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INVESTIGATING THE DURATION AND EFFECT OF CYCLOHEXIMIDE ON LOSER EFFECT IN GNATOCERUS CORNUTUS

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INVESTIGATING THE DURATION AND EFFECT OF CYCLOHEXIMIDE ON LOSER EFFECT IN *GNATOCERUS CORNUTUS*

by

AUSTIN TRUONG

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April 22, 2021

ABSTRACT

INVESTIGATING THE DURATION AND EFFECT
OF CYCLOHEXIMIDE ON LOSER EFFECT
IN *GNATOCERUS CORNUTUS*

Austin Truong, B.S. Biology

The University of Texas at Arlington, 2021

Faculty Mentor: Jeffery Demuth

The loser effect is a phenomenon that occurs when an individual who loses an aggressive encounter with a conspecific has an increased probability of losing future encounters. Behaviors associated with the loser effect include self-isolation, avoidance, and submissiveness, eventually leading to a shutdown of physical activity. Cycloheximide (CHX) is a protein synthesis inhibitor often used in behavioral experiments to test long-term memory. The losers of aggressive competition were tested for their response under the effects of CHX. We exposed male broad horned flour beetles who recently lost a male-male competition to CHX for 16 hours. Following treatment, we retested these "loser" beetles. Typically, males of this species can display loser effects for up to 4 days after losing contests. However, our preliminary results show that losers treated with CHX do not

display the typical loser behaviors. Our results suggest that CHX blocks the synthesis of proteins that perpetuate loser behaviors.

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CHAPTER 1

INTRODUCTION

1.1 Loser Effect

Members of a species typically participate in contests that compete for limited resources in the form of mates, territory, or food (Okada et al., 2006). During combat, individuals in a contest assess their resource holding power (RHP) during a fight. This RHP helps them decide whether to retreat or continue attacking based on how quickly their RHP budget expends. Typically, individuals with a higher RHP budget are more likely to win their fight (Parker, 1974). A particular phenomenon called the loser effect results when the individual self-assessed their RHP during combat. If an individual perceives that their holding potential is less than their opponents, they will consider themselves less dominant and likely retreat from combat. Individuals influenced by the loser effect are more likely to lose future aggressive bouts because the individual will have assessed that from experience if it is worth engaging in the behavior (Hsu et al., 2006). In the beetle species *Gnatocerus cornutus*, the aggressive males fight to gain access to females to mate. Upon losing a fight, the defeated male may avoid fighting for up to 4 days (Okada et al., 2010).

1.2 Cycloheximide

Cycloheximide (CHX) is a fungicide that has protein synthesis inhibition effects on eukaryotes. CHX targets the E-site of ribosomes, preventing the translation of proteins, which stops protein synthesis (Oksvold et al., 2012). In previous studies, cycloheximide impairs long-term memory formation in eukaryotes. In mice trained in high-stress

conditions, after being injected with cycloheximide 30 minutes before training displayed increased amnesiac tendencies towards the training 48 hours later (Gold and Wrenn., 2012). In *Drosophila melanogaster*, fruit flies exposed to CHX while in loser effect no longer displayed visible signs of loser effect. The flies that were not exposed to CHX continued to display obvious signs of the loser effect (Trannoy et al., 2016). In praying mantises, CHX can cause memory disruption for up to 2-3 hours after injection (Jaffé, 2003).

1.3 *Gnatocerus cornutus*

For our research, we used the beetle species *Gnatocerus cornutus*. Male and female *G. cornutus* are easily distinguishable, with only the males having enlarged mandibular horns present (Figure 1). The males use these horns to aggressively fight with other males for mating opportunities with the females. Also, losers from these aggressive bouts show distinct loser effect symptoms, with the loser beetle seen retreating from the fight, isolating itself, and eventually shutting down physical activities for an extended period (Okada et al., 2006). This phenomenon of the beetles shutting down their physical activity is what we will call "LE shutdown." The high reproduction rate of *G. cornutus*, and the short time for larvae to reach adulthood in a month, also allow us to have many beetles to gather results.

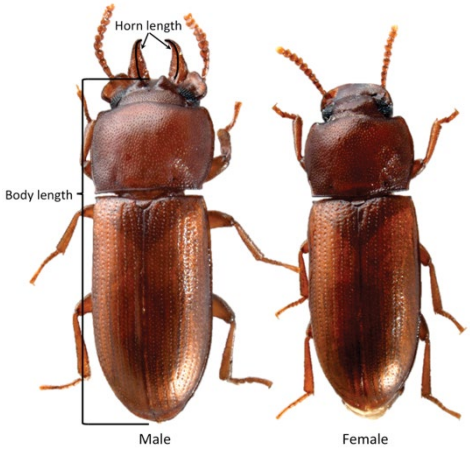

Figure 1.1: The Difference in Characteristics of Male and Female *Gnatocerus cornutus* (Demuth et al., 2012)

In this study, we investigated the changes to the behavior of *G. cornutus* related to the loser effect after being exposed to CHX. We compare the time it takes for individuals to first retreat from an aggressive encounter to when they enter a physical shutdown of activities. Our hypothesis is that inhibiting protein synthesis with CHX will delay loser behavior or remove the loser effect altogether, as seen with *D. melanogaster* flies (Trannoy et al., 2016). From this, we will be able to observe if the results of cycloheximide on loser effect is consistent with research on other species.

CHAPTER 2

METHODOLOGY

2.1 Collecting the Males

The Gnatocerus cornutus males used in the lab were extracted from stock populations present within the Demuth lab. These G. cornutus were maintained in a 24 h Percival Scientific Incubator at a constant temperature of 29.8 °C and 70% relative humidity in darkness. G. cornutus were reared on media consisting of 95:5 ratio of whole-wheat organic flour: brewer's yeast by weight. Stock cultures are kept in 45L x 30W x 8D cm covered plastic trays filled, 3-centimeters deep with media. To obtain the virgin adults necessary for experimentation, larvae in the final instar stage were collected from the stock population and placed into glass vials 25Diam x 95H mm with a gram of standard media as food for sustenance for the next two weeks (Demuth et al. 2012).

We identify the males by the presence of mandibular horns after eclosion or when the larvae become adults. The females were placed in another vial labeled with a female symbol. Two weeks after the eclosion, the beetles were prepared for competition. First, we took their body weight (+/-0.001mg) using an AT261 Delta Range precision balance (Mettler-Toledo Inc., Columbus, OH). After recording the weight, the beetle was given an identification number and labeled for CHX. Males that were given an odd number identification were also given a white dot on their elytra, using Wite-Out®, to be distinguished from the other contestant while fighting (Figure 2.1). The beetles were then placed back into their glass vials until it was their time for their trial.

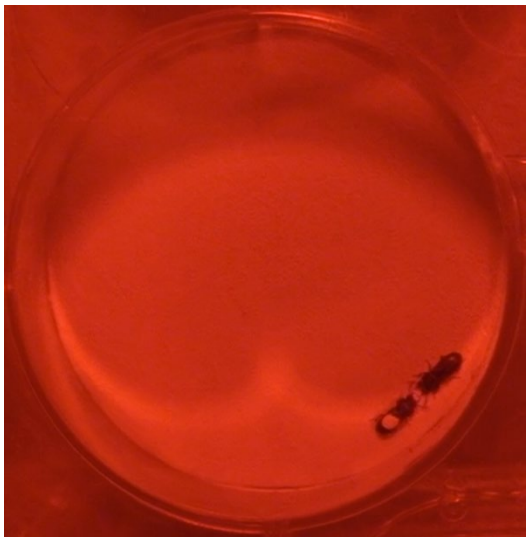

Figure 2.1: The Competitors Fighting (one having a white dot to make it distinct)

2.1.1 Beetle Observations

The fighting arena was 2.5 cm in diameter with a new filter paper (Whatman® qualitative filter paper) added to the bottom for traction before each fight. The fighting arena was in a dark incubator (at 30˚C and 70% relative humidity), recorded on a Sony Handycam HDR-SR5 under red lighting. Prior to being put into the dark incubator, we released a female beetle into the arena to release pheromones for 20 minutes before returning to their vial. This would motivate the males to fight one another with the presence of the female pheromones in the arena. We paired the beetles to their competitors based on the presence of a white dot and placed both of them into the arena. We recorded the times when they performed each behavior and quantified the individuals' behavior by counting the number of aggressive encounters per trial. We also recorded the time of the first retreat and when the beetle entered the loser effect shutdown. See Table 2.1 for the ethogram used to score behaviors.

For the CHX trials, we made our CHX by using 50g/L of sucrose with 1 gram of CHX from Sigma Aldrich in the ventilation hood of the lab. After mixing thoroughly while wearing protective gear, we used a Rainin pipette to deposit 125μL of CHX solution onto filter paper at the bottom of a small plastic 3.5 cm wide plastic container. Media and a single loser beetle from the earlier fights were placed in shortly after. We moved these plastic containers into a 45L x 30W x 8D cm tray container and placed it into the dark incubator to expose the beetles within to CHX for 16 hours. After 16 hours of exposure, we re-paired the beetles to their competitors and recorded the results.

We report mean±SD and range in seconds for each of our measures. We test for differences between average time to first retreat and average time to LE shutdown for -CHX and +CHX treatments using a one-tailed t-test (i.e. testing whether times were longer for +CHX treatment). Means were considered significantly different at $\alpha=0.05$.

Table 2.1: Ethogram of Behaviors Used During Observations of Beetles

Type of Behavior	Behavior	Code	Description
Social	Approach	A	Beetle initiates physical contact with the opponent
Aggressive	Attack	X	Beetle forcefully initiates physical contact with the opponent
Aggressive	Attack – Head Contact	HC	Beetle initiates forceful physical contact by using his mandibles to strike the opponent in the head area.
Aggressive	Attack – Body Contact	BC	Beetle initiates forceful physical contact by using his mandibles to strike the opponent in the body area.
Aggressive	Climb Over	CO	Beetle approaches the opponent from the side or the back and climbs onto the dorsal area of the opponent.
Aggressive	Lift	L	Beetle forcefully lifts opponent off substrate during combat
Aggressive	Flip	F	Beetle forcefully flips the opponent onto his back during combat
Aggressive	Chase	C	Beetle pushes or chases opponent from the fighting area
Avoidance	Retreat	R	Beetle runs or flees from opponent and fighting area
Solitary	Travel	T	Beetle moves from place to place without any interaction with the opponent

CHAPTER 3

RESULTS

We observed 34 contests in total, each lasting about 2 hours. It was observed that the beetles exposed to CHX continued to be "losers" but did not exhibit symptoms of the loser effect. Based on the results, the beetles exposed to CHX (+CHX) take longer to first retreat from an aggressive encounter than beetles that were not exposed (-CHX) (Figure 3; +CHX = 443±286, range 85-1082sec; -CHX = 112±87sec, range 10-345sec; $t(17) = -2.39$, $p<0.001$). The +CHX beetles also took longer to enter LE shutdown (Figure 4; +CHX = 2768±1704, range 567-5570sec; -CHX = 541±273, range 73-1192sec; $t(17) = -2.69$).

It was observed that the beetles exposed to CHX continued to be "losers" but did not exhibit symptoms of the loser effect and would even approach the winning beetle multiple times. Based on Figure 3, beetles not treated with CHX tended to retreat earlier than beetles treated with CHX. Based on Figure 4, beetles not exposed to CHX tended to enter LE shutdown much quicker than beetles treated with CHX.

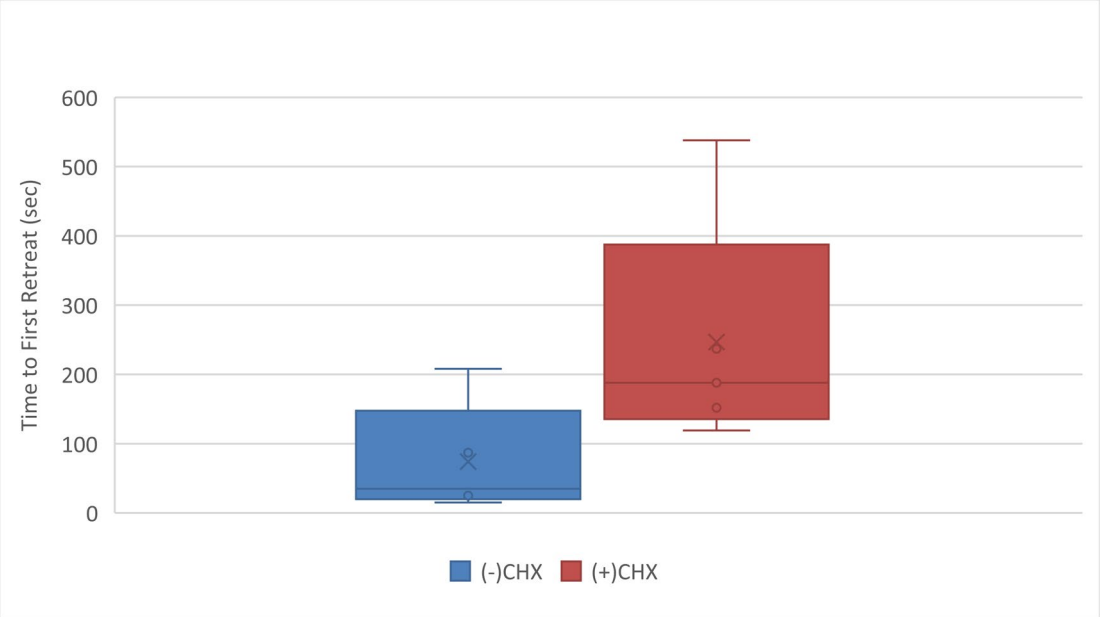

Figure 3.1: Comparison of Times of First Retreats of (-) CHX and (+) CHX

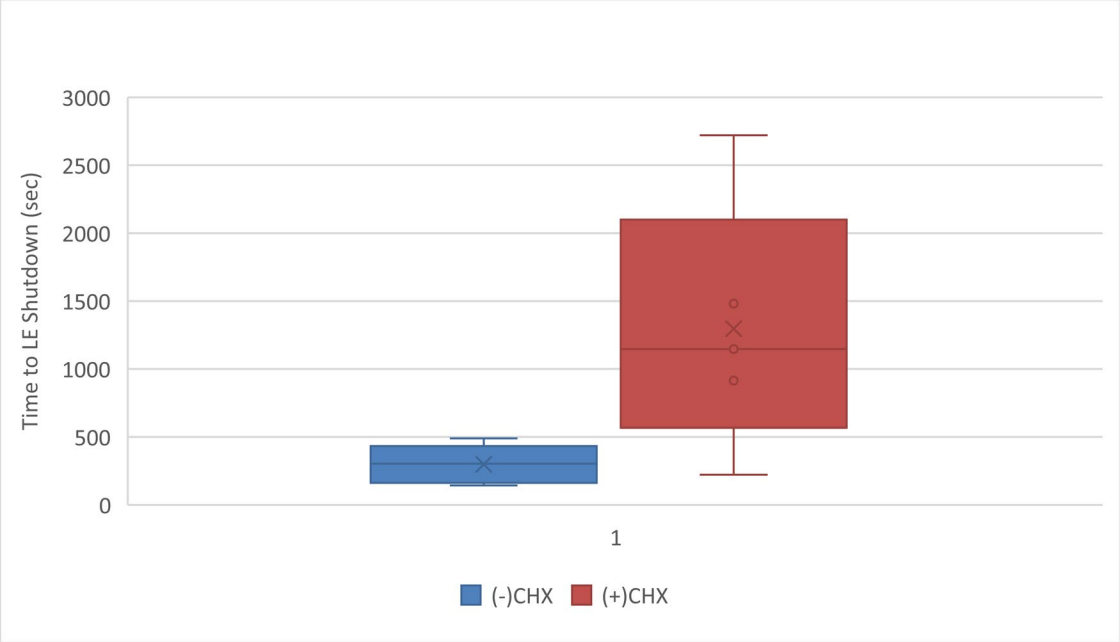

Figure 3.2: Comparison of Times to LE Shutdown of (-) CHX and (+) CHX

CHAPTER 4

DISCUSSION

Based on our results, we reject the null hypothesis that CHX does not delay or inhibit the loser effect behavior. We show that beetles exposed to CHX take longer to perform their first retreat (Figure 3) and enter LE shutdown (Figure 4) than beetles unexposed to CHX. From our results we understand that some aspect of loser effect is regulated at the level of translation. We know this because when we administered a protein synthesis inhibitor loser effect behavior was altered.

Some difficulties that occurred when watching the beetles were that sometimes the white dot on the back of the beetle would be scraped off by the other beetle, making it difficult to differentiate between the two beetles during observations. Another problem would be that some beetles would attempt to fight their reflection instead of chasing after the other beetle present in the arena.

If we were to redo this experiment, having the videos last for longer would be beneficial to document their behaviors over a more extended period to see how long the CHX effect lasts. The sides of the arena would also be covered with non-reflective material so that there would be fewer instances of the beetles fighting their reflection. More care could be taken to place the dots more flatly on the beetle's back to lessen the chance of it being scraped off. Follow-up research would include discovering which specific proteins

are being affected. Comparisons of inhibiting that gene in other species to attempt to replicate the same effect would confirm the specific gene that causes the loser behavior, to eventually lead to treatments in humans suffering from depression.

In our study, we explored the effects of losing on behavior and the resulting loser effect was affected when under the influence of cycloheximide. From our observations, we see that our results are consistent with prior research in other species, with *G. cornutus* displaying a lack of loser effect for a period of time that could last for some minutes to up to an hour. This was supported by our observations that although the beetles continued to run away from their opponent when fighting, they did not lose their aggression and continued to approach their opponent.

CHAPTER 5

CONCLUSION

The loser effect is a behavioral phenomenon that suggests that an individual is more likely to lose future fights after losing a fight. This behavior can result from an individual's self-assessment that their RHP is inferior to their opponent's. Under the effects of CHX, a protein inhibitor, long-term memory formation can be impaired. Consistent with our hypothesis, observations of beetle behavior show that individuals under the influence of CHX during the loser effect take longer before their first retreat. Individuals under the influence of CHX also enter LE shutdown almost five times later than a beetle that was not exposed to CHX. Further analysis of the topic could bring awareness to which specific proteins are being stopped, which could possibly lead to new genetic behavioral research in humans. For example, if we were able to apply chemical-safe treatments to target genes to temporarily remove the loser effect in humans, we may be able to help individuals suffering from depression. Our research shows that cycloheximide provides consistent results on aggression in different species, targeting the same proteins that cause loser effect. From earlier research of *D. melanogaster* flies, where cycloheximide was shown to maintain aggression in flies that had lost aggressive encounters (Trannoy et al., 2016), we show similar results in *G. cornutus*.

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BIOGRAPHICAL INFORMATION

Austin Truong began his academic career at the University of Texas at Arlington in the fall of 2017. In addition to being an Honors student, he is an officer of Circle K International. He is also involved with his local community, where he volunteers with the Green Oaks Physical Therapy, Meals on Wheels of Tarrant County, Arlington Urban Ministries, and Mission Arlington Medical Clinic. He joined Dr. Demuth's research lab in April 2020. Austin will graduate magna cum laude with honors this May and plans to apply to PT school this upcoming 2022 cycle.