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Studying the Effects of Hypothermia on Memory in the Gray Slug, *Deroceras reticulatum*: A Paradigm Suitable for Undergraduate Comparative Research

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Identifying instructional and manageable research ideas suitable for classroom or thesis projects in comparative learning and memory can be challenging, especially in the context of time constraints, facilities restrictions, and financial limitations. Nevertheless, the ubiquitous nature of many learning related process evident throughout the animal kingdom—e.g., the mechanisms involved in operant and classical conditioning—reinforces the consideration of diverse species in the investigation of learning and memory. Indeed, despite the apparent differences from commonly studied vertebrates (e.g., rodents), *invertebrates* offer a compelling model for the study of various memory mechanisms. In the present study, locally collected gray slugs (*Deroceratus reticulatum*) were used to investigate the amnestic impact of rapid hypothermia on the retention of classically conditioned aversive learning. Results of this study provide directional support for the influence of hypothermia on such learning, and reflect the need to consider multiple dependent measures when examining behavioral results in invertebrates. Moreover, this approach offers an economical, readily available, and highly useful species for studying methodological and conceptual issues in the acquisition, storage, and retention of conditioned learning in less complex invertebrate systems.

Keywords: *Invertebrate, hypothermia, slugs, conditioning, learning*

The study of learning and memory represents an exceptionally diverse field within the discipline of psychology (and biology), and includes research involving a great many species (Dewsbury, 1990). Most notably, psychology enjoys a rich history in comparative research, with a rather impressive tradition in the study of animal behavior in its own right (e.g., journals in *Comparative Psychology*; *Learning and Memory*; *Animal Behavior Processes*). More specifically, the continuity of memory process between animals and humans is intriguing, and the comparative study of memory mechanisms has both scientific and pedagogical merits.

Pedagogically, the breadth of issues related to learning *or* memory would seem to provide an unlimited range of options for undergraduate research projects. Nevertheless, identifying appropriate projects for individual or group research purposes can sometimes be difficult. Naturally, project selection is constrained somewhat by lab-specific interests; however, the challenges are increased when attempting to conduct comparative research in a context where physical and financial resources are limited.

Many aspects of learning and memory are commonly studied in rodents and smaller mammals, thus requiring suitable facilities with which to conduct such work. Nevertheless, in some circumstances facilities are naturally limited (or even unavailable) for such animal studies. In other cases, material costs are prohibitive, which curtails the purchase, maintenance and use of animals for comparative research. Accordingly, a primary consideration for ‘facilities-challenged’ researchers—or for those simply interested in alternative approaches to behavioral science research—is to identify cost-effective avenues for quality comparative research that promotes experimental and behavioral work with live species.

A Brief Rationale for a Program of Invertebrate Comparative Research

Fortunately, the majority of learning and memory processes typically discussed in the context of human, non-human primate, and smaller mammal research can be examined in even *lesser* species including—despite their marked difference from higher order animals—the class of *invertebrates* (Abramson, 1986). Accordingly, a specific goal of projects like the one described here is to provide students (typically in departments of psychology) an opportunity to engage in theory guided research with an emphasis on hypothesis testing through comparative experimental means.

It should be noted that an extensive historical and contemporary literature reveals numerous opportunities for students to investigate learning and memory phenomena in a diverse range of invertebrate species. In particular, the work of Abramson and colleagues offer suggestions for excellent demonstration projects involving many invertebrates, including worms, flies, and ants (see Abramson, 1990; 1986; Abramson, Collier, & Marcucella, 1977; Abramson, Onstott, Edwards, & Bowe, 1996). Beyond applications for classroom teaching, such work has laid a foundation for considering invertebrates as a viable means for studying behavioral processes throughout the animal kingdom. Indeed, a review of this literature provides many points of intersection between mammals and invertebrates, identifying the means with which to study various behavioral phenomena such as habituation, sensitization, classical conditioning, and even spatial navigation (see Abramson, 1986; Abramson, Milner, & Mann, 1982).

The similarity of processes seen across species with very different levels of complexity reinforces the phylogenetic conservation of various memory related mechanisms. Inasmuch as mammals and invertebrates (e.g., mollusks such as slugs and snails) are believed to have diverged from a common ancestor nearly 600 million years ago (Watanabe, Kirino, & Gelperin, 2008), similarities in learning and memory processes (and underlying mechanisms; see Krasne & Glanzman, 1995) are naturally expected among modern species. As such, an invertebrate comparative perspective encourages students to discover species-specific adaptations, while speculating on the utility and origins of shared behaviors and underlying mechanisms (see also Abramson et al., 1996).

In my own lab, various types of insects, spiders, common slugs, and other 'bugs' (e.g., crustaceans) have emerged as viable research subjects presenting a range of fascinating behavioral adaptations worth studying. By their very nature, studies involving these simpler organisms typically bring together a range of interesting issues in ethology, evolutionary biology (and evolutionary psychology) and animal behavior—to name just a few. Not to be misconstrued as merely 'simple', the less complex invertebrate nervous system *and* the limited behavioral repertoire seen in such organisms, still offer evidence of rather sophisticated mechanisms subserving learning, memory, and plasticity (see Alcock, 2009; Kandel, 1979). Accordingly, invertebrates remain valuable and helpful in studying many principles of learning (see Burrell & Sahley, 2001), and results of such studies continue to offer insights into more complex issues relevant to human psychology and neuroscience (e.g., anterograde and retrograde amnesia; Sekiguchi, Suzuki, Yamada, & Mizukami, 1994; Yamada, Sekiguchi, Suzuki, & Mizukami, 1992).

An Illustrated Invertebrate Research Study

In recent years we have tried in this lab to identify a program of research suitable for student projects addressing various questions of comparative behavioral interest. As a neuroscientist, my own familiarity with the work of Eric Kandel and the sea snail *aplysia* (1979) provided a starting point, later extended by undergraduate interests in invertebrate learning and memory (see Coffey & Kaut, 2010; Coffey, Meehan, & Rock, 2004). I came to recognize the extensive history of *gastropod* (e.g., snails, slugs, mollusks) behavioral research (e.g., Gelperin, 1975), now yielding impressive insights into the neural systems underlying behavioral responses to *visual* (Andrew & Savage, 2000; Crow, 1983, 2004), *tactile* (Kawai, Sunada, Horikoshi, & Sakakibara, 2004), *gustatory* (Sahley, Martin, & Gelperin, 1992), and *olfactory* cues (Inoue, Muramaki, Watanabe, Inokuma, & Kirino, 2006; Nikitin & Balaban, 2000; Yamagishi, Ito, & Matsuo, 2008).

Given the growing understanding of the slug nervous system—including how parts of its brain (i.e., the procerebrum) are altered in response to new learning (e.g., CS-UCS pairings; see Kimura, Toda, Sekiguchi, & Kirino, 1998; Nikitin & Balaban, 2000; also Ermentrout, Wang, Flores, & Gelperin, 2001)—it is intriguing to think of the many ways this invertebrate system can be used to investigate learning processes across the behavioral to molecular perspective. Much like contemporary research examining cockroach spatial navigation and the role of the invertebrate analogue of the hippocampus, i.e., the mushroom bodies (Mizunami, Weibrecht, & Strausfeld, 1998), the gastropod can be a model species for use in undergraduate research projects examining learning and memory. Indeed, the invertebrate system can be manipulated, even surgically altered (Kasai, Watanabe, Kirino, &

Table 1. Issues to Consider when Developing an Invertebrate Comparative Lab Agenda

Issue/Question	Response(s) for *this* Project
What behavioral issues are of particular interest to the lab?	Memory; amnesia; consolidation; classical (Pavlovian) conditioning
How have these issues been studies in *this* and *other* labs previously?	Rats; water maze; sand digging task; fear conditioning; lesions; hypothermia[a]
Have the behavioral issues been studied *directly* in an invertebrate species?	Slugs; worms (nematodes)
Is there an invertebrate species appropriate for research in *this* lab?	Snails; slugs; worms
What is the general availability of the species?	Local: regional forestation areas Distributors: e.g., California supplier[b] (restrictions on transport?)
What are the housing requirements for this species?	Refrigeration; vegetation/food; housing containers; lighting; ambient temperture[c]
What are the relevant behaviors in this species that can be utilized in an experimental paradigm?	Feeding (appetitive) Avoidance (aversive)
Are there stimuli of particular relevance for the study of behavior in this species?	Sucrose (high molarity) Sodium Chloride (NaCl) Bitter solutions (quinidine)
Consideration of manageable experimental paradigm for the study of behavior.	Slugs Aversive learning Classical Conditioning Hypothermia

Notes:

[a] An interesting and still relevant literature regarding the amnestic effects of hypothermia in rats can be found in the works of Riccio and colleagues, in addition to some intriguing human applications (see Riccio, Hodges, & Randall, 1968; Richardson & Riccio, 1987; also, Castellani, Young, Sawka, Backus, & Canete, 1998).

[b] Slugs obtained from California have been shown in this lab to not handle the hypothermic intervention well. Attrition rate was exceptionally high.

[c] There are no special arrangements necessary for the maintenance of slugs. Normal refrigeration can prolong lifespan in captivity.

Matsuo, 2006), in ways that are remarkably similar to paradigms used with mammals, albeit with less cost, easier access, and modest restrictions with regard to subject care, maintenance, and experimental use.

Translating an Existing Research Agenda

In the process of adapting a program of research based on mammals (e.g., rats) to a comparable invertebrate research agenda, it has been helpful to answer a series of scientific and logistical questions (see suggestions in Table 1). It can be helpful to begin with a review of the previous literature detailing demonstration projects involving invertebrates (e.g., Abramson, 1986; Abramson et al., 1996) inasmuch as these offer a rich source of ideas, required materials, experimental methods, and potential species of interest (see also Abramson et al., 1977, 1982). In my own lab, establishing an invertebrate plan of research first involved identifying behavioral similarities across vertebrates (e.g., rats) and potential invertebrates (e.g., snails) then considering how my own research expertise with the former could be adapted to exploring comparable behaviors and mechanisms in the latter. In the field of learning and memory research, numerous possibilities exist that promote such considerations.

One issue receiving considerable attention in the behavioral neuroscience literature, and was a primary focus of my own work with rats, is the notion of a consolidation period in memory development (e.g., see Kaut & Bunsey, 2001; McClelland, McNaughton, & O'Reilly, 1995). As a concept, consolidation posits that newly acquired information remains labile until strengthened or otherwise transferred to a more durable, long-term form. Arguably, during an immediate post-learning period, newly acquired information is most vulnerable to disruption, whereas remotely experienced events have undergone the gradual consolidative mechanisms resulting in more permanent representations within the existing neural circuitry.

Viewed as a time-dependent process, the specific temporal parameters of memory consolidation are likely to vary among species (see discussion in Yamada et al., 1992). Of particular relevance here, investigations into such temporal parameters have been extended to invertebrates with interesting implications. For example, the application of heat shock to nematodes (*c. elegans*) within the first 30 minutes after habituation training (i.e., 32° C for 45 minutes) has been shown to disrupt long-term memory of this response (i.e., > 24hr; Beck & Rankin, 1995) suggesting that interference with post-training cellular processes (e.g., cyclic AMP, protein synthesis; see Burrell & Sahley, 2001; Krasne & Glanzman, 1995) disrupts consolidation mechanisms.

Yamada et al. (1992) employed a variant of the thermal shock technique in their effort to identify the post-learning consolidative period in the terrestrial slug (*Limax flavus*), but did so using a hypothermic intervention (see also Sekiguchi et al., 1994). In their study, slugs rapidly cooled within 1-2 minutes after the pairing of carrot juice (CS) with quinidine sulfate (bitter UCS) failed to express avoidance of the CS at testing. Delaying hypothermia five minutes after initial CS-UCS pairing preserved the conditioned avoidance response at testing. Spared conditioned learning following a 5-minute delay underscores the idea that even in slugs, memories may be vulnerable to disruption within a limited period of time after learning, yet become strengthened with time and therefore less affected by trauma or shock to the organism.

A Retrograde Amnesia Paradigm—with Gastropods

As a first attempt at implementing a gastropod-based research model, the present study attempted to replicate the work of Yamada et al. (1992), with a principal goal of applying a retrograde amnesia paradigm to the study of learning and memory in a related invertebrate—the gray slug, *Derocerus reticulatum*. More specifically, slugs were exposed to hypothermia immediately after aversive conditioning (i.e., carrot juice CS + high sucrose solution UCS) or following an 8-minute post-conditioning delay. In addition, a related goal of this study—and quite possibly the most challenging—was to evaluate various dependent measures for purposes of better investigating invertebrate learning. Typically, post-training measures of preference for a CS+ or avoidance of a CS– are represented by time spent in contact with a given stimulus or associated surface (see Hopfield & Gelperin, 1989; Nikitin & Balaban, 2000; Sekiguchi et al., 1994; Yamada et al., 1992). Such a measure might not always reflect the full range of potentially relevant learning indicators present in certain invertebrates (see Andrew & Savage, 2000). Accordingly, identifying a range of ethologically relevant behaviors (e.g., head movements; escape latencies; other spatio-temporal activity patterns) was considered to be of particular value here in characterizing how invertebrates initially respond to stimulus materials and later perform when re-exposed to all or part of the original training context. Indeed, as noted in Table 1, the identification of relevant behavioral responses—particularly when the range of behaviors appears to be somewhat limited—is a worthwhile challenge in studying the adaptive behaviors of a given species.

Method

Subjects

For this study, forty-two gray slugs (*Deroceras reticulatum*), were obtained from a northeastern Ohio local forestation area in early fall (i.e., local metropolitan park, late September-early October). Obtaining slugs typically required a few hours of foraging through wooded areas, searching trees, and sifting through surrounding ground cover. For those unable to identify a region suitable for slug collection, various species can be obtained from commercial vendors and typically shipped with minimal restriction (e.g., Niles Biological, Sacramento, CA).

Slugs were initially group housed in a plastic container (i.e., Tupperware® or similar brands) with soil, sediment, and vegetation from their natural environment, and fed a diet of lettuce (other vegetables also suitable). Group housed slugs were initially refrigerated at approximately 44° *F*, then transferred to individual containers at room temperature approximately 1-3 hours prior to conditioning. Attrition among slugs in captivity eventually reduced the overall sample size to a total of 25 at the conclusion of this study.

Materials

For purposes of conditioning, carrot juice was used as the conditioned stimulus (CS) and a 1.0 molar sucrose solution was used as the naturally aversive unconditioned stimulus (UCS; see Coffey et al., 2004). To create the CS, packaged carrots were coarsely chopped and then more finely emulsified in deionized H_2O. This carrot solution was then centrifuged for 10 minutes and the excess water was drawn off leaving the supernatant carrot juice which was refrigerated throughout the study. Natural carrot juice can also be purchased, and has recently worked well in similar studies. Also, and quite possibly even more effective, has been the use of baby food (e.g., Gerber® carrots, peas, plums) as a CS. These commercial products are more convenient and have proven highly palatable to this species.

Procedures

As shown in Figure 1, slugs were randomly assigned to one of four experimental groups, three of which received a single trial of the CS-UCS pairing (conditioning) followed by either a) immediate hypothermia (Imm-Hypo, n = 6), b) delayed hypothermia (Del-Hypo, n = 6), or c) no hypothermia

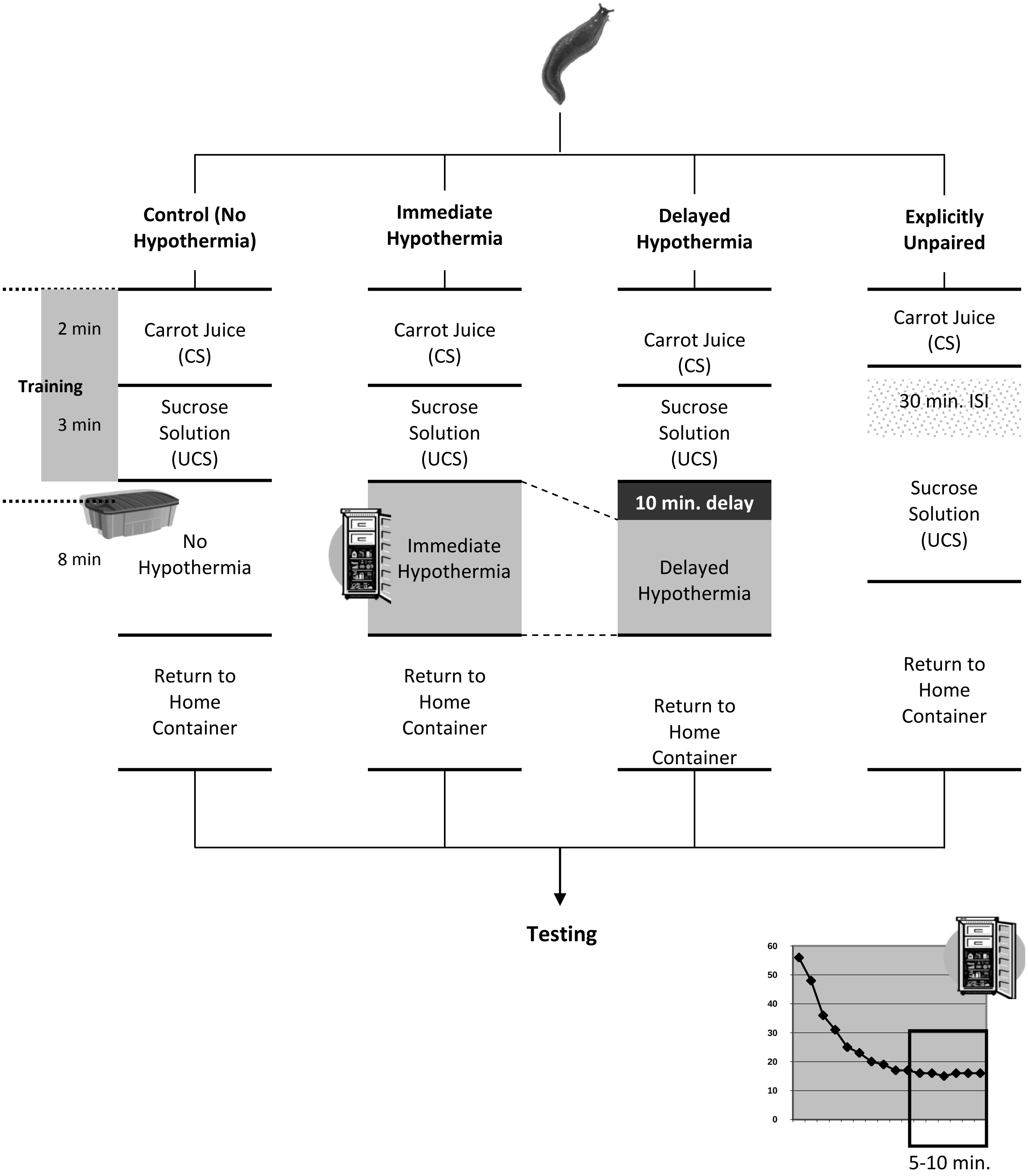

Figure 1. Experimental Design for Hypothermia Study. Inset at lower right indicates temperature probe readings compared to room temperature (~75° F) over a 10 minute recording period. The freezer compartment was maintained at approximately 16° F.

(No-Hypo, n = 5). Slugs assigned to the fourth group—an explicitly unpaired condition—received exposure to the CS and UCS separated by a 30-minute interstimulus interval (Exp-Un, n = 6). All conditioning and memory testing was typically conducted within a 2 hour period of time.

Immediate Hypothermia (Imm-Hypo). Procedural elements are identified in Figure 2. During conditioning (i.e., training), slugs were placed onto a transparent surface (i.e., 3M transparency film paper CG5000) placed over a concentric-field indicator (i.e., concentric circles printed on 8.5 x 11 in paper), with the slug's head positioned in the center of a 0.5 ml bolus of carrot juice (CS) previously delivered via syringe. Typically, the surface tension of carrot juice on the transparency film resulted in a well-defined fluid ring for slug placement. Upon contact with the CS, slugs were expected to sample and ingest the juice (i.e., appetitive behavior), typically showing minimal evidence of avoidance behavior.

After a 2 minute exposure to the CS alone, 1 ml of a 1.0 Molar (M) sucrose solution (UCS) was delivered via syringe to the head region and upper body. The delivery of the 1.0M sucrose solution had previously been shown to elicit a noticeable aversive response (i.e., pilot testing had revealed head rearing; body twisting; avoidance/escape behavior). Slugs were exposed to this solution (CS + UCS) for an additional 3 minutes (i.e., total conditioning trial was 5 minutes), after which they were removed from the transparency surface, rinsed in cool water, then positioned on a separate piece of concentric-field paper and placed in a freezer compartment for 8 minutes (i.e., hypothermia period). At the conclusion of the hypothermic exposure, the distance of the slug's head from its original placement was noted, and the slug was returned to its individual container for a period of 30 minutes prior to the testing phase. During this 30 minute period, physical activity was typically restored in these slugs as indicated by peristaltic movement up the sides of the container and onto the inner surface of the container lid.

Delayed Hypothermia (Del-Hypo). Following the same general procedures as described above, slugs in this condition were rinsed in cool water after the 5 minute conditioning trial (2 minutes CS alone + 3 minutes of CS/UCS exposure), then returned to their individual containers for 10 minutes (delay period). Following this delay, slugs were positioned on concentric-field paper and placed in the freezer compartment for 8 minutes. After hypothermia, head distance was recorded, and slugs were returned to their individual containers for 30 minutes.

No Hypothermia (No-Hypo). At the conclusion of the 5 minute conditioning trial, slugs were rinsed in cool water, positioned on concentric-field paper, and then placed inside a darkened rectangular compartment (35 cm L x 23.5 cm W x 12 cm H) at room temperature for a period of 8 minutes. Placement into this compartment was intended to control for the abrupt shift to the dark freezer compartment as in the hypothermia treatments. After 8 minutes, head location was noted relative to the initial placement, and slugs were returned to their individual containers for 30 minutes prior to memory testing.

Explicitly Unparied (Exp-Un). Slugs in this condition were first exposed to the 0.5 ml bolus of carrot juice then rinsed in cool water and returned to their individual containers for a period of 30 minutes. Slugs were then returned to the transparency surface and exposed to 1 ml of 1.0M sucrose solution. At the end of this exposure, slugs were rinsed and returned to their individual containers prior to memory testing.

Memory Testing. Each slug was placed onto a transparency sheet with its head in contact with a 0.5 ml bolus of carrot juice (CS) positioned to line up directly with the center of the underlying concentric field indicator (see Figure 2). The testing period lasted for 5 minutes, after which the slugs were rinsed in cool water and returned to their individual containers.

All conditioning and testing trials were videotaped with a Panasonic camcorder positioned approximately 1m above the training/testing field, and were later reviewed for data collection. For each subject, observations during the 2 minute CS exposure at conditioning were compared with the first 2 minutes of CS exposure at testing. In this way, direct comparisons of behavioral responses to the CS could be made across conditioning and testing sessions.

Dependent Measures. Expectations based on group assignment are provided in Table 2, and can be compared with the methods illustrated in Figure 2. It should be noted here that one of the challenges of working with invertebrates is the need to consider the potential range of behaviors suitable for analysis. It was anticipated that a single measure of avoidance might not capture the subtleties of this global response; accordingly, three measures of behavioral avoidance were selected, each believed to represent one component of an integrated avoidance response.

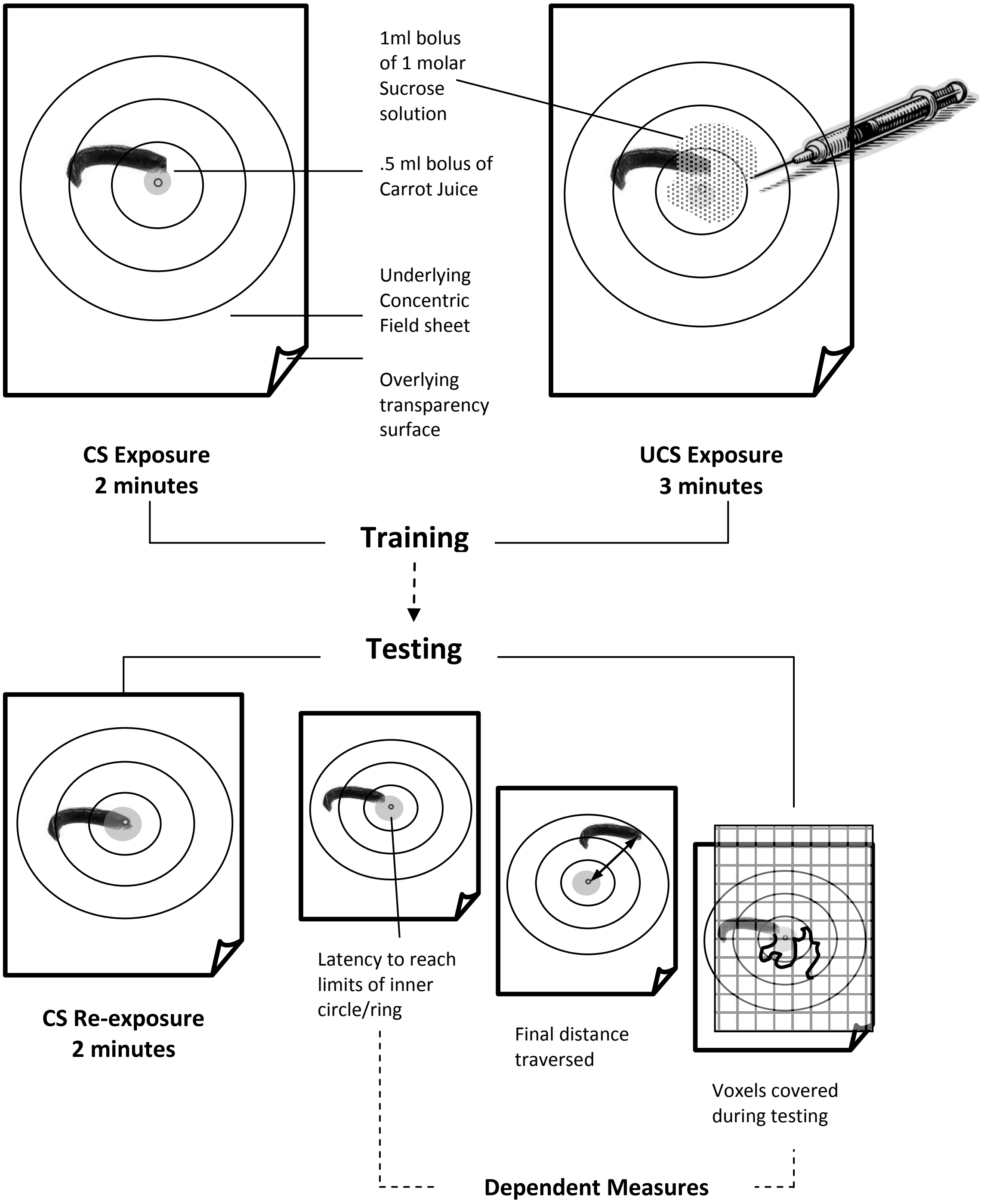

Figure 2. Procedures used in Training and Testing. The CS re-exposure at the testing trial involved a 2 minute period of videotaping from which the three dependent measures were taken.

Table 2. Expected Behavioral Responses to the CS at Testing Compared with Responses to the CS at Conditioning

	Latency to Reach Inner Circle	**Linear Distance Traveled**	**Voxels Traversed**
Control Group/No Hypothermia	Expected to be *Faster*, reflecting rapid avoidance of CS	*Further*, reflecting more vigorous avoidance of CS	*Large number* traversed reflecting more vigorous escape behavior
Immediate Hypothermia	*Slower*, reflecting forgetting of previous CS-UCS pairing	*Less Distance*, reflecting weaker avoidance of CS	*Fewer* voxels traversed, reflecting weaker response to CS
Delayed Hypothermia	Comparable to Controls (i.e., *consolidation*); or slower if delay is not long enough[a]	Comparable to Controls; or less distance if delay is not long enough[a]	Comparable to Controls; or fewer voxels if delay is not long enough[a]
Explicitly Unpaired	Slower, reflecting no aversion to CS	Less distance, reflecting weak avoidance of CS	Fewer voxels traversed, reflecting no learned aversion to CS

Note: [a]If the delay period (i.e., 10 minutes) is not long enough to permit consolidation of memory, the resulting behavioral pattern in the Delayed Hypothermia group is expected to be similar to the Immediate Hypothermia group.

First, *withdrawal latency*, or the latency for a slug's head to reach the boundary of an artificial ring encircling the head (i.e., this ring was drawn on the monitor screen during videotape analysis) was recorded from the start of CS exposure at both the conditioning and testing trials. For slugs in the control condition, it was anticipated that head withdrawal would be faster at testing, thus reflecting memory for the carrot (CS) + high sucrose (UCS) pairing during conditioning. A slower latency (i.e., reduced avoidance response to CS exposure) at testing compared to conditioning was taken here to reflect weakened or poorer memory for the initial CS-UCS pairing (i.e., specifically anticipated in the immediate hypothermia condition, Imm-Hypo). It should be noted that slower latencies were also expected in the explicitly unpaired group (Exp-Un), thereby demonstrating the absence of any conditioned aversion (i.e., not vigorously avoiding the CS at testing).

Second, the *linear distance* of head movement at the end of the 2 minute CS exposures during training and testing was also used as an index of CS avoidance (i.e., measurements obtained from videotape recordings). With this measure, better memory for the CS-UCS pairing at training was expected to result in longer distances at testing (i.e., showing greater movement away from the CS), whereas hypothermia groups (group Imm-Hypo in particular) were expected to show less linear distance covered at the testing trial.

Third, and to control in part for limitations with the aforementioned linear distance measure, a measure of absolute head movement was developed. For this measure, the head movement patterns of each slug were traced directly on transparency film during videotape analysis, and a grid of 1cm x 1cm 'voxels' was later laid over the movement tracings allowing for a determination of the number of voxels traversed during a trial (i.e., *movement voxels*). This measure was intended to control for the possibility that the linear distance covered by a slug might have been fairly short, whereas vigorous head movements and twisting motions (i.e., more voxels traversed during testing) might better capture some elements of behavioral avoidance.

Results and Discussion

Hypothermic Manipulation

Ambient temperature in the lab was approximately 75° F (74.8° , 47% humidity), with the freezer compartment maintained at approximately 16° F (i.e., lowest range = 16.0-16.9° F). A temperature probe, when placed inside the freezer compartment, registered the rapid drop in temperature over the first 8 minutes (see Figure 1 inset), after which time the temperature held fairly constant within the 16° -19° F range. Accordingly, 8 minutes was the time chosen for the hypothermic manipulation. When removed from hypothermia slugs were contracted in length, noticeably more solid to the touch, and immobilized. Indeed, when linear distance covered during an 8 minute non-hypothermia period ($M = 4.17$ cm, $SD = 2.49$) was compared with distance covered during 8 minutes of hypothermia ($M = 1.71$ cm, $SD = 1.05$), a significant reduction in movement was noted, $t(12) = 3.107$, $p < .05$. Movement was typically restored within 15-20 minutes.

Despite the behavioral impact of hypothermia on the slugs in this study, the failure to obtain statistically significant memory differences among groups suggests a number of methodological limitations, including the specific influence of

Table 3. Pattern of Support for Group Performance at Testing Compared to Training Trials Across Three Dependent Measures[1]

	Latency to Inner Circle	**Linear Distance Traveled**	**Voxels Traversed**
Control Group/No Hypothermia[2]	○	○	●
Immediate Hypothermia[3]	●	●	●
Delayed Hypothermia[3]	●	●	●
Explicitly Unpaired[4]	○	●	●

○ Denotes lack of directional support
● Denotes directional support for hypothesized finding
[1] Patterns based only on mean differences at testing compared to training
[2] Control slugs expected to show shorter latency, more linear distance, and more voxels traversed at testing
[3] Hypothermia slugs would show longer latency, less linear distance, and fewer voxels travers at testing (each suggestive of weakened CS-US memory)
[4] Explicitly unpaired slugs expected to show longer latency, less linear distance, and fewer voxels traversed (each reflecting no aversive CS-US association)

hypothermia itself, as well as the possibility of weaker learning of the CS-UCS pairing with the 1-trial procedure used here. Identifying limitations in experimental design has been of instructional value for students of this work, and has shaped subsequent iterations and modifications of methodology described here (i.e., with considerable success, see Coffey & Kaut, 2010). Accordingly, a more liberal consideration of the statistical data, and openness to trends in the results (refer also to Table 3) provides encouraging directional support for ongoing studies examining learning and memory in this species.

Behavioral Measures

The first of the behavioral measures, *withdrawal latency*, yielded mixed results (refer to Figure 3, see inset for methods). Statistically significant differences were not obtained, although the hypothermia groups showed longer withdrawal latencies at testing (ImmHypo *M* = 34.00 ± 42.95 sec.; DelHypo *M* = 46.00 ± 57.61 sec.) compared to the training exposure (ImmHypo *M* = 14.67 ± 13.00 sec.; DelHypo *M* = 36.00 ± 42.27 sec.). Nevertheless, this implication of ‘forgetting’ is mitigated somewhat by the less-than-predicted performance group NoHypo (training *M* = 32.60 ± 49.13 sec, testing *M* = 36.20 ± 47.08 sec.) and group ExpUn (training *M* = 32.83 ± 35.95 sec., testing *M* = 18.50 ± 41.08 sec.). Naturally, the small number of subjects, compounded in part by notable individual differences (variability within groups), limit interpretation here. In addition, it is possible—and considered likely—that this measure is not a sensitive indicator of avoidance inasmuch as slugs vary in the latency to sample a substrate and then react to the stimulus.

For the second behavioral measure, *linear distance* traveled, hypothermia was expected to result in less distance traveled at testing compared with training, suggesting a weakened memory for the previous CS-UCS pairing (i.e., training – testing = *difference*; see 4a). Inspection of Figure 4 suggests all groups performed in the anticipated direction. Group ImmHypo traveled less distance at testing (*M* = 3.23 ± 1.50 cm) compared with training (*M* = 4.72 ± 3.41 cm), as did DelHypo (test *M* = 2.75 ± 2.34 cm; training *M* = 3.75 ± 3.35 cm). Group ExpUn performed as predicted inasmuch as they received no paired conditioning and were expected to show less aversion to the CS at testing (*M* = 3.07 ± 2.36 cm) compared with training (*M* = 4.40 ± 1.98 cm). Group NoHypo showed the smallest mean difference score, although the distance traveled at testing (*M* = 3.36 ± 2.11 cm) was hoped to have been larger than the observed distance at training (*M* = 3.44 ± 2.48 cm).

Of the behavioral measures used here, the *number of voxels traversed* was possibly the most useful performance indicator. The mean difference in voxels traversed at the end of the 2 minute CS exposure for training and testing trials is shown in Figure 5 (i.e., test voxels – training voxels = *difference*). As anticipated, group NoHypo showed a positive difference score, reflecting slightly more head movement—presumed to reflect vigor of avoidance—at testing (*M* = 30.20 ± 18.13 voxels) compared with training (*M* = 27.60 ± 18.86 voxels). Immediate hypothermia resulted in fewer voxels traversed at testing (*M* = 27.67 ± 12.34) compared with training (*M* = 32.33 ± 9.46), as did the delayed hypothermia group (testing *M* = 18.0 ± 13.46; training *M* = 23.17 ± 16.13). Group ExpUn was expected to show minimal avoidance of the CS at testing, thus fewer voxels traversed upon re-exposure to the carrot juice (see Table 2). Indeed, this group showed the largest mean difference (Figure 5), reflecting quantitatively fewer voxels covered at testing (*M* = 24.0 ± 10.95) compared with training (*M* = 35.0 ± 14.03).

Summary

Previous work has shown that slugs (and gastropods in general) are quite capable of forming classically conditioned associations in appetitive

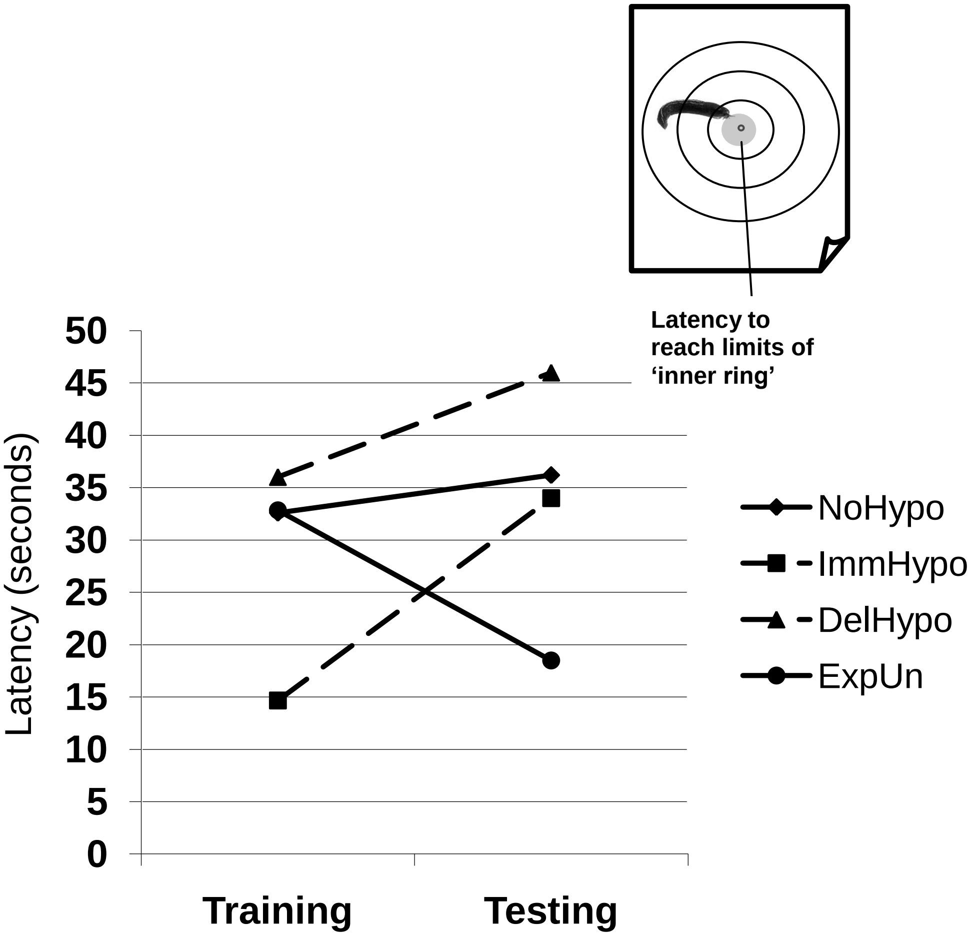

Figure 3. Latency of Initial Head Movement. This measure represented the latency for slugs to move the head from the original starting location to the limits of an 'inner ring drawn around the head on the video monitor. A 4 (group) x 2 (training vs testing) repeated measures ANOVA failed to identify a significant difference between groups in overall latencies (F 3, 19 = .298, P > .05), while finding no differences in latencies at training (overall M = 28.82 ± 35.4 sec.) or testing (overall M = 33.57 ± 41.08 sec.) (F 1, 19 = .333, p > .05), and no interaction between group and training or testing latencies (F 3, 19 = .815, p > .05).

tasks (Crow, 2004; Kimura et al., 1998; Yamamoto et al., 2008), the mechanisms of which have now been nicely detailed at the neural circuit and even molecular levels (see Ermentrout et al., 2001; Inoue, Inoduma, Watanabe, & Kirino, 2004; Inoue et al., 2006; Shirahata et al., 2006; Watanabe et al., 2008). At a behavioral level, hypothermia in slugs has been shown to disrupt the memories of previously acquired conditioned responses (Sekiguchi et al., 1994; Yamada et al., 1992), likely reflecting the disturbance of temperature sensitive metabolic processes associated with memory development (Krasne & Glanzman, 1995).

In this study, hypothermia appears to have had the intended effect of rapidly cooling the slugs, and rendering them largely immobile. However, the amnestic effects of hypothermia were less robust than expected, although the directional pattern of findings was quite promising. It would appear that the hypothermic procedure itself was not necessarily ineffective; rather, behavioral variability evident on certain dependent measures was possibly due to the weaker effects of the UCS (sucrose solution). Indeed, we have found that a NaCl solution is a more effective UCS, yielding more reliable behavioral reactions/aversion in this species (Coffey & Kaut, 2010). Nevertheless, the use of multiple dependent measures proved beneficial here, providing further insight into the behaviors of greatest interest in assessing learning and memory in this species (e.g., voxels traversed). In addition, it is noteworthy that the hypothermia groups (immediate and delayed)

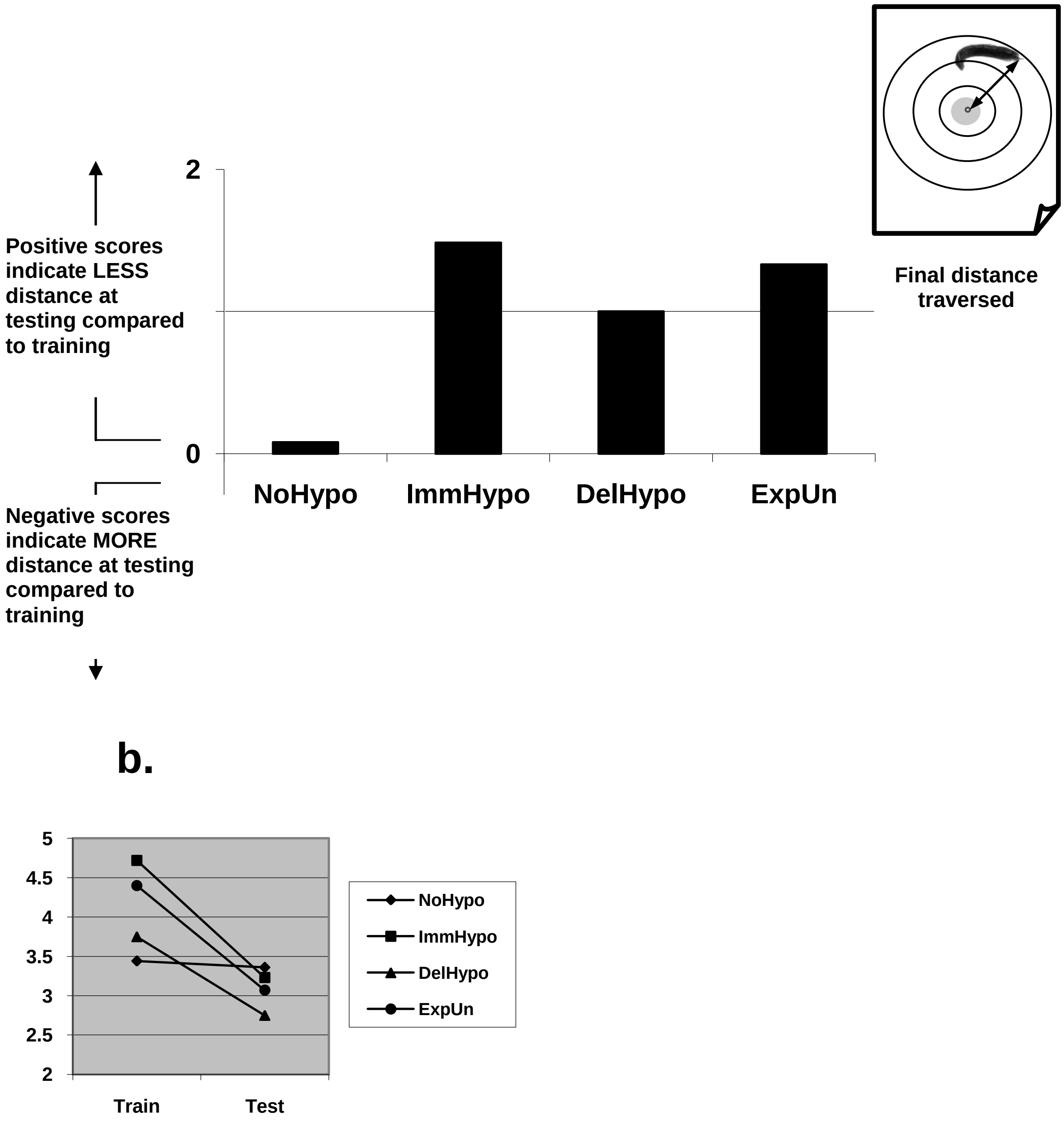

Figure 4. Final Distance Traversed. *4a.* Linear distance covered during 2 minute exposure to CS at Training *minus* the linear distance covered during the 2 minute exposure at Testing. *4b.* A comparison of mean distances covered at Training and Testing. Hypothermia groups showed less avoidance, as did the Explicitly Unpaired group (as expected). A 4 (group) x 2 (training vs testing) repeated measures ANOVA revealed no significant group difference (F 3, 19 = .140, p > .05) and no group x training/testing interaction (F 3, 19 = .275, p > .05); as reflected in 4b, there was a trend toward less distance at testing (overall *M* = 3.09 ± 1.97 cm) compared to training (overall M = 4.10 ± 2.73 cm) (F 1, 19 = 2.841, p = .108).

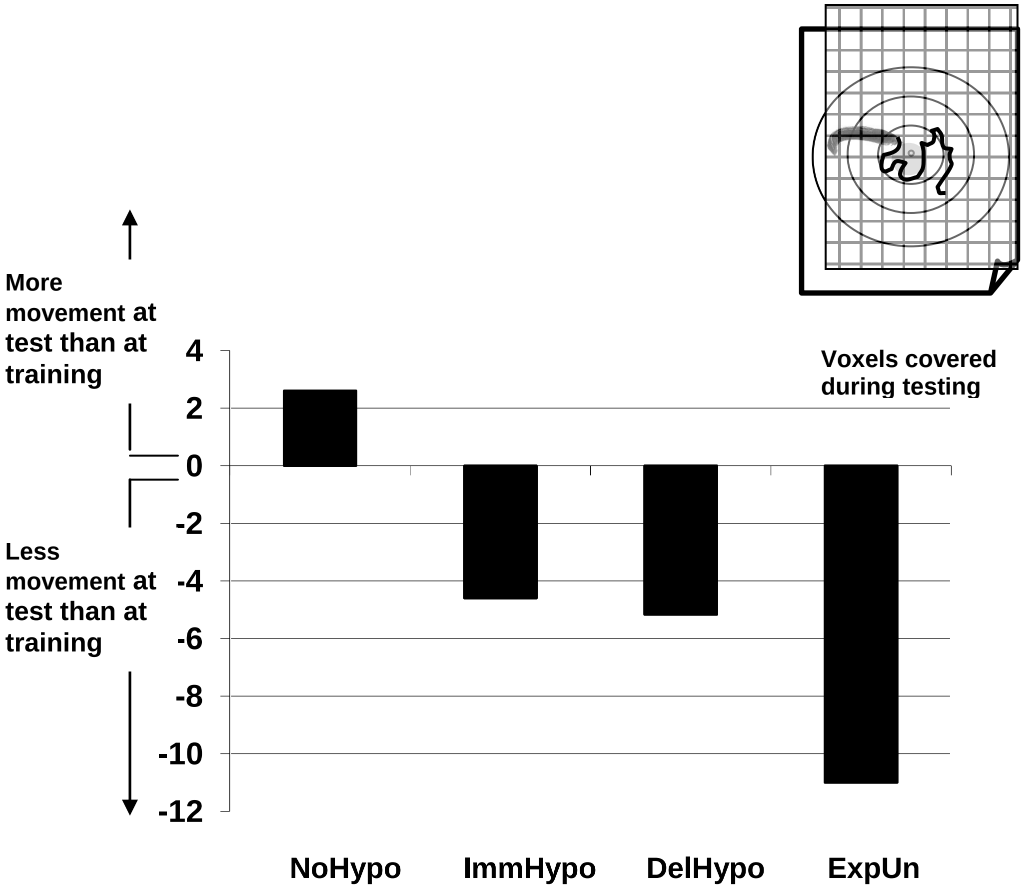

Figure 5. Voxels Covered During CS Exposures. Number of voxels covered during the 2 minute CS exposure at Testing *minus* the number of voxels covered during Training. A 4 (groups) x 2 (training vs testing) repeated measures ANOVA failed to identify a significant group effect (F 3, 19 = .772, p > .05), and no significant difference in voxels traversed during training (overall *M* = 29.60 voxels) or testing (overall *M* = 24.74 voxels) (F 1, 19 = 2.376, p > .05). Also, no group x train/testing interaction was evident (F 3, 19 = .843, p > .05).

performed similarly across the different performance measures used here, suggesting the delay period (i.e., 10 min.) might not have differentially affected these groups as anticipated. Such a finding is also of particular interest given recent work investigating the consolidation timeframe for memory establishment in this species (see Fulton, Kemenes, Andrew, & Benjamin, 2008). Moreover, and of particular interest to students of neuroscience, the potential to lesion parts of the gastropod nervous system (e.g., the procerebrum) presents new and intriguing ways to manipulate the nervous system and study the effects on information processing in this species (see Kasai et al., 2006).

Author Note

The University of Akron (Akron, Ohio) is a large Midwestern university, with a Psychology department of nearly 700 majors and three doctoral programs. Despite its size, there are no departmental facilities for animal research, and lab rooms are not equipped for animal maintenance and experimentation. Even in programs where animal research has been encouraged, financial and even political issues have placed limitations on vertebrate animal work.

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