Marine and Freshwater Behaviour and Physiology
Vol. 43, No. 4, July 2010, 297–303

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Long-duration anesthetization of squid (*Doryteuthis pealeii*)

T. Aran Mooney[a,b]*, Wu-Jung Lee[b] and Roger T. Hanlon[a]

[a]*Marine Resources Center, Marine Biological Laboratory, Woods Hole, MA 02543, USA;*
[b]*Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*

(*Received 4 May 2010; final version received 15 June 2010*)

Cephalopods, and particularly squid, play a central role in marine ecosystems and are a prime model animal in neuroscience. Yet, the capability to investigate these animals *in vivo* has been hampered by the inability to sedate them beyond several minutes. Here, we describe methods to anesthetize *Doryteuthis pealeii*, the longfin squid, noninvasively for up to 5 h using a 0.15 mol magnesium chloride ($MgCl_2$) seawater solution. Sedation was mild, rapid (<4 min), and the duration could be easily controlled by repeating anesthetic inductions. The sedation had no apparent effect on physiological evoked potentials recorded from nerve bundles within the statocyst system, suggesting the suitability of this solution as a sedating agent. This simple, long-duration anesthetic technique opens the possibility for longer *in vivo* investigations on this and related cephalopods, thus expanding potential neuroethological and ecophysiology research for a key marine invertebrate group.

Keywords: anesthesia; sedation; squid; *Loligo*; neurophysiology; giant axon

Introduction

Although cephalopods are key oceanic organisms used extensively as experimental animals in a variety of research fields (Gilbert et al. 1990), there is a relative paucity of information on maintaining them under anesthesia for prolonged durations. Lack of established protocols for sedation beyond several minutes constrains experimental conditions for many neurobiological and physiological preparations. This limits one's ability to investigate animals that act as key predators and prey (e.g., Boyle and Rodhouse 2005) and premiere biomedical model organisms, especially for neurobiology (Gilbert et al. 1990; Llinás 1999). There has been a clear need for establishing suitable extended-duration sedation procedures for cephalopods to facilitate research applications and to address experimental, husbandry, and ethical concerns.

Several studies have described cephalopod anesthetics for short durations; common solutions included urethane (ethyl carbamate), ethanol, and cold seawater. Yet, the use of all these solutions was discontinued as they proved problematic for various reasons. Urethane use (Messenger 1968; Young 1971) decreased when it was

*Corresponding author. Email: amooney@whoi.edu

ISSN 1023–6244 print/ISSN 1029–0362 online

DOI: 10.1080/10236244.2010.504334
http://www.informaworld.com

determined to be carcinogenic. Ethanol exposures often induced adverse reactions including jetting and inking upon initial immersion (Froesch and Marthy 1975; Andrews and Tansey 1981). Finally, cold water can be difficult to maintain within temperature ranges (3–7°C) necessary for quiescence and these low temperatures can affect the desired physiological responses (Weight and Erulkar 1976). One cephalopod sedative for which use has continued is magnesium chloride, $MgCl_2$. First demonstrated as narcotic by Pantin (1946), $MgCl_2$ sedation has subsequently been applied successfully for short-duration experiments (<25 min) on several cephalopod species (Messenger et al. 1985). None of these reports, however, involved *Doryteuthis* (formerly *Loligo*) *pealeii*, the model organism used for much of the principal neurobiological research on cephalopods. Furthermore, sedation durations remained limited, thus potentially constraining various experimental protocols. The goal of this study was to determine extended duration sedation (stage 1 anesthesia) procedures for *D. pealeii*. Here, we describe careful administration procedures of $MgCl_2$ to sedate the model species, *D. pealeii*, and protocols in which sedation was maintained under healthy conditions for up to 5 h.

Materials and methods

Anesthetic trials (38) were conducted over 4 months in 2008. The live squid were maintained in flowing, chilled seawater tanks at the Marine Biological Laboratory (Woods Hole, MA). These animals were collected *via* trawler from nearby ocean waters 4 days/week and held 0–2 days prior to experiments. All tested animals were examined visually and were deemed in good physical condition. For $MgCl_2$ sedation, squid (mean mantle, $L = 15.1$ cm; range 9.1–19.3 cm) were removed from the holding tank and placed in a small plastic bin $30 \times 18 \times 12$ (depth) cm, filled with 10 L of 14°C seawater. The bin was then covered to allow the animals to settle. After 5 min the cover was removed and the squid's respiration rate, baseline behavior, and coloration pattern assessed based on criteria established by Hanlon et al. (1999). The animals were then transferred gently, by hand, to an adjacent bin with sedating solution. After several minutes, the animals were returned to fresh, flowing seawater to monitor sedation times and latent effects of the anesthetic. "Sedation time" was designated as the duration that animals were (1) unresponsive to handling; (2) not swimming; and (3) failing to right themselves when turned ventral side up. When animals recovered from sedation, they were reintroduced into the anesthetic bath to extend the sedation time. Wet weight (g) was measured after each trial. In addition to $MgCl_2 \cdot 6H_2O$, solutions of benzocaine, ethanol (95%), clove oil, cold water, and gallamine triethiodide (Flaxedil®, Sigma), all in sand-filtered seawater were also examined (Table 1). Benzocaine, ethanol, and clove oil inductions followed procedures similar to above. Cold water sedation was conducted by maintaining the animal in chilled seawater and not returning it to a separate, uncooled container. Gallamine was administered at $1\ mg\ kg^{-1}$ by intramuscular injection into the arms, head or mantle of the three squid, respectively. The injection solution consisted of gallamine powder dissolved in a 1:1 ratio of 0.5-μm filtered seawater (consistent with teleost fish dosages; Suzuki et al. 2004). Of the 38 animals, 26 were tested with $MgCl_2$ and 24 of those were also used in a separate experiment.

Results

Squid responses varied with the type of anesthetic and all solutions but $MgCl_2$ proved unsuccessful in long-duration sedations. Benzocaine and clove oil are used regularly to sedate fish (Griffiths 2000; Laird and Oswald 2008); however, the two squids subjected to these substances reacted severely by jetting, inking, and flashing chromatophore skin patterns within seconds of induction and, in both cases, died within 4 min. For gallamine, all three squid and injection sites had similar results; all animals died within 15–20 min. The cold seawater reduced squid movement for up to 100 min; however, the low temperatures also reduced amplitudes of physiological evoked potentials, which may be a result of reduced synaptic potential transmission (Weight and Erulkar 1976). Ethanol (EtOH) effectively anesthetized the squid for periods up to 74 min, but in all five trials, there was the adverse reaction of some muscle tension, specifically that of attaching their suckers to the side or bottom of the bin. Three of the five animals exhibited jetting and repeated dramatic color/body pattern changes, from a deep, rust-colored red to pale and blanched, not normally seen in unsedated *D. pealeii* (Hanlon et al. 1999).

Magnesium chloride ($MgCl_2$; 0.15 mol; 30.5 g L^{-1} hexahydrate; Table 1) was found most suitable for mild, long-term sedation of squid. With repeated immersions, squid remained sedated for at least 5 h (Figure 1) without inducing adverse behavioral reactions (Figure 2). The level of anesthesia was suitable for both experimental and surgical purposes. Solutions were used for sedations of multiple animals without a noticeable detriment to efficacy. Within 3–4 min of immersion into the $MgCl_2$ solution, respiration rates began to decrease from 59 min^{-1} ($\pm$8 SD) to 44 min^{-1} ($\pm$9). Breathing quality generally changed to a more laborious or shallow breath at this point as well. Once this 25% drop was observed, the animal was then transferred to a tank of seawater (either 14 or 22°C) without added magnesium chloride. Initial sedations were relatively of short duration (10–20 min; Figure 1) and squid would become gradually more active (swimming or mild jetting). However, repeated inductions (i.e., administration of an anesthetic and establishment of anesthesia) into the $MgCl_2$ solution provided consecutively longer sedation periods; thus, increasing the number of inductions increased the sedation time ($r^2 = 0.61$; $p < 0.001$). For example, after a second 3-min induction, the squid would remain sedated for ca. 30 min (mean value). Third, fourth, and fifth inductions increased the duration of anesthesia to 53, 58, and 111 min, respectively. Respiration rates prior to later inductions were 60 min^{-1} ($\pm$9). By continuously monitoring the respiration rates and transferring the squid between the $MgCl_2$ solution (when respiration rates increased) and fresh seawater (as respiration rates decreased), it was possible to maintain sedation for long durations without apparent harmful effects.

Discussion

These are exceptionally long sedation times for any cephalopod and perhaps the longest recorded by nearly an order of magnitude. The previously reported longest sedation time was 25 min for *Sepia officinalis* and 5 min for the squid *Loligo forbesi* using $MgCl_2$ (Messenger et al. 1985) and 44 min in EtOH (Harms et al. 2006). While anesthetizing a new squid species, *D. pealeii*, is not surprising, sedating this particular species demonstrates applicability to a key model animal for various neurobiological studies. Clearly, Messenger et al.'s discovery of $MgCl_2$ as a suitable agent has proved accurate and useful, and this study extends its usefulness. Squid remained calm when

Table 1. Anesthetic solutions tested, parameters of squid (*D. pealeii*) subjects and solution effects.

Solution	Manufacturer	Concentration (dose)	*N*	Wet weight (g)[a]	Mantle L (cm)[a]	Mean induction time (min)[a]	Mean sedation time (min)[a]	Maximum sedation time (min)	% Mortalities	General reaction
Ethanol	Pharmco-AAPER	0.22–0.65 mol (10–30 mL L^{-1})	1[b]/4	68.6	18.5	4.0[b]/11.6	43.1	74[c]	0	Attached to container/jetting
Benzocaine (ethyl *p*-amino-benzoate)	Fisher scientific	1.67 mmol (0.28 g L^{-1})	1	55.0	10.0	N/A	N/A	N/A	100	Traumatic (death)
Gallamine triethiodide (Flaxadil®)	Sigma	2.37 mmol (1 mL kg^{-1} of 1 : 1 H_2O_{sw} solution)	3	68.5	16.4	N/A	N/A	100	100	Traumatic (death)
Cold seawater	N/A	N/A	2	57.7	22.4	86.0	86.0	100[c]	0	Mild to none
Clove oil	Now foods	1 mL L^{-1}	1	83.4	19.0	N/A	N/A	N/A	100	Traumatic (death)
$MgCl_2$ ($MgCl_2 \cdot 6H_2O$)	Fisher scientific	0.15 mol (30.5 g L^{-1})	26	54.6	14.8	3.1	162.8	302[c]	3.8	Mild to none

Notes: [a]Indicates mean values; [b]3% ethanol solution; [c]Stopped sedation due to end of experiment.

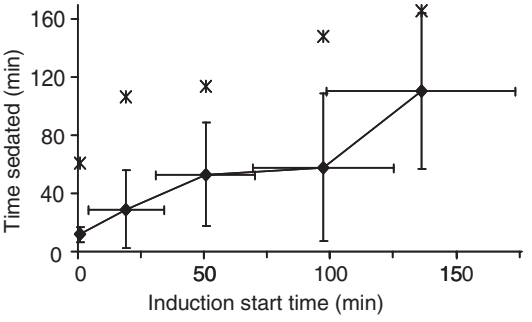

Figure 1. Mean time sedated (±SD) after each respective $MgCl_2$ induction. Time is relative to the initial anesthetic induction. Note that as number of inductions increases (abscissa), so does the duration of sedation. Stars indicate maximum time sedated for each induction.

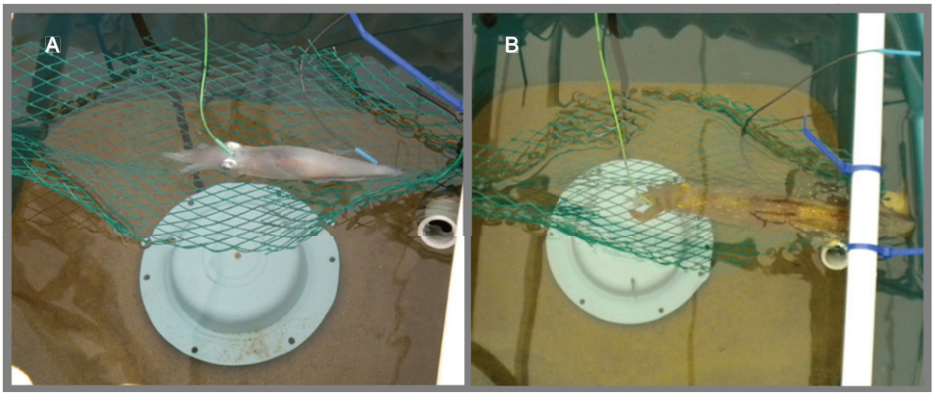

Figure 2. (A) Sedated and (B) unsedated squid. The unsedated animal has revived from anesthesia procedures that took place approximately 90 min prior to the photo and physiological recordings in Figure 3C. Visible in both photos are the response-recording electrode cables (green and blue), a speaker below the squid which is playing 150 Hz acoustic stimuli, and the green mesh netting which encapsulates the squid.

transferred into the $MgCl_2$ solution. Creating an $MgCl_2$ solution was relatively inexpensive and easy. Solutions were used repeatedly without altering the efficacy of the solution (thus the animal's metabolic system did not appear to substantially degrade the solution concentration). As with other anesthetics, induction duration and respirations were carefully monitored as overexposure could be lethal. However, such reactions allowed the 0.15 mol $MgCl_2$ solution to be used on four occasions as a humane euthanizing agent by keeping the squid within the solution for 3–4 min after respirations ceased. The mode of action of $MgCl_2$ has been hypothesized in cephalopods to work at the postsynaptic membrane in the central nervous system (Katz 1966; Messenger et al. 1985). Sedation did not affect the amplitude or latency of at least some neurological evoked responses (Figure 3), indicating that it is reliable for physiological experiments. These responses were recorded from nerve bundles associated with the statocyst of the squids in response to low-frequency acoustic stimuli during squid hearing examinations.

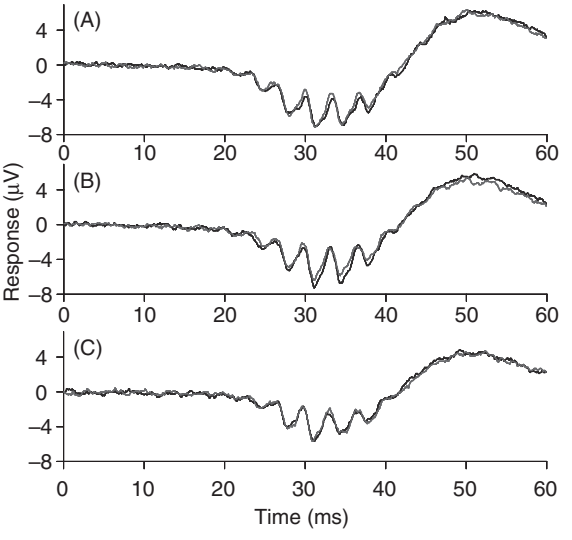

Figure 3. Acceleration generated physiological evoked responses from 150 Hz acoustic stimuli recorded from statocyst nerve bundles of two squid (represented by black and grey traces) at (A) initial sedation, 5 min after $MgCl_2$ induction, (B) 60 min into sedation, and (C) when the squid are no longer anesthetized, 90 min after the initial induction. In (A) and (B), squid are ventral side-up, rest on the experimental surface and do not generate typical coloration patterns (Figure 2). In (C), squid are dorsal side up, swimming and demonstrate typical coloration patters and startle responses. The evoked responses were recorded in an experiment examining squid hearing capabilities (unpublished data).

While this work is not a comprehensive study of anesthesia applied in squid, the results do represent a novel method of long-term sedation in squid. As squid have been referred to as a ''keystone'' species, this sedation procedure may broaden capacities to examine important ecophysiological processes. Furthermore, the technique was applied to a model species of neurobiological research, thus suggesting a new *in vivo* means of investigating the squid nervous system and giant axon. Relative to other means of anesthetizing squid, $MgCl_2$ proved superior in ease of application, fiscal economy, availability, and its minimal distressing effect on the animal subject.

Acknowledgments

This study was supported by a postdoctoral fellowship from the Grass Foundation to TAM. We thank the trustees and Grass Lab for their support. Assistance was also provided by the Marine Biological Laboratory including S. Lindell, L. Mäthger, J. Allen, R. Probyn, E. Enos, and J. Gardiner. L. Mäthger, D. Ketten, and two anonymous reviewers provided valuable comments on the earlier versions of this manuscript.

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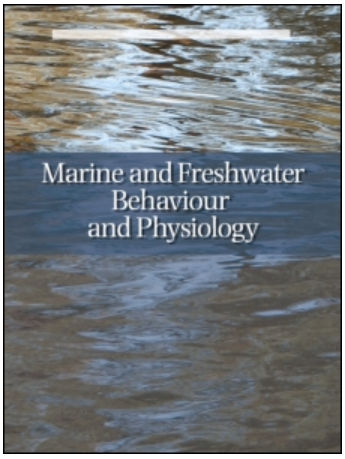

Marine and Freshwater Behaviour and Physiology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713644420

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T. Aran Mooney[ab]; Wu-Jung Lee[b]; Roger T. Hanlon[a]
[a] Marine Resources Center, Marine Biological Laboratory, Woods Hole, MA 02543, USA [b] Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

First published on: 03 August 2010

To cite this Article Aran Mooney, T. , Lee, Wu-Jung and Hanlon, Roger T.(2010) 'Long-duration anesthetization of squid (*Doryteuthis pealeii*)', Marine and Freshwater Behaviour and Physiology, 43: 4, 297 — 303, First published on: 03 August 2010 (iFirst)
To link to this Article: DOI: 10.1080/10236244.2010.504334
URL: http://dx.doi.org/10.1080/10236244.2010.504334