term monitoring of these restoration projects and to evaluate their restoration success. Adult female *Ostrea lurida* brood their fertilized embryos for 7–14 days; during this brooding period, the reproductive status and stage of females can be determined by visually examining the brood chamber. Nonlethal sampling of reproductive activity and tissue collection would improve both project efficacy and the ability to study this species of oysters. The ability to anesthetize *O. lurida* would allow for nonlethal examination of the internal structures, examination and removal of brooded larvae, tissue sampling, and experimental procedures on live bivalves without harming the individual. An optimal anesthetization method would be simple and quick for field applications, allows the animal to recover quickly to reduce stress, and have minimal to no mortality.

The goal of this study was to develop a cost effective, efficient anesthetization method that can be applied in the laboratory, hatchery, or field to assess the reproductive status of *Ostrea lurida*. The use of magnesium salts has been shown as an effective anesthetic in numerous species of oysters [*Crassostrea gigas* (Suquet et al. 2009), *Ostrea edulis* (Culloty & Mulcahy 1992, Suquet et al. 2010), *Ostrea chilensis* (Alipia et al. 2014), *Saccostrea glomerate* (Butt et al. 2008), *Pinctada maxima*

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(Mamangkey et al. 2009) and scallops (*Pecten fumatus* [Heasman et al. 1995])].

In the current study, a nondestructive method of anesthetization was developed for relaxing *Ostrea lurida* using $MgSO_4$, sold commercially as common Epsom salts. Laboratory trials were carried out to examine dose, pretreatment, and posttreatment mortality, followed by field trials to assess effectiveness as measured by the proportion of oysters successfully anesthetized.

MATERIALS AND METHODS

Laboratory Trials

Wild adult Olympia oysters from south Puget Sound (mean shell height 42.5 ± 2.6 mm and 95% CI) were held in ambient seawater at the Puget Sound Restoration Fund shellfish hatchery in Port Gamble, WA, before experiments. Oysters were submerged in concentrations of 0, 75, 85, and 100 g/L of $MgSO_4$ [Ultra Epsom Premium Epsom Salt, unscented (SaltWorks, China)] to characterize optimal anesthetic conditions. Preliminary trials revealed that concentrations $\leq$50 and $\geq$125 g/L were unsuccessful in anesthetizing high proportions of *Ostrea lurida* in less than 2 h (data not shown). Oysters were randomly distributed among three replicate treatments ($n = 10$ per treatment), with coded labeling to remove observer bias at each concentration plus controls. Treatments were carried out in clear 5.7-L plastic bins with 50:50 freshwater/seawater to maintain approximate salinity of seawater. The $MgSO_4$ was initially dissolved in freshwater before combining with equal parts seawater. Oysters were monitored at 15 min intervals for more than 2 h for response to the $MgSO_4$ anesthetic. Oysters were considered successfully anesthetized when valves opened (gaped), did not close in response to light tapping, and remained open after removal from anesthetic bath for at least 10 sec. The goal was to create an anesthetization method that was quick and easily applied during low tides in the field; therefore, immersion times >1 h were not viable options. Survival of oysters in all treatment and control groups was monitored for 1 mo after the trial. The magnesium salt, magnesium chloride ($MgCl_2$), was not included in the trials; although effective with *Ostrea chilensis* (Alipia et al. 2014) and *Ostrea edulis* (Culloty & Mulcahy 1992, Suquet et al. 2010), $MgCl_2$ did not quickly anesthetize *O. lurida* in preliminary trials (data not shown).

Field Application

Anaesthetization of *Ostrea lurida* using $MgSO_4$ was tested in the field for sampling the reproductive status of oysters. This method was applied to 14,262 *O. lurida* from 11 locations throughout Puget Sound, WA, from June to August 2015. After collection, oysters were exposed to (ambient temperature) air for 45 min, which was shown to increase the proportion of oysters that successfully responded to the anesthetic in preliminary studies (data not shown). After air exposure, oysters were transferred to an insulated bath containing 100 g/L $MgSO_4$ 50/50 seawater/freshwater solution for 45 min. The $MgSO_4$ was dissolved in freshwater in the laboratory and was subsequently combined with seawater collected on site. The ambient air temperature ranged from 12.8°C to 21.7°C at sampling events and the $MgSO_4$/seawater anesthetic ranged from 17°C to 25°C, reflecting the seawater temperature at the sampling locations. Shell height (defined as the distance from the umbo to the opposing valve margin), response to anesthetization (open or closed), and reproductive status (brooding or not, based on visual inspection) were recorded for each oyster.

To determine if the anesthetization method introduced a bias in reproductive data collection, reproductive rates from anesthetization to lethal methods of assessing reproductive status were compared. During June 2016 in Fidalgo Bay, WA (48.477810°N, –122.574217°W), oysters were collected from fourteen haphazardly placed 1/16 m^2 quadrats in aggregations of *Ostrea lurida*. Seven of the quadrats were anesthetized and the other seven were lethally examined for reproductive status.

Data Analysis and Statistics

Differences in the success rates of anesthetization across treatments in laboratory trials were determined by transforming proportional data using the arcsine-square root transformation (Zar 2010) followed by a two-way analyses of variance (ANOVA) and Tukey's posthoc tests. Linear regression analysis weighted by sample size was used to evaluate the success of anesthesia as a response to oyster shell height. To determine if there were statistically significant differences in the success rate of anesthetization spatially, one-way ANOVA was performed to compare the 11 different populations sampled. Similarly, to determine if there was a temporal effect, 15 sampling events were pooled from the two locations sampled most frequently, Fidalgo Bay and Mud Bay, and a one-way ANOVA was performed on anesthetization success. One-way ANOVA was also performed on the arcsine-square root transformation proportion of reproductive oysters found in each quadrat to compare the two methods of assessing reproductive status. Average anesthetization success is reported with 95% confidence intervals.

RESULTS

Laboratory Trial

Magnesium sulfate was found to be a successful anesthetic for *Ostrea lurida* in laboratory trials. After submersion for 45 min, the 100 g/L $MgSO_4$ treatment successfully anesthetized the highest proportion (26.7% ± 6.5%, 95% CI) of the oysters

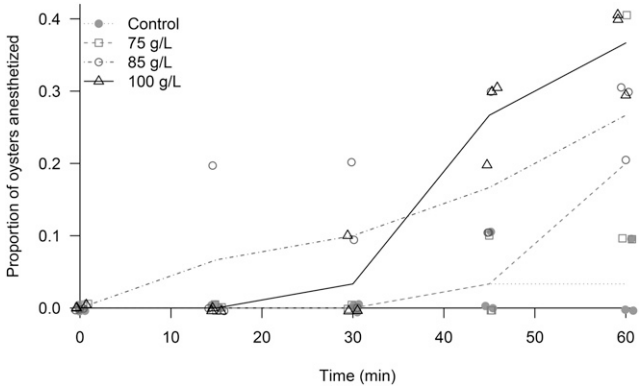

Figure 1. Anesthesia success of $MgSO_4$ in laboratory trials, three concentrations of $MgSO_4$ were assessed and compared with a control for their anesthesia success of *Ostrea luria* and monitored over time. Replicates are represented by individual data points, the average of each treatment is represented by the lines. Jitter was used to add a small amount of noise to the data points along both axis to improve visualization of overplotting.

TABLE 1.

Anesthesia success of three concentrations of $MgSO_4$ on *Ostrea lurida* over time in laboratory trials.

Time (min)	$MgSO_4$ concentration	Mean proportion anesthetized	
60	100	0.37	a
45	100	0.27	ab
30	100	0.03	c
15	100	0.00	c
0	100	0.00	c
60	85	0.27	ab
45	85	0.17	abc
30	85	0.10	bc
15	85	0.07	bc
0	85	0.00	c
60	75	0.20	abc
45	75	0.03	c
30	75	0.00	c
15	75	0.00	c
0	75	0.00	c
60	0	0.03	c
45	0	0.03	c
30	0	0.00	c
15	0	0.00	c
0	0	0.00	c

Different letters by column represent significant differences among treatments by Tukey HSD test ($P < 0.05$).

compared with controls (3.5% ± 6.5%) and the 75 g/L treatment (3.5% ± 6.5%) (Fig. 1, Table 1). After 60 min of treatment, 100 g/L was still the most successful treatment with 36.7% (± 6.5%) of the oysters anesthetized (Fig. 1, Table 1). Both time (ANOVA, $F_{(4,\ 40)} = 22.185$, $P < 0.0001$) and concentration of $MgSO_4$ (ANOVA, $F_{(3,\ 40)} = 11.099$, $P < 0.0001$) were shown to be significant factors in the success of the anesthetic. The interaction of time and concentration was significant (ANOVA, $F_{(12,\ 40)} = 3.198$, $P = 0.0028$), indicating that some of the earliest responses were from oysters exposed to lower concentrations. The general trend was greater anesthesia success with less time at higher concentrations of $MgSO_4$. The treatment concentration of 100 g/L $MgSO_4$ was most successful as an anesthetic (Tukey's post hoc HSD test, $P < 0.001$, Table 1). The proportion of oysters successfully anesthetized in each treatment continued to increase over the 2 h of treatment reducing the differences in response among $MgSO_4$ concentrations. No posttreatment mortality was detected in oysters over one month of observations.

Field Application

The anesthesia method applied in the field had a high overall success rate, with 11,595 of the 14,262 oysters (81.3%) successfully anesthetized after treatment. The success of 43 sampling events ranged from 42.4% to 97.9%, averaging 82.0% ± 0.1% anesthetization success among sampling events. No spatial effect was detected among the 11 locations (ANOVA, $F_{(10,\ 38)} = 1.862$, $P = 0.0948$) (Fig. 2); likewise no temporal effect was detected among 15 pooled sampling events between June and August [$F_{(2,\ 14)} = 2.0892$, $P = 0.1665$]. No effect of reproductive status (brooding or not brooding) on efficacy of the anesthetic was observed [$F_{(1,\ 13)} = 0.7953$, $P = 0.3901$]. As shell height increased, the success of anesthesia decreased significantly from 98% to 76% at 15 and 55 mm shell heights, respectively [$F_{(1,\ 52)} = 229.1$, $P < 0.001$, $R^2 = 0.8114$, 95% CI (0.7261, 0.8967)] (Fig. 3).

DISCUSSION

This study developed and field-tested a nonlethal method for accessing and sampling the mantle cavity of *Ostrea lurida*. A combination of 45 min air exposure with subsequent immersion in 100 g/L $MgSO_4$ in a 50:50 mix of fresh and seawater proved

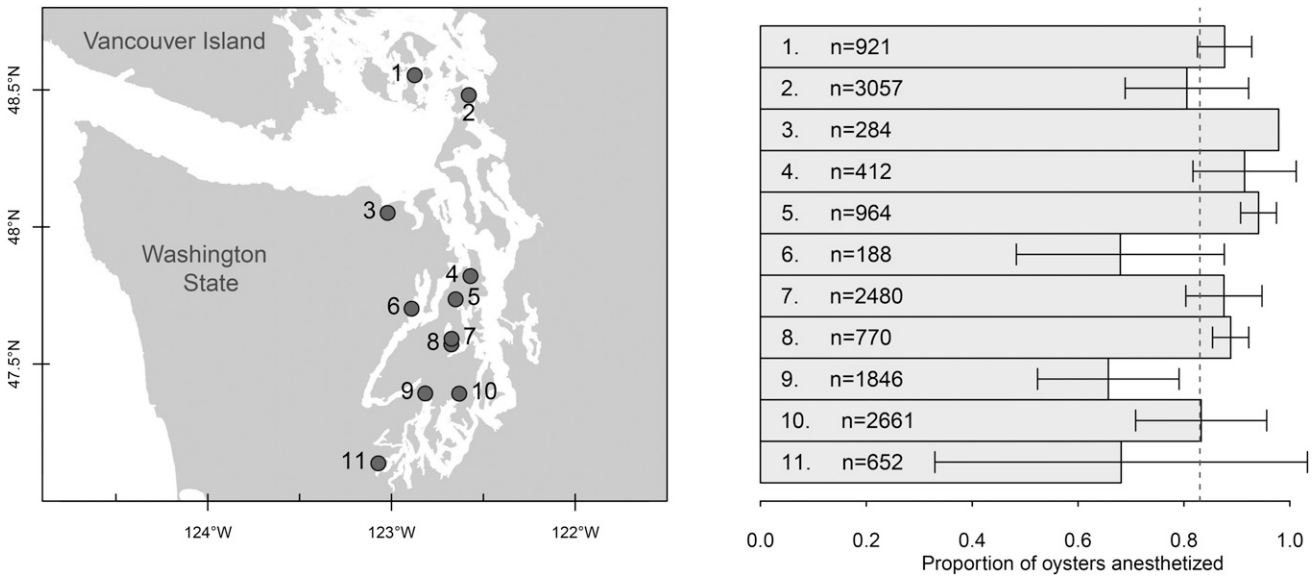

Figure 2. Olympia oyster anesthesia success at each population of oysters sampled in Puget Sound, WA. Left, a map of all populations sampled; right, average proportion of oysters successfully anesthetized at each population. Error bars represent 95% CI, and dashed line represents the average anesthesia success. Location of populations sampled (1) Lopez Island, (2) Fidalgo Bay, (3) Sequim Bay, (4) Port Gamble, (5) Liberty Bay, (6) Dosewallips, (7) Mud Bay, (8) Oyster Bay, (9) North Bay, (10) Burley Lagoon, (11) Little Skookum Inlet.

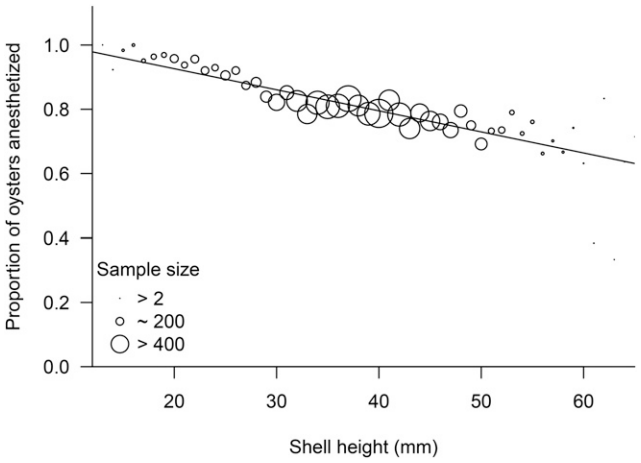

Figure 3. Proportion of Olympia oysters successfully anesthetized in field application as a response to oyster shell height, each bubble represents the number of oysters sampled with that shell height. Fit with a linear regression weighted by the sample size [$y_i = 1.057 + -6.5571E^{-3} x_i$, $R^2 = 0.8114$, $F_{(1, 52)} = 229.1$, $P < 0.001$].

to be an effective method for the anesthetization of *O. lurida*. This method induced rapid anesthesia, no detected mortality, and was easily applied in the field during the course of a low tide. Although there were varied responses in field applications, an average of 82.0% of the individual oysters exposed were successfully anaesthetized and at least 40% were anaesthetized in every trial. The addition of a 45 min desiccation before submersion in the $MgSO_4$ anesthetic (45 min) increased the anesthesia success from 27% in the laboratory trials to 82.0% in the field application. There were no temporal, spatial, or reproductive status effects on anesthetization effectiveness detected in field trials.

Larger oysters were slightly less likely to be anesthetized (Fig. 3), possibly because of their larger adductor muscles. Magnesium sulfate relaxes the adductor muscle of the oyster. The two valves are connected via the adductor muscle that contracts to close the shell; when the adductor muscle is relaxed the ligament is the acting force opening the two valves. Oysters can keep their adductor muscle contracted and their valves closed tightly for long periods of time allowing them to live intertidally. It is likely that the larger individuals with larger adductor muscles require more exposure to $MgSO_4$, either by an increase in concentration or exposure time to relax the adductor muscle and induce gaping. It is also possible that larger individuals are less exposed to the anesthetic because they do not require the intake of new seawater as often as smaller individuals.

Magnesium sulfate has been shown as a successful anesthetic in a variety of other molluscs, but is often not the recommended method of anesthesia. The European flat oyster (*Ostrea edulis*) was effectively anaesthetized by $MgSO_4$ with a 74% success rate but the large concentrations (300 g/L) and time necessary (24 h) were cited as disadvantages of using $MgSO_4$ as a method (Table 2; Culloty & Mulcahy 1992). Magnesium sulfate was shown to induce high mortality (>90%) after anaesthetization in scallops (*Pecten fumatus*) with a similar concentration of 80 g/L $MgSO_4$ (Heasman et al. 1995) and juvenile abalone (Sagara & Ninomiya 1970; Table 2). In the current study, $MgSO_4$ was not found to induce mortality after anaesthetization in laboratory trials on *Ostrea lurida*. In addition, Heare (2015) applied this anaesthetization method at a slightly lower concentration of 75 g/L $MgSO_4$ to the same Olympia oysters repeatedly with no mortality reported associated with anesthetization.

This study developed an *Ostrea lurida* anesthetic procedure that shows a high anesthetization success rate with minimum mortality. This nonlethal method of accessing the mantle cavity and brood chamber is a valuable tool with many potential applications. An anesthesia method allows for the nonlethal sampling of *O. lurida* tissue for genetic analysis, biopsies for disease, checking the stage of gametogenesis, and collection of brooding larvae. Each of these tools can be applied to answer scientific questions and remove the limitations of lethal sampling. The $MgSO_4$ anesthesia protocol reported here was recently used by Barber et al. (2016) to monitor reproduction of *O. lurida* restoration sites in northern Puget Sound. Whereas applied to a species of conservation concern for this study, this technique is broadly applicable to bivalve research. The use of an anesthetic is widely applied in aquaculture for tissue biopsies and for monitoring gamete development and could be applied to the aquaculture of *O. lurida*. This nonlethal sampling method allows for a large sample size of wild populations without harming active restoration efforts as well as repeated sampling of individuals.

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TABLE 2.

Comparing anesthesia methods on molluscs using magnesium salts.

Species	Anesthetic	Concentration (g/L)	Time	Success rate	Source
Ostrea lurida	$MgSO_4$	100	45 (min)	82%	*Current study*
Ostrea edulis	$MgSO_4$	300	24 (h)	75%	Culloty and Mulcahy (1992)
Ostrea edulis	$MgCl_2$	35	90 (min)	100%	Culloty and Mulcahy (1992)
Ostrea edulis	$MgCl_2$	50	2–3 (h)	80% ± 20%	Suquet et al. (2010)
Ostrea chilensis	$MgCl_2$	30–50	3 (h)	100%	Alipia et al. (2014)
Crassostrea gigas	$MgCl_2$	50	16 (h)	100%	Suquet et al. (2009)
Pecten fumatus	$MgCl_2$	30	~5 (min)		Heasman et al. (1995)
Pecten fumatus	$MgSO_4$	80	60 (min)	high mortality (>90%)	Heasman et al. (1995)
Saccostrea glomerata	$MgCl_2$	50	6 (h)	100%	Butt et al. (2008)

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