AGGRESSION AND PARENTAL CARE OF *NICROPHORUS ORBICOLLIS*

by

SHANNON D. HARRIS

(Under the Direction of Patricia Moore)

ABSTRACT

This study seeks to understand flexible paternal care of *Nicrophorus orbicollis* beginning with a synthesis of parental care within *N. orbicollis* (Chapter 1). I transition into investigating intra-specific competition and its connection to parental care (Chapter 2) by encouraging male-male competition prior to reproduction. Competitive males participated in parental care more than non-competitive males. Male experience had no bearing on female behavior, ensuring the integrity of offspring. This progressed into investigating aggression's role in parental care and winning/losing effects (Chapter 3). I do this by sampling males from different reproductive stages and look at subsequent expression of the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*. The *octopamine α receptor* was differentially expressed across stages implicating its role in aggression and parental care. I found no evidence of winner/loser effects in *N. orbicollis*.

INDEX WORDS: Parental Care, Intra-specific, Competition, Flexible, Aggression, *Nicrophorus orbicollis*, *octopamine α receptor*, *octopmanine/tyramine 1 receptor*

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SHANNON D. HARRIS

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by

SHANNON D. HARRIS

Major Professor: Patricia Moore

Committee: Jill Anderson
Kelly Dyer
Carmen Blubaugh

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
August 2022

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1:

[1]PARENTAL CARE OF *NICROPHORUS ORBICOLLIS*

Evolution of Parental Care

Parental care is defined as any “parental trait – behavioral or other - that increases the fitness of a parent’s offspring and that is likely to have originated and/or is currently maintained for this function” (Royle, Smiseth & Kölliker 2012). The evolution of parental care is a coevolutionary process. Parental fitness and offspring fitness are both determined by how parents and offspring interact with each other with respect to phenotypic and genotypic characteristics displayed by both participating parties (Royle, Smiseth & Kölliker 2012). The evolution of parental care could be attributed to combat stressors or hazards like predation, harsh conditions, stable environments, and competition for resources (Trivers 1972; Tallamy 1984; Trumbo & Sikes 2021). Numerous species across taxa have seen repeated convergence of behaviors associated with parental care (Tallamy 1984). Although beneficial to offspring, parental care can render physical injury or death for parental figures who actively perform parental duties that involve the defense of their offspring (Tallamy 1984). Usually, a parents’ decision to participate in parental care renders an energetic cost. This decision is strongly dependent on whether the benefits of parental care outweigh the energetic and physical costs of being an actual parent (Royle, Smiseth & Kölliker 2012). In most taxa maternal care predominates due to evolutionary

[1] S.D. Harris & P.J. Moore. To be submitted to *Insects*

constraints like sperm competition and paternity insecurities in males (Kahn, Schwanz & Kokko 2013) that favor uniparental maternal care.

Evolution of Biparental Care

Although maternal care dominates, paternal care exists in many species like fishes, birds, insects, some mammals, and anurans. Paternal care is a rare occurrence and is suggested to have evolved as a potential form of mate assistance (Fetherston, Scott & Traniello 1990) although there are many instances of male only uniparental care. Species that exhibit male parental care show a great deal of variation of degree and mode in which males participate in parental care. Males can participate in a range of behaviors associated with parental care like nest construction, provisioning, and even male pregnancy. Male-only care is a rare occurrence in which it only occurs in about 2% of all species (Gross 2005). In the instance of male-only care, males are often limited to playing the role of the protector of their offspring (Kahn, Schwanz & Kokko 2013). Male-only care can be selected for in environments in which males experience fewer costs than their mate or when males can care for multiple families without having to sacrifice the opportunity to experience multiple mating events (Gross 2005; Kahn, Schwanz & Kokko 2013). Additionally, sexual selection of caring males drives the evolution of male-only care (Kahn, Schwanz & Kokko 2013). In general, it seems as if the costs/benefits between each parent determines which mode of care a species will predominate.

The evolution of biparental care becomes of significant interest. Biparental care is likely to be selected for in species where both parents will directly benefit from maximum lifetime reproductive success. Realistically, most offspring have two parents which adds a certain level of complication. Biparental care is considered rare due to variation in confidence of paternity in comparison to maternity (Suzuki 2013). The degree of paternal care is often variable. This happens because the degree of paternity assurance often correlates with the degree of paternal investment across species, within species as well as individually (Kvarnemo 2006). Often a species will resort to biparental care, care provided by both the male and female, if care from a singular parent is insufficient or not as good as biparental care (Smith 1977; Jenkins, Morris & Blackman 2000). Labor associated with resource preparation and territorial defense selects for an increase in paternal investment. In many cases, proper exploitation of reproductive resources requires a cooperative effort of both parents to ensure reproductive success (Tallamy & Wood 1986).

Other factors such as competition come into play, as the evolution and maintenance of biparental families is more complex than mate assistance alone. Intraspecific competition for resources is proposed to play a significant role in promoting and maintaining monogamous families (Trumbo 1994). Post-hatch paternal care is believed to have evolved to defend the brood against conspecific competitors and ensuring reproductive success (Scott 1989). Conspecific competitors pose a direct danger to offspring, as they often commit infanticide and usurp reproductive resources to use for the development of their own offspring (Clouse 1995; Trumbo 2006; Choe 2010). In such competitive environments, enhancement of parental attentiveness through prolonged biparental care may be crucial to mitigate unsuccessful reproductive events (Tallamy & Wood 1986).

Biparental care confers quite a few benefits like reduction in female reproductive costs, exclusive mating opportunities and high paternity assurance (Jenkins, Morris & Blackman 2000). Alternatively, the costs of biparental care of each parent are reliant on the amount of care each parent provides (Royle, Smiseth & Kölliker 2012). From an unbiased standpoint, both males and females will experience the same degree of loss of opportunity to engage in additional mating events (Kvarnemo 2006). In general monogamy is a rare phenomenon across taxa (Brown, Morales & Summers 2010) suggesting the costs of biparental care outweigh the benefits. Realistically, within biparental care, comes variation in the degree of which both parents engage in reproductive efforts. Many biparental families participate in flexible compensatory biparental care. Here flexible compensatory parental care happens when the primary parent deserts or reduces parental care which primes the secondary parent to step in and compensate for the absence of the other parent (Ringler et al. 2015). This mode of biparental care could serve as a comfortable compromise for species that engage in biparental care.

Parental Care of *Nicrophorus orbicollis*

Social behavior involves cooperation, organization, communication, and coordinated actions amongst a set group of individuals. For social interactions to persist, at least one individual must provide a limiting resource to some extent to another individual or set of individuals (Choe & Crespi 1997). Subsociality, the simplest form of social behavior, involves prolonged, highly developed parental care of offspring in the absence of specialization (Roy-Zokan et al. 2015; Benowitz, McKinney & Moore 2016; Pizzi & Rehan 2021). Subsocial families consists only of parents and offspring and are typically defined by the types of behaviors expressed as parental care such as brood defense and provisioning/regurgitation (Linksvayer 2010) *N. orbicollis* are a biparental, subsocial carrion beetle in which both parents participate in all behaviors associated with parental care (Trumbo 1990; Royle, Smiseth & Kölliker 2012; Cunningham 2020). Biparental care in the *Nicrophorus* genus likely evolved as a response to their dependance of rare and ephemeral and easily accessible/monopolized resources (Jenkins, Morris & Blackman 2000).

Nicrophorus orbicollis, and burying beetles in general, are intriguing model organisms to study parental care as they have a complex system of parental care. Although *N. orbicollis* is commonly biparental, evidence of maternal uniparental care, paternal uniparental care and flexible biparental care can be shown in *N. orbicollis* (Benowitz, McKinney & Moore 2016). Within biparental families, burying beetles often participate in flexible compensatory parental care in the event of desertion or reduction of care by the secondary parent (Smiseth et al. 2005; Ringler et al. 2015). In general, burying beetle offspring may only require one parent to survive, causing the second parent to remain as insurance should any harm come to the primary parent (Trumbo 2012) priming males to desert their broods well before offspring have fully developed (Scott & Traniello 1990) when the primary parent remains healthy and an active parent.

Modes of Parental Care & Male Flexibility

The most common form of parental care exhibited by burying beetles is biparental care. In nature a male may pair with a female provided the male finds reproductive material (small vertebrate carcass) to progress towards biparental care (Trumbo 1990). Both male and females are attracted to and will participate in intraspecific competition for small vertebrate carcass. These competitive events continue until one male and one female remain to then use the small vertebrate carcass as a reproductive and nutritional resource for offspring (Scott 1990). Males will then release sex pheromone to attract the female to engage in copulation/mating activity (Panov 2012). Prior to mating, the pair will reposition, remove fur or feathers, ingest internal organs, roll into a ball, and bury the small vertebrate carcass (Scott 1990;). Here both males and females cooperate in burying carrion (Scott 1990). As burying of the carrion occurs, the pair engage in copulation and within 48 hours, eggs are oviposited in the surrounding soil (Trumbo

1990). After the vertebrate carcass is rolled into a ball, both males and females can participate in using their oral and anal secretions to coat the surface of the carrion (Scott 1990). Within 4 days of oviposition, eggs will hatch (Fetherston, Scott & Traniello 1994) and larvae will colonize the prepared vertebrate carcass. When larvae colonize the brood, parents will continue performing parental care behaviors by projecting anal and oral secretions to maintain the integrity of the carcass, brood guarding, and provisioning larvae by directly regurgitating pre-digested carrion (Scott 1990).

In biparental families of *N. orbicollis*, both males and females can remain to actively participate in all behaviors associated with post-hatch parental care (Trumbo 1990) however, there are sex-specific differences within the level of parental investment provided. In typical biparental families, males flexibly participate in parental care and usually desert the carcass before larvae development concludes. Males will typically desert the brood within the first three days of when larvae hatch. Unlike males, females will participate in post-hatch parental care until larvae have fully developed which typically takes about 6-8 days (Scott & Traniello 1990). In past studies, females have been observed to directly provision pre-digested carrion to offspring more frequently than males while male parents participate in brood guarding more than female parents. The general idea is that male presence and brood guarding reduces the likelihood of conspecific competitors (Fetherston, Scott & Traniello 1994). Male conspecifics become a threat as they discover and will attempt to kill offspring, expel one or both parents from the carcass, and hijack the carcass resource to then use as their own (Robertson 1994).

Although less common in *N. orbicollis*, both male and female parents can individually participate in uniparental care if needed. Males have been known to engage in flexible compensatory parental care provided the female mate has encountered injury or death

(Fetherston, Scott & Traniello 1990). An abundance of mating opportunities primes males to engage in flexible paternal care. In general males show an affinity to continuing to mate with other females provided the opportunity presents itself and almost certainly will not pass up the opportunity (Robertson 1994). Flexible behavior allows opportunity for individuals to appropriately respond to unpredictable environments and social interactions (Cunningham, Douthit and Moore 2014). Alternatively, an unpaired female may mate and use previously stored sperm to establish a brood if unpaired at the time of carrion discovery (Trumbo 1990).

Just as females, males can independently participate in all parental behaviors associated with pre- and post-hatch parental care needed for offspring to fully develop (Fetherston, Scott & Traniello 1990; 1994) however, key differences can be observed between male and female parents providing evidence of a potential sexual conflict. For instance, sex of the parent plays a role on brood size. Females typically have larger broods per breeding attempt than males suggesting differences in approaches to parental care (Smith, Creighton & Belk 2015) despite possessing the same parental care capabilities, introducing the concept of sexual conflict between males and females in brood size. The key difference here is that females prime their brood size to match resource availability while males remain relatively consistent in maintaining a brood 60% of its original size (Smith, Creighton & Belk 2015). Offspring number is regulated through direct means such as reducing the number of eggs before they hatch or reducing the number of larvae via filial cannibalism. Through regulation of brood size, parents indirectly control body size of offspring in which smaller broods typically have larger body sizes and vice versa. An added factor of regulation of brood size is reliant on the female adaptive response to the local environment. Specifically local population densities have a significant role in final determination of brood size/larvae number in which low population densities would yield larger broods and high population density yields low brood sizes (Creighton 2005).

Social Immunity

Burying beetles have developed a form of immunity that protects their offspring from microbial species. Due to their biparental nature, both parents contribute to social immunity which has its benefits. Involvement of both parents increases genetic diversity thus, increasing the effectiveness of social immunity efforts (Cotter & Kilner 2010). Here burying beetles have been found to cover their carcass with oral and anal secretions. Doing this prevents bacteria and other microbial species from growing on the surface of the carcass, maintaining palatability of the reproductive resource (Steiger et al. 2011). Because burying beetles reposition their reproductive resource below the soil, they subject their resource to opportunistic microorganisms within the soil that are looking to readily make contact with the breeding resource. More importantly, *N. orbicollis* is associated with wet soils, increasing the diversity and abundance of microbial activity (Hoback et al. 2004) increasing the need for social immunity for offspring survival. Hoback and colleagues (2004) found that the anal secretions of *N. orbicollis* contain the ability to reduce microbial growth.

Selection could potentially favor the evolution of antimicrobial agents within anal and oral secretions as a response to natural microbial species and their competitive nature to colonize carrion to render them unpalatable (Hall et al. 2011). Many insect species produce antimicrobial peptides (AMPs) that allow them to reclaim infected carrion from microbial populations. In a study conducted by Hall and colleagues, both native soil microbial species and yeast isolates were inhibited when treated with oral and anal secretions of *N. orbicollis*. Additionally, they found that although microorganismal growth was not inhibited, *Bacillus cereus* colony showed to be sufficiently inhibited by *N. orbicollis* and other *Nicrophorus* beetle species. They were able to confirm that the antimicrobial components of anal and oral secretions are proteins. (Hall et al. 2011). Anal secretions of burying beetles have been found to contain components that inhibit gram - positive, gram- negative, antifungal agents as well as other various antimicrobial species (Trumbo 2016).

Physiology of Parental Care

Juvenile Hormone (JH)

Juvenile hormone (JH) is an essential component in reproduction in insect species (Scott et al. 2001). JH plays a central role in ovarian development and promotes production of Vitellogenin (Vg) (Paiaitof & Scott 2006). Vitellogenin's main function is to provide the embryo with amino acids and other nutritional needs (Scott, Panaitof & Carleton 2005). Burying beetles experience significant fluctuations in levels of JH titers throughout the reproductive cycle (Scott et al. 2001).

Fluctuations of JH begin with a gradual increase for the first 20 days of adult females (Trumbo, Borst & Robinson 1995) with a sharp increase following the discovery of a carcass, a required reproductive resource. Upon the discovery of this resource, JH titers show an immediate two-fold increase, a further increase three to four-fold in JH titers and a subsequent two to three-fold increase in ovarian mass (Scott et al. 2001; Panaitof & Scott 2006). The conditions for ovarian development are restricted to the true detection of a carcass. Ovarian development does not persist even with available mates or nutritional contents of a carcass resource (Scott, Panaitof & Carleton 2005). Additionally, JH titers show to be high when the female engages behaviors of active parental care that include preparation of the previously discovered carcass, feeding larvae and maintenance of the carcass (Trumbo & Robinson 2008).

JH titer levels in males paint a picture that varies from females during parental care. Specifically, discovery of carcass stimulated a rise in JH of 10-15-fold within the first hour of initial discovery. However, levels of JH titer were not maintained and showed to decline shortly after. JH titers continue to decline as the parental care cycle proceeds. Specifically, by day three JH titers were nine-fold lower and remained consistently low throughout the remainder of larvae development (Trumbo & Robinson 2008). It is likely that JH titers show this pattern as males are more flexible parents and typically desert their broods by day three (Scott & Traniello 1990).

Monoamines

Variation in parental care can show to be a direct reflection of variation in the expression of molecular regulators of behavior, such as neurotransmitters, within the parent-offspring system of interactions. Parent-offspring traits are determined by their respective genotypes (Royle, Smiseth & Kölliker 2012). Parent-offspring interactions many times share a common genetic basis in which genes responsible for regulation of parental behavior also play key roles in regulating the ability of offspring to solicit resources (Champagne & Curley 2012). It is suggested that when parental care evolved, underlying genes associated with parental behavior were likely co-opted from precursor behaviors (Cunningham et al. 2021). Monoamines are neurotransmitters derived from aromatic amino acids. This class of neurotransmitters includes examples such as dopamine, norepinephrine, and serotonin that have been shown to play roles in regulation of animal behavior (Swallow et al. 2016). Monoamines have functions specific to regulating social behaviors like parental care through functionality of receptors and circuitry systems of insects (Kamhi et al. 2017). In animal species monoamines like octopamine and dopamine play an important role in animal behavior like aggression, mating, and reproduction (Swallow et al. 2016).

Dopamine plays the role as a neurotransmitter, neuromodulator and neurohormone and has key involvement in reproduction and behavior in social insects (Sasaki & Harano 2010). The dopaminergic system has a significant role in direct parental care and aggression in many insect species (Cunningham et al. 2021). Dopamine has been implicated in its involvement in parental care in *N. orbicollis*. Here evidence of upregulation of dopamine in parenting beetles when compared to non-parenting beetles can be shown. Specifically, a trend of increasing expression levels of dopamine in breeding females compared to nonbreeding females can be observed (Panaitof et al. 2016) indicating dopamine's potential role in switching from non-parenting to parenting behaviors.

Octopamine in insects also functions as a neuromodulator, neurotransmitter and neurohormone. The octopaminergic system plays a crucial role in behavior in invertebrates such as learning and memory in insect species (Farooqui 2007; Cunningham, Douthit & Moore 2014). Specific receptors like the *octopamine α receptor* have been implicated. Octopamine has been shown to regulate the 'fight or flight' response in crickets (Stevenson & Rillich 2016). Octopamine modulates the effect of social and other experiences on aggression and thus plays a role in resource possession-inducing aggression. Interestingly, octopamine has been shown to be involved in parental care in a closely related burying beetle species, *N. vespilloides*, in which expression of the *octopamine α receptor*, is downregulated at the resource preparation stage and gradually increases as males progressed towards post-parental care (Cunningham et al. 2021).

In this thesis, I explore the hypothesis that previous social experience of competition will impact the propensity of males to engage in biparental care. It is established that winning a competitive increases the likelihood that a male will win a subsequent encounter (the winner effect: Stevenson & Rillich 2016, Rutte et al. 2006). But it is less clear how this experience will translate to other social experiences, such as parental care. Given the role of octopamine in both motivation for aggression and parental care discussed above, I predict that previous experience of male-male competition, and the outcome of that competition, will affect the likelihood that males will participate in parental care. I first will examine the role of previous competitive experience on male parental care behavior. I will then examine the underlying molecular physiology by looking for differential expression of octopamine receptors, *octopamine α receptor* and *octopamine/tyramine 1 receptor*, in males that have had different social experiences prior to parenting, adding to our growing understanding of the molecular physiology underlying variation in parental care behavior.

CHAPTER 2:

[2]MALE-MALE COMPETITION AND IT'S IMPACT ON PARENTAL CARE OF *NICROPHORUS ORBICOLLIS*

[2] S.D. Harris, J.B. Moss & A.J. Moore. To be submitted to *Insects*

Abstract

Parental care is believed to have evolved to protect offspring against unpredictable hazards in their environment. When care provided by a single parent is sufficient to ensure reproductive success, a secondary parent may benefit from being flexible and providing care only when it is necessary to offspring survival. Intra-specific competition occurs unpredictably in the social environment and is predicted to favor flexible parenting in secondary parents. I test this hypothesis in a biparental beetle, *Nicrophorus orbicollis*. I investigated the effect of male-male competition on the duration and behaviors of care of males, the duration and behaviors of females, and offspring fitness. Here we show socially experienced males parented more than socially inexperienced males. Despite this, female parents are consistent with the amount of care provided, ensuring the integrity of the quantity and average mass of the larvae. In the end competition is one of the many identified variables contributing to flexible parenting in burying beetles.

Introduction

Parental care is thought to have evolved to combat stressors or hazards that can potentially harm offspring (Trivers 1972; Tallamy 1984). In the vast majority of species offspring only need a single parent to survive, such that secondary parents in biparental systems often appear redundant (McNamara et al. 2003; Trumbo 2012). However, because many stressors are unpredictable in nature offspring may benefit from having flexible secondary parents that provide care only when it is necessary (Royle, Russell & Wilson 2014). Some of the most unpredictable aspects of the environment that parents must overcome stem from the social environment. For example, flexibility in parental investment can serve as an ‘insurance policy’ against partner death or desertion (Eggert & Müller 1997; Smiseth et al. 2005; Rubenstein & Lovette 2007; Trumbo 2012; Ringler et al. 2015), or variable quality of care provided by the partner (Møller & Thornhill 1998; Smiseth & Moore 2004). Thus, a major function of secondary parents may be to compensate for the unexpected loss or reduced capabilities of their partner. Paternal care is suggested to have evolved as a potential form of mate assistance (Fetherston, Scott & Traniello 1990). Here this is shown in many species that exhibit biparental care where the removal of one parent, primes the other parent to increase parental investment that otherwise would not happen provided the primary parent remained (Leffelaar & Robertson 1986; Wright and Cuthill 1989).

Another social variable that arises unpredictably and could favor flexible parenting in secondary parents is competition. Many species face intense intra-specific competition for breeding resources before and during periods of parental care. Aggressive interactions among adults pose direct risks to young, as usurpers frequently commit infanticide to secure resources for the development of their own offspring (Clouse 1995; Trumbo 2006; Choe 2010). In such competitive environments, redoubling parental attentiveness through the increased involvement of a secondary parent may be crucial to mitigate unsuccessful reproductive events (Tallamy & Wood 1986). Perceived intensity of competition can inform and prime parents about likely future breeding opportunities, as well as best strategies to protect current investments (Moczek 1999). For example, male dung beetles (*Onthophagus taurus*) face a high level of competition for ephemeral breeding resources such that reproductive success among dominant males is secured primarily through extended defense (Moczek 1999). While these behaviors are not a part of direct care of offspring, fathers who extend their time at the nest to protect their investment are indirectly benefitting females and offspring. This has also been seen in many avian species in which males participate in defending a particular area to ensure the female is able to feed and lay her eggs (Trivers 1972).

Conversely, parental responses to competition can have deleterious effects for offspring. This is because for some species, the likelihood that an individual succeeds in thwarting a reproductive usurpation attempt is negatively correlated with its ability to perform caregiving duties (Schrader at al. 2021). For example, in western bluebirds, male aggression is inversely correlated with participation in parental behaviors such as egg incubation (Duckworth 2006). Some authors have suggested that specific parental behaviors including brood guarding and offspring attendance were likely evolutionarily co-opted from pre-existing aggressive behaviors unrelated to care (Tallamy 1984). Thus, it remains unclear whether environments that select for aggression make for better or worse parents.

Burying beetles (*Nicrophorus spp.*) are an ideal system to study the role of competition in shaping flexible parental behaviors. Not only do they have an elaborate system of parental care (Eggert and Müller 1997; Scott 1998) but burying beetles breed on highly ephemeral resources and participate in both inter-specific (Trumbo 1990) and intra-specific competition over carcass resources (Eggert and Müller 1997; Safryn & Scott 2000). In addition, biparental burying beetle species, often participate in flexible compensatory parental care in the event of desertion or reduction of care by the secondary parent (Smiseth et al. 2005; Ringler et al. 2015). I will investigate the effect of male-male competition on the duration of care and parental behavior of males, the duration of care and parental behavior of females, and offspring fitness. To sufficiently investigate these questions, I will simulate two environments: one in which males are given a breeding resource without ever being exposed to a conspecific, and one in which two males are encouraged to compete over a breeding resource. My specific predictions are: 1) socially experienced males will remain with their offspring longer than socially inexperienced males because of a perceived threat of conspecific competitors; 2) socially experienced males will participate in more direct parental behaviors and parent longer than the socially inexperienced males. If these predictions hold, I further predict that: 3) female partners of socially experienced males will flexibly reduce their parenting effort behaviors in response to increased male effort; and 4) offspring of socially experienced males will be more numerous and larger than those of socially inexperienced males.

Methods

Nicrophorus orbicollis

Nicrophorus orbicollis is a biparental species of subsocial carrion beetle, where both males and females can participate in all stages of parental care (Trumbo 1990; Royle, Smiseth & Kölliker 2012). Before hatching, parents work together to bury and defend a breeding resource (i.e., a small vertebrate carcass). After larvae hatch, parents continue to perform resource maintenance (projecting anal and oral secretions on the carcass surface), brood defense (standing directly on carcass without moving mouthparts) and provision pre-digested carrion (feed pre-chewed carcass directly to larvae by connecting mouthparts) (Trumbo 1990; Robertson 1993; Eggert & Müller 1997). Male parents are usually the first to desert their brood while the female remains until the larvae have dispersed (Scott and Traniello 1990; Scott 1990). Males that stay with the brood show greater variability in parental participation and duration when compared to females (Robertson 1993; Rauter & Moore 2004; Benowitz & Moore 2016).

Field Collection and Stock Colony

Our stock colony of *N. orbicollis* originated from Whitehall Forest, Athens GA. Beetles were collected with Japanese beetle traps baited with salmon. An outbred laboratory colony was maintained in incubators at the University of Georgia under a 14:10 light cycle, simulating a long-day summer photoperiod. The incubators were programmed to ramp between 19–20 degrees Celsius over 12-hour intervals. Adult beetles were fed organic raw ground beef twice per week. Healthy sexually mature (>14 days) virgin beetles were selected for the experiment.

Competition and Breeding Trials

My experiment consisted of one treatment with two conditions: socially experienced and socially inexperienced males. Socially experienced males were exposed to intra-specific resource competition prior to breeding. Socially inexperienced males had no contact with other males prior to breeding. Both treatments were set up at the same time. To set up the socially experienced treatment, two size-matched males with pronotum lengths within half of a standard deviation of each other (population mean: 5.31mm; SD: 0.509 mm; n =180) were paired on a single breeding resource to encourage competition. For each socially experienced male, a time-matched socially inexperienced male was also given a breeding resource. The period for competition was one hour, after which an escape hatch was revealed allowing the ‘losing’ male to freely exit the arena.

To effectively monitor the effects of competition on male parenting behavior and time to desertion, I enlisted a unique two-tiered breeding box design (Figure 2.1). Clear plastic containers (6.5in x 4.5in x 2in) were used to simulate a subterranean breeding environment with an above-ground escape chamber. Lower levels were prepared with ~2cm of soil and a thawed (overnight) mouse carcass (20-25 g). Leaving open space between the soil and the top enables behavioral observations of *N. orbicollis* without causing disturbance as well as maintaining visibility of the carcass.

The box's upper levels were constructed from inverted empty containers of the same size, which were divided from the lower levels by rectangular pieces of cardboard. Inverted plastic cups (37ml) anchored into circular holes in the cardboard ceiling formed the entrances to the escape chamber, and 3.5" x 1.5" aluminum wire mesh 'ladders' were secured to the insides of the cups to facilitate movement between the breeding chamber floor and the escape chamber's interior. To ensure movements were only one-way, petroleum jelly was applied around the sides of the plastic cup before every use. The petroleum jelly creates a slippery surface preventing beetles from climbing on the sides of the plastic cup to re-enter back into the breeding box.

After males were introduced to the two-tiered breeding box, the boxes were monitored once every two hours between the hours of 0900 and 1700. Upon detection of an escape, the 'losing' male was removed. Immediately thereafter, the 'winning' male and his matched socially inexperienced male were each given an unrelated virgin female. The escape chamber was again replaced with a regular lid to encourage mating. Boxes were monitored daily for the presence of eggs. Once the eggs hatched and the larvae colonized the brood ball, the escape chamber was placed back onto the breeding box. The two-tiered breeding box was monitored daily to estimate the timing of parental desertion.

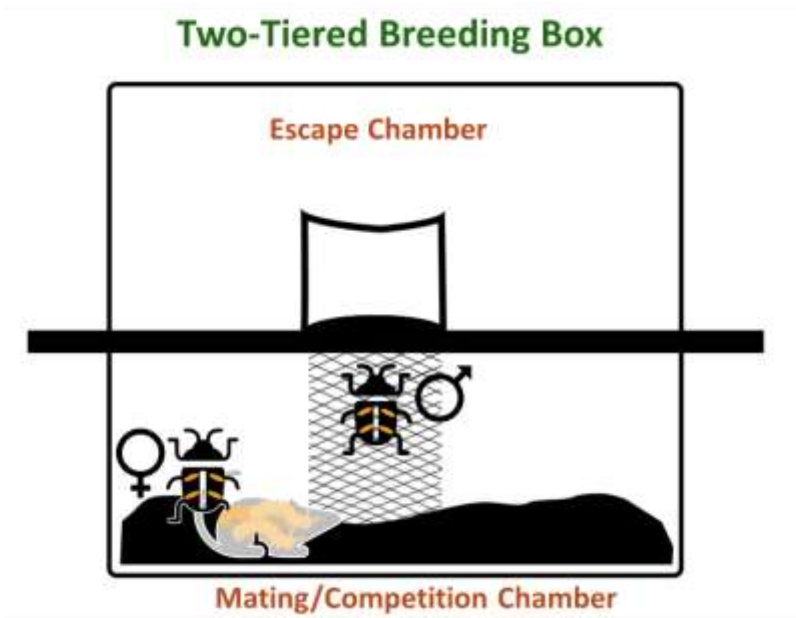

Figure 2.1. Diagram of the two-tiered breeding box to be used during the competition and parenting stages. The second tier (wire ladder, cardboard barrier and above) is the escape chamber. The first tier (clear container with about 2cm of soil and mouse vertebrate carcass) is the mating chamber.

Behavioral Observations and Performance Measurements

Once the eggs hatched and larvae colonized the brood ball, I initiated thirty-minute behavioral observations under red light. To ensure larvae were of similar stage, only broods discovered between 1400 the previous day and 0900 the day of were included in my observation data. Larvae discovered after this time period were observed the following day. All observations took place at 1400. Prior to performing observations, a designated 5-minute acclimation period was established where the lid of the two-tiered breeding boxes was removed, and the families were allowed to recover from disturbance. During each observation period, I recorded the behaviors performed by each parent once every 5 minutes *via* instantaneous scan sampling. Behaviors were assigned to one of four categories, which were treated as mutually exclusive and of which only one was recorded at each time interval: self-feeding, brood maintenance, regurgitating food to larvae, and bouts away from offspring/carcass (Eggert and Müller 1997; Eggert, Reinking & Müller 1998; Scott 1998; Smiseth & Moore 2004). Self-feeding is an essential behavior in our analysis as that is a key step towards the parents' ability to regurgitate towards offspring. This gave each individual four scores which together summed to six. Observations continued every day at the same time until parents deserted and/or the larvae dispersed and began the wandering larval phase. Once the larvae dispersed into the surrounding soil, number of surviving larvae and total brood mass (in grams) were recorded and used to estimate average larval mass.

Statistical Analysis

All statistical analyses were performed in R Studio programming software (R Core Team 2021). To test whether the experience of male-male competition could predict male and/or female parenting effort, I fit a series of linear models and generalized linear models followed by ANOVA, with significance of fixed effects reported based on F tests. My first model examined the effect of male social experience on overall duration of parenting. Days until desertion was found to approach normality and so was fit to a Gaussian distribution with treatment as a main effect. I next examined the effect of social experience on the performance of specific parental behaviors. Because individual behavioral categories often had low numbers of observations, I divided my analysis into two steps: first modeling the number of observations of regurgitating, the only form of direct offspring care, and second modeling total parenting of any type, calculated as the sum of all direct care behaviors (regurgitating) and indirect care behaviors (carcass maintenance and self-feeding). For each analysis, I fit generalized linear models to

Poisson distributions for count data. Parenting day was included as a covariate to test for possible temporal variation in behavior. Finally, to test the effect of treatment on reproductive success I fit linear models of number of larvae and average larval mass with treatment as a main effect.

Results

In my sample of 21 socially experienced breeding pairs and 26 socially inexperienced breeding pairs, I found no difference in the duration of care (parenting days until desertion) between males that experienced competition (socially experienced) and those that did not (socially inexperienced males; ($F_{1,44}$=*0.6052*, *p = 0.437*) (Figure 2.2).

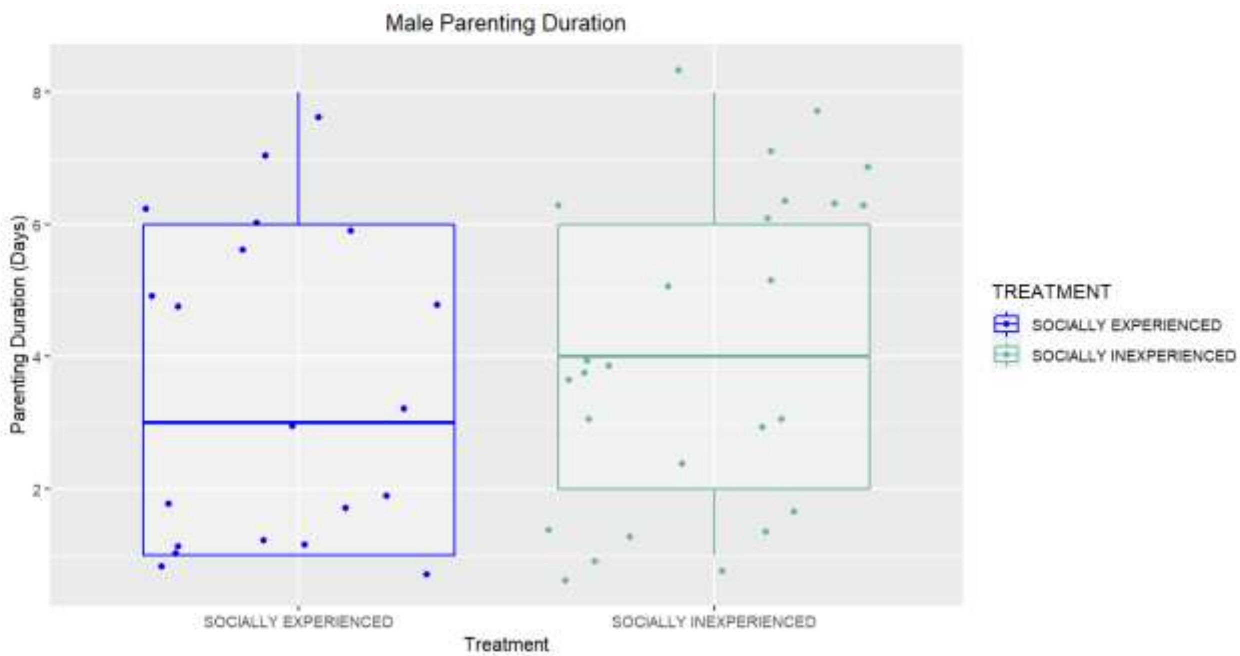

Figure 2.2. Duration (number of days) of total parenting (regurgitating, maintaining carcass and self-feeding) of males over the course of larvae development.

When investigating whether competition impacts parenting behavior *per se*, I found that socially experienced males spent significantly more time parenting overall (engaged in self-feeding, carcass maintenance, or regurgitating) than socially inexperienced males ($F_{1,198}$=*7.2833, p=0.007*) (Figure 2.3) and that total parental care declined over time ($F_{1,197}$=*22.1077, p < 0.001*) (Figure 2.3). Moreover, socially experienced males regurgitated (direct parenting) to offspring significantly more than socially inexperienced males ($F_{1,198}$=*8.9007, p=0.002*) and this behavior did not change over time ($F_{1,197}$=*3.1821, p=0.074*).

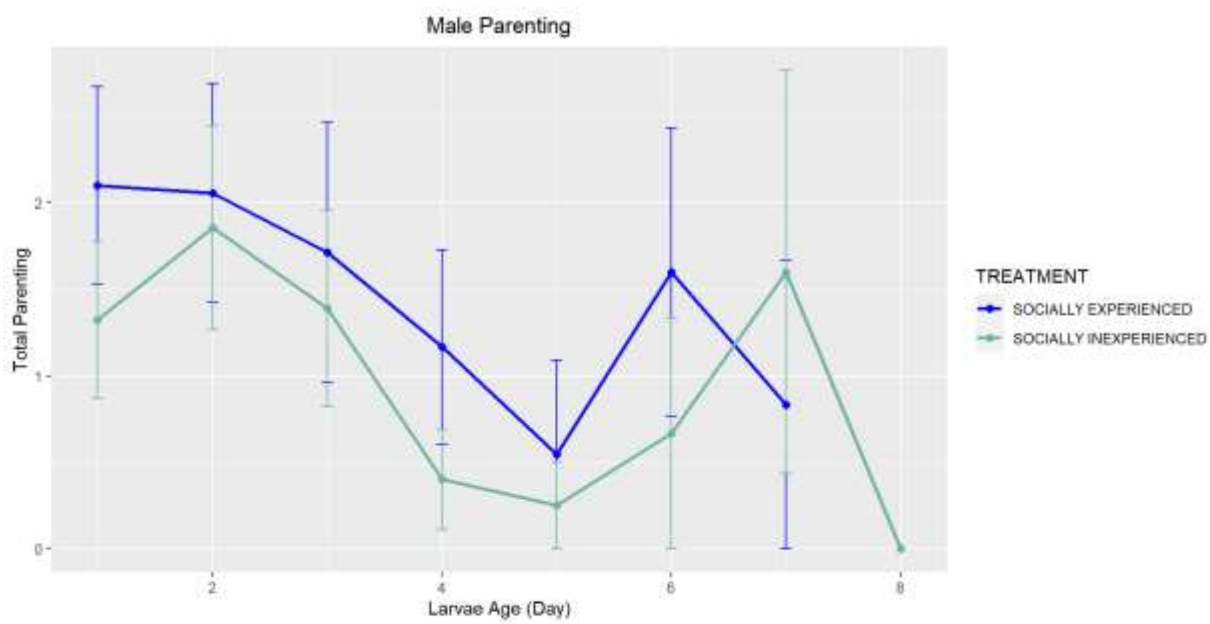

Figure 2.3. Number of observations of total parenting (regurgitating, maintaining carcass and self-feeding) of males in a thirty-minute period over the course of larvae development.

I also investigated whether social experience by males influenced female parenting behavior. I found that females did not significantly change their time in total care ($F_{1,198}$=*0.0211, p = 0.885*) or direct care ($F_{1,198}$=*1.3501, p=0.2453*) in response to partner social experience, although total parenting declined over time ($F_{1,197}$=*3.9302, p = 0.047*) (Figure 2.4).

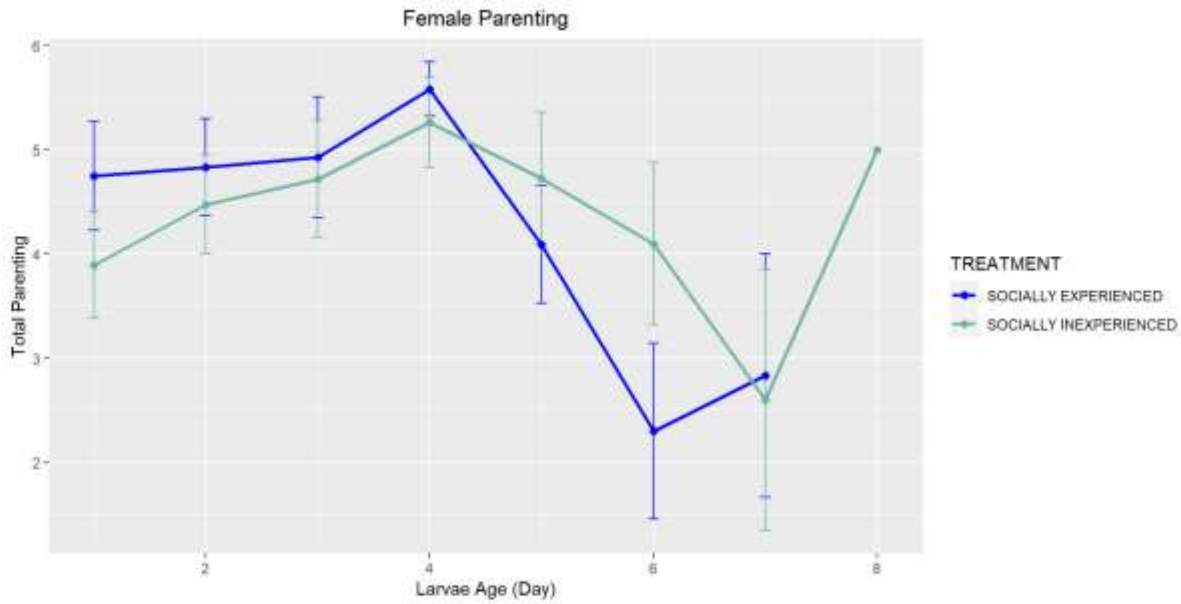

Figure 2.4. Total parenting; regurgitating, maintaining carcass and self-feeding, of female mates of males from each treatment group over the course of larvae development.

Finally, I examined whether social experience impacted larval number or average larval mass. I found no significant difference in either larval number ($F_{1,44}$=*0.017, p=0.897*) or average larval mass ($F_{1,44}$=*0.0444*, *p=0.834*) between families of socially experienced males and socially inexperienced males.

Discussion

In this study I investigated the effect of male-male competition on the duration and parental behavior of males, females, and offspring fitness. I predicted that male burying beetles that experienced competition would stay longer and participate in more direct parenting behaviors than males who did not. I further predicted that if socially experienced males participate more at the nest, then their female mates would withhold more direct parental care than female mates of socially inexperienced males. Lastly, I predicted that if socially experienced males increase parental care, then their broods and offspring will be larger on average than those of socially inexperienced males.

I found some support for these predictions: while social experience had no effect on the duration of male attendance, male parents that previously experienced male-male intra-specific competition participated more in all care behaviors than male parents who did not. Crucially, socially experienced males participated in provisioning more, a behavior directly influencing offspring fitness (Trumbo 1990, Robertson 1993, Eggert & Müller 1997), than socially inexperienced males. In general, participation of care decreased as larval development progressed which is consistent with the timeline of parenting in burying beetles (Smiseth et al. 2003). Regardless of the variation in parental care provided between male groups, both groups of males eventually succumbed to their flexible nature of concluding parental efforts before the conclusion of larval development. Here this can additionally be shown to occur in *N. vespilloides* in which males with no prior competitive experience deserted their broods much earlier than males with prior experience of intrasexual competition (Hopwood et al. 2015). Additionally, dung beetles show a connection between competition of reproductive resources and parental care. Behaviors like rolling and tunneling in dung beetles are perhaps an evolutionary response to reproductive competition (Simmons & Ridsdill-Smith 2011), supporting the idea that parental care can be an evolutionary response to ensure reproductive success. Similarly, to *N. orbicollis*, paternal care in dung beetles concludes prior to nest formation while the maternal figure remains to ensure offspring reach adulthood (Simmons & Ridsdill-Smith 2011).

Following the first prediction, I projected females would flexibly reduce the amount of care shown in the presence of increasing male parenting behavior, however, female parents remained consistent in the quality and quantity of care given. Male social experience had no bearing on the parental behavior of their female partner. This finding coincides with other studies in burying beetles that have also confirmed that the removal of males does not have a major effect on female parental behaviors (Fetherston, Scott & Traniello 1994; Trumbo 2006; Moss and Moore 2020). This is further confirmed in another study showing that duration of provisioning between male and female parental figures showed no evidence of a negative correlation. Indicating lack of a conscious effort to adjust in relation to their mate's parental investment (Fetherston, Scott & Traniello 1990).

Lastly, I predicted that offspring would increase in brood weight and larvae number as a response to an increase in paternal care, however I was not able to confirm this prediction. There are conflicting conclusions on whether biparental care in burying beetles is beneficial or detrimental to offspring. It has mainly been determined from similar studies that paternal care in addition to maternal care, leading to biparental care, does not increase the quantity or individual size of larvae (Trumbo 1991; 1994). In another study it was concluded that prolonged biparental care showed to be detrimental to offspring. Families with the paternal figure removed produced more abundant broods and a larger average mass (Scott 1989). While it may provide immediate benefits for offspring to experience added protection from intruding conspecifics by the secondary parent, prolonged presence of the paternal figure can lead to detrimental effects like infanticide (Eggert & Müller 1997) and over exploitation of reproductive resources (Robertson 1993). In this study female parents remained consistent and provided care until the conclusion of larval development, preserving the integrity of larvae despite the flexibility shown by the paternal figure. Continued feeding from a parental figure directly results in an increase of larval mass but is not specified whether biparental care is specifically needed to see this result (Eggert & Müller 1997).

Although we were able to determine that competition favors increased parental care in male burying beetles, this flexibility had no bearing on female parenting effort or on any measures of parental or offspring fitness. Much of this data produced slight effects. It's possible that competition had such weak effects on paternal care and subsequent familial performance because laboratory conditions were unrealistically benign. In addition, our behavioral observations covered only a small fraction of the entire duration of the reproductive cycle. Thus, our measure of parental investment is an approximation, not an absolute.

CHAPTER 3:

[3]PARENTAL CARE AND AGGRESSION OF *NICROPHORUS ORBICOLLIS*

[3] S.D. Harris, C.B. Cunningham, E.C. McKinney, J.B. Moss, A.J. Moore. To be submitted to *Insects*

Abstract

Parental care can be defined as any expressed trait that enhances offspring fitness while simultaneously mitigating unpredictable hazards. Aggression is notably involved in ensuring successful parenting efforts in many taxa. Prospective genes associated with parental behavior include genes that were likely co-opted from precursor aggressive behaviors during the evolution of parental care. Aggressive behavior typically renders temporal and energetic costs potentially selecting for flexible parental care in species where aggression plays a role in ensuring offspring survival. Winner/loser effects often show to be a key accessory of aggressive behaviors like competition. To understand aggression's role in parental care and possible winner/loser effects, I investigate male intra-specific competition's impact on expression of two key gene receptors, the *octopamine α receptor* and the *octopamine/tyramine 1 receptor* of *Nicrophorus orbicollis* and if winning/losing could be reflected in those expression patterns. I found differential expression for *octopamine α receptor* and no evidence for winner/loser effects in either the *octopamine α receptor* or the *octopamine/tyramine 1 receptor*. Implicating *octopamine α receptor's* involvement in both aggression and parental care.

Introduction

Parental care encompasses any trait that enhances offspring fitness when expressed in parents (Royle, Smiseth & Kölliker 2012) and is thought to have evolved in response to predation, competition and/or harsh physical conditions (Tallamy 1984). Because such challenges are often unpredictable in nature, parents benefit from being flexible. Yet the mechanisms that account for this behavioral flexibility are poorly understood. Potential gene candidates associated with parental behavior include genes that were likely co-opted from precursor behaviors during the evolution of parental care (Cunningham et al. 2021). For many species, a key component of a successful parenting strategy is aggression. Aggressive behavior, while advantageous, tends to invoke a tradeoff of time and energy (Kelly & Wilson 2020) potentially favoring flexible parental care in species where aggression is a component of care. In general, the state of the current environment directly influences the degree of parental investment in flexible parents (Archer 1988). Flexible parental care could be a direct reflection of variation in expression of key genes associated with aggression (Cunningham, Douthit & Moore 2014). In previous studies, variation in parental care could be attributed to an experience of competition for mating resources. In *Nicrophorus orbicollis*, competition was found to directly impact flexible parental care, provoking males to parent offspring more (Chapter 2; Trumbo 1990, Robertson 1993, Eggert & Müller 1997). This suggests that the opportunity for aggression; that is, the social environment, may influence genes that influence both parental care and aggression.

Biogenic amines offer mechanistic links between aggressive, reproductive, and social behaviors like brood guarding and offspring attendance. These amines include octopamine, dopamine and serotonin (Panaitof et al. 2016, Weitekamp et al. 2017). Octopamine and Tyramine are both associated with reproduction and aggression of *Nicrophorus vespilloides*. In *N. vespilloides*, expression of the *octopamine α receptor* and the *octopamine/tyramine 1 receptor* showed gradual upregulation beginning from resource preparation until post-parental care in males with expression lowest at resource preparation for both receptors (Cunningham et al. 2021).

Many species that compete for resources, often are impacted by the outcome: win or lose, of the competitive event. This is especially true for species that provide resources to offspring;

often there is competition for these resources (Royle, Smiseth & Kölliker 2012). Winner/loser effects are not studied in the realm of parental care, but instead are subjected to investigation of subsequent competitive events. A key biogenic amine, octopamine, has been explicitly implicated in both aggressive behavior and winner/loser effects in *G. bimaculatus* (Adamo, Linn & Hoy 1995). Male crickets that lost in their interaction with conspecifics, suppressed agonistic behavior whereas winners increased agonistic behavior (Adamo & Hoy 1995). With aggression playing a key role in both competitive and reproductive behavior, one could question whether the winner/loser effects carry over to the reproductive cycle. I inquire whether aggression and parental care connect and whether winner/loser effects impact expression of genes that connect aggression and parental care specifically in species who initiate the reproductive cycle by first competing for mating resources like *N. orbicollis* (Trumbo 1990).

Here I investigate the effect of male competition on expression levels of gene receptors, *octopamine α receptor* and *octopamine/tyramine 1 receptor*, throughout the parental care cycle of *Nicrophorus orbicollis*. Additionally, I will investigate whether the outcome of competition has any effects on expression patterns in male parenting at the post-competition stage, pre-parenting/post-mating stage (72 hours following mating), and the post-parenting stage. To investigate these questions, I will allow males to compete, mate, and proceed through the stages of parental care. Males will be collected at the post-competition, post-mating and post-parenting stage for losers, winners, and non-competitive males to be further investigated for expression levels of the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*. I predict that 1. there will be differential expression in the *octopamine α receptor* and the *octopamine/tyramine 1 receptor* between behavioral state checkpoints. 2. The outcome of competition (win, loss, no experience) will alter expression levels of the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*.

Methods

Nicrophorus orbicollis

Nicrophorus orbicollis engage in both intra- and interspecific competition for mating resources. *N. orbicollis* are a biparental species of subsocial carrion beetle where both males and females can participate in all stages of parental care (Trumbo 1990; Royle, Smiseth & Kölliker 2012). Before hatching, parents work together to bury and defend a breeding resource (i.e., a small vertebrate carcass). After larvae hatch, parents continue to perform carcass maintenance (placing excretions on the carcass surface), brood defense (standing directly on carcass without moving mouthparts) and regurgitate pre-digested carrion (feed pre-chewed carcass directly to larvae by connecting mouthparts) (Trumbo 1990, Robertson 1993, Eggert & Müller 1997). Male parents are usually the first to desert their brood while the female remains until the larvae have dispersed (Scott and Traniello 1990; Scott 1990; Moss & Moore 2021). Males that stay with the brood show greater variability in parental participation and duration when compared to females (Robertson 1993; Rauter & Moore 2004; Benowitz & Moore 2016; Moss & Moore 2021). Additionally, specific parental behaviors like brood guarding and offspring attendance show to be fueled by aggression (Panaitof 2006).

Field Collection and Stock Colony

The stock colony of *N. orbicollis* originated from Whitehall Forest, Athens GA. Beetles were collected with Japanese beetle traps baited with salmon. An outbred laboratory colony was maintained in incubators at the University of Georgia under a 14:10 light cycle, simulating a long-day summer photoperiod. The incubators were programmed to ramp between 19–20°C over 12-hour intervals. Adult beetles were fed *ad libitum* organic raw ground beef twice per week. Healthy sexually mature (>14 days) virgin beetles were selected for the experiment. Beetles were held in round clear plastic containers (5 oz) filled halfway with Miracle-Gro Performance Organics All Purpose In-Ground Soil. Small mouse carcass used in competition and breeding trials were ordered from RodentPro.com.

Competition Trials and Breeding Trials

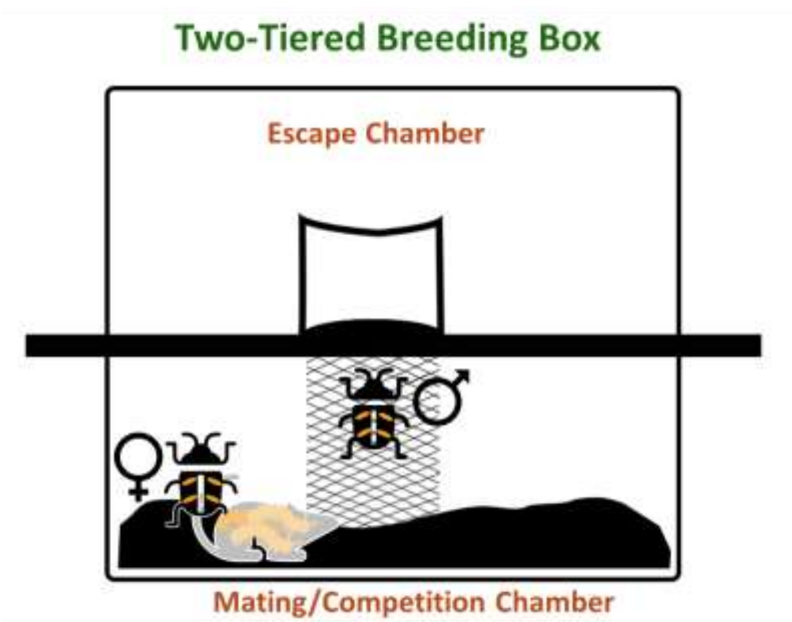

Figure 2.1. Diagram of the two-tiered breeding box to be used during competition stage and parenting stage. The second tier (wire ladder, cardboard barrier and above) is the escape chamber. The first tier (clear container with about 2cm of soil and mouse vertebrate carcass) is the mating chamber.

I performed an experiment to assess the effect of a competitive outcome on the expression of genes know to influence aggression. Males either had an initial competitive experience or no competitive experience. Males with an initial competitive experience were separated by winning/losing that interaction, and then sampled at three behavioral state checkpoints: post-competition, post-mating, and post-parenting. Therefore, my experiment consisted of 3x3 experiment consisting with three conditions: competitive interaction winners, competitive interaction losers and non-competitive males. Competitive males were exposed to intrasexual resource competition prior to breeding. Non-competitive males had no contact with other males prior to breeding. Both treatments were set up at the same time. To set up an aggressive interaction (competition), two males with pronotum lengths within half of a standard deviation of each other (population mean: 5.31 mm; SD: 0.509 mm; n =180) were paired on a single breeding resource to encourage competition (Trumbo 1990). For each competitive encounter pairing, a time-matched competitively inexperienced male of similar size was also given a breeding resource. The period allotted for competition lasted for 24 hours, in which the escape chamber was readily available for use while males compete for a singular mouse resource. Anytime within this 24-hour period, could the loser male escape via escape chamber. After which the 'loser' male and 'winner' male was determined by escape/abandonment of the resource by the 'loser'.

Following competition, all males (winner, loser, or no competition) were paired with an unrelated female. The female-male pair were placed inside an empty clear round container (5 oz) to visually confirm copulation. Following copulation, the pair were placed in a clear plastic container (6.5 in x 4.5 in x 2 in) and provided with a small mouse carcass (18g-23g). The container was closed and left undisturbed until eggs hatched, and larvae colonized the small mouse carcass.

To effectively monitor desertion during competition and parental care, I used a custom two-tiered breeding box design (Figure 2.1) that provides an effective escape chamber (higher level). Clear plastic containers (6.5 in x 4.5 in x 2 in) were utilized to simulate a subterranean breeding environment with an above-ground escape chamber. Lower levels were prepared with ~2 cm of soil and a thawed (overnight) mouse carcass (20-25 g). The box's upper levels were constructed from inverted empty containers of the same size, which were divided from the lower levels by rectangular pieces of cardboard. Inverted plastic cups (37ml) anchored into circular holes in the cardboard ceiling formed the entrances to the escape chamber, and 3.5" x 1.5" aluminum wire mesh 'ladders' were secured to the insides of the cups to facilitate movement between the breeding chamber floor and the escape chamber's interior. To ensure movements were only one-way, petroleum jelly was applied around the sides of the plastic cup before every use. The petroleum jelly creates a slippery surface preventing beetles from climbing on the sides of the plastic cup to re-enter back into the breeding box.

Sample Collection

We assayed gene expression from male whole head samples collected at specific points throughout the reproductive cycle for winners, losers, and control (Cunningham et al. 2021); post-competition, post-mating, and post-parenting. These collection points provide a clear picture of changes in gene expression as males engage in all the major steps that encompasses the reproductive cycle accounting for the very beginning and the very end (Cunningham et al.

2021). Post-competition represents a behavioral state checkpoint directly after males experience an aggressive encounter. Post-competition samples were collected 24 hours after two males are allowed to compete for a mouse carcass. Post-mating represents the period of preparation for the arrival of offspring after mating was confirmed *via* observation. They were collected 72 hours after a chosen male and female mate and begin carcass preparation. Post-parenting represents the period in which social interactions have concluded. This was either when the sire deserts the brood by climbing to the second tier of the two-tiered breeding box or larvae disperse. Heads of male beetles were collected and frozen in liquid nitrogen. Samples were stored at -80°C until RNA extraction of whole heads using the Quiagen RNAeasy Mini Kit (REF: 74106) following Roy-Zokan et al. (2015). cDNA synthesis was done using qScript Quantabio followed by storage at 20°C.

Quantitative real-time PCR (qRT-PCR)

We identified our target genes, *octopamine α receptor and octopamine/tyramine 1 receptor*, for *N. orbicollis* using orthologs from *Drosophila melanogaster, N. vespilloides* and *Tribolium castaneum.* We used BLASTp (v2.2.25+; default settings to search the *N. orbicollis* genome (Cunningham et al. 2021). The process of designing primers followed (Cunningham, Douthit & Moore 2014). Briefly, we ensured that each selected primer contained a PCR efficiency of 1.95 with a five-point series of four-fold dilution. Additionally, we ensured that the selected primers generated only a single amplicon to ensure specific amplification of the selected gene.

We utilized an established qRT-PCR protocol previously used for *N. vespilloides* (Cunningham, Douthit & Moore 2014; Cunningham et al. 2021), using a Roche LightCycler 480 with Roche SYBR I Green Master Mix, 45 cycles of amplification and 60℃ annealing temperatures. Following amplification, we performed melting curve analyses to confirm the presence of a single amplicon (Monis, Giglio & Saint 2005). *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) was used as the endogenous reference gene.

Data Analysis

To perform our analysis, we used the $\Delta\Delta C_T$ method to estimate standardized, relative gene expression (Livak & Schmittgen 2001). I performed an analysis of variance (ANOVA) to investigate changes in gene expression across nine groups encompassing two treatments at three levels. A two-way analysis of variance (ANOVA) was used to determine the key factor(s) driving changes in gene expression modeled as Behavioral State Checkpoints x Experience (win, lose or no competition). We used Dunnett's post-hoc test to compare the control group mean to the means of the remaining eight treatments (Mukerjee, Robertson & Wright 1987) to our control group, post-competitive socially inexperienced males' group. All figures and analyses were performed using R studio (v2021) running R (v4.1.2).

Results

octopamine α receptor (octαr)

I investigated the effect of behavioral state and competition experience on expression of the *octopamine α receptors*. There was an overall effect in the two-way ANOVA ($F_{8,36}$ *=2.846, p = 0.015*). I looked to answer whether behavioral state, natural progression through the reproductive cycle, drive differential expression of the *octopamine α receptor*. Expression of *octopamine α receptor* (*octαr*) changed significantly as behavioral state changed ($F_{2,36}$ = *8.819, p < 0.001*) (Figure 3.1). Next, I investigated whether there was a winner/loser effect on *octαr* expression by comparing expression levels between three outcomes of competition – win, lose or no experience. There was no statistically significant effect indicating the presence of a winner/loser effect on *octar* expression ($F_{2,36}$= *1.380, p=0.265*). Lastly, I investigated whether behavioral state and experience interacted to drive differential expression and found no evidence of interaction ($F_{4,36}$= *0.593, p=0.670*).

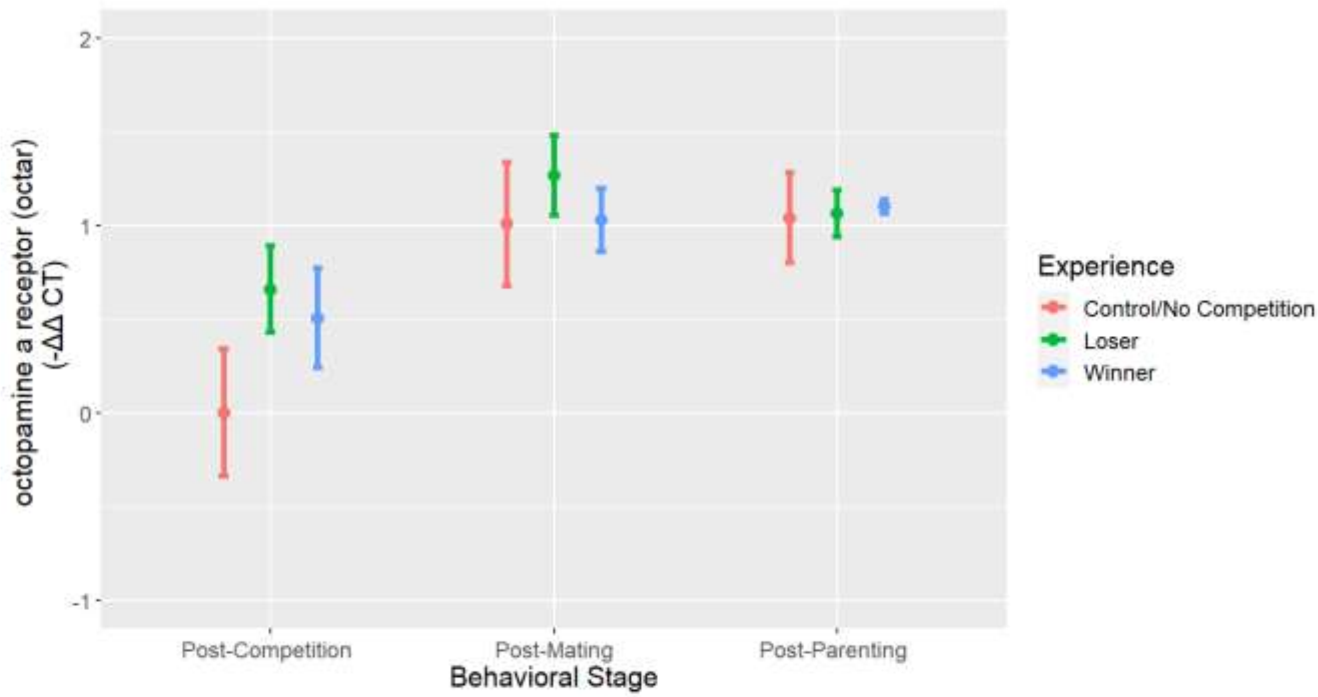

Figure 3.1. Normalized relative expression of the octopamine α receptor of the octopaminergic system of N. orbicollis at different behavioral states, post-competition, post-mating, and post-parenting. Males were separated by the outcome of their competitive interaction (Win, lose or no competition).

*Table 3.1: Dunnett's Post-Hoc test compares all other groups/treatments to the non-competitive post-competition male group. The rows are color coded by the range of values. *** = $p \leq 0.001$, **= $p \leq 0.01$, *= $p \leq 0.05$, NS = not significantly different; $p > 0.05$.*

Level	P-value	Significance Level
Loser Post-Mating	0.003	**
Winner Post-Parent	0.013	*
Loser Post-Parent	0.018	*
Control Post-Parent	0.021	*
Winner Post-Mating	0.023	*
Control Post-Mating	0.027	*
Loser Post-Comp	0.260	NS
Winner Post-Comp	0.532	NS
Control Post-Comp	1.000	

octopamine/tyramine 1 receptor (tyrr)

I investigated the effect of behavioral state and competition experience on expression of the *octopamine/tyramine 1 receptor*. There is no overall effect in the two-way ANOVA ($F_{8,35}$ =*0.463, p = 0.873*). I looked to answer whether behavioral state or natural progression through the reproductive cycle drive differential expression of the *octopamine/tyramine 1 receptor*. Expression of the *octopamine/tyramine 1 receptor* (*tyrr*) showed no evidence of differential expression as behavioral states change ($F_{2,35}$= *0.102, p=0.904*) (Figure. 3.2). Next, I investigated whether the outcome of competition (win, lose or no competition) had any effects on expression patterns of the *octopamine/tyramine 1 receptor*. I found no statistically significant effect of competition outcome on expression ($F_{2,35}$= *0.998, p=0.379*). Lastly, I measured whether experience and behavioral state interacted to drive variation in expression patterns and found no relationship between these two factors ($F_{4,35}$= *0.375, p=0.825*).

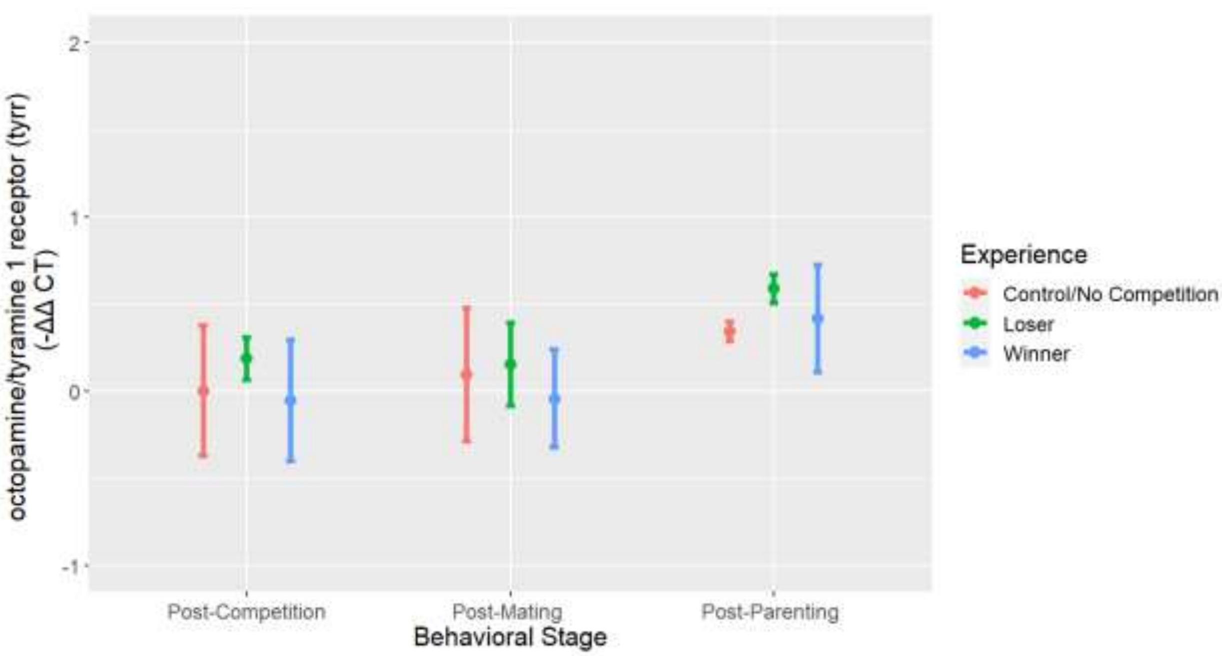

Figure 3.2. Normalized relative expression of the octopamine/tyramine 1 receptor of the octopaminergic system of N. orbicollis at different behavioral states, post-competition, post-

mating, and post-parenting. Males were separated by the outcome of their competitive interaction (Win, lose or no competition).

Discussion

In this study I examined if aggression influences the subsequent expression of parental care and the impacts of winner/loser effects. I specifically investigated the effect of male competition on expression levels of the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*, as males progressed through the parental care cycle. Increased expression of these genes is associated with parental care in a related burying beetle, *N. vespilloides*, and known to be associated with aggression in insects (Cunningham et al. 2021). I also investigated whether the outcome of competition has any effects on expression patterns in male parenting. I predicted that there would be differential expression in both the *octopamine α receptor*, the *octopamine/tyramine 1 receptor* and that winner/loser effects will carry over and influence expression of both the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*. I found partial support for my first prediction, as expression of the *octopamine α receptor* increased significantly between the post-competition and post-mating stages regardless of the outcome of competition, but this trend was not observed for the *octopamine/tyramine 1 receptor*. I found no evidence for winner/loser effects in the *octopamine α receptor* nor for the *octopamine/tyramine 1 receptor*. I conclude that the *octopamine α receptor* is involved in aggression and parental care and the *octopamine/tyramine 1 receptor* is not involved in aggression. Additionally, if males of *N. orbicollis* are subjected to winner/loser effects, it does not impact aggression and/or parental care.

An involvement of the *octopamine α receptor* in parental care in *N. orbicollis* is consistent with patterns observed in the closely related species, *N. vespilloides*, in which the *octopamine α receptor* also showed differential expression as individuals entered and concluded parental care (Cunningham et al. 2021). One key difference here is resource preparation of *N. vespilloides* is significantly lower than all other behavioral states (Cunningham et al. 2021) while that is not the case for *N. orbicollis*. Specifically, the post-mating behavioral stage, where both parents actively prepare their resource, is upregulated.

Unlike *N. vespilloides,* I did not find differential expression in the *octopamine/tyramine 1 receptor.* This could be indicative that the two receptors vary in functionality and/or conditions in *N. orbicollis*. Expression of one receptor could directly translate to increased aggressive behavior while expression of another could convey the opposite effect, or one receptor could not be involved while the other is. It's also possible that the two receptors act in an antagonistic way, meaning the activation of one receptor could inhibit the expression of another (Liu, Han & Smith 2013). Lastly, timing/behavioral state of which the males were sampled impacted expression of one of two receptors. Expression of the two receptors could differ between active participation and conclusion of a particular behavior. The methods used here differed from those used by Cunnigham et al. (2021). As males were collected after rather than during the competition stage, this may explain a difference in expression levels. One key aspect of this study is that males are flexible parents suggesting that the flexibility of parental care could be reflected in expression of the *octopamine α receptor* while the same cannot be assumed for the *octopamine/tyramine 1 receptor*.

Winner/loser effects are of particular interest because *N. orbicollis* often compete for mating resources before engaging in reproduction (Trumbo 1990). Winner/loser effects can be defined as an increase/decrease in agonistic/aggressive behavior after winning/losing a competitive and/or aggressive bout (Adamo & Hoy 1995). I expected to find an effect on expression of both the *octopamine α receptor* and the *octopamine/tyramine 1 receptor*. I found no evidence of winner/loser effects. Lowest expression of the *octopamine α receptor* occurred at the post-competition stage regardless of winner/loser status followed by upregulation in subsequent behavior states. It's possible that a single competitive interaction may be insufficient when investigating winner/loser effects since experience of competition primes males to participate in parental care more confirming competition's impact on paternal care (chapter 2). It is also possible that specific receptors regulate specific aggressive behaviors and that changes in behavior triggers a switch in receptor activation. Perhaps, receptors expressed during an active competitive event differ from receptors expressed during parental care, making it difficult to see effects of winning or losing a previous competitive event.

One of the complications of studying behavior is sampling at the critical timepoint. It is my hypothesis that sampling individuals while they actively engage in competitive and reproductive behaviors will yield a more robust expression profile amongst selected genes/receptors. I predict there is a minimum threshold of competitive events that an individual must experience before evidence of winner/loser effects appear and that it will carry over to parental care if that threshold is met. In past studies winner/loser effects have not been looked at beyond subsequent competitive events, so it's hard to say that *N. orbicollis* experience winner/loser effects at all. To have a true understanding of how aggression and parental care relate to each other in *N. orbicollis*, additional work could be directed towards genes that have been implicated in aggression and parental care. To expand on what is currently known, collection of males as they actively engage in both competitive and reproductive behavior, could potentially provide more information on how these genes play a role in parental care. To better understand winner/loser effects, multiple competitive events per individual before reproduction could give a better idea of whether males experience winner/loser effects.

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