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Conditioned Territory Defense in Madagascar Hissing Cockroaches (Gromphadorhina Portentosa)

Macy R. Albaitis
Bucknell University, mra011@bucknell.edu

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Conditioned Territory Defense in Madagascar Hissing Cockroaches (*Gromphadorhina Portentosa*)

By

Macy R. Albaitis

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Approved by:

Adviser: Regina Gazes

Second Evaluator in major: Kevin Myers

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Abstract

In male blue gourami fish (*Trichogaster trichopterus*), signaled presentation of a male rival produces an aggressive conditioned response that results in increased likelihood of the male winning the contest (Hollis, 1984). I replicated this classic study in a species which also shows territorial aggression: the Madagascar hissing cockroach (MHC; *Gromphadorhina portentosa*). During training, four adult male MHCs were designated as "CS+" animals and received a light (NS) followed by visual access to a rival male (US). Simultaneously, another group of four adult male MHCs designated as "UNC" animals received the light (NS) and visual access to a rival male (US) at random intervals, such that the light was not predictive of the appearance of the male. During testing, each CS+ animal was paired with an UNC animal, the light was presented, and the animals were given direct physical access to each other. CS+ animals won all four of these test fights, indicating conditioned territorial aggression in MHCs. Follow up experiments revealed that signaled presentation of a rival male may produce an aggressive conditioned response in male MHCs. These results suggest an important ecological role for classical conditioning in MHCs.

Keywords: classical conditioning, insect cognition, entomology, territory defense, social rank, conditioned territorial aggression

Introduction

Classical conditioning, often referred to as Pavlovian conditioning, is a well-studied and well-documented form of learning (Domjan, 2005). Conditioning hinges on a learned association between a previously unknown neutral stimulus, such as a tone or a light, and an ecologically relevant unpaired stimulus, like food, mates, or rival conspecifics. Unpaired stimuli naturally elicit associated unpaired responses, for example, salivation, arousal, or aggression respectively. During conditioning, the neutral stimulus is repeatedly paired with the unpaired stimulus, such that the neutral stimulus is predictive of the occurrence of the unpaired stimulus. Learning is ascribed when presentation of the neutral stimulus alone elicits the unpaired response. Once this occurs, the neutral stimulus has become the conditioned stimulus and the behavior it elicits is the conditioned response (Domjan, 2005).

Classical conditioning has been demonstrated in a variety of species including rodents, fish, humans, and birds (Bouslama et al., 2005; Mahometa & Domjan, 2005; Thompson & Sturm, 1965; Woodruff-Pak et al., 1993), and is even demonstrable in simpler organisms such as amoebas and sea slugs (Carew et al., 1981; Ildefonso et al., 2012). That classical conditioning is such a widespread phenomenon suggests that it likely confers some fitness benefit to animals that employ it. For example, a dog that salivates upon receiving a signal indicating that food will soon arrive should show better or faster digestion than a dog who does not learn this association. However, until 1984, the vast majority of literature on classical conditioning was focused on characterizing the details of learning processes and did not explore the benefits of conditioning to the subjects themselves.

In 1984, Hollis conducted a study with blue gourami fish (*Trichogaster trichopterus*) to determine whether males that were conditioned to expect the appearance of a rival male would

be more likely to win a territorial interaction than males without that training. The blue gourami has a specialized mating behavior that requires territorial access to create a "bubble nest" that attracts females for mating. Territory defense therefore has major ecological implications on reproduction in this species (Clifton, 1990; Krebs, 1980). In Hollis's (1984) study there was a conditioned group that was classically conditioned that a light (neutral stimulus) predicted the presence of an intruder (unpaired stimulus). Importantly, the study included an unpaired group that received exposures to both the light and the intruder but separated in time such that the light was not predictive of the intruder (Hollis, 1984). The conditioned subjects showed more aggressive behaviors and were more successful in territory defense compared to unpaired subjects (Hollis, 1984), indicating that conditioning provides an advantage in biologically relevant situations like territory defense in this species.

Despite the vast research on classical conditioning across species, there is a lack of research on classical conditioning in invertebrates such as insects, with the exception of bees (Aquino et al., 2004; Laloi & Pham-Delègue, 2004; Mamood et al., 2009; Mc Cabe et al., 2007). Given that invertebrates vastly outnumber vertebrates in both the number of species and in total biomass (Yinon, 2018), this represents a large gap in our knowledge of classical conditioning (Menzel, 1993). Cockroaches are considered to be among the oldest insects on earth and understanding learning in cockroach species could reveal the earliest mechanisms and purposes of classical conditioning (Schweid, 2021). The few studies of classical and operant conditioning that exist in cockroaches have primarily used scent as a neutral stimulus and taste as an unpaired stimulus (Dixon et al., 2016; Watanabe & Mizunami, 2007). While olfactory cues are important, some species, like the Madagascar hissing cockroach (*Gromphadorhina portentosa*), show complex social behavior similar to the blue gourami that may be fruitful avenues for studying

classical conditioning. Male Madagascar hissing cockroaches defend territories from rival males, and these territories have important implications for resource acquisition and reproduction (Guerra & Mason, 2005). Territorial disputes are settled through signals, displays, and physical fights between males (Guerra & Mason 2005, Clark et al. 1995). Behaviors seen in physical fights range from overt aggression, such as headbutting or pushing, to acoustic sounds, or "hisses" (Mowles et al., 2021). Fights are energetically and physically costly, but the benefits of the fight are also high, including increased access to females and territory (Guerra & Mason, 2005; Mowles et al., 2021). During a fight, the decision to submit or attempt to dominate typically depends on the information provided by the opponent at the outset of the interaction (Mowles et al., 2021). Therefore, a male may be more likely to win a territorial contest by being faster to show aggressive behavior.

This study is a replication and extension of Hollis's (1984) study to conditioned territorial defense in Madagascar hissing cockroaches. Following modified procedures, this thesis study will provide insight into the learning capabilities of insects in the ecological context of defending territories. If conditioned male Madagascar hissing cockroaches show more and faster aggression and are more likely to win their fights than unpaired males, then it will indicate that Madagascar hissing cockroaches can be classically conditioned using ecologically relevant stimuli and will suggest an ecological advantage to classical conditioning in this species.

General Methods

This study took place between May 31, 2021, and August 5, 2021.

Subjects and Housing

Subjects for this study were eight full-grown, captive-born adult male Madagascar hissing cockroaches (*Gromphadorhina portentosa*) housed individually at Bucknell University in Lewisburg, PA, USA. Subjects weighed approximately 8 grams at the beginning of the study. The average length of the subjects was 61.12 ± 2.24 mm, and the average girth was 21.10 ± 0.68 mm.

There were four stimulus animals, all full-grown adult male Madagascar hissing cockroaches, socially housed with each other. Like the subject animals, stimulus animals weighed an average of 8 grams at the beginning of the study. The average length of the stimulus animals was 64.24 ± 3.22 mm, and their girth was 21.09 ± 1.18 mm.

Subjects were housed individually in clear, acrylic holding enclosures that also served as the apparatuses for this experiment. Each enclosure was equipped with an inch-thick layer of Zoo Med Eco Earth loose Coconut Fiber substrate, two sections of an egg carton were used as shelter, and petri dishes for food and water. All stimulus animals were housed in a 10-gallon glass aquarium equipped with Zoo Med Eco Earth loose Coconut Fiber substrate, a coconut shell hut, and two petri dishes for food and water.

The housing room was kept at a set temperature of 26°C with a 12-hour dark to 12-hour light cycle (dark from 3:30 AM to 3:30 PM). Testing occurred during the dark cycle, and a red light was used to illuminate the testing room during research to not disturb the subjects' nocturnal activity. The cockroaches had ad libitum access to food and water but did not have access to food and water during training or testing sessions. All subjects were fed a mixture of monkey chow crumbs and wheat germ.

Apparatus

The subjects' housing enclosures served as the apparatuses for this study. Individual stimulus animals were placed in a similar housing enclosure during training and testing sessions. The side walls of the housing enclosures were covered with black vinyl to prevent subjects from seeing other training or testing pairs. Each housing enclosure had an opaque and a clear sliding door at each end that could be lifted to provide visual or tactile access to another conspecifics (Figure 1). The enclosures were positioned so that one end of the subject's enclosure was flush with the stimulus animal's testing enclosure. A simple circuit containing a battery pack with switch soldered to a small light bulb was adhered to the left side of each subject's housing enclosure. This light served as the neutral stimulus for this experiment.
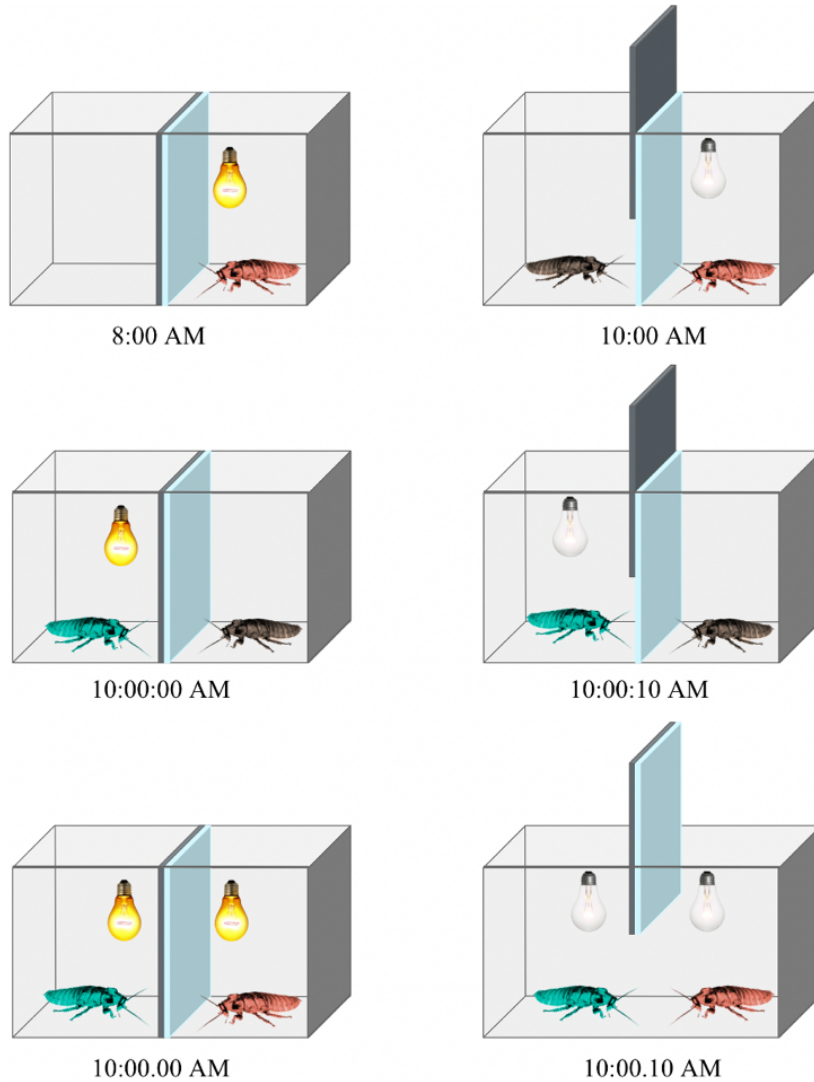
Figure 1. *Top left.* Training procedure for the UNP roaches (in red). *Center.* Training procedure for the CS+ roaches (in green). Stimulus roaches appear in dark brown. *Bottom.* Testing procedure for the CS+ and UNP roaches.

Procedure

Before training and testing sessions, a clear in-enclosure divider was placed at the midpoint of the subject's housing enclosure. All objects in the enclosure (egg carton, food dish, and water dish) were placed behind the divider away from the subject to create a clear area for

training and testing. The experiment was divided into three phases: habituation, training, and testing, presented in that order.

Habituation. The habituation phase for this study took place between May 31, 2021, and June 4, 2021. The purpose of this phase was to acclimate the subject animals to handling, to the test environment, and to the movement of the door. Habituation procedures took place for five days before training began. For the first two days of habituation, all subjects and stimulus roaches were each handled for one minute, then placed in their respective training locations. The stimulus animal was placed in the stimulus animal apparatus, flush against the door to the subject animal's enclosure. A one-minute acclimation period was followed by lifting the opaque door slowly to allow for twenty minutes of uninterrupted visual exposure to the stimulus animal. The clear door was still in place, preventing the animals from physically accessing one another. For the final three days of habituation, the experimenter followed the same procedures of handling and acclimation as in the first two days of habituation. However, instead of 20 minutes of uninterrupted visual access to the partner roach, the experimenter lifted the opaque door for 15 seconds with an intertrial interval of 60-120 seconds (M=90 seconds). The door was lifted 10 times for each session in the final three days of habituation. Importantly, the light was never turned on during this phase of training.

Training. The training phase of this study took place between June 7, 2021, and July 28, 2021. Subjects received training five days per week. As in habituation, for each training trial the assigned stimulus cockroach was removed from its home tank and placed in the stimulus enclosure adjacent to the subject's housing enclosure. Subjects and stimulus animals were handled for 1 minute, then given a 1-minute acclimation period in their respective enclosures before any procedures related to the experiment began. The opaque and clear doors were

removed from the stimulus roach's enclosure before the start of all sessions, leaving the clear and opaque doors for the subject roach's enclosure in place.

Four subjects were semi-randomly assigned to the conditioned (CS+) group, and four assigned to the unpaired (UNP) group. During training, the CS+ group received the neutral stimulus (light) followed by the presentation of the unpaired stimulus (visual access to the stimulus animal). In contrast, the UNP group received the neutral stimulus (light) followed by a long delay (M=90 min, range: 40-120 minutes), then the presentation of the unpaired stimulus (visual access to the stimulus animal). Importantly, both groups received equal exposure to both the neutral and unpaired stimulus; however, the neutral stimulus was only predictive of the unpaired stimulus in the conditioned group. The clear door separating the subject from the stimulus animal was never lifted during training and prevented subjects from physically interacting with the stimulus animal. All subjects received 10 training trials per day for 35 days of training, for a total of 350 training trials.

CS+ Group. Beginning at 10 am, an in-enclosure divider was placed in the midpoint of the subject animal's housing enclosure, and all objects were removed, the light bulb in the subject's enclosure was lit for 10 seconds. The light was then turned off and the opaque door separating the subject from the stimulus animal was lifted, allowing visual, but not physical, access to the stimulus animal through the clear door. The opaque door remained open for 15 seconds. The clear door separating the subject from the stimulus animal was never lifted during training, preventing subjects from physically interacting with the stimulus animal. This was repeated for 10 trials per session, with an intertrial interval of 60 to 120 seconds (M=90).

UNP Group. Training for the UNP group was divided into two phases, separated by an average of 90 minutes: presentation of the neutral stimulus, and presentation of the unpaired

stimulus. Like the CS+ subjects, each UNP subject received 10 presentations of the neutral

stimulus (the light) on each test day. However, unlike the CS+ subjects, the UNP subjects

received these presentations an average of 90 minutes before the presentation of the NS.

Beginning between 8:00 and 9:20 AM, the in-enclosure divider was placed in the midpoint of the

subject animal's housing enclosure and all objects in the enclosure were removed. Following

handing and acclimation, the light was illuminated for 10 seconds and then turned off. No

stimulus animal was placed in the stimulus animal enclosure, and the opaque door was not lifted.

As in training for the CS+ group, this was repeated for 10 trials with an intertrial interval of 60 to

120 seconds (M=90 seconds) between trials.

Each UNP subject also received 10 presentations of the unpaired stimulus (visual access

to the stimulus animal) each day. These presentations took place at the same time as the training

for the CS+ subjects, 10:00 AM. For these sessions the set up and procedures were identical to

that used in training for the CS+ group, with the exception that the light was not turned on prior

to the opaque door being opened. Following handling and acclimation, the opaque door was

opened, allowing for the subject to have visual, but not physical, access to the stimulus animal

for 15 seconds. After 15 seconds of visual access, the door was closed. This procedure was

repeated 10 times with an intertrial interval of 60 to 120 seconds (M=90 seconds).

Test 1

Methods

Test 1 took place at 10:00 AM on July 29, 2021. The territorial defense test occurred after

350 trials of training. The CS+ and UNP subjects were paired, and their home enclosures were

placed flush door-to-door. All subjects were weighed prior to the testing phase. As in training,

prior to testing, the in-enclosure dividers were placed at the midpoint of each enclosure and

subjects were picked up and handled for 1-minute and then placed on the testing side of their

enclosure. Handling was followed by a 5-minute acclimation period.

Unlike in training, before the test began, the clear doors were removed from both

enclosures, leaving only one opaque door in place. The lights in both enclosures were turned on

for 10 seconds. The lights were then turned off and the opaque door opened, allowing the CS+

and UNP subjects to have physical access to each other for the first time. The door remained

open for 15 minutes. After 15 minutes, the animals were picked up and separated to their

respective home enclosures and the opaque doors closed. Experimenters live coded agonistic and

submissive behaviors of both animals during the test using an ethogram (Table 1). All test trials

were filmed.

Ethogram

Behavior	Code	Description	Corresponding Behavior in Previous Paper
		Dominant Physical Behaviors	
Approach	Ap	Walk or run toward another animal. Only antennae contact made.	Approach
Climb On	CO	Climb on top of another animal.	Sit on
Head Butt	HB	Lower head and rapidly hit another animal with horns.	Butt
Push	P	Lower head and slowly push on another animal with horns.	N/A
Attack	At	Repeatedly push, head butt, and approach another individual.	Lunge (combined with others)
Wrestle	Wr	Two animals engage in attack behavior towards one another often accompanied by spinning around each other. Neither animal retreats, and both animals must engage in attack/head butting behavior.	N/A
		Dominant Display Behaviors	
Posture	Po	Stand tall, raise head and front of body up, abdomen stays down.	Stilt
Head Down	HD	Standing still, lower head below carapace. Similar to the position used in Head Butt or Push.	N/A
Waggle	W	Vigorously shake abdomen.	Abdomen Thrash
Hiss	H	Emit a hissing sound.	Agonistic Hiss
Abdominal Thrust	AbTh	Lift lower abdomen up in quick motion.	Abdomen Extension
		Submissive Behavior	
Retreat/Withdraw	Wd	Walk or run away from an approaching animal.	Retreat
Freeze	F	Lower head and body, do not move.	Crouch
Side Tilt	ST	Lower head, lean body toward other	Sideways-Lean

Table 1. Ethogram used for aggressive and submissive interactions during the testing phase.

Data Analysis. All data were analyzed using R statistical software (R Core Team, 2021).

The total wins for both the CS+ and UNP subjects were summed and graphed. The winner and loser of the aggressive contest was determined by a count of the dominance behaviors, the winner being deemed by the subject with the most dominance behaviors between the testing pair. Because of the low subject numbers (4 in each condition) statistical analyses were not conducted on the win/lost data.

Dominant and submissive behaviors were summed for each subject. A generalized linear mixed model was conducted on the total number of behaviors shown, with behavior type (dominance, submissive) and training condition (CS+, UNP) as independent variables, and subject as a random factor.

The latency to the first dominance and first submissive behavior for each subject in Test 1 was recorded as the number of seconds from when the test began until the behavior occurred. If a dominant or submissive behavior never occurred, a value of 900 seconds was recorded. Another generalized linear mixed model was conducted on the latency to initiate first behavior, and likewise included behavior type (dominance, submissive) and training condition (CS+, UNP) as independent variables, and subject as a random factor.

Results and Discussion

Subjects and Stimulus Animals. In Madagascar hissing cockroaches, aggressiveness is a function of size in which intermediate sized subjects are most aggressive compared to smaller and larger individuals (Logue et al., 2011). The average weight of the UNP at test day was 7.39 $\pm$ 1.27 g and the average weight of the CS+ at test day was 7.95 $\pm$ 1.39 g. Due to the importance of size in aggressive interactions, it is pertinent that the subjects were size matched to reduce the room for size influencing the result of the aggressive encounters. An independent samples t-test revealed that the weights of the CS+ and UNP subjects on the test day did not differ significantly

from one another (t (6) = 0.60, p = 0.573). Similarly, independent t-tests comparing both length

and girth revealed that the CS+ and UNP subjects did not differ significantly in either of these

size variables (length: t (6) = 0.72, p = 0.498; girth: t (6) = 1.62, p = 0.154). Because the

measurements of the two groups did not differ significantly on test day, size differences cannot

explain any contest outcome differences in Test 1.

Total Wins. In all four test contests, CS+ subjects won the contest and UNP subjects lost

the contest (Figure 2). Due to small sample sizes, it was not possible to conduct statistical tests

on these data. However, the consistency of this trend suggests that CS+ training may have

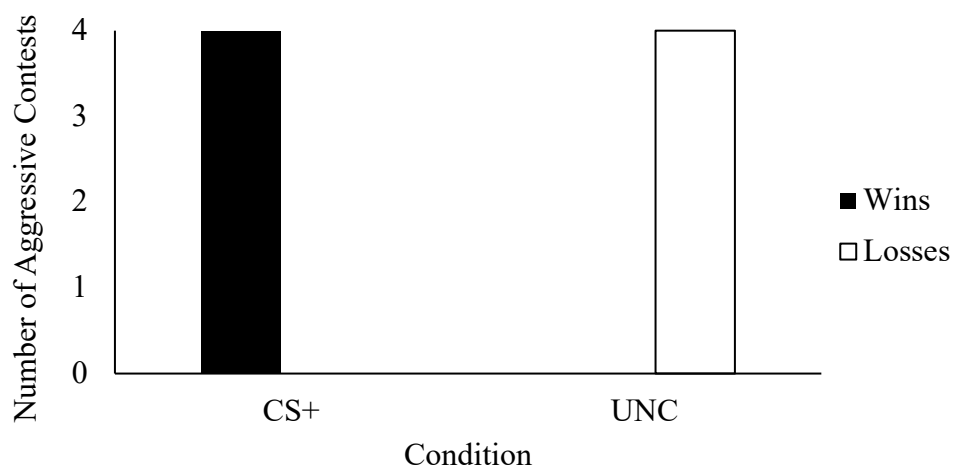conferred a competitive advantage to these animals.

Figure 2. Sum of wins and losses for Test 1 between animals trained in the CS+ and UNP

conditions.

Average Behaviors. A generalized linear mixed model comparing submissive and

dominant behaviors between CS+ and UNP conditions for Test 1 revealed no main effect of

condition (Figure 3., F (6) = 1.14, p = 0.327). There was a significant main effect of behavior,

such that subjects showed more dominant behaviors than submissive behaviors during the

contests (Figure 3., F (6) = 40.57, p < .001). Most importantly, the model revealed that there was

a significant interaction between behavior and condition (Figure 3., $F(6) = 16.35$, $p = 0.007$), such that CS+ subjects showed more dominant behaviors and less submissive behaviors than did UNP subjects. These results suggest that the mechanism that allowed the CS+ subjects to have a competitive advantage over the UNP subjects in contest outcomes was increased aggressive behaviors in response to the conditioned stimulus. This result is similar to the behaviors seen in Hollis's (1984) paper, in which the conditioned animals showed more aggressive behaviors in comparison to the unpaired animals.
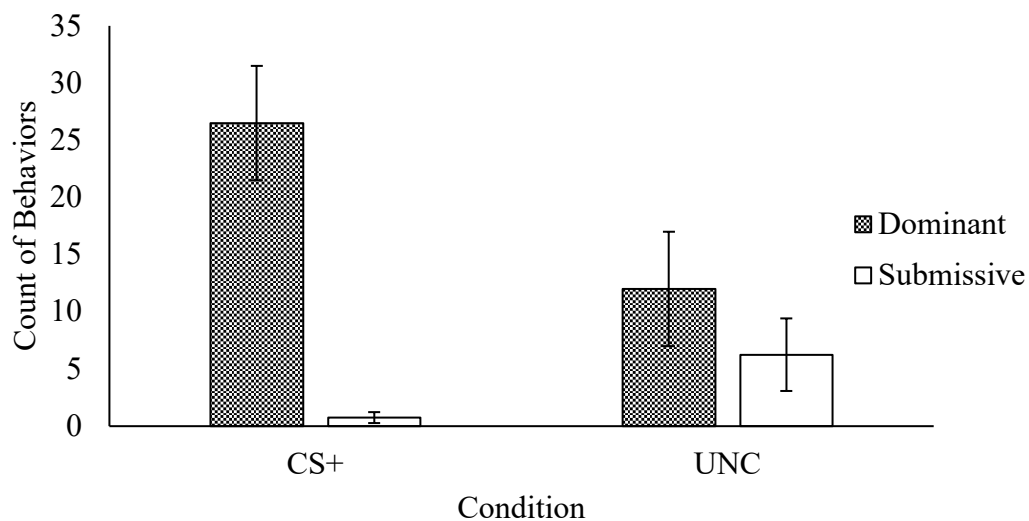

Figure 3. Average count of submissive (S) and dominant (D) behaviors for CS+ and UNP subjects in Test 1.

Latency. A generalized linear mixed model comparing latency to initiate submissive and dominant behaviors for both CS+ and UNP subjects revealed a significant main effect of behavior such that dominance behaviors occur significantly earlier in the contest than did submissive behaviors for both CS+ and UNP subjects ($F(6) = 12.23$, $p = 0.013$). The generalized linear mixed model revealed no main effect of condition on latency ($F(6) = 1.51$, $p = 0.265$), and no interaction between condition and behavior type (Figure 4., $F(6) = 2.60$, $p = 0.159$) for Test

1. This result shows that regardless of training condition, dominance behaviors occurred significantly earlier in a contest than did submissive behaviors. This provides insight into the nature of aggressive interactions for this species. Hollis (1984) suggested that for her fish, quicker initiation of dominance behaviors by CS+ animals contributed to a competitive advantage over the UNP. However, the results of the present study suggest that the speed of behavior initiation may not play as significant a role in territory defense in Madagascar hissing cockroaches. Instead, in Madagascar hissing cockroaches, the conditioned stimulus may simply elicit more, rather than faster, dominant behaviors.
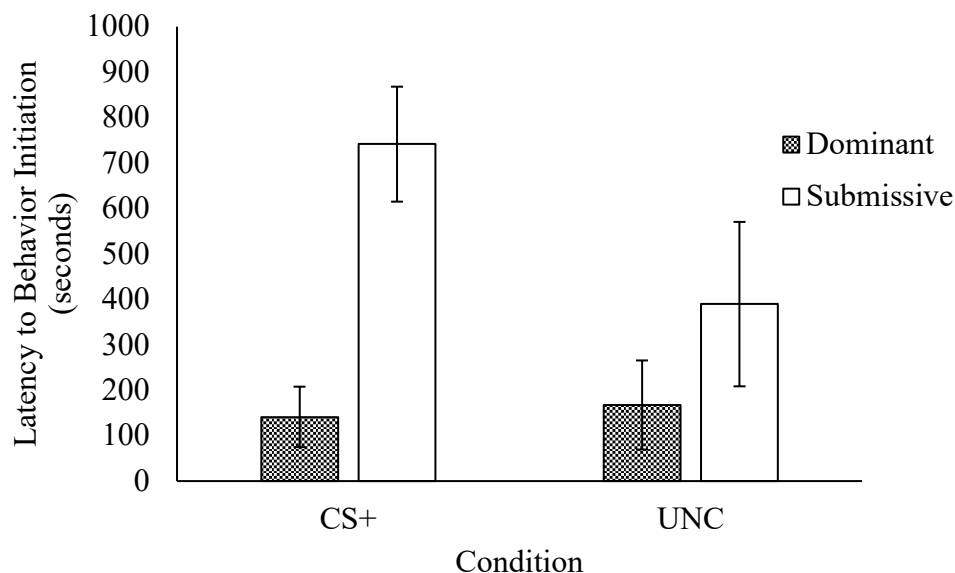
Figure 4. Average latency to initiate submissive and dominant behaviors for CS+ and UNP subjects in Test 1.

Overall, CS+ animals won 100% of their test contests and showed more dominance and less submissive behavior than UNP animals. These results suggest that Madagascar hissing cockroaches have the ability to be classically conditioned such that neutral stimuli in the environment can become conditioned stimuli predictive of a territory intruder. Furthermore, they

indicate that the ability to be classically conditioned confers a competitive advantage in territory defense.

However, there are alternative explanations for these results. First, it is possible that, rather than being classically conditioned during training, the CS+ animals were showing pseudo conditioning. Pseudo conditioning is a case in which the neutral stimulus of the light conditioned the CS+ subjects to be generally more aggressive. If this were the case, it would result in the CS+ subjects winning all of their contests and having high levels of dominance behaviors without necessarily having learned anything about the relationship between the light and the appearance of a rival male. If pseudo conditioning occurred, then the presentation of a rival without a light should also result in similarly high contest wins and dominance behaviors by the CS+ subjects. However, if classical conditioning did occur, then the presentation of a rival without a light will result in fewer contest wins and dominance behaviors by the CS+ subjects. This is because, if the CS+ subject is classically conditioned, the condition without an early indicator of an aggressive contest will not allow the CS+ subject to prepare for a fight and will even the playing field for an aggressive interaction.

Second, because the UNP animals received 350 training trials in which the light was never followed by a rival male, it is possible that they were conditioned that the light was predictive of the *absence* of a rival male. If the UNP subjects were conditioned in this way, they would have lost the contests in Test 1 regardless of the opponents' training. This would mean that the CS+ animals could have won their contests even without being conditioned. If this is the case, then UNP subjects should continue to lose their contests when presented with a new, untrained rival male at test. However, if this was not the case, then UNP subjects would be

expected to win approximately 50% of their contests against an untrained male, as both subjects would be equally unprepared for the contest.

Test 2 was conducted to address these alternative explanations.

Test 2

To better understand the mechanisms behind the performance patterns seen in Test 1, two types of tests were conducted in Test 2. To assure that subjects' behavior was driven by their own learning during training rather than a reaction to their partner's behavior at test, both UNP and CS+ animals were paired with a new untrained opponent during test contests.

To test for pseudo conditioning, CS+ subjects were presented with a similar test to Test 1, with two major changes. First, their partner was a novel untrained rival male. Second, and most importantly, the light was not presented before the appearance of the opponent. If the results of Test 1 can be explained by pseudo conditioning, then CS+ subjects should show similar outcomes and behaviors to those shown in Test 1, winning their contests, and showing more dominance and less submission than their opponents. However, if the results of Test 1 were explained by classical conditioning, CS+ subjects would be expected to lose most of their Test 2 contests, and to show less dominance behavior and more submissive behavior than they did in Test 1.

To see if the behavior of UNP subjects alone could explain the outcomes of Test 1, UNP subjects were likewise presented with a similar test to Test 1, but with one major change. As in Test 1, UNP animals were presented with a light before the appearance of the opponent. However, in Test 2, the opponent was a new untrained male. If UNP animals lost their contests in Test 1 because they had learned that the light predicted the *absence* of a rival male, they

would be expected to lose 100% of the contests against the new untrained opponents in Test 2. Alternatively, if UNP animals were truly "unpaired" and had learned nothing about the relationship between the light and the appearance of a rival male during training, they would be expected to win approximately 50% of the contests against the new untrained opponents in Test 2.

Methods

Follow up tests were conducted between August 3, 2021, and August 4, 2021, 5 days after the completion of Test 1. Subjects received 1 training trial between the end of Test 1 and the start of Test 2.

CS+ Subjects. Each of the four CS+ subjects were paired with a novel male of approximately equal size that had received no training. There was no difference in the average weights of the novel and CS+ males (independent samples t-test: $t(6) = 0.27$, $p = 0.795$; novel males: 7.71 ± 1.10 g; CS+ males: 7.95 ± 1.39 g). Methods were the same as those used in Test 1, with the important exception that the light was not turned on in the 10 seconds before the door between the subject and the rival was opened.

UNP Subjects. Each of the four UNP subjects were paired with a novel male of approximately equal size who had received no training. There was no difference in the average weights of the novel and UNP males (independent samples t-test: $t(6) = 0.25$, $p = 0.810$; UNP males: 7.21 ± 0.70g; UNP males: 7.39 ± 1.27g). Methods were otherwise the same as those used in Test 1. Importantly, as in Test 1, the light was presented for 10 seconds before the door between the subject and the rival was opened.

Data Analysis. The total number of wins and losses for both the CS+ and UNP subjects were summed and graphed to reveal preliminary trends. Because of the small subject numbers (4 in each condition), statistical tests were not conducted on these outcomes.

The behavior of each subject on Test 2 was compared to their behavior in Test 1. Two separate generalized linear mixed models were conducted, one for CS+ subjects and one for UNP subjects. Both generalized linear mixed models compared the number of behaviors, with test (Test 1, Test 2) and behavior type (dominance, submission) as independent variables, and subject as a random factor.

Results and Discussion

Total Wins. Unlike in Test 1 in which all CS+ subjects won their contests, in Test 2 when no light was present, only one of four CS+ subjects won their contests (Figure 5). This supports the hypothesis that CS+ animals were classically conditioned during training.

As in Test 1, UNP animals continued to lose their contests, with three of four losing their contests and the one remaining subject engaging in an inconclusive contest in which no dominance or submissive behaviors occurred (Figure 5). These results are therefore difficult to interpret, as the 75% loss rate falls between the two theoretically supported outcomes of 50% and 100% losses.
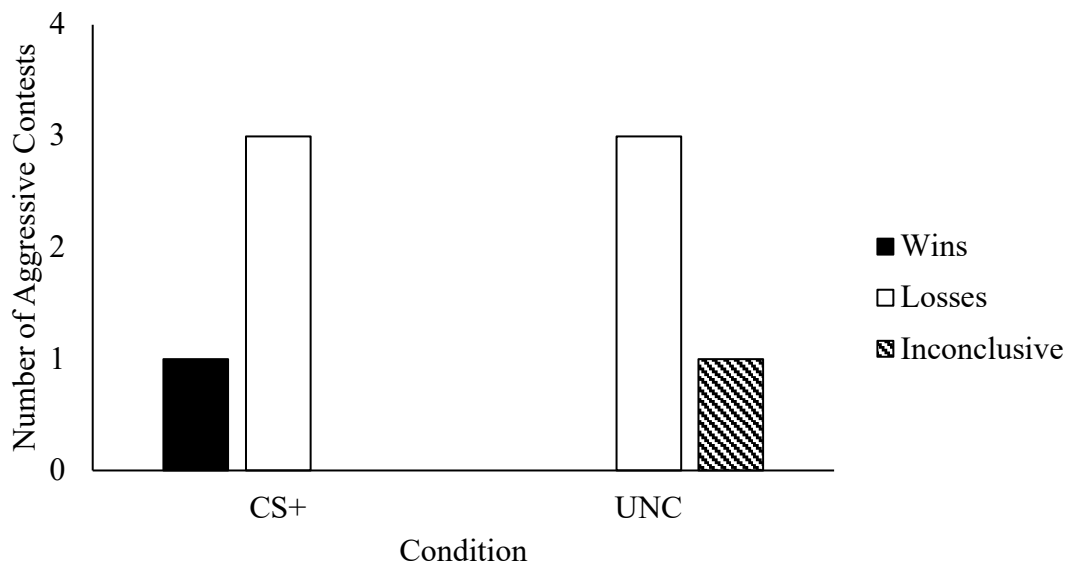
Figure 5. Sum of wins, losses, and inconclusive aggressive contest results for Test 2 for CS+ and UNP subjects.

Comparing Test 1 and Test 2. A generalized linear mixed model comparing the count of submissive and dominant behaviors for CS+ subjects between Test 1 and Test 2 revealed no main effect of test ($F (1, 12) = 4.25$, $p = 0.062$), but a significant main effect of behavior (Figure 6., $F (1, 12) = 20.66$, $p < 0.001$) such that all subjects performed more dominance behavior compared to submissive behavior. Importantly, the model revealed a significant interaction between test and behavior, such that CS+ subjects displayed fewer dominant behaviors in Test 2 compared to Test 1 (Figure 6., $F (1, 12) = 4.80$, $p = 0.049$). Since more dominant behaviors were elicited in the presence of the light signal compared to the absence of the light signal, this result indicates that the behavior displayed by CS+ subjects in Test 1 cannot be explained by pseudo conditioning, and instead suggest that CS+ subjects were classically conditioned to recognize the light signal predicted the presence of a rival. This is consistent with the observed patterns of wins and loses between Test 1 and 2 for these subjects.
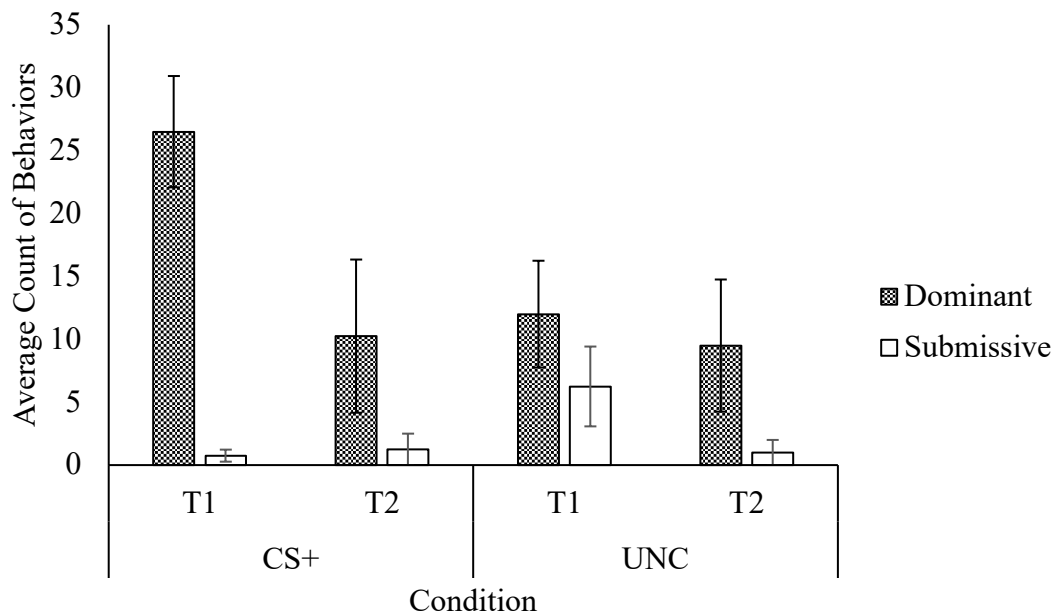

Figure 6. Comparison of submissive and dominant behaviors between Tests 1 and 2 for CS+ and UNP subjects.

A generalized linear mixed model comparing the count of submissive and dominant behaviors for UNP subjects between Test 1 and Test 2 revealed no main effect of test number (Figure 6; $F(1, 9) = 1.44$, $p = .261$), no interaction between test number and behavior type ($F(1, 9) = 0.182$, $p = .680$), and no main effect of behavior type ($F(1, 9) = 4.87$, $p = .055$). Together these findings indicate that UNP animals did not change their behavior when presented with an untrained partner compared to a CS+ trained partner.

Although there was no significant main effect of test, there was a trend towards UNP subjects showing less submission in Test 2 compared to Test 1, making it difficult to rule out the possibility that they were responding to the increased aggression of CS+ subjects in Test 1. The inconclusive results of both the contest outcomes and the behavioral data analyses for the UNP animals do not allow strong conclusions about whether they experienced conditioning during training. Because the UNP subjects were not tested in the absence of the light, it cannot be ruled

out the UNP subjects were conditioned such that the light predicted the absence of a rival male. However, Hollis (1984) did explicitly test this, by training an additional group of subjects without any experience with the light. These males showed more aggression at test when the light was presented than did the UNP subjects, suggesting that the UNP subjects had indeed learned that the light predicted the absence of a rival. It is therefore likely that the same is true for the UNP subjects.

One additional explanation of the results of Test 2 for UNP animals is that loser effects may have played a role in the similarity of behavior they showed between Tests 1 and 2. Loser effects exist when an animal is more likely to lose a subsequent contest after losing an initial contest. This effect has been shown to exist in other social cockroach species (Kou et al., 2019). Because all UNP subjects lost their aggressive contests in Test 1, their losses in Test 2 may not have indicated anything about their conditioning experience but could instead been due to that initial loss. Given that CS+ animals won their first contests but mostly loss their second contests, winner effects are unable to account for their performance. Winner and loser effects are governed by separate physiological mechanisms and exist independent from one another (Dugatkin, 1997). Furthermore, loser effects have not been studied extensively in this species; therefore, further studies are needed to determine if this possibility can explain the results.

General Discussion

In this study, signaled presentations of territory intruders resulted in the conditioning of aggressive behavior in adult male Madagascar hissing cockroaches. When given the opportunity to defend their territories against an intruder in a signaled encounter, the males that received conditioning training (CS+) won all of their contests and demonstrated significantly more

dominant and less submissive behavior than rivals that did not receive this training (UNP). In contrast, when presented with untrained rivals that were not signaled with a light, the CS+ subjects lost their contests and showed less dominance behavior, indicating that their behavior in contests that were signaled by the light was due to classical conditioning rather than pseudo conditioning. These results suggest an important role for classical conditioning in this species, in which the ability to be classically conditioned may provide a means by which territorial males increase the likelihood of a successful territory defense.

Hollis found similar results in her study on conditioned territory defense in blue gourami fish (Hollis, 1984). Classically conditioned blue gourami subjects performed more aggressive behaviors in a contest preceded by a conditioned stimulus compared to an unpaired or unpaired subject (Hollis, 1984). Hollis suggests that for blue gourami, "the best defense is a good offense," such that the most effective way of defending a territory is to enhance the offense of that territory through increased aggression or dominance behaviors (Hollis, 1984). In Experiment 1 of Hollis's study, the conditioned subject delivered significantly more aggressive behaviors in 3-minute, 5-minute, and 10-minute intervals compared to their control pair mates. Likewise, CS+ cockroaches in the present study showed more dominance and less submissive behavior than their UNP opponents. That the classically conditioned subjects showed more aggressive behavior and were more likely to win their contests in both studies suggests that classical conditioning bestows an ecological benefit to animals that defend territories.

In blue gourami, males who were aggressive early were more likely to win the contest. Classical conditioning may therefore have conferred an advantage by allowing males to begin aggressing sooner, thereby winning the contest. The results from the present study differ from Hollis's study in this aspect, as the CS+ and UNP subjects did not differ significantly in latency

to initiate an aggressive interaction. Indeed, there was no relationship between the subjects that initiated the first dominance behavior and the subject who won the contest in the cockroaches.

The ability to successfully defend a territory or maintain social rank is important to Madagascar hissing cockroaches in terms of reproductive benefits and overall fitness. The proposed benefits of territory defense have included access to females and food (Ewing, 1972; Ritter, 1964), both of which enhance ecological fitness and chances of survival. Male-male aggression maintains social ranking within a colony (Clark & Moore, 1994; Guerra & Mason, 2005). Social rank within a social colony of Madagascar hissing cockroaches confers similar reproductive and, thus, fitness benefits (Clark, 1998). For example, female Madagascar hissing cockroaches can discriminate between males of different social status on the basis of olfactory cues (Leibensperger et al., 1985), and prefer to mate with high-ranking males. As a result, subordinate males mate significantly less than dominant males (Clark, 1998). Due to the ecological significance and reproductive benefits of maintaining a high rank and territory, the ability to learn signals that are predictive of rival males and to act more aggressively upon the presentation of those signals would be advantageous for winning aggressive interactions and establishing a high rank (Domjan, 2005).

While in the present study the conditioned stimulus was a light bulb, it may still inform the understanding of how Madagascar hissing cockroaches might use visual cues in their natural environment. Visual cues such as shadows and light changes might serve as a conditioned stimulus for Madagascar hissing cockroaches in predicting the approach of a rival. This adds to the understanding of this species, which has been primarily known to perceive and learn most effectively through olfactory cues (Clark et al., 1995; Leibensperger et al., 1985). These inferences about sensory perception are difficult to make for invertebrates, as their sensory

systems differ vastly from vertebrate animals (Loy et al., 2021). Studies have shown that Madagascar hissing cockroaches have sensitivity and an ability to detect a plane of linearly polarized light (Mishra & Meyer-Rochow, 2008), suggesting that light cues specifically, rather than visual cues generally, may be important in this species.

In addition to the findings of the present study, previous studies indicate that classical conditioning and learning confer fitness advantages for invertebrates. Such advantages include oviposition, mate choice, diet, and resource acquisition (Dukas & Bernays, 2000; Leibensperger et al., 1985; Matías Gámez, 2018; Riffell et al., 2008). These advantages are crucial components for survival and reproduction. The results of the present study contribute to this important body of work by advancing the general collective knowledge on the function of learning in invertebrates, an understudied group of animals (Dukas, 2006; Yinon, 2018).

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