Chemosensory-based contextual conditioning in *Hermisenda crassicornis*

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In two experiments, the marine mollusk *Hermisenda crassicornis* was exposed to context discrimination training. In one context, defined by the presence of a diffuse chemosensory stimulus (shellfish extract A), brief, unsigned, unconditioned stimuli (USs; high-speed rotation) were presented; in a second context, defined by the presence of shellfish extract B, no USs were presented. Animals were then tested (at both 1.5 and 24 h) by exposing them to small pieces of the shellfish meat used to define the two contexts. The latency to strike at the meat served as an index of the context-US association. In Experiment 1, the latency to strike at the cue associated with rotation was reduced relative to both preconditioning strike latencies and the associatively neutral cue. However, in a two-choice test where the animals could approach the conditioned or neutral stimulus, the animals regularly avoided the stimulus paired with rotation. Moreover, if, following conditioning, the animals were presented with an unsigned rotation in the conditioned context or the neutral context, the animals exhibited more effective defensive clinging (an unconditioned reflex normally elicited by rotation) in the conditioned context, suggesting that it "prepared" the animal for the aversive US. In total, these results demonstrate that *Hermisenda* is capable of making associations to diffuse background (contextual) stimuli. Moreover, the results suggest that pairing the chemosensory cue with an aversive US elicits a strike response in *Hermisenda* when the animal is placed in forced contact with the cue and an active avoidance response when the animal can choose between that cue and a neutral cue.

The background stimuli that define an experimental context are known to have a profound influence on both the acquisition and the expression of associations formed within that context (e.g., Balsam & Tomie, 1985). For instance, unsigned unconditioned stimulus (US) presentations, both prior to and during CS-US pairings, attenuate the conditioned response elicited by the discrete CS (for a detailed discussion, see Durlach, 1989). These effects, as well as other detrimental effects of unsigned US presentations, are at least in part attributable to the capacity for the experimental context to acquire direct associations to the US (e.g., Balsam & Schwartz, 1981; Hinson, 1982; Matzel, Brown, & Miller, 1987; Randich & Lolordo, 1979; Tomie, 1976), which then competes with the discrete CS for the acquisition of associative strength (Pearce & Hall, 1980; Rescorla & Wagner, 1972) or the capacity to elicit an acquired response (Gibbon & Balsam, 1981; Miller & Matzel, 1988). Despite the impact of contextual stimuli on associative processes (see also Bouton, Rosengard, Achenbach, Peck, & Brooks, 1993; Grahame, Hallam, Geier, & Miller, 1990; Rescorla, Durlach, & Grau, 1985), many "model" systems employed for neurobiological examinations of learning have been limited to the analysis of

interactions between discrete stimuli. A consequence of this limitation is the failure, both behaviorally and neurophysiologically, to account for the full complexity of associative interactions (Bitterman, 1994; Miller, 1989; Rescorla, 1988).

Contextual modulation of associative processes is not without precedent in the invertebrate literature (Abramson & Bitterman, 1986; Sahley, Rudy, & Gelperin, 1981), although direct investigations of contextual associations have been extremely limited. Only recently has a direct demonstration of both context-US associations and the ability of that association to modulate subsequent CS-US conditioning been reported in an invertebrate system (Colwill, Absher, & Roberts, 1988a, 1988b). Although this work with *Aplysia californica* extended the already impressive conditioning repertoire of the invertebrates (Carew & Sahley, 1986), its translation to a cellular analysis is complicated by the complex array of stimuli used to define the experimental context (e.g., visual, tactile, and chemosensory).

Another gastropod mollusk, *Hermisenda crassicornis*, may serve as an appropriate model system for the study of contextual associations for several reasons. First, *Hermisenda* has been utilized extensively as a subject in the behavioral (Crow & Alkon, 1978; Farley, 1987a; Grover & Farley, 1987; Lederhendler, Gart, & Alkon, 1986; Matzel, Collin, & Alkon, 1992; Matzel, Schreurs, & Alkon, 1990; Matzel, Schreurs, Lederhendler, & Alkon, 1990; Richards, Farley, & Alkon, 1984; Rogers, Talk, & Matzel, 1994) and neurophysiological (Crow, 1988; Crow & Alkon, 1980; Farley, 1987b, 1988; Fryszak & Crow, 1993; Matzel, Lederhendler, & Alkon, 1990; Matzel & Rogers, 1993;

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Rogers et al., 1994) analyses of associative learning. Second, although the majority of this work with *Hermisenda* has focused on associations between discrete visual (light) and vestibular (rotation) stimuli, *Hermisenda* also exhibits the complex chemosensory responsivity characteristic of most mollusks (Agersborg, 1922, 1925), providing for the opportunity to use chemosensory stimuli as the defining feature of the experimental context. As with many other invertebrates (Colwill et al., 1988a; Croll & Chase, 1977, 1980; Mpitsos, Collins, & McClellan, 1978; Sahley, Martin, & Gelperin, 1990, 1992; Sahley et al., 1981), *Hermisenda* is highly responsive to chemosensory cues and will rapidly learn about their role as discriminative stimuli (Farley et al., 1990). Finally, the visual, vestibular, and chemosensory pathways in *Hermisenda* have been subjected to extensive neurophysiological analysis and are known to interact synaptically (e.g., Alkon, Akaike, & Harrigan, 1978), thus providing the opportunity for future examination of the interaction between discrete visual-vestibular associations and chemosensory-based contextual cues.

The present research was conducted to achieve two goals. First, this series of experiments investigated the capacity of *Hermisenda* to form associations between diffuse chemosensory cues (context) and an aversive US (rotation) presented in that context. Second, demonstration of the capacity for such learning will provide the foundation for investigations directed toward understanding the neurophysiological basis for contextual modulation of associative processes.

EXPERIMENT 1

Experiment 1 investigated whether *Hermisenda* could form associations between an aversive US (rotation) and the context in which that US was presented. Previous work with *Aplysia californica* has shown the capacity of an invertebrate to make such an association but did so using a complex set of contextual cues (e.g., visual, tactile, and chemoreception; see Colwill et al., 1988a). Although this approach was successful in demonstrating contextual conditioning at the behavioral level, its extension to a neurophysiological examination is hindered by the lack of sensory system specificity. Consequently, the present work attempted to demonstrate contextual conditioning using a narrowly defined contextual modality. Chemosensory cues were chosen as the defining characteristic of the context given the widely accepted importance of chemoreception in molluscan behavior. This is particularly true for the identification of potential food sources, initiation of feeding, and the detection of predators and noxious stimuli (Audesirk & Audesirk, 1985; Croll, 1983; Kohn, 1961; Kupfermann, 1974; Preston & Lee, 1973). Furthermore, the capacity of chemosensory systems to participate in the conditioning of invertebrates is well established (Audesirk, Alexander, Audesirk, & Moyer, 1982; Farley et al., 1990; Sahley et al., 1990; Sahley et al., 1981; Walters, Carew, & Kandel, 1981).

In the present experiment, animals were exposed to two different contexts defined by the presence of one of two

distinct chemosensory cues, shrimp or scallop extract (counterbalanced within groups). For each animal, one context was reinforced by unsignaled bouts of rotation (US), and the second context was never paired with US presentation (i.e., discrimination training). Rotation of *Hermisenda* has served as a US in a wide range of studies of associative learning using distinct, punctate visual (Crow, 1988) and chemosensory cues (Farley et al., 1990). The extent to which contextual associations were formed in the present experiment was determined based on (1) changes in the latency to strike at the shellfish meat that served as the source of the contextual cue and (2) the ability of the reinforced context to facilitate a defensive clinging response to US presentation (Rogers et al., 1994).

Method

Subjects

Seventeen *Hermisenda crassicornis* were obtained from Sea Life Supply Co. (Sand City, CA) and housed individually in perforated centrifuge tubes (50 ml). The animals were maintained on a 10:14-h light:dark cycle in a recirculating tank of artificial sea water (ASW; 12°C). During the light phase, a 25-W light was filtered through yellow acetate such that a uniform intensity of $20 \mu\text{W} \cdot \text{cm}^{-2}$ was recorded at the surface of the water. In this and subsequent experiments, behavioral testing began after at least a 2-day acclimation period in the laboratory, but no later than 1 week following the arrival of the animals. All behavioral training and testing was conducted during the middle 8 h of the light phase. The animals were fed daily throughout behavioral training and testing with a portion (~1.5 mg) of Hikari Gold Fishfood 1 h before the dark cycle. The animals were not food deprived in Experiment 1 (cf. Farley et al., 1990) for two reasons. First, we wished to dissociate changes in consummatory behavior as a function of deprivation state from changes in bite latencies resulting from a conditioned behavioral state (i.e., aversion). Second, previous research has demonstrated that food deprivation, while having little effect on the initiation of agonistic behaviors between two *Hermisenda*, can increase the occurrence of biting behaviors within these encounters (Zack, 1974a, 1974b). Our desire was to separate these nonspecific effects of food deprivation from behavioral changes resulting from the development of a context-US association.

Apparatus

The conditioning apparatus consisted of six circular chambers milled into a single piece of clear Plexiglas mounted atop a white Plexiglas base. Each chamber was partially filled with 25–30 ml of 12°C ASW (or ASW + extract), with 1 animal confined to each chamber. A clear Plexiglas cover was fastened over the chambers, thereby isolating each cell. These chambers were then mounted atop an orbital mixer (Model 4600, Lab-Line Instruments, Inc., Melrose Park, IL) that, when operated at ~300 rpm, produced a 4-mm orbital displacement and served as the US. All apparatus were housed in a light- and sound-proof incubator maintained at 13°C.

Procedures

Food sample and extract preparation. Two chemosensory cues were used: scallop extract and shrimp extract. A stock paste of these cues was prepared on the first day of training by blending 50 ml of ASW with ~110 mg of either shrimp or bay scallop. This stock was frozen after preparation and thawed each day to make fresh solutions. The final conditioning solutions consisted of 3:100 (v/v) dilution of the stock paste in ASW, which was then filtered to remove large food particles, and were kept refrigerated throughout the day of conditioning. Fresh portions of food were prepared each day to use as stimuli during testing. Whole scallop and shrimp were cut into

~2.0-mm squares and stored in separate, covered 5-ml petri dishes. A small amount (~0.5 ml) of ASW was placed into each dish in order to maintain freshness.

Behavioral conditioning and testing. Prior to any conditioning, each animal's response to the food cues was determined by a pre-conditioning bite-latency test (see below). All animals were then exposed to two contexts, defined by the presence of shrimp or scallop extract, for 25 min per day for 3 days. The two exposures were separated by an interval of 3 h. For each group, one of the two contexts was reinforced with 50 presentations of a 3-sec rotation (US) per day for the 3 days, with an interstimulus interval of 30 sec. The animals spent an equivalent amount of time in the second context, but without US exposure (nonreinforced). Thus, Group 1 was reinforced in the context made up of scallop, with the shrimp context serving as the nonreinforced context, whereas Group 2 was reinforced in the context made up of shrimp, with the scallop context serving as the nonreinforced context. Each day, training order was counterbalanced by running each group in the opposite order (nonreinforced before reinforced) and alternating this across days. During each delay, the animals were returned to their respective home tubes. All animals were trained in total darkness and tested under red-filtered light (630 nm, $2 \mu\text{W} \cdot \text{cm}^{-2}$), allowing the experimenter to observe their behavior with only minimal stimulation of the visual system.

Ninety minutes after the second conditioning session of each day and 24 h after the final session (i.e., retention test), the animal's bite latencies to either shrimp or scallop were measured. This test has been previously employed to assess conditioning to discrete chemosensory cues by Farley et al. (1990). Testing always occurred in two phases separated by a 15 min delay during which the animals were returned to their respective home tubes. During each phase, the latency to bite at a single food cue was measured, with the order of presentation balanced across groups and days of testing. To test the bite latency, an animal was placed, ventral surface up, into a Pyrex petri dish (100 × 15 mm) partially filled with ASW (~25–30 ml) and was presented with a small sample of either shrimp or scallop. Cue presentation was accomplished by gently contacting the food cue with the lip and anterior tentacular region of the head using forceps. During preliminary work, the forceps alone were used to apply tactile stimulation to the anterior tentacles and oral veil region. Despite precedence for the elicitation of feeding responses in mollusks by mechanical stimuli (Preston & Lee, 1973), a bite response was never observed to this tactile stimulation (see also Farley et al., 1990).

The bite response in *Hermisenda* follows a set sequence of behaviors (described initially by Farley et al., 1990). Upon opening of the lips and jaws, the buccal mass is extended and two radula halves are protracted toward the food, producing a distinct "strike" at the target. Following the convergence on the food source, the radula are closed and retracted, concluding with the closing of the lips. A bite response was therefore defined by this protraction of the radula. The animals were never allowed to consume the food cue. Responses were video taped, and a single latency score (interval between touching the food stimulus to the lips and the first bite response) was obtained for each animal.

Previous work (Rogers et al., 1994) has demonstrated the development of a defensive (i.e., conditioned) clinging response to a discrete CS following pairings of light (CS) and rotation (US; see also Lederhendler et al., 1986). In light of this, Experiment 1 also addressed whether a previously reinforced context was sufficient to facilitate preparatory responding (clinging) to the US, relative to responding within a nonreinforced context. To that end, all animals were administered a single US presentation in the presence of shrimp extract 120 min following the final bite test (i.e., 24-h retention). For half the animals, this context was excitatory, and, for half, it was neutral. The latency for an animal to fall off a Plexiglas substrate during this US presentation was used as our measure of defensive responding (see Perkins, 1968).

Statistical Analysis

The acquisition data assessed with bite-strike latencies were subjected to a three-factor analysis of variance (ANOVA) with one repeated measure, where Factor 1 was the treatment (i.e., reinforced or nonreinforced), Factor 2 was the contextual cue tested (i.e., shrimp or scallop), and Factor 3 was the number of training trials (i.e., 0, 50, 100, or 150). Data for this ANOVA were combined within each treatment condition (reinforced or nonreinforced) with regard to the type of stimulus used (i.e., shrimp or scallop) such that each animal contributed a single score to both levels of the treatment. This resulted in 17 observations (i.e., 17 subjects) for each treatment level. Data for the reinforced treatment condition were also analyzed independently for shrimp and scallop using a within-subject single-factor ANOVA to identify changes in latencies over trials. Retention data (i.e., the 24-h measure) were compared using a two-factor ANOVA, where Factor 1 was the treatment and Factor 2 was the contextual cue used. Finally, differences in the defensive clinging response were assessed using a Student's *t* test. Planned comparisons of individual means were conducted on the basis of the overall mean square error term of the ANOVA.

Results and Discussion

Changes in Bite-Strike Latencies During Acquisition: Combined Data Without Regard to Chemosensory Cue

Two behavioral measures of context-US associations were used in this experiment: (1) the bite-strike latency, and (2) the ability of a previously conditioned context to facilitate preparatory clinging as measured by the latency for an animal to be dislodged from a substrate during a US presentation. Figure 1A presents mean bite-latency data for all animals ($n = 17$) to their respective reinforced and nonreinforced shellfish meat (contextual stimuli) at 0 (pretraining), 50, 100, and 150 trials postconditioning. Prior to any conditioning, the animals did not differ significantly in their baseline preference for the two cues. In response to the nonreinforced shellfish meat, the animals displayed increasing bite latencies throughout conditioning, resulting in a 51% increase by Trial 150. This apparent habituation to the nonreinforced food stimulus over repeated exposures is consistent with previous reports on *Hermisenda* (Farley et al., 1990). In contrast, the animals exhibited a 20% decrease in their bite latency toward the reinforced cue following 50 training trials that persisted throughout testing. Comparison of these data using an ANOVA showed an effect of treatment (reinforced or nonreinforced) [$F(1,30) = 26.89, p < .001$] and number of training trials [$F(3,90) = 3.39, p < .05$], and an interaction between treatment history and training trial [$F(3,90) = 8.82, p < .001$]. All other interactions or main effects failed to reach significance ($F_s \leq 1.73$).

Planned comparisons were conducted between treatment means on Trials 50, 100, and 150. The animals' bite latencies were found to be faster for reinforced, relative to nonreinforced, stimuli at Trial 50 [$F(1,30) = 4.79, p < .05$], Trial 100 [$F(1,30) = 11.98, p < .01$], and Trial 150 [$F(1,30) = 21.57, p < .001$]. In contrast, a comparison of means between Trial 150 and the pretreatment measure for the reinforced cue failed to reach significance [$F(1,90) =$

Figure 1. Differential contextual conditioning in Experiment 1. Context-US associations were evaluated using bite latencies as an indication of preference for the chemosensory contextual cue. The animals were given equivalent exposures to two independent contexts each day. For each animal, one context was reinforced by US presentations (reinforced), and the other context was never reinforced (nonreinforced). Panel A presents mean latency data (in seconds) for reinforced and nonreinforced contextual cues across training trials and at a 24-h retention interval. The data have been combined without regard as to which cue served as the reinforced or nonreinforced context. Panels B and C display mean acquisition and retention data with the data separated in regard to the contextual cue used (i.e., scallop or shrimp). Brackets indicate standard errors.

3.42, $p < .10$]. This latter comparison is of some importance in that a demonstration of a significant decrease in bite latency relative to baseline response latencies is a particularly good demonstration of an excitatory association between the context and US as opposed to just a failure to habituate over trials. Given that these data were collapsed across the two types of reinforced cues, we were interested in determining whether differences in the relative salience of shrimp or scallop could in fact be sublimating a facilitated response to one of the stimuli. In order to address this issue, the collapsed data were separated into the corresponding cue conditions.

Changes in Bite-Strike Latencies During Acquisition: Data Separated Into Respective Chemosensory Cues

Figures 1B and 1C depict the separated mean bite latencies for the scallop ($n = 8$) and shrimp ($n = 9$) contexts, respectively. Behavior toward either contextual cue in isolation exhibited the same basic trend of responding (i.e., habituation of nonreinforced cues and decreased bite latencies for reinforced cues). However, differences were observed in regard to the magnitude of these trends. By Trial 150, the animals displayed a more pronounced decrease in bite latencies for shrimp (26%) relative to those for scallop (13%), as well as a more pronounced habituation in responding to the shrimp contextual cue (71%) relative to that for the scallop contextual cue (43%). Consequently, the data from reinforced animals were compared across acquisition trials for each contextual cue in isolation using a single within-factor ANOVA. Under these conditions, a significant reduction in bite-strike latencies was observed for the reinforced shrimp context [$F(3,24) = 13.8$, $p < .001$], but not for scallop [$F(3,21) = 0.548$, n.s.].

Bite latencies were also examined at a 24-h retention interval for reinforced and nonreinforced contextual cues. Mean data for treatment conditions collapsed in regard to the contextual cue are presented in Figure 1A. These data were compared using a two-factor ANOVA, in which Factor 1 represented treatment condition (reinforced or nonreinforced) and Factor 2 represented the contextual stimulus (shrimp or scallop). The ANOVA found a main effect of treatment condition [$F(1,30) = 23.31$, $p < .001$], as well as an interaction between treatment and context [$F(1,30) = 16.54$, $p < .001$], but no effect of context was detected [$F(1,30) = 0.012$, n.s.].

Bite-Strike Latencies at the 24-h Retention Interval

As with the acquisition data, a difference in retention was observed between the two contextual stimuli at the 24-h interval. When tested in the shrimp context, reinforced and nonreinforced animals exhibited the same pattern of behavior at 24 h as that observed at the end of training (Trial 150). In contrast, no difference in bite latencies was observed between reinforced and nonreinforced contextual conditions using scallop. The potential for distinct food preferences is supported by investigations of the chemosensory abilities of a broad range of intertidal gas-

tropods (Audesirk & Audesirk, 1985; Kohn, 1961). For instance, Farley et al. (1990) found that *Hermisenda* displayed different baseline response patterns to chemosensory stimuli (shellfish), thus suggesting a between-stimuli difference in salience. Similar differences between conditioning stimuli have been documented in *Pleurobranchia* using chemosensory aversion learning (Davis et al., 1980).

Facilitation of the Preparatory Clinging Response by a Reinforced Context

Given the differential treatment of shrimp and scallop by *Hermisenda*, a single contextual cue (shrimp) was selected to investigate the defensive clinging response in order to maximize statistical power. Figure 2 displays the results obtained when we assessed the defensive clinging response to a single US presentation for all animals in the context containing shrimp. Thus, half the animals had received nonreinforced experience in this context, and the other half had received reinforced experience. Plotted are the mean durations that the animals remained fixed to a Plexiglas substrate during a single US (rotation) presentation. As the data suggest, prior unsignaled presentations of the US in this context facilitated the animal's ability to remain fixed to the substrate during a single US presentation relative to animals with no prior conditioning history in that context [$t(12) = 2.68, p < .05$].

As indicated by two different behavioral measures, these findings demonstrate the ability of *Hermisenda* to

Figure 2. Preparatory clinging response in Experiment 1. A context-US association was evaluated using a preparatory clinging response to the US. To the extent that the context predicted the occurrence of a US (reinforced group), facilitated clinging during a US presentation should be observed. Mean latency data (in seconds) for an animal to be dislodged from the Plexiglas substrate during a 3-sec US presentation are presented for each group. Two groups were used and differed as to their experience with the US within the shrimp context. All animals were tested in the presence of shrimp extract. Brackets indicate standard errors.

form context-US associations using a contextual cue with a single distinct characteristic. The animals in the present experiment appeared to develop a conditioned bite response, in that they exhibited faster bite latencies to the reinforced contextual cue, relative to both the neutral cue and the pretraining levels. Previous work with *Hermisenda* has shown the capacity of this animal to acquire aversions to chemosensory cues following discrete CS-US pairings of shellfish extract and rotation (Farley et al., 1990). The present results extend these findings to include the ability of chemosensory cues to function as salient contextual stimuli. While these results demonstrate contextual conditioning, the direction (i.e., decreases in latencies) of the bite-test result is inconsistent with previous work using discrete CS-US conditioning. Specifically, Farley et al. (1990) found that animals receiving paired presentations of discrete chemosensory and vestibular stimuli displayed significantly slower latencies for bite strikes than did their unpaired controls. Farley et al.'s findings, as well as several similar patterns of results (Davis et al., 1980; Mpitsos et al., 1978; Sahley et al., 1981), have been interpreted as resulting from a conditioned aversion to the chemosensory cue. In contrast, our findings indicate that a diffuse chemosensory cue (context) reinforced with vestibular stimulation results in significantly faster bite latencies relative to those for nonreinforced controls.

Although the source of this discrepancy is not immediately apparent, it may be dependent on the behavioral requirement imposed upon the animal at the time of testing. For instance, the decrease in bite latency observed in Experiment 1 may represent one of several possible behavioral responses of a larger conditioned state (i.e., aversion). When directly confronted with an aversive stimulus during testing, an animal may choose an aggressive response (i.e., bite-strike) as the most appropriate. However, this hypothesis does not account for the discrepancy between the present finding and those of Farley et al. (1990) given the similarities in testing procedures. Although a single factor does not appear to account for this discrepancy, several methodological differences between this work and that of Farley et al.—most notably, the use of contextual versus discrete cues—may have contributed to the difference (see General Discussion).

EXPERIMENT 2

Thus far, evidence has been presented indicating the capacity for contextual conditioning in *Hermisenda*. This is evidenced by the development of faster bite latencies to a cue used to define an excitatory context and the ability of that context to facilitate a defensive clinging response to US presentations. The conditioned bite response was somewhat surprising in that one might have instead predicted the development of conditioned avoidance based on aversion to the contextual stimulus, given the similarity of the present procedure to other aversive conditioning procedures reported with mollusks (see Davis et al., 1980; Farley et al., 1990; Sahley et al., 1981). One hypothesis to account for these findings is that, within the broader con-

ditioned state (i.e., aversion), there exist several possible conditioned responses, the expression of which depends on the behavioral demand placed upon the animal at the time of testing. For instance, if we assume that an overall aversion is conditioned to the contextual cue, then we might hypothesize that an aggressive response is appropriate when the animal is forced to confront the cue (i.e., bite-strike test), whereas the animal may choose to avoid the contextual stimulus when given the choice between confrontation and retreat. To test this possibility, Experiment 2 employed the differential conditioning procedure of Experiment 1 and tested the animals' preferences for the contextual cue in a modified Y maze.

Method

Subjects

Twenty-three naive *Hermisenda crassicornis*, housed and maintained under the same general descriptions as in Experiment 1, served as subjects in this experiment. Prior to conditioning, each animal was fed with daily portions (~1.5 mg) of Hikari Gold Fishfood 1 h before the dark cycle. Twenty-four hours prior to the first training session, the animals were food deprived and were maintained in a state of deprivation throughout the remainder of behavioral conditioning and testing (3 days). The animals were food deprived because locomotor activity in *Hermisenda* is diminished in satiated, relative to food-deprived, animals (Zack, 1974a). The animals were therefore food deprived in order to ensure a high rate of locomotion and consequent sampling of the maze.

Apparatus

The animals were conditioned in the same apparatus described in Experiment 1. Preference testing occurred in a Y maze. Three independent mazes were milled into a single piece of clear Plexiglas mounted atop a thin sheet of white Plexiglas. Tracks in each maze measured 12 × 7 mm (width × depth), with each arm extending 75 mm at a 45° angle from a 25-mm start arm. The end of each arm contained a single sample of either shrimp or scallop, with access restricted by a nylon-mesh barrier. This allowed the unrestricted diffusion of the chemosensory cue, without allowing the animals to gain access to the food. A single animal was placed into each maze while submerged in 12°C ASW and was confined using a clear Plexiglas cover fastened over the chambers. The apparatus was then mounted 35 cm below a CCD video camera (Model AVC-D7, Sony Corp.) for observation throughout the probe trial. All apparatuses were housed in a light- and sound-proof incubator maintained at 15°C. All animals were tested in the presence of a red-filtered light (GE 7.5 W nominal, 630 nm, 2 μ W · cm⁻²), allowing the experimenter to observe their behavior in the dark. This light has been tested electrophysiologically and was found to elicit virtually no photoresponse (Matzel et al., 1992).

Procedures

Food sample. Two contextual cues were used, scallop and shrimp. Solutions were prepared in the same fashion as described in Experiment 1.

Behavioral conditioning and testing. The animals were conditioned with 150 US presentations (50 per day for 3 days) using the differential conditioning procedure described for Experiment 1. Briefly, each animal received two 25-min exposures to shrimp and scallop extract each day. One context was reinforced, with bouts of rotation (US; 3 sec) separated by 30 sec; the second context was nonreinforced, with no US exposures. The chemosensory cue serving as the reinforced context was counterbalanced across animals, as was the order of training both within and across days. No bite tests were administered in this experiment.

The animals were tested following 150 training trials in a Y maze using a 15-min test trial in which the percent of total time spent in each arm (i.e., start, left, and right arms) was determined. Prior to any behavioral conditioning, baseline arm preference was measured for each animal. Small squares (~5 mm) of scallop and shrimp were prepared from fresh supplies and placed at the end of each ASW-filled arm. The arm designation (i.e., scallop or shrimp) was counterbalanced across animals but remained the same for each animal across the pre- and postconditioning test. The percent time (arm time/15 min) spent in each arm served as our measure of preference and was determined from video tape by an investigator who was blind to each animal's conditioning history. An animal was said to have entered (or exited) an arm when the front one third of its body passed the arm opening (as indicated by marks on the maze). The animals that did not sample both arms during the preconditioning test trial were removed from the study. Ninety minutes following the final training trial (Trial 150), the animals were returned to the ASW-filled Y maze, and preference for each chemosensory cue was again measured.

Statistical Analysis

All analyses were conducted on scores reflecting the change in arm preference between pre- and postconditioning. To obtain these, three scores were computed (one for each arm) for each animal by subtracting the percent time spent in each arm during the postconditioning test trial from that of the preconditioning test trial. Changes in arm preference across 5-min trial blocks were determined using a two-factor ANOVA with a single repeated measure. Factor 1 (between subjects) of the ANOVA was the treatment of the chemosensory cue associated with the maze arm (i.e., reinforced, nonreinforced, or start arm), and Factor 2 (within subjects) was the time during the test trial (i.e., 5, 10, or 15 min). In addition, the total change in arm preferences across the entire 15-min test trial was analyzed using a one-factor ANOVA (i.e., reinforced, nonreinforced, or start arm).

Results and Discussion

Three animals were removed from the study due to their failure to sample both arms during the preconditioning test. Context-US associations were evaluated using changes in arm (stimulus) preference as a measure. The percent time spent in each arm during the 15-min test trial was calculated, and the differences between pre- and post-conditioning scores were determined. Change in arm preference was analyzed in two ways. First, in order to visualize the temporal dynamics within the 15-min session (Rogers, Opello, Stackman, & Walsh, 1991), the test trial was divided into change in preference over three 5-min blocks (Figure 3A). Second, scores were collapsed across the three time blocks of the pre- and posttraining test to give an overall indication of the animal's change in preference for the contextual cues relative to pretraining preference (Figure 3B).

Figure 3A presents the mean percent change in preference for each arm (i.e., reinforced, nonreinforced, and start). These data have been collapsed in reference to which arm or stimulus (shrimp or scallop) served as the reinforced context. When viewing the data in this manner, systematic behaviors become evident. First, the animals displayed an initial decrease in their preference for the reinforced stimulus within the first 5 min. As sampling of the stimuli continued, an additional decrease was observed during the 10- and 15-min blocks. In contrast to this progressive decrease, the animal's behavior toward the nonreinforced and start arm exhibited marked dynamics throughout the 15-min test. Initially (5-min block), the animals spent close to 20%

Figure 3. Preference test using differential contextual conditioning in Experiment 2. Context-US associations were evaluated using changes in arm (stimulus) preference as a measure. Following a 15-min test trial, the percent time spent in each arm was calculated, and the differences between pre- and postconditioning scores were determined. Panel A presents the mean change in arm preference (i.e., difference in percent time between pre- and postscores) across the test in 5-min blocks. The data are in reference to the animal's behavior towards the context cue (shrimp or scallop) that was either reinforced by US presentation (reinforced arm) or was never paired with US presentation (nonreinforced arm). In this manner, each animal contributed a data point to each mean. Panel B presents the same data as in Panel A except that now it has been collapsed across the three time blocks, thus displaying overall means for the change in arm (stimulus) preference between pre- and posttraining scores. Brackets indicate standard errors.

more time sampling the nonreinforced arm. This initial increase in preference exhibited a gradual diminution to essentially no change in preference by the end of the 15-min block. In contrast, their behavior toward the start arm changed in the opposite fashion, initially decreased, with a progressive increase in preference as the test trial continued. Although changes in preference for the reinforced arm and the start arm were observed throughout the 15-min test, the stable reduction in preference for the reinforced cue suggests the development of a conditioned contextual aversion in these animals. These data were compared using an ANOVA, which revealed an effect of treatment (i.e., reinforced, nonreinforced, or start arm) [$F(2,38) = 3.19, p < .05$] and an interaction between treatment and time during the test trial (i.e., 5, 10, or 15 min) [$F(4,76) = 2.49, p < .05$]. In contrast, the effect of time during the test trial failed to reach significance [$F(2,38) = 0.96, n.s.$].

Figure 3B presents mean data for change in arm preference collapsed across all three time blocks of the post-training test. Here, the overall decrease in preference for the reinforced stimulus during the postconditioning test relative to the pretraining test can be seen. The change in arm preference for the reinforced, nonreinforced, and start arms differed [$F(2,57) = 4.77, p < .01$]. Planned comparisons were conducted between treatment means, and the animals' preference for the arm containing the reinforced cue was found to be significantly decreased relative to that for the nonreinforced cue [$F(1,57) = 5.95, p < .05$] and start arms [$F(1,57) = 8.16, p < .05$].

GENERAL DISCUSSION

Associative processes in *Hermisenda crassicornis* are in many respects similar to those exhibited by higher vertebrates (Crow, 1988; Farley, 1987a; Matzel et al., 1992; Rogers et al., 1994). One factor that has limited the capacity of this model system's applicability to vertebrate learning has been the focus on discrete CS-US associations, and the consequent exclusion of complex interactions between multiple conditioning stimuli (i.e., CS and/or contextual stimuli). The present experiments begin to address this limitation through the demonstration of contextual conditioning in *Hermisenda*. Pairings of a US (rotation) with a context defined by a distinct chemosensory stimulus led to the development of an association between that US and the context. This was demonstrated using a conditioning procedure in which two distinct chemosensory cues were differentially reinforced.

In Experiment 1, shrimp and scallop extract served as contextual cues in a differential conditioning procedure. It was determined that animals develop faster bite latencies for the chemosensory cue that had served as the reinforced contextual background. Responding to the reinforced cue was faster relative to preconditioning bite latencies and to the nonreinforced controls and was found to persist for at least 24 h following conditioning. This general pattern of results was detected for contexts based on both shrimp or scallop extract, although the latencies were only signifi-

cantly reduced relative to baseline rates for animals trained in the shrimp context. Further support for contextual conditioning came from the demonstration that the reinforced context was sufficient to facilitate a defensive clinging response 24 h postconditioning when a US was presented in that context relative to a neutral context. Experiment 2 evaluated the hypothesis that these animals were indeed developing an aversion to the conditioned context, but that the expression of that aversion was a function of the behavioral task. It was hypothesized that when animals are unavoidably confronted with an aversive stimulus they will strike, but when given a choice not to confront the aversive stimulus, they will withdraw from it. Using the same conditioning parameters as in Experiment 1, Experiment 2 tested the animals' cue preference in a Y maze. A decrease was found in their preference for the reinforced contextual cue relative to preconditioning baseline preferences and to an associatively neutral cue. These results are consistent with our speculation that animals develop an overall aversion to the contextual cue during conditioning that may be expressed as aggression when forced to confront the stimulus (i.e., bite-strike test) and a withdrawal when given an opportunity to avoid the stimulus (i.e., Y maze). In this regard, our findings are consistent with much of the literature in that other studies employing discrete chemosensory cue conditioning have shown a decrease in preference with a choice or preference test (Sahley et al., 1981).

Our contention that the conditioned bite response reflects the behavioral demand placed upon the animal at the time of testing does not immediately account for the inconsistency between our findings and those of Farley et al. (1990). Using a similar testing procedure, Farley et al. reported that pairings of a punctate chemosensory CS with rotation produced longer bite-strike latencies. Aside from the major difference of conditioning a diffuse contextual cue versus a discrete chemosensory CS, several other methodological differences may have contributed to this discrepancy. First, despite the common use of vestibular stimulation as a US, qualitative differences exist in regard to the nature of that US. For instance, Farley et al. used long-duration (30 sec) turntable-induced rotation to achieve vestibular stimulation. In contrast, we have successfully used brief (2–3 sec) orbital rotation as a US in numerous studies (Matzel & Rogers, 1993; Matzel, Schreurs, Lederhendler, & Alkon, 1990; Rogers, Fass, & Matzel, 1992; Rogers et al., 1994). Although both techniques result in the stimulation of hair cells within the vestibular organ, differences arise as to the pattern of stimulation. For example, turntable rotation induces a constant and unidirectional force (~2 g) that results in the mechanical stimulation of a subset of vestibular hair cells. In contrast, orbital rotation results in more general stimulation of hair cells throughout the statocyst. It is unclear whether this differential stimulation of hair cells could contribute (e.g., activation of different cellular networks) to the observed differences in bite response between these studies. This appears unlikely in that both forms of the US have been shown to result in a similar conditioned response (i.e., foot contraction), as well as similar cellular correlates of a light-rotation as-

sociation (e.g., a reduction of neuronal potassium conductance in B photoreceptors). Another possibility is that orbital rotation results in more than just vestibular stimulation (e.g., tactile information resulting from a turbulence-induced stimulation of the rhinophores), and that the US within these studies represents a more complex array of sensory inputs than those used by Farley et al. Finally, the long-duration US (30 sec) employed by Farley et al. is more likely to produce a state of general malaise than is the 4-sec US used here. If so, it is reasonable to expect the development of a behavioral response more consistent with classic food-aversion learning.

Another difference between the present work and that of Farley et al. is the behavioral expression of the bite response. Specifically, Farley et al. report that the reliable occurrence of a bite-strike response was dependent on maintaining the animals in a state of food deprivation. This was not so in our hands. Although pilot studies in our laboratory utilized a deprivation state comparable to that of Farley et al., we found no significant difference between those studies and Experiment 1 in the baseline levels of biting behavior (~2.5 sec) or in the behavior of animals toward the reinforced cue. However, differences were observed in response to the nonreinforced cue in that a much larger habituation (a mean latency of ~6.0 sec by Trial 150) to the nonreinforced scallop cue was observed in food-deprived animals relative to the nondeprived animals of Experiment 1 (see Figure 1B). Additionally, Farley et al. report baseline bite latencies of 5.4–8.2 sec, depending on the chemosensory cue used. In contrast, we have consistently obtained pretraining bite latencies of 2.0–2.75 sec, regardless of the cue, across multiple replications. It is unclear whether these differences merely reflect differences in the maintenance of our animals or whether they are attributable to differences in the populations of the animals used.

Some consideration should be given to the role of post-stimulus interference in our findings. Pfautz, Donegan, and Wagner (1978) have proposed that the process of habituation may be interfered with by poststimulus events that compete for processing in short-term memory, a process called *protection from habituation*. For instance, although we have attributed the differential responding in reinforced and nonreinforced contexts in Experiment 1 to the development of a context-US association, one might also argue that the animals failed to habituate to the reinforced context due to a disruption in processing imposed by repeated US presentations. Therefore, assuming post-stimulus interference, the sustained responding to the reinforced context could potentially be accounted for without reference to the formation of a context-US association. Three pieces of evidence argue against this interpretation as a general explanation of our results. First, the animals trained in the context made up of shrimp exhibited *reduced* strike latencies relative to their pretreatment levels, whereas the control groups underwent habituation. The occurrence of poststimulus interference cannot readily account for this reduction but would have instead predicted that the latencies would remain near baseline levels. Second, the capacity of the animals to remain fixed to a sub-

strate during a single US presentation (a defensive response to water turbulence) was facilitated by the presence of the conditioned contextual cue. This finding promotes the suggestion that the context has been endowed with excitatory associative strength and again supports the conclusion of a context-US association. Finally, the demonstration of decreased contextual cue preference in Experiment 2 is characteristic of a conditioned chemosensory aversion, the occurrence of which is dependent on the animal's forming an association between the relevant chemosensory contextual cue and the aversive event that occurs within it. In total, these three observations suggest a *change* in the topography of the response to the CS following CS-US pairings, as opposed to merely an absence of change against a background of habituation. However, one could suppose that a protection from habituation (or even sensitization) to the CS might be occurring against a background of sensitization produced by the local presentation of the US. Although one might expect differential responding to the paired and unpaired CSs to develop under these conditions, we would conclude that the pattern of responding on three separate behavioral tests can be more parsimoniously described as associative learning. At present, however, we cannot definitively distinguish between these alternative possibilities.

Finally, attention should be given to the extension of this work to a cellular analysis of associative processes in gastropods. It is now clear, on the basis of the present results and those of other investigators (Colwill et al., 1988a, 1988b), that invertebrates possess the capacity to form associations between static contextual stimuli and aversive events embedded within those contexts. These findings have important implications for activity-dependent models of associative learning (e.g., Alkon, 1989; Hawkins, Abrams, Carew, & Kandel, 1983; Walters & Byrne, 1983). In general, activity-dependent models of learning rely heavily on the temporal convergence (i.e., temporal contiguity) of short-lived CS- and US-induced intracellular events to account for synaptic plasticity. For instance, associative learning in both *Aplysia* and *Hermissenda* is said to occur when a CS-induced rise in intracellular Ca^{2+} is potentiated by the temporal convergence of synaptic activity mediated by the US, such that Ca^{2+} -sensitive enzymes are stimulated (Matzel & Rogers, 1993; Rogers et al., 1994; Yovell & Abrams, 1992). These forms of "cellular learning" are temporally constrained in that the Ca^{2+} signal induced by the CS dissipates rapidly owing to an inactivation of Ca^{2+} currents and rapid intracellular buffering mechanisms. Since a diffuse CS (i.e., context) would presumably produce tonic activation of the sensory neurons, no Ca^{2+} signal would persist to "converge" with the US during its repeated presentation. Although these models can account for conditioning using punctate CSs, they fail to predict the formation of associations between these static, temporally diffuse stimuli (see related discussion by Colwill et al., 1988a). Our ability to distinguish whether the formation of context-US associations represents a different cellular mechanism or, instead, a reformation of existing cellular models will rely on a cellular analysis of the present phenomena.

The identification of contextual conditioning in invertebrates presents an exciting opportunity to evolve from a simple cellular analysis of discrete, binary stimuli to a more refined analysis that begins to address some complexities of associative interactions. For instance, a sensitivity to CS-US contingencies has been reported for *Aplysia* (Hawkins, Carew, & Kandel, 1986) and *Hermissenda* (Farley, 1987b) in that conditioning of a CS is diminished by the addition of unsignaled US presentations. Although explanations of these effects have traditionally been of a nonassociative nature, the present results suggest that conditioning to the context could also play some role.

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