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Classical Fear Conditioning in Turtles (*Chrysemys picta*)

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In all organisms, fear conditioning is a behavior that would be expected to be adaptive for anticipating dangerous stimuli. It therefore should have been selected for in the course of evolution. Although fear conditioning has been demonstrated in many different species, it had only been shown in one reptile group, lizards, prior to this study. We developed a paradigm to study fear conditioning in painted turtles (*Chrysemys picta*). In each of two experiments, there were three groups: an Experimental group in which a red light (the conditioned stimulus, or CS) was paired with a footshock (the unconditioned stimulus, or US), an Unpaired control group given unpaired presentations of the red light and footshock, and a CS Only control group presented with the red light only. The experiments differed in the number of days of training: In Experiment 1, the animals were trained for 9 days and were then run on extinction for 6 days. The results appeared to show that turtles in the Experimental group learned to stop moving when the red light came on, but this was not statistically significant. There were significant group differences during extinction, however. It appeared that the behavior was not asymptotic during acquisition, so we ran a second, longer experiment (Experiment 2) for 15 days of acquisition training and 8 or 9 days of extinction. In this experiment, the Experimental group learned to stop moving during the CS during acquisition and reversed that behavior in extinction. These results demonstrate that classical conditioning with footshock is a suitable method for studying fear learning in turtles. Further, they shed light on the evolution of fear behavior. Turtles are closely related to the stem amniotes that gave rise to both mammals and reptiles. Thus, the results demonstrate that fear conditioning is present in turtles and suggest that it was present in the stem amniote ancestors of mammals as well.

Keywords: extinction, fear learning, freezing, reptile, stem amniote

カメ（*Chrysemys picta*）における古典的恐怖条件付け

全ての生物において、恐怖条件付けは危険を予測するために適応的だと期待されている行動である。そのため、進化の過程で選択されてきたはずである。恐怖条件付けはさまざまな生物種で実証されているが、爬虫類ではトカゲ類でしか実証されていない。 我々は、ニシキガメ（*Chrysemys picta*）の恐怖条件付けを研究するためのパラダイムを開発した。2つの実験それぞれにおいて、赤色光（条件刺激：CS）と足への電気ショック（無条件刺激：US）を対にした実験群、赤色光と足への電気ショックを対にせずに提示した非対照対照群、赤色光のみを提示したCSのみ対照群の3つのグループがあった。それぞれの実験で、トレーニングを行った日数は異なった：実験1では9日間トレーニングを行い、その後6日間消去した。その結果、実験グループのカメは赤いライトが点灯すると動きを止めることを学習したように見えたが、これは統計的に有意ではなかった。しかし、消去期間中にはグループ間で有意な差が見られた。獲得期間中の行動は漸近的なものではないと思われたので、獲得訓練を15日間、消去訓練を8、9日間とした、より長期間の2回目の実験（実験2）を行った。この実験では、実験グループは獲得期間のCS提示中に動きを止めることを学習し、消去期間にその行動を逆転させた。これらの結果は、足への電気ショックを用いた古典的条件付けが、カメの恐怖学習を研究するのに適した方法であることを示している。さらにそれらは恐怖行動の進化の過程を明らかにしている。カメは、哺乳類と爬虫類の起源となったステム有羊膜類に近縁である。 したがって、カメに恐怖条件付けが存在することを示した本研究の結果は、恐怖条件付けが哺乳類の祖先であるステム有羊膜類にも存在したことを示唆している。

キーワード：消去、恐怖学習、すくみ反応、爬虫類、ステム羊膜類

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Condicionamiento clásico en tortugas (*Chrysemys picta*)

Para todos los organismos, el condicionamiento de miedo es un comportamiento que se esperaría que fuera adaptativo para la anticipación de estímulos peligrosos. Por lo tanto, debería haber sido seleccionado en el curso de la evolución. Aunque el condicionamiento de miedo ha sido demostrado en especies diferentes, solo se había reportado en un grupo de reptiles, lagartos, antes del presente estudio. Se diseñó un paradigma para estudiar el condicionamiento del miedo en tortugas pintadas (Chrysemys picta). Se evaluaron tres grupos en dos experimentos: un grupo experimental en el que una luz roja (el estímulo condicional, o EC) se asoció con una descarga eléctrica en la pata (el estímulo incondicional, o EI), un grupo de control no apareado que recibió presentaciones no apareadas de la luz roja y la descarga eléctrica en la pata, y un grupo de control en el que el EC se presentó independientemente. Los dos experimentos se diferenciaron en el número de días de entrenamiento: para el Experimento 1, los animales fueron entrenados durante 9 días y luego se les realizó una prueba de extinción durante 6 días. Los resultados sugieren que las tortugas del grupo experimental aprendieron a detenerse al encenderse la luz roja, pero esto no fue estadísticamente significativo. No obstante, se detectaron diferencias significativas entre los grupos durante la extinción. Dado que el comportamiento podría no haber alcanzado un nivel asintótico durante la adquisición, se diseñó un segundo experimento (Experimento 2) con una fase de adquisición extendida de 15 días y una fase de extinción de 8 o 9 días. En este experimento, el grupo experimental aprendió a detenerse durante la prueba de adquisición durante la adquisición y revirtió ese comportamiento en la extinción. Los resultados demuestran que el condicionamiento clásico con descarga eléctrica en las patas es un método adecuado para estudiar el aprendizaje del miedo en tortugas. Además, arrojan luz sobre la evolución de la conducta de miedo. Las tortugas están estrechamente relacionadas con los amniotas basales que dieron origen tanto a los mamíferos como a los reptiles. Por lo tanto, los resultados demuestran que el condicionamiento del miedo está presente en las tortugas y sugieren que también estaba presente en los amniotas basales de los mamíferos.

Keywords: extinción, aprendizaje del miedo, congelamiento, reptil, amniota basal

Classical fear conditioning has been studied extensively in mammals and has aided in the understanding of learning and of brain mechanisms of emotion (e.g., Ledoux, 2015). In order to understand the evolution of this kind of learning, however, studies of nonmammalian vertebrates are needed. A variety of nonmammalian vertebrates has been studied, but studies of non-avian reptiles are lacking. Classical fear conditioning has been demonstrated in fish (e.g., Agetsuma et al., 2010; Barela, 2022; Carpenter & Summers, 2009: Roy et al., 2024; Yoshida & Hirano, 2010) and birds (Brito et al., 2019; Faria et al., 2013), but there are almost no studies on non-avian reptiles. This lack is problematic because all birds and mammals evolved from a group of extinct early animals called the stem amniotes, the common ancestors of reptiles, birds, and mammals. Non-avian reptiles are closely related to the stem amniotes, and demonstration of behavioral continuity between birds and mammals cannot be accomplished without demonstration of the behavior in non-avian reptiles. Further, among reptilian groups, turtles are considered to be an early diverging line of reptiles leading to the archosaurs, which include crocodilians and birds (Crawford et al., 2012; Rieppel, 1999). Thus they are an important group to study when we are interested in the lineages of birds and mammals.

When we talk about the concept of fear here, we use an operational definition: fear is the behavior change induced by pairing a neutral stimulus with an aversive stimulus. Many more elaborate definitions of fear have been used (e.g., Perusini & Fanselow, 2015; LeDoux, 2014).

In turtles, appetitive classical conditioning has been shown, but little research on fear conditioning has been done. In our laboratory, we demonstrated that painted turtles (*Chrysemys picta*) are capable of autoshaping, a form of appetitive classical conditioning (Powers et al., 2009; Yeh & Powers, 2005).

There is a model of eyeblink conditioning *in vitro* (Keifer, 2003, 2023), using red-eared turtles (*Pseudemy scripta*), that has yielded a great deal of interesting detail on synaptic changes during learning, but no *in vivo* demonstration of such conditioning in a behavioral paradigm. In any case, eyeblink conditioning is probably not a fear-motivated behavior. We wished to develop a paradigm for studying fear conditioning in turtles in order to understand the evolution of brain mechanisms of fear.

The need for a demonstration of classical fear conditioning in turtles was highlighted by a recent paper (Szabo, Noble, & Whiting, 2020) that reviewed the literature on learning in non-avian reptiles in the past 40 years and did not cite any studies of classical conditioning. Of note, they describe three avoidance studies in lizards (Punzo, 1985; Rothblum et al., 1979; Yori, 1978) as "classical conditioning," but categorize it under the heading *Operant Conditioning.* Avoidance conditioning is indeed operant conditioning, not classical, because there is a response contingency (i.e., there is something the animal can do to reduce the probability of an aversive consequence).

Some earlier studies of fear learning were done in turtles in the 1960s, but these were not demonstrations of classical conditioning. Mrosovsky (1964) studied juvenile Colombian slider turtles (*Trachemys callirostris*) and trained them to reverse a choice of one visual intensity for another by using 2-s shocks through water as a punishment. Almost all turtles learned to reverse their choices. Spigel and Ellis (1965) studied passive avoidance in painted turtles and punished the animals with electric shock for climbing on the walls of the chamber. They found a negative correlation between the shock intensity used and the mean number of seconds of climbing before criterion was reached. All animals learned not to climb except a control given zero shock. Spigel and Ellis (1966, 1967) used the same learning situation to study the effects of small forebrain lesions and amphetamine and found, perhaps surprisingly, that both reduced the amount of climbing.

To our knowledge, Davidson and Richardson (1970) did the only study of classical conditioning in reptiles, using collared lizards (*Crotaphytus collaris*). The conditioned stimulus was a 10-s compound stimulus of light and sound, and the unconditioned stimulus was a 1-s shock to the leg starting 9 s after the CS and terminating at the same time. The lizards were restrained, so that heartrate and breathing could be measured; they also measured leg contraction. They were able to condition both the leg contraction and breathing; heartrate was less reliable. They also demonstrated extinction of all responses.

An understanding of the evolution of fear conditioning requires demonstration of such conditioning in a variety of non-avian reptiles. To that end, we developed a classical conditioning paradigm for turtles. We used a red light as the conditioned stimulus (CS) and footshock, scrambled and delivered through a grid floor, as the unconditioned stimulus (US). The reason we had not tried footshock earlier was that we expected turtles to pull their legs in if they received shock to the feet. We found, however, that painted turtles did not do this.

In two different experiments, we ran three groups of turtles: Paired, Unpaired, and CS Only. The Paired groups received 10 trials per day of the 5-s red light paired with a 1 amp footshock. The Unpaired groups had both lights and shocks, but they never overlapped (explicitly unpaired). The CS Only groups had only red lights, never any shocks. In the first experiment, we gave 9 days of acquisition training and 6 days of extinction (no shock). In the second experiment, there were 15 days of acquisition and 9 days of extinction. The results showed that turtles in the Paired groups learned to stop moving during the CS during acquisition and began to reverse that behavior during extinction. The Unpaired and CS Only groups showed no change in behavior over the course of the experimental training. Although the behavior was similar in both experiments, the acquisition results were statistically significant only in Experiment 2.

At the end of Experiment 2, we changed the learning context to test whether a change of context would disrupt the turtles' behavior. We did so by lining the walls of the chamber with blue and white checked plastic drawer liner. The change did not have a significant effect on performance.

Method Experiment 1

Subjects

Twelve naïve turtles of both sexes, obtained from The Turtle Source, Fort Myers, FL (https://theturtlesource.com/) were used. They were housed in individual plastic tanks with water roughly 5 cm deep and a platform for basking, in a room with 14 hours of light per day and a temperature of 27-29ºC. This light schedule and temperature prevent the turtles from trying to estivate in the winter months. They were fed turtle pellets (Mazuri) twice a week and pieces of fish (tilapia) once per week.

Because the animals' brains would be used subsequently to study adult neurogenesis, they were given nine injections of 5-bromo-2'-deoxyuridine (BrdU) (50 mg/kg in a 1.0 ml/kg solution of 80% DMSO and 20% water), over a three-week period starting one week before the experiment, one each on Monday, Wednesday, and Friday. BrdU is a thymidine analog that is incorporated into DNA during the S-phase of mitosis. The results of the adult neurogenesis study will be reported separately.

Apparatus

The apparatus (Figure 1) was a Coulbourn Instruments standard rat chamber (Habitest), modified for this experiment (28.5 × 24 cm × 28 cm). It had a grid floor, 2 aluminum walls, and one clear plastic wall. A clear plastic door on the front, covered with white paper to prevent the animal's seeing the experimenter, allowed access to the chamber. A red lamp 2 cm in diameter (Coulbourn Instruments white cue light, painted red with Essie nail polish #576) was mounted in the center of one wall 15 cm from the floor (light intensity 20 lux). A Coulbourn Instruments standard house light was mounted in the center of the opposite wall, 2 cm from the ceiling (light intensity 25 lux). The house light was illuminated throughout the training sessions. A shock scrambler (Coulbourn Instruments Precision Animal Shocker) was attached to the rods on the grid floor. The level of shock was set at 1.0 mA, the minimum level that elicited a consistent flinch response in pilot work. A video camera was mounted on the wall behind the chamber to record the behavior of the turtles during the experiment.

Figure 1

Photograph of Turtle in Conditioning Chamber. The Light on the Left Served as the Conditioned Stimulus and Was Red When Illuminated.

Procedure

Three groups of turtles were run in this experiment: Paired, Unpaired, and CS Only. The Paired group received 10 trials per day of the 5-s red light paired with the footshock. The Unpaired group had both 10 5-s lights and 10 shocks, but they never overlapped (explicitly unpaired). The CS Only groups had 10 5-s red lights each day, but never any shocks. These parameters were chosen based on unpublished experiments on avoidance behavior in fish that had been run by the senior author. Two replications were run, one in Fall 2020 and the other in Spring 2021, with 6 turtles in each (total $N = 12$). They were run in two identical chambers, such that two turtles from each group were run at the same time. The order of running the three groups was different in Fall and Spring.

For all groups, the experimental sessions started with a two-min period with no stimuli, to allow the turtle to acclimate to the chamber. Then there were ten presentations of the red light, separated by a variable intertrial interval (ITI), averaging 2 min. At the end of the session there was again a two-min period without any stimuli before the turtles were removed. The boxes were cleaned with 70% alcohol after each animal was run.

Nine days of acquisition and six days of extinction were run. For the Paired group during Acquisition, each trial consisted of a 5-s red light, followed after 4.5 s by a 1 mA shock lasting 0.5 s (delay procedure). For the Unpaired group, the 5-s red light was presented alone, and one shock (1 mA, 0.5 s in duration) was presented during each ITI, so that the session length was the same as for the Paired group, but the lights and shocks were never presented together. For the CS Only group, only the red light was presented; shock was never presented. During Extinction, the procedure was identical for all groups and was identical to the CS Only Acquisition procedure.

Video recordings were made for each session, and observers coded the behavior after the sessions. Because we did not know what response to expect as an index of fear conditioning, we measured a range of responses: moving, climbing, pulling in the head, pulling in the feet, and location in the chamber. We recorded each behavior in the 5 s before each CS came on and during the 5-s CS. The location of the turtle (which of four quadrants they were in), whether they moved, whether they pulled in their heads or their legs, and whether they climbed (put their front feet on the side walls) were recorded, both during the red light and in the five-second period before the red light came on. The observers were not blind to the group the turtles were in (because they could see the reaction to the shock), but they did not know what response would turn out to be conditioned, at least in the first replication.

In order to assess reliability, two observers rated the movement from one animal over the 15 days of the experiment. The correlations between their ratings were 0.98 for the movement before the CS and 0.95 for the movement during the CS.

All procedures were performed according to the guidelines established by the American Psychological Association and the Society for Neuroscience and were approved by the Institutional Animal Care and Use Committee at Stony Brook University (protocol #335685).

Statistical Analysis

For each measure taken from the videos, we calculated the total number of trials with a response each day (maximum = 10) and the total number of pre-CS periods with a response. We then subtracted the first number from the second. Thus, if the response during the CS was less than during the pre-CS period, the values would be negative, and if the CS responses declined over time, the difference scores would decline. If the response increased over time, the difference scores would increase. The measurement of movement proved to be the one measure that changed in the Paired group as a function of training in the experiment. The other three measures showed no change over time for any group. The data were analyzed using mixed design ANOVAs with Blocks (or Days) as the within-subjects factor and Groups as the between-subjects factor and post-hoc tests when needed. We used SPSS version 28.0 for all analyses.

Results Experiment 1

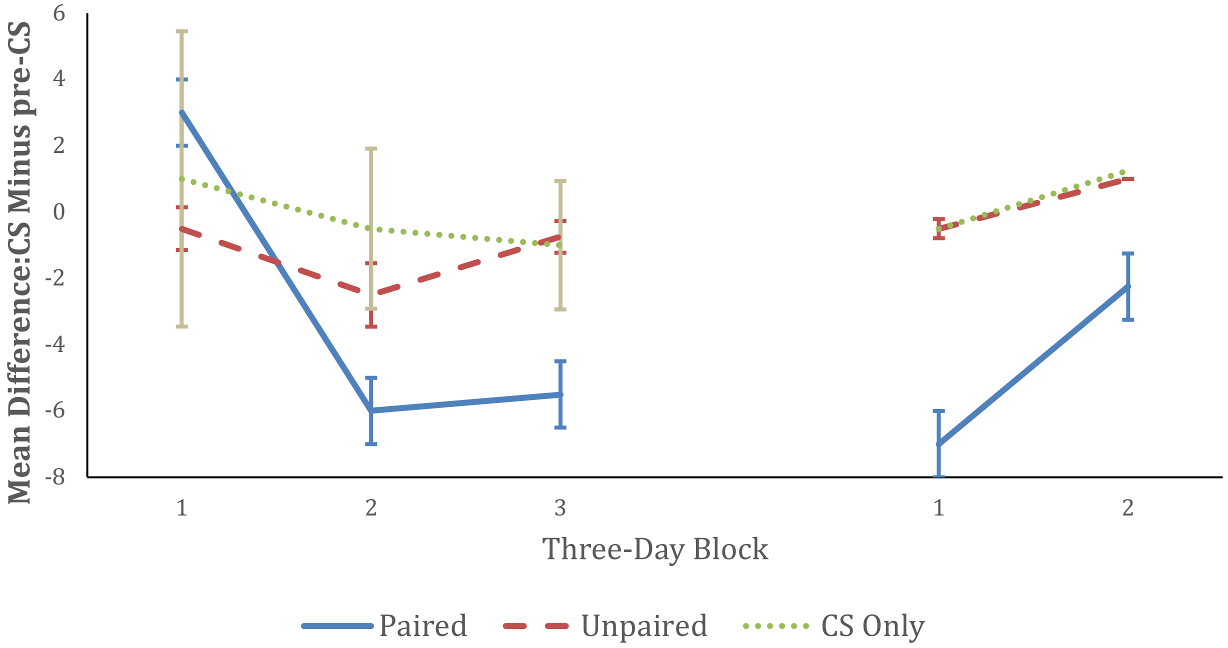
Although we recorded four different behaviors, only movement showed change over the course of the experiment. The other behaviors, location in the box, pulling in their heads or limbs, and climbing, rarely occurred and did not change over the course of the experiment (all p values > .05).

We analyzed all data both day by day and in three-day blocks. The results were the same for both, but we present three-day blocks because the progress is more easily seen. The turtles in the Paired group appeared to learn to stop moving when the CS came on (Figure 2). In an ANOVA of three-day blocks during acquisition, there was a significant Blocks effect ($F(2, 18) = 5.43$, $p = .014$), but no Groups effect ($F(2, 9) = 0.92$, $p = .432$), and no significant interaction of Blocks × Groups ($F(4, 18) = 2.24$, $p = .106$). ANOVA of the groups on Block 3 showed a trend toward significance ($F(2, 9) = 4.17$, $p = .052$).

During extinction, the freezing behavior of the Paired group declined (Figure 2). There was a significant Blocks effect (*F*(1,9) = 15.21, *p* = .004), a significant Groups effect (*F*(2, 9) = 7.48, *p* =.012), and a nonsignificant interaction of Blocks × Groups (*F*(2, 9)=2.33, *p* = .153). Post-hoc Tukey HSD tests on the differences between groups showed that the Paired group differed significantly from both the other groups (Paired vs. Unpaired, *p* = .022; Paired vs. CS Only = -5.00, *p*= .020), which did not differ from each other *(p* = .99).

Figure 2

The Mean Difference Score for Movement by 3-day Block in Experiment 1. Error Bars are SEMs.



Discussion Experiment 1

The results of Experiment 1 were promising, but it appeared that there had not been sufficient days of training to establish a statistical difference between groups during acquisition. In addition, the performance of the Paired group did not appear to be asymptotic at the end of acquisition. We had no prior data to rely on to determine how many days of training were necessary, but this experiment suggested that, perhaps with more training, the turtles could learn to freeze at the presentation of the conditioned stimulus. Therefore, we ran Experiment 2 with more days of acquisition (15 instead of 9) and more days of extinction (9 instead of 6).

Method Experiment 2

Subjects

Twelve more naïve turtles of both sexes were run in Experiment 2. Their housing was identical to that of Experiment 1.

Apparatus

The apparatus was the same as that used in Experiment 1.

Procedure

Again three groups of turtles were run ($n = 4$, each) in two replications, one in Summer 2021 and the other in Fall 2021, with 6 turtles in each. The order of groups being run was different in summer and fall.

For the most part, the procedure was the same as in Experiment 1, including the injection of BrdU for analysis of adult neurogenesis. The main difference was that there were 15 days of acquisition for all animals and, for replication 1, 9 days of extinction. Because of holidays, turtles in the second replication were run for only 7 days of extinction. In addition, on the last day of Extinction, all animals were run with a different context, to assess how context change might affect their learning. The walls of the box were covered with blue and white checked plastic drawer liner sheets for that day.

Statistical Analysis

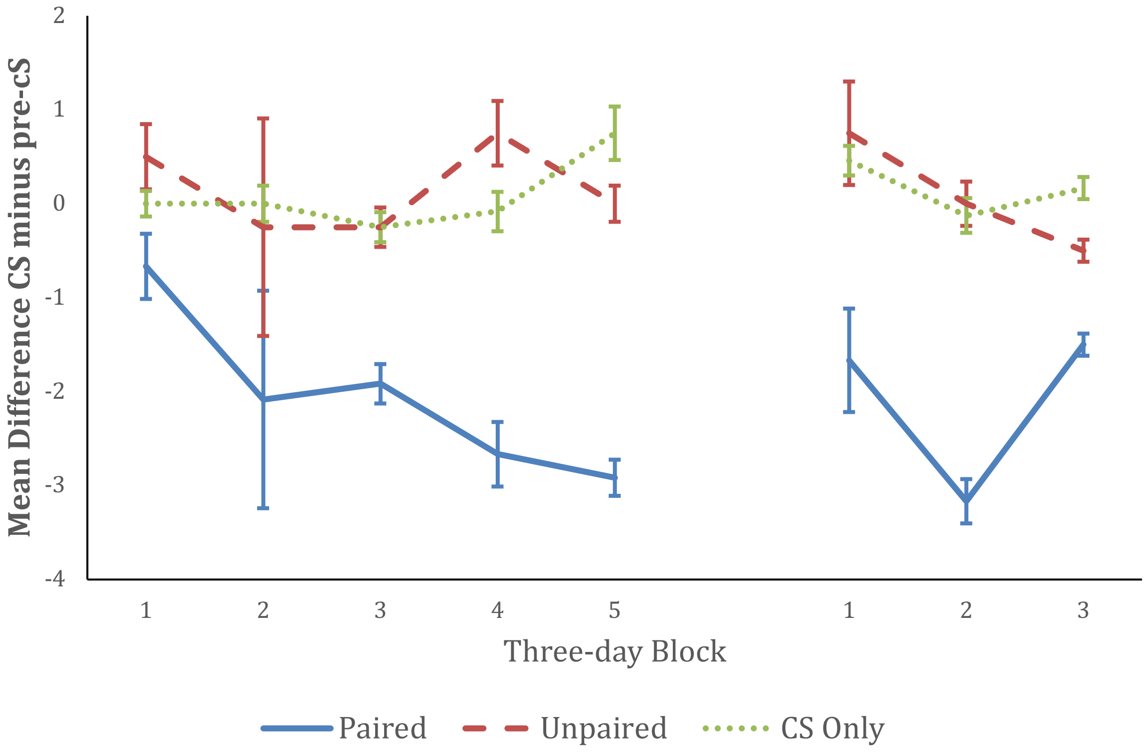
The statistical analyses were carried out in the same way as for Experiment 1. We measured only movement in this experiment, however.

Results Experiment 2

Again, in Experiment 2, the Paired group showed a decline in movement over the course of acquisition (Figure 3), but again it did not appear to have reached an asymptotic level. In this experiment, unlike in Experiment 1, there was a significant effect of Groups ($F(2, 9) = 4.92$, $p = .036$). For the repeated measures, Mauchley's test of sphericity was significant, so we used the multivariate solution in SPSS (McCall and Applebaum, 1973). Blocks showed a significant effect, Pillai's Trace ($F(4, 6) = 10.51$, $p = .007$), and the interaction between Groups × Blocks was also significant, Pillai's Trace ($F(8, 14) = 3.29$, $p = .025$).

Figure 3

The Mean Difference Score for Movement by 3-day Block in Experiment 2. Error Bars are SEMs.



In extinction, the Paired group showed some variability in behavior, but did not really show an increase in movement over the two blocks, as would be expected if they were extinguishing (Figure 3). For the statistical analysis, we analyzed only two blocks because some points were missing from the third block. There was a significant effect of Groups ($F(2, 9) = 19.35, p = .016$), and a significant effect of Blocks ($F(1, 9) = 8.76, p = .016$). The interaction between Groups × Blocks was not significant ($F(2, 9) = 0.78, p = .49$). Post-hoc Tukey HSD tests showed that the Paired group was close to significantly different from both the Unpaired group ($p = .053$), and the CS Only group ($p = .06$).

On the last day of the experiment, when the context changed to blue and white checked walls, the behavior of the turtles did not change. An ANOVA comparing groups on the last day before the context change and the context day showed no significant Group differences ($F(2, 9) = 1.82, p = .22$), and no Days effect ($F(1, 9) = 3.33, p = .10$), or interaction of Groups × Days ($F(2, 9) = 1.48, p = .28$).

Discussion Experiment 2

Although a longer acquisition period was successful in obtaining significant differences in this experiment, the behavior at the end of acquisition seemed not to be asymptotic, and more training might have increased the performance even more. In extinction, the behavior of the Paired group did not decline as much as we expected. Nonetheless, in this experiment, group differences were significant both in acquisition and extinction.

General Discussion

This is the first demonstration of classical fear conditioning in turtles. We use an operational definition of fear conditioning: a behavior change induced by pairing a neutral stimulus with an aversive stimulus (electric shock). The learned behavior was similar to what is seen in rodents, a cessation of movement when the conditioned stimulus (CS) was presented. The behavior occurred only in the Paired group, not in the Unpaired group or the CS Only group. The behavior of the latter two groups did not change over the course of the experiments.

In extinction, the behavior of the Paired group did not reverse completely, but it appeared to be doing so. Again more training would be required to demonstrate complete extinction.

We did not run summation and retardation tests (Rescorla, 1969) to examine whether the Unpaired group learned inhibition. These tests involve presenting the CS in another context to determine whether it will reduce responding, as evidence of inhibition. Summation tests involve presenting the CS with an excitatory CS. Retardation tests involve reinforcing the CS to determine whether the acquisition of responding is slower than it would be to a neutral stimulus. Inhibition would be expected to develop in the Unpaired group because the CS predicts absence of shock (the shock occurred only in the intertrial interval). Future work should examine inhibitory processes in conditioning in turtles.

The behavior we saw change with training was movement in the chamber. Turtles stopped moving during the conditioned stimulus. In Experiment 1, we looked for other behaviors as evidence of conditioning: climbing, pulling in the limbs or the head, or location in the chamber. None of the other behaviors changed over time. We do not have an explanation of this. The turtles “froze” in the presence of a stimulus for fear, a behavior observed in many other species, including, of course, rodents.

Aversive fear conditioning has been studied in teleost fish (Agetsuma et al., 2010; Barela, 2022; Carpenter & Summers, 2009: Roy et al., 2024; Yoshida & Hirano, 2010) and birds (Brito et al., 2019; Faria et al., 2013), but until now there has been no paradigm for studying classical fear conditioning in non-avian reptiles, except for one study on lizards (Davidson & Richardson, 1970). Study of the evolution of fear learning should include the non-avian reptiles, as they are the group closest to the stem amniotes that gave rise to mammals. The research on fish and birds suggests continuity in vertebrates, but studies of non-avian reptiles were needed. The findings of this study demonstrate such continuity in a group that is close to the stem amniotes that gave rise to non-avian reptiles, birds, and mammals. Further, they provide a paradigm for studying fear conditioning in turtles.

Future work in our laboratory will focus on the brain mechanisms involved in fear learning. Our understanding of the evolution of the amygdala, for example, which would be expected to be involved in fear conditioning, will benefit greatly from data on fear conditioning and the amygdala's role in non-avian reptiles. We hope to delineate the areas of the brain involved and the mechanisms by which those areas mediate fear learning. The demonstration that classical conditioning of fear can be studied in turtles opens a new area of investigation for those interested in the evolution of mechanisms of aversive conditioning.

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