ELUCIDATING THE MOLECULAR GENETIC BASIS OF BEHAVIORS RELATED TO THE LOSER EFFECT

by

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DISSERTATION

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Dedication

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Abstract

Elucidating The Molecular Genetic Basis of Behaviors Related to the Loser Effect

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Across the animal kingdom, when individuals experience social defeat (losing an aggressive conflict with a conspecific), it elicits stereotypical loser behavior such as isolation or submissiveness. In many species, social defeat is a precursor to the "loser effect" (LE), the propensity for individuals who lose a contest to lose subsequent competitions. Experiments in rodent systems reveal physiological consequences of social defeat and loser behaviors but underlying genetic mechanisms of the LE remain elusive. Furthermore, of the recent studies investigating gene expression changes associated with social defeat, there is little focus on the recovery of LE, wherein some animals naturally exit loser behaviors. In the following four chapters, we present research on observed behavior that stems from social defeat to identify changes in gene expression that correlate with the duration, the related protein synthesis activity, and the gene regulation of the LE. We first present a synthetic perspective of LE by discussing both the behavioral and genetic components from onset to recovery and identify current gaps in our understanding. In chapter two, we investigate the effect of a broad-spectrum protein synthesis inhibitor, cycloheximide (CHX), on the maintenance of LE. Using the broad-horned flour beetle (*Gnatocerus cornutus*) as a model system, we show that CHX

treatment eliminates stereotypical LE behaviors and prolongs the time to re-initiate LEs. In chapter three, we present gene expression data that reinforces the current understanding of how vital certain neurotransmitters are in regulating long-term depression. LE shutdown appears to be initiated by shutting down neurotransmitter production, while recovery mediates by elevating the expression of anti-stress pathways associated with increased synapse potential. Finally, in chapter four, we present a behavioral study highlighting the potential for *G.cornutus* to be a model system for behavior studies by demonstrating how environmental stress can impact the probability of success in aggressive conflict.

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Chapter 1

Using Insect Systems to Elucidate the Molecular Basis of the Loser Effect: A Review of Social Defeat Stress and the Loser Effect

Abstract

Across the animal kingdom, when individuals experience social defeat (losing an aggressive conflict with a conspecific), elicits stereotypical loser behavior such as isolation or submissiveness. In many species, social defeat is a precursor to the "loser effect," the increased probability that the loser of a single fight will continue to lose subsequent fights. Experiments in rodent systems reveal physiological consequences of social defeat and loser behaviors but underlying genetic mechanisms of the loser effect remain elusive. Furthermore, of the recent studies that investigate gene expression changes associated with social defeat, there is little focus given towards the recovery of the loser effect, wherein some animals naturally exit loser behaviors. In this review, we aim to present a synthetic perspective of loser effect by discussing both the behavioral and genetic components from onset to recovery. We begin with a summary of established methods used to observe behavioral and physiological consequences of social defeat stress (SDS). We then review what is known about molecular mechanisms that regulate SDS and loser behaviors. Finally, we offer a promising avenue for future study using an insect system to investigate the molecular mechanisms responsible for LE behaviors and the termination of the loser effect to help fill the gaps in our current understanding.

Introduction

When animals compete, resulting in a winner and a loser, the loser experienced a social defeat (SD) (Rillich & Stevenson, 2014). Immediately following SD, losers often manifest a syndrome of stereotypical depression-like behaviors, such as avoidance or submissiveness (Hsu et al., 2006). These behaviors are markers of social defeat stress (SDS), and for many species individuals who experience SDS will go on to exhibit the loser effect (LE) (Iwasaki et al., 2006; Rose et al., 2017b), which is the increased probability that the loser of a fight will continue to lose subsequent fights (Hsu et al., 2006; Hsu & Wolf, 1999; Rutte et al., 2006). The LE is a phylogenetically widespread phenomenon that leads to significant penalties regarding the control of resources, establishment of hierarchal relationships, and reduced relative fitness (Dugatkin, 1997; Kura et al., 2015).

For decades, rodent systems have been models for studying the behavioral and physiological consequences of SDS because their depression-like responses reliably parallel human responses to social stress (Golden et al., 2011; Hollis & Kabbaj, 2018; Kudryavtseva, Madorskaya, et al., 1991; Martinez et al., 1998). Various approaches have been employed to study SDS in rodents, including the colony model, which is meant to reflect SDS under natural social dynamics (Martinez et al., 1998), and the forced swim test, which uses desperation to induce depression-like symptoms (Porsolt et al., 1977). However, the most common approach employs the resident/intruder paradigm, wherein a male in their established territory (resident), will dominate physical interactions with a newly introduced male (intruder)(Koolhaas et al., 2013). Typically, once the intruder accepts defeat, he goes to a separate compartment, protected from further physical harm, but remains exposed to the resident and consequently experiences ongoing SDS. The aim of the resident/intruder approach is

to ensure predictable SDS, so resident males are often from a more aggressive strain, older, heavier, and or more experienced fighters than intruder males (Bartolomucci et al., 2009; Golden et al., 2011). Resident intruder experiments have successfully elucidated much about the physiological underpinnings of depression-like behavior in rodents and have also been adapted to study SDS in other systems such as in zebrafish (Nakajo et al., 2020), tree shrews (Jing et al., 2011), and in model insect systems such as fruit flies (Penn et al., 2010) and crickets (Rillich & Stevenson, 2014, 2018; Rose et al., 2017a).

Based on the above methods for studying SDS, rodent studies show that repeated exposure to stress leads to lack of motivation, decreased locomotion, despondency (despair), anhedonia (inability to feel pleasure), unusual patterns of grooming, social avoidance, and anxiety-like behavior (Denmark et al., 2010; Hammels et al., 2015; Razzoli et al., 2009, 2011). Also affected are sexual behaviors. For instance, in defeated mice, subordinate males have a decreased sexual response even in the absence of dominant males (D'Amato, 1988a). Physiological changes induced by social defeat include changes to hormone levels mediated by activity of the hypothalamic-pituitary-adrenal (HPA) axis (Hammels et al., 2015; Koolhaas et al., 2011). Specifically, following both acute and chronic social defeat, the HPA axis triggers elevated adrenocorticotropic hormone (ACTH) and corticosterone in mice (Keeney et al., 2006). Similarly, within an hour following an aggressive conflict, loser rats show high corticosterone levels in the bloodstream compared to decreases in the winners (Koolhaas et al., 2011). Other physiological modifications in response to social defeat include rapid increases in blood pressure and heart rate, compromised temperature regulation, and changes in circadian amplitude (Meehan et al., 1995; Meerlo et al., 1999; Tornatzky & Miczek, 1993).

Studies in other taxa show that behavioral and physiological responses like those seen in rodents are phylogenetically widespread (Table 1). However, studies in non-rodent systems often focus on LE, rather than SDS *per se*. In LE experiments, competitions often involve two opponents simultaneously placed in an enclosed arena, where interactions follow a natural escalation of conflict that is part of normal behavior associated with resource competition. To investigate how differences in resource holding potential (RHP) affect the probability of defeat, pairs of competitors with a range of trait dissimilarities compete against each other in contests (Okada et al., 2006; Sneddon & Taylor, 1997). Common traits that determine constants' RHP include body size, weapon size, age, fighting experience, and motivation (the value of the resource) and often smaller, and or less experienced contestants have lower RHP and enter LE (Rutte et al., 2006).

The symptoms, and duration of LE varies across taxa. For example, when fruit flies and house crickets accept defeat, they retreat and become submissive (Stevenson & Rillich, 2012; Trannoy et al., 2016). After withdrawing from the fight, broad-horned flour beetles cease regular activity and disperse (Okada & Miyatake, 2010). Big-clawed snapping shrimp and zebrafish flee to avoid potential conflict (Obermeier & Schmitz, 2003; Oliveira et al., 2011). Reptiles also experience depression-like phenotypes. After losing to another male, green anole lizards become sexually inactive and subordinate (Larson & Summers, 2001). Sometimes losers enter a severe "shutdown" phase, where normal activities are extremely curtailed. However, losers typically recover from shutdown after a species-specific duration, regaining pre-LE levels of activity and aggression**.** For example, fruit flies have a LE duration of 24 hours, house crickets recover within 3 hours, and copperhead snakes remain in LE for 7 days (G.W., 1997; Stevenson & Rillich, 2013; Trannoy et al., 2016).

Although SDS and LE research reveals many shared behavioral and physiological phenotypes, the two have largely been studied in isolation due to the disparate model systems and experimental approaches employed. We propose that valuable insights may be gained by looking at the neurological and molecular homologies of SDS response and LE at the molecular level. More specifically, the wealth of rodent SDS studies may recommend hypotheses about the pathways involved in other taxa. Also, investigating loser behaviors and the consequences of LE in invertebrate models such as fruit fly, crickets and beetles, where social conflict behavior is often highly ritualized and predictable, and genetics are easily manipulated, may be an ideal way forward to understand the molecular genetic mechanisms behind responses to SDS in rodents and humans. Following, we first review what is known about the neurological and molecular homologies involved in stress responses between vertebrates and invertebrates. From these insights we then conclude by making hypotheses about the genetic mechanisms that may be involved in regulating extreme LE behaviors.

Neurological and Molecular Homologies Underlying SDS (Figure 1)

Vertebrates

When vertebrates face danger or a threat, the brain sends a stress signal to the hypothalamus, activating the fight-or-flight response in the sympathetic nervous system. The fight-or-flight response happens within seconds and involves two events beginning with the hypothalamus signaling the adrenal glands to secrete epinephrine (adrenaline) into the bloodstream. When epinephrine binds to its membrane bound G-protein coupled receptor, a protein kinase pathway activates. Following a cAMP/PKA signaling cascade, glucose releases into the bloodstream to support a fight/flight response. Epinephrine release also leads to physiological modifications including expanding the lungs, and elevated respiration, heartbeat,

blood pressure, and glycogen production. These modifications prime the muscles and the brain to stay and fight or flee.

While stress persists, activation of the hypothalamus-pituitary-adrenal (HPA) axis results in elevated levels of glucocorticoids (GCs) (cortisol in humans, corticosterone in rodents) (Hosseinichimeh et al., 2015)**.** The HPA axis involves the hypothalamus first secreting corticotropin-releasing hormone (CRH), which signals the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH then signals the adrenal gland to release glucocorticoids (GCs), a primary stress hormone (e.g., cortisol in humans and insects, corticosterone in rodents) that helps the body to remain alert by increasing circulating glucose levels. During times of stress, CRH, ACTH, and GC concentrations increase. Under normal circumstances, feedback mechanisms in the HPA axis promote homeostasis by inhibiting CRH and ACTH secretion (Makino et al., 2002), allowing individuals to return to pre-stress levels. However, when individuals are chronically stressed, the HPA axis repeatedly activates, GCs build up, and the feedback loop becomes compromised.

The build-up of GCs has far-reaching consequences (e.g., depression) because of their indirect effect on the brain-derived neurotrophic factor (BDNF), an essential protein for learning and memory (Bathina & Das, 2015; Juruena et al., 2006). BDNF signaling begins when extracellular BDNF binds to a tyrosine kinase receptor (TrkB), a transmembrane receptor that binds as a dimer explicitly with extracellular mature BDNF (mBDNF)(Bathina & Das, 2015). After binding, the TrkB receptors activate multiple intracellular signaling pathways including phospholipase C (PLC-γ), mitogen-activated protein kinase (MAPK/ERK), and protein kinase B (AKT) (Jin et al., 2019; Lima Giacobbo et al., 2019).

Under stressed conditions, GC buildup mediates downregulation of BDNF signaling, impairs the downstream pathways (Yang et al., 2020) and results in depression. When GCs reach a neuronal cell, they bind with the GC receptor (GR) in the cytoplasm (Munck & Brinck-Johnsen, 1968). The GR is part of a multi-protein complex that only activates via GC-binding (Steckler et al., 1999). When activated, the complex undergoes a conformational change enabling the GR to translocate to the nucleus (Galigniana et al., 2010; Oakley & Cidlowski, 2013; Wittmann et al., 2019). Once inside the nucleus, GR directly downregulates the expression of BDNF (H. Chen et al., 2017) and interferes with BDNF signaling (Arango-Lievano et al., 2015). Low levels of BDNF correlate with mood disorders such as anxiety (Bergami et al., 2008) and depression (Taliaz et al., 2010). Accordingly, recent reviews attribute the success of various antidepressant treatments to their roles in raising BDNF expression levels (Colucci-D'amato et al., 2020; Jin et al., 2019; Yang et al., 2020).

Invertebrates

In response to acute stress, insect octopaminergic neurons release elevated levels of OA into the hemolymph, increasing arousal and alertness (Cinel et al., 2020). In the central nervous system, OA also regulates the responsiveness of sensory neurons, which has the effect of modulating initiation and maintenance of complex behaviors (Farooqui, 2007; Roeder, 1999). In peripheral nerves, OA affects the activity of the flight muscles, the peripheral organs, and the sensory organs (e.g., antennae, bristle hairs (Farooqui, 2007). This aspect of OA signaling is analogous to vertebrate epinephrine signaling and similarly activates glycogen production via the cAMP/PKA pathway in support of the fight or flight response (Cinel et al., 2020). Persistent exposure to stress leads to increased levels of OA (Adamo & Baker, 2011), which corresponds with eventual decreases in the enzymes involved in OA synthesis (Livingstone & Tempel, 1983;

Wright, 1987). These findings suggest that ongoing stress may eventually inhibit release of OA by octopaminergic neurons, which would be analogous to vertebrates no longer releasing adrenaline.

OA also signals the corpus cardiacum, a bundle of neurosecretory cells located posterior to the brain, to secrete adipokinetic hormone (AKH). AKH is a metabolic neuropeptide that regulates metabolism, mediating the mobilization of energy substrates, such as trehalose or diacylglycerol, from insect fat bodies (Gäde & Auerswald, 2003; Orchard et al., 1993). Interestingly, the binding of AKH to their receptors (AKHR-GPCR) initiates homologous intracellular pathways to those regulated by BDNF/TrkB signaling in vertebrates (Kodrík et al., 2015). However, the exact transcriptional target (i.e., BDNF's functional equivalent) remains unclear.

One possible transcriptional target may be within the highly conserved insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway. The IGF-1 is an insulin-like peptide (ILP). In insects, the ILPs can serve as hormones, neurotransmitters, and growth factors (Wu & Brown, 2006) and activate the same cellular signaling pathways as neurotrophins in vertebrates. For instance, after binding with cell surface receptors, both IGF-1 and neurotrophins (e.g., BDNF) activate the PI3K pathway (Fruman et al., 1998), followed by the activation of Akt signaling. Furthermore, in *Drosophila* studies IIS signaling contributes to stress resistance via FOXO, a forkhead box-O transcription factor (Broughton et al., 2005; Rauschenbach et al., 2014)

Insights on the Molecular Basis of LE Based on Homologous Stress Response Pathways

Which mechanism(s) initiate LE? (Figure2)

Across taxa, LE depends on changes in neuroendocrine levels and degraded expression of proteins associated with decline in synaptic signaling, which in turn plays a key role in the long-term depression-like phenotype observed in losers (Yang et al., 2020). In both vertebrates and

insects, the response to stress begins with the overexpression of hormones. In vertebrates the translocation of GRs into the nucleus, which downregulates BDNF, is the molecular switch that leads to changes in synaptic plasticity caused by stress (Xu et al., 1998). Likewise, for insects the molecular switch results from neuroendocrine effects on gene expression that are responsible for the strength or efficacy of synapse activity.

Neuronal communication involves an intracellular signal transduction cascade that is central to maintaining neural plasticity (Yang et al., 2020). However, stress influences neuronal plasticity at the synapse (J. Kim & Yoon, 1998; Krishnan & Nestler, 2008), impeding subsequent long-term potentiation (LTP), which is necessary for maintaining strength in synaptic transmission. J. Kim et al., 2006). The link between synaptic transmission and LE involves the principal excitatory neurotransmitter, glutamate (GLU), which is essential for the maintenance and repair of the brain's neural networks. In normal conditions, stimulation of presynaptic glutamate release results in postsynaptic glutamate receptor-induced activation of the BDNF/TrkB and intracellular PLC-γ pathways (Numakawa et al., 2009). Because BDNF participates in synapse formation and regulation of activity-dependent changes in synapse structure and function, activation of this pathway is necessary for maintaining synaptic spine density. Under stressful conditions when BDNF expression diminishes, there is a decrease in the density of dendritic spines and consequently decreased neural connectivity (Radley et al., 2006). The decreased connectivity between neurons leads to decreased glutamate signaling, disruption of synaptic activity, and depression-like behavior observed in vertebrates (reviewed in Yang et al., 2020). Glu may play a similar role in insects. Investigations of *Drosophila* larval neurons show that activating PI3K decreases glutamate excitability causing the phosphorylation and

inhibition of FOXO, a transcription factor associated with the IIS pathway that when inhibited subsequently decreases neuronal excitability (Howlett et al., 2008).

Which mechanism(s) regulate recovery from LE?

Under normal (non-stressed) conditions, IIS inhibits FOXOs from entering the nucleus via Akt phosphorylation (Jünger et al., 2003; Matsuzaki et al., 2003; Wu & Brown, 2006). However, when experiencing oxidative stress or starvation FOXO undergoes a conformational change and translocates from the cytosol to the nucleus to induce gene expression of anti-stress related proteins (Jünger et al., 2003)**.** Thus, when FOXO is inhibited, anti-stress proteins cannot be expressed, which may lead to increased circulating stress hormones and the depression-like phenotype observed throughout the duration of LE.

In vertebrate's recent investigations show that FOXO transcription factors are essential in the physiology and etiology of depression (Rana et al., 2021). For example, mice express the FOXO1 transcription factor in brain areas related to stress (Biggs et al., 2001), and FOXO1 deficient mice exhibit a depression-like phenotype (Polter et al., 2009). Additionally, neurotransmitters and glucocorticoids can regulate FOXO transcription factors (Liang et al., 2006; Qin et al., 2014), which suggests a link with the HPA axis stress pathway. Furthermore, as mentioned earlier, the IIS pathway associated with FOXO's is highly conserved across vertebrates and invertebrates, suggesting a strong potential that a FOXO transcriptional response to SDS exists in insects. We hypothesize that upregulation of a FOXO transcription factor is the "molecular switch" that initiates recovery from LE and loser behaviors.

Future Studies on the Molecular Basis of SDS and LE

Elucidating the genetic basis of complex behaviors associated with social defeat, depression-like phenotype, and loser effect requires using organisms that exhibit multi-faceted behavioral phenotypes. As such, rodent species have long been a choice system for observing

and conducting social defeat experiments as they are attractive models to address behavioral and physiological similarities with humans. However, observations of insect systems can also provide an understanding of complex behavior, even with insects having a more simplistic anatomy compared to vertebrates. This review demonstrated how comparative analysis of vertebrate and insect systems offers a consolidated view of the molecular and cellular underpinnings of social behavior across taxa.

Insights about the neural mechanisms related to LE have been gained by investigating model insect systems like fruit flies and crickets. Insect studies have revealed that certain neuromodulators either promote or mediate post-conflict behavior depending on the degree of external stimuli. For instance, cricket and fruit fly aggression experiments show that OA plays a crucial role in arousal and mediates behavior in response to stress (Baier et al., 2002; Crocker & Sehgal, 2008; Hoyer et al., 2008; Rillich & Stevenson, 2015; Stevenson et al., 2005). An emerging model insect system demonstrating ritualized and predictable social conflict behavior is the broad-horned flour beetle, *Gnatocerus cornutus*. These flour beetles might be an ideal way forward in identifying the underlying molecular mechanisms of loser behavior. *G. cornutus*, are easily manipulated to perform stereotypical social behaviors associated with mate competition. They are also easy to rear and have a short development time (~1.5 months from egg to adult). Males possess large mandibular horns, widened gena, and a pair of small horns on the vertex of the head, whereas females do not have any of these traits (Okada et al., 2006). Males use their mandibular horns to fight other males for access to females (Okada et al., 2006, 2010). Typically, male-male contests resolve into a clear winner and loser within 20 minutes of introducing males to the arena (Demuth et al., 2012). Losers of these contests exhibit a well-defined loser effect where they retreat, cease normal activities, and remain in a malaise. The predictability of easily

definable loser phenotypes makes studying the genetic basis of systems like *Gnatocerus* appealing.

The similarities in physiological mechanisms (e.g., signaling pathways) that insect systems share with vertebrates also mean that findings in insects have the potential to inform our understanding of important mental health issues in humans. For example, insect neuroendocrine related signaling molecules such as hormones and neuropeptides have counterparts in mammals (Chowanski et al., 2017), suggesting that they are highly conserved. Highly conserved signaling molecules allow us to understand animal physiology better. Furthermore, insects are cost-effective, easy to rear, and in most cases, easy to manipulate for behavioral observations. Compared to insects, vertebrates are expensive to study, have higher level housing needs, have a long generation time, have low fecundity, and have ethical considerations.

There is an even broader impact. This review presents an overarching perspective on how social defeat is the precursor for the loser effect across taxa, wherein aggressive competitions for resources result in dominant/subordinate or winner/loser relationships. The involuntary defeat strategy (IDS) also examines social contest outcomes. IDS suggests that all members of the animal kingdom have a genetic-based tactic activated when an individual can recognize that defeat in social competition is inevitable (Sloman, Gilbert, & Hasey, 2003). This strategy reduces injury or death and induces submissiveness even in the face of losing valuable resources (Sloman et al., 2003). Investigating loser behavior and the consequences of the loser effect complements IDS studies for understanding social behavior within and across species.

Table 1. Shared behaviors and physiologies between Loser Effect (LE) and Social Defeat (SD). The physiological and behavioral responses documented from rodent observations can be seen across vertebrate and invertebrate taxa. Abbreviations: BDNF, brain-derived neurotrophic factor; DA, dopamine; 5-HT, serotonin; HPA, hypothalamic-pituitary-adrenal axis; LE, loser effect; OA, octopamine; NO, Nitric Oxide; SD, social defeat

Study System	Response	Response Type	Study Focus		References
			LE	SD	
Mice *(C57BL/6J strain)*	Grooming patterns	Behavioral		√	(Denmark et al., 2010)
Fruit flies *(Drosophila melanogaster)*	Submissiveness	Behavioral	√		(Trannoy et al., 2016)
Copperhead snake *(Agkistrodon contortrix)*	Submissiveness	Behavioral	√		(Schuett, 1997)
Mice *(C57BL/6J strain)*	Submissiveness	Behavioral		√	(Kudryavtseva, Bakshtanovskaya, et al., 1991)
Fruit flies *(Drosophila melanogaster)*	Retreat, Withdraw	Behavioral		√	(Chen et al., 2002)
Cricket *(Gryllus bimaculatus)*	Retreat, Withdraw	Behavioral	√		(Adamo & Hoy, 1995)
Cricket *(Gryllus bimaculatus)*	Isolation	Behavioral	√		(Stevenson & Rillich, 2013)
Mice *(Mus musculus)*	Decreased sexual response	Behavioral		√	(D'Amato, 1988b)
Mice *(C57BL/6J strain)*	Avoidance	Behavioral		√	(Iñiguez et al., 2014)
Broad-horned Flour Beetle *(Gnatocerus cornutus)*	Reduced locomotor	Behavioral	√		(Okada & Miyatake, 2010)
Mice *(C57BL/6J strain)*	Despondency	Behavioral		√	(Iñiguez et al., 2014)
Rats *(Hooded Long-Evans)*	Defense Posture	Behavioral		√	(Tornatzky & Miczek, 1993)
Mice (C57BL/6J strain)	Anhedonia	Behavioral		√	(Iñiguez et al., 2014)
Rats *(Sprague-Dawley)*	Anhedonia	Behavioral		√	(Razzoli et al., 2009)
Mice *(C57BL/6J strain)*	Immobile Posture	Behavioral		√	(Kudryavtseva, Bakshtanovskaya, et al., 1991)
Rats *(Sprague-Dawley)*	Anxiety-like	Behavioral		√	(Hollis & Kabbaj, 2014)
Rats *(Hooded Long-Evans)*	Tachycardia	Physiological		√	(Tornatzky & Miczek, 1993)
Rats *(Wistar)*	Hyperthermia	Physiological		√	(Hayashida et al., 2010)

Rats *(NMRI)*	Hyperactivity of HPA	Physiological		√	(Keeney et al., 2006)
Rats *(Sprague-Dawley)*	Hyperactivity of HPA	Physiological		√	(Razzoli et al., 2009)
Rats *(NMRI)*	Changes in 5-HT levels	Physiological		√	(Keeney et al., 2006)
Cricket *(Gryllus bimaculatus)*	Changes in 5-HT levels	Physiological	√		(Dyakonova & Krushinsky, 2013)
Big-clawed snapping shrimp *(Alpheus heterochaelis)*	Changes in 5-HT levels	Physiological	√		(Obermeier & Schmitz, 2003)
Mice (C57BL/6J strain)	Changes in metabolism	Physiological		√	(Razzoli et al., 2011)
Convict cichlids *(Amatitlania nigrofasciata)*	Changes in plasma glucose concentration	Physiological	√		(Copeland et al., 2011)
Green Anole Lizard (*Anolis carolinensis)*	Lower androgen levels	Physiological		√	(Greenberg & Crews, 1990)
Cricket *(Gryllus bimaculatus)*	Changes in DA levels	Physiological	√	√	(Rillich & Stevenson, 2014)
Mice (C57BL/6J strain)	Increased BDNF protein	Physiological		√	(Berton et al., 2006)
Copperhead snake *(Agkistrodon contortrix)*	Changes is corticosterone levels	Physiological	√		(Schuett et al., 1996)
Copperhead snake *(Agkistrodon contortrix)*	Changes in testosterone levels	Physiological	√		(Schuett et al., 1996)
Cricket *(Gryllus bimaculatus)*	Changes in OA levels	Physiological	√		(Stevenson et al., 2005)
Cricket *(Gryllus bimaculatus)*	Changes in NO levels	Physiological	√		(Stevenson & Rillich, 2015, 2016)

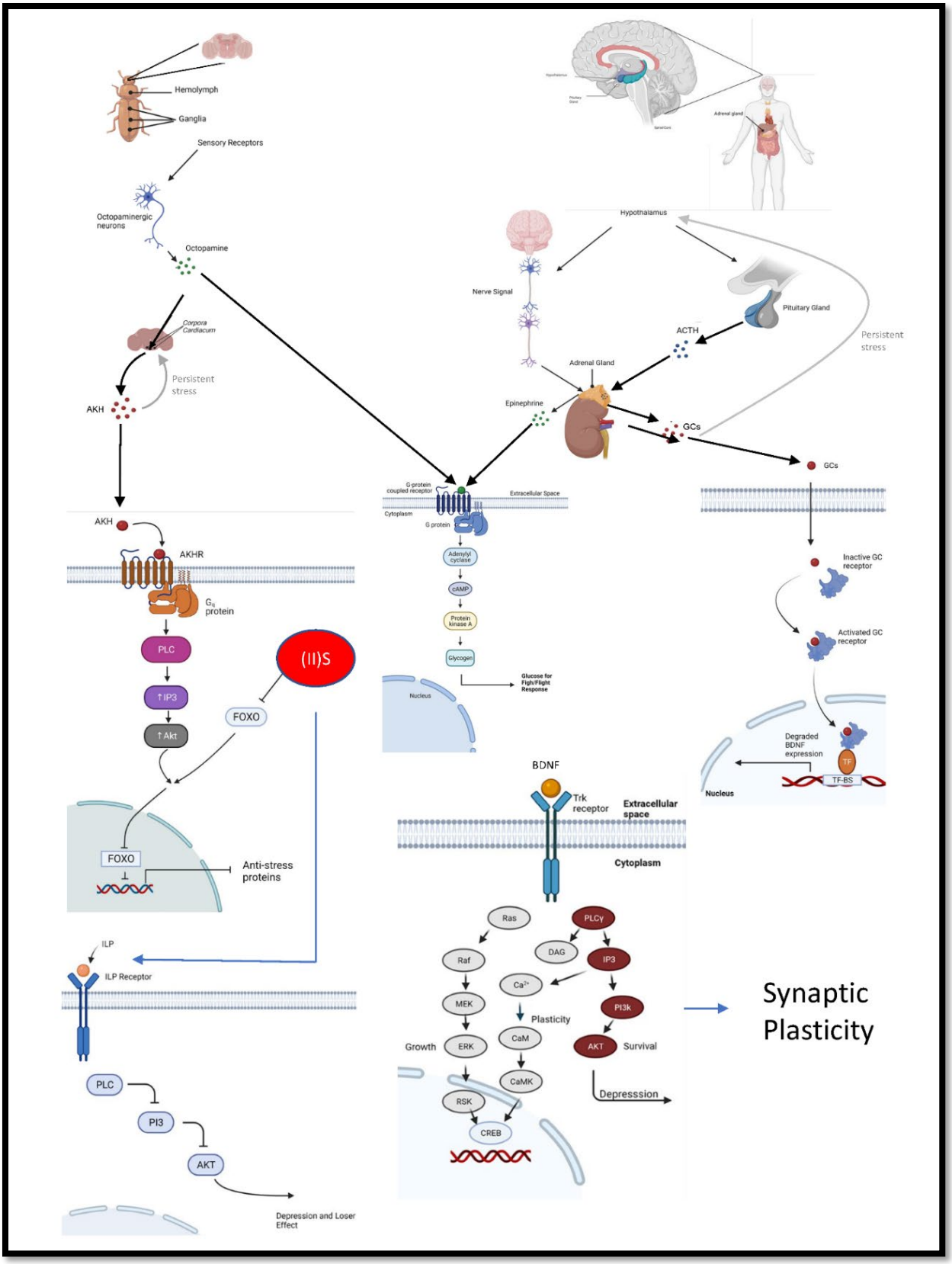
Hemolymph
Ganglia
Sensory Receptors
Octopaminergic neurons
Octopamine
Corpora Cardiacum
Persistent stress
AKH
AKH
AKHR
G_q protein
PLC
↑IP3
↑Akt
(II)S
FOXO
FOXO
Anti-stress proteins
ILP
ILP Receptor
PLC
PI3
AKT
Depression and Loser Effect
Hypothalamus
Nerve Signal
Pituitary Gland
ACTH
Persistent stress
Adrenal Gland
Epinephrine
GCs
GCs
G-protein coupled receptor
Extracellular Space
Cytoplasm
G protein
Adenylyl cyclase
cAMP
Protein kinase A
Glycogen
Nucleus
Glucose for Fight/Flight Response
Inactive GC receptor
Activated GC receptor
Degraded BDNF expression
TF
TF-BS
Nucleus
BDNF
Trk receptor
Extracellular space
Cytoplasm
Ras
PLCγ
DAG
IP3
Raf
Ca^{2+}
MEK
PI3k
Plasticity
Growth
ERK
CaM
AKT
Survival
CaMK
Depresssion
RSK
CREB
Synaptic Plasticity

Figure 1. (Above). Homologous Stress Response Pathways between Vertebrates and Invertebrates. Across vertebrate and invertebrate systems when individuals are faced with threat or danger a stress response is activated, the hypothalamus-pituitary-adrenal (HPA) axis in vertebrates (right) and the octopamine-adipokinetic hormone (OAH) axis in invertebrates (left). Both the HPA and the OAH responses involve increased arousal/awareness, a quick release of energy, activation of the fight/flight response, and subsequent increase of circulating hormones activated by stress, glucocorticoids (GCs) in vertebrates and adipokinetic hormone (AKH) in invertebrates. Cellular pathways and subsequent transcriptional activity are altered because of ongoing stress. In vertebrates the glucocorticoid receptor (GR) undergoes a confirmational change, translocates into the nucleus, and downregulates expression of the brain-derived-neurotrophic-factor (BDNF). The homologous pathway in invertebrates involves the binding of AKH to its receptor (AKHR-GPCR); however, the transcriptional target in invertebrates is unclear. We suggest that insulin-like peptides, which serve as hormones, neurotransmitters, and growth factors in insect systems, may play a homologous role to BDNF in vertebrates. Created with BioRender.com.

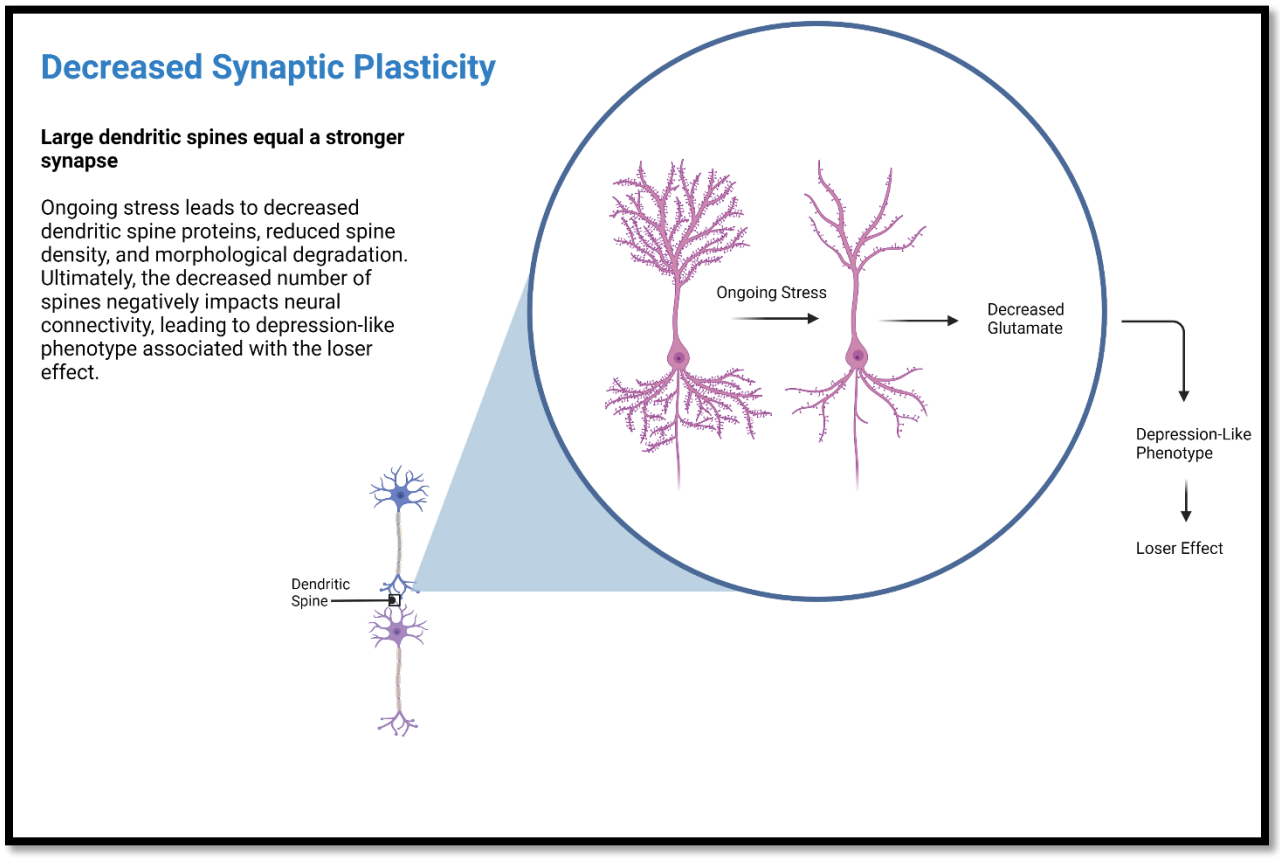

Figure 2. Persistent stress leads to synaptic spine degradation resulting in depression-like phenotypes. Stress influences neuronal plasticity at the synapse and the link between synaptic transmission and LE involves the principal excitatory neurotransmitter, glutamate (GLU), which is essential for the maintenance and repair of the brain's neural networks. Created with BioRender.com.

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Chapter 2

Evidence for Regulation of Loser Effect Memory at the Level of Protein Synthesis

Abstract

The loser effect (LE) is the propensity for individuals who lose a contest to also lose subsequent contests. In this study, we investigate the effect of a broad-spectrum protein synthesis inhibitor (cycloheximide) on the maintenance of LE. Using the broad-horned flour beetle (*Gnatocerus cornutus*) as a model system, we show that CHX treatment eliminates stereotypical LE behaviors and prolongs the time to re-initiate LEs. More specifically we show that CHX treated beetle take longer to make a first retreat and longer to enter an extreme form of LE we call LE shutdown. By comparing CHX treated beetles that are familiar or unfamiliar with their opponent, we also show that there is an additional aspect of when LE behaviors are initiated that depends on opponent recognition. We interpret our findings in the context of memory acquisition and consolidation.

Introduction

Aggression is a highly complex social behavior used to compete for resources. Across taxa, aggressive contests result in a winner and a loser. In many cases, the loser will experience the loser effect (LE), a phenomenon wherein once an individual loses a fight, they will have a higher probability of losing subsequent conflicts. Behaviors associated with LE are broadly like those of depression, such as submissiveness, isolation, and avoidance. LEs are phylogenetically widespread, being observed in fish (Hsu & Wolf, 1999), crustaceans (Huber et al., 2002), birds (Drummond & Canales, 1998), mammals (Kahn, 1951), and insects (Rose et al., 2017). Further, the duration of LE can last from hours to weeks, depending on the species.

When studying LE, the losers are defined based on the outcome of the competition. Often, experimental competitions involve two opponents simultaneously placed in an enclosed arena, where interactions follow a natural conflict escalation that is part of normal behavior associated with resource competition. Often smaller and less experienced contestants have a lower ability to win a fight and ultimately enter LE (Parker, 1974; Rutte et al., 2006). The duration and severity of LE vary across taxa. In some cases, losers enter a severe phase we will call "LE shutdown," where regular activities cease. In some species, losers may then change fighting strategies or adjust fighting tactics in subsequent contests (Okada & Miyatake, 2010; Rutte et al., 2006). LE observations are ideal for investigating the molecular basis of behavior associated with learning and memory. More specifically, the behaviors that lead to establishment of LE represent the long-term memory (LTM) phase of learning and memory.

Consolidation theory describes the two primary phases of memory formation. Short-term memory (STM) is the first phase. STM is produced in the first moments after acquiring the memory, lasts minutes to hours, and depends on post-translational modification of post-synaptic

proteins (Hernandez & Abel, 2008). In contrast, LTM is the second phase; it can last from hours to weeks and depends on intracellular signaling and the regulation of transcription and translation (Hernandez & Abel, 2008). In other words, memory consolidation is when a temporary memory becomes long-lasting (Lechner et al., 1999) whereas long-lasting memory, or LTM, requires protein synthesis.

Protein synthesis plays a role in memory formation by transforming the new information into stable synaptic modifications (McGaugh, 2000). Furthermore, the impact of protein synthesis on LTM formation may involve ongoing exposure to specific proteins (Bekinschtein et al., 2007). One of the proteins associated with memory in the adult mammalian brain is the *brain-derived neurotrophic factor* (BDNF). BDNF is essential for the development of a form of synaptic plasticity that is related to LTM and for modulating synapse formation and dendritic spine growth in the hippocampus (Bamji et al., 2006; Morris et al., 2003; Tyler & Pozzo-Miller, 2003). Repeated exposure to stress leads to downregulation of BDNF, which promotes dendritic spine degradation (reviewed in (Yang et al., 2020) and in turn may maintain LE (Bekinschtein et al., 2007).

Using protein synthesis inhibitors may allow us to better understand the molecular and genetic mechanisms of learning and memory. The argument that memory depends on protein-synthesis relies on evidence that protein-synthesis inhibitors impair memory (Davis & Squire, 1984) . Accordingly, the use of protein synthesis inhibitors to investigate learning and memory has been highly consistent (Bourtchouladze et al., 1998; Tiunova et al., 1998), making them an ideal tool for investigating molecular mechanisms involved in LTM. For example, after receiving protein synthesis inhibitor anisomycin (Ani) injections, rats are unable to remember recently learned tasks (Bekinschtein et al., 2007).

Studying competitive interactions between conspecifics is an ideal way to elucidate the molecular mechanisms associated with learning and memory because learning may involve acquiring a skill or tactic, and then memory expresses what was learned. For instance, if fighting experience leads to acquiring a new behavior, then some losers may learn to modify their behavior in subsequent contests, such as using dispersal techniques or changing levels of aggression towards opponent (Yamane et al., 2010). Aggression is an inherent and potentially adaptive trait used in competition for limited resources like food, water, and territory. Furthermore, aggression levels typically dictate who wins and who loses, such that losers will adjust their fighting strategy for subsequent fights (Okada & Miyatake, 2010). Thus, observing the behavior of losers of aggressive competitions helps to elucidate the molecular mechanisms underlying complex social behaviors associated with learning and memory.

Insect systems (e.g., fruit flies, crickets, flour beetles) exhibit complex social behaviors related to aggressive interactions making them ideal for studying LE. Research on *Drosophila* provides an insect consolidation model wherein STM lasts minutes to hours, and LTM lasts days with repeated stimuli exposure and requires ongoing protein synthesis (Tully, T., Preat, T., Boynton, S.C., Del Vecchio, 1994; Yin et al., 1994). *Drosophila* studies also reveal that the protein synthesis inhibitor cycloheximide (CHX) can block LTM formation (Trannoy et al., 2016; Tully, T., Preat, T., Boynton, S.C., Del Vecchio, 1994; Yin et al., 1994).

In the following study, we use CHX to test whether inhibiting protein synthesis affects loser behaviors. CHX targets the E-site of ribosomes and prevents the translation of proteins (Oksvold et al., 2012). CHX exposure remains a commonly used assay to clarify whether specific biological processes require *de novo* protein synthesis (Wiepz et al., 2006). In *Drosophila melanogaster*, defeated males exposed to CHX show no visible signs of LE, whereas

losers not exposed to CHX display the typical LE behaviors (Trannoy et al., 2016). Similarly, praying mantises undergo memory disruption for 2-3 hours following CHX exposure (Jaffé, 1980). To investigate the behavioral changes, we use *G. cornutus* males subject to LE. We hypothesize that ongoing protein synthesis is required to maintain the LTM that is manifested as LE shutdown. Consequently, we expect CHX treatment to erase LE behaviors. More specifically, we predict that when treated with the protein synthesis inhibitor CHX, the time it takes for individuals who recently lost a fight to make their first retreat from a new aggressive encounter will be delayed and it will also take them longer to enter LE shutdown.

Materials and Methods

Study System

The broad-horned flour beetle, *Gnatocerus cornutus* is an emerging model for observing behavior. With easy manipulation, *G. cornutus* performs stereotypical social behaviors associated with mate competition. They require simple accommodations and have a relatively short development time (~1.5 months from egg to adult). Additionally, males possess large mandibular horns, widened gena, and a pair of small horns on the vertex of the head, whereas females do not have any of these traits (Figure 1). Males use their mandibular horns to fight other males for access to females. Experimental fights usually begin with one male contacting an opponent and usually ensue within the first 20 seconds of contestants being placed in an arena. Within 2-3 minutes, the fighting escalates and involves more locomotor movements, more attacks, and increasingly

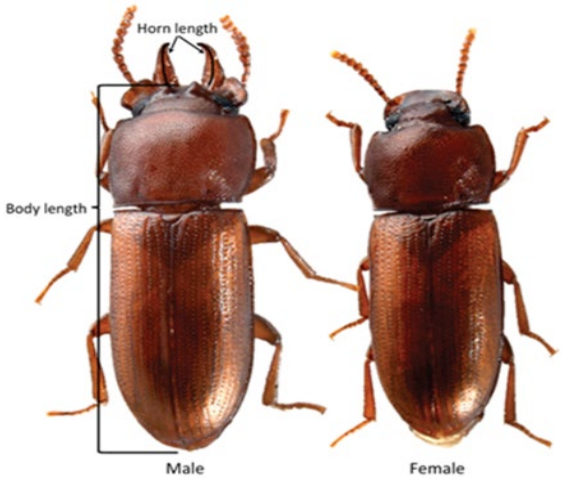

Figure 1. Male and Female *Gnatocerus cornutus*. *G.cornutus* males possess large mandibular horns, widened gena, and a pair of small horns on the vertex of the head, whereas females do not have any of these traits.

aggressive posturing. Ultimately, the loser avoids the winner and becomes increasingly less aggressive as they seemingly attempt to isolate themselves. Depending on the number and intensity of repeated attacks from the winner, the loser enters LE shutdown, where most physical activity ceases. Typically, contests resolve into a clear winner and loser within 20 minutes of introducing males to the arena and LE shutdown may last from 1 to 4 days (Okada et al., 2006; Demuth et al., 2012).

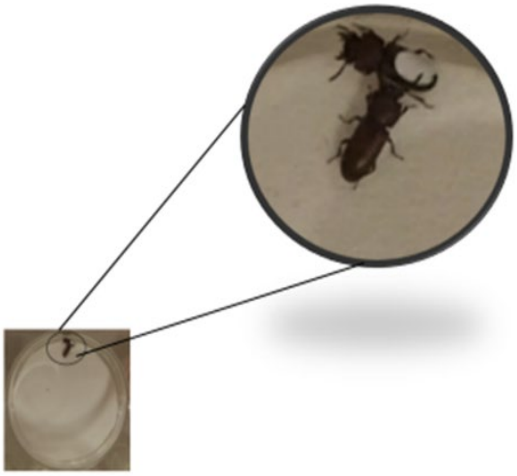

Figure 2. Male contestants distinguished by white marking. Two weeks after eclosion male beetles were given ID numbers wherein odd numbered individuals received a white m dot on their elytra, using Wite-Out®, to be distinguished from the even-number contestants while fighting.

Animal Husbandry

We extracted *G. cornutus* beetles from stock populations in the Demuth lab. Stocks are maintained in a Percival Scientific Incubator at 30 °C and 70% relative humidity with a 24h dark photoperiod and reared on media consisting of a 95:5 ratio of whole- wheat organic flour: brewer's yeast by weight. Stock cultures remain in 45L x 30W x 8D cm covered plastic trays filled, 3-centimeters deep with media. Virgin, naïve adults necessary for experimentation were isolated as larvae in the final instar stage 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).

Competition Environment

We identify the males by the presence of mandibular horns after eclosion. Two weeks after eclosion we weighed each male (+/-0.001mg) using an AT261 Delta Range precision balance (Mettler-Toledo Inc., Columbus, OH). The beetle was then given an identification number. Those assigned an odd number received a white dot on their elytra, using Wite-Out®, to be distinguished from the even-number contestants while fighting (Figure 2). Previous studies

show that this marking scheme does not affect contest outcome (Demuth et al., 2012). Males were then individually placed back into glass vials with standard media to remain isolated until it was time for their trial.

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 housed in a dark incubator (at 30˚C and 70% relative humidity), where contests were observed and recorded on a Sony Handycam HDR-SR5 under red lighting. To help stimulate male aggression, we placed a female beetle into the arena to release pheromones for 20 minutes. Females were then removed prior to simultaneously adding one odd and one even numbered male beetle to the arena. Losers were identified by the onset of LE shutdown and immediately moved to one of the following experimental groups.

Males that were losers in the initial round of contests were placed into one of two groups, CHX exposed (+CHX) or CHX unexposed (–CHX). CHX was prepared by combining 50g/L of sucrose with 1 gram of CHX (Sigma Aldrich SX1075) under a fume hood. After thoroughly mixing the solution, we pipetted 125µL of CHX solution onto filter paper (2.5cm Whatman® qualitative filter paper) at the bottom of a 3.5 cm diameter plastic container. Each container received 10g of media, followed by a single loser beetle. We then placed all containers into a 45L x 30W x 8D cm plastic tray in a dark incubator for 16 hours. None of the winners from the initial contests were exposed to CHX.

Behavior Trials

Two types of trials were conducted, one where +CHX or –CHX losers were paired with the same male they lost to initially (i.e., familiar contests) and another where the +CHX or –CHX losers were paired with a winner that they had not previously encountered (i.e., unfamiliar

contests). For each set of trials, we quantified the Time of First Retreat, Time to Enter LE Shutdown. Typically, first retreat happens within minutes of the initial contact between the two males, and LE behaviors within 20 minutes. Therefore, the videos are at least 20 minutes in length to observe the first retreat and up to 40 minutes in length to observe LE behaviors. Statistical analyses were performed using Intellectus Statistics (*Intellectus Statistics [Online Computer Software]*, 2022). Statistical test results were considered significant at alpha=0.05. Means are reported ± SD unless otherwise noted.

Results

We conducted 29 contests between unfamiliar opponents (12 +CHX, and 17 -CHX) and 12 trials where opponents were familiar (all +CHX). Contestants performed typical fighting maneuvers for this species, such as interlocking mandibles, shoving or pushing the opponent, and lifting an opponent from the fighting arena (Okada et al., 2006; Okada & Miyatake, 2010). The most common form of aggression observed was the beetles locking their mandibular horns in head-to-head contact for long durations, with one beetle pushing and propelling forward. The second most observed form of aggression was characterized by one male forcibly making head contact to the body of a second male, which often resulted in flipping the second male onto his back. After regaining upright posture, the second male either attacked the initiating beetle or adopted a retreat tactic. Finally, climbing over, where one beetle would climb on top of the other as if attempting copulation, was also frequently observed following prolonged head contact. The contestant that was mounted eventually became the loser.

Unfamiliar Combatants

Our results show that CHX treatment does not determine contest outcome, but significantly increases losers' Time to First Retreat and Time to Enter LE Shutdown. There were

no cases where a loser from the initial contests became a winner. This is likely explained by the fundamental effect of size on contest outcome. Losers weighed significantly less than winners in both the exposed (+CHX: $t(22) = 2.97$, $p = .007$) and unexposed (-CHX: ($t(32) = 4.30$, $p < .001$) treatment groups (Table 1). To account for the potential effects of size difference between contestants, we incorporated the Weight Difference (winner weight – loser weight) as a parameter in our models for subsequent analyses.

Table 1. Average weight of winners and losers in +CHX and –CHX treatments

		Winners		Losers		Two-tailed t-test results		
Treatment	n	mean weight (g)	SD	mean weight (g)	SD	*t*	*p*	*Cohen's d*
+CHX	12	3.17	0.26	2.80	0.34	2.97	**.007**	1.21
-CHX	17	2.95	0.24	2.60	0.24	4.30	**<0.00**	1.47

t-test for difference in weight between winners and losers within each CHX treatment group

Time to First Retreat

A generalized linear model (GLM) including Weight Difference, CHX Treatment (+CHX and -CHX), and the Weight Difference * Treatment interaction show that Time to First Retreat (measured in minutes) was significantly affected by CHX treatment (F (3,25) = 11.64, $p < .001$, $R^2 = .58$) explaining approximately 58.27% of the variance (Table 2). The CHX Treatment significantly predicted the time of First Retreat (B = -4.74, t (25) = -2.85, p = .009; Figure 3A). On average +CHX beetles delayed their first retreat 5.86 minutes (~126%) longer than beetles that did not experience CHX (Table 2). Weight difference was not a significant predictor (Table 2) nor was there an interaction between weight difference and CHX treatment on the Time to First Retreat.

Table 2. Time to First Retreat (A): summary statistics (B): GLM results

(A)

Treatment	n	mean (minutes)	SD	SE_M	Min	Max	Mdn
+CHX	12	7.59	3.96	1.14	1.33	14.27	8.12
-CHX	17	1.73	1.43	0.35	0.10	5.45	2.02

(B)

Variable	*B*	*SE*	95.00% CI	β	*t*	*p*
(Intercept)	6.09	1.26	[3.48, 8.69]	0.00	4.81	< .001
Weight Difference	4.05	2.66	[-1.43, 9.52]	0.27	1.52	0.140
CHX Treatment	-4.74	1.67	[-8.18, -1.31]	-0.60	-2.85	0.009
Weight Difference * CHX	-2.74	3.93	[-10.84, 5.36]	-0.16	-0.70	0.493

Overall GLM Result: F (3,25) = 11.64, $\mathbf{p < .001}$, R^2 = 0.58

Time to Enter LE Shutdown

The GLM for Time to Enter LE Shutdown shows that beetles exposed to CHX also take significantly longer to shut down than beetles that were not exposed (F (3,25) = 14.22, p < .001, R^2 = 0.63), with the model explaining 63.05% of the variance. CHX exposure slowed Time to Enter LE Shutdown, by 46.19 minutes (~145%) (Table 3). Like the Time to First Retreat, Weight Difference did not affect Time to Enter LE Shutdown, but -CHX Treatment did (Table 3 and Figure 3B). The interaction between Weight Difference and -CHX Treatment also did not have a significant effect on Time to Enter LE Shutdown, B = 21.55, t (25) = 0.78, p = 0.442.

Table 3. Time to Enter LE Shutdown (A): summary statistics (B): GLM results

(A)

Treatment	n	mean (minutes)	SD	SE_M	Min	Max	Mdn
+CHX	12	55.01	29.42	8.49	9.27	92.50	58.80
-CHX	17	8.82	4.52	1.10	1.13	19.52	9.19

(B)

Variable	*B*	*SE*	95.00% CI	β	*t*	*p*
(Intercept)	64.23	8.87	[45.97, 82.50]	0.00	7.24	< .001
Weight Difference	-24.87	18.64	[-63.27, 13.53]	-0.22	-1.33	.194
CHX Treatment	-54.42	11.69	[-78.50, -30.34]	-0.92	-4.65	< .001
Weight Difference * CHX	21.55	27.59	[-35.27, 78.36]	0.17	0.78	.442

Overall GLM Result: F (3,25) = 14.22, $\boldsymbol{p < .001}$, R^2 = 0.63

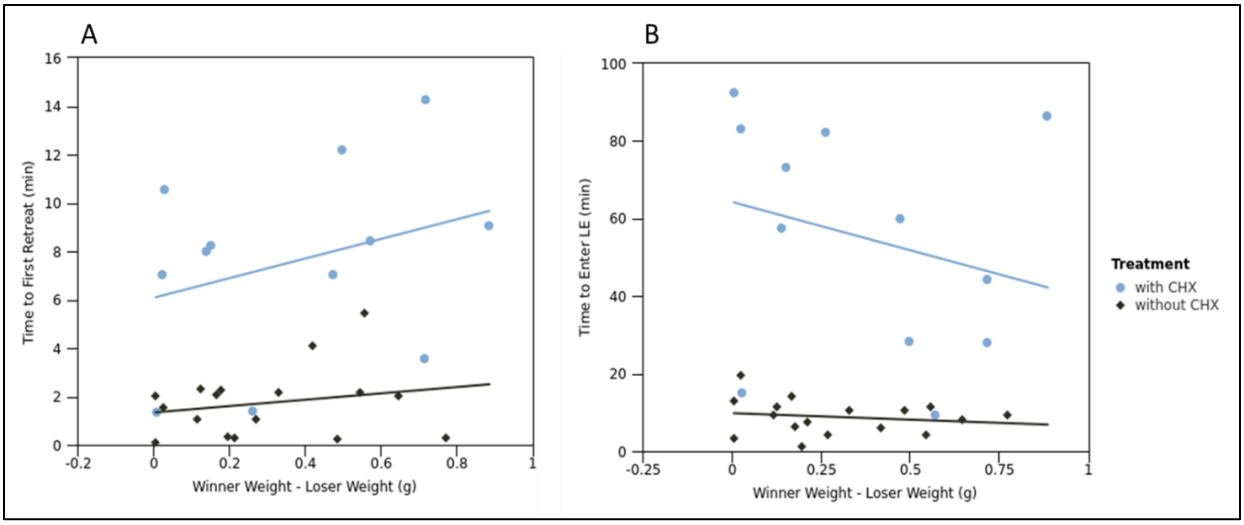

Figure 3. Effects of CHX treatment and weight difference on the time to (A) first retreat, and (B) start of LE shutdown.

Since larger beetles tend to fight longer and win more contests, the above results could be explained by unintentional bias in the size distribution of beetles between +CHX and -CHX treatments. However, we found no significant difference in weight between treatment groups for either the losers (Figure 4A) or the winners (Figure 4B) as presented in Table 1.

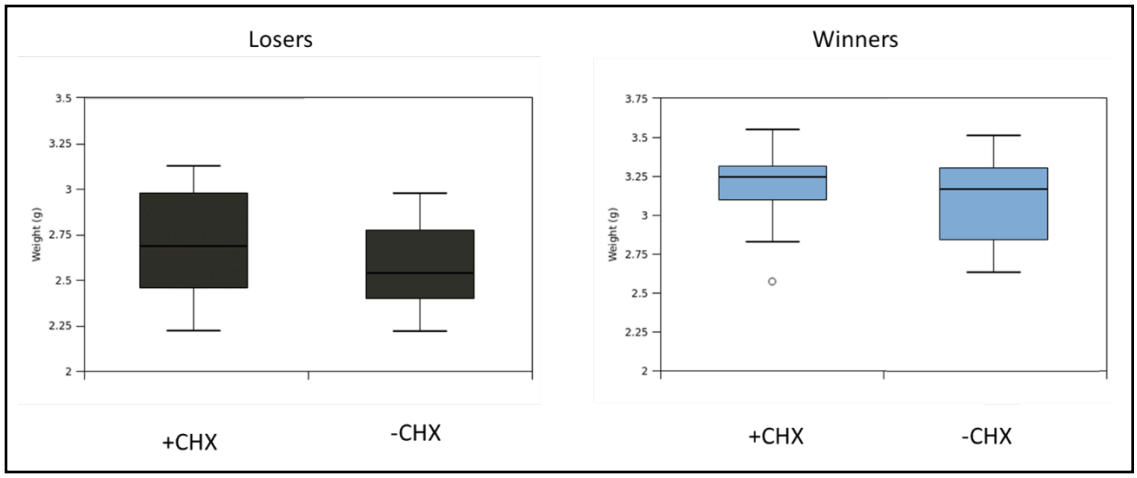

Figure 4. Comparison of weight between treatment groups for (A) Losers and (B) Winners. (Note: winners were not exposed to CHX, the +/- indicates the treatment group of the loser they were paired with).

Familiar Combatants

To determine whether the effects shown for CHX treatment above would still hold when losers were paired with an opponent that they had previously lost to, we paired familiar opponents after treating the loser with CHX. The Time to First Retreat was not significantly different between losers treated with CHX when fighting a familiar or an unfamiliar male ($t(22) = -0.13$, $p = .896$; Table 4). However, the Time to Enter LE Shutdown was significantly shorter for males fighting a familiar opponent ($t(11.58) = -4.10$, $p = .002$; Table 4).

Table 4. Two-Tailed Independent Samples t-Test for Time to First Retreat and LE Shutdown

	Familiar			Unfamiliar					
	n	*Mean (min)*	*SD*	*n*	*Mean (min)*	*SD*	*t*	*p*	*Cohen's d*
First Retreat	12	7.26	7.8	12	7.59	3.96	-0.13	.896	0.05
Time to LE Shutdown	12	24.64	4.79	12	60.01	29.53	-4.10*	.002	1.67

t-statistic df = 22. * *Welch's t-test was used instead of Student's t-test due to unequal variances*

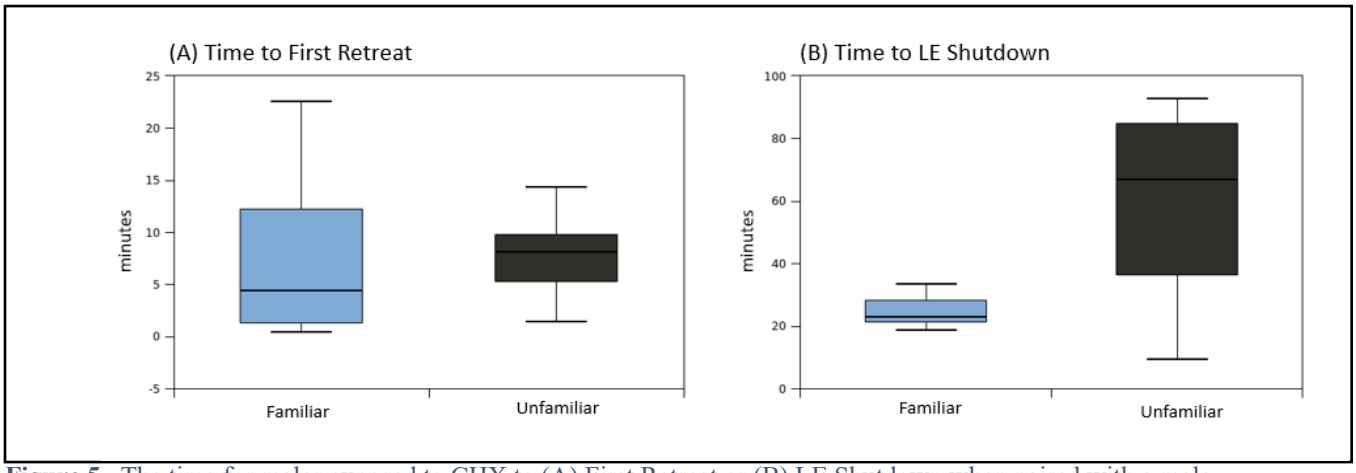

Figure 5. The time for males exposed to CHX to (A) First Retreat or (B) LE Shutdown when paired with a male they previously lost to (blue: familiar) or a male they had no prior experience fighting (grey: unfamiliar).

Discussion

Our study shows that broad-horned flour beetles with inhibited protein synthesis: i) stop behaving as losers, ii) delay initiation of new loser behavior (time to first retreat), and iii) delay the consolidation of STM to LTM after recurrent losses (time to enter LE shutdown). These results indicate that ongoing protein synthesis is required for males to acquire STM and maintain LTM, indicating that there is at least some aspect of the LE that depends on newly synthesized protein. The results of our study are consistent with previous findings in *Drosophila* where long-term loser effects are maintained by ongoing protein synthesis (Trannoy et al., 2016).

Across taxa, the onset of LE depends on changes in neuroendocrine levels and consequent degradation of proteins expression associated with a decline in synaptic signaling (Yang et al., 2020). For instance, in vertebrates the translocation of glucocorticoid receptors into the nucleus downregulates BDNF leading to decreased synaptic plasticity (Xu et al., 1998). In insects, similar neuroendocrine effects on gene expression are also responsible for the variation in stress-related synapse activity. However, the functional homolog of BDNF in insects remains unknown.

Stress influences neuronal plasticity at the synapse (J. Kim & Yoon, 1998; Krishnan & Nestler, 2008). Glutamate (GLU), the principal excitatory neurotransmitter that is essential for the maintenance and repair of the brain's neural networks, is the link between synaptic transmission and LE. The process is well-defined in vertebrates, wherein stimulation of presynaptic glutamate release results in postsynaptic glutamate receptor-induced activation of the BDNF/TrkB and intracellular PLC-γ pathways (Numakawa et al., 2009). When BDNF expression diminishes under stressful conditions, there is a decrease in the density of dendritic spines and consequently decreased neural connectivity (Radley et al., 2006). The decreased connectivity between neurons leads to decreased glutamate signaling, disruption of synaptic activity, and depression-like behavior (reviewed in Yang et al., 2020).

Glu may play a similar role in insects. Investigations of *Drosophila* larval neurons show that decreased glutamate excitability causes phosphorylation and inhibition of a FOXO transcription factor (Howlett et al., 2008). Under normal, non-stressed, conditions FOXO is bound by proteins in the insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway, preventing its translocation to the nucleus. We hypothesize that by blocking protein synthesis we are inhibiting the production of anti-stress proteins typically activated by the FOXO transcription

factor. However, our study cannot differentiate whether we are blocking the anti-stress proteins directly induced by FOXO, or we are inhibiting proteins in the IIS pathway from stress-induced release of FOXO, which would ultimately also prevent synthesis of FOXO associated anti-stress proteins.

Our comparison of familiar and unfamiliar beetles with inhibited protein synthesis suggests that two aspects of memory, i) competitor recognition and ii) losing, are regulated by different molecular mechanisms. First, we showed that there is no difference in the initial time it takes to establish the first defeat between beetles that are familiar or unfamiliar with their competitor (Figure 5A). However, +CHX losers that were familiar with their opponent initiated LE shutdown much quicker (24.64 ± 4.79 minutes; Table 4) than their +CHX unfamiliar counterparts (55.01 ± 29.42 minutes; Table 3 and Figure 5B) but still slowed initiation of LE shutdown relative to –CHX unfamiliar competitors (8.82 ± 4.52 minutes [Table 3], $t(27) = 9.06$, $p<0.001$). These results suggest that there is a competitor recognition component of LE that is not completely erased by inhibiting protein synthesis. This raises the question, how does recognizing an opponent override the memory-impeding effects of CHX treatment? An alternative explanation is that losers are not affected, and it is winners who have previously defeated their opponent, which drive our observations. Additional studies where winners are treated with CHX are needed to further understand this potentially novel aspect of memory.

Conclusion

We hypothesized that CHX treatment would erase the memory of being of loser by blocking the protein synthesis necessary to maintain LE behaviors. We predicted that this would lead to an increase in the time it takes for losers to make their first retreat in a new contest and delay the onset of LE shutdown. Our results confirm these predictions, demonstrating that

inhibiting protein synthesis eliminates the memory of losing and impedes forming new memories of losing. In the context of learning and memory theory, these results support previous work showing that ongoing protein synthesis is required to maintain LTM and consolidate STM into LTM.

Our study also potentially shows a novel aspect of memory that depends on recognition of the opponent, because LE shutdown begins sooner when contestants are familiar with each other. Since this pattern is true in the presence of CHX-induced protein inhibition it suggests that the recognition and losing aspects of memory may be regulated by different molecular mechanisms. This raises interesting questions about how similar the trigger for recognition must be, and the extent to which recognition by winners influences when their opponent enters LE.

Future studies using RNA-Seq to understand gene expression at the transcriptional level could help identify the specific proteins that are upregulated during LE. Such studies would provide potential candidates for targeted protein inhibition rather than the broad-spectrum inhibition of CHX. More broadly, additional studies such as these could inform behavioral genetics research in humans. For example, identifying the proteins that link learning, memory, and behavior could potentially lead to novel therapies for mental health related conditions such as depression and post-traumatic stress disorder (PTSD).

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Chapter 3

Elucidating the Molecular Basis of the Loser Effect:

Analyzing Gene Expression in Flour Beetles

Abstract

When losing an aggressive conflict with a conspecific one is said to have experienced social defeat. Social defeat elicits stereotypical loser behaviors such as isolation or submissiveness and, in many species, it is a precursor to the loser effect (LE). LE is the increased probability that the loser of a single fight will continue to lose in subsequent conflicts. Model insect systems help reveal physiological changes associated with social defeat; however, underlying genetic mechanisms of loser behavior remain unknown. For example, fruit fly aggression experiments show short-term loser behavior after single fights. Repeated defeats induce a prolonged loser behavior that depends on ongoing protein synthesis (Trannoy et al., 2016). No studies have documented the changes in gene expression levels from onset to recovery from LE behavior. This study aimed to observe behavior that stems from social defeat to identify changes in gene expression that correlate with the duration, the related protein synthesis activity, and the gene regulation of LE.

Introduction

When animals compete, resulting in a winner and a loser, the loser experiences a social defeat (SD) (Rillich & Stevenson, 2014). Immediately following SD, losers often manifest a syndrome of stereotypical depression-like behaviors, such as avoidance or submissiveness (Hsu et al., 2006). These behaviors are markers of social defeat stress (SDS), and for many species, individuals who experience SDS will go on to exhibit the loser effect (LE) (Iwasaki et al., 2006; Rose et al., 2017), which is the increased probability that the loser of a fight will continue to lose subsequent conflicts(Hsu et al., 2006; Hsu & Wolf, 1999; Rutte et al., 2006). The onset of the LE begins with the experience of losing; however, activation of the associated loser behaviors can start at the first sign of a threat or danger with activation of the evolutionarily conserved fight-or-flight response.

When vertebrates are threatened or stressed, the sympathetic nervous system activates the fight-or-flight response. The hypothalamus of the hypothalamus-pituitary-adrenal (HPA) axis signals the secretion of adrenaline into the bloodstream triggering an intracellular signaling cascade leading to the release of glucose for the energy needed to fight or flee. In a typical scenario, once the stress leaves, feedback mechanisms in the HPA axis promote homeostasis by inhibiting CRH and ACTH secretion (Makino et al., 2002). The parasympathetic nervous system returns the body to pre-stress status. However, if the threat persists, repeated activation of the hypothalamus-pituitary-adrenal (HPA) axis results in elevated levels of glucocorticoids (GCs), also known as the stress hormone cortisol (Hosseinichimeh et al., 2015).

The build-up of GCs has far-reaching consequences (e.g., depression) because of their indirect effect on the brain-derived neurotrophic factor (BDNF), an essential protein for learning and memory(Bathina & Das, 2015; Juruena et al., 2006). When GCs reach a neuronal cell, they

bind with the GC receptor (GR) complex in the cytoplasm (Munck & Brinck-Johnsen, 1968). When activated, the complex undergoes a conformational change enabling the GR to translocate to the nucleus(Galigniana et al., 2010; Oakley & Cidlowski, 2013; Wittmann et al., 2019), where GR directly downregulates the expression of BDNF(Chen et al., 2017) influencing mood disorders such as anxiety (Bergami et al., 2008) and depression (Taliaz et al., 2010).

In invertebrate systems, the response to acute stress involves elevated octopaminergic neurons releasing elevated levels of octopamine (OA), the invertebrate version of adrenaline, into the hemolymph, increasing arousal and alertness (Cinel et al., 2020). This aspect of OA signaling is analogous to vertebrate epinephrine signaling and similarly supports the fight-or-flight response as it activates glycogen production via the cAMP/PKA pathway (Cinel et al., 2020). OA also signals the *corpus cardiacum*, a bundle of neurosecretory cells located posterior to the brain, to secrete an adipokinetic hormone (AKH). AKH is responsible for mobilizing energy from fat bodies to the hemolymph, and the binding of AKH to their receptors (AKHR-GPCR) initiates homologous intracellular pathways to those regulated by BDNF/TrkB signaling in vertebrates (Bednářová et al., 2015). However, the exact transcriptional target (i.e., BDNF's functional equivalent) remains unclear.

Initiation of and Recovery from LE

LE depends on changes in neuroendocrine levels and degraded expression of proteins. These alterations lead to a decline in synaptic signaling, which induces the long-term depression-like phenotype observed in losers (Yang et al., 2020). An intracellular signal transduction cascade maintains the change in the strength of synaptic transmission, wherein high levels of stress can have a negative impact (J. J. Kim et al., 2006; J. Kim & Yoon, 1998; Krishnan & Nestler, 2008).The link between synaptic transmission and LE involves glutamate, the principal

excitatory neurotransmitter essential for maintaining and repairing the brain's neural networks. With diminished BDNF, decreased connectivity between neurons leads to reduced glutamate signaling, disruption of synaptic activity, and depression-like behavior observed in vertebrates (Yang et al., 2020). Similarly, *Drosophila* larval neurons show that activating PI3K decreases glutamate excitability, causing the phosphorylation and inhibition of FOXO, a transcription factor responsible for the gene expression of anti-stress proteins during a stressful event (Howlett et al., 2008).

In vertebrates, recent investigations show that FOXO transcription factors are essential in the physiology and etiology of depression (Rana et al., 2021). For example, mice express the FOXO1 transcription factor in brain areas related to stress (Biggs et al., 2001; Polter et al., 2009), and FOXO1 deficient mice exhibit a depression-like phenotype. Additionally, neurotransmitters and glucocorticoids can regulate FOXO transcription factors(Liang et al., 2006; Qin et al., 2014), which suggests a link with the HPA axis stress pathway. Furthermore, the highly conserved insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway associated with FOXOs is highly conserved across vertebrates and invertebrates, suggesting a strong potential that a FOXO transcriptional response to SDS exists in insects. We hypothesize that the upregulation of a FOXO transcription factor is the "molecular switch" that initiates recovery from LE and loser behaviors.

What maintains LE?

To fully understand LE, discussing its connection with memory formation is essential. There are two primary phases, short-term memory (STM) and long-term memory (LTM). Short-term memory, the first phase, is produced in the first moments after acquiring the memory, lasts minutes to hours, and depends on post-translational modification of postsynaptic proteins

(Hernandez & Abel, 2008). In contrast, LTM is the second phase; it can last from hours to weeks and depends on intracellular signaling and the regulation of transcription and translation (Hernandez & Abel, 2008). Thus, the behaviors that lead to the establishment of LE represent the long-term memory (LTM) phase of learning and memory.

In broad-horned flour beetles, *Gnatocerus cornutus*, male losers with inhibited protein synthesis: i) stop behaving as losers, ii) delay initiation of new loser behavior (time to first retreat), and iii) delay the consolidation of STM to LTM after recurrent losses (time to enter LE shutdown). These results indicate that ongoing protein synthesis is required for males to acquire STM and maintain LTM, indicating that at least some aspect of the LE depends on newly synthesized protein. Similar *Drosophila* results show that ongoing protein synthesis holds long-term loser effects (Trannoy et al., 2016).

This study used RNA-Seq to try to understand gene expression of LE at the transcriptional level to help identify the specific proteins that upregulate during LE. The intent was to correlate loser behaviors throughout LE with the upregulation and downregulation of genes expressed simultaneously when the behaviors occur. The onset of loser behavior defines the moment when one male makes a retreat and displays a significantly reduced activity level (e.g., dramatically reduced movement, lethargy, etc.). The termination of loser behavior is then the point at which normal activity levels resume (e.g., activity displayed before loser behavior onset). We expect the duration of loser behavior to range from one to four days (Okada & Miyatake, 2010).

Potentially, the results of this study could identify candidates for targeted protein inhibition rather than the broad-spectrum inhibition of cycloheximide (CHX). More broadly, additional studies could inform human behavioral genetics research. For example, identifying the

proteins that link learning, memory, and behavior could lead to novel therapies for mental health-related conditions such as depression and post-traumatic stress disorder (PTSD).

Materials and Methods

Study System

The study system used in this experiment is the broad-horned flour beetle, *Gnatocerus cornutus*. *G.cornutus* is ideal for conducting behavioral experiments with easy manipulation to perform stereotypical social behaviors associated with mate competition. They require simple accommodations and have a relatively short development time (~1.5 months from egg to adult). A sexually dimorphic species, the males possess large mandibular horns, widened gena, and a pair of small horns on the vertex of the head. The females lack these traits. The males use their horns to fight other males for access to females. The losers in these bouts show well-defined loser behaviors where they retreat, exhibit avoidance behavior, and isolate themselves for the duration of their LE.

Animal Husbandry

The *G. cornutus* used in this study come from populations reared in the laboratory for many generations with occasional temporary bottlenecks brought on by overcrowding. Stocks are raised and maintained on standard media made up of a 95:5 ratio of whole wheat organic flour: brewer's yeast by weight. Stocks are in 30ºC and 70% relative humidity (RH) incubator with a 24D:0L photoperiod. Stock cultures remain in 45L x 30W x 8D cm covered plastic trays filled ~3 centimeters deep with media. Single final instar larvae from the stock cultures were extracted and transferred to individual glass vials 25Diam x 95H mm containing 2g of media.

We identify males by mandibular horns' presence after eclosion when the larvae become adults. Two weeks after eclosion, we prepare them for competition. First, we measured body

weight (+/-0.001mg) using an AT261 Delta Range precision balance (Mettler-Toledo Inc., Columbus, OH). After recording the weight, the beetle received an identification number. We then arbitrarily chose one of the males to receive a white dot on the elytra, using Wite-Out®, to be distinguished while fighting. All participants remained in isolation until being used for contests described below.

Behavior Observations

The fighting arena was 2.5cm in diameter, and before each contest, a new filter paper (Whatman® qualitative filter paper) was added to the bottom to provide traction. The fighting environment was in a dark incubator (at 30ºC and 70% RH), and we used a Sony Handycam HDR-SR5 under red lighting to record behavior. Every 8-hour timepoint, one dotted and one plain competitor entered the fighting arena, and video recording began immediately after. Each contest lasted 20-minutes which gave ample time for a loser to be declared.

Sampling regime

All males were naïve contestants (contestants who had never participated in competition) and had never mated with a female. The control samples were males who remained naïve and not used in intrasexual contests. In total, there are 15 timepoint samples and one control sample. The first time point is T-0. Losers from T-0 were sacrificed (frozen in liquid nitrogen) immediately after the loser entered LE shutdown, where losers suffer from so many repetitive defeats that they avoid further contact and isolate to the best of their ability, ceasing all regular locomotor activity. For the second time point T-8, losers were separated for 8 hours after entering LE shutdown and immediately sacrificed. For the third time point, T-16, losers were isolated for 16 hours after entering LE shutdown and immediately sacrificed. This sampling regime repeats in 8-

hr increments until 96hrs. An additional timepoint sampled, T-120, represents when LE duration has passed.

We used the Promega SV Total RNA Isolation System Kit and protocol for small tissue samples of ≤30mg for RNA extractions. We used the Agilent 2100 BioAnalyzer and the Nanodrop to analyze for quality control. Each RNA sample consists of three beetles as Controls and three at each time point. All extracted samples were put on ice and sent to Novogene for 150PE library prep and sequencing.

Data Analysis

Expression quantification

To quantify expression, we first uploaded data to Galaxy (usegalaxy.org). We then used salmon quant (v1.5.1; (Patro et al., 2017)) in Reads mode to pseudoalign paired reads from each timepoint to a reference transcriptome. Since *G. cornutus* does not have an annotated genome available, we used a transcriptome previously generated from the same beetle stocks as our reference (Ramesh & Demuth, 2020). Based on busco analysis, the reference transcriptome is approximately 98% complete (1622 single copy + 464 duplicated, out of 2124 total endopterygota buscos). The Galaxy workflow history is available at (LE Time Series Galaxy History).

Differential Expression of Time Series

Normalized (TPM) gene level expression data were exported and compiled into a table where each gene is a row and each timepoint a column. This table was used as input for time series analysis using the R package maSigPro (v 1.68.0; (Nueda et al., 2018)). We tested gene expression changes over time using linear and polynomial models of degree 1-6 and compiled a list of significant genes for each model. The design matric and expression data were imported using the RStudio *import dataset* tool. For each file we specified to use included header and get

row names from the first column. We used a Single Time Course design matrix where each timepoint was a single replicate (i.e., unreplicated design). We also ran the models using a pseudoreplicated design where control was its own timepoint, T0-T16 = Day 1 (3 replicates, T24-40 = Day2 (3 replicates), T48-T64= Day 3 (3 replicates), T72-T88=Day 4 (3 replicates), and T96-T120=Day5 (2 replicates; only 96, 120). Genes were considered significant at FDR = 0.05. The R markdown notebook and input files are included as Appendix 1.

Functional Analysis

To get functional annotations for genes with significant changes in gene expression over time we used blastx to query translated *G. cornutus* transcripts against the *Tribolium castaneum* protein database via the NCBI blast website. For each, transcript we extracted the accession number for the best hit in *T. castaneum*. We then used Biomart (Ensemble Metazoa Genes v 54; *Tribolium castaneum* genes (Tcas5.2)) to extract Stable Gene IDs and functional annotations for each *T. castaneum* gene that is homologous to a differentially expressed gene in *G. cornutus*. Finally, we analyzed our results for functional enrichment using the Gene Ontology webtools ((geneontology.org; (Ashburner et al., 2000; Mi et al., 2019) Gene Ontology Consortium 2021)). We submitted the list of GeneIDs (TC#), specified the GO category to analyze (biological process, molecular function, or cellular component), and the GO database for *Tribolium castaneum*. From the results page we further performed pathway enrichment analysis using Annotation Data Set = PANTHER Pathways.

Results

We generated an average of 45.7M reads per sample (range: 40.5M – 50.6; Table 1) across each of the 15 time points (including the control time point). The control time point was

collected prior to fighting, while the Time 0 (T0) was collected as soon as the beetle entered LE shutdown.

Table 1 Data Quality Summary

Sample	Raw reads	Raw data	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
Control	48,644,468	7.3	98.00	0.02	98.63	95.55	41.48
T0	50,536,658	7.6	98.02	0.03	97.87	94.13	40.42
T8	50,636,232	7.6	98.38	0.03	97.75	93.70	37.35
T16	40,763,352	6.1	97.75	0.03	97.74	93.70	36.89
T24	43,352,978	6.5	98.20	0.03	97.70	93.76	41.17
T32	42,932,480	6.4	98.44	0.03	97.83	93.97	37.83
T40	45,440,902	6.8	98.21	0.02	97.98	94.37	41.54
T48	48,171,918	7.2	98.19	0.03	97.83	94.01	39.74
T56	41,836,994	6.3	98.23	0.03	97.85	94.10	39.41
T64	46,551,078	7.0	98.37	0.03	97.89	94.02	38.78
T72	47,029,772	7.1	98.10	0.02	98.59	95.47	40.89
T80	43,938,124	6.6	98.04	0.02	98.54	95.36	41.16
T88	45,360,434	6.8	97.48	0.02	98.33	94.65	39.06
T96	40,499,570	6.1	98.22	0.02	98.62	95.35	37.81
T120	49,302,186	7.4	97.79	0.02	98.58	95.27	37.90

Sample: Timepoints in hours after entering LE shutdown
Raw data: (Raw reads) * (sequence length=150bp), calculating in GB
Effective: (Clean reads/Raw reads)*100%
Error: base error rate
Q20, Q30: (Base count of Phred value > 20 or 30) / (Total base count)
GC: (G & C base count) / (Total base count)

Behavior Observations

Losing contestants are not sacrificed until they enter the LE shutdown phase. So, the gene expression data that reveals the natural duration and termination of LE should also coincide with behavior observed across time points. Before sacrificing each loser, we visually watched him with care not to re-introduce stress. To get a quick and easy sense of variation in activity, we compare what we observe visually to what we previously documented using grid lines (Fig 1). We use grid lines to record baseline behaviors across a spectrum of situations (e.g., naïve males, losers, winners, etc.). The earlier time points (T-0 to T-32) showed losers in full LE shutdown mode, where they did not move. Losers in time points T-40 to T-72 showed some movement but remained within one or two grid squares. However, from T-80 to T-96, we noticed increased activity across multiple grid squares.

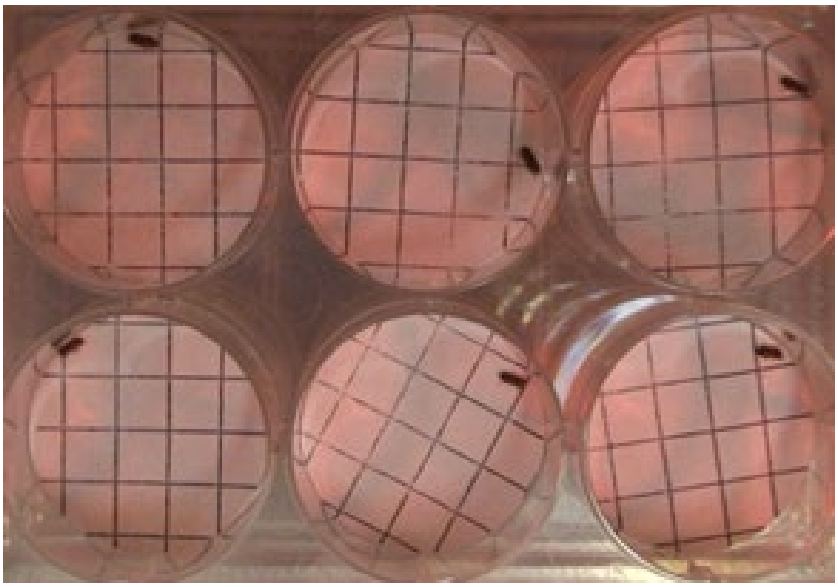

Figure 1. Grid lines used to establish baseline behavior.

Time series analysis where each time point was treated as a single replicate identified 2,3,1, and 38 genes as significant at FDR<0.05 for the polynomial regressions with degrees 3,4,5, and 6 respectively. In each case the lower degree polynomial identified a subset of the genes identified by higher degree models. No genes were found to have a significant association between expression and time under a simple linear or quadratic model (i.e., degree 1 or 2). Additionally, pooling timepoints into sets of 24-hour time periods to create pseudo replicates did not identify any additional significant associations. The complete list of significant genes with normalized expression values (TPM) and associated model significance are included in Supplementary Material (Table S1).

Differential Expression and Functional Enrichment

Significant genes fall into five broad categories (Figure 3, A-E). The most common pattern (17/38 = 44.7%) are genes that are expressed in control males but not expressed throughout LE shutdown (Figure 3B). The second most common pattern (11/28 = 28.9%) belongs to genes that are only transcribed late, as beetles come out of LE shutdown (Figure 3A). Four genes showed a

pattern wherein expression was upregulated in the first 8 hours of LE shutdown, then declined to control levels by 24 hours, then increased again late (Figure 3C). Three genes were expressed in controls the downregulated or silenced early in LE shutdown until 120 hours, when they returned to control levels or higher (Figure 3D). Finally, three genes showed intermediate peaks during LE shutdown with a strong upregulation late (Figure 3E).

Our Gene Ontology (GO) analysis revealed several functional categories significantly enriched among our differentially expressed genes (Complete annotated list presented in Supplemental Table S2). The most common expression pattern (Figure 3, Cluster B), where genes are detected in controls and downregulated throughout LE shutdown, is functionally characterized primarily by enrichment for the octopamine and glutamate receptor homologs, as well as neuronal ion channel transport (blue bars in Figures 4-6). Biological process analysis also indicates spermatogenesis homologs are enriched in this cluster. Interestingly, homologs involved in meiosis and DNA repair pathways are enriched in cluster D (red bars in Figures 4-6), where we see expression return to control levels at the 120-hour timepoint.

For genes expressed primarily in the recovery from LE shutdown (Figure 3, Cluster A; yellow bar in Figure 4) we find significant enrichment for only a histone deacetylase based on GO term analysis alone. However, PANTHER pathway analysis indicates enrichment for the p38 MAPK and Oxidative Stress pathways owing to G_corn_DN1316 (DN1316). DN1316 encodes a homolog of MYOCYTE-SPECIFIC ENHANCER FACTOR 2, a MADS box containing transcription factor. Interestingly, the MAPK signaling pathway is known to interact with the transcription factor nF-*k*B, which modulates a variety of anti-stress and anti-inflamatory proteins in mammals (Falcicchia et al., 2020). Our data also show an additional nF-*k*B binding protein homolog in expression cluster C (Figure 3, green) where genes have peaks of expression

early in LE shutdown (0-8 hours) and late (120 hours). The final expression cluster, E, shows GO term enrichment for the mitotic cytokinesis Biological Process (purple bars in Figure 5) and several PANTHER pathways involved in Axonal plasticity, all owing to G_corn_DN24096_c0_g1_i2.p1.p1 (DN24096) a homolog of the gene Actin (Figure 2).

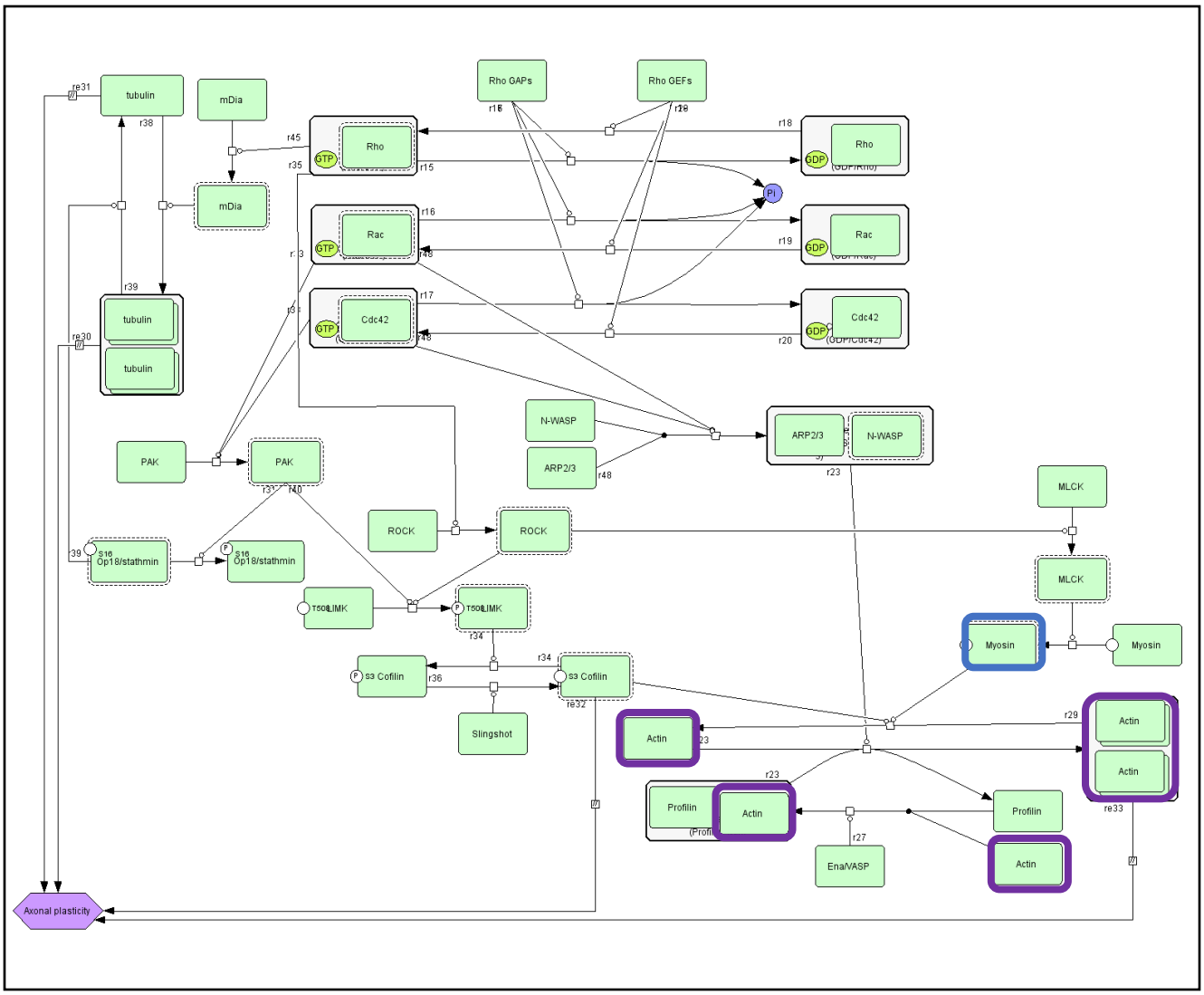

Figure 2. PANTHER Axonal plasticity pathway highlighting homologous genes that are differentially expressed in our analysis.

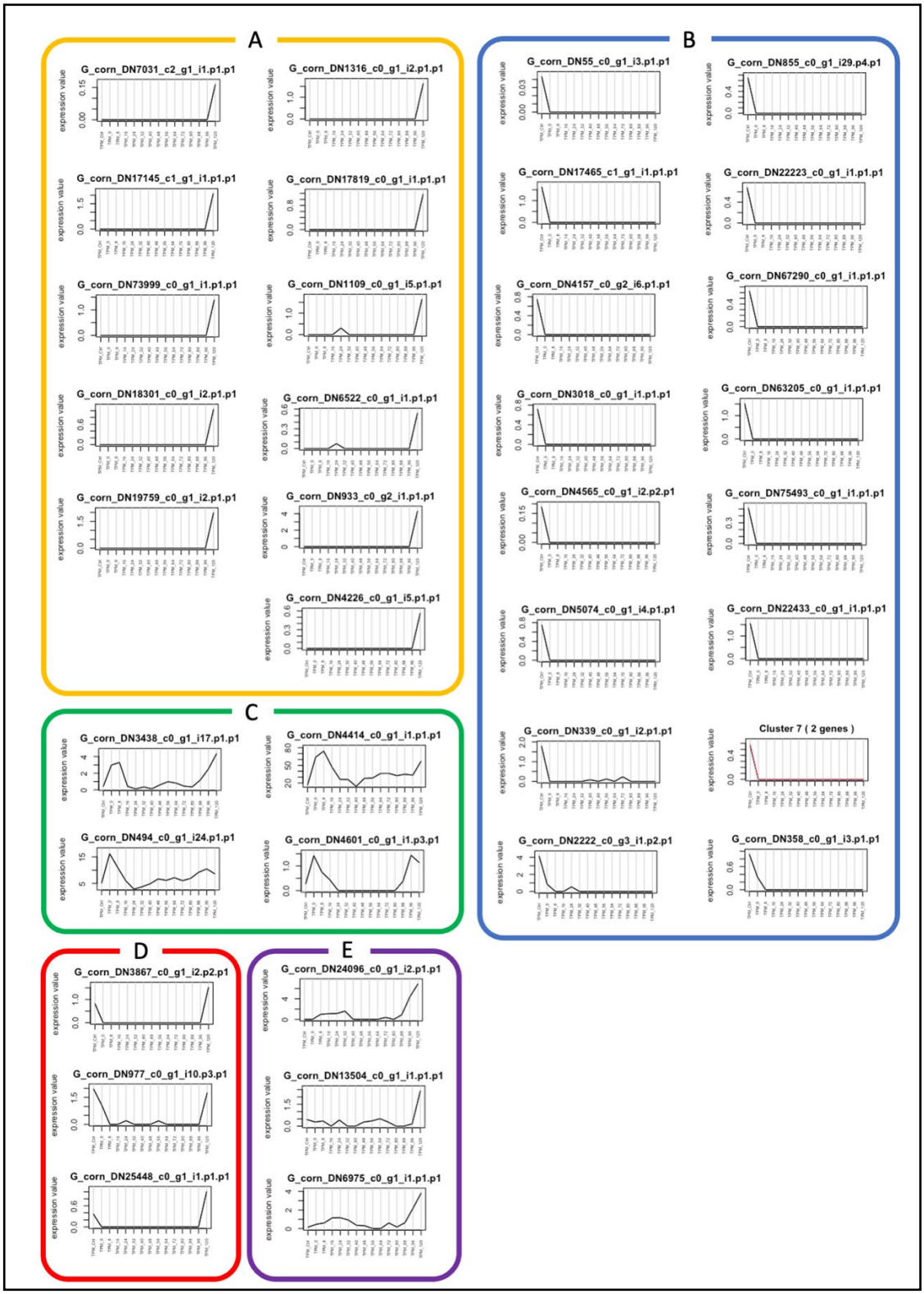

Figure 3. Expression pattern clusters for genes with differential expression across time. Expression values are TPM.TPM_Ctrl = control beetles that were not used for male-male competitions. TPM_0 – TPM_120 = Time points 0 (immediately upon entering LE shutdown) to 120 hours post initiation of LE shutdown.

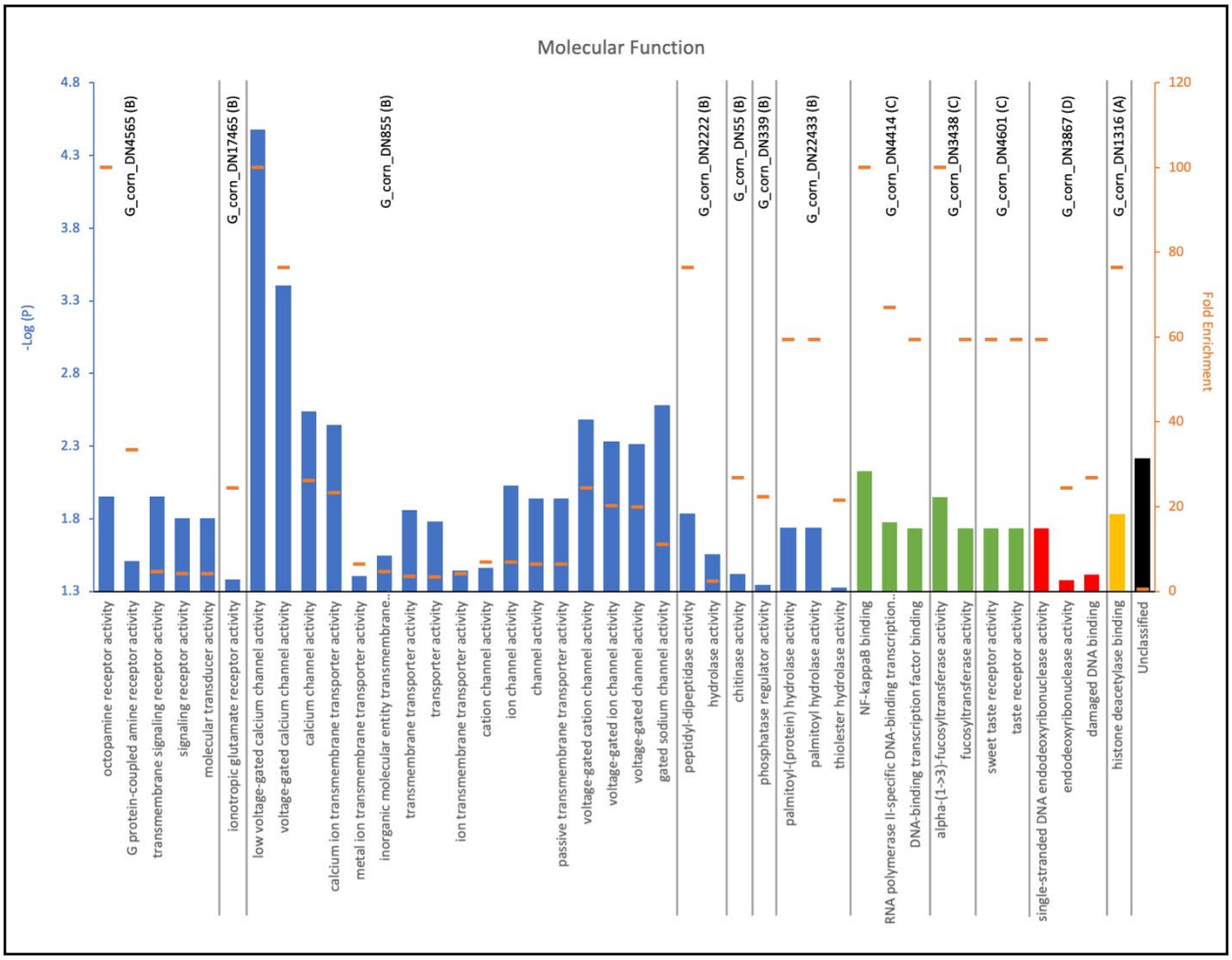

Figure 4. Results of Gene Ontology (GO) enrichment for Molecular Function. -Log(0.05) = 1.3. Grey vertical bars separate hierarchically defined GO terms and include overlapping gene sets. GO terms within a group are listed from most specific (left) to most general (right). Orange bars indicate fold enrichment (# of differentially genes / expected number for each GO term). Expected number compared to proportion of that GO term in *T. castaneum*. Bars indicating negative log p-value (left axis) are color coded to match Figure 3, and a *G. cornutus* homolog representing each GO term cluster is indicated along with the expression pattern cluster's letter.

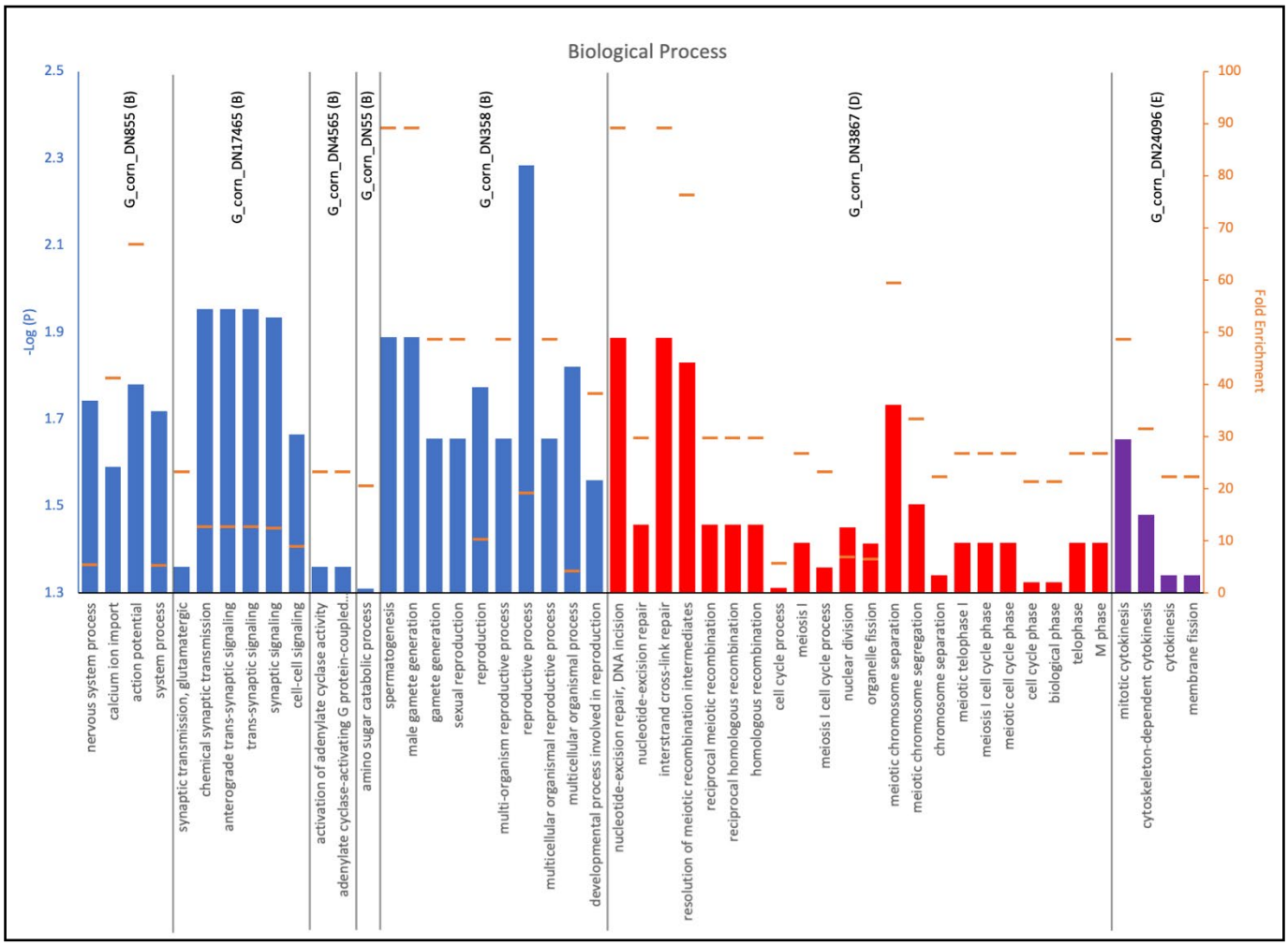

Figure 5. Results of Gene Ontology (GO) enrichment for Biological Process. -Log(0.05) = 1.3. Grey vertical bars separate hierarchically defined GO terms and include overlapping gene sets. GO terms within a group are listed from most specific (left) to most general (right). Orange bars indicate fold enrichment (# of differentially genes / expected number for each GO term). Expected number compared to proportion of that GO term in *T. castaneum*. Bars indicating negative log p-value (left axis) are color coded to match Figure 3, and a *G. cornutus* homolog representing each GO term cluster is indicated along with the expression pattern cluster's letter.

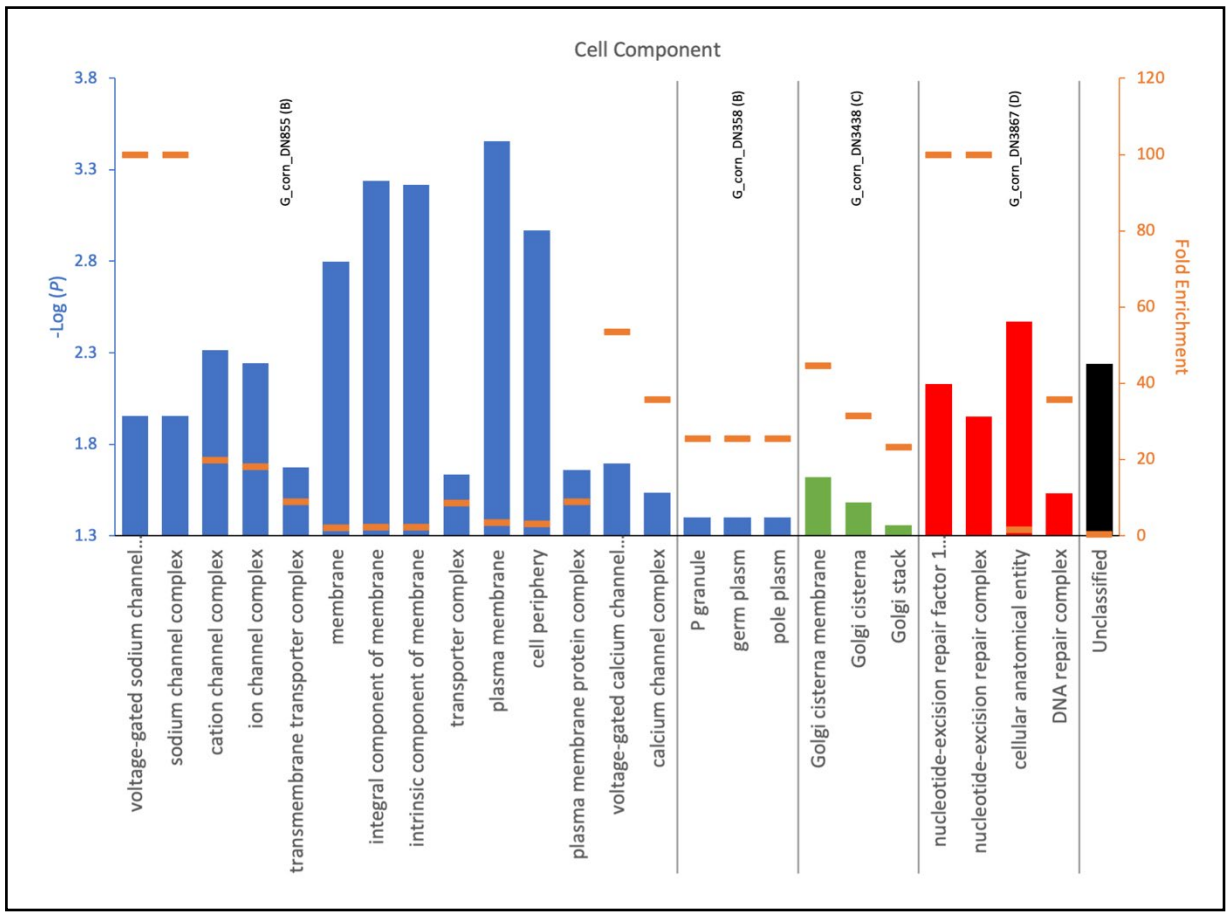

Figure 6. Results of Gene Ontology (GO) enrichment for Cellular Component. -Log(0.05) = 1.3. Grey vertical bars separate hierarchically defined GO terms and include overlapping gene sets. GO terms within a group are listed from most specific (left) to most general (right). Orange bars indicate fold enrichment (# of differentially genes / expected number for each GO term). Expected number compared to proportion of that GO term in *T. castaneum*. Bars indicating negative log p-value (left axis) are color coded to match Figure 3, and a *G. cornutus* homolog representing each GO term cluster is indicated along with the expression pattern cluster's letter.

Discussion

The results of this study demonstrate potential genes associated with the onset and recovery from LE shutdown. Because all contestants enter LE at T-0, we can infer that the genes turned either off or on at this point relative to controls are associated with LE onset. In contrast, genes that change expression from 96 to 120 hours may represent molecular signals for individuals to exit LE shutdown and return to normal activity. Following, we discuss our findings regarding

the molecular genetic mechanisms regulating the onset and recovery from LE shutdown in *G. cornutus* in the context of the neurophysiology of behavior.

Onset of LE shutdown

The results of this study show the consequences of LE by revealing the molecular changes associated with the regulation of LE shutdown. Our data show that the onset of LE shutdown is characterized by dramatic downregulation of glutamate and octopamine neurotransmitter receptors (DN17465 and DN4565 respectively). Glutamate is the principal excitatory neurotransmitter key to synaptic transmission across neurons. In normal conditions, stimulation of presynaptic glutamate release results in postsynaptic glutamate receptor-induced activation which is necessary for maintaining good synaptic spine density (Numakawa et al., 2009). Downregulation of glutamate signaling causes reduced dendritic spine density (Kasai et al., 2010). Therefore, our observed silencing of the *G. cornutus* glutamate receptor homolog (DN17465) is expected to cause a negative feedback loop consistent with the long-term depression-like state experienced by losers.

Octopamine (OA) -- the insect member of the catecholamine family of neurotransmitters that includes epinephrine (adrenaline) and norepinephrine in vertebrates -- partially mediates the fight-or-flight response. In mammals, chronic exposure to stress results in continuous adrenaline production to maintain the energy required to keep going until the threat passes (Koolhaas et al., 2017). The *G. cornutus* OA receptor (DN4565), which is downregulated at the outset of LE shutdown, is a typical rhodopsin-like G protein-coupled receptor with a sequence like that of vertebrate adrenergic receptors (Maqueira et al., 2005). In response to OA, adrenergic-like OA receptors typically induce the generation of intracellular secondary messengers such as cyclic adenosine monophosphate (cAMP) and calcium ions (Ca2+) (Huang et al., 2012; Verlinden et

al., 2010). As a consequence of reduced OA signaling intracellular Ca2+ increases, which may lead to disruption of insulin signaling (Guo & Kang, 2016). In insect systems, a disturbance in insulin signaling inhibits the FOXO transcription factor from translocating across the nucleus and activating anti-stress proteins (See Chapter 1 Review). Our data also shows downregulation of the calcium import homolog DN855 during this initial phase of LE shutdown, but it is unclear whether this is a protective mechanism against too much intracellular calcium, which may damage cells, or it is a secondary mechanism to further modulate insulin signaling. In summary, our results indicate that even though chronic conflict may continue to elicit neurotransmitters production, at some point critical receptor production shuts down in the loser, thereby short-circuiting the signal to continue fighting.

An interesting but unexpected finding among the genes that are expressed in control but silenced in LE shutdown, is a gene involved in spermatogenesis (DN358). Intriguingly, the downregulation of spermatogenesis during LE shutdown also corresponds with changes in expression of meiosis related DNA repair (DN3867) and transposable element related genes (DN7031 and DN19759). Changes in expression of these genes across LE shutdown suggest a model wherein losers have diminished sperm production while in LE shutdown but as they recover, transposons and DNA repair are simultaneously activated. Future studies will be needed to confirm this pattern but reducing sperm production during a phase where mating efforts cease, could be an adaptation to conserve energy.

Recovery from LE Shutdown

We find 17 genes whose upregulation mark the exit of LE shutdown (Figure 3 A, D, and E). All these genes show dramatic upregulation from 96 to 120 hours. Several of them also show minor spikes at earlier timepoints (e.g., 24 hours); however, it is possible that these early signs of

upregulation may represent a subset of the individuals in our sample initiating the path to exiting LE shutdown. One of the genes in this category, DN1109, is especially interesting. It is homologous to a group of secondary transporters called the Major Facilitator Superfamily (MFS), which are members of solute carrier family 18 (DN1109 is homologous to SLC18B1). Members of the SLC18 family perform active transport into synaptic vesicles for neurotransmitters acetylcholine, dopamine, serotonin, and epinephrine (octopamine in insects) with polymorphisms possibly linked to neuropsychiatric disorders (Lawal & Krantz, 2013). An insect-specific member, SLC18A4, is also associated with insect learning and memory in *Drosophila* (Brooks et al., 2011). The SLC18 proteins are associated with neurons and neuroendocrine cells and localize to secretory vesicles responsible for storing neurotransmitters before release. Furthermore, molecular genetic analyses of vesicular transporters have shown that presynaptic changes in their expression can regulate neurotransmitter release and postsynaptic signaling and that loss of transport activity can have profound behavioral consequences (Lawal & Krantz, 2013). Assuming DN1109 is an octopamine transporter, our data suggests that regaining its expression between 96 and 120 hours reverses the neurotransmitter blocking mechanism described above for the onset of LE shutdown and results in a return to normal activity.

Several of the genes upregulated at 120 hours are associated with the neuronal mitogen-activated protein kinase (p38 MAPK) pathway, including: DN1316 (myocyte enhancer factor 2), DN24096 (Actin), and DN4414 (NF-kB binding protein). In vertebrates, the p38 MAPK pathway responds to neuroendocrine signaling, and helps govern synaptic plasticity, and cellular stress responses(Falcicchia et al., 2020). The p38MAPK pathway is crucial for regulating synaptic function related to learning and memory. It also modulates long-term potentiation and

long-term depression of synaptic transmission via effects on synaptic plasticity. Our data show that activation of the pathway in *G. cornutus* coincides with recovery from LE shutdown, likely as a consequence of returning synaptic transmission to normal. Additionally, in neurons, astrocytes, and microglia the p38MAPK pathway mediates the production of proteins necessary for future stress responses. More specifically, nF-kB and other p38MAPK initiated transcription factors regulate production of both iNOS and IL-6, which may play roles in rapid response to stress via nitric oxide release and cytokine pathways respectively. iNOS and IL-6 are not found in our list of differentially regulated genes, but that may be because their functions are primarily governed by post-translational modifications, while their transcription remains relatively stable.

Other potential signaling genes

In contrast to those genes above that have relatively clear association with either the onset or recovery from LE shutdown, there are four genes that show more complex patterns. They are upregulated from controls to T0, suggesting they are part of the signal that initiates LE shutdown. However, they remain active at low levels throughout shutdown, until late, when they increase in a way that suggests they may also provide a signal to recover normal behavior. One of the genes, DN3438 is a fucosyltransferase homolog. In mice, fucosyltransferase deficiency promotes neuroinflammation by increasing the sensitivity of glial cells to inflammatory mediators (Lu et al., 2019). The second gene is an NF-kB binding protein, whose neuron related functions were discussed above in relation to p38MAPK signaling. The third gene, DN494, is a homolog of *spinster* (*spin*). In *Drosophila, spin* is involved in programmed cell death (PCD) of neuronal cells, where mutants for *spin* fail to undergo appropriate PCD resulting in elongated central nervous systems (Nakano et al., 2001). While we can envision a role for increased PCD accompanying the downregulation of synapses prompted by the deteriorating glutamate and or

octopamine signaling, it is unclear how upregulation of a PCD gene would signal recovery. The final of the four genes is DN4601, a homolog of gustatory receptor for sugar taste.

Conclusions

The gene expression data in this study largely reinforce current understanding of how vital certain neurotransmitters are in regulating long-term depression. LE shutdown appears to be initiated by shutting down neurotransmitter production, while recovery is mediated by elevating expression of anti-stress pathways associated with increased synapse potential. We also see that spermatogenesis and sensory receptors (taste and smell) are diminished throughout LE shutdown but are upregulated late, indicating a return to normal bodily functions upon recovery.

We initially hypothesized, based on literature review and our CHX experiments that a possible explanation for LE shutdown duration could be mediated by FOXO. Highly stressful situations wherein stress hormones are repeatedly released are known to inhibit FOXO activity. Inhibiting FOXO means that anti-stress hormones are not activated, which is predicted to directly contribute to the duration of LE shutdown. Although FOXO was not in our list of genes whose expression was significantly associated with time, we used tblastn to identify the *T. castaneum* homolog of FOXO in the *G. cornutus* transcriptome (G_corn_DN92= 97% identical). We found that FOXO is expressed throughout LE shutdown and therefore, if FOXO is playing an important role in the onset and maintenance of LE shutdown, then it must be regulated at the level of translation or by post-translational modification.

The present results of transcriptome analysis throughout the course of LE shutdown, combined with our earlier work blocking protein synthesis via CHX treatment (see Chapter 2), suggest that LE shutdown is substantially regulated at the level of translation or post-translational modification. The CHX experiment found that ongoing protein synthesis is

required to maintain LE shutdown. Beetles exposed to CHX shortly after entering LE shutdown effectively had their memory "reset" and did not readily reenter LE shutdown. However, our RNA-Seq data do not clearly recommend a candidate gene or genes that are upregulated at the onset of LE shutdown, which would be blocked by CHX. DN4414 (NF-kB binding protein) seems like the most likely candidate, but future studies are necessary to tease apart the specific genes and regulatory mechanism(s) governing LE shutdown.

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Chapter 4

Effects of starvation on aggression levels in the broad-horned flour beetle,

Gnatocerus cornutus

Abstract

Animals often compete directly for access to food, where nutritional state of the competitors strongly determines the probability of success. Classic competition theory proposes that food scarcity will lead to increased intraspecific aggression. The self-assessment hypothesis further suggests that weaker competitors will be first to reach an energy threshold and accept defeat. Here we investigate the effect of starvation on levels of aggression in a system where males engage in male-male competition for mates. We recorded aggressive activity in 20-minute bouts between size-matched naïve males from fed and starved treatments, where the starved competitor was food-deprived for up to 264 hours. Our results show that starved beetles initially show reduced aggression but over the course of prolonged food deprivation levels of aggression increase to those comparable with individuals who were fed continuously. These findings are difficult to explain with either classic competition or self-assessment theories.

Introduction

Across taxa, food deprivation can affect typical social behavior and is known to cause varying levels of aggression. In humans, low glucose levels cause an imbalance of equilibrium in the body's cells leading to irritability and aggravation, a phenomenon commonly referred to as being "hangry" (Benton, 2002; Bushman, DeWall, Pond, & Hanus, 2014; Craig & Halton, 2009). Behavior experiments with mice have demonstrated how the intensity of interaction between individuals can vary based on the hunger level and whether food is readily available (Fredericson, 1950). In siblicidal avian species (e.g., blue-footed booby, black-legged kittiwake), if parental feeding is scarce, dominant nesting siblings that are hungry will increase aggression towards younger nestmates with violent pecking bouts (Drummond, 2001; Machmer & Ydenberg, 1998; Mora et al., 2010).

When individuals fight directly for access to food, the degree of hunger may influence chief characteristics that help determine fighting success, resource holding power, and motivation. Resource holding power (RHP), or the ability to win a fight, along with motivation influences fighting success for a limited resource (e.g., food) (Enquist & Leimar, 1987; Parker, 1974a). RHP consists of intrinsic components such as body size and physiological state (Marden & Waage, 1990; Riechert, 1978), and the motivation to fight involves how willing the individual is to expend energy or take risks and how valuable the resource is (Enquist & Leimar, 1987; Riechert, 1988). For example, faced with depleted foraging sites, female chamois have been observed to adopt more alert postures and increase vigilance, the interruption of chewing while scanning, and behave more aggressively towards others (Fattorini et al., 2018a). Similarly, seabird observations have shown adjustments towards aggressive behaviors (e.g., wing-displays and foraging distance) depending on the amount of available food and the extent of competition

involved (Enquist et al., 1985; Lewis et al., 2001). Even insects experience hunger driven aggression. In a study on house crickets, Nosil, 2002, found that larger male house crickets are usually the winners, but after 72 hours without food, large size only increases fighting success when both contestants are equally food-deprived (Nosil, 2002).

Survival depends on the availability of food, and competition for food can be expensive as it costs time and energy to acquire and keep it. Because selection should favor individuals who make the right decisions to survive (Maynard Smith & Parker, 1976; Parker, 1974b), we might ask what tactics/strategies are used by those who survive starvation. Additionally, what effect does starvation have on levels of aggression as time progresses without acquiring food (e.g., increase or decrease aggressive activity)?

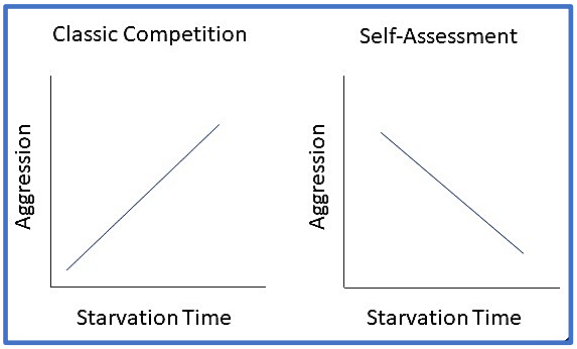

Figure 1. Classic competition theory vs self-assessment hypothesis

Classic competition theory proposes that the availability of a resource is inversely proportional to competitive interactions (Fattorini et al., 2018b; Schoener, 1973). That is, when food absence leads to increasingly hungry individuals, intraspecific aggression will increase. Various studies document such experiences. Hungry crayfish will use the cheliped extension display, a typical signal of motivation and aggression, considerably more following extended periods without food and a hermit crab that starved for seven days will throw caution aside and use risky moves against a larger conspecific (Brian et al., 1966; Hazlett et al., 1975; Liu et al., 2017; Stocker & Huber, 2001). Similarly, lab observations of seed bugs reveal increases in the frequency and magnitude of aggressive maneuvers, namely fighting and chasing, against an opponent after ten days without food (Himuro & Fujisaki, 2012b). Further, observations of

honeybees show more impulsiveness as starvation develops (Mayack & Naug, 2015)**.** Collectively, these observations presume a correlation between increased hunger, which elevates the motivation to fight for food, and increased aggression needed for competitive interactions (Fig 1). Thus, the longer an individual starves, the expectation is that there would be a steady increase in aggressive activity over time. Initially, individuals will display typical aggressive behavior that eventually leads to a state of frantic aggression or a state of being, ''hangry."

An alternative consideration is the self-assessment fighting strategy (Taylor & Elwood, 2003), where contestants assess their own ability to fight without taking the opponents' RHP into account. RHP (e.g., body size, weapon size, physiological differences) correlates with contest outcome (Okada et al., 2006; Riechert, 1978), and asymmetrical differences between contestant's aid in determining victory. Large differences tend to yield quick outcomes, and small differences lead to longer contest durations (Prenter et al., 2006). The contestant with the lower RHP will reach an energy limit and accept defeat based on evaluating its own ability to win (Arnott & Elwood, 2009a; Rutte, Taborsky, & Brinkhof, 2006a). This determination involves the decision, if or when, to engage the opponent and for how long. If fighters are weak from starvation, there may be an outcome where levels of aggression decrease as starvation increases across Time (Fig 1). We would initially expect to see typical levels of aggression when food is first absent. Following, we expect to see a steady decrease in aggression as time passes and food absence is prolonged.

Conventional intraspecific aggression, such as intentional head or body attacks, makes the broad-horned flour beetle, *Gnatocerus cornutus*, an ideal system for this area of behavioral research. These consistent physical maneuvers provide a secure platform to measure a range of starvation-induced aggressive activity. The *G. cornutus* displays a strong sexual dimorphism

(Chapter 2 Figure 1) where the adult males have sexually selected traits that females lack, including enlarged mandibular horns, flat, full cephalic dorsal projections, and small horns on the vertex of the head. Males utilize the mandibular horns as weapons to fight other males for access to mating opportunity (Okada et al., 2006). Losers of these aggressive male-male contests exhibit a well-defined loser effect, which is the high probability that once an individual loses an aggressive conflict, subsequent conflicts will also be lost(Rutte, Taborsky, & Brinkhof, 2006b). *G. cornutus* losers retreat from the fight, avoid interaction, and cease normal activities for an extended period (Okada et al., 2006). The loser effect does not determine the fighting ability of an individual but instead allows a self-assessment on the probability of a win or loss (Arnott & Elwood, 2009b; Hsu & Wolf, 2001; Huber et al., 2002; Rutte et al., 2006a).

This study examines the behavioral changes associated with prolonged food deprivation. We present a group with typical levels of aggressive activity (control) to compete with a group that has atypical circumstances (starved). Counts of initiated aggressive activity measure the response as food limitation increases across a 264-hour duration (i.e., 11 days). This experiment seeks to document the changes in aggression levels across time and present a data-driven analysis to explain the behavioral observations. Providing these answers will add to the current understanding of how nutritional status influences social behavior.

Materials and Methods

Animal Husbandry

The *G. cornutus* used in this study come from populations reared in the laboratory for many generations with occasional temporary bottlenecks brought on by overcrowding. Stocks are raised and maintained on standard media made up of 95:5 ratio of whole wheat organic flour:

brewer's yeast by weight. Stocks are in 30ºC and 70% relative humidity (RH) incubator with a 24D:0L photoperiod. Stock cultures remain in 45L x 30W x 8D cm covered plastic trays filled ~3 centimeters deep with media. Single final instar larvae from the stock cultures were extracted and transferred to individual glass vials 25Diam x 95H mm containing 2g of media.

We identify males by the presence of mandibular horns after eclosion, which is when the larvae become adults. Two weeks after eclosion, we prepare them for competition. First, we measured body weight (+/-0.001mg) using an AT261 Delta Range precision balance (Mettler-Toledo Inc., Columbus, OH). After recording the weight, the beetle received an identification number and was then designated as fed or starved (fed = food available *ad libitum;* starved = deprived of all food). To the extent possible, each fighting pair was size-matched, and we arbitrarily chose one of the males to receive a white dot on the elytra, using Wite-Out®, to be distinguished while fighting (Chapter 2 Figure 2). All participants were maintained in isolation until being used for contests described below.

Behavior Trials

The fighting arena was 2.5cm in diameter, and before each contest, a new filter paper (Whatman® qualitative filter paper) was added to the bottom to provide traction. The fighting environment was in a dark incubator (at 30ºC and 70% RH), and we used a Sony Handy Cam HDR-SR5 under red lighting to record behavior. The starved individuals were food-deprived between 24 and 264 hours (i.e., 1 to 11 days) and visually confirmed to be alive before each competition. Every 24-hour timepoint, in sync with the starved groups' food absence, one starved and one fed competitor entered the fighting arena (Fig 2), and video recording began immediately after.

Each trial was recorded for 20-minutes. To avoid bias, the person watching the recordings and scoring the competitions did not know which beetle belonged to the starved or fed treatment. We quantified the starved individuals' behavior by counting the number of aggressive encounters per trial where every 5-seconds we assigned a 1 (present) or 0 (absent) to mark whether each behavior in Table 1 was performed or not (Edwards et al., 2006). We then calculated the sum of each behavioral activity and calculated the average aggression for each fighter. Observed behaviors varied across a spectrum from no movement to aggressive initiations; however, only the dynamic changes scored (Table 1). Aggressive initiations included: head contact (HC) - characterized an aggressive behavior when one beetle initiates the connection of the horns and mandibles to the opponent's head area.; body contact (BC) - defined an aggressive behavior wherein a beetle will initiate contact of the horns and mandibles to the opponent's body; and climbing over (CO) – one beetle forcefully positions himself over the opponent. The two passive methods of avoidance behavior were turning away and moving away from the situation or resting as if avoiding all confrontation entirely.

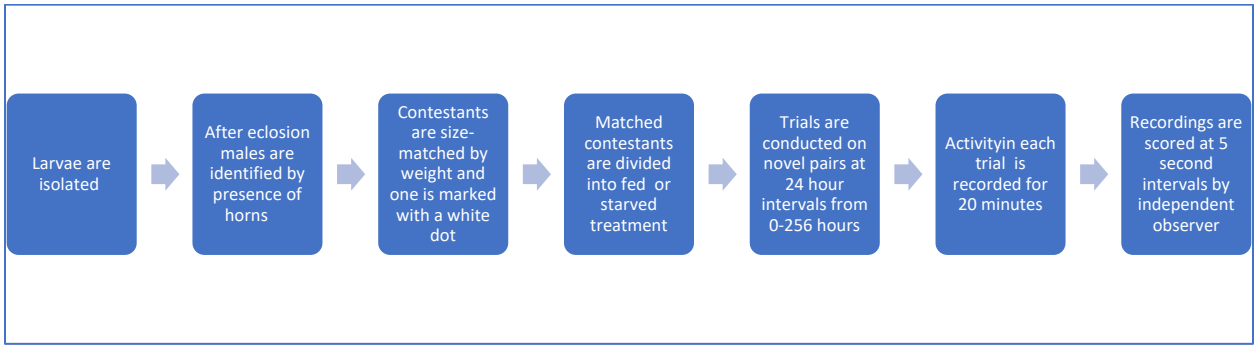

Figure 2. Flowchart of Behavior Trials. Males fought only once so that both participants were naïve fighters for all contests. Starved individuals were visually confirmed to be still alive. Each beetle was then placed in the arena with a divider to keep them separated. Removing the divider signaled the beginning of the 20-minute competition.

Table 1. Ethogram of Behaviors.

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

Statistical Evaluation

Descriptive statistics assessed each group, fed, and starved. A Pearson correlation analysis showed the association between the levels of aggression ('aggression') and hours of starvation ('time') for both the fed and starved groups. The effect size was measured using Cohen's d standard. A polynomial regression analysis was conducted on both fed and starved groups to assess whether the relationship between time and starvation were related in a non-linear way. Differences among models were evaluated using the AIC criterion determined the best polynomial model to represent the data. Statistics were computed using Intellectus Statistics (*Intellectus Statistics [Online Computer Software]*, 2022) , and RStudio desktop (v1.0.136).

Results

We scored behavioral observations by playback of the video recording for 33 contests in total. A multivariate regression analysis was conducted to assess whether Time and Status significantly predicted Aggression. The results of the regression model were significant, F (3,62) = 14.21, $p < .001$, $R^2 = .41$, indicating that approximately 40.75% of the variance in aggression is explainable by Time and Status (Table 2). On average, aggression increases over time, driven primarily by the increase observed in starving beetles (Figure 5). Status also significantly

predicts aggression, because across all timepoints, fed males performed 42.9% more aggressive maneuvers per trial than starved males (Table 2: Summary statistics; Table 3: Status effect). The interaction between Time and Status did not have a significant effect on aggression. However, at 24 hours of starvation the distribution of aggressive maneuvers is clearly lower in starved beetles than in fed beetles, but it the middle of the experiment the distributions overlap, suggesting fed and starved beetles are not responding the same way. Furthermore, simple linear regression of time on aggression for starved beetles is significant ($F(1,31) = 4.30$, $p = .047$, $R^2 = .12$).

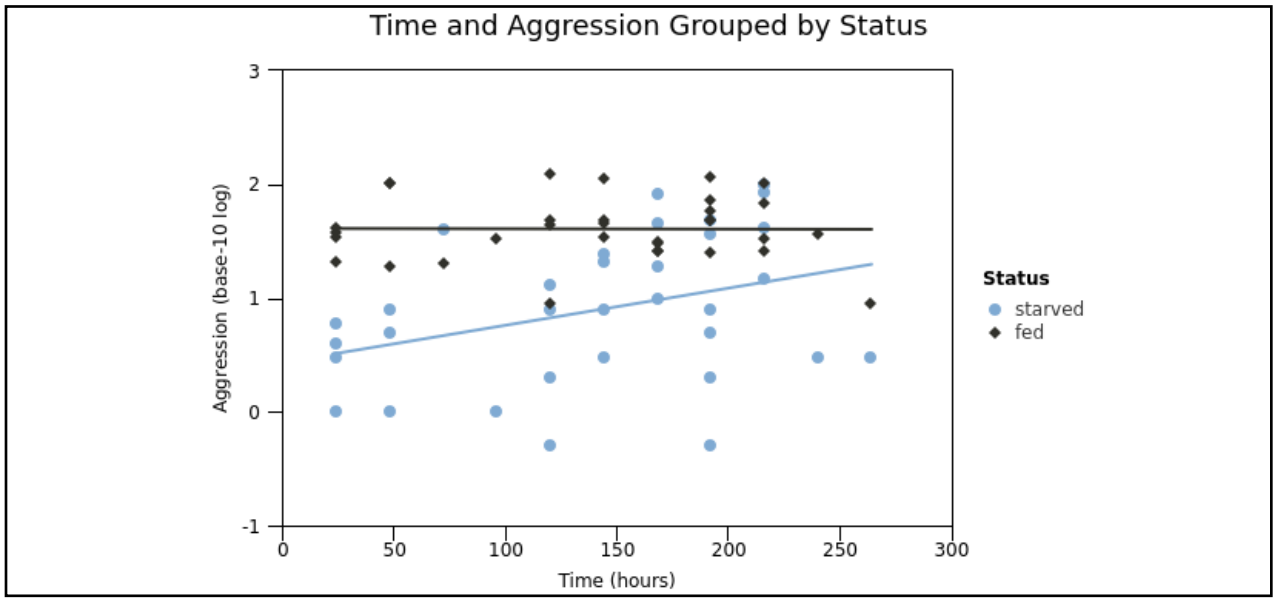

Figure 3. Increased activity across time. These results showing increased aggressive activity with time are similar to results found in Himuro and Fujisaki, 2012 (Himuro & Fujisaki, 2012a) wherein aggressive behaviors between males occurred more frequently after ten days starvation. Note: Calculations based on α=0.05; n=33.

Table 2. Summary Statistics Table for Interval and Ratio Variables

Variable	M	SD	n	SE_M	Min	Max	*Mdn*
fed	49.61	32.41	33	5.64	9.00	125.00	38.00
starved	19.82	26.44	33	4.60	0.50	98.00	8.00

Note. '-' indicates the statistic is undefined due to constant data or an insufficient sample size.

Table 3. Results for Linear Regression with Time, Status, and Time:Status predicting Aggression

Variable	B	SE	95.00% CI	β	t	p
(Intercept)	0.44	0.19	[0.05, 0.82]	0.00	2.25	.028
Time	0.003	0.001	[0.0008, 0.006]	0.37	2.65	.010
Status (Fed or Starved)	1.18	0.27	[0.63, 1.72]	0.96	4.31	< .001
Time:Status	-0.003	0.002	[-0.007, 0.0002]	-0.46	-1.89	.063

Note. Results: F (3,62) = 14.21, $p < .001$, $R^2 = .41$
Unstandardized Regression Equation: Aggression = 0.44 + 0.003*Time + 1.18*Status - 0.003*Time:Status

Linear Model	*Aggression ~ Time + Ɛ*
Quadratic Model	*Aggression ~ Time² + Ɛ*
Cubic Model	*Aggression ~ Time³ + Ɛ*

Summary of AIC scores for Three Polynomial Regression Models for Starved and Fed Groups

Group	Linear Model	Quadratic Model	Cubic Model
Starved	-113.456	-111.7641	-112.0589
Fed	-116.5611	-115.7848	-114.4748

Table 4. Akaike Information Criteria. The linear model received the lowest AIC score in both groups ($AIC_{starved}$ = -113.456; $AIC_{control}$ = -116.5611), indicating that this is the most parsimonious model for the given data

The Akaike Information Criteria (AIC) was applied to the regression analysis to compare the best model for the given data set, where the lowest AIC indicates the "best" model to explain the specified data (Akaike, 1973). Three models were estimated using R, and their associated *AIC* scores calculated (See Table 3 for AIC summaries).

Behavioral observations

Contestants performed typical fighting maneuvers for this species, such as interlocking mandibles, shoving or pushing the opponent, and lifting an opponent from the fighting arena

(Okada et al., 2006, 2010). On occasion, the beetles would lock horns in head contact for long periods, wherein they seemed to lock their mandibles onto one another, but only one beetle appears to be primarily pushing and making the forward motions. The second most frequent aggression observed was body contact, which often resulted in flipping an opponent onto his back. Following body contact, the opponent responds with one of three responses. The opponent either took an aggressive approach and turned toward the initiating beetle and engaged in head contact or adopted a maneuver. Climbing over would often happen in the context of after a prolonged head contact, one beetle would climb on top of the other beetle as if to attempt copulation. All three aggressive behaviors happened throughout the entire experiment.

At the beginning of the trial, the fed group had a higher amount of aggressive activity than the starved group and maintained this same level of aggression throughout the experiment. The starved group, however, had much lower initial aggression that gradually increases until the individuals could no longer put up a good fight, eventually dying. Although all individuals were size-matched and age-matched, there were differences in survival and performance within the starved group. Mortality was higher for fighters set to compete from 72 hours – 96 hours and between 240 hours -264 hours, and all individuals set to fight after 288 hours without food expired before their scheduled competition. Some of the individuals after 24, 48, and 96 hours of food deprivation maintained a lower level of aggression compared to the starved individuals at 240 and 264 hours. Additionally, there are two situations at 120 hours and 192 hours where individuals failed to display any aggression at all where other competitors at the same time points displayed substantially higher aggression, even compared to those in the first 24 hours. Alternatively, individuals in the starved group after 72 hours, 168 hours, 192 hours, and 216 hours that met or exceeded the aggression levels of counterparts within the fed group.

Discussion

While our results demonstrate characteristics of both classic competition and self-assessment theories, neither provide a complete explanation for *G. cornutus* behavior over the course of the experiment. As classic competition theory predicts, we see an increase in aggressive activity across time with increased starvation. However, starved beetles were not more aggressive than beetles fed continuously, which is counter to the expectations of competition theory, since competition theory predicts that more resource-rich environments should produce less aggressive individuals. Indeed, early in starvation (less than 120 hours) aggression levels are substantially reduced. This early reduction is reasonably explained by self-assessment, which predicts that starved individuals, having lower RHP, will be less aggressive because they are more likely to lose encounters. However, starved beetles' aggression increased to the same level as fed beetles after 120 hours, which is counter to the prediction of self-assessment since beetles starved longer should have lower RHP and continue to decrease their aggression as their physiological condition deteriorates. Thus, neither competition nor self-assessment provides a comprehensive context to interpret our results.

Our observations are best explained by the physiological response wherein individuals with low glucose levels become "hangry," a colloquial term used to describe increased anger and aggression when individuals get are overly hungry. In vertebrates, the association between low energy state is relatively well understood (Fokidis et al., 2013; Janson & Vogel, 2006; Lischinsky & Lin, 2020; Rohles & Wilson, 1974). Food deprivation triggers the release of a gastric peptide ghrelin, while low glucose and lipids levels block insulin and leptin release. Although the primary function of this pathway is to stimulate feeding, in vitro studies show that that ghrelin can directly activate, while insulin inhibits regions of the hypothalamus that control

aggression. For instance, infusion of ghrelin promotes male-male aggression (Vestlund et al., 2019). However, the association between "hunger" and aggression is less well understood in invertebrates.

Physiological responses to starvation stress are similar across vertebrates and invertebrates. Both experience a shift from metabolizing carbohydrates, to fat, and then to protein (reviewed in McCue 2020). Indeed, the preferential shift to fat metabolism rather than protein metabolism after carbohydrates are depleted is thought to be an adaptive mechanism that protects muscle digestion and prolongs activity that may help organisms persist when faced with prolonged food deprivation Our data are consistent with an adaptive response where in starvation-induced aggression may be linked to the shift toward lipid metabolism in a way that helps weaker individuals maintain competitiveness despite starvation's physical and physiological costs. We also see that once these fat reserves depleted, after ~200 hours in *G. cornutus*, they were unable to maintain aggression, which would be consistent with the necessary shift to metabolizing muscle in the final stages before death (Wang et al., 2006).

Given the gross similarity in physiological and behavioral responses to starvation between what is known from vertebrates, and what we observe in G. cornutus, these beetles may provide an excellent model for future studies of the molecular basis of aggression. Identifying the functional homolog of ghrelin may provide a promising avenue for future work.

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