Assessment of Various Anesthetic Agents on *Octopus vulgaris* Paralarvae

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Abstract

European Directive 2010/63 addresses the welfare of cephalopod species used in scientific projects under European jurisdiction and covers experimental procedures likely to cause pain, suffering, distress, or lasting harm. These procedures require authorization from the National Competent Authorities. In aquaculture research, some procedures require the temporary immobilization of individuals to allow for measuring body parameters (i.e., weight, sex, gonad condition, and others), avoiding any damage. This study compares three anesthetics used on common octopus, *Octopus vulgaris*, paralarvae to reach a state of sedation. The anesthetics were tested for their effects at different concentrations: magnesium chloride (6.8, 13.6, 20.4, and 27.4 g/L), ethanol (8, 10, and 12 mL/L), and clove oil (0.2, 0.3, and 0.4 g/L). Three variables were monitored: induction, recovery, and stressful behavior of paralarvae during treatments. Significant differences were found between anesthetics, both in the induction and recovery time, and between concentrations of the same anesthetic. The shortest times of induction and recovery corresponded to ethanol at low concentrations, 10 mL/L (20 ± 1 sec) and 8 mL/L (19 ± 2 sec), respectively. Clove oil at 0.2 g/L was the slowest to reach sedation (340 ± 7 sec). Magnesium chloride at 20.4 g/L showed longer recovery times (554 ± 201 sec) with increasing concentrations. Octopus paralarvae showed adverse behavior only under clove oil treatments, whose use is therefore considered inadmissible. This study shows that ethanol at 10 mL/L may be suitable as a reliable anesthetic for octopus paralarvae, diminishing the induction and recovery time without showing any stressful behavior.

KEYWORDS

anesthesia, cephalopods, clove oil, ethanol, induction and recovery, magnesium chloride, *Octopus vulgaris* paralarvae

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Directive 2010/63/EU on animal welfare establishes measures for the protection of animals used for scientific or educational purposes. This Directive addresses the welfare of cephalopod species used in scientific projects under European jurisdiction and covers experimental procedures likely to cause pain, suffering, distress, or lasting harm (PSDLH) throughout the life cycle. National administrations are involved in the fulfillment of the Directive 2010/63/EU because the project authorization is approved by the National Competent Authorities.

Cephalopods are considered of utmost importance as experimental animals, especially in neurophysiology (Young 1971, 1974; Williamson and Chrachri 2004). Recently, several species have been identified as potential candidates for aquaculture, resulting in an increase of experiments with live cephalopods (Nesis 1987; Barnabé 1996; Baltazar et al. 2000; Segawa and Nomoto 2002; Okumura et al. 2005; Rosas 2007; Solorzano et al. 2009). The culture of cuttlefish, *Sepia officinalis*, and common octopus, *Octopus vulgaris*, are currently the leading research edge in cephalopods aquaculture in Europe (Iglesias et al. 2014). Achieving maintenance and mass production of cephalopods requires experimentation with live individuals. This research has developed a number of techniques to care for and manage cephalopods during their life cycle, including paralarvae, juvenile, and adult stages, although in several species (e.g., *S. officinalis*) there is no need for anesthesia for handling. Handling cephalopods for research procedures such as biological sampling (i.e., weighing, measuring, and tissues collection) is a delicate step and so stressful for animals that it may cause death after manipulation, especially in the first stages of the life cycle (Fiorito et al. 2015).

Various anesthetics have been tested in cephalopods, including *Sepioteuthis sepioidea* (García-Franco 1992), *S. officinalis* (Gonçalves et al. 2012), *Doryteuthis pealeii* (Mooney et al. 2010), *Eledone moschata* (Sen and Tanrikul 2009), *Octopus minor* (Seol et al. 2007), and *O. vulgaris* (Estefanell et al. 2011). The anesthetics used extensively to date are magnesium chloride ($MgCl_2$), ethanol, and clove oil (Messenger

et al. 1985; Seol et al. 2007; Estefanell et al. 2011; Andrews et al. 2013; Fiorito et al. 2015), although clove oil is not acceptable in species such as *S. officinalis* and *D. pealeii* (Mooney et al. 2010; Gonçalves et al. 2012). As these anesthetic agents have only been used on juvenile and adult cephalopods (Sykes et al. 2012; Gleadall 2013), there is an information gap regarding their effects on paralarvae.

This study assesses the use of different concentrations of the aforementioned anesthetics in *O. vulgaris* paralarvae and analyzes their effects on induction, recovery time, and behavior.

Materials and Methods

Ethical Implications

Experimentation was performed according to Spanish regulations, Law 6/2013 and European Directive 2010/63/EU, for the protection of animals used for experimentation and other scientific purposes.

Newly hatched paralarvae of *O. vulgaris* from spawns of wild broodstock were used in this experiment (see Reis et al. 2014, for octopus culture details). One hour before the test, 1-d-old paralarvae were removed from a 1000-L hatching tank to a 4-L holding tank with filtered seawater and continuous aeration.

Tested concentrations were clove oil (Acofarma, Barcelona, Spain; concentration 1.027 g/mL) 0.1, 0.2, 0.3, and 0.4 g/L; pure ethanol (100°) (Panreac Química, Barcelona, Spain) 5, 8, 10, and 20 mL/L (v/v); and magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$) (Acofarma) 6.8, 13.6, 20.4, and 27.2 g/L, diluted exclusively in seawater. The different anesthetic concentrations were prepared in 100-mL individualized containers with previously filtered and aerated seawater, which was renewed after six trials. All tests were conducted at a temperature of 22.2 ± 0.5 C and salinity of 36.8 ± 0.14 Practical Salinity Unit; thus, salinity was not corrected throughout the experiment, and this needs to be pointed out with special emphasis on treatments using magnesium chloride.

A total of 18 octopus paralarvae were used as replicates to evaluate each concentration of each anesthetic agent. Each paralarva was

pipetted from the holding tank (4 L) to the anesthetic container. Octopus paralarvae were considered sedated when they lost the ability to swim and remained perfectly still on the bottom; the elapsed time was registered as being the time of induction (in seconds). The paralarvae that reached this stage were then immediately removed from the anesthetic container and placed in a 4-L recovery tank with filtered seawater and aeration. Recovery time was considered from this moment until complete recovery of swimming ability, as observed before the treatment. In octopus paralarvae, inking is the most detectable negative reaction to stress, because other stressing behaviors reported in juveniles and adults of cephalopod species, such as depression of ventilation, and decrease of chromatophores or skin paling (Andrews et al. 2013), are difficult to observe, principally due to the small size of new hatchling paralarvae and the natural variability of the chromatophore contraction between individuals in the hatchling tank, previous to any treatment.

Thus, inking was recorded as a stressful behavior by direct observation during the induction and recovery periods.

Data Analysis

Linear regression analysis was used to evaluate the time of induction and recovery, and each treatment was considered separately (magnesium chloride, ethanol, and clove oil). Nonlinear fits were also tested but had equal or lower r^2 values in almost all cases; hence, the statistics for linear fits are reported.

Data were checked for normality with the Kolmogorov–Smirnov test (Zar 1999) as well as for homogeneity of the variances with the Levene test (Zar 1999), and when necessary square-root transformation was performed. If all assumptions were met, a one-way nested ANOVA was run to test differences among anesthetics considering concentrations used in each treatment (0.1, 0.2, 0.3, and 0.4 g/L for ethanol; 5, 8, 10, and 20 mL/L for magnesium chloride; and 6.8, 13.6, 20.4, and 27.2 g/L for clove oil) to check differences in the time of induction

and recovery among anesthetics, followed by Tukey's post hoc test (Zar 1999).

Multivariate analysis among treatments based on times of induction and recovery were established using nonmetric multidimensional scaling, untransformed data, and the Bray–Curtis similarity index (Clarke 1993). Multivariate analyses were carried out using the PRIMER statistical package 6.0 (Clarke and Gorley 2006).

Results

The only anesthetic agent that induced stressful behavior in octopus paralarvae concentrations was clove oil. It is therefore considered inappropriate as an anesthetic agent for octopus paralarvae. In contrast, ethanol and magnesium chloride treatments caused no signs of stress.

The mean time of induction varied greatly between treatments: minimum using ethanol 10 mL/L (20 ± 1 sec) and maximum using clove oil 0.2 g/L (340 ± 7 sec) (Table 1, Fig. 1). The mean time of recovery also varied greatly between treatments: minimum using ethanol 8 mL/L (19 ± 2 sec) and maximum using $MgCl_2$ 20.4 g/L (554 ± 201 sec) (Table 1, Fig. 1). Concentrations within each treatment showed shorter induction times with the highest $MgCl_2$ and clove oil concentrations (Fig. 1A, C). In contrast, the ethanol treatment showed a positive correlation between induction times and ethanol concentrations (Fig. 1B).

Significant differences for induction time were observed between anesthetics, considering

TABLE 1. *Mean and SD, maximum and minimum induction, and recovery times of each treatment, in seconds.*

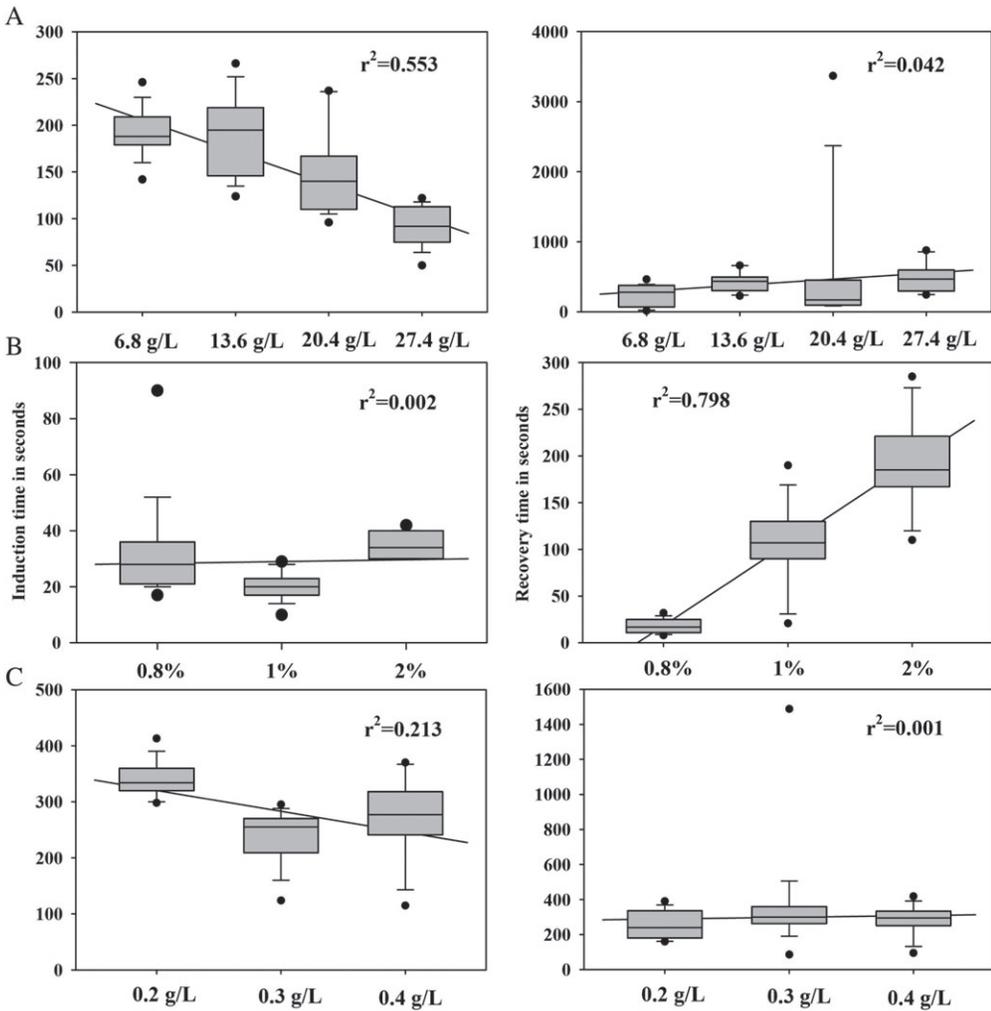| Anesthetic agents		Time of induction (sec)	Time of recovery (sec)
Magnesium chloride (g/L)	6.8	191 ± 25 (142–246)	243 (18–463)
	13.6	190 ± 40 (124–266)	418 (228–664)
	20.4	143 ± 41 (96–237)	554 (85–3368)
	27.4	91 ± 22 (50–122)	483 (245–879)
Ethanol (mL/L)	8	33 ± 17 (17–90)	19 (8–32)
	10	20 ± 5 (10–29)	106 (21–190)
	20	34 ± 5 (30–42)	194 (110–285)
Clove oil (g/L)	0.2	340 ± 31 (298–413)	255 (160–390)
	0.3	243 ± 45 (124–295)	362 (86–1488)
	0.4	266 ± 73 (115–370)	276 (96–418)

FIGURE 1. *Induction and recovery time (in seconds) of each treatment. (A) Magnesium chloride; (B) ethanol; (C) clove oil. Box-plot showing median (black line) and upper and lower quartiles of data. Black dots represent outliers. Regression line r^2 values are shown.*

concentrations (one-way nested ANOVA, $F = 0.672$, $P = 0.006$), mainly due to the dissimilarity between ethanol and the remaining two anesthetics (magnesium chloride and clove oil) (Tukey post hoc test, ethanol–magnesium chloride, $F = 0.398$, $P = 0.028$; ethanol–clove oil, $F = 0.658$, $P = 0.012$). The same pattern was observed for the recovery time among anesthetic concentrations (one-way nested ANOVA, $F = 0.289$, $P = 0.008$), and differences were also mainly explained by the dissimilarity between ethanol and the other two anesthetics (magnesium chloride and clove

oil) (Tukey post hoc test, ethanol–magnesium chloride, $F = 0.312$, $P = 0.032$; ethanol–clove oil, $F = 0.523$, $P = 0.021$).

Spatial representation showed ethanol treatments on the left of the ordination, while magnesium chloride and clove oil treatments obtained similar times of induction and recovery (Fig. 2).

Discussion

Our criteria for defining the efficiency of a given anesthetic were based on the time of induction and recovery, as well as on the
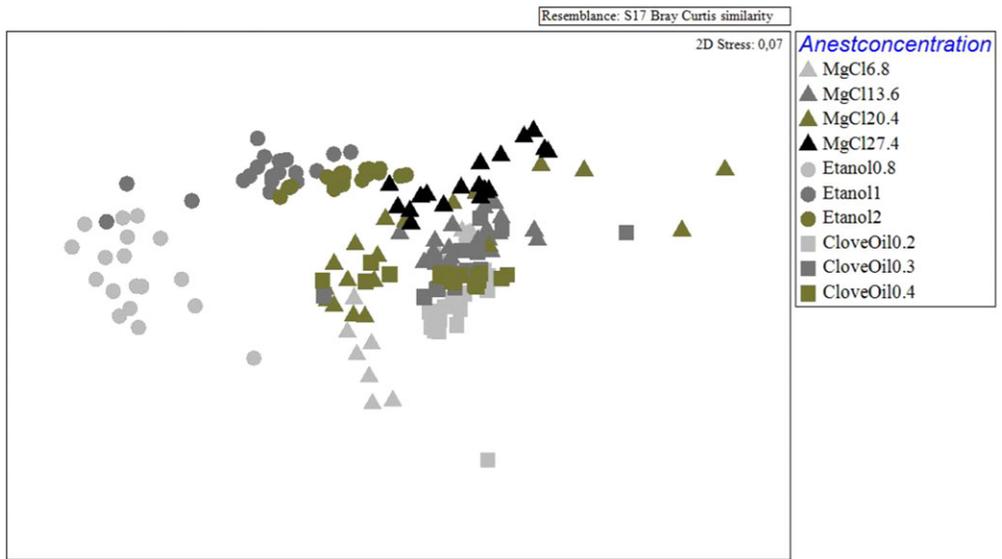
FIGURE 2.   *Two-dimensional solution of nonmetric multidimensional scaling (n-MDS) of all treatments.*

stressful signals (inking) of paralarvae during treatment. A treatment is considered useful when it allows paralarvae to be handled for approximately 180 sec, provokes rapid immobility (<200 sec), enables rapid total recovery (200–500 sec), and does not cause death or severe stressful behaviors. We established 180 sec as a suitable time based on previous experiments carried out with paralarvae. These criteria were fulfilled by some of the treatments tested (see Table 1). However, we observed stressful behaviors (inking) in all clove oil trials (100% paralarvae treated); clove oil is therefore considered inappropriate for use with octopus paralarvae. Clove oil has been previously used with good results as an anesthetic compound in adult cephalopods, for example, *Sepia elongata*, *D. pealeii*, *O. minor*, and *O. vulgaris*, producing minimal mortality after treatment and rapid induction and recovery times (Seol et al. 2007; Mooney et al. 2010; Estefanell et al. 2011). However, as some authors add ethanol to clove oil to facilitate its dissolution in seawater (Seol et al. 2007; Darmaillacq and Shashar 2008), we believe that the ethanol masks the real anesthetic effect of clove oil. Stressful behavior, including inking, jetting, chromatophore flashing, and death have only been reported by Mooney

et al. (2010) in *D. pealeii*, when using clove oil without ethanol. We conclude that clove oil and probably its active constituent (eugenol) cannot be recommended as an anesthetic compound for *O. vulgaris* paralarvae.

In general, $MgCl_2$ can be considered as a good anesthetic, without showing any stressful behavior on paralarvae (0% inking). Its induction times were slower compared to those observed with ethanol; this trend also occurred considering recovery times of paralarvae, with the longest times of the three anesthetics analyzed. This agent is of interest when long-time handling octopus paralarvae is necessary. The economic cost of $MgCl_2$ solutions is approximately six times more than ethanol solutions. In cases where paralarvae handling is <2 min, ethanol is a good anesthetic agent, but if longer periods of time are needed, $MgCl_2$ solutions are more useful. However, salinity variations on microcosms need to be under control throughout the treatments because additions of $MgCl_2$ may trigger consistent variations of this parameter.

This study reveals that low concentrations (10 mL/L) of ethanol in seawater are useful, producing quick sedation and immobilization in *O. vulgaris* paralarvae, at temperatures around 20 C, with no apparent signs of PSDLH. These

results concur with the findings of former studies in cephalopod adults and juveniles, including *O. vulgaris* (Lange and Hartline 1974; Patterson and Silver 1983; Silver et al. 1983; García-Franco 1992; Miyan and Messenger 1995; Ikeda et al. 2009; Mooney et al. 2010; Estefanell et al. 2011; Shomrat et al. 2011; Gleadall 2013), in which anesthetic treatments with ethanol were successful without any type of adverse reaction to treatment. The effectiveness of ethanol to produce sedation appears to be related to water temperature: ethanol is ineffective at low temperatures (10 C) and effective at temperatures ranging from 19 to 26 C (Gleadall 2013). Despite the use of several substances as general anesthetics for cephalopods (e.g., magnesium chloride and ethanol), few studies have focused on whether their mechanisms of action (i.e., insensitivity and unconsciousness) produce anesthesia in the nervous system.

We have preferred to use the term anesthesia rather than sedation in order to avoid the current controversy on the true or false perception of pain, suffering, and distress by the animal during anesthesia with these compounds. Future research is needed to clarify this aspect, because international efforts are being made to develop guidelines for the care and welfare of cephalopods in order to meet the requirements of European regulations on animal welfare (Fiorito et al. 2014, 2015).

Acknowledgments

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