Mechanisms of Navigation in Fiddler Crabs: An Analysis of Allocentric and Egocentric Contributions

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MECHANISMS OF NAVIGATION IN FIDDLER CRABS:
AN ANALYSIS OF ALLOCENTRIC AND EGOCENTRIC CONTRIBUTIONS

by

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Navigation in biological systems is a complex task-set that involves learning processes and may include constructing representations of features of their environment. Across the animal kingdom, different learning mechanisms have evolved to similar spatial problems. The extent to which mechanisms are conserved across taxa are an important research area that can guide our understanding of the cognitive dimensions of navigation. Recent studies of mammals, birds, and arthropods has found that these animals often attend to multiple forms of sensory cues, and to either integrate the solutions generated by these cues, or at times prefer one form of cue over another. This dissertation examines the fiddler crab (Uca pugilator), a burrow-homing arthropod whose ecology and behavior engender evolutionary pressures that favor spatial memory to determine which these kinds of multi-modal integrative processes are they employ. Previous field studies give indications of complexity beyond simple route reversal methods. U. pugilator are a species that share and likely resemble a basal ancestor to the insect taxa that have proved fruitful to the study of navigation. The results of this dissertation suggest that the ability to employ and integrate solutions from multiple navigational mechanisms is evolutionarily old and conserved across a wide range of taxa. Four experiments are presented that employ a place learning paradigm to examine the roles of externally (allocentric) and internally (egocentric) generated sensory cues
in the construction of fiddler crab navigational strategies. Three of these experiments provide evidence for a preexisting taxis in these animals that dictates they approach certain visual stimuli, and two of these experiments provide evidence of an allocentrically informed associative process in navigating fiddler crabs, a finding not before seen in a laboratory study of these animals. Taken together the results of this dissertation suggest that fiddler crabs possess some form of cognitive representation of the external world, which is informed by multiple sensory modalities, and extends beyond response learning and path integration.
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Chapter 1 – Introduction

Natural navigation by animals is a complex task-set that is often solved by animals learning about and constructing representations of features of their environment (Kramer, 1957). Across the animal kingdom, evolution has produced different solutions to similar spatial problems, and also conserved solutions across multiple taxa. Different approaches to navigation employ different perceptual, cognitive, and motor abilities that are adapted to specific spatial challenges an animal must overcome to increase their fitness. Using a comparative approach that evaluates the similarities and differences in navigational strategies across species, we gain insight into how different ecologies, body forms, and brain architectures guide the development and evolution of cognitive mechanisms used to solve similar spatial problems (Striedter, 2005). The navigational and integrative mechanisms used by vertebrates are well understood (Robinson & Berridge, 1993). This understanding has created an opportunity to apply the comparative method to understand invertebrate navigational mechanisms and through comparing these mechanisms to those employed by other animals, including vertebrates, determine to what degree these mechanisms are a general product of the basic properties of neural networks, and conversely to what degree these are specific, individually evolved solutions.

Invertebrate animals show a variety of navigation strategies and are therefore a good source of model systems for understanding the range and variety of cues and information processing that inform and guide navigation (M Giurfa, 2007; Wehner, 2003). In addition, as their brains are relatively small, and their spatial orientation behaviors are robust (M Giurfa, 2007; Wehner, 2003), invertebrate animals, and specifically arthropods, rely on fewer neural resources to navigate with a degree of competence comparable to the better-studied vertebrates
(mammals are especially well studied). Where mammals are able to employ their relatively advanced and plentiful neural processing power and capacious memory systems (i.e., hippocampus) to solve spatial navigation tasks the smaller brains of invertebrates, though structurally complex in their own ways, highlight what can be done without a comparatively large investment in neural resources (Brusca & Brusca, 1990; Bullock & Horridge, 1965). Honey bees, for example, have approximately 850,000 neurons, compared to 40 million in mice, and 85,000 billion in humans (Azevedo et al., 2009; Herculano-Houzel & Lent, 2005; Miklos, 1998). Further, their brain architectures lack the homologous organization common to vertebrates (Brusca & Brusca, 1990). Given that invertebrates often occupy the same environments and compete with vertebrates for the same sources they offer opportunities for ‘natural experiments’ in brain design and computational mechanism. Put another way, the dramatic differences in brain organization beg the questions of convergent evolution or unique mechanism innovation (Hochner, Shomrat, & Fiorito, 2006).

There is a long standing debate about just how “cognitive” invertebrate animals are. Historically, invertebrates have sometimes been characterized as “well programmed robots” (Gould, 1982), implying that the majority of their behavior is the result of simple fixed (possibly instinctive) stimulus response reflex arcs. The English Oxford dictionary defines cognition as “The mental action or process of acquiring knowledge and understanding through thought, experience, and the senses” (English Oxford Dictionary, 2016). Under this definition, stimulus response reflex arcs once thought to be responsible for most invertebrate behavior could not be called cognitive. While invertebrates are capable of complex behaviors, it is currently not known if complex invertebrate behavior is the result of isolated and highly modularized instinctual neural systems, or is there are plastic, experience dependent cognitive systems supporting
complex invertebrate behavior. There is growing evidence within the ethological literature that addresses these possibilities, through research programs aimed at searching for complex forms of learning within the invertebrate classes (Ken Cheng, Shettleworth, Huttenlocher, & Rieser, 2007; Fahrbach, Moore, Capaldi, Farris, & Robinson, 1998; M Giurfa, 2007), which may qualify as “cognitive”.

It has been known for many decades that invertebrates are capable of learning simple Pavlovian and operant associations, as far back as Eric Kandel’s groundbreaking work with the sea slug, *Aplysia* (Carew, Walters, & Kandel, 1981; Hawkins, 2006). Beyond simple associative mechanisms, flying insects such as bees and wasps integrate navigationally relevant cues from multiple modalities to aid their path integration system, such as using olfactory cues to guide themselves toward feeding sites on final approach, and using view matching to recognize and return to their home nests (Thomas S Collett & Collett, 2002; Möller, 2001). Such sequential sensory guidance strategies, while not navigation per-se, illustrate the hierarchical control of orientation that is common in advanced insect brains.

In recent years there is evidence emerging that invertebrates are also capable of learning more complex associative relationships. Studies done by Martian Giurfa have demonstrated that honey bees are capable of learning associative relationships among conditioned stimuli that are non-linear, such as the negative patterning occasion-setting task (A+,B+,AB-), and a delayed matching to sample task (a forced choice task where the “correct” cue varies from trial to trial). Both of these learning paradigms do not allow animals to simply learn “cue A is reinforced, and cue B is not”, as during training each cue is equally reinforced and not reinforced. This task can only be successfully completed by attending to the context in which cues are presented, and learning a “rule” to determine when a cue is likely to be reinforced (Martin Giurfa, 2007).
These findings argue against Gould’s “well programmed robot” concept, as these learning tasks cannot be solved with simple stimulus-response circuits, and instead demand an increased degree of computational processing, relying on more complex patterns of association.

Arthropods, and specifically insects, have long been a fruitful and productive group of subjects in research on the mechanisms of navigation, and of the interaction and competition between navigational mechanisms. Path integration, the navigational mechanism by which animals track their current position relative to some previously visited location by monitoring and integrating their own movements, is known to be present in many species (Etienne & Jeffery, 2004). The method of tracking movement used differs across species. Ants and other terrestrial animals rely on proprioceptive feedback from their legs or possibly efference copy of commands sent to their legs (Wehner, 2003; Wittlinger, Wehner, & Wolf, 2007) Bees, wasps, and other flying insect species, on the other hand, rely on optic flow (Hrncir, Jarau, Zucchi, & Barth, 2003; Srinivasan & Gregory, 1992), but the overall strategy of path integration is found in many arthropods (Hrncir et al., 2003; Layne, Barnes, & Duncan, 2003b; Wehner, 2003). In addition to path integration, ants are sensitive to a diverse set of navigationally relevant cues such as visual landmarks, tactile, olfactory, vibrational, electrical, and magnetic cues (Bühlmann, Cheng, & Wehner, 2011; Collett & Collett, 2000; MacKay, Majdi, & Irving, 1992; Wehner, 2003; Wohlgemuth, Ronacher, & Wehner, 2001). Current understanding of the interaction of these different sources of spatial information in ants suggests that path integration is the primary mode of navigation (Martin Müller & Wehner, 2010; M Müller & Wehner, 1988). Path integration is of finite practical utility because it is vulnerable to cumulative errors, termed “integration errors”, that result in increasingly inaccurate solution vectors as an animal’s distance from their target increases (Barnes, Johnson, Horseman, & Macauley, 2002; McNaughton et al.,
1996; Martin Müller & Wehner, 2010; M Müller & Wehner, 1988). It is believed that the other forms of navigational cues are used by ants to overcome this tendency toward integration error, by providing opportunities for animals to “reset” their path integrator, and effectively shorten the length of their “home vector” by using salient landmarks as waypoints between themselves, their foraging/mating sites, and their ultimate target, their nest (Martin Müller & Wehner, 2010). These, and many similar results have demonstrated not only that ants are capable of using multiple modes of navigational information, but also that they are sensitive to the usefulness or accuracy of these different sensory information streams, based on their previous experience, an advanced learning mechanism that fits the definition of cognition used above. (Bühlmann et al., 2011; Ken Cheng, Middleton, & Wehner, 2012; Ken Cheng et al., 2007; Collett & Collett, 2000).

This kind of interaction between navigational mechanisms and multi-modal sensory systems, has led me to the thesis that multi modal integration, often involving weighting based a given modalities performance history, is characteristic of how arthropods move through and understand space. This is an assertion that requires more experimental support and providing this support is one of the overall goals of this dissertation.

The domain of spatial learning has a long and productive history of providing insights to the nature of central representation and navigational information processing in invertebrates(Basil & Sandeman, 2000; Beugnon, Pastergue-Ruiz, Schatz, & Lachaud, 1995; Perry, Barron, & Cheng, 2013; Wehner, 2003), as it is possible to present animals with a diverse set of cues of different sensory modalities, and examine whether and to what extent animals are able to use cues from different modalities in order to solve spatial problems.

There are two different frameworks through which one can conceptualize how animals use cues from different modalities to navigate. Animals may use different cues in a “modular”
way, meaning that cues from one modality are exclusively in control of the animal’s behavior unless some condition is met causing the animal to switch to cues from a different modality simply ignoring other modalities when they come into conflict. Conversely, animals may “integrate” information from multiple navigational modalities, combining the behavioral dictates (solution vectors) generated by multiple systems into one overall solution vector. It is important to note that these two frameworks, integration and modularity, are not mutually exclusive, and in fact modularity can be viewed as a special form of integration where weights are binary, either 0 or 1 (all or nothing).

Desert ant navigation was long thought to be an exclusively modular process, with egocentric navigational information only being relied on as a “backup system” for when visual information is unavailable or produces a navigational error (Müller & Wehner, 2010). In extreme cases animals may not learn about other certain spatial cues at all when their preferred cue is present, as is the case in certain blocking effects found in rats navigating in a Morris water maze (Roberts & Pearce, 1999). These types of strategies could be referred to as stimulus-triggered or modular (K Cheng, 1986) mechanisms, as the competing navigational systems are separate from one another, and simply switched, or gated, between as the need arises.

Recent work with several species of ants has found that an integrative view describes their navigational choices better than a modular one (Bühlmann et al., 2011; Ken Cheng et al., 2012), with ants weighting this integration according to the predictive usefulness of specific modalities over the animals evolutionary and personal history. The solution produced by different cue modalities can be combined or averaged with one another, in order to create a unified solution. Studies have found that a wide range of animals, including humans, pigeons, and ants all employ integrative strategies in cue completion tasks, under certain conditions (Ken
Cheng et al., 2007). Additionally, it has been shown definitively for humans that this integration is performed in an optimal Bayesian fashion, with each sensory modality being weighted in inverse proportion to its previous performance variance (Ken Cheng et al., 2007). In other words, sensory modalities with a history of inaccurate solutions to spatial problems are weighted less, when averaged with better performing modalities. Other research suggests that this kind of optimal integration may also be being performed by pigeons and ants, although there is no definitive evidence demonstrating this as of yet (see Cheng, Shettleworth, Huttenlocher, & Rieser, 2007 for review).

Integrative strategies are more cognitive (under the above definition) because they require an animal to attend to multiple sources of information simultaneously, and actively reconcile the directional information provided by these sources, and in the optimal case, attend to and remember the performance history of different cues. The discovery of complex learning abilities in invertebrates encourages researchers to seek out more of these mechanisms, however one must use caution to rule out simple, instinctual mechanisms as the explanations of any potential learning effects uncovered. Complicated behaviors are prone to being interpreted as necessarily requiring generalized information processing mechanisms, however hierarchically organized fixed behaviors (finely tuned by evolution to specific purposes) might act in concert to produce complicated behavior that appear cognitive.

If invertebrates are solving navigational problems in a modular way, one would expect them to only be able to use conditioned stimuli (CSs) that resemble those that they encounter under natural conditions to solve these spatial problems, or at least are of a modality they typically encounter. However, if the spatial navigation abilities of these animals are general rather than phylogenetically inherited stimulus-triggered processes, then even relatively
unnatural stimuli, and possibly stimuli from modalities not typically used by these animals for
the purposes of navigation, should be able to support spatial learning under laboratory conditions
as integrative mechanisms are by definition plastic. Additionally, because of the plethora of
conditioned stimuli that are available to researchers to present to subjects in a spatial learning
paradigm, it is possible to design experiments that examine the possibility of animals combining,
or integrating, spatial information from these multiple sources of information, as opposed to
simply defaulting to one source. This sort of multi-modal integration would be strong evidence
against the modularity hypothesis, and would support the view that invertebrate learning abilities
are integrative and cognitive.

One approach which has been used to demonstrate allocentrically informed place
learning, and could be used to examine issues of cue integration is the conditioned place
preference experimental paradigm. In this procedure cues are paired with an outcome, either
aversive or appetitive, and learning is detected as a change in the animals preference (measured
by proximity) for or against these cues, in the absence of the outcome. One of the strengths of
this paradigm is the ability to present animals with stimuli from a diverse set of modalities
(visual, egocentric, olfactory, etc.), in order to determine which of these modalities subjects
process to in order to learn about space, and selectively devalue specific cues to determine if
subjects are sensitive to the “usefulness” or solution variance of these cues (“solution variance”
meaning the difference between the solution vector generated by a navigational system, and the
true solution vector, which an animal is only aware of after carrying out the behavioral dictates
of a particular solution vector.). Additionally, if it is found that subjects are capable of learning
to avoid places based on more than one of the these modalities, the place learning paradigm
(after training) allows researchers to put the spatial information provided by these different cues
into conflict, in order to analyze how subjects reconcile this conflict, a procedure known as “cue competition”.

As the body of evidence supporting cognitive integration and decision making in navigating insects has increased in recent years, important questions have emerged about whether or not these abilities are recent evolutionary developments in the eusocial insects (ants and bees specifically), or if they originate from a shared ancestor. The common ancestor thesis would explain why it is relatively ubiquitous amongst the existent invertebrate taxa (Perry, Barron, & Cheng, 2013) the eusocial insects however have elaborated this ability far beyond what is currently thought to be the capacities of non-eusocial arthropods. Answers to these questions will be found by searching for cognitive spatial abilities outside of the well-studied Hymenoptera (wasps, bees and ants), as has been done with the non-eusocial insect, Diptera. A natural group would be the invertebrate order Decapoda given that the crustaceans are phylogenetically basal to the insects (Brusca & Brusca, 1990). In this connection, fiddler crabs have great potential for the purposes of comparative studies navigation and spatial memory in arthropods. As a basal comparison group (a representative species similar to species that insects evolved from) to other navigating invertebrates, studies of fiddler crab spatial navigation behavior may provide insight on evolutionary and mechanistic questions about the origins and neural substrates of these abilities.

First and perhaps most importantly, fiddler crabs are relatively good at navigational tasks in the wild. Fiddler crabs construct and maintain burrows which they rely on for protection from predators, and for access to water during low tides (Hemmi & Zeil, 2003; T. W. Kim & Christy, 2015). Fiddler crabs leave these burrows regularly to forage and mate, and quickly and directly return to their burrow when presented with looming stimuli, which simulate the approach of a
Experiments done with foraging fiddler crabs have provided strong evidence for which cues these animals rely on to construct a representation of space. In these experiments, crabs are allowed to freely forage, typically until they walk onto a section of substrate that an experimenter has rigged to “passively” (i.e., not under its’ own voluntary movement) move the crab some distance, this gives the subject access to visual and vestibular cues that indicate it’s new position, but not proprioceptive cues. This type of experiment is typically referred to as a “translocation experiment.

In the context of translocation experiments, fiddler crabs are typically then shown a predator like stimulus, prompting them to try to “escape”. In one example, the experimenter would allow a crab to follow a circuitous path in search of food. At some point after the crab was out of sight of the burrow the experimenter would relocate the crab 10 cm to the west. The direction and length of the home run of the animal is then recorded. The results of translocation experiments demonstrate that fiddler crabs have a representation of the location of their burrow that they carry with them. Furthermore these types of experiments provide evidence that suggests which sensory cues are informing this representation. If the subject is relying on egocentric cues, one would expect the subject’s home run to terminate at a point 1 foot to the west of its burrow (commonly referred to as the “fictive burrow”). Contrarily, if external, or allocentric cues are being employed, such as visual landmarks or gradient following, one would expect the subject to be able to account for the translocation and accurately return to their burrow’s location.

Translocation experiments with fiddler crabs consistently find that subjects initially home on the “fictive” burrow location, suggesting that fiddler crabs rely primarily on path integration to track the location of their burrows, and generate an escape vector (T. W. K. Kim & Choe, 2010; Layne et al., 2003b; Walls & Layne, 2009a; Zeil, 1998). In this they are similar to the
well-studied ants discussed above. There are two separate components that must be integrated in order for fiddler crabs to be able to generate a vector describing the most direct path back to their burrow via path integration: the distance they have traveled, and the direction of return from their current location.

There is evidence of flexibility in the path integration mechanism in fiddler crabs that differs from the typical ant homing model (Walls & Layne, 2009b). It has been demonstrated that fiddler crabs are able to accurately compensate for experimenter-induced changes in the distance between their current location and their burrows due to vertical dimension changes in their homebound path. They appear to adjust the distance component of their home vector. This manipulation was achieved by allowing fiddler crabs to forage away from their burrows in the typical way, and then inflating a dome shaped balloon between foraging crabs and their home burrows (Walls & Layne, 2009b). The crabs home run was the initiated by exposing crabs to a simulated looming “predator” stimulus. Under these conditions, if a crab were simply running out the distance it calculated during their outbound trip, it would stop short of its burrow, as walking on top of this inflated dome requires more travel distance than walking across the corresponding flat surface. Walls and Layne surprisingly found that their subjects were able to account for this artificially introduced change in distance, and accurately returned, traversing the full distance to their home burrows. This suggests that fiddler crabs are tracking their body angle relative to the y (vertical) axis during navigation, and integrating changes in the angle of the substrate on which they are running into their calculation of the amount of distance they have traveled.

To understand how fiddler crabs encode the directional aspect of their perception of space, researchers have conducted “rotational experiments” similar to the translocation experiments
described above. Instead of translating subjects before initiating their home run, subjects are rotated instead. When rotated on an experimenter-operated spinning disk, fiddler crabs exhibit a compensatory response, rotating themselves against the direction of the imposed rotation (Layne, Barnes, & Duncan, 2003a). Layne et al. found that crabs often undercompensated for imposed rotation, and miss-calculated the direction of their burrow by an angle that matched the error in their compensatory volitional rotation. This suggests that fiddler crabs to not include these compensatory rotational movements when calculating their home vector. If they did, they would miss their home burrow by the full amount of imposed rotation.

In a 2003 study, Layne et al. made a theoretical treatment of the issues engendered by these findings to explore possible mechanisms. He compared the homeward paths of these rotated crabs with paths produced by seven models of the crab’s putative computations. These models differed in what frame of reference determined the crabs orientation, allocentric or egocentric, and whether or not the crabs integrated imposed and self-generated rotation and translation (small translations are an unintended consequence of disk rotation, as animals rarely position themselves at the center of the experimental disk). They found that the models that best fit their behavioral data were those that relied on egocentric directional information, and did not integrate compensatory self-generated rotation into the path integration module. Any possible effect of self-generated translation (linear movement on the disk during compensation) was not distinguishable in the models, because they produced small differences in the simulated paths. The modeling and empirical results taken together tell us Fiddler crabs are capable information stored in spatial memory to gate proprioceptive information in a way that does not include movements that were compensatory. Fiddler crabs are capable of gating when proprioceptive information is passed into their path integrator, in a way that rightfully does not include
movements that were not “intentional”, and instead were attempts to resist motion caused by and outside force.

Recent research has found that although fiddler crabs do seem to rely on egocentric information, allocentric visual information may also play a role in their homing abilities, either being combined with path integration information, or by serving as a backup, in situations where the path integration system is likely to be error prone (T. W. Kim & Christy, 2015; T. W. Kim & Choe, 2010). These recent results further suggest recommend fiddler crabs as a useful subject to study allocentric and egocentric cue competition and integration in animal navigation systems.

Fiddler crabs have access to several unusual visual cues that can be brought to bear on navigational tasks. Fiddler crabs have an almost cyclopean visual field in the horizontal plane, just shy of 360 degrees (Zeil & Al-Mutairi, 1996). This optical arrangement makes it possible for them to navigate by using an instantaneous, holistic, image of their surroundings. It also relieves them of the need for memory systems which, in vertebrates are supported by the use of eye movements but require the central integration (“stitching together”) of several stored views to understand the current visual context (Melcher, 2001). The visual representation can be quite coarse and still be useful to the animal as a input to a view matching strategy of navigation, as computational studies with mechanisms such as the “block match” algorithm show (Brown, 2013; Franz, Schölkopf, Mallot, & Bülthoff, 1998). Visual navigation stratagems that rely on 360 degree coarse impressions of the surrounding visual scene have recently exhibited strong explanatory power when attempting to explain the navigational abilities of other invertebrates (Baddeley, Graham, Husbands, & Philippides, 2012). It has also been demonstrated that fiddler crabs are able to perceive celestial cues, such as polarized light and that they likely use a time compensated mechanism to infer bearing from the orientation of the suns polarization
(Herrnkind, 1968; How et al., 2012; Meyer-Rochow, 2001). In this they are similar to bees and ants, and this similarity may be due to a shared inheritance amongst arthropods.

As discussed above, fiddler crabs navigate using strategies that share numerous commonalities with other well studied invertebrates, ants and bees, both of which are eusocial animals. Fiddler crabs are gregarious in that they live in large social groups, but are by no means eusocial. As noted above this raises interesting opportunities for contrast with eusocial insects. For eusocial species, extreme natural selection forces shaped the evolution of their cooperative and large-scale social spatial abilities. A foraging trip for a eusocial animal is only “successful” if they are able to return to the nest with the food item they have procured. In comparison, fiddler crabs and other non-eusocial animals have the option of abandoning their old burrow and constructing or finding a new one, if their navigational mechanisms fail and they are unable to return to their own burrow. A fiddler crabs investment in their home burrow is only in the construction of the burrow, as they do not store food or other resources there. This places an entirely different set of constraints on the evolution of fiddler crab spatial learning mechanisms than those involved in the evolution of spatial learning in eusocial animals, again making fiddler crabs and interesting comparison group in understanding invertebrate cognition in general.

Another reason fiddler crabs are a suitable model species to further our understanding of the cognitive aspects of invertebrate behavior is their posture during foraging and mating trips. Biomechanically, fiddler crab’s fastest mode of locomotion is a sideways run that uses the legs on the side of the body facing the direction of travel to pull on the substrate, and the opposing legs to push. Since fiddler crab’s legs and muscles make them relatively slow to rotate their bodies or move at angles oblique to their long axis. They therefore forage with one side of their body pointing toward their burrow at all times, permitting a rapid escape from any threat (Layne
et al., 2003b). This behavior demonstrates that foraging fiddler crabs are aware of their homing direction. Additionally fiddler crabs rotate themselves while moving radially relative to their burrow, providing evidence that fiddler crabs are aware of the distance between themselves and “home”. This adjustment to the angle of rotation that varies with distance between a fiddler crab and its’ burrow has been repeatedly verified in several quantitative studies (Land & Layne, 1995; Layne et al., 2003a, 2003b; Zeil, 1998).

A notable exception to the tendency of fiddler crabs to keep their body aligned with their burrow suggests an even more complex cognitive representation of space than a solitary home vector, and may suggest something akin to a spatial “map”. When there is an obstacle, such as a mangrove tree, between a foraging crab and its burrow, the fiddler crab realigns itself to align its body toward a navigable edge of the obstacle. This suggests that central mechanisms in the crab’s brain respond to the presence obstacles, to adjust their alignment to a direct run around the obstacle should the need for escape present itself (Layne et al., 2003b). This would entail a more complex representation of space than a single home vector. One possibility is the storage in memory of chains of a series of at least two vectors, one to the proximal target (the edge of the obstacle, and another to the final goal location. The fiddler crabs’ maintained body alignment during navigation behavior allows for a constant behavioral “read out” of the animals sense of direction toward home at all times while the animal navigates. (Layne et al., 2003a, 2003b).

In summary, recent findings from studies of insect cognition have revealed a surprising amount of cognitive flexibility in these animals traditionally thought to possess behavioral repertoires that were built exclusively from instincts (Gould, 1982). The domain of spatial cognition provides an lens through which one can examine the cognitive abilities of invertebrate animals. Here we can ask questions regarding where these animals are cognitively flexible, and
where they are behaving according to genetically programmed instincts. Additionally, in order to understand the phylogenetic history of both behavioral (possibly cognitive) flexibility and instinctual spatial strategies in invertebrates, there is a need for lab studies of invertebrate animals outside of the well-studied groups Hymenoptera and Diptera. The Decapod crustacean taxa of fiddler crabs provide an suitable model species to fill this role, as they are skilled navigators in the wild, owing to their ecological need to accurately navigate the between their burrows and environmental resources. Fiddler crabs also have the unique characteristic of navigating in such a way as to continuous indicating their internal sense of where “home” is through their behavior. These factors make fiddler crabs an interesting and potentially fruitful species to compare to the eusocial navigating insects.

The vast majority of previous spatial learning work with fiddler crabs has occurred in the field, in the natural ecology of these animals. These studies have demonstrated a great deal of what typical, naturalistic fiddler crab behaviors is composed of, but does not allow us to draw conclusions about what these animals are capable of. Field studies of fiddler crab behavior have benefited from the fact that these animals naturally maintain a home burrow, which they leave in a predictable manner (at the onset of low tide) and have a limited range (on the order of a few meters), which is a beneficial dynamic to the study of navigation in these animals. This has resulted in many experiments where fiddler crabs are first marked for individual identification, followed over the course of a foraging or mating excursion, and then typically manipulated in some way. These manipulations have varied from a simple translocation experiment (moving the animal before its homeward journey, to interfere with its egocentric frame of reference) (Cannicci, Fratini, & Vannini, 1999), adding and manipulating visual cues at the animals home burrow (T. W. Kim & Christy, 2015; T. W. Kim & Choe, 2010), to inflating balloons buried
under the sand to increase the overall distance the animal needs to travel to get home (Walls & Layne, 2009b). These field experiments have been highly informative, and have produced a body of knowledge concerning the navigation strategies of fiddler crabs. However, due to the constraints of field research methodology, these studies are limited, and in my opinion, have led to pre-mature conclusions in the literature regarding the central information processing mechanism fiddler crabs during navigation. Specifically, studying navigating fiddler crabs within their natural environment can potentially bias conclusions about fiddler crabs cognitive flexibility in that fiddler crabs may simply rely on non-plastic egocentric mechanisms unless they are experimentally forced to rely on other more complex mechanisms.

The study of fiddler crab spatial learning in a laboratory setting provides the typical benefits of experimental control. They are trackable via automated video observation, due to the consistency of their body shape when viewed from above, making it relatively easy to design spatial conditioning chambers and tracking software for these animals, in which reinforcement can be automatically delivered, contingent on the crab’s location.

There is also a question about our view as a field on the general spatial abilities of fiddler crabs. As discussed above, fiddler crabs are thought to be largely egocentric in their navigation strategy. It is only in the last six years that any evidence of allocentric navigation in these animals has begun to emerge (T. W. Kim & Christy, 2015; T. W. K. Kim & Choe, 2010). This view however, may be founded on biased field methodology, in that almost all studies of these animals spatial learning has focused on one specific task, homing. We as a field know very little about the generality of fiddler crab spatial abilities outside of this context. The establishment of a set of procedures to allow the study of the generality of the spatial abilities these animals (and other decapod crustaceans), for the use of researchers to search for the importance of multiple
sources of sensory information (egocentric, visual, chemosensory, magnetic) in terrestrial invertebrates’ spatial abilities.

It has been well established in many field studies that fiddler crabs are capable of homing to their burrows, and that they accomplish this by primarily relying on egocentric stride integration (step counts which factor in stride length) in order to establish a path integration home vector (Cannicci et al., 1999; Layne et al., 2003a, 2003b, Walls & Layne, 2009a, 2009b; Zeil, 1998). The laboratory procedures I employed were designed to aid in the continuing study of the spatial abilities of fiddler crabs in a way that is inaccessible to field research because I could completely control the stimuli and contingencies that the freely navigating fiddler crabs encountered. Through these methods the general question I address in this dissertation is if the spatial learning abilities of fiddler crabs are specific to egocentrically informed homing, or if it extends to a more general spatial navigation system that is sensitive to allocentric modalities of spatial information. A related question is if fiddler crabs create a cognitive representation of space at all outside of the context of homing, or if they simply rely on hierarchically organized instinctual behavioral modules in order to accomplish non-homing related spatial tasks they encounter.

In order to address the above questions, I designed a set of spatial place learning tasks that use electric shock as an aversive stimulus. The literature is largely silent on examples of successful appetitive conditioning with crustaceans. It is generally understood that this is a product of the “file drawer problem”, in that when experimenters are unsuccessful in demonstrating significant effects, reports of these failures are rarely published. Another reason I chose to work within an aversive paradigm because there is evidence in the literature that fiddler crabs may rely on allocentric spatial cues more when they are in situations that require non-
navigation related movements. Researchers found that when male fiddler crabs perform ritualistic mating “dances” during trips away from their burrow, they are more likely to approach visual stimuli that had previously been pair with their burrows location, as opposed to the burrow itself (T. W. K. Kim & Choe, 2010). They believe that this is due to non-navigational movements introducing error into the path integration system, and that fiddler crabs are sensitive to this increase in error. When exposed to electric shock, I have found that fiddler crabs tend to quickly flee, and undergo body “spasms” during the time period of the shock. It is my theory that if fiddler crabs are capable of attending to visual stimuli to solve a spatial problem, they are more likely to do so after experiencing these spasms, as they are likely to also introduce error into their egocentric representations of space.

This dissertation also includes an experiment that employs an appetitive paradigm, using access to an artificial burrow as an US as opposed to electric shock. This experiment addresses an alternative, although complementary hypothesis to the one outlined above. It is possible that in the lab setting, when placed into a spatial arena only during training and testing periods, fiddler crabs do not “reset” their path integration system, and therefor do not have access to an egocentric home vector. While we know that rats attend to the starting position at which they are placed into experimental mazes, there is currently no evidence that suggest fiddler crabs do the same, except when they are occupying their home burrow. If this is the case, it is possible that in any maze in which a fiddler crab does not reside in on a semi-permanent basis, their path integration system might contain a high amount of error, leading them to attend to allocentric cues, regardless of the averseness or appetiveness of the US being used.

The reason I have chosen to focus on visual stimuli to use as conditioned stimuli is twofold. Firstly, to my knowledge there is only one demonstration of visually guided navigation
in fiddler crabs (discussed above). It was a field study, with relatively little control over the visual experience of the animals. Under controlled laboratory conditions it is possible to demonstrate that these animals do not simply ignore the plethora of visual information available to them in the wild, by exposing them to spatial tasks that are only solvable using experimenter selected visual information. Secondly, this dissertation tests the hypothesis that non-navigation related movements, or the lack of a home burrow at which the path integration system can be “reset”, increases the fiddler crabs reliance on visual information. This finding represents an important first step towards discovering if fiddler crabs are capable of “optimal” Bayesian multi-modal cue integration of spatial information, and therefore a sensitivity to the error of navigational systems is a prerequisite for this type of cognitive cue integration (Ken Cheng et al., 2007).

As stated above, another goal of this dissertation was to attempt to detect cue integration phenomena in navigating fiddler crabs. If fiddler crabs are capable of combining multiple modes of sensory information in order to learn about space, then the generality question demands that we ask: to what extent and when do fiddler crabs rely on one information source? Do they combine information from two different modalities (integration), or do they use each modality for distinct tasks (modular)? The paradigms employed in this dissertation are suited to address this question. They allow for spatial information from different modalities to be placed into conflict (after training), and detect changes in the animal’s navigation behavior in response to these changes.

By measuring the way in which an animal responds to a spatial manipulation that occurs after subjects have learned to complete a spatial task, one can discriminate between three behavioral strategies the animal may be employing: 1. The animal may respond to only
allocentric cues, or the animal may respond to only egocentric information, 2. The animal may show signs of responding to both stimuli by performing an intermediate behavior and 3. The animal might fail to successfully navigate. Strategy 1 would indicate that these animals preferentially rely on only one source of information (modularity). Strategy 2 would suggest that animals are combining information from both of these sources, and one could begin to estimate how they weight these sources, based on how often they visit the egocentric arm and the allocentric arm. Strategy 3 could indicate that subjects construct a gestalt-like representation of space, and are incapable of dealing with the individual atomic aspects of this representation changing without further training. This type of cue manipulation during testing makes a spatial paradigm ideal to examine fiddler crabs for evidence of integration of sensory information.

The remainder of this dissertation will report on four separate experiments. Each of these experiments are designed to provide evidence of visually guided navigation in fiddler crabs, outside of the context of homing, and without providing subjects with a home burrow within the experimental apparatus. The denial of a home burrow hypothesized to prevent subjects from generating a “zero point” for their path integration system, making it more likely for subjects to rely on allocentric cues consistent with the theories of both Kim and Choe and Cheng et. al. (Ken Cheng et al., 2007; T. W. K. Kim & Choe, 2010). Experiments 1 and 3 were carried out using a passive avoidance paradigm, while experiment 2 employed a conditioned place preference paradigm, all using electric shock as a US that was contingent only on allocentric visual stimuli. Experiment 4 employed a Y-maze paradigm, and allowed subject both visual cues, and egocentric cues that could be learned about according to a response learning strategy, to determine if fiddler crabs rely on non-path integration based egocentric cues. Taken together, the subsequent experiments will provide evidence for: 1) an unlearned taxis to approach certain
visual stimuli in fiddler crabs, 2) an associative mechanism by which fiddler crabs learn to avoid certain visual cues, 3) an interaction of these two navigational modalities and finally, 4) the inability of fiddler crabs to use egocentric information outside of the homing context. These findings and their meaning are discussed in detail in chapter 6.
Chapter 2: Passive Avoidance Learning in Fiddler Crabs

It has been well established that fiddler crabs in the wild are capable of egocentrically informed navigation to their home burrow via path integration (Cannicci et al., 1999; Layne et al., 2003a, 2003b; Walls & Layne, 2009a; Zeil, 1998). As discussed in previous chapters, fiddler crabs leave their home burrow in order to forage and mate daily during low tide. When a threat is perceived by fiddler crabs, they are capable of returning along a direct path to their home burrow; this is contrary to the expectation that the animals might memorize their outward path and on return re-enact it in reverse order, (i.e., a route reversal strategy). It has been generally accepted that this is ability relies on a step counting strategy, which includes the combination of counts of steps and their lengths in order to determine the distance the animal has traveled (Walls & Layne, 2009a). In addition the crab’s statocyst system (vestibular system) is involved in sensing the angle relative to gravity in which the animal is moving (Walls & Layne, 2009b). It is also thought by researchers that a time compensated sun compass is used by fiddler crab for orientation information, as these animals are capable of perceiving polarized light, and maintaining their orientation relative to rotated patterns of polarization (Herrnkind, 1968; How et al., 2012; Meyer-Rochow, 2001).

This path integration ability has been argued to be a relatively hardwired servo mechanism, specifically evolved to allow crabs to avoid predation to quickly return to their home burrow, and not to be part of a general spatial learning ability that is sensitive to non-egocentric place information, such as visual landmarks (Cannicci et al., 1999). However, some recent findings call this idea into question. In 2010, Kim and Choe reported evidence that suggests that under certain conditions, navigating fiddler crabs use local visual landmarks in order to compensate for error introduced into their path integration system by courtship related
movements (T. W. K. Kim & Choe, 2010). Additionally, the closely related but distinct taxa of shore crabs have been demonstrated to be capable of learning to avoid an artificial burrow that has been paired with electric shock, when labeled with a black and white, checkered, visual stimulus (Magee & Elwood, 2013). These findings suggest the interesting possibility that fiddler crabs may be more plastic and versatile in their learning and spatial abilities than previously thought, and may in fact be capable of generalized spatial learning.

The present study aims to begin to address this possibility of plastic navigation behavior in fiddler crabs, by determining if they are capable of successfully learning an association between a visual conditioned stimulus (CS) and an unconditioned stimulus (US), as a passive avoidance behavior. A simple apparatus can be used to test for the development of a conditioned passive avoidance behavior. A shuttle-box like alley, with distinct visual stimuli displayed on either side of the alley provide allocentric cues. The US is realized by exposing subjects to electric shock on one randomly assigned side of the alley. No previous study, to my knowledge, has provided evidence for the presence of passive avoidance behavior learning in fiddler crabs.

In the present study, an aversive paradigm was employed because the literature (T. W. K. Kim & Choe, 2010) suggests aversive stimuli cause fiddler crabs to make non-navigation related movements, which Kim and Choe suggest encourages fiddler crabs to attend to and learn about visual stimuli. To realize this aim the present study presented subject crabs with 3, 3-hour training trials in which they were free to move anywhere in the experimental alley with the condition that they were given a 10-volt electric shock every 10 seconds during the period of a trial during which they remained in the “shock paired” half of the alley. An extinction test was given on the fourth day, in which subjects were returned to the alley, and again allowed to freely explore, in the absence of shock. Avoidance of the “shock” side during this extinction test
provides evidence that fiddler crabs are capable of learning to avoid the CS that had been paired with shock, and were not simply escaping from the shock itself during the 3 days of training. This experimental design improves on ambiguous results reported in the literature using a similar paradigm to study learning in crustaceans (Bhimani & Huber, 2015).

Due to the operant nature of this task (subjects are free to enter and exit the shock side of the alley during all trials), individuals subjects’ exposure to shock is determined by that subject’s own behavior and is not under experimenter control. To account for this, I chose to employ a yoked-pairs experimental design. One member of each pair was then chosen to serve as the “Master” animal, who is exposed to electric shock only on one half of the alley, and the other member of the pair served as a “Yoked” animal, who is exposed to electric shock only when their master animal was. This results in the administration of shock being correlated with the position of the animal within the box only for master animals, while allowing us to control for potential effects of overall exposure to shock across different master group animals. I matched the pairs of yoked animals according to the size of their carapace, in order to match both the distance electric current needed to travel within the subjects’ body, and the subjects’ ability to move about the alley.

The design of this experiment allows for several potential insights into the learning abilities of fiddler crabs. If subjects do show avoidance of the side of the alley that was previously paired with shock during the day 4 extinction test, it will be the first time that fiddler crabs have been shown to be capable of passive avoidance learning. This potential discovery of allocentric avoidance learning in fiddler crabs would suggest and justify a plethora of follow-up studies of how and when allocentric avoidance learning operates in these animals. A positive result would also suggest studies concerning how representations of allocentric cues may (or
may not) interact with, integrate, or override egocentrically informed place learning in these animals. This line of research that has proven to be very fruitful in both mammals and insects (Ken Cheng et al., 2007), and the addition of crustacean results would provide comparative contrast and depth to our understanding of spatial learning generally.

A second potential benefit from this experiment is the validation of electric shock as an effective punisher in these animals. To my knowledge electric shock has only been used as a successful US in crustaceans twice (Carcinus maenas and Orconectes rusticus), and never with a fiddler crab of any species (Bhimani & Huber, 2015; Magee & Elwood, 2013). Several researchers have found that fiddler crabs respond aversively (by fleeing) from “looming” stimuli, which are visual stimuli intended to simulate the approach of a predator from above (Ebie, 2012; Hemmi & Zeil, 2003; How et al., 2012; Smolka, Zeil, & Hemmi, 2011). However, fiddler crabs quickly habituate to repeated presentations of looming stimuli, limiting their utility in the context of learning experiments (Smolka et al., 2011; Zeil & Hemmi, 2006). Electrical shock could have a different habituation time course which would also open up new avenues of research. Additionally, because looming stimuli are presented externally to the crab (unlike electric shock), experimenters have much less control of the subjects’ perception of a looming stimulus in an operant situation where the animal is freely behaving. These two factors make electric shock a potentially useful US in crustacean learning studies. Previous attempts to use electric shock as a US with crabs have attempted to wrap electrical leads around the legs of animals, as opposed to implanting electrodes directly into the bodies of animals. The wrapping approach has two drawbacks, that implanting electrodes directly into animals may overcome. Wrapping wire around the legs of animals has the potential to limit the freedom of movement of those animals’
joints. It also has the liability of additionally, producing autonomy in subject (the spontaneous casting off of an appendage) when shock is applied directly to a leg.

A separate and equally important goal of this experiment is the development of automated procedures to study the spatial learning and navigatory behaviors of crustaceans. Crustaceans, being hard-shelled animals, are an excellent subject for automated video tracking technologies, as their body-form when viewed from above is largely invariant. This simplifies the algorithms needed to track their position within a confined experimental arena, and makes automated video track algorithms more accurate. Additionally, the electrode implantation procedure developed for this experiment, which allows reliable and automatic delivery of USs, makes it computationally simple to develop software that automatically delivers USs to subjects, dependent on their location, with any arbitrary contingency the experiment requires. It is my hope (and the hope of others in this field (Bhimani & Huber, 2015)), that this technology will allow for widespread, inexpensive, and efficient experimental examination of the sensory cues and learning strategies employed by many species of crustaceans as they explore space.

This study manipulates three independent variables. The between groups factor “contingency”, comprised of the “master” group, whose experience of shock is completely correlated with their position within the experimental alley, and the “yoked” group, whose experience of shock is independent of their position within the alley. The second independent variable in this study is the within subject factor “trial”, which consists of 4 levels, the three days of training, and the subsequent extinction test. The series trials was be included as a third within subject factor. The dependent variables in this study are the number of seconds each subject spends on the side of the alley that is assigned to the shock condition for each given master
subject, the amount of total movement in centimeters carried out by each subject, and the average speed of movement of each subject, during all four days of the experiment.

The hypotheses of this experiment are as follows. The “Avoidance Hypothesis” is that subjects in the master group will spend less time in the shock paired side of the alley than subjects in the yoked group, during all 3 days of training, and the fourth day extinction test. This would produce a main effect of the contingency variable, and would indicate that subject in the master condition escaped the electric shock successfully during training trials, and continued to avoid the visual stimuli on that side of the alley during the extinction test, indicating that they have associated the visual stimuli available to them (CS) with the experience of electric shock US, and had developed a conditioned avoidance. A second hypothesis that is mutually exclusive to the avoidance hypothesis is the “Escape Hypothesis” where subjects in the master group may instead spend less time on the side of the box paired with electric shock than yoked subjects, but only during the 3 days of training, and not on the fourth day when shock is not present. This would produce an interaction effect between the contingency and trial variables, and would indicate that Master group subjects successfully escaped the application of shock, when shock was present, but did not associate the presence of shock with the available visual stimuli, and had instead simply responded to the shock itself during training trials. This hypothesis being confirmed would indicate that while subjects did not develop a conditioned avoidance, they did experience the electric shock as an aversive stimulus and responded accordingly, validating electric shock as a useful US for future studies of learning in these animals.

Finally, I hypothesized that subjects in the master condition will show a decrease in time spent in the shock paired side of the alley over the course of each training session, while yoked subjects will not, which I refer to as the “Within Session Avoidance Hypothesis”. This
interaction effect between the contingency and block variables would indicate that subjects whose experience of shock was spatially correlated learned to avoid the shock side of the box indicating that subjects in the master condition had developed a conditioned avoidance.
However, if the block and contingency variables were found to also interact with the trial variable, that may again (as discussed above) indicate that master subjects were only escaping the application of shock when shock was present, and had not developed a conditioned avoidance behavior, although in this situation electric shock would again be confirmed as a useful US for future studies (the “Within Session Escape Hypothesis”).

Methods

Subjects

48 Male fiddler crabs of the species *Uca pugilator* were obtained from Carolina Biological Supply. Only male subjects were used because male fiddler crabs leave their burrows for both mating and foraging trips, unlike females who remain at their burrow during mating encounters. This makes male fiddler crabs more likely to be more reliant on visual cues, as previous research suggests that after mating trips egocentric information may be less accurate (T. W. K. Kim & Choe, 2010). Subjects were housed in individual plastic tubs (13in x 8 in x 4 in) each of which contained an artificial beach of aquarium gravel and sand. Each housing tub also contained 1000 ml of brackish artificial sea water (ASW) at a salinity of 23 parts per thousand (ppt), which was constantly aerated by air-stone bubblers. Every two weeks the water in each subjects tub was changed, and the gravel was rinsed in fresh water. Subjects were fed 1 pellet of fiddler crab food (also obtained from Carolina Biological Supply) every other day.

Electrodes
Electrodes were constructed of 20 gauge stainless steel solid core PVC insulated wire. Each electrode was ¾ of an inch long. The end that was implanted in subjects was stripped to expose 1/8 of an inch of wire, and was then inserted through and attached to a ¼ inch diameter plastic disc using cyanoacrylate (A.K.A. “SuperGlue”). This disc aided in the insertion and attachment of these electrodes; they maintained a constant angle of the electrode relative to the crab’s carapace, and limited its depth of penetration. These discs also provided additional surface area to adhere to subjects’ carapace. The non-insertion end of each electrode was stripped on a length of ¼ inch, in order to allow attachment of the electrode to the output of the shock generator.

Electrode implantation

Subjects were held in individual housing for at least one week after being transported to the lab from the supplier. This assured that they were not suffering negative health effects due to the shipping process.

20 minutes before the electrodes were implanted, subjects were placed in a clean plastic bin filled with 2 cm of 23 ppt salinity artificial sea water. 500 ml of crushed ice was added to these bins once every 5 minutes during this 20 minute period, in order to anesthetize subjects. During and prior to this period all electrodes and tools were cleaned with acetone and allowed to air dry before being applied to subjects.

Once anesthetized, animals were restrained using a 4mm plastic strap, and a 1mm in diameter section of carapace above the gills of the animal was slowly removed using a Dremel rotary tool, until that area of carapace was thin enough to puncture with an electrode. This process was repeated on both sides of the animal symmetrically around the midline. The
mounting disc of each electrode was then coated in cyanoacrylate and inserted through this thin section, and held in place for approximately 10 seconds until it was securely glued. Subjects were then allowed to recover while the adhesive set for 20 minutes in dry housing tubs. Brackish water and an air-stone bubbler was then returned to these tubs. Subjects were then maintained in individual housing for at least 7 days post-surgery before being included in the experiment to verify that they had recovered from the implantation. The state of the electrode attachment was monitored during this period and animals with insecure electrodes were not used placed in trials. 28 of the original 48 animals met this criteria and were included as subjects in this study.

Place learning arena

Trials were conducted within a sound-proof room that was sealed during all trials in order to prevent auditory and visual extra-maze cues from reaching animals within the arena. The arena that subjects were trained and tested in was constructed from ¼ inch acrylic panels, and assembled using cyanoacrylate. The testing apparatus consists of 4 individual alleys, which are 18 inches long by 5.5 inches wide, with walls that are 8 inches high. Each alley is completely isolated from the others, allowing 4 subjects to be trained or tested simultaneously. The walls of the arena were painted white. Paper visual stimuli were attached to these walls using cyanoacrylate (See figure 2.1). The floor of each alley was covered in a ½ inch thick layer of clean, white sand to enhance the contrast of dark animals moving atop, and provide animals with traction for walking (Fiddler crabs slip on acrylic surfaces). A series of 4 dual lead wires (1 per alley) were fixed to a mounting rack above the arena. Each lead terminated in a small alligator clip, which was attached to the magnet wire leads coming from each subject, in order to deliver shocks. 2 video cameras (Sony CCD model #CB8440) were mounted above the arena, pointed directly down with a field of view that completely spanned two of the training/test alleys of a
pair of yoked animals. The output lines from these cameras and the 4 shock lines all exited the sound-proof room in which the experiments occur, and terminate at the trial control area (~20 feet away). A 60w incandescent light was attached to the center of the above arena mounting rack and, pointed upwards to completely illuminate the arena with diffuse light, and prevent any shadows from appearing within the arena.

Electric shock

The unconditioned stimulus, electric shock, was generated by 2 Grass S88 dual channel stimulator units, providing a total of 4 output lines. The output channel of each stimulator is attached to the “shock lines”, which enter the experimental booth and terminate above each experimental alley in alligator clips. These alligator clips are then attached to twisted pair magnet wires (30 gauge), which are in turn attached to the electrodes mounted into each subject. These thin gauge magnet wires allowed current to be passed to subjects without impeding their ability to move about the arena. Shocks were delivered on a 50/50 duty cycle oscillating at 180 Hz for 200 ms with an amplitude of 10 volts. These values reproduce those of Magee & Elwood (2013) who successfully obtained responses to shocks applied to freely moving shore crabs. These parameters were observationally verified to ensure that they reliably produced a visible behavioral response in animals in our own lab.

Trial Monitoring and Control

The video feeds from both cameras were fed into a computer located outside the sound proof room. They were processed by custom code implemented in Matlab 2013. Images were retrieved from this video stream once every 2 seconds (.5 fps). Images were processed by first converting them to a binary image, in which only the dark subject was visible. A Gaussian blur
filter was then applied to these binary images, and the maximum value of the blurred fiddler crab was used as a measure of the animal’s position within the alley. Whenever the animal was on the side of the arena designated as the “shock side”, the Matlab code would close an electronic relay (KMTronic 2-channel USB relay), which in turn was connected to the shock generator. Whenever this relay was closed, the shock generator would output the 200 ms shock (as described above), once every 10 seconds, according to the internal clock of the shock generator. Because the internal clock of the shock generators is constantly running, the first shock an animal received could occur at any time between 0-10 seconds after the animal had entered the shock side. When an animal exited the shock side, Matlab opened the electronic relay, stopping the shock generator from producing further output. All video processing occurred in real time, and after each trial Matlab would save the animal’s positions in their alleys in x, y coordinates. The status of both shock generators, and the threshold “x” value for the shock and non-shock side, were recorded as time series for every frame of the trial.

Experimental design

In order to control for possible effects of electric shock exposure, I employed a yoked pairs’ experimental design. Subjects were paired according to carapace size, and one subject from each pair was randomly chosen to serve as the “master” subject, and the other member of the pair was assigned to the “yoked” condition. Each pair of subjects was placed in the experimental arena for 3 hours, for 4 consecutive days. At the start of each trial, subjects were placed in the center their assigned alley, and allowed a 5 minute acclimation period before the trial began. After this acclimation period, the Matlab code that monitored each trial would deliver shock to the master subject whenever it occupied the side of the alley designated as the “shock side”. The shock side in each trial was randomly assigned and counterbalanced across
subjects. The yoked subject received shocks whenever the master animal received shocks. This resulted in the yoked animals receiving an equal amount number of shocks, which were not correlated with spatial position of the yoked animals within their alley.

Trials began at noon each day for each pair of animals. Days 1-3 were training trials, in which shock was present for the full 3 hours of the trial. On day 4 an extinction test was conducted. This test consisted of animals again being placed in the experimental alley and connected to the shock generator, with shock generator turned off. The independent variables were the group subjects were assigned to (master and yoked), the day of the trial (1, 2, 3, and 4).

Analysis

For analysis purposes, each 3 hour trial was divided into 20 minute blocks (9 per trial). The primary dependent variables were the amount of time subjects spent on the side of the alley paired with shock, the amount of distance traveled by the subject, and the speed at which the subject moved.

In order to determine the amount of time subjects spent on the shock and non-shock side of their alley, the x coordinate of each subject’s position over the course of each trial was recorded. All frames in which this coordinate was on the side of the alley that had been paired with shock were then counted. These values were then summed within each block of each daily trial.

The distance traveled within the alley by each subject was calculated by computing the distance between each x and y coordinate of each subject for each consecutive pair of frames. These values were then converted from units of pixels to units of cm, by measuring in pixels the
overall length of the alley floor, the actual length of which was known in cm. These values were then summed within each block of each daily trial.

The speed at which subjects moved between each frame of the experiment was calculated using the x and y coordinates of the subjects’ position during each frame. These speed measurements were then separated into those that occurred when shocks were and weren’t being delivered. An average speed measurement was then calculated of each subject, on each day, during shock periods and no shock periods.

All three of these dependent variables discussed above were then analyzed using mixed model three way ANOVAs, with the within subject factors “day” and “block” and the between subject factor “treatment” (master vs yoked groups).

Results

Time spent on the side of the alley paired with shock

This analysis revealed no main effect of the day of the experiment on the subjects occupancy of the “shock” side of the arena (F(3,60)=2.089 p=.111). There was a significant effect of condition, with master animals spending much less time overall on the shock side of the alley (F(1,20)=38.689 p < .001). This main effect however is best understood in light of the significant interaction effect between condition and the day of the trial (F(3,60)=4.416 p=.007). This interaction was produced by the main effect of condition declining on the fourth day of the trial, the day of the extinction test (Figure 2.2).

The block variable showed no main effect (F(8,160)=.910 p=.510), no interaction with condition (F(8,160)=.266 p=.976), and no interaction with day of the experiment (F(24,480)=1.012 p=.448). Additionally, there was no three way interaction between day block and
condition ($F(24, 480) = .587, p = .942$). This lack of significant effects of block indicated that subjects’ behavior did not change within each trial (see table 2.1 for full report of all statistics).

To determine if the time span of a block was simply too large to capture any behavioral changes, the first 20 minute block of each was then broken into 1 minute blocks. This analysis demonstrated that even in the early minutes of each day, master group subjects did spend significantly less time on the side paired with shock than the yoked group ($F(1, 26) = 6.522, p = .017$). However, this analysis also revealed that during the first several minutes of each day subjects in the master group did spend more time on the side of the arena that was paired with shock than they did later in the first block. Although this analysis did not produce any significant effects (main effect of 1 minute blocks $F(19, 494) = .220, p = .977$, blocks by treatment interaction $F(19, 494) = 1.078, p = .370$) (see figure 2.3), this could be interpreted as a fast learning curve, with animals learning to avoid the shock side of the alley within the first 5 minutes of trial 1. However, this effect does not persist across any days, including the 4<sup>th</sup> day extinction test, therefore there is insufficient evidence to conclude that this is a learning effect (days $F(3, 78) = .5, p = .684$, days by treatment $F(3, 78) = 2.164, p = .099$, days by 1 min block interaction $F(57, 1482) = .912, p = .661$, days by 1 min blocks by treatment interaction $F(57, 1482) = 1.196, p = .154$) (see table 2.2 for full report of all statistics). Nevertheless this possibility is worth entertaining as our analysis is not necessarily sensitive enough to detect learning that occurred in less than one minute.

**Distance traveled**

The significant effects revealed by this analysis were a main effect of the day of training ($F(3, 60) = 3.232, p = .029$), and a day by 20 minute block interaction ($F(24, 480) = 3.358, p < .001$) (see table 2.3 for full report of all statistics). Averaged across treatment groups, subjects traveled
progressively less distance within the arena during each of the three days of training, until the fourth day, when shock was no longer administered and both groups of animals traveled within the arena more (see figure 2.4). Additionally, on days 1-3 subjects tended to travel within the arena more as the trial progressed, but showed the opposite pattern on day 4, traveling the arena proportionally less during the later blocks of the trial (see figure 2.5).

**Speed of Movement**

During periods when subjects were being presented with shock they moved at a faster speed, as indicated by a significant effect of shock state on average speed (F(1,26)=16.484, p<.001). There was also a significant main effect of day of the experiment on movement speed, with subjects moving faster on days 1-3 of the experiment, than they did on day 4 when shock was not presented (F(3,78)=3.173, p=.029).

The two main effects discussed above are best understood in the context of the significant interaction effect between the day of the experiment and the shock state (F(3,78)=7.759, p<.001). Subjects in both the master and yoked groups moved at a higher speed when shock was being administered, except on day 4 when no shock was administered (note: since no shocks were delivered on day 4 the shock state variable for that day instead indicates when shock would have been delivered had that been a training trial, i.e., when the master subject was on the side of the box that had been paired with shock). This result indicates that subjects were increasing their speed due to shock, and that this behavior was not persisting in the absence of shock (see table 2.4 for full report of all statistics).
Discussion

Overall these results do not support the hypothesis that fiddler crabs are able to associate visual cues with aversive shock stimuli. While there were significant between group effects that indicated that fiddler crabs can successfully escape electric shock, the lack of persistence into the extinction test indicates that this escape response did not map onto the visual stimuli to produce conditioned avoidance. The data from the first twenty minutes of the first training trial show evidence of what could be considered a learning acquisition curve (although this interpretation is not statistically supported), but this result can more parsimoniously be explained via a simple instinctual taxis, without invoking more complicated learning systems. Subjects may have been following a simple movement taxis along the lines of “move faster after experiencing shock”. Assuming that subjects’ initial choice of movement direction is random, the “acquisition” curve seen in figure 2.3 may have simply been the result of animals in the master group moving in the “wrong” direction several times before exiting the shock side of the arena. This sort of behavior would produce the between groups effects we observed, as subjects in the master group would only be moving at a higher speed on the side of the arena paired with shock, while yoked subjects would have these periods of increased speed distributed across both sides of the arena. This post hoc hypothesis is supported by the results of our analysis of our subjects’ movement speeds, as both groups showed significantly faster movement during bouts of shock. Taken together these results support the idea that fiddler crabs are using an automatic, hardwired kinesis to escape shock, but are not (in this particular paradigm) learning to avoid the visual cues that had been paired with shock.

The results discussed above also support the conclusion that electric shock delivered via implanted electrodes does serve as an effective US in fiddler crabs. An aversive unconditioned
stimulus is defined as a stimulus that produces withdrawal, escape or avoidance behavior in an organism without previous experience with that stimulus. Subjects in the present experiment showed escape behaviors when subjected to electric shock within the first few minutes of their first trial, which argues strongly that they were able to sense the electric shock, and that the application of electric shock caused them to attempt to escape subsequent shocks (figure 2.3). Additionally, subjects showed increased average speed during periods of shock administration, indicating that they were altering their behavior in the presence of shock. These two results allow us to conclude that electric shock is a perceivable and aversive US to fiddler crabs, and that these animals did not need to learn that this stimulus is negative. This has been demonstrated before in other crustaceans (Bhimani & Huber, 2015; Magee & Elwood, 2013), but this is the first demonstration in fiddler crabs.

This experiment validated this method of automatic tracking of the animals’ position within the arena and automatic, computer controlled delivery of electric shock. Although no evidence of association between electric shock and visual stimulus was revealed, the fact that subjects in the master group displayed spatially correlated behavior during training trials (avoiding the side of the arena paired with shock), and yoked animals did not, indicates both that the delivery of shock in both groups was appropriately controlled via our trial control computer, and was spatially correlated for master group animals, but not for yoked group animals. This kind of automatic trial monitoring and stimulus delivery is an inexpensive and efficient method for exposing crustacean animals to experimenter controlled, spatial contingent stimuli, that may greatly increase the productivity of investigators interested in the learning abilities of the animals.
Had this experiment not included an extinction test, where subjects were returned to the experimental arena after training and allowed to explore without any administration of shock, it would have likely concluded, as Bhimani and Huber did, that these subjects had learned to avoid the available stimuli within the arena. Especially within spatial learning contexts, extinction tests are required to demonstrate that any associative processes is involved in observed phenomena. However, even if the subjects in this experiment had persisted in avoiding the side of the alley paired with shock during the extinction test, that would only indicate that they had associated some sensory aspect of the conditioning environment with electric shock, not necessarily the visual stimuli that we provided. These animals have a very different umvelt from our own, and we must exercise caution when assuming that the cues that are obvious to us humans (i.e. the visual stimuli we provided these animals) are the most salient cues available to the animal. For example fiddler crabs are capable of highly accurate polarized light perception (How et al., 2012). In order to demonstrate that subjects in an operant place conditioning paradigm have formed an association with a specific subset of stimuli, and not with some form of uncontrolled extra maze cue, a rotation test is required. In this paradigm a rotation test would consist of switching the placement of the visual cues along the horizontal axis before an extinction test, producing a mirrored version of the training environment. This would put the explicit experimenter-controlled cues in direct competition with uncontrolled extra maze cues. If subjects persisted to avoid the stimuli that had previously been paired with shock after this rotation, then the argument that they had associated those specific cues with shock would have direct support.

The fact that this experiment did not produce evidence of spatial learning abilities in fiddler crabs does not allow us to directly argue that these abilities do not exist. The instinctual kinesis proposed above is not mutually exclusive with associative spatial learning, and could in
fact support an associative spatial learning mechanism, in that the “move more” rule may acquire through associative learning a new releaser stimulus other than electric shock. There are several potential reasons that this experiment may have not uncovered evidence for such a learning system, even if it is present. First, subjects in this experiment may not have been able to discriminate the visual stimuli on either end of the alley from one another. The specific visual stimuli used in this experiment were chosen because they are equated in overall intensity, and we hoped to avoid complicated counterbalance effects that could have been produced by fiddler crabs’ tendency to move toward darker areas. This however may have made the visual discrimination too difficult for our subjects, hindering their ability to learn about these stimuli. In an attempt to address this possibility, current work in our laboratory has uncovered visual stimuli that fiddler crabs can in fact discriminate from one another, which we plan on using in future spatial learning studies of these animals. This increases the importance and utility of the master/yoked control used in this study, as it allows us to account for instinctual preferences for particular visual stimuli. A second potential reason that animals failed to show a learning effect in this experiment is that our retention interval (24 hours between the final training trial and the extinction test) may have been too long for the memory of our subjects. This possibility is also being addressed by further work in our lab, which is attempting to both train and test animals within this paradigm in a single day.
Chapter 3 – Pavlovian Place Preference Learning in Fiddler Crabs

This experiment is an attempt to demonstrate visually-informed place preference learning in fiddler crabs using a Pavlovian paradigm. The motivations behind this experiment are similar to those discussed in Experiment 1. A demonstration of visually informed spatial learning in fiddler crabs would allow for the study of the interaction of visual and egocentric information in fiddler crab navigation, and allow us to examine the possibility that multi-modal cue integration in animals learning about space is an evolutionarily “old” phenomena. As discussed in earlier chapters, the presence of similar “Bayesian” multimodal integration phenomena in a wide range of species such as ants, pigeons, and humans (Ken Cheng et al., 2007) suggests that either this phenomena is a product of shared inheritance amongst these wide ranging taxa, or a surprisingly repeated product of convergent evolution. Crustaceans are a reasonable “next step backward” in light of the animals in which multi modal integration in spatial tasks have already been demonstrated. Establishing that fiddler crabs attend to and learn about sensory information, outside of egocentric homing contexts, is a first step towards this goal. A positive finding on this point would indicate that the presence of multi-modal cue integration in diverse taxa is likely a product of shared inheritance, and not of convergent evolution.

Another motivation for this experiment, again very similar to the motivations of experiment 1, is to establish lab-based paradigms to study place preference and general spatial learning abilities of crustacean animals. As many researchers of animal learning report, specific methodological issues, and the parameters of the situation and contingencies that animals are presented with, can greatly influence the degree to which animals display learning in their measurable behavior. Although experiment 1 did not produce any evidence of CS-US association that does not allow one to argue that these animals are incapable of creating any such
associations. This experiment is aimed at addressing several such methodological issues that may have prevented experiment 1 from providing evidence for visually informed place preference learning in fiddler crabs.

One potential issue that may have prevented the detection of learning in experiment 1 is the operant nature of the paradigm employed in experiment 1. Operant paradigms by definition give subjects direct behavioral control over their own rate of stimulus - response – shock exposure. This may have caused relatively high levels of across-subject variability in Experiment 1, as some animals may have received very few shocks (and therefore few learning opportunities) while other subjects may have received many shocks. In turn, this variable rate of reinforcement across subjects may have produced variable amounts of conditioned avoidance, which the analysis of experiment 1 might not have been sensitive enough to detect. Experiment 1 controlled for this possibility by using a yoked groups design, but it is still possible that some subjects developed a place preference, that was hidden by subjects that had developed a weak or no preference. Additionally, the fact that a significant escape effect was detected on days 1, 2, and 3, implies that the analysis of Experiment 1 did have enough power to overcome subject variability, but it is possible that subjects who did not experience many shocks during the training trials (which would reinforce the escape effect on days 1, 2, and 3), randomly entered the shock side on day 4, to which these subjects had not had sufficient opportunity to learn to avoid.

This experiment addresses the potential problem described above by shifting to a Pavlovian place preference learning paradigm. In this experiment, subjects are not free to travel between both sides of the experimental alley during training, and are instead isolated for a set period of time on one side of the alley and then another, and either exposed to shock or not,
depending on the assignment of that given side of the alley for that subject. Subjects receive several cycles between being isolated on the “shock” side of the alley and the “non-shock” side of the alley each day of training. This results in all subjects receiving an equal number of shocks, over an equal amount of time, removing any potential variability issues in our dependent measurements due to differential numbers of CS-US exposures.

Additionally, the lack of evidence of visually informed place preference learning in Experiment 1 may have been due to fiddler crabs being unable to discriminate between the visual stimuli provided. Experiment 1 employed intensity equated visual stimuli (a black circle, black cross, and black rectangles, all of equivalent area) in order to avoid preference effects of animals preferring to be near darker stimuli. Although there is behavioral evidence in the literature indicating that fiddler crabs can discriminate between simple shapes (Langdon & Herrnkind, 1985), it is possible that subjects in Experiment 1 were not able to discriminate the presented visual stimuli, and were therefore unable to learn to avoid the side signaling the response-shock contingency. To address this possibility, the present experiment uses visual stimuli that were designed to differ from one another in as many perceptual dimensions as possible. The stimuli used were total black coverage on the walls of one half of the alley, and a checkerboard pattern of 1” by 1” black squares on a white background. These two stimuli differ in their overall intensity, degree of contrast, and spatial frequency. This is intended to make these stimuli maximally discriminable to the fiddler crab visual system.

Subjects in Experiment 1 were presented with three, three-hour training trials, but because of the operant nature of Experiment 1 subjects were capable of fleeing the side of the alley in which shocks were administered, and were therefore unlikely to experience continuous bouts of shock for an entire session. Because subjects in the present experiment are isolated on
one side of the box at a time, they are unable to escape presentations of shock. To avoid damaging subjects, Experiment 2 limited the total amount of time per day that animals spent on the shock side of the box to 40 minutes (at 1 shock every 30 seconds, resulting in 80 shocks per day). In order to roughly equate the total amount of training between subjects in Experiment 1 and 2, Experiment 2 included 6 (as opposed to 3) total days of training.

The final potential feature of Experiment 1 that may have prevented the detection of a conditioned place preference that Experiment 2 is designed to address is possibility that the day 4 test in Experiment one may have occurred too long after subjects’ final training session. The Experiment 1 retention interval of 24 hours, although seemingly ecologically reasonable, may have allowed for too much retroactive interference to develop in subjects’ memory. Subjects were returned to their home tanks during the retention interval, which may have caused a loss of any avoidance learning during the three days of training. There is currently no behavioral data in the literature to my knowledge that demonstrates across day retention of learned associations in these animals. To address this possibility, Experiment 2 includes daily probe tests of subjects’ place preferences, which take place after each day of training. These probe tests are described in detail in the methods section below, but to briefly summarize, they consisted of each subject being placed on the center line of the alley without barriers in place, and exposed to a single electric shock. The direction in which the subject fled after this shock exposure, toward the “safe” side of the alley, or the side paired with shock, was used as a measure of place preference. This allows for the detection of potential learned CS-US associations during each day of training, before the opportunity for retroactive interference of the memory trace had the opportunity to occur (These probe tests may have inadvertently paired the shock with both sides of the arena, but these probe exposures were comparatively short relative to the training interval). Subjects
were also tested 24 hours after the final training session with an open alley test, in which they were free to explore and occupy either side of the box for 3 hours, identical to the extinction test employed in Experiment 1.

The present experiment includes two independent variables, the within subject factor of trial sequence, and the between subject factor of stimulus. Trial sequence has six levels, each corresponding to one of the six days of training subjects’ experience. Stimulus is a counterbalance variable with two levels comprised of randomly assigned groups of subjects who have shock paired with the checkerboard side of the alley, and randomly assigned groups of subjects who have the black side of the alley paired with shock. Two dependent variables are included in this study, direction of fleeing during the daily probe trials (movement toward the “safe” side or shock paired side of the alley after being exposed to a single electric shock), and alley occupancy during the day 7 extinction test (where subjects are free to explore the arena for 3 hours 24 hours after their final training session). A yoked condition was not included in this study, as all subjects received an equal number of CS-US pairings.

I hypothesize that there will be an increase in the proportion of probe trials in which subjects flee towards the side of the alley paired with safety across days of training. This would produce a significant effect of trial sequence, which would indicate that subjects had formed and association between the visual stimuli and electric shock. If subjects are capable of retaining this CS-US association for the 24 hour retention interval before the day 7 extinction test, I would also hypothesize that there will also be a significant difference in side occupancy during the extinction test. This would take the form of subjects spending significantly more time on the side of the alley paired with safety, and would indicate that they had formed and retained a conditioned place preference.
I also hypothesized that there would not be an effect of the between-groups stimulus variable, in either the daily probe tests or during the day 7 extinction test. However if the stimulus counterbalance variable did produce a significant result, it would indicate that subjects had a preference for one of the two visual stimuli, which is a possibility raised in previous studies that suggests that fiddler crabs prefer to approach certain shapes, and may prefer to occupy areas near darker stimuli (Herrnkind, 1968; Langdon & Herrnkind, 1985). Although demonstrating a stimulus preference is not an explicit goal of this study, it would indicate that subjects were capable of discriminating between the visual stimuli that are employed in this study, which would address one of the potential limitations of Experiment 1.

Finally, an alternative possible hypothesis to those discussed above is that the stimulus variable may produce a significant interaction effect with the trial sequence variable in the daily probe test data. This would indicate that subjects were able to learn to move towards the side of the alley paired with safety, but only when that side of the arena was paired with one of our two visual stimuli. This could result if subjects do have a strong preference for one of the visual stimuli, and this preference can only be strengthened or weakened by pairings of that stimuli with shock, but not overridden completely. This result would be of great interest, as it would indicate that fiddler crabs modify a (likely instinctual) taxis that dictates “move toward preferred stimulus”, through experience with an aversive stimulus. This would imply, in turn, that subjects modify the expression of taxis-based navigational information with learned CS-US associations. The detection of this kind of multi-modal integration is one of the overall long term goals of this dissertation.
Methods

Subjects

20 male fiddler crabs were purchased from Carolina Biological Supply. Electrode implantation and animal housing were identical to Experiment 1. 8 of the subjects used in this experiment were not naive, as they were also used in Experiment 1, with a minimum of 1 month between the completion of their experiment 1 trials and the beginning of their Experiment 2 trials. In total 20 subjects were used, although 2 passed away before completing all 7 days of the experiment.

Place learning arena

The arena used was the same as described above, with two important modifications. The visual stimuli were changed to stimuli that were designed to be salient to fiddler crabs. One half of each alley was covered in a checkerboard pattern that consisted of alternating white and black squares, with sides of 1 inch. The other half of each alley was covered in flat black. These two stimuli differed in both overall intensity and spatial frequency, two perceptual factors that previous research has indicated fiddler crabs are sensitive to (Langdon & Herrnkind, 1985). Of the four alleys within the testing arena, two were had the checkerboard placed on the “south” side of the alley, and two had the checkerboard placed at the “north” end of the alley. The second modification was that Plexiglas dividers were constructed that could be placed in the center of each alley, in order to isolate animals on the “checkered” side or “black” side. The surfaces of these dividers were marked with the stimuli corresponding to the stimuli on the side of the alley that each surface was facing (see figure 3.1).

Experimental Procedure
One side of each subjects training alley was randomly assigned to be their “shock side” (SS), and the other side was considered their “Non-Shock side” (NS). The transparent divider was placed in the center of the alley each day before training began, in order to prevent animals from leaving their SS or NS area. When placed on the SS, animals were exposed to a 200 ms, 10V shock (same as described in experiment 1) every 30 seconds for 10 minutes, for a total of 20 shocks per exposure. This was followed by a 1 minute rest period outside of the arena, during which animals were individually isolated inside of a closed holding area. This was then followed by a 10 minute exposure to the NS side, during which animals were not exposed to shock. Subjects were then again placed in isolation for one minute. This training cycle of SS – isolation – NS – isolation was repeated 4 times consecutively each day, for 6 days.

*Experimental testing - Probe trials*

At the end of each day of training 4 probe trials were conducted on each subject in order to detect the development of any preference to the visual stimuli. Each probe trial consisted of subjects being placed in the center of the experimental alley with the center divider removed, allowing subjects access to both halves of the alley. Immediately after being placed in the arena, one 200ms shock was administered, and the direction of the subjects first movement was recorded. Each probe trial lasted for 60 seconds, with a 75 second rest period in between each probe, during which subjects were placed in isolation. These probe trials were also filmed from above, in order to allow more detailed analysis of subjects’ responses.

*Experimental testing - Choice test*

On the 7th day of the experiment, after subjects had received 6 days of training and probe tests, subjects were administered a free choice test. This test consisted of subjects being placed
into the experimental alley, with the center dividing wall removed. They were then allowed to freely explore the alley, in the absence of shock, to determine if they had developed a place preference that was dependent on the visual stimuli. Subjects were attached to the shock terminals during these trials, as they were during training, in order to avoid any context effects of carrying the shock lines, but shock was never administered. Choice trials lasted for 3 hours, and were filmed from above. The subjects’ position within the alley was determined using the same Matlab based tracking methods described above in experiment 1.

**Results**

*Subject Mortality*

Three subjects died during the course of the experiment, before completing all 6 days of training and the extinction test on day 7. Data from trials with these three subjects were removed from all analyses.

*Daily Probe Tests*

After each of the six days of training, each subject was presented with 4 probe tests. The behavior of subjects during each of these probe tests was scored as “moved toward side paired with shock”, “moved toward side not paired with shock”, or “did not move”. “Movement” to either direction was considered moving at least one body length away from the subjects starting position on the center line of the alley.

Subjects moved towards one side of the alley or the other on 64.86% of probe trials. Trials in which the subject did not move were removed from this analysis, as these trials do not indicate any behavioral choice on the part of the animal. For each day of training, all probe trials in which the subject moved toward the side of the arena that had been paired with safety were
divided by the total number of probe trials in which that subject moved at all on that day. This resulted in a proportion for each subject and each day of training that indicates the amount of that subject's total movements in response to shock that were oriented towards the side of the arena paired with safety.

These proportions were then subjected to a mixed model ANOVA to determine if there was any effect of the 6 days of training on the subjects’ tendency to move toward the side of the arena not previously paired with shock. The model included both the within subject factor “Day of training” and the between subject factor “stimulus”, intended to examine any counterbalance effects due to shock having been paired with the checkerboard or black side of the alley.

I found no significant effect of day of training, indicating that subjects did not increasingly moved towards the side of the alley that had not been paired with shock (F(5,30)=0.718 p=.615). I did however find a significant main effect of stimulus (F(1,6)=13.824 p=0.01). Subjects who had shock paired with the checkerboard side of the arena, moved toward the safety paired side of the alley in less than 50% of probe trials, while subjects who had shock paired with the black side of the alley moved toward the safety paired side of the alley in more than 50% of probe trials (See figure 3.2). I also found no significant interaction between these two variables, indicating that there was no effect of the day of training that varied systematically between the two counterbalance conditions (F(5,30)=1.04 p=0.413).

In order to examine the significant effect of stimulus discussed above, I calculated for each animal on each day of training the proportion of probe trials in which the animal moved, where those movements were directed towards the side of the arena marked with the checkerboard stimulus. I then calculated a single sample t-test for each day of training, comparing the average proportions of movement towards the checkerboard stimulus across
animals to a hypothesized population mean of .5 (a null hypothesis of random movement). On
days 1, 2, and 6 of training, subjects’ showed a significant preference toward moving toward the
checkerboard side of the arena (t(14) = 2.71 p=0.017, t(13)=2.535 p=0.025, t(12)=4.292
p=0.001, respectively). On days 3, 4, and 5, subjects movements did not significantly differ from
the null hypothesis of random movement, but on all of these days subjects still moved toward the
checkerboard stimulus more often than not (t(13)=1.953 p=0.073, t(14) = 1.653 p=0.121,
t(12)=0.739 p=0.474, respectively). These results are plotted in figure 3.3 and reported in table
3.1.

Day 7 – 3 hour extinction test

Subjects’ movements during extinction testing on day 7 was measured as Cartesian
coordinates. These coordinates were then grouped according to which side of the midline of the
arena they were positioned. Out of 2700 frames (.25 frames per second, for 3 hours), subjects
spent an average of 1165 frames on the side of the alley that had been previously paired with
shock. We employed a single sample t test to compare this value to a population value of 1350
frames, which would represent random movement within the arena, and found no significant
difference (t(13)=−0.573 p=.577), although subjects tended toward spending more time on the
side of the arena that had been previously paired with shock. Data for each individual subjects
can be seen in figure 3.4.

We also examined subjects’ positions within the arena to determine if they were spending
more time on the side of the alley that was marked with the checkerboard pattern, regardless of
that sides training history. Out of 2700 frames (.25 fps for 3 hours), subjects spent an average of
1769 frames on the side of the alley marked with checkerboard patterning. We employed a single
sample t test to compare this value to a population value of 1350 frames, which would represent
no preference for either side of the alley, and found no significant difference (t(13)=1.369 p=.194), although subjects tended toward spending more time on the side of the arena that was marked with the checkerboard stimulus. Data for each individual subject can be seen in figure 3.5.

It is possible that subjects may have displayed a learned avoidance of the side of the arena paired with shock during the beginning of the three hour extinction test, which the overall measure was too temporally coarse to detect. In order to address this possibility I analyzed only the first ten minutes of the day 7 extinction test separately. This analysis was performed by blocking the number of frames subjects spent on the side of the arena paired with safety into 10 one minute blocks, each consisting of 15 frames. This data was then subjected to a two way mixed-model ANOVA, with the within subject factor “minute”, which had 10 levels, and the between subject factor “stimulus”, which consisted of two levels, subjects that had safety paired with the black side of the arena, and subjects that had safety paired with the checkerboard side of the arena. This analysis did not detect any significant effects of block or stimulus (F(9,108)=0.494 p=0.875 and F(1,108)=0.759 p=0.401, respectively). This analysis also did not find any significant interaction of these two factors (F(9,108)=0.705 p=0.703). These results can be seen in figure 3.6.

**Discussion**

The overall hypothesis of this study, that fiddler crabs would develop a conditioned place preference for the side of the arena that had not been paired with shock during training, was not supported. Results from both the daily probe tests and the day 7 extinction test failed to provide any evidence of a learned association between the visual CS and the electric shock US.
The secondary hypothesis, that fiddler crabs would not show a preference for either of the counterbalanced visual stimuli was directly disconfirmed. The significant effects of stimuli found in the daily probe tests (discussed above) indicate that subjects in both counterbalance conditions moved more often towards the checkerboard stimulus, regardless of the training history of that stimulus. This effect was significant for 3 out of 6 days of training, and was marginally significant, or at least in the right direction, for all days of the daily probe test. The day 7 extinction data confirmed this result, in that subjects did spend more of their time during the 3 hour test on the checkerboard side of the alley, regardless of the training history of that side of the alley for that subject, although this effect was not significant.

The lack of evidence of learning provided by this experiment, is not evidence that these animals are not capable of developing a conditioned place preference. There are several parameters of the experimental design that may have prevented subjects from displaying behavior that would provide evidence of learning. Taken together the results of experiment 1 and 2 form a body of evidence that one might use to argue that these animals do not attend to visual cues in order to avoid aversive stimuli, but there are still several possible specific parameters that would need to be explicitly manipulated in future studies in order to strengthen this argument. For example, electric shock was delivered every 30 seconds in regular intervals in this experiment, and it is possible, that subjects may have learned to expect shock based on this timing cue, and that this learning may have overshadowed or blocked any potential learning about the CS-US contingency. It is also possible that subjects simply did not have sufficient time in training to develop a CS-US association, and that given longer training sessions, or more days of training, they would have developed a conditioned place preference. This is by no means an exhaustive list of the potential factors that may have prevented the detection of learning in this
experiment, as issues such as the inter-stimulus interval, US duration, US intensity, or opportunities to consolidate learning (to name just a few) may have also played a role in this experiments lack of significant leaning related results. This is unfortunately one of the defining characteristics of learning experiments, as the presence of a learning ability is a statistically simple thing to demonstrate, whereas the inability to learn a specific category of contingency is very difficult, if not impossible, to demonstrate to a logical certainty, as you must attempt to “argue from the null hypothesis”.

One of the goals of this experiment was to address the limitation of experiment 1 that subjects may not have been able to discriminate between the visual stimuli used in experiment 1. The significant effect of the counterbalanced visual stimuli produced by this experiment confirms that fiddler crabs can discriminate between the checkerboard pattern and black stimuli. Fiddler crabs in both counterbalance conditions showed a preference for moving toward the checkerboard stimulus. In order for animals to be able to show this preference, they must have been able to discriminate between the stimuli, as the physical location of these stimuli was also counterbalanced across animals. This result does not speak to fiddler crabs’ ability to discriminate between the intensity equated stimuli in experiment 1, but it does demonstrate that the lack of a result that would indicate learning across trials in the present experiment is not due to an inability to discriminate the visual CSs. This form of preference is not ideal for visual stimuli that are intended to be used in learning experiments, as it requires that all future experiments using these stimuli be completely counterbalanced, but it does provide us with stimuli that we know these animals can discriminate, making the formation of association between these visual stimuli and a suitable US possible.
Strategies of invertebrate navigation are often thought of as taxis. A taxis is a navigation strategy where animals move toward a specific target stimuli, in order to maximize their perception of that target stimulus (Ken Cheng, 2006; Ken Cheng et al., 2007). The preference for the checkerboard stimulus discovered in this experiment could indicate that fiddler crabs have an instinctual taxis that dictates that when presented with a uniform stimulus (in this experiment the black stimulus), and a more visually complex stimulus (the checkerboard stimulus), move toward the more complex visual target. It is possible that this taxis may have overwhelmed evidence of potential CS-US associations in the present study, if this possibly innate bias was simply too “strong” to allow potential learning effects to be detected in a sample of this size. From a less methodological point of view, this taxis is of interest in the context of this dissertation for two reasons.

Firstly, a taxis of this form would make sense in the context of fiddler crab ecology. Fiddler crabs’ primary mode of avoiding predation is to flee to either their home burrow, or to physical structures that provide safety, such as mangrove trees and rocks (Smolka et al., 2011). The more complex checkerboard stimulus may be perceived as more likely to provide spaces into which a fiddler crabs body could fit, and therefore provide the animal with shelter. It is particularly interesting to me that fiddler crabs are showing this preference in the context of an aversive learning study, as the application of electric shock may activate the same navigational systems used by fiddler crabs to avoid other aversive stimuli, such as the presence of a predator. Previous research does suggest that fiddler crabs may follow different navigational schemas depending on the situational context, such as relying on visual information only when their path integration system is error prone (T. W. K. Kim & Choe, 2010). The possibility that fiddler crabs
rely on this taxis only in aversive situations is one that could be directly tested in future experiments.

Secondly, an interest that has been gaining popularity amongst those that study animal navigation, is how animals use multiple, and especially reconcile, conflicting sources of navigational information (Ken Cheng et al., 2007). This taxis could be thought of as a Bayesian prior in the context of fiddler crab navigation, in that either through previous experience or genetic inheritance, these animals seem to have a predisposition toward moving to checkerboard stimuli. This hypothetical taxis could be experimentally put into opposition with other sources of navigational, or place identifying, information in exploring fiddler crabs in order to attempt to detect and describe potential conflicts between this “prior” and other current sources of information. This kind of cue competition or cue integration study has been successfully performed in many species from ants to humans (Ken Cheng et al., 2007; Giard & Peronnet, 2006; Sakata, Yamamori, & Sakurai, 2004; Wessnitzer & Webb, 2006), and it would be of great interest to determine what strategies the evolutionarily distant fiddler crab is employing in situations where multiple types of navigational information are conflicting with one another. To put it simply, the taxis that the results of this experiment point to is a real feature of fiddler crab navigation, can this taxis be modified via learning processes, or is it an inflexible, hardwired behavior?

The tendency of fiddler crabs to move toward the checkerboard stimulus in this experiment certainly indicates that fiddler crabs have a preference for this specific stimulus. What the results of this experiment cannot provide direct evidence for is the specific stimulus properties that fiddler crabs are motivated to approach. However, examining the stimuli used in this experiment can provide some insight. The visual stimuli covered all of the walls of the
experimental alley completely, and were the same width and height. We can therefore conclude that the overall three dimensional structure of the stimuli was not the stimulus element that informed these animals’ preference. In fact the two stimuli (black and checkerboard) only differed along 3 perceptual dimensions: their overall average intensity, the amount of contrast in the image, and their spatial frequency. Based on the results of the present study, overall intensity seems an unlikely feature to be motivating this preference, as fiddler crabs tend to prefer darker areas when moving, and the checkboard stimulus has a brighter overall intensity than the black stimulus. Contrast and spatial frequency both could be the relevant stimulus element(s) that fiddler crabs are attending to, as they both correlate, in the fiddler crab ecology, with areas into which fiddler crabs could hide from a predator. Spatial frequency in this case seems like a particularly likely candidate, as the checkerboard pattern used in this experiment was 1 inch by 1 inch, which is the approximate size of a fiddler crab carapace, meaning that in the wild, locations with this spatial frequency would offer crabs ideal hiding locations. The relative convolution of these stimulus elements to the approach preference seen in this experiment is an issue that could be discerned in future studies using the same automatic animal tracking protocols described in this dissertation.

Overall this experiment did not provide evidence of fiddler crabs being capable of forming a conditioned place preference informed by the pairings of visual conditioned stimuli and electric shock. However as discussed above, the lack of evidence does not provide evidence of a lack of ability. This experiment did provide evidence of a stimulus specific navigational mechanism in fiddler crabs, which I have identified as a behavioral taxis, in the form of a taxsis that dictates “move toward checkerboard stimulus”. The discovery of this taxis provides direction for future research to address several questions such as: Is this mechanism context
specific (only displayed in aversive conditions)? How can researchers use this apparent preference to our advantage in designing future learning experiments? What are the stimulus features that underpin this mechanism (logically, such a mechanism did not evolve specifically for 1 inch by 1 inch checkerboard patterns), and most interesting to me personally, is this taxis plastic, meaning can it be modified through experience? This final question is one of multi modal integration of navigational information, which is the type of phenomena now known to exist in animals as diverse as ant, pigeons, and humans, which inspired this dissertation project.
Chapter 4 – Passive Avoidance Learning in Fiddler Crabs II

This experiment attempts to capitalize on the conclusions drawn in experiments 1 and 2 in order to modify the Operant avoidance learning paradigm employed by Experiment 1, in an effort to detect visually-informed conditioned avoidance learning in navigating fiddler crabs. This experiment employed the same apparatus as experiment 1, a shuttle box like alley that contained visual stimuli, and the same video processing and stimulus delivery software that automatically locates subjects within the alley and delivers electric shock contingent upon their location. The primary differences between this experiment and Experiment 1 are the visual stimuli that are presented to the subjects, the inter-stimulus interval between each presentation of the shock US, the length of training trials, and the retention interval between training and the first extinction test.

Experiment 1 demonstrated that fiddler crabs escape the application of electric shock by moving away from the area of the experimental alley where the shock was delivered, as indicated by the significant difference between master and yoked group subjects in that study. Experiment 1 did not however provide any evidence of an acquired avoidance learning contingent on the visual stimuli provided. There are two categories of explanations for experiment 1’s lack of learning effects. One possibility is that fiddler crabs are simply incapable of learning a passive avoidance task with visual cues and electric shock as the punisher. The other category of possibilities is that there was some methodological issue with the design of experiment 1 that prevented subjects from acquiring, or behaviorally displaying such learning. This second category of possibilities is what this experiment is designed to address.

The visual CSs used in experiment 1 were designed to be equal in overall visual intensity. I believed a priori, based on personal observations of fiddler crab behavior, that subjects would
show a preference to approach stimuli that were darker. This choice of dark-light discrimination pair may have resulted in subjects being unable to visually discriminate the visual stimuli provided in experiment one from one another. Experiment 2 attempted to address this issue by using a checkerboard and a black visual stimulus, which were designed to be maximally discriminable by varying in form, overall intensity and spatial frequency. While experiment 2 did not provide evidence of learning in a Pavlovian task, it did demonstrate a significant preference for fiddler crabs to approach a checkerboard visual stimulus when opposed with a plain one and the corollary that these stimuli are discriminable by the fiddler crab visual system. The present experiment employed these same visual stimuli, to determine whether fiddler crabs are capable of associating checkerboard or black visual patterns with electric shock under an Operant learning paradigm. Additionally, in order to increase the discriminability of these visual stimuli, the current experiment reduced subjects’ line of sight to the visual stimuli on the opposite side of the testing alley.

A further adjustment involved the timing of shock delivery. In both experiments 1 and 2 timing of the shock was consistent across presentations whenever subjects occupied the shock paired side of the experimental alley (every 10 seconds in experiment 1, and every 30 seconds in experiment 2). The regularity of the inter-stimulus interval (ISI) might have provided a timing cue to subjects that they could use to predict when subsequent shocks would be delivered. If subjects did in fact learn this timing cue in experiments 1 and 2, this learning may have overshadowed any potential associative learning between the visual stimuli and shock, resulting in the lack of learning effects seen in those experiments, as is the case in shock training with rats (Williams, Frame, & LoLordo, 1992). In order to address this potential issue, the present
experiments randomizes the ISI between presentations of the shock using a uniform random
distribution between 1 and 10, with a mean ISI of 5 seconds.

Another potential limitation of experiments 1 that the present study is designed to address
is the possibility that the 24 hour period between each training session and the extinction test
used in that experiments may have simply been too long for fiddler crabs to retain any learned
associations. If fiddler crabs are capable of learning a passive avoidance task, it is likely that
such a learning system evolved in conjunction with their general predation avoidance
mechanisms. Crabs have previously been shown to be very reactive to visual cues of predation,
but to quickly habituate to repeated presentations of predation cues (Lozada, Romano, &
Maldonado, 1990; Smolka et al., 2011; Zeil & Hemmi, 2006). However, habituation to predation
cues has only been described in within session experiments with these animals, and no evidence
of any long lasting, across session habituation has ever been demonstrated in fiddler crabs (to my
knowledge). It is reasonable that if an avoidance learning ability exists within fiddler crabs, that
it too may be a relatively short term learning system, if crabs use this system only to avoid
predation, as seems to be the case with the fiddler crab habituation mechanisms. To account for
this possibility, the present experiment includes all training trials on a single day, separated by
half hour periods during which subjects are returned to their individual housing units. The last
trial was followed by an extinction test, in order to detect avoidance learning in the same day as
training. Additionally, to examine the duration of any formed association the present experiment
includes an additional subsequent extinction test 24 hours after training, to address the possibility
that avoidance learning may still be detectable after 24 hours.

The dependent variable of this study was the time each subject spent on the shock side of
the alley during each trial. This variable was shown in experiment 1 to provide a good measure
of escape of electric shock as it produced large effect sizes during training trials \((\eta_p^2 = .9)\), and therefore should provide a robust measure of any potential learned associations between the visual stimulus and the response-shock contingency in this experiment.

This experiment manipulated three independent variables: Visual stimulus, Treatment, and Trial. The “visual stimulus” is a manipulation of which visual stimulus (checkered or plain black) is paired with the electric shock US. The design is counterbalanced so that the levels “black” or “checkerboard”, are “safe” for half of the animals and paired with shock for the other half.

The second between groups independent variable “treatment” consisted of the levels “master” and “yoked”, in the same form as used in experiment 1. Subjects in the master condition received the electric shock contingent on their spatial position within the experimental alley, while subjects in the yoked condition are shocked whenever their sized-matched master animal is shocked, regardless of the yoked animals’ spatial location within the alley.

The third independent variable used in this study is the within subjects factor of “trials”. This variable has 5 levels, corresponding to the 5 one hour trials that each subject experiences within the experimental alley. The first three levels of this variable are training trials, in which subjects were exposed to electric shock according to their assigned groups on the stimulus and treatment variables, and the final two levels of this variable were the same day and next day extinction tests, in which subjects were not be presented with electric shock.

The primary hypothesis of this experiment is that subjects in the master condition spend less time in the side of the alley paired with shock (and therefore receive less shocks) than subjects in the yoked condition. This hypothesis would be confirmed by a significant main effect
of the master/yoked variable which would indicate that subjects in the master group learned to avoid the shock paired side of the alley. A critical test was whether this association is retained through the day 2 extinction test (trial 5), otherwise a treatment effect could be interpreted as simple escape from shock, but not avoidance learning.

The presence of a significant interaction between treatment and trial could suggest several potential interpretations related to the hypothesis that fiddler crabs are capable of forming allocentrically informed conditioned avoidance learning. It is highly likely, based on the results of experiment 1, that subjects in the master group will show an escape effect, spending less time than yoked animals on the shock paired side of the alley during the three training trials. Depending on which trial (3 4 or 5) any differences between master group and yoked group subjects persisted to, the interpretation of a treatment by trial interaction would change. Master/yoked differences persisting to only the final training trial, would indicate that subjects had not developed a learned avoidance behavior. If Master/yoked differences persisted only to the 4th trial, it would indicate that subjects had learned to associate the visual stimuli and the US creating a conditioned avoidance response, but that this preference either could not be maintained for the 24 hour retention interval before trial 5, or that this preference had been extinguished during the first extinction test (trial 4). To distinguish between a significant trial by treatment interaction that does or does not indicate learning, the analysis of the design of this experiment included a set of orthogonal a priori contrasts that compare the performance of master and yoked subjects, separated by stimulus condition, on levels 4 and 5 of the trial variable.

The sensory bias for checkerboard patterns found in experiments 1 and 2 needed to be accommodated in this experimental design. Were this experiment to replicate this stimulus
preference effect it could complicate the interpretation of potential main effects. Such a preference could result in a main effect of stimulus, such that subjects who have checkerboard paired with shock spend more time on the shock side of the alley than do subjects who have black paired with shock. Additionally, if the preference were strong enough and the training trials insufficient in number this preference could result in a stimulus by condition interaction, in that yoked subjects may be the only ones to show this preference effects, as the spatial contingency of shock in master subjects may hide this effect. A preference for checkerboard may also result in a stimulus by trial interaction, as subjects in the black stimulus condition may avoid the shock side of the arena across trials 4 and 5 not due to a formed association, but instead due to simple stimulus preference. This experiment include planned a priori contrasts of master/yoked subjects’ performance during the extinction trials separately for each level of stimulus. Additionally, because of the yoked pairs experimental design of this study, the critical comparisons to detect conditioned avoidance should be immune from potential confounds due to inherent preferences to approach checkered stimuli.

In summary, this experiment is a modified replication of experiment 1, aimed at addressing several potential issues that may have prevented the formation or detection of successful avoidance learning in experiment 1, namely: 1) The possibility that fiddler crabs are incapable of maintaining such learning over 24 hours. 2) The possibility that consistent timing between shock deliveries may have overshadowed learning about the visual cues. 3) The possibility that fiddler crabs were unable to discriminate between the visual stimuli used in experiment 1.
Methods

Subjects

134 total animals were obtained from the Carolina biological supply company (Burlington, NC). As with previous experiments, the crabs were then maintained in the laboratory for 1 week following delivery to ensure that they were not suffering ill effects due to the shipping process. Animals were maintained in individual tubs containing sand, brackish water, and an artificial beach, identical to the housings discussed in all previous experiments. All 134 animals were implanted with electrodes following the protocol described in experiment 1. 81 of these animals survived the 1 week post-implantation criteria to be eligible for inclusion in this study. Among these 81 successfully implanted animals, the average survival time post implantation was 64.9 days, with a standard deviation of 52.0 days. 48 animals that survived the electrode implantation process in good condition (all 8 legs intact) post-surgery were randomly selected to be used as subjects in this experiment. As with all previously discussed experiments, only male animals were used.

Place learning arena

The same place learning arena described in experiment 1 was used in this experiment. The arena consisted of two pairs of identical alleys in which four individual subjects could be trained at the same time (two master/yoked pairs). Each isolated alley was 18 inches long by 5.5 inches wide, with walls that were 8 inches high. The alterations that were made to these alleys for the purposes of this experiment were: 1) 1 inch occluding walls were added at the center line of each alley. These walls were intended to limits subjects’ line of sight to the opposite side of the alley, to limit their exposure to the alternate visual stimulus when they were not on that side.
of the alley and 2) The visual stimuli used in this experiment were the 1 inch by 1 inch checkerboard pattern and solid black stimuli demonstrated to be effective in that experiment 2 and 3) the patterned visual stimuli covered the entirety of the walls of the experimental alleys and were not limited to just the terminal ends. This included the central occluding walls. See figure 4.1 for a schematic diagram of a single alley of the place learning arena.

The place learning arena was lit with four, 50 watt lamps from above, and filmed with 2 cameras (Sony CCD model #CB8440) from above. Both the lamps and camera were positioned directly over the dividing wall between the first and second, and third and fourth alleys, resulting in each camera being responsible for monitoring 2 of the 4 alleys in the arena. The cameras and lights above each pair of alleys were placed symmetrically relative to the center line of the alleys, in order to prevent these devices from providing animals with extra maze cues that could be used to discriminate either side of the alley from one another.

Electric shock

The punisher used in this experiment, electric shock, was again generated by 2 Grass S88 dual channel stimulator units, providing a total of 4 output lines (providing a separate output channel for each alley of the place learning arena). Shocks were again delivered as a 50/50 duty cycle oscillating at 180 Hz for 200 ms per shock. Each stimulator unit’s two output channels were yoked to one another, in order to deliver shocks simultaneously to each pair of “master/yoked” subjects.

The amplitude of the shock (10 volts in experiment 1) was varied across subjects, in order to attempt to achieve an equal amount of current across animals when shock was delivered. The resistance of each subject was measured in the week between their surgeries and their inclusion
in the experiment, by attaching their electrodes to a Radio Shack digital multi meter (model 22-812) with computer digital logging for offline analysis. The resistance between the electrodes of each subject was then sampled once per second for an hour, in order to calculate an average and variance of resistance value for each subject. From this resistance estimate the voltage to be applied to that subject during their training sessions was then calculated to produce an average 50 micro amps of current. This resulted in variable voltage between subjects but kept the average current constant. An average resistance across all subjects of 314.9 Kohms was observed, with a standard deviation of 217.68 Kohms. The highest observed average resistance for a single animal was 978 Kohms, and the lowest was 32 Kohms. These average resistance values results in a minimum applied voltage of 1.6 volts and a maximum of 48.9 volts. As each subjects measured resistance was variable across time, each shock may have deviated from the desired 50 micro amp target value. The average within subject standard deviation of resistance was 209.4 Kohms, and the average applied voltage was 15.7, resulting in a 95% confidence interval of average delivered current of 50 +/- 151.26 micro amps. The resistance measurements were positively skewed, resulting in this confidence interval that inaccurately implies the possibility of negative current values.

As noted above the crabs in experiment 1 might have been cued by the regular timing of shock delivery from the Grass stimulator. In order to address this possibility in experiment 2, the timing of each electric shock was randomized with a uniform probability distribution that varied between a 1 second ISI and a maximum of a 10 second ISI, and a mean ISI of 5 seconds.

Trial monitoring and stimulus control

As was the case in experiment 1, the video feeds from the video cameras were fed into a computer in a separate room running custom object tracking code implemented in Matlab 2016b.
This software would sample a frame from both video feeds once every second. The software would then located the fiddler crab within each alley, and record the subjects’ positions in pairs of x y coordinates (image analysis described in full in the experiment 1 methods section). The software would then determine if the master subject was on the “shock side” (SS) or “non-shock side” (NS) of their alley. When subjects were detected on the SS of the alley, Matlab generated a random integer between 1 and 10 as the delay to shock delivery. When the delay interval elapsed the program would trigger the grass stimulator (via a 2 channel relay (KMTronic 2-channel USB relay) to deliver a shock to the pair (yoked and master) of subjects.

Procedure

All training sessions began at 10 am, in order to avoid effects of circadian and ultradian rhythms known to exist in crustacean (Aréchiga, Fernández-Quiróz, Fernández de Miguel, & Rodríguez-Sosa, 1993). Subjects electrodes were attached to the mobile magnet wires (which were in turn connected to the stimulator units), 30 minutes before the start of their first training trial. This was accomplished by stripping the insulation from a 1 cm length of the end of the magnet wire, wrapping this end around the hooked tip of each of the subject’s electrodes, and crimping the electrode to tightly hold the magnet wire in place. A resistance measurement was then taken from each animal with a multi-meter, in order to ensure that there was a reliable electrical connection across the internal ends of each subjects’ electrodes. Subjects were then returned to their home tubs for 30 minutes. During this period each pair of magnet wires were attached to leads emanating from the stimulator units, suspended above the middle of testing arena, via pairs of alligator clips. The final portion of the shock delivery lines that connected to the electrodes implanted in subjects were constructed of lightweight, flexible magnet wires,
which applied very little weight to subjects, allowing them unfettered access to all points within the alley, and avoiding any side biases that may have occurred due to shock line attachment.

After the 30 minute holding period the first training trial was begun. Each of the four subjects being trained that day were then placed by hand in the center of their assigned alley, with their mouth parts facing the “north” long wall of the alley. The researcher then sealed the door to the testing chamber (sound proof booth) in which the experimental arena was situated and initiate the software controller to commence the trial. Each trial lasted for 1 hour, and was followed by a 10 minute inter-trial interval, during which subjects were returned to their home tubs and allowed access to water, but feeding trays were removed during this period. Following this ITI, the next trial would then begin, following the same methods above. Each subject received three, one hour training sessions in a single day, followed by a one hour extinction test, which followed the same procedure described above, with the exception that the stimulator units was powered off to prevent shocks delivery. After the extinction test, subjects were returned to their home tanks for 24 hours, with their electrodes and magnet wires still attached. 24 hours later, the same for subjects were returned to the alley for a final extinction test, which again lasted for 1 hour.

Data Collection

The dependent variables considered in this study were measured and recorded automatically via the Matlab trial controller. The timestamp of each shock delivered to a master/yoked pair was logged by matlab as time value of the video frame during which it was delivered. The position of each subject was recorded as a pair of x y coordinates during each of the 3600 frames of the trial, with coordinate origin at the center line of the alley, (the border of the “shock” and “non-shock” sides of the alley). The time each subject spent on the shock side of
the alley was calculated as the sum of the number of frames in which a given subject’s x position within the alley was above or below (depending on their counterbalance condition) relative to the midline of the alley. At the conclusion of each trial all of these values were automatically saved as a matlab data file, and subsequently imported to excel and SPSS for data analysis.

Results

Data Pre-Screening

Before any analyses were performed data was pre-screened to correct any anomalous data points. The data showed no outlier values that needed correction. All of the dependent variables considered in this experiment were calculated up to the final second/frame (3600) of the allotted trial time. No collected trials were excluded from this experiment.

Time Spent on Shock Paired Side of Alley

The total number of seconds that subjects spent on the side of the alley that had been assigned to shock was subjected to a three-way mixed model ANOVA with the between group factors of “stimulus”, comprised of the levels “black” and “checkerboard”, and “treatment”, comprised of the levels “yoked” and “master”. For yoked group subjects shock was not spatially contingent, so their “shock side” was determined by the shock side assigned to their master counterpart. The third factor in this model was the within subjects factor of “trial”, comprised of 5 levels (the first 3 level being training trials, and the last 2 being the extinction tests).

This three way ANOVA produced significant main effects of all three independent variables. The main effect of trial (F(4,176)=3.38 p=0.011) was produced by subjects, when
averaged across all groups, spending more time on the shock-paired side of the alley during trial 5, relative to all other trials (see figure 4.2). The main effect of the treatment variable \( (F(1,44)=42.844 \ p<0.001) \) was produced by subjects in the yoked condition spending more time on the shock side of the arena (as would be expected as shock was not spatially contingent for these subjects) (see figure 4.3). Finally the main effect of the stimulus variable \( (F(1,44)=26.495 \ p<0.001) \), was produced by subjects who had shock paired with the checkerboard stimulus spending more time on the shock side of the alley then subjects who had shock paired with the black stimulus. Interpretations of these main effects are complicated, because all of these variables were also found to significantly interact with one another.

This ANOVA also produced significant two way interactions between each pair of independent variables. The interaction of treatment and stimulus \( (F(1,44)=8.505 \ p=0.006) \) was generated by subjects in the yoked condition who were paired with master animals assigned to the checkerboard-shock condition spending significantly more time in the shock side of the alley, relative to all other groups. The interactions of trial and treatment \( (F(4,176)=2.534 \ p=0.042) \) and also trial and stimulus \( (F(4,176)=5.368 \ p<0.001) \) both are best understood as a product of the increase in occupancy of the shock-paired side of the alley found in the master – checkerboard condition between trials 4 and 5 (the two extinction tests, see figure 4.2).

In order to better understand the interactions between treatment and trial, and stimulus and trial, and to provide evidence of learning based on the extinction tests, a set of orthogonal post-hoc contrast were performed. These contrasts compared master and yoked group shock side occupancy, within each level of stimulus, and within trials 4 and 5. These contrasts revealed that within the black level of the stimulus variable, Master and yoked group subjects did not significantly differ from one another during either trial 4 or 5. However, master and yoked
subjects in the check level of stimulus did significantly differ from one another in shock side occupancy during trial 4 (F(1,176)=24.627), but not during trial 5 (F(1,176)=3.3 (see table 4.2 and 4.3 for full report). The reported critical values for these contrasts have been adjusted using the Scheffé correction.

The three way interaction between trial, treatment, and stimulus was not found to be significant (F(4,176)=1.211 p=0.308). See table 4.1 for a full report of all statistics.

Discussion

The primary hypothesis of the current study, that subjects in the Master condition would spend less time in the side of the arena that had been paired with shock than their yoked counterparts during extinction testing was confirmed under certain conditions. As can be seen in figure 4.2, fiddler crabs who had the checkered stimulus paired with shock avoided the checkered side of the testing alley during the extinction test (trial 4), but this effect had largely disappeared 24 hours later during the subsequent extinction test (trial 5). Subjects who had the black stimulus paired with shock also spent very little time in the black side of the alley during testing, but this was not significantly different from their yoked counterparts, who also avoided the black half of the alley during testing. Thus subjects developed a conditioned avoidance when shock was paired with the checkered stimulus, and possibly also when shock was paired with the black stimulus, although evidence of this possibility is hidden due to a floor effect.

For fiddler crabs in the checkered stimulus – master group, the significant difference between themselves and their yoked counterparts in shock side occupancy during trial 4 was generated by a learned, allocentrically informed conditioned avoidance response that they had developed over the course of the training sessions. As can be seen in figure 4.2, the
yoked subjects under both levels of stimulus showed a tendency to spend more time on the checkered side of the arena. Unsurprisingly, during training, the checkered master group subjects spent most of their time on the black side of the arena, successfully escaping the application of shock during training, similar to the results produced by experiment 1. The persistence of avoidance of the checkered side during trial 4 by these animals, was not a direct result of US responding, as no US was presented during this trial, and therefore must have been due to a learned association between the checkered stimuli, the response, and the US.

Although fiddler crabs in the checkered master group did show a significant difference from their yoked control group during the first extinction test, this difference had disappeared by the second extinction test 24 hours later. There are two possible explanations for this lack of persistence of the avoidance effect. First, it is possible that fiddler crabs are incapable of retaining a learned avoidance response over a 24 hour interval. This interpretation is consistent with the lack of learning effects produced in experiment 1, in which the retention interval was also 24 hours. It is possible that given more extensive training, these animals may be able to retain a learned avoidance response over 24 hours, but addressing this possibility would require future studies that directly address this parametric question of fiddler crab retention. Secondly, it is possible that the first extinction test (trial 4) may have extinguished the learned avoidance response in these subjects, and had they not been tested in trial, they would have displayed an avoidance effect 24 hours later. To begin to address this possibility, both trials 4 and 5 were broken down into 60 blocks of 1 minute each, to attempt to determine if there was evidence of extinction within either trial 4 or trial 5. As can been seen in figure 4.4, subjects in the checkered – master condition began trial 4 spending
less than 50% of their time on the shock assigned side of the alley, and continued to do so across the entire hour of the extinction trial, suggesting that these animals did not extinguish their avoidance of the checkered stimulus over the course of the trial. In figure 4.5, it is clear that subjects in the checkered – master condition began the fifth trial (the second extinction test 24 hours after the first) already with a bias toward spending time on the checkered, previously shock paired, side of the alley. These results alone are not enough to conclude that extinction was not the cause of the lack of an avoidance effect in trial 5, but are consistent with the hypothesis that a 24 hour retention interval is too long for fiddler crabs to maintain a learned avoidance response in this paradigm, or possibly that extinction due to trial 4 exposure did not express itself until after consolidation.

As mentioned above, fiddler crabs in the black stimulus – master group also spent very little time on the side of the alley paired with shock, producing a situation where on first inspection the behavior of these animals seems to indicate a avoidance response. However, their yoked counterparts also avoided the black side of the arena, leaving me unable to argue that subjects in the black – master condition had learned based on their experience of spatially contingent shock. This is likely due to a preexisting preference to approach checkered visual stimuli, a phenomena that has been observed in all three experiments of this dissertation that employed checkered stimuli.

There are several results from this experiment that support the secondary hypothesis of this study, that fiddler crabs have a preexisting preference to approach checkered stimuli compared to plain black stimuli: 1) The main effect of stimulus in which subjects who had shock assigned to the black stimulus spent less time on the shock side of the alley. 2) The interaction of treatment and condition in which yoked subjects in both stimulus groups spent
more time on the checkered side of the alley. Taken together these separate effects both support the conclusion that in the absence of a spatially contingent experience of shock, fiddler crabs default to approaching the checkered stimulus (figure 4.3). Additionally, the results of the extinction tests for both the checkered and black stimulus groups support this hypothesis. In the black stimulus groups, both the master and yoked subjects spent very little time on the black, shock-paired, side of the arena, instead spending the vast majority of their time on the checkered side. As shock was not spatially contingent for yoked animals, this group’s tendency to avoid the black side of the arena must be due to a pre-existing bias toward checkered stimuli. If true these result can be explained by a floor effect: these animals had no way to improve performance., The checkered – master group also displayed evidence of a preference for the checkered stimuli during the second extinction test, as they not only stopped avoiding the checkered side, but in fact spent more than half of the trial on the checkered side of the arena. This finding is best interpreted as a preference to approach checkerboard that reasserts itself quickly, presumably as the avoidance response learned during the training sessions the previous day has dissipated.

This experiment was very similar to experiment 1, with several methodological changes. Experiment 1 failed to produce any evidence of allocentric learning in fiddler crabs, while the present experiment provided evidence that subjects in the checkered master condition did form a learned avoidance response informed by allocentric visual stimuli. The differences between the design of the present experiment and that of experiment 1 are: a random inter-shock interval, different, more salient visual stimuli, and the compression of all training and testing sessions to a single day. Determining which of the changes resulted in positive evidence of learning in the present study is not possible with the data obtained. The
possibility that consistent timing in the delivery of electric shock may have overshadowed avoidance learning in experiment 1 is an interesting one that should be addressed by future research in this paradigm, although this explanation is less parsimonious than the idea that animals could not visually discriminate the CSs used in experiment 1.

This experiment, for the first time, has provided evidence of allocentrically informed place learning in navigating fiddler crabs under the right conditions. This result is consistent with previous research that has found that in situations where the fiddler crab path integration system is not informative, they instead rely on visual cues (T. W. K. Kim & Choe, 2010). This result is also consistent with findings in other invertebrates that suggest a hierarchal organization of navigatory modalities, dependent on the variability, or usefulness, of the solution vectors produced by these modalities (Bühlmann et al., 2011; Ken Cheng et al., 2012, 2007; Schultheiss et al., 2016). A logical next step would be to replicate the current experiment in a form that allows subjects to establish an egocentric home vector within the experimental arena, such as placing a burrow at the center of the alley, and allowing subjects to occupy this burrow on a semi-permanent basis, exiting the burrow and entering the alley itself under their own power and at their own volition. This would allow the generation of a useful path integration based home vector in animals navigating the alley, and allow for the direct testing of the hypothesis that allocentric place learning only occurs in fiddler crabs when egocentric information is unavailable or uninformative. In such an experiment, it would also be possible to directly manipulate the informational value of both egocentric and allocentric cues by varying the spatial consistency of both the allocentric cues, and or the location of the shock side of the arena. This would allow researchers to directly test for the presence of, and describe, a hierarchically organized system of navigatory modality
preference, and determine if there is at any point integration of cues emanating from different modalities in crustaceans.
Chapter 5 – Allocentric and Egocentric Cue Use by Fiddler crabs in a Y-maze

The previous experiments discussed in this dissertation all attempted to detect allocentrically informed navigation behavior in fiddler crabs using an aversive unconditioned stimulus, electric shock. The present study was designed to conversely detect allocentrically guided navigation behavior using an appetitive unconditioned stimulus, access to water and darkness. This experiment goal of this experiment was to determine if both the previously navigation mechanisms, an attraction towards checkered stimuli, and the ability to learn to navigate using allocentric visual cues, are specific to the shock paradigm used in the previous three experiments, or if these effects are general and possibly to study in other lab based paradigms.

The primary predators on fiddler crabs are sea birds that forage in littoral areas during low tide for crabs that have exited their burrows to forage (or seek mates) (Layne, 1998; Zeil & Hemmi, 2006). Burrows offer fiddler crabs refuge from these predators, and there is extensive research on the ability of these animals to remember the location, and quickly flee to their burrows when threatened (Layne et al., 2003a; Zeil, 1998), or when their burrow is approached by another fiddler crab (Hemmi & Zeil, 2003). This argues that access to these burrows in an important, appetitive stimulus for fiddler crabs. While much research has been done on the ability of these animals to locate and return to burrows that they themselves constructed and occupy regularly, to my knowledge no studies have ever attempted to use access to a burrow as an unconditioned stimulus.

This study employed a Y-maze paradigm to determine if fiddler crabs were capable of learning to locate and enter an artificial burrow, using either allocentric or egocentric cues. The Y-maze paradigm has a long history of being used to study spatial behavior in a wide range of
taxa, including but not limited to mice, fish, and starfish (Castilla, 1972; Sumpter et al., 2011; Yamazaki et al., 1979). In the Y-maze paradigm a researcher situates a subject in a forced choice situation. The subject may explore the starting arm of the maze and on reaching the point at the central intersection of the “Y” the subject is able to simultaneously perceive the stimuli that the researcher has placed in the two “terminal” arms of the maze. By recording which of the two target arms subjects enter, occupy, and explore more, researchers can infer which of the stimuli paired with the target arms subjects prefer. By exposing subjects to repeated trials within a Y-maze configured with a choice stimuli, and pairing the stimuli on a particular arm with a unconditioned stimulus, the Y-maze can also be used to detect learning effects, in that when a subject learns that a given US is associated with one of the two CSs on either arm, they enter and occupy that arm faster, and with a higher frequency across trials. Another useful feature of the Y-maze paradigm is that the locations of the target stimuli may be altered across trials, which allows investigators to address questions of cue competition. For example, if the location of the US remains constant across trials, but the researcher varies the location of any allocentric cues they have provided subjects, it is possible to detect the relative contribution of egocentric cues in guiding the subject to the US. Conversely, if one alters the location of both the target CS and US between trials, but maintains the pairing between allocentric CS and US, the relative allocentric contribution in guidance can be determined, by searching for a reduction in subject performance when egocentric cues are uninformative. This is the approach taken in this experiment.

The present study uses the Y-maze paradigm to answer two primary questions. First, the results of experiments 2 & 3 indicate that fiddler crabs have a preference to approach the checkerboard stimuli preferentially over the solid black stimuli used in that experiment. This experiment attempts to replicate that preference effect in an appetitive paradigm to determine its
generality. By pairing the same checkerboard and black stimuli used in experiment 2 with each of the two target arms, and measuring which of these arms subjects move toward and occupy more often, this experiment aims to detect if a taxis toward checkerboard patterns exists in fiddler crabs outside of an aversive context.

The second question this experiment is designed to answer is if fiddler crabs are capable of learning to navigate and escape the Y-maze more efficiently over repeated trials, and if they can, to determine if they employ egocentric and/or allocentric cues to accomplish this task. To answer this question, this experiment employs an artificial burrow that leads to an “escape cup” as a US. The artificial burrow may be situated in either arm of the Y-maze on a given trial, and is not visually detectable to the subjects until they are within one inch of the burrow hole. The Y-maze itself is brightly lit and dry, conditions that should be aversive to fiddler crabs. The artificial burrow and escape cup contain brackish water, sand, and are kept dark, which should make the artificial burrow appetitive to fiddler crabs. To my knowledge access to a wet dark burrow has not been previously used as a US in any studies of decapod behavior. If subjects reach and enter this artificial burrow with increased efficiency across trials, it will indicate for the first time both that artificial burrows are in fact an appetitive US for these animals, and that fiddler crabs are capable of learning to escape a Y-maze. The spatial location of the burrow was varied across trials for half of subjects, while the visual stimulus maintained its’ pairing with the burrow, allowing this experiment to additionally determine if subjects are attending to allocentric or egocentric cues in order to locate the burrow.

This experiment sought evidence for the effects of three independent variables: the repeated trials within a subject to reveal learning effects; the type of visual cue in the terminal arms to reveal effects of stimulus preference and; the consistency of stimulus arm location to
reveal difference between allocentric versus egocentric information processing by the subjects in this task. The following paragraphs detail these features of the experimental design.

Measures of animal performance across repeated trials was used to detect learning effects across the within subject factor. Each subject was placed into the y-maze and allowed to move about it freely for up to 30 minutes. If a subject had not entered the escape burrow after 30 minutes the experimenter would guide them into the escape cup manually. Subjects that did enter the escape cup before the 30 minute mark were allowed to remain there until the 30 minutes of trial time had elapsed. In between each trial, subjects were removed from the escape cup, any necessary adjustments to the Y-maze were made (such as relocating the visual stimuli and escape cup, see below), and subjects were then replaced at the starting position of the maze. This process continued until a given subject had been presented with 10, 30-minute trials.

The between groups independent variable “Visual stimulus” was manipulated by pairing the US with a uniform black panel visual stimulus for half of all subjects, and with a checkerboard stimulus for the remaining half, placed at either terminal arm of the Y-maze. Effects related to this visual stimulus variable allow for the detection of behavioral changes related to the allocentric visual stimuli, which can verify the existence of a taxis toward checkerboard stimuli in fiddler crabs.

The second between groups independent variable is the location of the escape burrow in one or the other the terminal arms of the maze, which we refer to as the “Spatial consistency” of the artificial burrow. For half of all subjects the location of the escape burrow and its’ associated visual stimulus was consistent across all ten trials for each subject (i.e. if the burrow is on the left arm of the Y-maze during the first trial, it remained on that arm for all subsequent trials), and for the other half of subjects, the location of the escape burrow was inconsistent across trials,
switching positions on each consecutive trial. Effects related to this variable of spatial consistency allow for the detection of egocentrically guided learning in these animals. Logically if animals in the consistent group show a learning effect across trials, but the animals in the inconsistent group do not, it would indicate that subjects are using some form of egocentric strategy in order to locate the escape burrow. Conversely, in this paradigm, an egocentric strategy would consist of some kind of response learning, such as “always go left at the Y”, whereas the typical egocentric strategy seen in fiddler crabs within the context of homing is the more complicated strategy of path integration.

The dependent variables measured in this study were the distance traveled by subjects before entering the escape cup, the amount of time spent within the maze before entering the escape cup, the average speed moved by animals before entering the escape cup, and the number of times subjects moved from one arm of the y-maze to another. The time, distance, and speed variables were all included in order to detect changes in the efficiency with which subjects were locating the escape burrow. The number of arm to arm transitions was measured in order to detect changes in the area of the Y-maze that subjects were exploring across trials, regardless of whether or not they were changing the efficiency of how they were moving within the maze. Each of these dependent variables were sensitive to different aspects of fiddler crabs behavior that might change with either experience, training or visual stimulus preferences.

Following many learning studies with Crustacea I hypothesized a main effect of “trials” as a decrease in time, number of arm to arm transitions, or distance traveled, or an increase in average speed across trials as an indication of learning the cup location. Confirmation of this hypothesis would provide evidence for the ability of fiddler crabs to learn to complete this y-maze, and would validate artificial burrows as an appetitive US in these animals. If a main effect
of trials occurs in isolation, that is to say without the presence of any significant interactions, it would demonstrate that fiddler crabs are capable of learning to use allocentric cues to locate the burrow regardless of conflicting egocentric information, which would agree with the field work performed by Kim and Choe (2010), and would be the first demonstration of allocentrically guided navigation in a laboratory study of these animals. If a main effect of “trials” occurred in conjunction with a significant interaction of “trials” and “visual stimulus”, depending on the nature of the interaction, it could indicate that subjects were only able to learn about one of the two visual stimuli. Conversely, if subjects display the expected preference for checkerboard, it would confirm the previous result (experiments 2 & 3) and, further, a significant interaction between “trials” and “visual stimulus” could provide deeper insights into the specifics of any learning processes identified as an effect of trial.

Finally, I’ve hypothesized a main effect of the consistency variable, and that would be indicated by subjects in the consistent group completing the maze with significantly less time and distance traveled, and/or with fewer movements between the different arms of the y-maze than those in the inconsistent group. This would indicate that subjects attended to the egocentric cues available in this task, and when these cues were not useful, there performance was compromised. This would for the first time demonstrate egocentric cue use in fiddler crabs outside of the context of homing, and for the first time in a laboratory based study.

**Methods**

**Subjects**

64 Male fiddler crabs of the species *Uca pugilator* were obtained from Carolina Biological Supply. Only male subjects were used, as discussed in the previous experiments.
After delivery subjects were housed in individual plastic tubs (13in x 8 in x 4 in) each of which contained an artificial beach of aquarium gravel and sand, as fiddler crabs are semi terrestrial. Each housing tub also contained 1000 ml of brackish artificial sea water at a salinity of 23 ppt, which was constantly aerated by air-stone bubblers. Subjects were fed 1 pellet of fiddler crab food twice a week in a plastic dish filled with sand. This food dish remained in the housing tubs at all times. The food dishes and housing tubs were cleaned every two weeks: the water in each subject's tub was changed, and the gravel was rinsed in fresh water. Subjects were maintained in the laboratory for a minimum of 1 week after receipt before being included in this experiment, to verify that they were not experiencing health problems due to the shipping process or pre-existing conditions.

**Y-maze arena**

The Y-maze arena that subjects were tested in was constructed of 1.3 cm plywood. The primary, or starting, arm of the maze was 32 cm long, and 10 cm wide. Each of the two terminal, or “target”, arms of the arena were 23 cm long and 6 cm wide. All vertical walls of the maze were 13 cm tall (See figure 5.1). The interior walls of the maze were all painted uniformly white (Krylon Acrylic 1502), to prevent the influence of visual cues generated by differences in the grain of the plywood (although any such cues are likely imperceptible to a fiddler crab's comparatively course resolution visual system, which has a resolution of ~1.5 cycles per second within +/- 15 degrees of the horizon (Zeil & Al-Mutairi, 1996)). The floor of each target arm contained a 5 cm hole, under which was a 5 cm diameter PVC pipe, oriented at 45 degrees. Each pipe had 2 mm holes drilled in it in a grid pattern with a spacing of 1 cm, in order to provide traction for crabs entering. The holes and pipes together served as artificial burrows to provide animals with access to a dry, wet area, which I hypothesize to be appetive to fiddler crabs.
any given trial, the artificial burrow in one target arm of the Y-maze would lead into an “escape cup” that contained sand and brackish water (23 ppm salinity, changed daily) and the other arm contained a hole that was blocked in a way (see below) that was not visible to the crab from the y-maze choice point. The escape cup was surrounded by a black curtain in order to make the escape cup dark, and appealing to fiddler crabs. The target arm that is not connected to the escape cup on any given trial was blocked with a 5 cm diameter, 30 mm thick wooden disk that could be inserted into either escape hole. This disk rested 9 mm under the walking surface of the Y-maze, making it impossible for animals to visually determine which escape hole was blocked and which was open until after they had entered that arm and approached the escape hole.

Two identical foam core inserts were placed vertically in the terminal arms of the maze in order to hold and display visual stimuli to subjects from behind the artificial burrow hole. Both of these inserts had one side covered in black card stock, and the other side covered in cardstock on to which had been printed a 1 by 1 inch checkerboard pattern. These cards were equal in size and shape to one another, and could be removed from the target arms and reversed, in order to change the visual stimuli displayed in that arm of the y-maze, according to the experimental design of a given subject’s group.

The y-maze arena was surrounded by a black, light blocking curtain in order to prevent subjects from perceiving uncontrolled extra-maze visual cues. This curtain was 100 cm tall, and had a diameter of 76 cm, which fit the y-maze itself with a small gap between the maze arms and the curtain. The curtain had a slit through which subjects could be placed into the maze that could be sealed with Velcro during trials (see figure 5.2).

Trials were filmed using a video camera (Sony CCD model #CB8440), who’s output was fed into a computer running Matlab 2016b with the image acquisition toolbox installed. The
matlab video controller sampled 1 frame from this video stream every two seconds (a frame rate of .5 frames per second), and saved these images sequentially. A total of 1800 frames were collected during each trial, representing the total trial time of 30 minutes. The arena was lit with two 50 watt incandescent lights.

Above the y-maze a wooden bar was suspended, in line with the central axis of symmetry of the y-maze. Suspended from this mounting bar were the black curtain, the camera, and two lights that illuminated the maze. The camera was positioned directly above the center of the y-maze, and the two lights were suspended 15 cm away from the camera also on the wooden bar, oriented downward, on both sides of the camera. This arrangement of lights, camera and mounting bar was designed to be symmetrical from the subjects’ perspective, in order to prevent these devices from providing the animal with visual cues that could be used to discriminate between the two target arms of the y-maze.

Procedure

Each subject experienced 10 sequential half hour trials, resulting in a five hour series of trials for each subject. Each subject’s series of trials was begun between 10 am and 12 am on the given day, in order to control for possible effects of circadian and tidal cycles. One subject was run per day. The order of subjects to be run was randomized in order to control for potential effects of month and batch of animals, as animals were received in batches of 12.

Before each subject began their series of trials, the brackish water in the escape cup was replaced with clean brackish water, to avoid cross-subject chemical cues. Subjects were removed from their home tub at the start of their first trial, and placed by hand into the y-maze arena at the base of the starting arm. Subjects were placed facing “forward”, with their mouth parts oriented
towards the target arms of the y-maze. Video recording commenced 1-2 seconds before the animal was placed into the y-maze. During the trial the subjects were free to move about the y-maze, without interference from the experimenter and in visual isolation from activity outside the maze. Trials were continued for the full 30 minutes allotted, regardless of when the subject entered the target burrow. Subjects were free to exit the target burrow and attached escape cup and return to the y-maze, although any animal movement after they had fully entered the escape cup were not considered in the final analysis of this experiment. At the termination of a trial (after 30 minutes had elapsed), subjects that were not inside the escape cup were guided by the experimenters hand to enter the escape cup under their own power. The escape cup was then removed from the y-maze and the subject was retrieved into the experimenter’s hand. The escape cup was then replaced in the appropriate position for that subject’s next trial, the visual stimuli were rearranged (if required for that trial), and the animal was again placed at the starting position of the maze, oriented forward, and the subsequent trial was begun. This process was repeated until that subject had experienced 10 trials.

**Analysis**

The movements made by subjects within the y-maze during each trial were measured using MetaMorph image analysis software v7.1 (Molecular Devices Corp., Sunnyvale, CA). The set of images representing each trial were first pre-processed, by subtracting the average value of each pixel across all frames of the trial from each individual frame. This “background subtraction” removed the constant background from each frame, resulting in only moving objects (the subject) being visible in each frame of the trial. The subject’s position within each of these processed frames was then determined using MetaMorph’s automatic track objects function. Subjects were tracked until they had entered the target escape hole. “Entering the target escape
hole” was defined as the subject being completely within the escape hole when viewed from above, including all of the subject’s legs. These tracked paths were then represented as a time series of x y coordinates and exported to a Microsoft Excel spreadsheet.

These series of x y coordinates were then imported into Matlab 2016 in order to calculate the dependent variables considered in this study. The number of video frames elapsed in each path from the start position to the first time it entered the cup was computed to determine to total amount of time each animal spent in the maze before entering the target burrow. The distance between each consecutive point on the same path was calculated and summed in order to determine the total amount of distance each subject traveled during each trial. The total distance measure was then divided by the total time measure in order to determine each subjects’ average speed over the course of the trial. The x y coordinate corresponding to the “center” of the y maze was measured for each trial, and then compared to the x y value of each subject for every frame of every trial in order to determine which of the 3 arms of the maze the subject was occupying that frame. The x y coordinates of each arm of the Y-maze was defined relative to the central point of the maze itself. Viewing the maze as it is oriented in figure 5.1, all subjects coordinates that were below the central point were considered to be in the “base” arm of the Y, all subjects coordinates that were above and to the left of the central point were considered to be in the “left” arm, etc. The number of transitions from one arm of the maze to another arm of the maze was then calculated, as a measure of to what degree subjects were exploring the maze on each trial. The arm of the maze that each subject first chose to enter, and the number of frames they spent within the target arm were also recorded. After being computed the dependent variables were then saved to excel files and subsequently imported into SPSS v24 (IBM, Armonk, New York) for statistical analysis.
Results

Data Pre-Screening

Before any analyses were performed data was pre-screened to correct any anomalous data points. The data showed no outlier values that needed correction. All of the dependent variables considered in this experiment were calculated up to the point at which subjects entered the escape burrow, with the consequence that for any trials in which the animal did not reach the cup (failed trials), it was impossible to calculate these values.

To account for subjects that failed to locate the escape burrow during their trials in the y-maze, any trial in which the subject failed to enter the escape burrow completely before the 30 minutes allowed had elapsed, was considered a failed trial. Subjects with failed trials were accounted for in two ways. Firstly, any subject that produced more than 2 failed trials were completely excluded from all further analyses. 2 trials was chosen as the exclusion criteria, as subjects who failed only 2 non-consecutive trials could still be included in our analysis without producing empty data values, as we grouped trials into blocks of two. These subjects appeared to not be motivated to escape the y-maze itself, and were therefore considered not useful in determining if they were capable of learning the location of the escape burrow. In total 7 subjects exceed this criteria, resulting in a total of 57 subjects which were included in most analyses (with the exception of the explicit analysis of failed trials themselves, discussed below).

To allow for subjects with fewer than 2 or fewer failed trials was to conduct the analysis in blocks of trials rather than the original trials. All subjects were given 10 trials within the y-maze, but for purposes of analysis the dependent variable values from multiple trials were
averaged together in order to create blocks of trials to analyze. This was done for two reasons. First, because we only included subjects who failed 2 or fewer trials, and because the 4 subjects who failed 2 trials never failed two trials consecutively, blocking trials resulted in our analysis containing no missing cells, and therefore we did not need to rely on data replacement methods. The second benefit of blocking trials was that by averaging together subjects’ performance during multiple trials, we were able to lessen the effects of within subject performance variance on our statistical procedure, and instead focus our analysis on the overall trends in changing performance over trials. Trials were grouped into blocks of both 2 and 5 trials for analysis purposes (resulting in either 5 or 2 distinct blocks, respectively). Both sets of blocked data will only be presented where deemed useful for understanding the results of this experiment.

Analysis of failed trials

The number of trials failed by each subject was summed into a single value. For this analysis all subjects were included regardless of the number of trials they failed, as failed trials were the explicit topic of this analysis. The total number of failed trials per subject was then subjected to a two-way ANOVA with the between groups factors of visual stimulus (the visual cue that marked the escape burrow for those subjects, with the levels “black” and “checkerboard”), and spatial consistency (whether the escape burrows location switched arms between trials, with the levels “consistent” and “inconsistent”). This analysis revealed that there were no significant differences between groups in the number of trials in which subjects failed to enter the escape burrow, driven by either the target visual stimulus (F(1,60)=0.207 p=.651), the spatial consistency of the target (F(1,60)=0.092 p=.763), or by an interaction of the two factors (F(1,60)=0.826 p=.367).
Time in the Y-Maze before Entering the Escape Burrow

The total amount of time each subject spent in the y-maze before entering the escape burrow on each trial was calculated by identifying the first video frame of the trial in which the subject’s entire body was within the escape burrow, including all of their legs (the “trial end frame”). As discussed in the methods section, video recording commenced several seconds before the subject was placed into the y-maze, requiring that the “trial start frame”, or the frame at which the subject was placed at the starting position within the y-maze, also be identified for each trial, and subsequently subtracted from the value of the trial end frame. This resulted in a value that represented the number of frames a given subject, on a given trial, spent exploring the y-maze. This value was then multiplied by 2 in order to convert it from units of “frames” at .5 FPS, to units of seconds. These values were then averaged together to create values for blocks of trials, as described above.

The total number of seconds each subject spent in the y-maze before entering the escape burrow during each block of trials was then subjected to a three-way mixed model ANOVA, with the between group factors of visual stimulus and spatial consistency, and the within group factor of blocks of trials. This analysis was performed twice, once using both five blocks of 2 trials each, and a second time using two blocks of 5 trials each. The analysis of five, 2-trial blocks revealed significant main effects of block of trials (F(4,212)=5.623 p= <.001) and spatial consistency (F(1,53)=4.419 p= .040) on the amount of time animals spent exploring the maze. The main effect of block resulted from subjects, when averaged across all groups, taking less time to enter the escape cup during later blocks (blocks 3, 4, and 5) than during blocks 1 and 2 (see figure 5.3). The main effect of consistency resulted from subjects in the inconsistent group
taking less time, on average, to enter the escape cup than subjects in the consistent group, where the spatial location of the escape burrow was invariant (see figure 5.4).

This analysis also revealed a significant interaction between the blocks of trials and the visual stimulus that marked the escape burrow (F(4,212)=2.804 p=.027). This effect is the product of subjects in the groups who had the escape burrow marked by the black stimulus showing a greater decrease in time within the y-maze across blocks than subjects in the groups who had the escape burrow marked with the checkerboard stimulus (see figure 5.5). This interaction is perhaps more clear when considered in two, 5-trial blocks. The overall analysis of two, 5-trial blocks revealed the same significant effects as discussed above for blocks, consistency and the block by visual stimulus interaction (F(1,53)=13.751 p=.001, F(1,53)=4.07 p=.049, and F(1,53)=6.87 p=.011, respectively). This analysis of 5 trial blocks is however useful in that it makes the nature of the block by visual stimulus interaction more clear when graphed, in that subjects in the black stimulus groups clearly have more negative slopes over trials, as compared with the checkerboard stimulus groups (see figure 5.6).

All other effects were found to be not statistically significant, both in the 2 trial and 5 trial block analyses (see table 5.1 and 5.2 for full report).

In order to understand the significant interaction between blocks of trials and visual stimulus, an analysis of simple effects of block at each level of the visual stimulus variable was performed, followed by a set of non-orthogonal contrasts that compared the first block of trials to each other block, at each level of the visual stimulus variable. The simple effects analysis revealed that there was a significant effect of blocks for subjects in the black stimulus groups
(F(4,108)=8.074 p<.001), but not for subjects in the checker board groups (F(4,104)=.912 p=.460).

A set of non-orthogonal post hoc contrasts were performed in order to further dissect the significant interaction between visual stimulus and blocks of trials, using the Scheffé correction to account for multiple comparisons. These contrasts revealed that within the check level of visual stimulus, the first block of trials did not differ from all 4 subsequent blocks pooled together, or from any of the 4 subsequent blocks individually (see table 5.3 for full report). However, at the black level of visual stimulus, this contrast analysis revealed significant differences between the first block of trials and all 4 subsequent blocks pooled together, and between block 1 and blocks 4 (marginally significant p=0.057) and 5 (see table 5.4 for detailed statistical report).

**Distance Traveled Within the Y-Maze before Entering the Escape Burrow**

The total amount of distance traveled by each subject during each trial was calculated by subtracting the x and y coordinate of the subject on each frame of the trial after the first frame from their x and y position on the previous frame. The Euclidean distance between each consecutive set of measurements were then calculated. The distances were then summed to generate the total amount of distance traveled by the subject, until the frame in which they completely entered the escape burrow. These values were in units of “pixels”, as the x and y coordinates of the animals were themselves measured in pixels. These values were converted into units of centimeters, using a conversion factor of 0.14978 cm per pixel, which was generated by measuring the overall length of the y maze in both pixels and cm, and dividing the total length
in cm by the total length in pixels. This conversion factor was measured repeatedly, on at least one trial of each subject, and never varied by more than 0.0001 cm.

The total number of centimeters each subject traveled within the y-maze before entering the escape burrow during each block of trials was then subjected to a three-way mixed model ANOVA, with the between group factors of visual stimulus and spatial consistency, and the within group factor of blocks of trials. This analysis was performed using both five blocks of 2 trials each, and two blocks of 5 trials each. The analysis of five, 2-trial blocks revealed a significant main effect of block of trials (F(4,212)=2.975 p=.020) on the amount of distance animals traveled within the maze. The main effect of block when averaged across all groups, was found to be due to subjects traveling less distance before entering the escape cup during later blocks (blocks 3, 4, and 5) than during blocks 1 and 2 (see figure 5.7). This analysis also produced a marginally significant interaction effect between block of trials and visual stimulus (F(4,212)=2.278 p=.062). This interaction appears to be produced by subjects in the “black” stimulus group traveling more distance within the maze during the first two blocks of trials, as compared with subject in the “check” stimulus group (see figure 5.8). The analysis of distance traveled within the maze using two, 5 trial blocks also revealed a significant main effect of block of trials (F(1,53)=6.784 p=.012), however the interaction effect of block of trial by visual stimulus that was marginally significant when examined in 2 trial blocks did not approach significance when examined in 5 trial blocks (F(1,53)=1.426 p=.238).

All other effects were found to be not statistically significant, both in the 2 trial and 5 trial block analyses (see table 5.5 and 5.6 for full report).
In order to understand the cause of the marginally significant interaction between blocks of trials and visual stimulus, an analysis of simple effects of block at each level of the visual stimulus variable was performed, followed by a set of non-orthogonal contrasts that compared the first block of trials to each other block, at each level of the stimulus variable. The simple effects analysis revealed that there was a significant effect of blocks for subjects in the black stimulus groups (F(4,108)=4.023 p=.004), but not for subjects in the checker board groups (F(4,104)=.544 p=.704).

A set of non-orthogonal post hoc contrasts were performed in order to further dissect the significant interaction between visual stimulus and blocks of trials, using the Scheffé correction to account for multiple comparisons. These contrasts revealed that within the check level of stimulus, the first block of trials did not differ from all 4 subsequent blocks pooled together, or from any of the 4 subsequent blocks individually (see table 5.6 for full report). Additionally, at the black level of stimulus, this contrast analysis did not reveal significant differences due to the loss of statistical power from the Scheffé correction, but did produce significance values approaching .1 between the first block of trials and all 4 subsequent blocks pooled together, and between block 1 and blocks 3 and 5 (see table 5.7 for full report).

**Number of Arm to Arm Transitions before Entering the Escape Cup**

The y-maze was composed of three distinct “arms”, the starting arm (the base of the “Y”), and the two target arms (the branching top of the “Y”). The arm occupancy of each subject on each frame of each trial was calculated by measuring the x and y coordinates of the center point of the y-maze, and comparing the subject’s position relative to this center point on each frame of the trial. If the subject was below the central point of the y-maze, it was considered to
be in the starting arm, if it was above and to the left of the center it was considered to be in the
left target arm, and if it was above and to the right of the center point it was considered to be in
the right target arm. The number of arm to arm transitions made by each subject was then tallied
by examining how often the arm occupancy measurement changed from one position to another
for each subject over the course of each trial. The minimum number of arm transitions possible,
which would be produced by a subject who moved directly from the start arm to the appropriate
target arm, was 1. There is no upper limit to this measurement, as subjects could conceivably
move from one arm to another many times given the 30 minute length of each trial.

The total number of arm to arm transitions made by each subject during each trial before
entering the escape burrow during each block of trials was then subjected to a three-way mixed
model ANOVA, with the between group factors of visual stimulus and spatial consistency, and
the within group factor of blocks of trials. This analysis was performed using both five blocks of
2 trials each, and two blocks of 5 trials each. The analysis of five, 2-trial blocks revealed
significant main effects of block of trials (F(4,212)=3.725 p= .006). The main effect of block
was produced by subjects, when averaged across all groups, making fewer transitions from one
arm to another before entering the escape cup during later blocks (blocks 3, 4, and 5) than during
blocks 1 and 2 (see figures 5.9 and 5.10). The analysis of arm to arm transitions using two, 5-trial
blocks also revealed a significant main effect of block of trials (F(1,53)=7.53 p = .008).

All other effects were found to be not statistically significant, both in the 2 trial and 5 trial
block analyses (see table 5.9 and 5.10 for full report).

Average Speed in the Y-Maze before Entering the Escape Cup
The average speed of movement of subjects within the y-maze before entering the escape burrow on each trial was calculated by dividing the total distance traveled by each subject (in cm), by the total amount of time spent in the y-maze. This resulted in an average speed of movement across an entire trial, in units of cm per second.

The average speed of movement of each subject during each trial before entering the escape burrow during each block of trials was then subjected to the same three-way mixed model ANOVA as the previous DVs. The analysis of five, 2-trial blocks revealed a significant interaction effect between the block of trials and visual stimulus (F(4,212)=2.818 p=.026). The analysis of two, 5-trial blocks also revealed a significant interaction effect between the block of trials and visual stimulus (F(1,53)=5.499 p=.023). This interaction was caused by subjects in the black stimulus groups increasing their average speed across blocks of trials, while subjects in the check stimulus groups decreased or did not change their average speed across blocks of trials (see figures 5.11 and 5.12).

All other effects were found to be not statistically significant, both in the 2 trial and 5 trial block analyses (see table 5.11 and 5.14 for full report).

In order to understand the nature of the significant interaction between blocks of trials and visual stimulus, an analysis of simple effects of block at each level of the visual stimulus variable was performed, followed by a set of non-orthogonal contrasts that compared the first block of trials to each other block, at each level of the visual stimulus variable. The simple effects analysis revealed that there was a significant effect of blocks for subjects in the black stimulus groups (F(4,108)=2.957 p=.023), but not for subjects in the checker board groups (F(4,104)=1.278 p=.283).
A set of non-orthogonal a priori contrasts were performed in order to further dissect the significant interaction between visual stimulus and blocks of trials. These contrasts revealed that within the both the black and check levels of visual stimulus, the first block of trials did not differ from all 4 subsequent blocks pooled together, or from any of the 4 subsequent blocks individually (see table 5.12 and 5.13 for full report). Again, the loss of statistical power due to the Scheffé correction is likely the cause of this contrast analysis failing to find significant differences, despite the significant simple effect at the black level of stimulus.

**Discussion**

The first hypothesis of this study was that fiddler crabs would learn over repeated trials to navigate the Y-maze more efficiently. This was confirmed by a significant decrease in the amount of time and movement that fiddler crabs took before entering the escape burrow as trials progressed. One possible interpretation of this main effect is that as opposed to learning about the contingency between the visual stimulus and the location of the escape burrow, subjects could have simply been learning to move faster when in the Y-maze, while still randomly moving about the arena, a possibility that is given credence by the significant interaction between blocks of trials and visual stimulus condition and the associated contrast sets, which demonstrated that subjects in the black stimulus condition did in fact move faster within the arena during their last block of trials, as compared with their first block of trials. The interpretation that subjects were simply moving faster is unsupported because the significant main effect of blocks of trials on the number of arm to arm transitions made by subjects. This effect demonstrates that subjects were not only navigating the maze more efficiently, but in fact were taking a more direct route to the arm containing the escape burrow, and therefore were exploring the maze less. These results taken together provide evidence that fiddler crabs are
capable of learning about the stimuli presented to them in the Y-maze, and using this learning in order to navigate the Y-maze more efficiently, and more accurately.

The significant main effects of blocks of trials on the three primary dependent variables of this study (time, distance, and transitions), indicates that subjects are learning about the stimulus characteristics of the Y-maze. There are two possible stimulus types that subjects may have been using in order to learn to escape the Y-maze, internal egocentric cues, or the visual stimuli that were paired with the location of the escape burrow. If subjects had relied on egocentric cues and followed a route memorization strategy (such as “go left at the junction of the Y”), then subjects in the spatially consistent groups would have escaped the maze faster and more accurately than subjects in the spatially inconsistent groups. The results of this experiment however, indicate that the opposite occurred. The time until escape variable produced a significant main effect of spatial consistency, but surprisingly this was produced by subjects in the spatially inconsistent group taking less time to escape than subjects in the consistent group. This effect was not significant in the other dependent variables, but the direction of the differences between spatially consistent and inconsistent did follow the same pattern, lending additional support to the hypothesis.

This effect of spatial consistency certainly demonstrates that subjects were not relying on egocentric cues alone in order to navigate the maze, but the presence of a significant effect in the wrong direction begs for interpretation. The idea that making the egocentric cues inconsistent, and therefore not useful for navigating the maze, made the task itself easier seems infeasible, as one would expect conflicting information to make the task inherently more difficult. In my view there are two plausible explanations of this effect. It is possible that subjects noticed in the first few trials that the spatial location of the escape burrow was varying, and quickly learned to
ignore the spatial location in favor of attending to the visual cues that moved with the escape burrow. This may have resolved a conflict between the egocentric and allocentric information streams, and simplified the task for subjects in the inconsistent group, as they no longer needed to process both information streams, and could instead focus their limited computational resources on the allocentric visual cue alone. In other words, spatial inconsistency may have in fact made this task easier.

A second plausible explanation of why subjects in the inconsistent group escaped the Y-maze faster may be that the varying position of the escape burrow may have made the Y-maze itself a more aversive experience for subjects. It is possible that after noticing that the location of the escape burrow was varying, subjects in that condition began to view the maze as a different, or at least an unpredictable, environment across each trial. This in turn may have motivated subjects in the inconsistent condition to escape the maze faster, as this kind of unpredictability may be associated with danger in these animals, while animals in the consistent condition may have been learning over trials that this environment was predictable, and therefore fundamentally safe. Interestingly, both of the above explanations of this effect require that the spatial position of the exit burrow was salient to the subjects of this experiment, despite the fact that there is no evidence that they used this information in order to more quickly or accurately escape the Y-maze. This may suggest that although subjects were aware of the spatial location of the escape burrow within the Y-maze, they were unable (or unmotivated) to employ their path integration mechanisms, as they did not have a “zero point” at which their path integrator could be reset, such as a “home” burrow at the starting position of the Y-maze.

The primary learning effect in this study, the main effect of blocks found in the time, distance, and transitions data, cannot be interpreted in isolation, as block was also found to
significantly interact with the visual stimulus variable in the distance and time data, and although not significant, the same trend was seen in the arm-transitions analysis. A set of a priori contrasts revealed that while the subjects in the black stimulus group did show a significant change in the distance and time variables across trials (comparing block one to each subsequent block, and to the average of all subsequent blocks), subjects in the checkered stimulus groups did not. This result might seem to suggest that only subjects in the black stimulus groups learned to escape the Y-maze, however it seems more likely that this result is an artifact of the paradigm and stimuli employed in this experiment. The checkered stimulus groups, during the first block of trials, produced time and distance values that are similar to those produced by the black stimulus groups during their final block of trials. In other words, the performance of the checkered groups did not begin inefficient and stay inefficient, but instead began at a level of efficiency comparable to the best performance displayed by the subjects in the black stimulus groups. This could have been produced by a floor effect, in that subjects in the checkered group began the experiment so efficiently that there was no space left in our performance metrics for them to display any further decline. Considered in the context of the preference to move toward checkered stimuli seen in experiments 2 and 3, as this preference may have driven subjects in the checkered groups to be more likely to choose the correct arm of the Y-maze before any learning had occurred.

The analysis of trials failed by subjects revealed no significant differences between any of the four groups of this experiment. A failed trial was defined as any trial in which the subject did not enter the escape burrow. The overall failure rate was relatively low, indicating that fiddler crabs were motivated to escape the surface level of the Y-maze. This is of great concern to researchers of invertebrate behavior, as it is not uncommon for invertebrate animals to not
move and display a general lack of motivation when placed within an experimental arena. The lack of differences in failure rate across groups indicates that this task was not prohibitively difficult for any single group, and additionally, that the removal of subjects who failed a large (>2) number of trials from subsequent analyses should not have differentially effected one group more than others.

The results of this experiment indicate that fiddler crabs are motivated to and capable of learning to locate an artificial burrow in order to escape a dry and brightly lit Y-maze. It can safely be concluded that subjects learned something about the visual stimuli in this experiment, as discussed above, the spatial consistency effect indicted that the absolute spatial location of the escape burrow was not helpful to subjects in escaping the arena, and subjects in the black inconsistent group showed a large learning effect, indicating that extra-maze allocentric cues were not the primary cue used either. The finding that subjects relied on visual cues in this experiment, where path integration cues were likely unavailable or uninformative to subjects due to the lack of a home burrow at the starting position of the Y-maze, is consistent with the hypothesis put forward by Kim and Choe (2010) that fiddler crabs rely more on allocentric spatial cues under conditions in which their path integrator system is error prone. This kind of sensitivity to the accuracy of a navigational information stream has been hypothesized previously in other invertebrates (Ken Cheng et al., 2007; Schultheiss et al., 2016).

It is unlikely that fiddler crabs could employ a true path integration strategy in this paradigm, as they were placed within the maze at the start point, and not exiting a burrow that they normally occupy, when entering the maze. Without a burrow of their own as a starting location, it is unlikely that these animals “reset” their path integrator, and therefore did not have a common reference point across trials. It is possible that entering the new context of the Y-maze
induced these animals to reset their path integrator, which would enable them to use a traditional path integration strategy to navigate this maze. The present study was not designed to separate these two possibilities, but if egocentric learning was detected, it would then be possible to design an separate “obstruction” experiment, where the obstacle is placed between subject and target after several training trials, which would dissociate response learning from true path integration.

The content of what specifically these animals are learning in regard to the visual stimuli is however still an open question, but based on the results of this experiment we can begin to enumerate some of the possibilities. Subjects in this experiment may be learning a direct association between the visual CS and the escape burrow US in a traditional Pavlovian sense. If this is the case, it is possible that subjects in both the checkered and black stimulus conditions formed this association, but this experiment was only able to detect the presence of this association in the black condition groups, due to the putative floor effect discussed above. In an exploratory learning study such as the present one it is important to be wary of misinterpreting results as being due to learning effects when they may instead be generated by performance or motivational issues.

Another possibility, beside a direct CS-US association, that could account for the learning effects seen in this experiment deal with the idea that these animals have an inherent preference to approach checkered stimuli. Perhaps the learning being performed by subjects in the black stimulus groups is taking the form of a modification of this inherent preference. It is ecologically and evolutionarily reasonable to hypothesize that the purpose of a preference to approach checkered stimuli serves the purpose of guiding fiddler crabs towards visual scenes that offer them locations in which they could shelter from potential predators, as the size of the
checkerboard squares used in this experiment were approximately equal to the size of a fiddler crabs carapace (1 inch). This makes it a reasonable possibility that subjects in the black stimulus group, while initially following this preference, learned across trials that approaching the checkerboard stimulus did not lead to shelter, but approaching the black stimulus did. This may have resulted not in an association between CS and US, but instead in a tuning down of the checkerboard preference, due to it not effectively serving its purpose in the experimental context. This kind of modification of a navigational “prior” is similar to the learning based changes in inherent spatial preferences in humans described by Cheng et al. in 2007, and would represent an interesting example of spatial strategy integration in fiddler crabs.

Future work using this Y-maze paradigm in fiddler crabs should address two potential limitations of the present study. First, subjects in this experiment were allowed to remain in the escape cup until the 30 minute maximum trial time had elapsed. This resulted in a situation where subject’s experienced differential amounts of reward (time in the wet, dark escape cup), and different proportions of “escape” time relative to the period of human handling between trials, which itself may have been aversive. To address this potential confounds future work should end trials and remove subjects from the escape cup and a constant time after the subject first escapes the Y-maze. The second potential issue that future research should address is including a control group in which the informational usefulness of the visual cue is explicitly devalued. By alternating which visual cue is paired with the escape burrow, investigators will be able to more soundly argue that it is in fact this cue that subjects in a non-alternating condition are learning about. The fact that subjects in the black inconsistent group showed a solid learning effect I argue also demonstrates this, but the presence of an explicit control in which both the
allocentric and egocentric information is explicitly devalued (a random control) would certainly make this argument stronger.

Overall, this experiment provides the first evidence for the use of allocentric cues for spatial navigation by fiddler crabs in a controlled laboratory setting. This opens the door to future work that can examine how allocentric cues are used by these animals, and how these cues compete with or are integrated with the egocentric mechanisms fiddler crabs are known to rely on in the context of homing. Future research could also use this paradigm in order to test the visual discriminate abilities of fiddler crabs, and attempt to identify stimuli that do not have provoke inherent preferences in fiddler crabs, and would therefore provide better CSs for future studies of fiddler crab learning. Additionally, this study has provided evidence supporting the use of access to an artificial burrow as an appetitive unconditioned stimulus that these animals are motivated to perform for, a critical aspect of studies of learning that has until now not been available, as fiddler crabs have not been found to perform for food or other appetitive rewards.
Chapter 6 – Conclusions

One stated goal of this dissertation was to establish a set of laboratory based methods for the study of navigation in fiddler crabs. As discussed previously, the vast majority of studies of navigation related learning in fiddler crabs, and more generally in invertebrates as a whole, has been performed in the field under naturalistic conditions. While field research is extremely useful in determining the types of learning and strategies typically used by animals to explore and navigate their environments, field research is limited in its’ ability to show us what these animals are truly capable of, and determining to what extant learning plays a role in cue reliance during navigation. Under laboratory conditions, the increased degree of control that researchers have over both environmental and individual subject history factors allows the asking of questions related not to what animals “typically” or “normally” do, but what they are capable of, given specific situations and circumstances. For instance, for more than 20 years, many studies of fiddler crab behavior in the field have concluded that fiddler crabs do not attend to allocentric cues when navigating (Layne et al., 2003a, 2003b, Walls & Layne, 2009a, 2009b; Zeil, 1998), and there is only a single study in the field that found effects of allocentric information on fiddler crab navigation in the field (T. W. K. Kim & Choe, 2010). While these findings certainly do argue that under typical conditions fiddler crab navigation is a largely egocentrically informed process, the common interpretation of these results that “crabs utilize idiothetic rather than allothetic direction information” (Layne et al., 2003a) is to my reading an over-interpretation of the experimental evidence. The fact that homing fiddler crabs seem to preferentially behave in a fashion consistent with egocentric navigation under field conditions, does not mean that these animals are not attending to, or in some way “utilizing” allocentric cues. This bias in
interpretation of experimental results is, in my opinion, a result of the lack of non-naturalistic controlled laboratory studies of these animals.

In order to address this potential bias in the current thinking about the types of sensory cues utilized by navigating fiddler crabs, this dissertation attempted to develop a set of laboratory based procedures that would allow for greater control of the modalities and quality of cues available to navigating fiddler crabs. Towards this goal, experiments 1 and 2 demonstrated that fiddler crabs can be efficiently studied using automatic subject tracking procedures, and that electric shock can be consistently and reliably delivered to these animals as an aversive US. The automatic subject tracking algorithms used in this dissertation are not computationally intensive, and can be run on any modern computer, making possible inexpensive and effective studies of fiddler crab navigation behavior in current and future laboratory studies. The electrode implantation procedure developed as part of this dissertation has made it possible to reliably and consistently deliver electric shocks to freely navigating animals, in a way which frees researchers from the problem of subject mortality due to the autotomization of subject legs that has been observed in previous studies that attempt to use electric shock as a unconditioned stimulus (US) in studies of crab learning (Magee & Elwood, 2013). Although the implantation procedure did produce a relatively high mortality rate itself (~64%), using this procedure, subjects that do not survive the implantation process are removed from the experiment before experimental time and resources are dedicated to them, increasing the overall efficiency of studies using a shock US. Experiments 1, 2 and 3 all demonstrated that electric shock can be automatically delivered to freely navigating fiddler crabs using light weight magnet wires that fiddler crabs are capable of carrying with them as they move about an area. The escape effects seen in all three of these experiments validate that subjects were capable of perceiving and responsive to the delivery of
electric shock over the course of several hours of training, demonstrating that electric shock is a valid and potentially useful US for future studies of learning in these animals. This finding is consistent with a recently published paper on spatial learning in crayfish which used a similar procedure to apply electric shock, which also found escape effects similar to those reported in this dissertation (Bhimani & Huber, 2015). Additionally, the effects of improved performance over trials seen in the Y-maze experiment reported in this dissertation (experiment 4), demonstrated that access to a wet, dark, artificial burrow can serve as an appetitive US in these animals, giving future researchers both an aversive and appetitive US that can easily be used in laboratory based studies of fiddler crab (and likely crustacean in general) learning and behavior.

Also furthering the goal of producing a set of laboratory procedures that allow for the study of learning in fiddler crabs, this dissertation has discovered a set of visual stimuli that are supported by the evidence to be discriminable by these animals. The visual stimuli used in experiment 2, a black and white checkered pattern and monotone black, have now in 3 separate experiments produced statistically significant effects that demonstrate that fiddler crabs preferentially approach the checkered stimulus. This preference effect demonstrates both that fiddler crabs can discriminate between these two visual stimuli, and that they have a pre-existing taxis that dictates they approach checkered stimuli preferentially over plain black stimuli. This finding provides future studies with a pair of visual stimuli that are discriminable to these animals, allowing for the examination of visually informed learning studies, spatial and otherwise, in these animals. In the context of a traditional learning study, stimuli for which subjects have a pre-existing preference are not optimal, as one would prefer to have discriminable, but neutral stimuli as conditional stimuli (CSs) in order to simplify potential counterbalance effects that complicate the methodology required to study visual learning, but as
experiment 3 and 4 showed, with a proper analysis of counterbalance conditions, learning can still be discovered and described using these visual stimuli. Taken together, it is my hope that the methodological advancements described above will aid myself and other researchers in efficiently and accurately addressing some of the open questions related to fiddler crab and crustacean learning, cue saliency, and cue integration, that I have.

Another stated goal of this dissertation is to begin to examine the diversity of mechanisms through which fiddler crabs learn about and navigate their environment. The research presented in this dissertation collectively has revealed two previously undescribed navigational strategies used by fiddler crabs. The first was revealed by the analysis of subjects’ average speed of movement during periods of shock application relative to other time periods, included as part of experiment 1 in this dissertation. Subjects moved significantly faster when shock was being applied to them, indicating that fiddler crabs attempt to escape aversive stimuli by increasing their rate of movement. Experiment 1 found this increase to be non-directional in nature, but given the limited area in which subjects could travel in that experiment, simply moving more typically resulted in subjects escaping the shock area of the alley. This strategy of “move more when experiencing aversive stimuli” is a sensible one for fiddler crabs to employ given their natural ecology, as it is likely to remove them from aversive situations.

An additional navigational strategy revealed by the results of experiments 2, 3 and 4 is that fiddler crabs possess an unlearned preference to approach 1 by 1 inch checkerboard visual stimuli over plan black visual stimuli. This preference is present in subjects regardless of the training history of checkered stimuli, and manifests itself early in training, suggesting that this is an unlearned, and possibly innate taxis in these animals. The specific stimulus characteristics that motivate this taxis are unclear given the evidence produced by these experiments, but two likely
candidates are the increased amount of contrast, and increased spatial frequency in the checkered stimulus relative to the black stimulus. These stimulus characteristics motivating this approach would make sound ecological sense given the environment inhabited by fiddler crabs. Navigating fiddler crabs are constantly vulnerable to predation by larger, fast moving avian species, and as such would be well served by a spatial mechanism that guides them towards areas that are likely to provide shelter and release from predation pressures. The contrast and spatial frequency of the checkered pattern used in these experiments is likely to mimic those that would be correlated with visual scenes in the fiddler crabs environment that are likely to provide physical spaces that a fiddler crab could hide and shelter in. This interpretation is consistent with that of previous researchers who found that fiddler crabs display a preference to move toward three-dimensional objects that are in contrast with their surrounding visual scene, such as mangrove trees (Herrnkind, 1968). This finding of a unlearned preference toward certain visual stimuli also calls into question the interpretation of the results produced by Kim and Choe (2010), as they assumed that their subjects had learned to return to burrows that had an artificial dome stimulus placed over them, when in fact this may have just been a manifestation of this unlearned taxis. Verifying that spatial frequency and relative contrast are the factors motivating this taxis, and that this taxis is an innate antipredator strategy is a potentially fruitful topic for future research. Using the Y-maze paradigm described in experiment 4, tests of visual stimulus preference in fiddler crabs is a simple thing to perform in the laboratory.

The third previously undescribed fiddler crab navigational strategy revealed by experiment 3 in this dissertation is the ability of fiddler crabs to associate a visual stimulus (checkerboard) with and aversive unconditioned stimulus (electric shock). Subjects in that experiment who had shock paired with the checkered side of the arena displayed a learned
avoidance of the checkered stimulus in the first extinction test, in which shock was not administered. This avoidance effect was transient, as in the second extinction test the following day the preference to approach the checkered stimulus reasserted itself. This is the first demonstration of the formation of an association between an allocentric CS and a US in fiddler crab to my knowledge. Previous thinking on fiddler crab navigation has argued that the only role allocentric cues play in these animals is as an orientation mechanism, using a time compensated sun compass (Herrnkind, 1968). This result suggest that fiddler crab behavior may be more plastic than previously thought, and that these animals are capable of learning to avoid certain regions based on their visual characteristics. The present results suggest that allocentric cues do guide fiddler crab navigation to avoid aversive stimuli, at the very least, when egocentric, path integration information is unavailable, and that fiddler crabs are capable of forming associations between aversive unconditioned stimuli and novel visual cues.

Taking the above results highlighting several distinct mechanisms at play in navigating fiddler crabs, we can begin to address the possible forms of organization of these mechanisms. A portion of this overall question is to ask specifically if fiddler crabs cognitively represent space as a unitary representation that is informed by multiple navigational strategies and perceptual modalities, or if fiddler crab navigational behavior is instead regulated by a hierarchically organized set of strategies. Given the findings of the experiments described in this dissertation, the results of experiment 4 (the Y-maze) speak best to this issue. As discussed above, fiddler crabs under natural conditions are largely egocentric in their navigational behavior. The results of experiment 4 suggest that fiddler crabs were solving that task in an allocentric fashion, and were in fact performing worse when egocentric cues were potentially informative. This finding is much more consistent with the view that fiddler crab spatial navigation mechanisms are
arraigned in a hierarchical fashion. If fiddler crabs were capable of forming a general multi-modal representation of space it would logically follow that a multitude of informationally useful cues would enhance the overall accuracy and usefulness of this representation of space. The fact that subjects performed worse when egocentric information was available and useful argues that this information may have created a situation of cue competition within a hierarchal organized set of behavioral strategies, which slowed overall performance in those animals, whereas subjects in the conditions where egocentric cues were not useful resolved this competition easier, producing more efficient navigation of the y-maze in general.

If indeed the navigatory mechanisms of fiddler crabs are arraigned in a hierarchal fashion, and not in a unitary, generalized representation of space, the next question to ask is how are these mechanisms organized, and is there any form of interaction between these mechanisms? Interaction between navigational mechanisms could take the form of cue competition effects, where cues from multiple modalities “compete” with one another in order to control behavior, integration effects, where navigatory solutions from multiple mechanism are weighted in some fashion and combined in order to control behavior, or learning effects, where one mechanism may come to modulate the output of another mechanism due to experience. Across the 4 experiments included in this dissertation, The Y-maze experiment is the only one designed to show possible effects of integration of multiple navigatory modalities. If fiddler crabs were capable of combining egocentric and allocentric place information into a single navigational solution, subject in the Y-maze in the spatially consistent groups should have shown enhanced efficiency of performance relative to subjects in the spatially inconsistent groups. This however was not resulted in experiment 4. To the contrary, as discussed above, subjects in the
spatially consistent groups performed worse than subject in the spatially inconsistent groups, supporting the view of cue competition in this paradigm, not cue integration.

On the issue of one hierarchically arraigned navigation modality modifying the expression of another through potential learning effects, the evidence generated by the 3 shock avoidance experiments discussed is most relevant. Experiments 2, 3 and 4 all provided evidence of a preexisting taxis to approach checkerboard in independent groups of fiddler crabs. Experiment 3 however also provided evidence of this preexisting taxis being either overruled, or possibly directly modified, via an associative learning mechanisms. As discussed above, the preference of fiddler crabs to approach checkerboard was reduced, and in fact reversed, in subjects that experienced this checkered stimuli in conjunction with electric shock. This result suggests that either associative avoidance mechanisms take precedence in the hierarchy of navigational strategies in these animals, or that the association between checkerboard and shock directly modified the preference from checkerboard itself. In either case, the effect of the associative mechanism was short lived in experiment 3, as the preference to approach checkerboard clearly reasserted itself by the second extinction test. This result suggests that the dynamics of interaction of fiddler crab navigational mechanisms may in fact be a plastic process, and more generalized than previously thought. The view that fiddler crab navigational mechanisms interact with one another depending on situation and training history is consistent with Kim and Choe’s view of allocentric learning as a constantly ongoing process in navigating fiddler crabs, that is only expressed under certain circumstances (T. W. K. Kim & Choe, 2010).

In the introduction of this dissertation I suggested that fiddler crabs may be more likely to attend to and learn about allocentric cues in an aversive, shock US, paradigm, as shocks introduce non-navigation related movements that previous researchers have argued are likely to
introduce error into the path integration system, and that fiddler crabs are sensitive to the amount of error present in their path integrator (T. W. K. Kim & Choe, 2010). However, the research discussed in this dissertation has found evidence of allocentric learning under both aversive (experiment 3) and appetitive (experiment 4) conditions. This argues for a reevaluation of what role the path integration system serves in these animals, and under what conditions the path integration system is “error prone”. In all of the studies that comprise this dissertation, it is possible that the path integration system of fiddler crabs was never engaged, and if it was, it may have been very noisy and error prone. In the wild, fiddler crabs are believed to create a zero point, or origin point for their path integration system while inhabiting their home burrow, in order to return to this point later. Because subjects in the present experiments were placed into these artificial contexts by hand, and did not have a known area or “home burrow” within the experimental arenas, it follows that the path integration system may have been useless for animals navigating these. In other words, in both the appetitive and aversive contexts, the experiments presented here may have all been situations where the path integration system was noisy and unreliable, leading subjects to instead rely on allocentric cues. This possibility is directly testable to future experiments using the same paradigms developed for and described in this dissertation, with the addition of a home burrow within the experimental arenas that subjects can inhabit on a semi-permanent basis, allowing them to establish zero points for the path integration system. If subjects in such an experiment did not attend to allocentric cues, it would provide solid evidence that these animals are sensitive to the relative error of their independent navigational strategies, which is a prerequisite for the “optimal” use and integration of multiple strategies (Ken Cheng et al., 2007).
As discussed in the introduction of this dissertation, there is a long history of using the translocation paradigm, in which a navigating animals is moved under experimenter control, and the position to which the animal returns (target or fictive target) is used to determine what cues that animal is attending to in order to navigate their environment (Perry et al., 2013; Schultheiss et al., 2016; Walls & Layne, 2009b). Relatively results from the wider invertebrate literature, and indeed from this dissertation, are beginning to suggest that an animals “primary” modality of navigation may not represent the entire set of cues they are attending to, but only the navigational modality they are expressing in that specific situation. In studies of mammalian navigation, it has long been clear that many modalities are integrated to form cognitive maps of space. Results from studies of invertebrate animals are beginning to suggest that although invertebrates do not seem to form true cognitive maps, they do combine, select from, and integrate multiple types of navigational information, likely from a set of hierocracy organized strategies. Evidence of this kind should prompt researchers of invertebrate navigation to reevaluate the previous interpretations of translocation experiments from “these are the cues these animals attend to”, to something more along the lines of “these are the cues these animals attend to in this specific context/paradigm”, and investigate directly pressures and contexts that may modify the expression of multiple navigational strategies.

A theme has emerged amongst the experimental findings reported in this dissertation. Navigation and spatial learning effects are not a unitary, simple, process, even in these relatively simple animals. We now have evidence for at least 3 distinct navigational mechanisms in fiddler crabs, suggested by the research of this dissertation, and present to some degree in the pre-existing literature. The perspective this dissertation takes to understand these multiple navigational strategies is as a set of hierarchically organized strategies, with egocentric path
integration likely serving as the primary modality by which fiddler crabs navigate, with a taxis toward certain visual stimuli serving as a backup system in situations when the path integrator is without a home vector, or is error prone. The role of the associative mechanisms uncovered in experiments 3 and 4 (and possibly supported by (T. W. K. Kim & Choe, 2010)) is currently unclear, although the results of experiment 3 suggest that associative mechanisms can modify either the form or expression of other navigational strategies, or possibly create new ones. It is my hope that future research using the methods I have described will attempt to untangle the interactions between these navigational systems, with a focus on understanding under what conditions egocentric information is and isn’t used by fiddler crabs, and how other allocentric mechanisms interact with the egocentric system.
## Tables

### 2.1

**Time spent of side of arena paired with shock**

<table>
<thead>
<tr>
<th>Effect</th>
<th>dfb</th>
<th>dfw</th>
<th>F</th>
<th>sig</th>
</tr>
</thead>
<tbody>
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<td>38.689</td>
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<tr>
<td>days</td>
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<td>60</td>
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<td>0.111</td>
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<td>days by treatment</td>
<td>3</td>
<td>60</td>
<td>4.416</td>
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<tr>
<td>blocks</td>
<td>8</td>
<td>160</td>
<td>0.91</td>
<td>0.51</td>
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<tr>
<td>blocks by treatment</td>
<td>8</td>
<td>160</td>
<td>0.266</td>
<td>0.976</td>
</tr>
<tr>
<td>days by blocks</td>
<td>24</td>
<td>480</td>
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<td>days by blocks by treatment</td>
<td>24</td>
<td>480</td>
<td>0.587</td>
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</table>

### 2.2

**First 20 min - 1 min blocks - Time spent on shock side**

<table>
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<tr>
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<th>F</th>
<th>Sig</th>
</tr>
</thead>
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<td>0.22</td>
<td>0.999</td>
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<tr>
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<td>494</td>
<td>1.078</td>
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<td>days by blocks</td>
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<td>0.912</td>
<td>0.661</td>
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<tr>
<td>days by blocks by treatment</td>
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<td>1482</td>
<td>1.196</td>
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### 2.3

**Distance Traveled**

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<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
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<td>0.454</td>
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<tr>
<td>days</td>
<td>3</td>
<td>60</td>
<td>3.232</td>
<td>0.029*</td>
</tr>
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<td>0.338</td>
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<td>blocks</td>
<td>8</td>
<td>160</td>
<td>0.642</td>
<td>0.741</td>
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<tr>
<td>blocks by treatment</td>
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<td>160</td>
<td>0.503</td>
<td>0.853</td>
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<td>24</td>
<td>480</td>
<td>3.358</td>
<td>&lt;.001*</td>
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<td>days by blocks by treatment</td>
<td>24</td>
<td>480</td>
<td>0.723</td>
<td>0.83</td>
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</table>
2.4

### Average Speed

<table>
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<th>sig</th>
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</thead>
<tbody>
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<td>0.545</td>
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<td>Shock state</td>
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<td>26</td>
<td>16.484</td>
<td>&lt;.001*</td>
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<tr>
<td>Days</td>
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<td>3.173</td>
<td>0.029</td>
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<tr>
<td>Shock state by Days</td>
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<td>78</td>
<td>7.759</td>
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<td>Shock state by Days by Treatment</td>
<td>3</td>
<td>78</td>
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3.1

### Movements to Checkerboard stimulus

<table>
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<tr>
<th>Day of training</th>
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<th>t</th>
<th>sig</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>14</td>
<td>2.71</td>
<td>0.017</td>
</tr>
<tr>
<td>Day 2</td>
<td>13</td>
<td>2.535</td>
<td>0.025</td>
</tr>
<tr>
<td>Day 3</td>
<td>13</td>
<td>1.953</td>
<td>0.073</td>
</tr>
<tr>
<td>Day 4</td>
<td>14</td>
<td>1.653</td>
<td>0.121</td>
</tr>
<tr>
<td>Day 5</td>
<td>12</td>
<td>0.739</td>
<td>0.474</td>
</tr>
<tr>
<td>Day 6</td>
<td>12</td>
<td>4.292</td>
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</table>

4.1

### Seconds on shock paired side of alley

<table>
<thead>
<tr>
<th>Effect</th>
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<th>dfw</th>
<th>F</th>
<th>sig</th>
<th>(\eta^2_p)</th>
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</thead>
<tbody>
<tr>
<td>Trial</td>
<td>4</td>
<td>176</td>
<td>3.38</td>
<td>0.011</td>
<td>0.071</td>
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<tr>
<td>Treatment</td>
<td>1</td>
<td>44</td>
<td>42.844</td>
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<td>0.493</td>
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<tr>
<td>Stimulus</td>
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<td>0.376</td>
</tr>
<tr>
<td>Trial by Treatment</td>
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<td>2.534</td>
<td>0.042</td>
<td>0.054</td>
</tr>
<tr>
<td>Trial by Stimulus</td>
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<td>5.368</td>
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<td>0.109</td>
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<tr>
<td>Treatment by Stimulus</td>
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<td>8.505</td>
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<td>0.162</td>
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<tr>
<td>Trail by Treatment by Stimulus</td>
<td>4</td>
<td>176</td>
<td>1.211</td>
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<td>0.027</td>
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</table>
4.2

Number of Seconds Spent on Shock Side of Alley - Trial 4

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<th>F</th>
<th>Fcrit</th>
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</thead>
<tbody>
<tr>
<td>Black Stimulus - Master vs Yoked</td>
<td>1</td>
<td>176</td>
<td>1.530</td>
<td>15.580</td>
</tr>
<tr>
<td>Check Stimulus - Master vs Yoked</td>
<td>1</td>
<td>176</td>
<td>24.627</td>
<td>15.580</td>
</tr>
</tbody>
</table>

4.3

Number of Seconds Spent on Shock Side of Alley - Trial 5

<table>
<thead>
<tr>
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<th>dfw</th>
<th>F</th>
<th>Fcrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Stimulus - Master vs Yoked</td>
<td>1</td>
<td>176</td>
<td>0.373</td>
<td>15.580</td>
</tr>
<tr>
<td>Check Stimulus - Master vs Yoked</td>
<td>1</td>
<td>176</td>
<td>3.300</td>
<td>15.580</td>
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</table>

5.1 2 trial blocks

Time Until Escape

<table>
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<tr>
<th>Effect</th>
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<th>F</th>
<th>sig</th>
<th>( \eta^2_p )</th>
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</thead>
<tbody>
<tr>
<td>Block</td>
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<td>212</td>
<td>5.623</td>
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</tr>
<tr>
<td>Stimulus</td>
<td>1</td>
<td>53</td>
<td>0.295</td>
<td>0.590</td>
<td>0.006</td>
</tr>
<tr>
<td>Consistancy</td>
<td>1</td>
<td>53</td>
<td>4.419</td>
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</tr>
<tr>
<td>Block by Stimulus</td>
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<td>2.804</td>
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<tr>
<td>Block by Consistancy</td>
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<td>212</td>
<td>0.632</td>
<td>0.641</td>
<td>0.012</td>
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<tr>
<td>Stimulus by Consistancy</td>
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<td>53</td>
<td>0.001</td>
<td>0.980</td>
<td>0.000</td>
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<tr>
<td>Block by Stimulus by Consistancy</td>
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<td>212</td>
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5.2 5 trial blocks

Time Until Escape

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<th>dfw</th>
<th>F</th>
<th>sig</th>
<th>( \eta^2_p )</th>
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</thead>
<tbody>
<tr>
<td>Block</td>
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<td>53</td>
<td>13.751</td>
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<td>Stimulus</td>
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<tr>
<td>Consistancy</td>
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<td>6.87</td>
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<td>Block by Consistancy</td>
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<td>0.976</td>
<td>0.074</td>
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<tr>
<td>Block by Stimulus by Consistancy</td>
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<td>53</td>
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5.3

### Time to Escape at Check level of Stimulus

<table>
<thead>
<tr>
<th>Contrast</th>
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<th>F</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block 1 vs Block 2-5</td>
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<td>104</td>
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<tr>
<td>Block 1 vs Block 2</td>
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<td>104</td>
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<td>0.947</td>
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<tr>
<td>Block 1 vs Block 3</td>
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<td>0.026</td>
<td>0.935</td>
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<tr>
<td>Block 1 vs Block 4</td>
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<td>Block 1 vs Block 5</td>
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<td>0.007</td>
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5.4

### Time to Escape at Black level of Stimulus

<table>
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</thead>
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<tr>
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<tr>
<td>Block 1 vs Block 2</td>
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<td>Block 1 vs Block 3</td>
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<td>104</td>
<td>10.530</td>
<td>0.108</td>
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<tr>
<td>Block 1 vs Block 4</td>
<td>1</td>
<td>104</td>
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<td>0.057</td>
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<td>Block 1 vs Block 5</td>
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5.5 2 trial blocks

### Distance Traveled Before Escape

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</thead>
<tbody>
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<td>0.833</td>
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### 5.6

**Distance to Escape at Check level of Stimulus**

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<td>0.145</td>
<td>0.849</td>
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<tr>
<td>Block 1 vs Block 2</td>
<td>1</td>
<td>104</td>
<td>0.012</td>
<td>0.957</td>
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<tr>
<td>Block 1 vs Block 3</td>
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<td>Block 1 vs Block 4</td>
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<td>Block 1 vs Block 5</td>
<td>1</td>
<td>104</td>
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### 5.7

**Distance to Escape at Black level of Stimulus**

<table>
<thead>
<tr>
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<tbody>
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<td>Block 1 vs Block 5</td>
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### 5.8 5 trial blocks

**Distance Traveled Before Escape**

<table>
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<tr>
<th>Effect</th>
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<td>53</td>
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<td>&lt;.001</td>
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<tr>
<td>Block by Stimulus</td>
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### 5.9 2 trial blocks

**Number of Arm Transitions Before Escape**

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<th>$\eta_{p}^{2}$</th>
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<td>53</td>
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<td>0.754</td>
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<td>53</td>
<td>0.279</td>
<td>0.600</td>
<td>0.005</td>
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<tr>
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<td>1.044</td>
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<td>0.019</td>
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</table>
5.10 5 trial blocks

### Number of Arm Transitions Before Escape

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<td>0.997</td>
<td>&lt;.001</td>
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<td>0.652</td>
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<td>53</td>
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</table>

5.11 2 trial blocks

### Average Speed Before Escape

<table>
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<td>1.158</td>
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<td>Block by Stimulus by Consistency</td>
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<td>212</td>
<td>0.827</td>
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</table>

5.12

### Average Speed at Check level of Stimulus

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<tbody>
<tr>
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<td>104</td>
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<tr>
<td>Block 1 vs Block 2</td>
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<td>Block 1 vs Block 3</td>
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<tr>
<td>Block 1 vs Block 4</td>
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<td>1.228</td>
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<tr>
<td>Block 1 vs Block 5</td>
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<td>0.702</td>
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5.13

### Average Speed at Black level of Stimulus

<table>
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<th>F</th>
<th>sig</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Block 1 vs Block 3</td>
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<td>104</td>
<td>0.270</td>
<td>0.796</td>
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<tr>
<td>Block 1 vs Block 4</td>
<td>1</td>
<td>104</td>
<td>2.552</td>
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<td>Block 1 vs Block 5</td>
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<td>8.232</td>
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5.14 5 trial blocks

<table>
<thead>
<tr>
<th>Effect</th>
<th>dfb</th>
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<th>F</th>
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<th>$\eta^2_P$</th>
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</thead>
<tbody>
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<td>0.697</td>
<td>0.003</td>
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</tbody>
</table>
Figures

2.1

A schematic diagram of an experimental alley within the place learning arena. Arrows represent the flow of information through the trial controller. Images are collected once a second by the overhead camera, sent to the Matlab controller where they are processed, and the location of the subject is determined. According to the location of the subjects, the stimulator unit is commanded to deliver electric shock to a pair of subjects. Stimuli on all walls of the alley are displayed. Experimental alley is not drawn to scale.
2.2

The percentage of each trial spent on the shock paired side of the alley, collapsed across all subjects. Black lines represent subjects in the Master condition, grey lines represent subjects in the yoked condition.

2.3

The percentage of each trial spent on the shock paired side of the alley, collapsed across all subjects, broken down into 1 minute blocks. Black lines represent subjects in the Master condition, grey lines represent subjects in the yoked condition.
The average distance in cm traveled by subjects during each trial, collapsed across all subjects. Black lines represent subjects in the Master condition, grey lines represent subjects in the yoked condition.

2.5

The average distance in cm traveled by subjects during each trial, collapsed across all subjects, broken down into 1 minute blocks. Black lines represent subjects in the Master condition, grey lines represent subjects in the yoked condition.
The average speed of subjects, in the presence and absence of electric shock application, across all 4 days of the experiment. Black lines represent subjects in the Master condition, grey lines represent subjects in the yoked condition.
3.1

A schematic diagram of an experimental alley within the place learning arena. Black and Checkerboard stimuli on all walls of the alley are displayed, as is the central dividing wall that isolated subjects on either side of the arena during training. Experimental alley is not drawn to scale.

3.2

![Graph showing the average proportion of probe trials in which subjects moved towards the “safe” side of the arena, across all 6 days of training.]

The average proportion of probe trials in which subjects moved towards the “safe” side of the arena, across all 6 days of training.
3.3

The average proportion of probe trials in which subjects moved towards the “checkered” side of the arena, across all 6 days of training. Error bars represent the SEM.

3.4

The average proportion of day 7 probe test that subjects spent on the “safe” side of the arena. Red line represents value expected according to chance (.5).
3.5

The average proportion of day 7 probe test that subjects spent on the “checkered” side of the arena. Red line represents value expected according to chance (.5).

3.6

The average number of seconds of the day 7 probe test that subjects spent on the “safe” side of the arena. Separated according to counterbalance condition.
4.1

A schematic diagram of an experimental alley within the place learning arena. Arrows represent the flow of information through the trial controller. Images are collected once a second by the overhead camera, sent to the Matlab controller where they are processed, and the location of the subject is determined. According to the location of the subjects, the stimulator unit is commanded to deliver electric shock to a pair of subjects. Black and Checkerboard stimuli on all walls of the alley are displayed, as are the central partially occluding walls. Experimental alley is not drawn to scale.
4.2

The number of seconds spent on the shock paired side of the alley, across all training (1-3) and extinction (4-5) trials. Solid lines correspond to Master groups and dotted lines correspond to Yoked groups. Black lines represent subjects who had shock paired with the black side of the alley and red lines represent subjects who had shock paired with the checkered side of the alley. 1800 on the Y-axis represents value expected due to chance (50%). Error bars represent the SEM.

4.3

The number of seconds spent on the shock paired side of the alley, collapsed across all trials. Solid lines represent subjects in the Master condition, dotted lines represent subjects in the yoked condition.
The number of seconds spent on the shock paired side of the alley, during each minute of the first extinction test (trial 4). Solid lines correspond to Master groups and dotted lines correspond to Yoked groups. Black lines represent subjects who had shock paired with the black side of the alley and red lines represent subjects who had shock paired with the checkered side of the alley. 30 on the Y-axis represents value expected due to chance (50%).
The number of seconds spent on the shock paired side of the alley, during each minute of the second extinction test 25 hours after training (trial 5). Solid lines correspond to Master groups and dotted lines correspond to Yoked groups. Black lines represent subjects who had shock paired with the black side of the alley and red lines represent subjects who had shock paired with the checkered side of the alley. 30 on the Y-axis represents value expected due to chance (50%).
Top view diagram of the Y-maze arena. A. The starting position of the subjects for all trials. B. The locations of the two target escape holes, which lead to wet, dark artificial burrows under the y-maze, and can be individually closed off. C. Representation of the visual stimulus holders that were placed vertically behind the escape holes on both target arms of the arena. All components drawn to scale.
5.3

The average time in seconds before subjects entered the escape burrow across blocks of 2 trials collapsed across all groups to highlight the significant main effect of block.

5.4

The average time in seconds before subjects entered the escape burrow across blocks of 2 trials collapsed across all trials to highlight the significant main effect of spatial consistency.
The average time in seconds before subjects entered the escape burrow across blocks of 2 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.

The average time in seconds before subjects entered the escape burrow across blocks of 5 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.
5.7

The average distance traveled before subjects entered the escape burrow across blocks of 2 trials collapsed across all groups to highlight the significant main effect of block.

5.8

The average distance in centimeters subjects traveled before they entered the escape burrow across blocks of 2 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.
The average number of transitions from one arm of the arena to another made before subjects entered the escape burrow across blocks of 2 trials collapsed across all groups to highlight the significant main effect of block.
The average number of movements from one arm of the Y-maze to another subjects made before they entered the escape burrow across blocks of 2 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.
The average speed of movement of subjects before they entered the escape burrow across blocks of 2 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.
The average speed of movement of subjects before they entered the escape burrow across blocks of 5 trials for all 4 groups. Red lines represent subjects in checkered condition, Black lines represent subjects in the black condition. Solid lines represent subjects in the spatially consistent condition, dashed lines represent subjects in the spatially inconsistent condition. Error bars represent the SEM.
References


