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Anesthetic Efficacy of Magnesium Chloride and Ethyl Alcohol in Temperate Octopus and Cuttlefish Species

Lisa A Abbo,[1,*] Nicole E Himebaugh,[2] Lindsey M DeMelo,[1] Roger T Hanlon,[1] and Robyn J Crook[3]

Cephalopods are important in biologic and biomedical research, yet relatively little objective information is available to guide researchers and veterinarians regarding the best methods for anesthetizing these animals for various experimental procedures. Recent studies demonstrate that ethyl alcohol and magnesium chloride are effective at depressing efferent and afferent neural signals in some tropical cephalopod species when measured via the pallial nerve. Here we used similar methods to test 2 temperate species (*Octopus bimaculoides* and *Sepia officinalis*) and demonstrate that (1) ethyl alcohol and magnesium chloride were effective at reversibly depressing evoked activity in the pallial nerve, (2) ethyl alcohol generally had shorter induction and recovery times compared with magnesium chloride, (3) both agents were associated with a latency between the behavioral and neural effects, and it was longer with magnesium chloride, and (4) senescent animals generally had longer induction or recovery times than young animals. Both agents successfully anesthetized both life stages; however, our data show that assessing anesthesia based solely on behavior may lead to premature commencement of invasive procedures. We conclude that temperate cephalopods can be humanely, effectively, and completely anesthetized by using these 2 agents and that the loss of neural signal we show here is consistent with true anesthesia and not merely paralysis. This relatively simple, nondestructive nerve recording technique can be applied to the study of other prospective anesthetic agents in cephalopods.

Abbreviation: EtOH, ethyl alcohol.

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Cephalopods have provided important models for biologic and biomedical research for more than 100 y,[11,24,35,43] and their use will likely continue to increase for the foreseeable future.[31] Unlike vertebrate animals, cephalopods have only recently been included in regulatory oversight of research animals in select countries. In 2010, cephalopods were included in regulations governing use of laboratory animals in the European Union[40] due to mounting evidence that their well-developed nervous system, which results in complex behavioral, cognitive, and learning capabilities, may indicate they are capable of experiencing pain and distress.[1,2,6,7,12] Studies are needed to (1) address the capacity of cephalopods to experience pain and distress and (2) rigorously test methods that enhance animal welfare during invasive procedures.

Anesthesia is required for the prevention of distress or pain in vertebrate animals used in research unless the withholding of anesthesia can be justified scientifically.[6] In its most basic definition, anesthesia is the loss of sensation to the body.[18] The term "general anesthesia" describes, in addition to analgesia (alleviation of pain), a state of CNS depression such that sensory and motor reflexes are attenuated. CNS depression renders the animal unaware and unarousable. For the purposes of the present study, "anesthesia" indicates (1) a state of neural depression and (2) lack of response to noxious stimuli.[18]

Historically, urethane (ethyl carbamate) and ethanol (EtOH) have been the most commonly used anesthetics in cephalopods.[3,43] Urethane has fallen out of favor due to its carcinogenic and mutagenic effects in laboratory animals,[10] but EtOH continues to be used widely despite reports that it can be aversive in some cephalopods.[3,9,15,16,29,37] A study comparing 3 anesthetic techniques in *Octopus vulgaris* concluded that EtOH satisfied the criteria for general anesthesia defined as "modifying transmission of information between the periphery and the CNS."[3] The observed criteria in that study[3] were respiratory depression, lack of evoked response to stimulus, periocular contraction of skin on touching of the eye, muscle relaxation, paling of chromatophores, and loss of righting reflex. Nerve stimulation was used to assess the presence or absence of electrical activity by stimulating the cut end of the pallial nerve that resulted in contraction of the mantle musculature, suggesting a failure of the anesthetics to block transmission.[3] The authors concluded that 2 substances were effective anesthetics.[3] A more recent publication confirmed EtOH, $MgCl_2$, and urethane as the most effective and reliable anesthetics with the fewest adverse reactions (termed "typical adverse response patterns" [TARP]).[15] In that study and in most others focused on anesthesia of cephalopods, anesthesia depth was assessed solely by behavioral indicators.[3,15]

The first publication to describe the use of $MgCl_2$ as an anesthetic in cephalopods suggested that it was effective as an

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[1]Marine Biological Laboratory, Woods Hole, Massachusetts; [2]College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina; [3]Department of Biology, San Francisco State University, San Francisco, California
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[*]Corresponding author. Email: labbo@mbl.edu

anesthetic agent in a range of genera and that it was preferable to both urethane and EtOH due to their adverse effects on the animals.[28] Since then, $MgCl_2$ has been used commonly in cephalopod research.[2,8,12,15–17,37] The criteria for anesthesia in the original $MgCl_2$ publication[28] were behavioral - muscle relaxation and failure to respond to evoked stimuli such as skin and arm pinch and loss of pupillary light reflex. However, the question remained of whether the animals experienced CNS depression and subsequent loss of consciousness (which are key features of general anesthesia), or if instead the behavioral effects were due solely to paralysis. The challenge is to demonstrate convincing blockade of sensory input. In vertebrate animals, the consensus is that general anesthesia comprises 5 criteria: (1) hypnosis, (2) amnesia, (3) analgesia, (4) muscle relaxation, and (5) lack of reflex responses to painful stimuli.[42] In the absence of a similar consensus for cephalopods, using these same criteria as a starting point to assess the effectiveness of an anesthetic agent in those species seems reasonable. The lack of evidence for anesthesia beyond simple immobilization (i.e., criteria 4 and 5) has been a concern with $MgCl_2$.[33,41] However, until recently, few systematic attempts have been made to evaluate effects beyond behavioral measures for any of the anesthetic agents commonly used in cephalopods. Other anesthetic agents, including eugenol, 2-phenoxyethanol, isoeugenol (AQUI-S), tricaine methanesulfonate (MS222), and hypothermia, have been tested in several cephalopod species with varying degrees of success,[2,4,8,16,37,39] yet $MgCl_2$ and EtOH remain the most commonly used in part due to their availability, reliability, and ease of use.[27]

Recently, the first evidence that $MgCl_2$ and EtOH produce complete and reversible blockade of evoked neural signals in response to noxious stimulation was provided for several tropical cephalopod species, indicating that these 2 substances function as "true" anesthetics for some cephalopods.[4] The study demonstrated a technique for measuring the neural signal in the pallial nerve, which innervates the mantle, by using a hook electrode that can be placed and removed with minimal effect on animal behavior, allowing minimally invasive recordings of nervous system activity during anesthesia induction and recovery. In that study[4], EtOH and $MgCl_2$ were effective at blocking efferent and afferent signals when measured via the pallial nerve in 3 tropical species of cephalopods, suggesting that 4 of the 5 criteria (amnesia was not tested) for general anesthesia were met. The study was restricted to small tropical species; effects on larger, temperate species might differ significantly. Although extrapolation from one species to another is reasonable when species-specific information is sparse, evidence suggests that in cephalopods environmental temperature has important effects on anesthetic efficacy (for example, failure of EtOH to induce anesthesia in cold-water species held at less than 15 °C).[27]

The aim of the current study was to investigate the effects of $MgCl_2$ and EtOH on the nervous system of 2 temperate species of cephalopods that are in common use in research in the United States: *Octopus bimaculoides* and the common European cuttlefish, *Sepia officinalis*. In addition, the current study compared life stages to evaluate the effectiveness of these agents in young animals compared with senescent adults.

Materials and Methods

Cephalopod species. *O. bimaculoides, S. officinalis,* and the squid *Doryteuthis pealeii* were used in an initial study. Two of these (*Octopus* and *Sepia*) produced ample reliable data and are reported here. The animals were all housed in the Marine Resources Center of the Marine Biological Laboratory, which has sophisticated seawater systems and multiple filtration and temperature control capabilities.

O. bimaculoides, the California 2-spot octopus, ranges from the Northeast Pacific, CA (San Simeon) and the California Channel Islands south to Guerrero Negro, on the Pacific coast of the Baja California Peninsula, Mexico.[30] This species is used to study camouflage, behavior, and learning.[13,14,25] We studied 2 distinct life-stage groups: senescent (post-egg-laying) females (average mantle length, 11 cm: average weight, 380 g; $n = 8$ total for both EtOH and $MgCl_2$ treatment groups) and lab-cultured juveniles (age, 3 to 4 mo; mantle length, 3 cm; average weight, 9 g; $n = 6$ total used for both EtOH and $MgCl_2$ treatment groups). Determining the sex of juvenile *O. bimaculoides* was not possible at this age. The sample size was based on availability of the animals and not, as would be ideal, on a pre-determined estimate of effect size or variability. Therefore, we caution that some effects are subject to low statistical power. These animals were acquired for an unrelated study involving the embryos, so these females were considered surplus and would have been euthanized otherwise. The natural life cycle of this species is such that females lay a single clutch of eggs and do not exit the den or feed during brooding. Once the eggs have hatched, the female senesces and dies. Female *O. bimaculoides* with eggs were collected by a commercial enterprise (Aquatic Research Consultants, San Pedro, CA) from southern California and shipped to Woods Hole. For this reason, the study here refers to this group as senescent adults, although the exact age and timing of egg laying was unknown. Before this study, none of the octopuses had been used in previous research. The animals were fed daily a diet of green crabs (*Carcinus maenas*) and marine snails (*Crepidula fornicata*). Animals were housed individually in glass aquaria (19 L; 40.64 cm × 20.32 cm × 25.40 cm) on an open seawater system. They were provided with a den and rocks for environmental enrichment. Tanks were siphoned daily, and tanks were completely changed and disinfected once weekly. The mariculture room in which they were kept was on a 12:12-h light:dark cycle. Water temperature was kept constant, at 21 to 22 °C. Total ammonia nitrogen, nitrites, nitrates, pH, dissolved oxygen, salinity, and temperature were monitored at least once weekly and remained within normal limits throughout the study.

Sepia officinalis, the common European cuttlefish, is native to the eastern Atlantic Ocean and Mediterranean Sea.[36] This species is frequently used to study camouflage, behavior, and learning.[5,20,21,23,44] Eggs were collected from the south coast of England by a commercial collector in April 2016 and 2017 and reared in the Marine Resources Center; each group was a first filial generation reared directly from normal, healthy, feral eggs.[32] Two distinct life-stage groups were used for the study: senescent cuttlefish that hatched in August 2016 (age, approximately 2 y; mantle length, 9 to 11 cm; weight, 103 to 173 g; $n = 5$ for EtOH group; $n = 3$ for $MgCl_2$) and subadult cuttlefish that hatched in August 2017 (age, approximately 1 y; mantle length, 4.7 to 6 cm: weight, 21 to 29 g; $n = 5$ for EtOH group; $n = 5$ for $MgCl_2$). Again, sample size was determined based on availability, and statistical power in some comparisons is low. Sex in *S. officinalis* is not possible by external exam, so the sex composition of both groups remained unknown. These animals were acquired for an unrelated study and therefore were deemed surplus and would have gone unused otherwise. *S. officinalis* typically survives in the wild to an age of 1 to 2 y, but the animals housed at the Marine Biological Laboratory are kept at a constant, low water temperature (16 °C) and constant 12:12-h light:dark cycle to prevent maximal growth and retard rapid maturation. Therefore, these animals can be kept in the labora-

tory and used longer than their normal lifespan in the wild.[32] The younger cohort was about a third the size of the older group and had not reached sexual maturity; we refer to these animals as subadults or juveniles throughout this study. The older group had exceeded its normal lifespan and was approximately 2 y old at the time of the study; some animals from the cohort had begun showing signs of senescence in the form of minor skin lesions, slight loss of normal skin patterns, anorexia, and in a few cases, death. None of the animals used in the study showed any signs of disease or illness. The 2-y-old group of cuttlefish had been previously used for noninvasive camouflage studies but had never been anesthetized or undergone other invasive procedures. The 1-y-old group of cuttlefish had never been used in any research prior to this project. Each animal was included in only one test group, either EtOH or $MgCl_2$.

Animals were trained to feed on food-grade, frozen penaeid shrimp (Trader Joe's brand) and were fed once daily. Animals were housed in a 54 L, 130 cm × 75 cm × 5.5 cm fiberglass open sea water table divided equally into 6 sections, isolating each animal. They were provided with a PVC den and plastic plants for environmental enrichment. Tanks were siphoned every day. The mariculture room in which they were kept was on a 12-h light:dark cycle. Water temperature was kept constant at 16 °C. Total ammonia nitrogen, nitrites, nitrates, pH, dissolved oxygen, salinity, and temperature were monitored at least once a week and remained within normal limits throughout the study.

Ethical note. In the United States, cephalopods are not included in federal regulations that govern the use of animals in research laboratories; consequently, no protocol or approval number was required for this study. However, the Marine Biological Laboratory voluntarily upholds a Cephalopod Care Policy, and the care of the animals in this study adhered to that policy.

Neural recordings. The pallial nerve in each species was selected for electrode placement due to ease of access and minimal tissue overlying the recording site. Animals were sedated in 1% to 2% EtOH in home tank water. Once the animal was unresponsive to touch, it was lifted out of the water and into a vertical position. The mantle flap was moved aside in cuttlefish, revealing the large pallial nerve visible on the inner dorsal surface. To place the hook electrode, the connective tissue holding this large nerve against the mantle was teased away carefully by using fine forceps, and the hook electrode was passed through a small window made in the connective tissue between the nerve and mantle, leaving the nerve fully intact. No bleeding was observed, and no wound closure was necessary. The hook allowed direct nerve contact with the electrode for clearest neural recording. In octopuses, the pallial nerve is surrounded by connective tissue and a thin muscular bridge and is free from the body wall in the mantle. The hook was simply placed around the nerve–muscle bundle and secured by gently twisting the wire's end. The electrode was made from insulated silver wire (diameter, 3.8 mm; item AFTI510, World Precision Instruments, Sarasota, FL), with the insulation stripped from the inner surface of the hook portion. Figures 1 A and B show the general arrangement and posture of animals for recording. Figures 1 C and D show the location of the hook electrode around the pallial nerve. One animal was removed from the study because nerve compression was suspected (indicated by ipsilateral skin paling).

A webcam (model VU0018, Logitech, Freemont, CA) was positioned above the experimental field to record audio and video of each trial. A digitizer (Powerlab 4/35, AD Instruments, Colorado Springs, CO) and an extracellular amplifier (model 1700, A-M Systems, Sequim, WA) were used to capture neural signals from the electrode attached to each animal's pallial nerve and recorded on LabChart software (AD Instruments) using the Video Capture module. A grounding cable was secured in the recording tank and positioned away from the animal.

Anesthesia protocol using EtOH. The subject was removed from its home tank by using a small seawater-filled container and transported to the experimental location. A sedative bath of 1% EtOH[2,9,16] (95% undenatured, Decon Laboratories, King

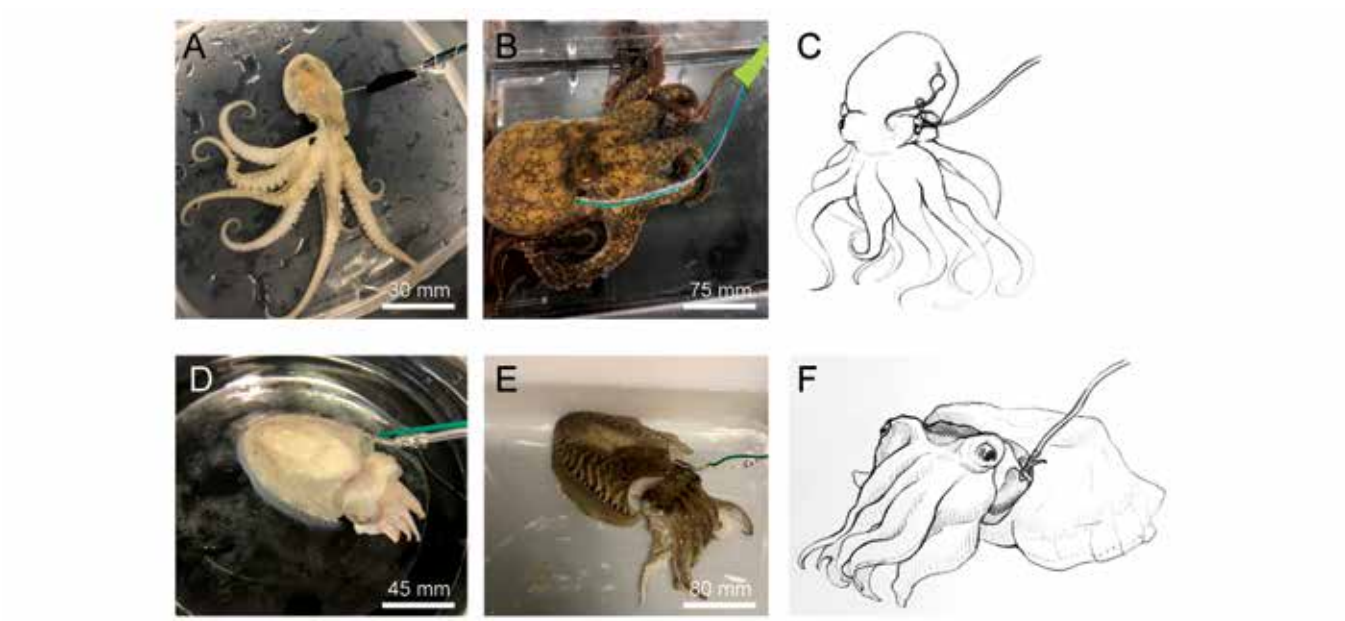

Figure 1. Electrode placement in temperate cephalopods. (A) Juvenile *Octopus bimaculoides*. (B) Senescent adult *Octopus bimaculoides*. (C) Illustration of where the U-shaped electrode is hooked gently around the large pallial nerve in *Octopus bimaculoides*, (D) subadult *Sepia officinalis*, and (E) senescent adult *Sepia officinalis*. (F) Illustration of where the U-shaped electrode is hooked gently around the large pallial nerve in *Sepia officinalis*.

of Prussia, PA) and home tank water was mixed in either a 1- or 3-L test chamber, depending on the age or size of the animal, and the animal was placed in the bath for sedation to allow electrode placement. If after 5 min the animal was not sedated sufficiently for electrode placement, an additional 1% of EtOH was added. All animals were sedated adequately by using 1% to 2% EtOH.

Once the electrode was placed on the pallial nerve, the water was changed completely to fresh seawater to allow the animal to recover from sedation. The time needed to change the water was less than 1 min. Recording began once the animal showed return of normal, spontaneous behaviors. Upon recovery from sedation (indicated by the return to normal skin patterning coloration and spontaneous movement), the experimental induction procedure began, and EtOH was added to the animal's container to create a 0.5% EtOH bath. At each minute after EtOH administration, the animal's skin was pinched by using fine forceps to measure behavioral and neural responses to this noxious sensory input. For cuttlefish, the pinch was performed in 2 locations: the rostral and caudal extremities of the fin ipsilateral to the electrode. For octopuses, the pinch was given on the rostral and caudal mantle ipsilateral to the electrode. If no behavioral or neural effect of 0.5% EtOH was apparent after 5 min, EtOH was added in 0.5% increments every 5 min until the animal was unresponsive to the pinch and the pinch-evoked neural signal was absent. Once full anesthesia was reached (defined as lack of physical reaction and lack of neural signal in response to pinching), the water was changed completely to fresh seawater and neural recording resumed, with pinches every minute until the animal was recovered fully. Electrode removal sometimes required additional sedation with EtOH. At the end of the trial, the animal was replaced in its home tank and observed over the next few days for any complications.

Anesthesia protocol using $MgCl_2$. Before each trial, a 7.5% (369mM) stock solution of magnesium chloride hexahydrate (Fisher Scientific, Waltham, MA) was prepared using distilled water and was chilled to match each animal's ambient seawater temperature.[28] Containers of equal volume and temperature were designated for premade baths comprising fresh seawater only, 1:3 $MgCl_2$:seawater, 1:2 $MgCl_2$:seawater, and 1:1 $MgCl_2$:seawater by using home tank seawater for each dilution.[2,16,28] For initial sedation to place the electrode, baths of 1% to 2% EtOH (concentration, 95%) were used, as described earlier. After application of the electrode, the animal was placed into fresh seawater for full recovery from sedation before starting the trial for $MgCl_2$. After the animal was fully awake from sedation, it was placed into the 1:3 $MgCl_2$ bath. The animal was pinched every minute by using fine forceps at the same locations as described earlier. The behavioral responses and neural signals were recorded after each pinch. When the animal still showed signs of consciousness or neural signal after 5 min in the 1:3 $MgCl_2$ bath, it was placed into the 1:2 $MgCl_2$ bath and subsequently (if awake after an additional 5 min) in the 1:1 $MgCl_2$ bath. Recordings and pinches continued until the animal was fully anesthetized, at which time the animal was placed into fresh seawater. Pinches continued every minute until full reversal and consciousness was apparent. After the subject was fully awake, the electrode was removed, and the animal was returned to its home tank for observation and signs of complications.

Determining points of full anesthesia and recovery. Neural tracings and video recordings were analyzed simultaneously to determine the period of anesthesia. Full anesthesia was based on the absence of evoked neural signal and a lack of physical response after a pinch stimulus. Sufficient electrode contact was assured through the consistent presence of signal associated with respiration (Figure S1). The recordings were analyzed in LabChart to determine the following elapsed time points: time from adding anesthetic to loss of behavioral response, time from adding anesthetic to loss of neural signal, time to return of neural signal after placement in fresh sea water, and time to behavioral response after placement in fresh seawater. The initial and endpoint concentrations of EtOH and $MgCl_2$ were recorded for each trial.

The animals were observed for signs of loss or regaining of alertness; these included (1) jetting behavior, (2) color patterning change, (3) spontaneous movement, (4) change in respiratory rate, (5) response to forceps in the visual field, and (6) a physical response when touched lightly by forceps. Wakefulness and periods of neural activity were characterized by bursts of neural signal (Figures S2, S3, S4, and S5) after a pinch stimulus on the fin or mantle.

Data analysis. Data were analyzed by using Prism 8.1.1 software (GraphPad, San Diego, CA). Tests of normality appropriate for small sample sizes (Kolmogorov–Smirnov test for 4 or more members per group; Shapiro–Wilk test for fewer than 4 members per group) were used to screen data prior to hypothesis testing, and all distributions passed the assumptions of normality.

A 2-tailed *t* test was used to compare anesthetics within species and between life stages. Paired *t* tests were used to evaluate significant differences between onset and recovery of behavioral compared with neural response within groups. Data are expressed as mean ± 1 SEM, and the critical α was set at a *P* value of 0.05.

Final disposition. At the conclusion of the study, adult cuttlefish and octopuses resumed normal feeding and behavior until they began to decline due to senescence and were euthanized by immersion in 2-3% ethanol for a minimum of 30 min past cessation of ventilation at which time the brain was destroyed to ensure complete euthanasia. The juvenile cuttlefish and octopuses were transferred to an unrelated study.

Results

EtOH compared with $MgCl_2$. Induction times (i.e., the time until loss of neural signal) were generally shorter for EtOH than $MgCl_2$. Octopus data are illustrated in Figures 2 A and B, and Figures S2, and S3; cuttlefish data are shown in Figures 3 A and B, Figures S4 and S5. For example, anesthetic induction of adult octopuses was 8.3 ± 1.9 min in EtOH as compared with 19.2 ± 1.6 min in $MgCl_2$ ($P = 0.001$). However, in juvenile cuttlefish the induction time for EtOH (20.7 ± 3.1 min) was significantly ($P = 0.004$) longer than for $MgCl_2$ (8.1 ± 0.7 min). In addition, recovery times were generally longer for $MgCl_2$ as compared with EtOH (Figures 2 C and D, 3 C and D), especially for senescent cuttlefish. Recovery (i.e., return of neural signal) was more rapid for EtOH (1.6 ± 0.1 min) than for $MgCl_2$ (13.0 ± 0.1 min; $P < 0.0001$).

Senescent adults compared with juvenile or subadult animals. In *Octopus*, induction (i.e., loss of neural signal) generally took longer in senescent adults as compared with juveniles for both EtOH and $MgCl_2$ (Figures 4 A and B). The difference was greater with $MgCl_2$: senescent *Octopus* took 19.2 ± 1.6 min to lose neural signal compared with 6.2 ± 0.7 min for the subadults ($P < 0.001$). Recovery time was similar between the senescent adult and juvenile *Octopus* (Figures 4 C and D).

Cuttlefish showed the same general pattern as octopus, with longer induction time in senescent adults as compared with subadults (Figures 5 A and B). The exception was the EtOH

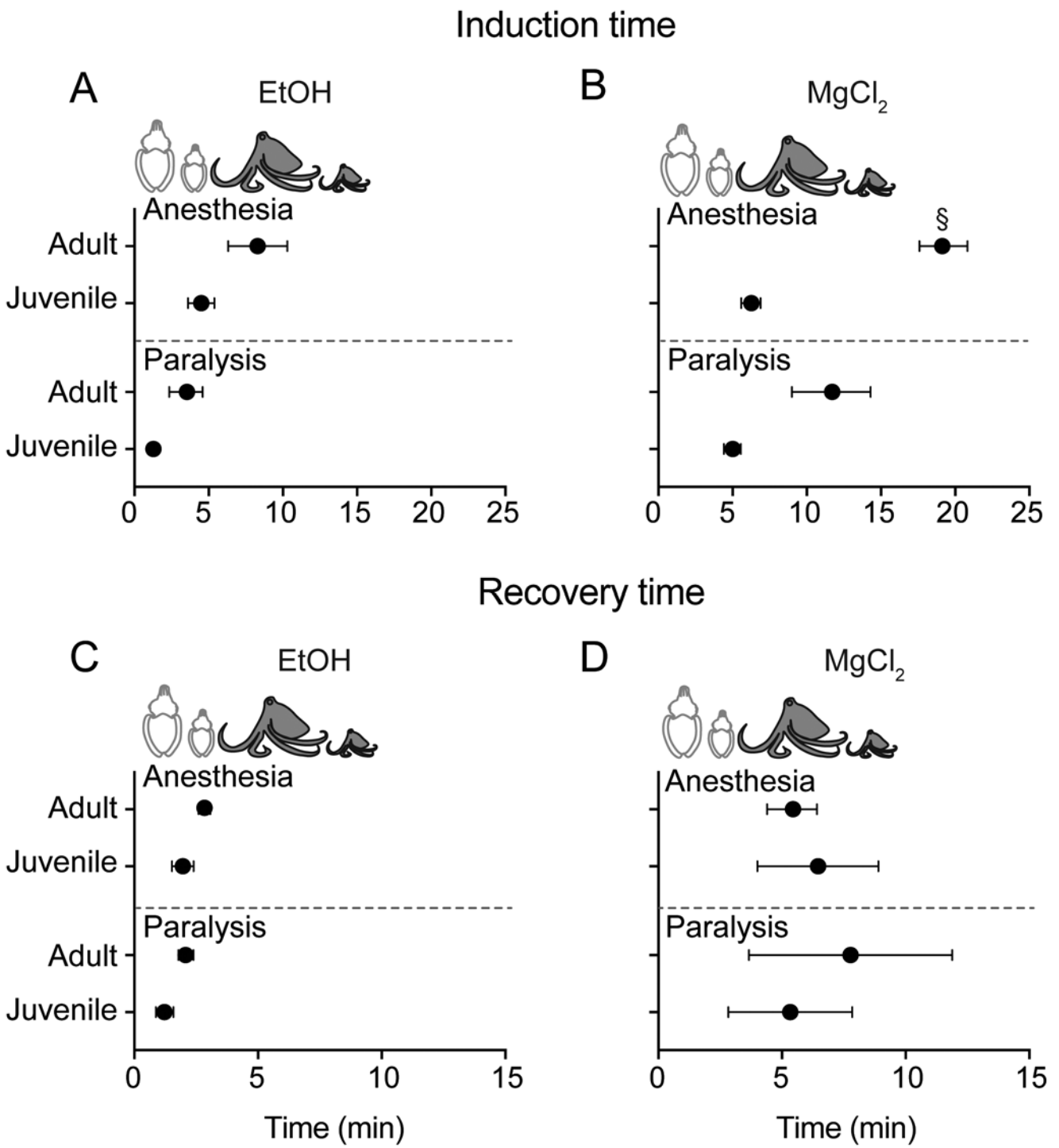

Figure 2. Responses of *O. bimaculoides* to anesthesia via EtOH and $MgCl_2$ (8 adult, 6 juvenile). (A) Juvenile and adult octopuses show no significant differences in time to loss of behavioral response to pinch (paralysis) or in time to loss of afferent neural signal (anesthesia). (B) Extended induction times were apparent for adults when exposed to magnesium chloride, compared with juveniles. (C) Recovery times from EtOH anesthesia were short and showed little variation in either adults or juveniles. (D) Recovery times from magnesium chloride were highly variable, but no significant differences were apparent. Data are given as mean ± SEM; the cartoons show the groups included in the figure in gray shading. Significant differences between age classes are indicated (unpaired *t* test, §, $P < 0.0001$).

induction time, for which *Sepia* subadults took significantly longer than senescent adults (20.7 ± 3.1 compared with 9.2 ± 1.3 min; $P = 0.009$) to lose neural signal. For $MgCl_2$, recovery was significantly longer for *Sepia* senescent adults (13.1 ± 0.1 min) than for subadults (6.1 ± 1.1 min, $P = 0.004$), recovery after EtOH anesthesia was similar between senescent adults and subadults (Figures 5 C and D).

Behavioral compared with neural onset and reversal of anesthesia (neural signal). Regarding the behavioral onset of anesthesia and neural signal loss (*Octopus*, Figures 2 A through D; *Sepia*, Figures 3 A through D), many subjects showed a significant delay between the onset of behavioral signs of anesthesia (paralysis) and the loss of neural signal (anesthesia). For *Octopus* juveniles in the EtOH group, the behavioral response to pinch

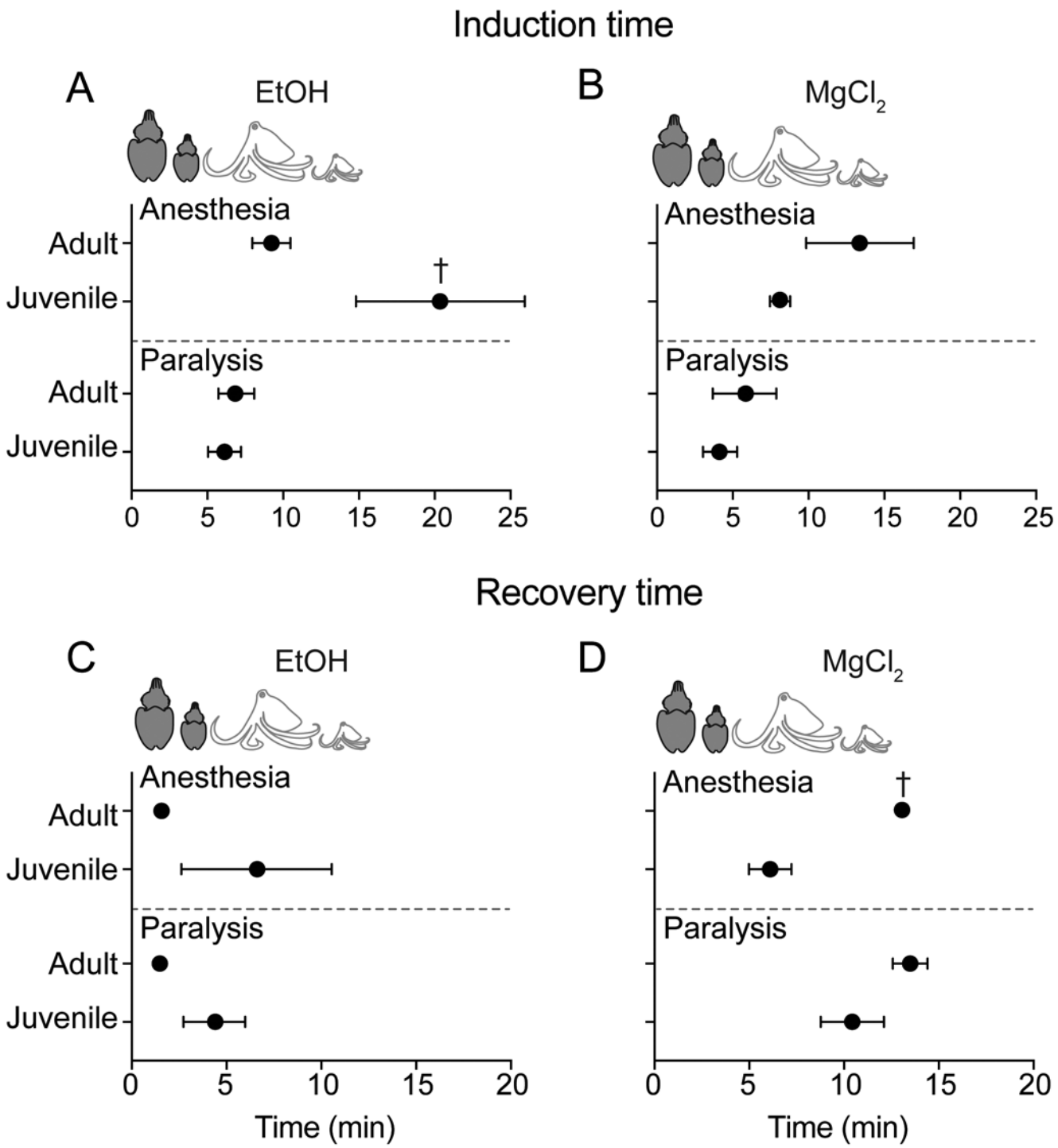

Figure 3. Responses of cuttlefish (*S. officinalis*) to anesthesia via EtOH and $MgCl_2$ (adults: 5 EtOH and 3 $MgCl_2$; juveniles: 5 EtOH, 5 $MgCl_2$). (A) Juvenile and adult cuttlefish show no significant differences in time to loss of behavioral response to pinch (paralysis), but juveniles had significantly longer latency to loss of afferent neural signal (anesthesia) compared with adults. (B) There were no differences in induction times when $MgCl_2$ was used. (C) Recovery times after EtOH anesthesia were more variable for juveniles than for adults but did not differ significantly between groups. (D) Recovery of neural signal after $MgCl_2$ anesthesia was significantly longer for adults, but behavioral recovery did not differ significantly between age groups. Data are given as mean ± SEM; cartoons show the groups included in the figure in gray shading. Significant difference between age classes are indicated (unpaired *t* test, †, $P < 0.01$).

was lost in 1.3 ± 0.3 min whereas 4.5 ± 0.9 min ($P = 0.011$) was required for the neural signal to disappear. Therefore, although the animal appeared to be anesthetized based on behavioral indicators, a neural signal was still present, indicating that the animal was still receiving nociceptive input from the mantle pinch. A similar but more pronounced effect occurred in the *Sepia* subadult group in EtOH; these animals required 6.1 ± 0.8 min to lose behavioral response to the pinch and 20.7 ± 3.1 min ($P = 0.002$) to lose neural signal response. *Octopus* senescent adults given $MgCl_2$ showed a similar pattern to *Sepia:* adults required 1.6 ± 2.7 min to behavioral loss and 19.2 ± 1.6 min to neural signal loss ($P = 0.002$), whereas *Sepia* subadults required 4.2 ± 1.1 min to behavioral loss and 8.1 ± 0.7 min to neural signal loss ($P = 0.016$).

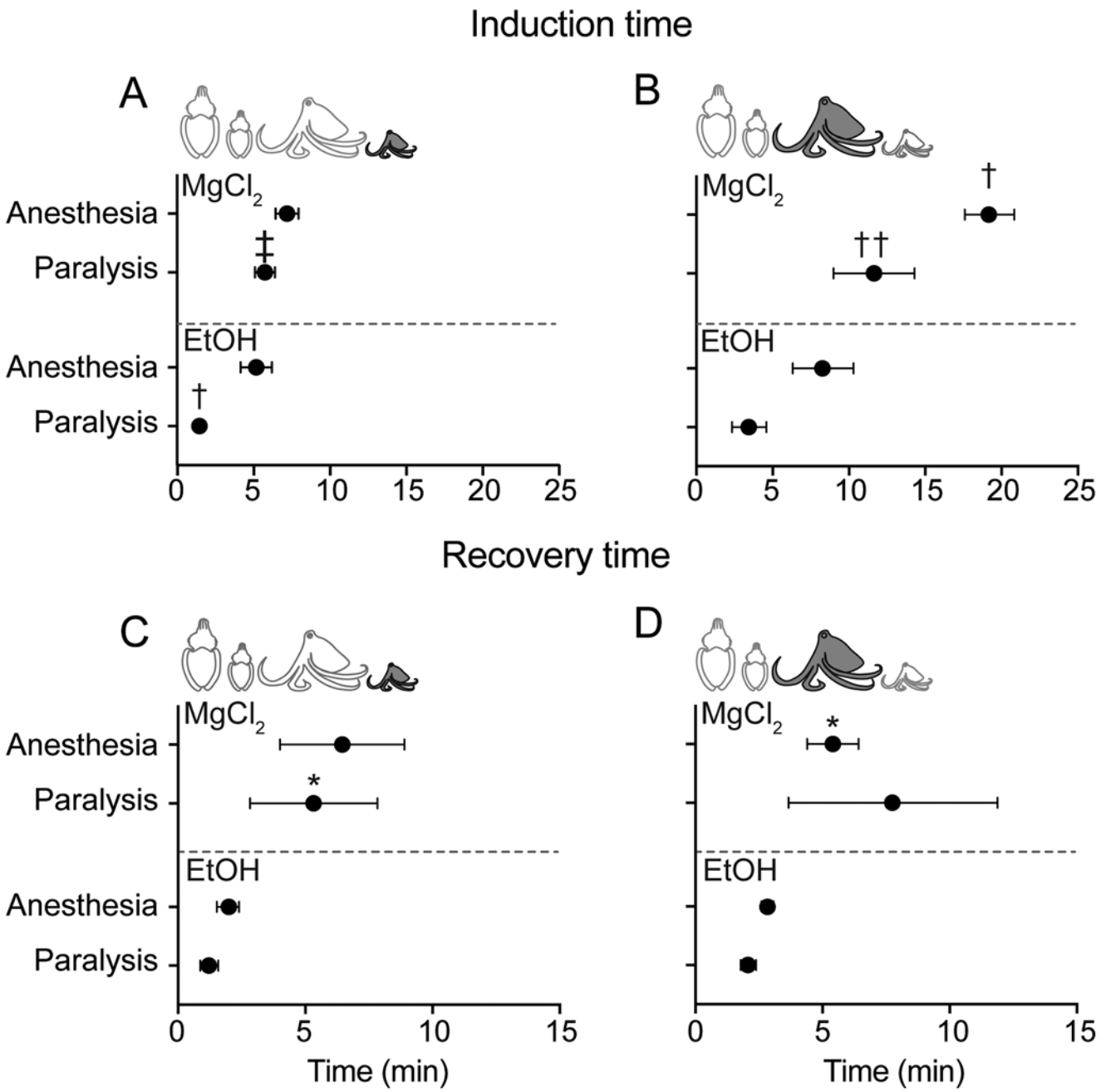

Figure 4. Comparisons of the 2 anesthetic substances plotted separately for juvenile (n = 6) and adult (n = 8) octopuses. (A) In juvenile octopuses, latency from application of the anesthetic agent until loss of behavioral response (paralysis) was significantly (P = 0.0003) longer for $MgCl_2$ compared with EtOH. There was also a significant (P = 0.011) lag in the EtOH group between the loss of behavioral response (paralysis) and the loss of neural signal (anesthesia). (B) In adult octopuses, both paralysis and anesthesia took longer (paralysis, 0.008, anesthesia, P = 0.001) in the $MgCl_2$ group compared with EtOH. There was also a significant lag between paralysis and anesthesia with $MgCl_2$ (P = 0.02) (C) Recovery of juveniles from paralysis (that is, return of behavioral responses to the stimulus) took longer for animals exposed to $MgCl_2$ compared with EtOH (P = 0.04). In adults, recovery of neural signal (reversal of anesthesia) took longer for $MgCl_2$ (P = 0.02). Data are given as mean ± SEM. Comparisons within groups according to agent made by using paired t tests; comparisons of paralysis or anesthesia times between substances were made by using unpaired t tests, *, $P < 0.05$. †, $P < 0.01$ ‡, $P < 0.001$

Discussion

This study demonstrates that 2 anesthetics used commonly in cephalopod research—$MgCl_2$ and EtOH—are effective at blocking afferent nerve signals in 2 temperate species of cephalopods. Comparisons of the 2 distinct life stages of *O. bimaculoides* and *S. officinalis* demonstrate that both agents act as true anesthetics despite temporal differences between young and senescent animals and different latencies between the onset of behavioral anesthesia and of neural signal loss. These results provide more detailed data with which to inform decisions about the choice and use of anesthetics for a given application or species. Moreover, such data can help refine regulations for animal care in different countries.

Both EtOH and $MgCl_2$ were effective at blocking the neural signal from the pallial nerve in all animals in this study. The longer induction time observed for $MgCl_2$ in most of the groups tested is consistent with other recent reports.[4,8,16] The one excep-

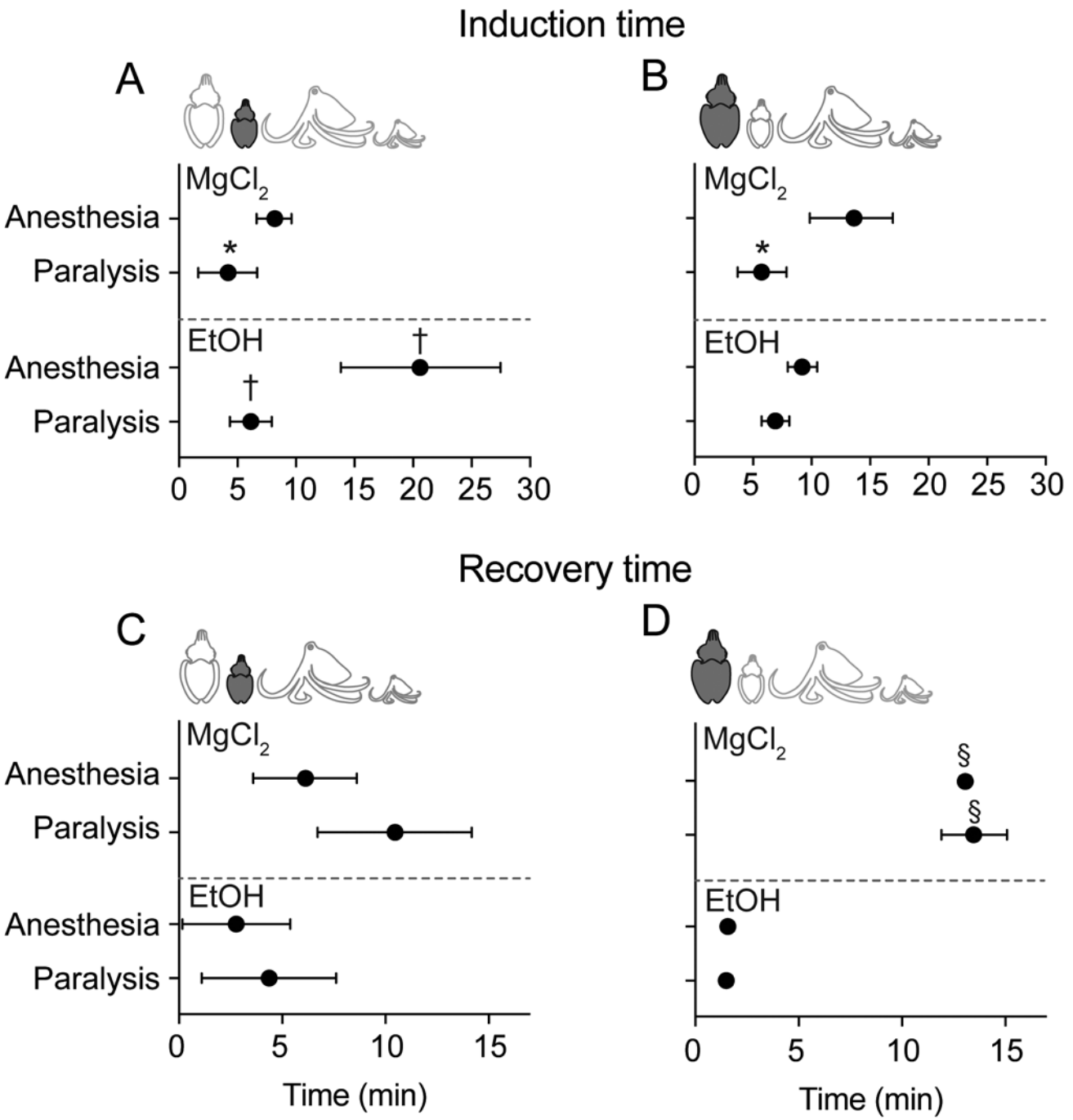

Figure 5. Comparisons of the 2 anesthetic substances plotted separately for juvenile and adult cuttlefish. (A) In juvenile cuttlefish, both anesthetic substances produced significant lag of anesthesia onset compared with the onset of paralysis (EtOH, $P = 0.002$, $MgCl_2$, $P = 0.02$). Time to anesthesia was also longer in the EtOH group compared with the $MgCl_2$ group ($n = 5$ per group, $P = 0.003$). (B) In adults, onset of anesthesia lagged significantly behind the onset of paralysis for $MgCl_2$ ($n = 3$, $P = 0.045$) but not for EtOH ($n = 5$). (C) Recovery from anesthesia (i.e., return of afferent neural signal) did not differ in juveniles in the $MgCl_2$ group compared with EtOH. (D) Recovery of both behavioral ($P < 0.0001$) and neural responses ($P < 0.0001$) were delayed significantly in adults treated with $MgCl_2$ compared with those anesthetized with EtOH . Data are shown as comparisons within groups according to agent made by using paired *t* tests; comparisons of paralysis or anesthesia times between substances were made by using unpaired *t* tests. *, $P < 0.05$. †, $P < 0.01$. ‡, $P < 0.001$

tion was the *Sepia* subadult group, in which the induction time was significantly longer for EtOH than for $MgCl_2$. This finding may be explained in part by the technique we used. Our study followed a similar protocol as an earlier study,[4] starting at a low concentration and titrating until full anesthesia was achieved. Starting at this low dose may have prolonged the induction phase for this group of animals; starting at 2% may have resulted in a similar or shorter induction time. Other authors[38] reported 50% mortality of the octopus *Eledone moschata* at the lower phenoxyethanol dose and suggested that this higher mortality could be related to the longer induction time required at the lower dose. A prolonged induction time for low-dose (1%) EtOH has been reported elsewhere.[9,16] However, this same protocol was used for all the groups tested in this study, so the reason for this difference is unclear.

The literature contains conflicting reports of the aversiveness of EtOH. Some reports describe distress behaviors such as escape attempts,[3] whereas others did not observe these adverse reactions.[8,15] The octopuses and cuttlefish in the present study did not show adverse reaction to 2% EtOH. Alternatively, signs of aversiveness (animals attempting to climb out of the experimental tank or moving quickly around the sides of the tank) were observed in some octopuses when administering $MgCl_2$ .This is in contrast to findings in tropical species in which neither substance produced aversive behavioral responses at any dose.[4] None of the octopuses inked at any time during the study, as has been reported elsewhere with 2-phenoxyethanol.[38] Occasional inking did occur with *Sepia officinalis* at various points during the EtOH trials and once during $MgCl_2$ trials.

A previous study found increased agitation in juvenile *Sepia* during recovery from 2% and 3% EtOH anesthesia.[16] As an indication of distress, inking may be species-specific or life-stage–specific.[8] In our experience, *O. bimaculoides* rarely inks in a laboratory setting unless heavily stressed, whereas lab-reared *S. officinalis* inks with some regularity when disturbed. The most common effects noted in $MgCl_2$ during induction were a subjective increase in ventilation by observing mantle and siphon contraction and increased activity in the octopuses. An increased ventilation rate has been quantified as increased oxygen consumption in *Octopus maya* but it was transitory and followed by a decrease in oxygen consumption.[37] It is unclear if the increased ventilation is of significance.

The osmolal differences between $MgCl_2$, EtOH, and seawater and their possible effects on nerve conduction or other physiologic parameters in cephalopods have not been studied in detail. The calculated osmolalities of the anesthetic solutions were 1.1067 Osm/kg for 1:1 $MgCl_2$, 1.177 Osm/kg for 2% EtOH in seawater; and an estimated 1.200 Osm/kg for natural seawater. These values were roughly equivalent to those reported for similar drug concentrations in other studies,[34] which showed dose–related depression of cardiac function. However, due to the scope of that study, this effect could not be attributed to differences in osmolality. Although differences in osmolality may have contributed to the depression of neural and behavioral responses that we report here, the exact mechanisms of anesthetic effects were beyond the scope of our study, and the role of osmolality in anesthetic substances should be investigated.

The animals in the current study showed a similar pattern of behavioral changes during induction and recovery as was reported in previous work,[4,9,15,37,38] namely mantle relaxation, loss of sucker intensity, initial increased then slowed respiratory rate, loss of righting reflex, and pale body color (that is, relaxation of skin chromatophores). In tropical cephalopods, a latency between loss of behavioral indications of anesthesia and absence of evoked neural signal in response to noxious stimulation was observed during $MgCl_2$ anesthesia.[4] In contrast, in the EtOH groups, behavioral and neural measures of anesthesia were coupled tightly, suggesting that using behavioral methods to estimate time to true anesthesia would be more reliable with EtOH as compared with $MgCl_2$. This finding, together with the different life stage responses under the 2 anesthetics, suggests that management of anesthesia by relying solely on behavioral indicators is likely to be challenging in temperate species and that further research into responses to anesthesia at various life stages is warranted.

Of the 6 senescent *Sepia* tested in the EtOH group, one had a prolonged recovery and died 3 d later; this animal was not included in the analysis. Another animal in this group laid eggs 5 d later and then died; we included this animal in the analysis. In the $MgCl_2$ group, 5 animals were tested, but one died during electrode placement, and another remained unresponsive for a prolonged period after being placed into $MgCl_2$; therefore, the trial was aborted, and the animal died 13 d later. These 2 animals were not included in analysis. These findings are similar to a recent study in which 4 of 7 juvenile *O. maya* died at the end of the study after $MgCl_2$ anesthesia.[37] However, comparing data between different species and life stages is difficult. These results differ from other studies in which no mortality was observed after using EtOH and $MgCl_2$ at the same doses.[4,16] The notable difference is the advanced age of the animals in our study, suggesting that senescent animals are more likely to experience high morbidity and mortality when anesthetized, and performing survival surgery on senescent animals may not be advisable. This outcome could be species-specific, given that none of the senescent octopuses died during any of the trials, and all animals survived for at least 2 wk after the final experiment.

The smaller subadult *Sepia* used in the study generally recovered well from the procedure if their respiration remained stable, but they were more sensitive than the adults to respiratory depression and cessation. Four subadult *Sepia* stopped breathing at some point during the trial, and in 2 cases, the animals died despite prompt manual ventilation and were not included in analysis. In some reports, loss of spontaneous respiration has been suggested to indicate surgical anesthesia,[3,15,37,38] but another study views this effect as a sign of excessively deep anesthesia.[27] The current study shows that cessation of behavioral and neural responses to noxious sensory stimulation can occur while breathing continues. Ventilation rate as measured by mantle contraction and relaxation or siphon contraction and relaxation rarely stopped in any of the animals that were included in the final analysis. This pattern suggests that cessation of respiration is not a necessary requirement for complete anesthesia. Given these results, we do not advise using cessation of respiration as an indicator of an adequate plane of anesthesia.

Although size differences between juvenile and fully adult stages is typical of cephalopods, size itself is not likely to be a factor in the observed differences. As animals move from somatic growth during the juvenile stage into reproductive growth, changes in metabolism may affect metabolism of the anesthetic, thus accounting for the life-stage differences that we observed. Further investigation into the effects of life-stage on anesthetic responses are needed. Likewise, the effect of sex on the observed differences cannot be evaluated based on our study. With the exception of the female adult *O. bimaculoides,* determining the sex of the other groups was not possible by observation alone, so the sex composition of the remaining groups is unknown. Further work in this area is needed also.

In the initial phase of the current study, the protocol included repeating the induction to recovery for 2 cycles in a single session, as done in a previous study.[4] The first cohort in our pilot study was a group of senescing *Sepia*, and higher than expected mortalities occurred. Of the 6 senescent *Sepia* in the first EtOH study, one died during recovery, and 2 died within 5 d of the trial. The second induction-to-recovery cycle therefore was omitted.

This anesthetic procedure was also attempted with the squid *Doryteuthis pealeii.* These animals were collected by trawl net and had signs of stress and with various degrees of skin injury from net abrasions when coming off of the collection boat. High rates of morbidity and mortality are typical in squid captured this way, with few animals surviving more than a few days after capture. Twenty-one trials were attempted but were mostly unsuccessful, resulting in the death of the animal

during induction or recovery, failure of the electrode to give a signal, or nerve damage. Attempts were made to change the conditions to improve the outcome, such as allowing the animals to acclimate for at least 24 h after capture rather than testing them immediately. In addition, attempts were made to select animals whose skin was in the best condition, given that the capture methods result in significant skin damage to many of the squid. None of the changes improved the poor outcome, so those data were not included in this study. The high stress to which these animals were exposed during capture likely contributed to the poor responses to anesthesia. In another study[29] performed at the Marine Biological Laboratory under similar conditions, animals were held for 0 to 2 d after capture before being used in experiments. They were then maintained under "mild" sedation with $MgCl_2$ for extended periods of time without reported adverse effects. One notable difference between that study and the current one is that in the current study, the squid underwent manipulation for electrode placement, which required deep sedation. That study[5] discusses evoked stimuli and electrophysiologic recordings via the statocyst nerve bundle but does not explain whether this monitoring was performed in every animal or in just a subset to show that $MgCl_2$ does not diminish the recording. The maintenance of evoked neural activity during sedation in a previous study[29] suggests that the anesthesia plane was much lighter than that needed to achieve true anesthesia, which was our goal in the current study. Allowing the squids to acclimate in the laboratory for longer periods of time could result in a better outcome when deep anesthesia is required. Jig-caught squids handled with particular care during shipboard transport and gingerly transferred to large tanks with adequate food and substrate can provide adult squids that remain healthy for many days and even weeks or months.[19,22] However, we were unable to obtain squids via this method for this study. Although we could not test the full anesthesia protocol in *D. pealei* in the current study, we suggest that the high mortality rate and associated complications were due to distress from capture and handling of feral squids and may influence the success of anesthetic procedures in other loliginid and oceanic squids as well, given that their skin is somewhat more delicate than that of benthic cephalopods such as octopuses and cuttlefish.

In conclusion, EtOH and $MgCl_2$ were effective at blocking the efferent and afferent neural responses to evoked stimuli in 2 representative species of temperate cephalopods that are commonly used in research laboratories. Both anesthetic agents showed temporal differences between cessation of behavioral reactions to stimulation and true anesthesia, thus indicating that sufficient time should be allowed between behavioral indications of anesthesia and the start of a potentially noxious procedure. Life-stage–associated differences were evident and should be considered when determining the choice of anesthetic and recovery goals. In particular, anesthesia should be used cautiously for senescent cephalopods, given that adverse outcomes were highest in these animals. In general, progressive increases to achieve effective doses were well tolerated by both species and may reduce adverse reactions to the introduction of the anesthetic agents to the water. Finally, recent stress should be considered, and wherever possible, animals should be allowed to acclimate after capture or another distressing event before attempting anesthesia. Although our study involved a small number of animals and statistical power was limited in some cases, both EtOH and $MgCl_2$ fulfill the necessary criteria to be considered as genuine anesthetic agents for these temperate species, and their use should be encouraged as a means of promoting cephalopod welfare in research laboratories.

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Supplementary Materials

Figure S1. Examples of background neural activity during awake periods of *O. bimaculoides* (top) and *S. officinalis* (bottom), showing rhythmic bursts associated with mantle contractions during respiration. Arrowheads on the bottom trace show 2 instances of jetting behavior, which is accomplished by strong mantle contraction after an extended inhalation.

Figure S2. Examples of electrophysiologic traces recorded during a pinch of the distal mantle of juvenile *O. bimaculoides* undergoing EtOH anesthesia. In this example, the animal's behavioral response to the stimulus persists until around 7 min after the introduction of EtOH, but a simultaneous cessation of both neural and behavioral response resulting in anesthesia (that is, loss of sensation) is accurately indicated by loss of behavioral response. Reversal in seawater was rapid, with simultaneous return of neural and behavioral responses to stimulation within 2 min.

Figure S3. Juvenile octopuses undergoing $MgCl_2$ anesthesia showed a somewhat different pattern than did those undergoing EtOH anesthesia. This animal was active and making spontaneous walking movements until 6 min after the introduction of $MgCl_2$, so responses to pinch were tested once the animal was quiescent, beginning at 6 min. The animal progressed rapidly into a state of behavioral unresponsiveness, but full anesthesia was slightly delayed. Recovery of neural response once the animal was placed in fresh seawater was almost immediate, although the traces suggest somewhat variable and disorganized neural activity through the recovery period. Behavioral responses returned after a considerable delay.

Figure S4. Examples of electrophysiologic traces recorded during a pinch of the distal mantle in juvenile *S. officinalis* undergoing EtOH anesthesia. In this example, intermittent spontaneous firing is overlaid with the evoked response to fin pinch, most notable at minute 4 in the induction sequence. This animal recovers neural response to the stimulus rapidly after anesthesia, although there is some delay in the return of behavioral response. Despite strong neural activity at minute 6 in the recovery period, the animals still showed impaired righting response, which returned by minute 7.

Figure S5. Induction and reversal for juvenile *S. officinalis* under $MgCl_2$ anesthesia. This subject showed progressive loss of behavioral response that was complete by 6 min, and the neural signal was completely absent by 9 min. Reversal was rapid, with neural signal in response to pinch apparent at 3 min and behavioral responses apparent at 6 min. In this example, recording was stopped as soon as the animal showed return of behavioral response.

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