Personality and plasticity across time, space, and context in forked fungus beetles (*Bolitotherus cornutus*)

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ABSTRACT

Individuals experience constant fluctuations in social contexts within a breeding season and across their lifetime, and these changes can have a large effect on the expression of behaviors. Behavioral plasticity allows individuals to instantaneously adjust their phenotype to changing contexts, but behaviors are not perfectly plastic. Animal personalities and behavioral syndromes quantify the proportion of behaviors that remain consistent in response to changing cues. My dissertation research used forked fungus beetles (Bolitotherus cornutus) as a system to explore the consistency of and correlations among competition and mating behaviors across contexts. In Chapters 1-3, I showed that behavior can be both plastic and repeatable in response to changing social contexts. Mating and competition behaviors in *B. cornutus* had high plasticity across social contexts, but were highly repeatable within contexts. In Chapter 1, I asked about some of the causes and consequences of behaviors initiated during competition. Larger, more aggressive males were more likely to win male-male competition, while females did not act competitively towards their female partners. In Chapter 4, I examined the potential fitness consequences of male competition outcome by asking if females preferred the chemical cues of males based on their competition experience. Females preferred to associate with the chemical cues of future losing males prior to competition, but switched their preference for winning males after competition. Social context extended beyond immediate surroundings to include past experiences in my female choice trials. I expanded the idea that past experience affects current behavior in Chapter 5 by asking if

age affects aggression. I determined that age explains some of the variation in expression of aggression among males. Older males were more aggressive than younger males. Overall, my dissertation research provides key first steps in understanding personalities across mating and competition contexts and shows that past experience is an important context that can affect the expression and consequences of behavior.

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INTRODUCTION

Social contexts are often in constant fluctuation as animals physically move through their populations and as populations change through time. Individuals must respond immediately to new contexts, but behaviors are not perfectly plastic in response to changes in cues (Sih et al. 2004). Despite being arguably the most plastic suite of traits at an animal's disposal, behaviors can be consistent in response to changing contexts. The field of animal personality was conceptualized as a way to understand the limitations to plasticity. Personality quantifies the proportion of animal behavior that remains consistent across contexts and can be defined as single repeatable behavior or include multiple, repeatable behaviors (Sih et al. 2004; Stamps and Groothuis 2010).

Personalities ultimately highlight important differences among individuals that can be targets for selection (Dingemanse and Réale 2005; Schuett et al. 2010). Personality traits may be maladaptive when individuals respond inappropriately to new social cues (Duckworth 2006; Smith and Blumstein 2010; Wilson et al. 2011). For example, previous studies found individuals that express high levels of aggression receive fitness benefits in intrasexual competition over resources, but incur fitness losses when acting aggressively in mating contexts (Duckworth 2006; Sih et al. 2014). Alternatively, personalities may represent adaptive strategies among individuals with different internal states (Luttbeg and Sih 2010; Sih et al. 2015; Näslund and Johnsson 2016). Higher quality individuals may invest more in costly behaviors, like aggression and boldness (Brockmann 2001; Sih et al. 2015). Individuals may also pursue different strategies depending on the frequency of other behavioral types in the environment (Brockmann 2001; Wey et al. 2014). Whether a personality is maladaptive or adaptive can only be determined by first quantifying behavioral correlations among contexts and potential differences in these correlations among individuals.

The current perspective on personalities includes the plastic portion of behavior in which plasticity is treated as part of the personality and not just error surrounding the personality itself (Dingemanse et al. 2010b, 2012; Dochtermann and Dingemanse 2014; Dingemanse and Wright 2020). This perspective highlights the unique intricacies of behavior by accounting for both within-individual differences in plasticity to social context and among-individual differences in overall behavioral expression. Amongindividual correlations quantify the extent to which individuals behave differently across contexts, and indicate personality when behaviors are, at some level, repeatable. Withinindividual correlations then quantify the remaining, plastic proportion of behaviors that allow individuals to adjust to changes in social context. Personality types may respond more or less plastically to changes in social context depending on the among- and withinindividual behavioral correlations (Dingemanse et al. 2010b, 2012). For example, one study found that bold versus shy sticklebacks differed in their tendency to explore new habitat, and bold sticklebacks were more like to increase their exploration through time compared to shy sticklebacks (Jolles et al. 2019).

Much of the work on animal personalities to date examines the causes and consequences of boldness, activity, and aggression (Wilson et al. 1994; Coleman and Wilson 1998; Van Oers et al. 2005; Smith and Blumstein 2010; Jolles et al. 2019). These well-documented personalities, however, do not encompass all behaviors with potential fitness consequences. I know surprisingly little about the consistency and correlations among mating and competition behaviors. Behaviors expressed in mating and competition represent meaningful differences among individuals in their abilities to achieve reproductive opportunities (Moore et al. 2001; Bailey and Zuk 2009; Sih et al. 2014; Boutin et al. 2016). Understanding the degree to which competition and mating behaviors are consistent across contexts will provide a more holistic approach to understanding the potential fitness consequences of important social behaviors.

Sexual selection involves a complex interplay between competition and mate choice in both sexes (Hunt et al. 2009). Winning competition provides individuals access to important food and mating resources (Berglund et al. 1996; Rosvall 2011), and potential mates can choose among competitors that vary in resource acquiring ability (Candolin 1999; Wong and Candolin 2005). Behaviors typically categorized as competition and or mating are often observed out of their expected contexts (Le Galliard et al. 2005; Bailey et al. 2013; Sih et al. 2014), but behavioral correlations across these contexts are rarely measured. For example, winning competition usually indicates high quality in mates (Zahavi 1975; Ditchkoff et al. 2001; Unrug et al. 2004), but mating success may be low when correlations across contexts cause aggression that is advantageous in competition to be inappropriately expressed during mating (Moore et al. 2003; Sih et al. 2014; Kiyose et al. 2015). Sexual selection may be misinterpreted if correlations among competition and mating behaviors differ across contexts and are only quantified in within context (Smith and Blumstein 2008; Wolf and Weissing 2012).

In this study, I use *Bolitotherus cornutus* (forked fungus beetles) as a system to ask how social context affects the expression of and relationship between competition and mating behaviors. I then assess the consequences of these behaviors for female choice and competition. *B. cornutus* live in discrete populations on dead, decaying logs

distributed through forest habitats spanning eastern North America (Liles 1956). The polypore fungi that grow on dead wood are used by the beetles as food and egg laying substrates. Beetles also have ecologically important interactions on fungus that influence their abilities to survive and reproduce (Brown 1980; Brown et al. 1985; Conner 1988), including competition, courtship, and mating (Figure 1B-D). Male-male competition, courtship, and mating (Figure 1B-D). Male-male competition, courtship, and mating have all been previously measured and impact beetle fitness (Brown 1980; Brown et al. 1985; Conner 1988, 1989; Formica et al. 2011), but the intricacies of these behaviors has not been characterized. In Chapters 1-3, I establish the paradigm for *B. cornutus* mating and competition behaviors to then compare these behaviors across contexts.

B. cornutus is sexually dimorphic – males have two sets of horns, one growing out of the thorax and one out of the clypeus at the front of the head, whereas females are hornless (Figure 1A). Males use their horns in male-male competition (Liles 1956; Conner 1988). Although I do not know exactly how horn size impacts fighting ability, previous studies found that males with larger horns have longer lifespans and more access to females (Conner 1988). Females do not appear to have control over which males court them, but do control who they copulate with (Brown et al. 1985). There is no previously published work on female social behaviors in this species.

In Chapter 1, I characterize behaviors performed during male-male competition and ask if specific behaviors predict competition outcome. I quantified the initiation of each behavior performed during dyadic competition trials (Figure 2). From these data, I constructed an ethogram that described transitions between behaviors and categorized initiations as aggressive, non-aggressive, and mounting. Overall, larger, more aggressive males were more likely to emerge as winners. Male aggression in *B. cornutus*, however, was not associated with individual differences in body size. Males of all body sizes initiated aggression towards their competitors and retained their level of aggressiveness and non-aggressiveness regardless of the identity of their competitor. These repeatable differences in expression of aggression highlight meaningful differences in male competitive abilities and may result in fitness differences through access to mates and potential consequences for female choice.

Female-female interactions are not often measured in the same species in which male-male interactions are well-defined, despite the potential importance of both for sexual selection (Wong and Candolin 2005; Hunt et al. 2009). In *B. cornutus*, females may compete over fungal resources to maximize number of eggs laid and offspring survival. Larval competition and cannibalism are common within the fungus brackets that house larvae (Liles 1956; Wood et al. 2014), and fungus quality differs within populations. In Chapter 2, I performed a similar experiment to Chapter 1 and constructed an ethogram of female-female interactions to characterize behaviors between females. I asked if females perform directly aggressive or indirectly competitive behaviors in the presence of a fungal resource. Females performed suites of non-aggressive and mounting behaviors towards female partners, but these behaviors were not repeatable. Additionally, females were not aggressive or exclusionary towards their female partners. Body size and expression of non-aggressive or mounting behaviors did not predict access to the provided fungal resource. I interpret these results as indicating fungus brackets are not a limiting resource for females and females likely do not compete over space on fungus brackets in the wild.

I show that males and females perform similar behaviors in intrasexual interactions, but these behaviors can differ in their elicited responses. Behaviors categorized as aggressive in male competition were categorized as non-aggressive in female competition trials. One surprising finding from Chapter 1 and 2 was the expression of mating-like, mounting behaviors during competition. Mounting behaviors were previously described as used during courting (Brown et al. 1985), but almost all males and females in our trials initiated mounting in the non-mating, same-sex contexts. The expression of mounting in competition led me to predict that other behaviors used in competition trials may also translate to a mating context.

In Chapter 3, I test for the presence of personalities in *B. cornutus* by asking if behaviors performed in competition and mating covary across these contexts. I placed the same individuals from Chapters 1 and 2 in dyadic, mating trials and compared their behaviors to the ones performed in competition trials (Figure 2). Similar to Chapter 1 and 2, I constructed an ethogram to gain a better understanding of the types of behaviors performed in mating contexts. I then quantified correlations among behaviors across contexts. Overall, male and female *B. cornutus* were highly plastic in their behavioral responses to differences in the sex of social partners, despite expressing mating behaviors in competition contexts, and vice versa. Behaviors likely had different roles in mating and competition contexts and these responses were not connected to their performance across contexts. Individual behavioral strategies in competition and mating contexts were not constrained by intrinsic personalities.

The first three chapters provide an in-depth view of social behaviors in *B*. *cornutus*. My findings reinforce previous work that suggests competitive behaviors

indicate meaningful differences among males (Brown 1980; Conner 1988). In chapter 4, I examined the potential fitness consequences of male competitive behaviors by asking if females preferred the chemical cues of winning males or males that perform specific suites of behaviors during male competition. Previous work on female preference in B. *cornutus* determined that females do not generally prefer larger males (Brown et al. 1985; Brown and Bartalon 1986). However, the potential use of coercive behaviors in courtship, highlighted in Chapter 3, may make female preference difficult to discern in the presence of males. Females may, instead, use chemical cues as a way to detect differences among male courters and block copulation from unfavorable mates. Overall, females preferred to associate with the chemical cues of future losers before males competed, but changed their preference to realized winners following male competition. These results were a little surprising, but exciting. I documented a change in female preference as a result of dynamic changes in social context. Whereas male behaviors themselves may not be plastic within a competition context, the timing of competition itself may influence which males have access to mating opportunities in the wild.

My female choice study showed that social context extends beyond immediate surroundings to include past experiences. Chapter 5 expanded this idea by asking if age affects aggression. Work on age-related changes in social behaviors is usually conducted on large, long-living species that can be easily marked and recaptured. The shorter life spans, smaller size, and elusiveness make it challenging to assess the same types of social behaviors in invertebrates. *B. cornutus*, however, provide an ideal invertebrate system to test age-related changes in behavior because they are relatively long-lived, iteroparous, are easily marked and recaptured, and experiences higher mortality as they age (Liles 1956; Pace 1967; Formica et al. 2012, 2021). Aggression is costly to express (Careau and Garland 2012), and therefore may correlate with age-specific differences in life history strategies (Sih et al. 2015), but few studies examine the relationship between aggression and age. I measured aggression in male competition trials using beetles of different age classes from a metapopulation surveyed annually for multiple years. Overall, older males initiated more aggressive behaviors towards male competitors than young males. My results provide support for the terminal investment hypothesis, which predicts that individuals increase reproductive effort as survival probability decreases. Chapter 5 provides further evidence that the effects of social context on behavior extend beyond an individual's immediate surroundings to include their partner's past experiences.

My study adds to the increasing body of work that characterizes animal personality across contexts. Animal personalities have historically been quantified using repeatability and principal component analysis in which behaviors are distilled down to simple consistency and correlations across contexts (Sih et al. 2004). More recently, a variances components framework has been used to assess personality in terms of both consistency and plasticity (Dingemanse et al. 2010a; Dingemanse and Dochtermann 2013; Dochtermann and Dingemanse 2014). This method, however, can be challenging because the generalized linear mixed models used to calculate variance components cannot always process the characteristically zero-heavy, Poisson-distributed behavioral dataset. I combat this problem in Chapter 3 by co-opting methods used in quantitative genetics and Bayesian statistics to create models with variance components outputs that do not violate model assumptions. I anticipate that researchers who study animal personalities will find these methods useful in their own work.

Current animal personality research is asking how temporal and spatial differences in environment affect personalities (Dingemanse and Wright 2020). Personality structure can vary spatially among populations and evolution of those personalities in different populations are expected to vary accordingly (Rudin et al. 2019; Royauté et al. 2020). My dissertation research can be expanded to ask how population differences in biotic and abiotic environment affect *B. cornutus* personalities. My methodology for calculating variance components of behaviors combined with parentage data can then be used to quantify the genetic and environmental components of behavior to understand how personalities evolve.

I show that behavior can be both plastic and repeatable, and that time can affect differences in expression of behaviors among individuals. Understanding the relationship between behaviors used in both competition and mating allows for a complete understanding of sexually selected social interactions that can vary across time and ecological context. Overall, my dissertation research provides key first steps in understanding personalities across mating and competition contexts and creates more exciting questions about the effects of social contexts on the expression and evolution of behavior.

Figures



Figure 1: A. *Bolitotherus cornutus* male (left) and female (right). B. Male *B. cornutus* courting a female by positioning himself anterior to posterior. The male will then rub his abdomen on the head of the female. C. Male *B. cornutus* mate-guarding a female. Mate-guarding usually occurs after successful copulation. Photo taken by Phoebe Cook. D. Male attempting to dislodge a mate-guarding male from the back of a female laying an egg.



Figure 2: A. Behavioral trial recording set up for Chapters 1-3. B. Screenshot of behavioral arena. Two beetles were placed on opposite sides of a square piece of fungus embedded in plaster at the start of the trail and allowed to interact freely for the duration of the 4-hour trial period.

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CHAPTER ONE:

Contest interactions and outcomes: relative body size and aggression independently

predict contest status¹

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Abstract

In species with pronounced male armaments, body and weapon size often determine success in agonistic interactions. However, the behavioral components of interactions also play a significant role in determining outcomes and individuals that win agonistic interactions may not be the ones that start contests. In this study, I used dyadic, intrasexual assays to characterize agonistic behaviors and determine if body size and/or the propensity to start interactions influences contest outcome. I characterized agonistic interactions from start to end in the sexually dimorphic forked fungus beetle (Bolitotherus cornutus). Males of the species are known to use their thoracic horns in intrasexual combat, but other aspects of male-male behavior are undescribed. I created an ethogram that described transitions between behaviors and categorizes actions as aggressive, nonaggressive, and mounting. Individual aggressive and non-aggressive behaviors were highly repeatable between trials while mounting behaviors were not repeatable. The initiation of non-aggressive and mounting behaviors was not predictive of contest outcome. Relative body size and absolute aggression independently predicted contest outcome. Our results indicate that traits important for establishing contest outcome are not always correlated. Considering either aggression or body size alone may be misleading when determining competitor abilities.

Introduction

In sexually dimorphic species, larger body size and armaments are selected for through male agonistic interactions (Berglund et al. 1996; Emlen 2008; Hoem et al. 2007), female choice (Bonduriansky & Rowe 2003; Oliveira & Custódio 1998), and mate coercion (Arnqvist & Rowe 2002; Haley & Gray 2012). Males use armaments in a variety of behaviors during agonistic competitions over resources and mates, usually resulting in the male with the largest armament winning the interaction (reviewed in: Emlen 2008). Agonistic competitions consist of both aggressive and non-aggressive behaviors; however, aggressive behaviors are often the determinants of contest outcome (McGhee & Travis 2010; Sih et al. 2014; Wilson et al. 2009; Wilson et al. 2013). Because aggression is an important behavior used during agonistic interactions, it is easy to assume that larger males are more aggressive, but this assumption is hardly ever tested. Instead, many studies examine how morphological traits correspond with contest outcome (Bonduriansky & Rowe 2003; Okada et al. 2006; Okada & Miyatake 2004; Schuett 1997), and not whether morphological variation is correlated with important combat behaviors. Additionally, studies on male-male competition focus on the effects of aggression on contest outcome, but rarely consider the other suites of behaviors performed during intrasexual interactions (but see: Bertram et al. 2011; McGinley et al. 2015). The totality of a male-male interaction from start to finish must be characterized to determine how the combined effects of morphology and intrasexual behaviors influence contest outcome.

Behaviors that seem unremarkable may be essential for the progression of an interaction or lead to contest outcome. Some species use ritualized transitions of approaches and touching to assess their competitor (Chen et al. 2002; Jang et al. 2008), allowing contest outcome to be determined in the absence of overt aggressive behaviors (Cooper 1987; Enquist 1983; Jaeger 1981; López & Martín 2001). For example, males may avoid costly aggressive interactions by assessing their competitors using non-aggressive means (Berglund et al. 1996; Holekamp & Strauss 2016; Jenssen et al. 2005). To understand what determines contest outcomes, I must understand the totality of agonistic interactions because of the importance of these complex suites of behaviors (Bertram et al. 2011; Chen et al. 2002; Jang et al. 2008; Oliveira et al. 2011).

Attributing specific agonistic behaviors to an individual is difficult because social behavior inherently involves input from a competitor. The identity of a competitor may cause individual behaviors to vary from one interaction to the next. However, some proportion of an individual's behavior will remain consistent and repeatable across social contexts (Bell et al. 2009; Brooks & Endler 2001; Dochtermann & Nelson 2014; McGhee & Travis 2010; Schuster et al. 2017). Repeatability, or the proportion of variance that can be explained by differences among individuals, varies depending on the behavior and species in question (Bell et al. 2009). Despite the expected importance of social context, agonistic behaviors exhibit especially high repeatability. Across all behaviors, repeatability averages 0.37 across taxa, whereas repeatability of agonistic behaviors averages 0.5 (Bell et al. 2009).

In this paper, I characterized male-male interactions in forked fungus beetles (*Bolitotherus cornutus*) to test the assumption that body size is correlated with aggression

and determine if agonistic behaviors and/or body size influence contest outcome. *Bolitotherus cornutus* are sexually dimorphic and promiscuous tenebrionid beetles that live on and consume polypore shelf fungus that grows on dead wood. Females mate multiply within a season and lay one egg per laying event (Pace 1967). Males in the wild are observed mounting females head to abdomen, engaging in a suite of courting behaviors, then transitioning to a head to head position for copulation (Brown & Bartalon 1986). Males remain in a head to head mounting position for several hours to ensure insemination (Conner 1988, 1989). Rival males can engage the mating male in agonistic contests at any point in this interaction. Longer head to head, 'mate guarding' is suggested to increase insemination (Conner 1995), so the ability of a rival male to remove a guarding male from the back of a female should decrease the likelihood of insemination for the first male and increase mating opportunities for the rival male.

Male *B. cornutus* perform suites of agonistic behaviors using two sets of horns (clypeal and thoracic) (Benowitz et al. 2012; Conner 1988). Horn size is a sexually selected trait that is tightly correlated with body size and assumed to correlate with aggression (Conner 1988, 1989; Formica et al. 2011). Males engage in agonistic interactions both in the absence and presence of females (Conner 1988), suggesting that interactions may be important for obtaining both mating and food resources. Male beetles are often observed touching other males in the wild without escalation to fighting or chasing. Few instances of male-male mounting, similar to male-female mating behaviors, have also been observed. However, the consequences of these behaviors are unknown. Non-aggression and male-male mounting behaviors may be performed to establish a

dominance hierarchy and could be indicative of contest outcome (Holekamp & Strauss 2016; López & Martín 2001; McGinley et al. 2015).

Methods

Beetle collection, husbandry, and ethical note

I collected beetles from a large metapopulation near Mountain Lake Biological Station in Pembroke, Virginia in May 2016. A total of 130 male beetles were housed individually in small, plastic containers (5 cm X 2.5 cm X 5 cm) containing a thin layer of plaster to retain moisture, mulch, and a piece of their host fungus *Ganoderma tsugae* as a food resource. Water was administered as needed to beetle containers via a small, squeeze wash bottle. Beetles were houses in natural light conditions with room temperature held constant at 20° C \pm 1.5. I isolated beetles for about a month before beginning trials.

Behavioral assays were performed in July of 2016. Before interaction trials, I photographed each beetle using an EPSON scanner, then measured elytra length using the photographs in ImageJ (Abramoff et al. 2004). Elytra length is a known indicator of body size in most coleopterans. The elytra of every beetle was then painted with a black or white stripe to identifying them in dyadic trials. I used non-toxic, acrylic paint for these stripes. Beetles physically interacted with competitors, however there were no signs of harm after trials. Beetles were housed in the small, plastic containers following the trials until the end of their natural lives.

Interaction trials

A total of 48 unique beetles were randomly paired in different combinations in three trials for a total of 72 trials. Beetles were paired with a different male competitor in each trial and allowed to interact freely. Half of the beetles (n = 24) were designated as 'focals' in each trial. Behaviors were assayed in 10 cm X 10 cm plastic containers filled approximately 2 cm deep with plaster. A 5 cm X 5 cm square piece of *G. tsugae* fungus was set in plaster in the middle of the arena as a resource for the beetles to fight over. I differentiated between beetles in each trial by painting either black or white stripes on their elytra. I randomly paired beetles with respect to body size. Beetles were paired with competitors collected from different wild populations. Beetles experienced no previous interactions with any of their trial competitors.

Trials started with each male placed on an opposite side of the *G. tsugae* bracket and ended after four hours of recording. I returned beetles to their husbandry chambers after each trial and waited two days before placing them in another trial. Between trials, the plaster and fungus were replaced and the plastic containers were washed. The beetles painted black were arbitrarily designated as focal beetles after the trials, and only observations of these focal beetles were included in analyses of ethograms and contest outcomes to avoid pseudoreplication.

Bolitotherus cornutus are most active in low light periods of the day (e.g. night and early morning), so I recorded trials in a dark, temperature-controlled room using Canon PowerShot G1 X digital camera on infrared setting placed 1 m above interaction arenas. I attached infrared lights to each camera to enhance visibility. Cameras recorded beetle activity by taking a picture every five seconds using a Neewer© LCD digital shutter release remote control. I then stitched images into a video using FFmpeg software (Version be1d324).

I recorded the initiation and duration of the following behaviors using Inqscribe® (version 2.2.4): touch, bump, head, mount, flip, chase, grapple, fungus patrolling, end. See Table 1 for description of behaviors. Mounting is typically a behavior performed during male-female courtship and mating (Conner 1988). Mounting in a male-male context may be interpreted as adaptive or non-adaptive (Bailey & Zuk 2009). Males may be establishing dominance by mounting (Lane et al. 2016). Alternatively, males may be more likely to perform mounting behaviors on other males as a byproduct of high activity (Bailey et al. 2013; Boutin et al. 2016). Therefore, I used mounting as its own suite of behaviors that is sometimes followed by courting and copulation. Male-male courting and copulation attempts were observed on rare occasions in our trials. I scored whether a beetle initiated each behavior and the duration of that behavior. I assigned initiation of a behavior to the individual that approached its competitor and progressed physically into the behavior. The end of an interaction was assigned to the individual that left the area before its competitor.

Creating ethograms

I constructed a matrix of transitions between behaviors in the first trial (n=24). The behaviors of each beetle were considered for this analysis, so the transition matrices are properties of the trial, not the individual. Behaviors in the transition matrix included: bump, touch, head, mount, chase, flip, grapple, and end. Fungus patrolling is a behavior conducted by only one individual (not an interaction), so I did not include it in the creation of our ethogram. I combined the transition matrices for dyadic trails for a total count of the number of times every behavior was followed by a subsequent behavior. Each of the three trials were conducted for every beetle on the same day with two days in between trials. I created an ethogram using only the first trial to avoid pseudoreplication. I then performed a contingency analysis to determine which transitions occurred at a greater likelihood than expected (Bertram et al. 2011; Chen et al. 2002). Using this matrix, I performed a Markov chain analysis to construct an ethogram of non-random transitions from one behavior to the next in a string of interactions (Bertram et al. 2011; Chen et al. 2002). Ethograms were constructed using the 'markovchain' package in R.

Characterizing winners and losers

I counted the number of behaviors that the focal beetle (black) initiated. From the clustering of behaviors in the ethogram analysis, I used the following behavioral categories (see below) in the analysis of winning/losing status: aggressive, non-aggressive, and mounting. I classified a male as losing the entire trial if it ended more interactions than its competitor. I chose the proportion of ends as a conservative measure because the time required to establish dominance in *Bolitotherus cornutus* is unknown. I alternatively could have designated losers as the beetle who ended the final interaction of the four-hour trial period. These two metrics of contest outcome were highly correlated (r = 0.61, p < 0.001). Results of analyses were qualitatively similar regardless of the outcome metric used in the models. Trials that ended inconclusively because both males ended an equal number of interactions were not included in the analysis. Using the 'lmer' package in R (Bates et al. 2015), I assessed the effect of the number of initiated behaviors

in a trial on winning/losing status with a generalized linear mixed model using a binomial distribution: winning/losing status ~ aggressive behaviors + non-aggressive behaviors + mounting behaviors + size difference (focal elytra length – competitor elytra length) + trial date. Winning/losing status was coded as 1 for winning and 0 of losing. I included focal beetle ID and competitor beetle ID as random effects. I assessed the components of this model with a type 3 Wald χ^2 test using the 'car' package in R.

Repeatability and correlations

To calculate repeatability for each behavior category (aggression, non-aggression, and mounting), I obtained measures of within- and between-individual variance and covariance using univariate generalized linear mixed models (Dingemanse & Dochtermann 2013). Our models included behavior as the dependent variable and focal and competitor identity as random effects. I used the 'MCMCglmm' package in R for our analyses (Hadfield 2010). MCMC chains used 500,000 iterations, a burn-in rate of 5,000, and thinning intervals of 100. I assumed a Poisson error model because many individuals performed zero or one behavior in each category. The described behaviors have not been quantified before in *Bolitotherus cornutus*, so I used non-informative priors with an assumed Poisson distribution in our MCMC models. Non-informative priors make no assumptions about how I would expect the variances to be structured. I calculated repeatability from model outputs of variance using methods described in Nakagawa & Schielzeth 2010. I calculated correlations using Pearson's correlation test between number of initiated behaviors within individuals and between focal males and competitor

males. I adjusted for multiple comparisons using Bonferroni adjustment for 28 tests (alpha of 0.002).

Results

Male-male interaction behaviors

To reduce the number of behavioral variables used in our subsequent analyses, I used our ethogram to group behaviors into categories of related interactions. I designated male-male interactive behaviors into three categories: aggressive, non-aggressive, and mounting (Figure 1). Following Holekamp & Strauss's (2016) definition of aggression, I categorized aggressiveness as the suite of behaviors that most often led to one beetle ending the interaction. Non-aggressive behaviors were more likely to transition to other behaviors than to ending an interaction. The head behavior is an island within the ethogram because no non-random transitions occurred to or from this behavior. Beetles initiated a 'head' interaction, then transitioned to any other behavior with random probability.

In the first trial, focal beetles interacted with their competitors for an average of 10.3% of the four-hour trial period (range = 0.0% - 86.6%). A total of 19% of interactions consisted of males coming into proximity of one another, then immediately ending the interaction. During the proportion of time beetles spent interacting with their competitor, focal males were most frequently seen mounting their competitor. I frequently observed males cycling through bouts of touching and mounting. Males spent 57.3% of their total interaction time mounting their competitor. Males initiated more non-aggressive

behaviors (301 of 415 total initiated behaviors) than other behaviors but the actual duration of non-aggressive behaviors accounted for only 16.5% of the time interacting. Males conducted a total of 48 aggressive behaviors which accounted for 26.2% of trial duration. In both focal and competitor beetles, individuals that performed more aggressive behaviors also performed more non-aggression and mounting behaviors (Table 2).

Repeatability

The number of initiated aggressive and non-aggressive behaviors were highly repeatable across the three trials. Aggression had an estimated repeatability of 0.79 (95% CI: 0.46,0.99). Non-aggressive behaviors had an estimated repeatability of 0.39 (95% CI: 0.06,0.68). The number of initiated mounting behaviors an individual conducted in a trail were not repeatable (0.00, 95% CI: 0.00,0.65).

Contest outcomes

The majority of trials resulted in conclusive winning/losing status (one beetle ended more interactions than the other). Only six (of 72) trials resulted in a 'draw' between both beetles; five of those inconclusive trials resulted when beetles performed no interactions in the four hours of recording. A total of 37 trials resulted in the focal beetle winning and 28 resulted in the focal beetle losing. Nine focal of the 48 beetles emerged as winners in all three of their trials. Four emerged as losers in all three of their trials.
The size difference between competitors and number of initiated aggressive behaviors predicted winning status (Table 3). Larger males who initiated more interactions were more likely to emerge as winners. Difference in competitor body size was not correlated with the number of initiated aggressive behaviors (Table 2). Nonaggressive behaviors, mounting behaviors, and trial date had no significant effect on winning status (Table 3). The focal male's winning status was also predicted by the number of aggressive behaviors initiated by its competitor. Losing focal males had highly aggressive competitor (Table 2).

Discussion

Behaviors exhibited by *Bolitotherus cornutus* during agonistic interactions were classifiable into three suites of related actions (aggressive, non-aggressive, and sexual) that identify important transitions for contest outcome (Figure 1). I found that males often cycled through bouts of touching and mounting, sexual behaviors did not lead to aggressive behaviors, and non-aggressive behaviors preclude aggression. Most of the interaction time consisted of one male mounting the other. Both the number of initiated aggression and non-aggression behaviors were significantly repeatable across trials.

Relative body size and number of initiated aggressive behaviors independently predicted trial outcome. Difference in competitor body size and number of initiated aggressive behaviors were not significantly correlated. Larger beetles are not necessarily more aggressive, which challenges previous assumptions of the link between male morphological traits and contest behaviors. It is unknown whether contest outcome results in access to more resources; however, I can infer that contest winners will gain more access to females. Because aggression and relative body size independently predict contest status, I would expect both larger and more aggressive individuals to frequently be seen mating with females.

B. cornutus males initiated a similar number of aggressive behaviors regardless of competitor identity (Table 3). Though non-aggression and mounting are not predictive of contest outcome, these behaviors are tightly correlated with aggression (Table 2). I observed a few instances of non-aggressive behaviors transitioning to the initiation of aggressive behaviors in our trials. However, these transitions were not significantly different from random in our contingency analysis. The suite of non-aggressive behaviors may be utilized for establishing male-male relationships without the escalation to aggression, which could explain the high correlation of aggressive and non-aggressive behaviors (López & Martín 2001; Maynard Smith & Parker 1976).

Competitor assessment may explain why *B. cornutus* males in the wild are frequently observed engaging in non-aggressive behaviors compared to aggressive behaviors. In asymmetric contests, males with different body and horn size must assess whether the cost of aggression is worth the resource benefit (Maynard Smith & Parker 1976; McGinley et al. 2015). Non-aggression is used by many species as a form of competitor assessment, where smaller males with less competitive abilities in a dyadic interaction can determine the costs of benefits of fighting versus fleeing (Jenssen et al. 2005; McGinley et al. 2015). Observations of wild *B. cornutus* males engaging in nonaggressive touches and bumps could be a similar form of contest assessment where males are assessing the probability of winning and the resource benefits compared to the costs of aggression. Differences in aggression within a population may be due to intrinsic individual phenotypes that are highly repeatable regardless of social contexts or plasticity of aggression that depends on the characteristics of an individual's social partners (Bell et al. 2009; Matessi et al. 2010; Santostefano et al. 2016; Wilson et al. 2009). If aggressiveness is dependent on social context, an individual's behavior will depend on its partner's morphology and competitive ability (McGlothlin et al. 2010; Moore et al. 1997; Santostefano et al. 2016; Wilson et al. 2009). Alternatively, if aggression itself is important for contest outcome, individuals should maintain their level of aggressiveness regardless of their competitor's characteristics. In our study, males retained their level of aggressiveness regardless of the identity of their competitor, supporting the latter explanation.

One surprising finding of our study was the frequency and duration of same-sex mounting behaviors. Mounting behaviors in *B. cornutus* have been observed only infrequently in wild populations (unpublished data) and yet, in our trials, mounting behaviors accounted for just over half of total activity time. Differences between observations in the wild and our lab study may be due to the inherent nature of catching these behaviors while they are occurring or could be an artifact of the spatially constrained dyadic contest arenas. However, if same-sex mounting behaviors do occur in natural settings, they may have important consequences. Same-sex mounting behaviors are hypothesized to provide a variety of functions in other taxa (Bailey et al. 2013; Bailey & Zuk 2009). Same-sex mounting behaviors can function as a means of establishing dominance, therefore limiting the cost of aggression, or as a way of demonstrating sexual prowess to a broad audience of viewers (Bailey et al. 2013; Dukas 2010; Lane et al. 2016). In insects, studies have found that same-sex mounting behaviors are a result of repeatable personality traits performed regardless of social context (Boutin et al. 2016; Levan et al. 2009). Individuals perform mounting behaviors in a variety of social situations, regardless of whether or not that behavior is adaptive (Duckworth, 2006; Levan et al. 2009; Wey et al. 2014).

High correlations between behavioral traits may indicate the presence of behavioral syndromes, or correlations among repeatable behaviors across social contexts (Brommer & Class 2017; Sih et al. 2004). Male aggression, non-aggression, and mounting behaviors were all highly correlated in our study. Non-aggression and mounting behavior used in other social contexts (mating or mate defense) are also expressed in male-male interactions despite having no effect on contest outcome. If aggression is correlated with non-aggression and mounting in mating contexts, then highly aggressive males may incur a fitness loss when initiating aggression towards potential mates (Dingemanse et al. 2012; Duckworth 2006; Réale et al. 2007; Wey et al. 2014). The fitness loss of aggression towards mates may however be counteracted by frequently mounting mates (Sih et al. 2004). Less aggressive phenotypes would also incur a potential fitness loss in mating contexts due to less initiation of mounting behaviors (Sih et al. 2004). The actual costs and consequences of aggression in *B. cornutus* are largely unknown. Future studies should examine aggression across multiple fitness contexts and its correlation with other social behaviors to determine the potential constraints of aggressive personality on behavioral plasticity.

Male-male interactions rarely resulted in physical aggression. In our trials, only 16.6% of all initiated behaviors were characterized as aggressive. Instead, male-male

interactions were largely non-aggressive (67.9%). Two nonexclusive explanations may underlie this result. First, individuals may be avoiding costly aggressive behaviors by assessing their competitors using non-aggressive means (Berglund et al. 1996; Coleman & Wilson 1998; Unrug et al. 2004). Second, our experimental context may not have reflected a limiting resource for our population of *B. cornutus*. In our trials, males were given a piece of fungus as a food resource to fight over, but individuals may not assess this context as one needing aggression. Male beetles in the wild utilize aggression in a variety of scenarios. Fighting occurs either between two males, in groups of many males, as well as during mating and courtship. Whether or not *B. cornutus* males are territorial remains unknown. Future studies should measure male-male interactions in scenarios that vary in resource value (i.e. food, mates, etc.) to further examine how context affects the proportion of aggressive versus non-aggression behaviors. Future studies should also explore the repeatability of correlated behaviors across multiple social contexts (i.e. male-female interactions versus contests over food or mates).

Both size and aggression are important in establishing contest outcome, but these traits are not correlated in this species. Our study does not support the assumption that body size is correlated with aggression in sexually-dimorphic species. Overall, I conclude that 1) difference in competitor body size is correlated with contest outcome, 2) difference in competitor body size and number of initiated aggressive behaviors are independently predictive of contest outcome, 3) aggressive and non-aggressive behaviors are highly repeatable, and 4) male-male interactions over food resources are mostly non-aggressive. Considering body size or aggression alone may be misleading in predicting contest outcome.

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Figures and Tables

Behavior	Description
Bump	Head of one beetle comes into contact with any part of
	the body of the second beetle
Touch	Any physical contact that is not characterized by another
	behavior
Head	Both beetles touch head to head
Mount	One beetle crawls onto the back of the second beetle
Chase	One beetle rapidly follows the second beetle
Flip	One beetle flips the second beetle onto its back
Grapple	Both beetles are in full contact and tumbling over each
	other
Fungus patrolling	One beetle moves around the perimeter of the provided
	fungus square
End	One or both beetles leave an interaction

Table 1: Behaviors observed in male-male interaction trials

Table 2: A) within-individual and B) between-competitor correlation matrices. Winning status was designated as the proportion of times a beetle 'ended' interactions within a trial, where losing beetles ended more interactions compared to their competitor. Behaviors (aggression, non-aggression, and mounting) are the total number of initiated behaviors for each individual beetle within a trial. A) within-individual correlations were quantified using focal, black-painted beetles. B) between-competitor correlations were quantified by comparing focal, black-painted beetles to the behaviors of their three competitors. Bolded values indicate statistically significant correlations Bonferroni adjusted for multiple comparisons (alpha of 0.002) as determined by Pearson's correlation test.

	Winning status	Aggression	Non-aggression	Mounting				
Winning status								
Aggression	0.391							
Non-aggression	0.241	0.770						
Mounting	0.286	0.797	0.800					
B) Between-Competitor Correlations								
	Winning	Aggression	Non-aggression	Mounting				
	status							
Winning status								
Aggression	-0.418	-0.171						
Non-aggression	-0.369	-0.139	-0.045					
Mount	-0 341	-0.180	-0.146					
	0.5 11							

A) within-individual Correlatio

Table 3: GLMM describing winning/losing status of focal male in each male-male trial using type III analysis of variance (Wald χ^2 test). Model includes 65 trials with unique pairs and 26 focal individuals. The number of initiated behaviors is the number of times a focal individual initiated a specific behavior towards their competitor. Trails where both focal and competitor beetles performed equally, resulting in an undetermined trial status, were excluded from the model.

Model term	Coef.	SE	χ^2	р
Winning/losing status				
# focal initiated aggressive behaviors	1.32	0.65	4.13	0.04*
# focal initiated non-aggressive behaviors	-0.13	0.08	2.55	0.11
# focal initiated mounting behaviors	0.19	0.29	0.41	0.52
# competitor initiated aggressive	-0.78	0.38	4.28	0.04*
behaviors				
# competitor initiated non-aggressive	-0.04	0.09	0.16	0.68
behaviors				
# competitor initiated mounting behaviors	0.36	0.38	0.93	0.34
elytra size difference (focal size –	9.76	4.62	4.47	0.03*
competitor size)				
trial date	-0.05	0.49	0.01	0.91



Figure 1: Markov chain diagram for male-male interactions. Circle diameter indicates the number of times that behavior occurs across all trials. Arrow width indicates the probability that one behavior transitioned to the following behavior. Blue = non-aggressive states, purple = mounting states, red = aggressive states, gray = terminal state.

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CHAPTER TWO:

Mycophagous beetle females do not behave competitively during intrasexual interactions

in presence of a fungal resource²

² Formatted as a co-authored manuscript: Mitchem, L.D., Formica, V.A, Debray, R., Homer, D.E., & Brodie, E.D. III. Females do not behave competitively during intrasexual interactions in the presence of a fungal resource in a mycophagous beetle species, *Bolitotherus cornutus*.

Abstract

Intrasexual interactions can determine which individuals within a population have access to limited resources. Despite their potential importance for fitness generally and mating success especially, female-female interactions are not often measured in the same species where male-male interactions are well-defined. In this study, I characterized female-female interactions in *Bolitotherus cornutus*, a mycophagous beetle species native to Northeastern North America. I used dyadic, behavioral assays to determine whether females perform directly aggressive or indirectly exclusionary competitive behaviors. Polypore shelf fungus, an important food and egg-laying resource for *B. cornutus* females, is patchily distributed and of variable quality, so I tested for competition over fungus as a resource. Behavior of females was assessed in three sets of dyadic trials with randomly paired female partners. Overall, females did not behave aggressively towards their female partner or perform exclusionary behaviors over the fungal resource. None of the behaviors performed by females were individually repeatable. I discuss our results in direct comparison to a similar study on male-male interactions in the same species and argue for measuring male-male and female-female interactions with the same experimental paradigm to avoid unintended bias and allow for better comparison between the sexes.

Introduction

Individuals, male or female, should engage in intrasexual competition when resources are limited or variable (Knell 2009; Rosvall 2011; Arnocky et al. 2014; Baniel et al. 2018), but research on intrasexual behaviors is biased towards studies on males. Male competition is described in many species while the intricacies of female-female interactions are less known, despite the fact that both processes are important functions of sexual selection (Candolin 1999; Moore and Moore 1999; Wong and Candolin 2005; Hunt et al. 2009; Zhu et al. 2016). There is no reason to expect females to also compete over resources in the same ways males do. Interactions among females can affect which individuals have access to resources and can ultimately lead to selection on behaviors exhibited in agonistic and competitive interactions (Goubault et al. 2007; Clutton-Brock 2009; Rosvall 2011; Cain and Langmore 2016; Hare and Simmons 2019). Characterizing and defining female-female interactions is critical for ultimately determining how individuals distribute themselves within a specific habitat and how specific behavioral phenotypes affect resource allocation (Stockley and Campbell 2013).

Female competition typically has not been characterized in species in which male competition is well-defined (Hunt et al. 2009; Stockley and Bro-Jørgensen 2011). In insects, female competition is largely unexplored, despite considerable work on male competition (Kemp and Wiklund 2001; Giron et al. 2004; Goubault et al. 2007; Dunn et al. 2015; Kaiser et al. 2019). In fact, research on female competition comes largely from mammals (Stockley and Bro-Jørgensen 2011; Arnocky et al. 2014; Baniel et al. 2018; Haunhorst et al. 2020), birds (Cain and Ketterson 2013; Cain and Langmore 2016; Thys et al. 2017), and anoles (Reedy et al. 2017). The bias towards characterizing male competition is largely due to a subset of species in which males display overtly aggressive interactions because overt physical competition is easily observed and therefore easily characterized (Berglund et al. 1996; Kemp and Wiklund 2001; Holekamp and Strauss 2016; Tinghitella et al. 2018). Conversely, female competition is often described as more lengthy or discrete (Clutton-Brock 2007, 2009; Stockley and Campbell 2013; Čokl et al. 2020), though this classification may reflect human biases and not direct quantification of female aggressiveness (Rubenstein 2012; Kamath and Wesner 2020).

When approaching studies of same-sex interactions, an objective definition of aggressiveness used for both sexes is required in order for observers to remain unbiased. Competition behaviors can be difficult to compare between sexes because they are categorized based on observational studies and the observer risks imparting their own judgement on the intention of those behaviors (Burghardt et al. 2012; Tuyttens et al. 2014). Aggression is broadly defined as any behavior that intimidates or harms a social partner to an extent that causes them to flee the immediate area (Holekamp and Strauss 2016). The base observation that one individual leaves the area can therefore be used as a means of categorizing behaviors, where any behavior that leads to an individual immediately fleeing is determined to be aggressive in nature (e.g. Chapter 1). Chapter 1 constructed an ethogram of male-male interactions using contingency analysis where only the significant transitions from one behavior to the ending of an interaction were considered aggressive. Using this definition, all behaviors utilized during same-sex interactions can be observed and characterized without unintended human bias. Moreover, measuring same-sex interactions using the same behavioral paradigm for both males and females is critical for determining the meaning of those behaviors.

Understanding the proportion of behavior that is attributed to differences among individuals is essential for ultimately determining the consequences of behavioral interactions. Despite high plasticity, behaviors often have a component of variance that remains repeatable from one context to the next (Sih et al. 2004; Kralj-Fišer and Schuett 2014), and it is this repeatable proportion of a behavior that is attributable to intrinsic differences among individuals providing a fixed phenotype that may be subject to sexual selection (Réale et al. 2007; Schuett et al. 2010; Wolf and Weissing 2012). Behaviors that are not repeatable may show no response to selection because their plasticity across contexts equalizes fitness outcomes for all individuals expressing that behavior (Boake 1989; Schuett et al. 2010). Therefore, estimating repeatability is an important first step to understanding evolvability of behavioral traits.

In this study I tested if female forked fungus beetles (*Bolitotherus cornutus*) compete aggressively over a food and egg-laying resource. I characterized and quantified their physical interactions and then tested for exclusionary competition by quantifying the proportion of time females monopolized the provided resource over their same-sex partner. I also tested if female social interactions were repeatable. I use the same behavioral trial paradigm as a similar study on male-male competitive behaviors in this species (Chapter 1) and argue for the importance of measuring male-male and female-female behaviors equally.

Bolitotherus cornutus is a sexually dimorphic, mycophagous beetle species native to Northeastern North America (Liles 1956). *B. cornutus* are found feeding and interacting on three species of polypore shelf fungus - *Ganoderma tsugae*, *Ganoderma applanatum*, and *Fomes fomentarius* - that grow on dead logs in forested habitats: (Liles 1956). Fungus quantity and quality is variable both within and across populations.

Documented social interactions including male-male competition, mating, and egg laying occur on these fungus shelves (Liles 1956; Pace 1967; Conner 1988). While territoriality is not documented in *B. cornutus*, males often engage in combat while on the fungal shelves even when no females are present (Chapter 1), and have been observed walking along the periphery of the shelves in a potentially patrolling-like manner (pers obs.). Beetles mate and females lay eggs continuously throughout their active season (mid-May to mid-October) (Pace 1967). Once females lay eggs on the fungus, larvae hatch inside the fruiting body and consume it until they eventually emerge as adults (Liles 1956; Pace 1967). Larval fitness differs depending on the fungus species they develop in, but females may be limited in their movement among populations as they are more likely to lay eggs on lower quality fungus than migrate (Wood et al. 2014). Larval competition is common within fungal brackets, as larvae often cannibalize each other as they develop within their fungus (Liles 1956; Wood et al. 2014), so females may compete over fungal resources to maximize offspring survival.

Male competitive behaviors are well characterized in *B. cornutus* (Chapter 1), but less is known about how females interact and whether they compete over resources. Males are distinguished by their two sets of horns, clypeal and thoracic, whereas females are hornless but have two small tubercles on the top of their pronotum where horns would be. Males use both sets of horns in competitive interactions to gain access to mates by prying courting or mating males off the backs of females (Brown et al. 1985). Males also engage in combat over access to fruiting bodies with no females present, and both aggressive and non-aggressive behaviors in this context are highly repeatable (Chapter 1). Males who win competitive interactions gain more access to females who allow males to passively court but can block attempted copulation by closing their anal sternite (Brown et al. 1985; Conner 1988). While past field observational studies did not detect the presence of competitive behaviors in females (Liles 1956; Conner 1988; Formica et al. 2012), variable mate quality (Conner 1988, 1995), patchy resource quality within populations (Costello 2020), and larval cannibalism (Liles 1956; Wood et al. 2014) all provide motive for female-female competition.

Methods

Beetle collection and morphological measurements

I collected beetles from a large metapopulation near Mountain Lake Biological Station in Pembroke, Virginia (May 2016). I housed 47 female beetles in natural light conditions with temperature held constant at 20 ± 1.5 °C. Beetles were isolated in 5 x 2.5 x 5 cm, plastic containers for one month before I conducted trials. Beetle containers consisted of plaster as a substrate to retain moisture, mulch, and a piece of *Ganoderma tsugae* fungus as food. I provided water to beetles as needed.

Following collection, I imaged beetles using a flatbed Scanner (Epson Perfection V600 Photo) and used those images to measure beetle elytra length to the nearest 0.01 mm in ImageJ (Abramoff et al. 2004). I then assigned each beetle a unique ID and painted a white or black stripe along the sides of both elytra using non-toxic Testors[®] Enamel paint so I could differentiate between individuals in trials.

Female-female interaction trials

I performed dyadic, female-female interaction trials in July 2016 following methods from Chapter 1. Each trial consisted of two beetles interacting freely in a small plastic container (10 X 10 cm) filled approximately 2 cm deep with plaster for four hours. I provided an embedded, 5 X 5 cm square of *G. tsugae* in the trial containers (hereafter referred to as arenas) as a resource for females to fight over. Because *B. cornutus* are most active at night, I conducted trails in a dark, temperature-controlled room held at 19 \pm 2°C. A Canon PowerShot G1 X digital camera on infrared setting placed 1 meter above the arenas recorded female-female interactions trials by taking snapshot images every 5 seconds for 4 hours. I controlled the cameras shutter speed using Neewer© LCD digital shutter release remote control.

A total of 47 females were paired in three different combinations of femalefemale interaction trials (71 trials total). I paired each female randomly with respect to body size but always paired females painted white with females painted black to differentiate between individuals in each trial. I returned females to their isolated housing containers after each trial and waited two days before any female was re-paired in a new trial.

To conduct behavioral observation of female-female interactions, I first stitched still images from each trial into time-lapse videos using FFmpeg software (version be1d324). Behavioral observations were completed by DEH and LDM who scored the initiation and duration of the following behaviors (described in Chapter 1): touch, bump, head, mount, grapple, chase, flip, and end. LDM and DEH trained together to ensure interobserver consistency in scoring of specific behaviors. I scored whenever a female came within proximity of her partner, which was scored as approaching to at least one body length of their partner without physically touching. Fungus patrolling, or the duration of time each beetle spent alone on the fungal resource, was quantified to determine if females perform any resource guarding or exclusionary competition behaviors. I remained objective about which behaviors were considered aggressive and non-aggressive and later used ethogram analysis to determine specific classifications for each behavior (see Ethogram construction).

Ethogram construction

Following Chapter 1, I constructed an ethogram containing statistically significant transitions among behaviors to describe the most probable sequences of interactions in female-female trials. To create the female-female ethogram, I first constructed a matrix of transitions among all behaviors in the first set of trials (N = 23 interaction trials). I then combined all trial matrices into one matrix representing the total number of transitions among behaviors for every female's first trial. The final matrix was tested using a contingency analysis to determine which transitions occurred at a greater likelihood than expected by random chance. I constructed the ethogram of probabilities of transitions among behaviors using Markov chain analysis on the final matrix (R package: markovchain (Spedicato 2017)). I used only the first set of trials to avoid pseudoreplication that would have been caused by using data from each female three times. Results did not differ based on which trial set I used and using the first allowed us

to directly compare the female-female ethogram to the related male-male study, Chapter 1, that also used the first set of trials.

Once ethograms were constructed, I characterized behaviors into categories based on their transitions with other behaviors. I followed the rationale used in Chapter 1 to define male-male competitive behaviors as either non-aggressive, aggressive, or mounting. Non-aggressive behaviors were more likely to lead to another behavior, aggressive behaviors most frequently resulted in the ending of an interaction, and mounting behaviors occur when one individual climbs on top of their partner's back (Holekamp and Strauss 2016; Chapter 1). Mounting behaviors are given their own specific designation because they were previously assumed to only occur in male-female courtship contexts, but were frequently observed in male-male competition (Chapter 1) and our female-female competition trials (see Results).

Statistical analysis

I calculated intraclass correlations (ICCs) to quantify within-individual repeatability of each observed behavior. ICCs require measurements of within-individual variance and between-individual covariance. I obtained these measurements of variance and covariance for each behavior from univariate linear mixed models implemented in a Bayesian framework. Using the 'MCMCglmm' package in R (Hadfield 2010), our models included individual behaviors (touch, bump, head, etc.) as the dependent variables and female ID as random effects. Our MCMC analysis included 500,000 iterations, thinning intervals of 100, and a burn-in rate of 5,000. I used noninformative priors with an assumed Poisson error model. MCMCglmm outputs variance components for fixed effects, random effects, and residuals. I used the variance outputs of our random effects, female ID, and residual variance to calculate ICCs. I created six total models - one model for each behavior observed in our female-female interaction trials.

Next, I assessed the effects of body size and interactive behaviors on access to the provided fungal resource. Only 23 of our females performed any fungus patrolling behavior, so I scored fungus patrolled as a binomial factor where females were categorized as either performing any fungus patrolling behavior (n = 23), or performing no fungus patrolling behavior (n = 24). I used a T-test to assess differences in body size and Mann-Whitney U test to assess differences in interactive behavior (# of initiated behaviors by that female in a trial) between females who did and did not perform fungus patrolling behaviors. To avoid pseudoreplication, I selected the first trial for each female to be represented as their score for interactive behaviors in our final analysis. I used a Mann-Whitney U test for our second analysis because our behaviors did not fit a normal distribution. I performed our T-test and Mann-Whitney U test in R v.3.6.0.

Results

Females spent an average of 3.1% (range: 0% - 47.8%) of their 4-hour trial period interacting with their female partners. Almost half of all initiated interactions (48%) consisted of females coming within proximity of their partner, and then ending the interaction without engaging in any physical contact. The most common physical interactions included touching, bumping, mounting, and head-to-head. Females frequently engaged in the bumping behavior (32.6% of initiated physical behaviors) and often cycled through bouts of touching and mounting (Figure 1). Females performed a small frequency of head-to-head (9.7%), chases (3.5%), and flips (1.1%), though these behaviors did not have any statistically supported transitions to other behaviors (Figure 1). Based on our definition of aggressiveness, I could not characterize any females' behaviors as either aggressive or non-aggressive. No behaviors transitioned to ending the interaction more frequently than to other behaviors. Lastly, behaviors in female-female interaction trials were not repeatable across the three trials (Table 1).

Fungus patrolling duration averaged 8.91 minutes [range: 0.00 - 218.95 minutes] among all female-female interaction trials. Females performed fungus patrolling behaviors in 17 of our 42 trials. A total of 23 females performed fungus patrolling behaviors in any of their three female-female interaction trials while 24 females did not patrol the fungus squares. Females who performed fungus patrolling behaviors did not differ in body size (t = 1.00, df = 45.11, p = 0.32) or total interactive behaviors (w = 275, p = 0.99) from females who performed no fungus patrolling behaviors.

Discussion

Female-female interactions in *B. cornutus* were neither overtly aggressive nor exclusionary in our study. Instead, females only interacted with their female partner 3% of the time and spent a majority of their interactive time either in close proximity of their female partner or cycling through bouts of bumping, touching, and mounting. No female behaviors were individually repeatable, so behaviors in our trials may be driven more by extrinsic variation in social and abiotic environments, including characteristics of social partners or environmental differences among trials. Behaviors that were categorized as aggressive in male-male interaction trials – grapple, chase, and flip (Chapter 1) – did not

lead to ending interactions in female-female trails and, therefore, were not categorized as aggressive.

One of the more surprising results in our study was the lack of repeatability in any specific behavior of females. Both aggressive and non-aggressive behaviors in male-male forked fungus beetle interactions are highly repeatable (Chapter 1), which led us to predict similar levels of repeatability for those behaviors in females. The lack of repeatability observed in females, however, aligns with what is already known about female-female interactions in other species. Females may be more plastic in their response to intrasexual stimuli compared to males (Stockley and Campbell 2013). Behavioral plasticity in response to potential competition is a more efficient strategy for females who require a greater energetic cost of gamete production (LeBas 2006; Clutton-Brock 2007; Stockley et al. 2013). Regulating costly aggressive behaviors depending on the context allows females to invest more in egg quality and/or quantity (Stockley et al. 2013). Alternatively, partner behaviors may have been too variable and therefore unable to elicit the same response from trial to trial (Dingemanse et al. 2010; Dingemanse and Dochtermann 2013). High variance in social partner behavior followed by variable focal female response would result in higher within-individual variance and overall lower repeatability (Nakagawa and Schielzeth 2010; Wolak et al. 2012).

Two possible scenarios may explain the lack of competition between females in our trials. First, competition is context specific (King 1989; Giron et al. 2004; Clutton-Brock 2009), and our trial context may not induce competition in female *B. cornutus*. Competition occurs when valuable resources at that given moment are limited or vary in quality within an environment (Clutton-Brock 2009; Rosvall 2011). If females in our trials were not physiologically ready for oviposition, then our provided egg-laying resource would be superfluous. Females in our experiment were isolated for a short period of time prior to behavioral trials and had little time to assess the trial environment, which could affect motivation for competition in our trials. The specific context is also likely important for female-female competition. Females may be more likely to engage in aggressive behaviors when mates are the limiting resource (reviewed in: Rosvall 2011), or resources are highly variable in quality (Elias et al. 2010; Cain and Ketterson 2013; Stockley and Campbell 2013). I also found no evidence for indirect or exclusionary competition, which is more often observed when food resources are scarce (Rosvall 2011). The quantity of fungus provided in our study may not be resource limiting for females.

A second explanation for our lack of observed competition may be that female *B. cornutus* simply may not behave competitively towards one another in the wild. If food, mates, and egg-laying resources are not limited, then I would expect competition and aggression to negatively affect fitness in females (Cain and Ketterson 2013; Stockley and Campbell 2013). Although fungal resources vary among wild *B. cornutus* populations (Ludwig 2008; Wood et al. 2018), that variation may not reflect meaningful differences in quality and abundance for females. Based on analysis of *B. cornutus* social networks, female-female interactions do not correlate with fitness effects even when resource distribution was manipulated (Costello 2020). Our results also support previous field studies that found, while male aggression is often observed in the wild, *B. cornutus* females do not engage in similar aggressive behaviors (Pace 1967; Conner 1989; Formica et al. 2012).

When studying female-female interactions, it is important to remain objective in the classification of behaviors. Both sexes should be tested for the presence of competition using the same guidelines to limit unintended observer bias. Classification of female competition as exclusionary or absent may be due to our own human bias in what I expect from behavior in different sexes (Rubenstein 2012; Kamath and Wesner 2020). Our lab assays allowed us to parse apart the individual behaviors performed by females and analytically determine if any of those behaviors were aggressive. Using our method, I was able to objectively test females in the same way as males but categorize behaviors differently by observing the interactions immediately following each behavior. I determined that some behaviors have different functions when performed by males versus females when using the same criteria to evaluate them. Both males and females perform chasing and flipping behaviors, but these behaviors appear to be aggressive in males (Chapter 1) and non-aggressive in females. Male aggressive and non-aggressive behaviors were highly repeatable (ICC's of 0.8 and 0.4 respectively, Chapter 1) whereas no female behaviors were repeatable, and females performed fewer interactions with their partners for a shorter amount of time compared to males (males: 10.3% of trial time, females: 3.1% of trial time)(Chapter 1).

Conclusions

Future studies should measure female-female interactions in different contexts including presence/absence of a male cue, and variation in fungal resource quality or quantity to further elucidate potential competitive behaviors. Measuring females under multiple contexts will aid in determining the level of plasticity for these female-female

interactive behaviors both within a specific context, as demonstrated in our present study, and across contexts. Overall, I show that female and male intrasexual interactions have similar behaviors, but these behaviors differ in their elicited responses. Using the same behavioral paradigm for both sexes allows for direct comparison of same-sex interactions and objective, unbiased quantification of behaviors.

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Figures and Tables:

Table 1: Repeatability (intraclass correlation coefficient) measurements for behaviors measured in female-female interaction trials. Bracketed values represent 95% lower and upper confidence intervals.

Behavior	Description (Chapter 1)	Female ICC
Touch	Any physical contact that is not characterized	0.001 [0.00 , 0.14]
	by another behavior	
Mount	One beetle crawls onto the back of the second	0.001 [0.00 , 0.26]
	beetle	
Bump	Head of one beetle comes into contact with	0.002 [0.00 , 0.33]
	any part of the body of the second beetle	
Head	Both beetles touch head to head	0.003 [0.00 , 0.61]
Chase	One beetle rapidly follows the second beetle	0.004 [0.00 , 0.70]
Flip	One beetle flips the second beetle onto its	0.005 [0.00 , 0.98]
	back	



Figure 1: Ethogram for female-female interactions. Size of the circle indicates the relative number of times a behavior occurred across all trials. Colors signify the type of behavior where blue is non-aggressive and purple is mounting. Arrow width indicates the probability a behavior transitioned to the next behavior where significant transitions are labeled black and non-significant transitions are labeled gray. Specific probabilities are noted on the arrow line of significant transitions.

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CHAPTER THREE:

Personalities are not expressed across intrasexual competition and intersexual mating contexts in forked fungus beetles (*Bolitotherus cornutus*)³

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Abstract

The major question in the field of animal personality asks about the extent to which behaviors covary across contexts. Competition and mating behaviors are often performed in multiple contexts, but correlations among these behaviors are rarely quantified across-contexts. In this study I ask if intrasexual competition and intersexual mating behaviors covary within and across contexts in the forked fungus beetle, *Bolitotherus cornutus*, and relate these results to potential tradeoffs in behavioral expression. I measured the behaviors initiated by each sex in three rounds of dyadic intrasexual competition and intersexual mating behavioral assays. For mating trials, I constructed an ethogram that classified initiated behaviors and compared these classifications to the same behaviors initiated in competition from previous studies. Behaviors initiated during mating trials were either non-aggressive or sexual in nature. Male mounting and courting behaviors were highly repeatable within individual while no female behaviors were repeatable. I next constructed a phenotypic covariance matrix for behaviors initiated within and across competition and mating contexts. Overall, behaviors across contexts were highly plastic and showed no indication of personality. Male behaviors, however, were highly correlated within competition trials, showing a strong signal of within-context syndrome. Females were plastic in their response to both competition and mating contexts. Behaviors likely had different roles in separate contexts and indicate different behavioral strategies between the sexes.

Introduction

Animals interact with a variety of social partners from both sexes and must immediately decide on how to respond to these as potential mates or competitors. The extent to which behaviors are consistent and correlated – often referred to as personality or behavioral syndromes – affects how animals respond to new cues in their environment. Behavioral plasticity allows individuals to quickly adjust their phenotype to the social context (Réale et al. 2007; Dingemanse et al. 2010b; Mathot et al. 2012; Mitchell and Houslay 2020); however, individuals do not always express different behaviors in response to changing social cues (Dall et al. 2004, 2012; Wolf and Weissing 2012; Sih et al. 2015). To determine the entirety of possible behavioral responses and subsequent selection on behaviors, it is essential to quantify within-individual correlations (plasticity) in conjunction with among-individual correlations (personality) (Dingemanse et al. 2010b). Understanding the degree to which competition and mating behaviors are consistent or plastic across contexts will reveal how well individuals can adjust to changes in different social partners.

When a personality is present, behaviors remain mostly consistent regardless of large-scale, social partner differences across contexts and small-scale phenotypic differences among social partners within a context (Sih et al. 2004b; Stamps and Groothuis 2010). In mating versus competition contexts, individuals have a clear, classified difference among social partners – sex. Species either respond similarly to both sexes or respond according to the specific cue (sex) that distinguishes the contexts of mating from competition (Stamps and Groothuis 2010). The differences in cues provided within context are more subtle. Individuals can either respond according to fine-scaled phenotypic differences among social partners or maintain one level of behavioral expression for all partners within a context (Sih et al. 2004b). Behaviors are generally expected to be less plastic to within context differences compared to across context differences (Sih et al. 2004b, 2015; Stamps and Groothuis 2010). However, previous studies do not measure mating and competition simultaneously, making it difficult to assess plasticity and personality at both the within and across context levels.

Suites of intrasexual competition and intersexual mating behaviors are often observed outside of their respective context (Le Galliard et al. 2005; Bailey et al. 2013; Sih et al. 2014). In males, aggressive behaviors can have negative fitness consequences in courtship contexts (Moore et al. 2001; Sih et al. 2012, 2014), and vice versa (Bailey and Zuk 2009; Bailey et al. 2013; Boutin et al. 2016). Alternatively, competition and mating behaviors can be utilized in ways that increase fitness across contexts. Some species use courtship behaviors as a dominance display in which individuals court same-sex partners during competition to achieve dominance (Wilson et al. 2013; Lane et al. 2016). Aggression can also be used to increase fitness as sexual coercion in mating contexts (Moore and Moore 1999; Moore et al. 2003; Le Galliard et al. 2005; Wey et al. 2014). Female fitness may be similarly affected by expressing disadvantageous traits in competition or mating (Clutton-Brock 2009), but most studies have focused on male behaviors. Examining behavioral correlations among competition and mating in males and females is necessary because both sexes actively participate in both contexts (Clutton-Brock 2007).

Quantifying within-individual behavioral correlations in competition and mating contexts allows us to determine if an individual responds plastically to changes in sex of

social partner (Dingemanse et al. 2010a; Dingemanse and Dochtermann 2013). Behavioral correlations can either consist of multiple behaviors within a context, the same behavior across contexts, or multiple behaviors across contexts. Some level of significant within-individual behavioral correlation is an essential prerequisite to defining a suite of behaviors as personalities. High within-individual correlations indicate low plasticity, and therefore high consistency in behavioral response (Dingemanse and Dochtermann 2013). In competition and mating contexts, within-individual correlations within context indicate consistency in response to social partners of the same sex, while across context correlations indicate consistency in expression elicited by social partners of either sex (Figure 1).

Among-individual behavioral correlations, when combined with strong withinindividual behavioral correlations, allow us to determine if behaviors represent meaningful personality, or behavioral syndrome, differences. Hereafter, I refer to correlated behaviors across contexts as personalities and syndromes as correlated behaviors within contexts. Our scheme for personalities and syndromes is modified from behavioral syndromes proposed by Sih et al. 2004. Among-individual behavioral correlations quantify the extent to which individuals behave differently within and across contexts (Dingemanse and Dochtermann 2013). Among-individual behavioral correlations within a specific context represent a within-context syndrome (Figure 2A). For example, males may act highly aggressive regardless of the phenotype of male competitors with no correlation to the same aggression in mating. Personalities occur when among-individual behavioral correlations extend across social contexts (Figure 2B-C). Extending the competition example, an aggressive personality is present when aggressiveness towards male competitors is correlated with aggressiveness towards potential mates. Personalities can involve individual behaviors (Figure 2A), such as aggressive acts, or include suites of multiple behaviors, like aggressive acts, nonaggressive acts, and sexual acts (Figure 2C).

Two scenarios may explain why competitive behaviors are expressed in mating contexts and vice versa. First, social stimuli in each context may be similar enough to stimulate similar expressions of behavior, even if the same level of behavior is disadvantageous in one context (Sih et al. 2004b; Careau and Garland 2012). In this case, behaviors are expected to have high, positive correlations across contexts and can be identified as aggressive, mating, or aggressive-mating personalities depending on their correlations with other, unrelated behaviors (Figure 2B-C). Alternatively, behaviors that appear similar to observers may have different roles in intra- and intersexual contexts (Sih et al. 2004a, 2015; Careau and Garland 2012; Bailey et al. 2013). In this case, behaviors are triggered by different social stimuli and expected to have high plasticity and little to no associated correlations across contexts (Figure 1A, Figure 2A).

In this study, I investigated variation in behavior of *Bolitotherus cornutus* (forked fungus beetles) to ask whether intrasexual competition and intersexual mating behaviors covary across contexts. *Bolitotherus cornutus* are sexually-dimorphic tenebrionid beetles native to Northeastern North America (Liles 1956). Males are characterized by two sets of horns (clypeal and thoracic) that they use in competition over access to females (Liles 1956; Conner 1988). In intrasexual interactions, male behaviors are highly repeatable and correlated (Chapter 1). Female behavior in comparable intrasexual interactions, however, is not repeatable or indicative of aggression or exclusionary competition (Chapter 2).

Larger and more aggressive males are more likely to win competitive interactions against male partners (Chapter 1). In the wild, winning males then have more access to female mates (Conner 1988). Males court females by mounting anterior-to-posterior and rubbing their abdomen on the heads of females, then position themselves posterior-to-posterior to attempt copulation (Brown et al. 1985). Females do not appear to have control over which males court them, yet they do control who successfully copulates with them (Brown et al. 1985). Females have an anal sternite (a hard covering on the ventral surface of the terminal abdominal segment) that acts as a lock system giving the female control over insemination (Conner 1988).

Previous findings suggest that male competition and mating are potential targets for sexual selection. Our study quantifies how behaviors expressed in competition and mating correlate within and across contexts. I provide an in-depth analysis of behaviors initiated during intersexual mating interactions and directly compare mating trials to the same behaviors in male and female competitive interactions from previous studies (Chapter 1 and 2). Understanding correlations in competition and mating behaviors across context will determine the extent to which these behaviors contribute to animal personality and provide insight to how these behaviors may respond to sexual selection.

Methods

Beetle collection

In May 2016, I collected 50 male and 50 female beetles from a large metapopulation near Mountain Lake Biological Station in Pembroke, Virginia. After collection I isolated beetles in small, plastic cups containing a small layer of plaster to retain moisture, mulch, and a piece of *Ganoderma tsugae* fungus as a food resource. I added water to beetle cups as needed using a plastic wash bottle. Beetles were housed within their beetle cups in natural light conditions with temperature held constant at $20 \pm$ 1.5°C until the time of their behavioral trials. I imaged each beetle using a flatbed scanner (Epson Perfection V600 Photo) and used those images to measure elytra length to the nearest 0.1 mm in ImageJ (Abramoff et al. 2004). I then used non-toxic Testors[®] Enamel paint to paint either a black or white stripe along each side of the elytra of every beetle to differentiate between individuals in dyadic behavioral trials.

Intrasexual competition trials

After a month of isolation in their individual housing chambers, males and females competed in a randomized set of three same-sex dyadic competition trials in which two individuals were placed in a plastic container layered with plaster holding a square of *G. tsugae* as a resource to fight over. I video recorded competition trials for four hours in the dark because beetles perform most of their interactive behaviors between dawn and dusk. Infrared lighting allowed us to see and differentiate between beetles painted with either black or white stripes. Beetles were placed back in isolation following one trial and held two days before their next competition trial. Body size of dyadic partners was randomized. After all trials were recorded, I scored the video recordings for the following behaviors using Inqscribe® (version 2.2.4): touching, bumping, head-to-head, mounting, grappling, chasing, and flipping. Specific details on the video recording set up and scoring procedure is described in Chapter 1 and 2.

Intersexual mating trials

Following competition trials, I paired males and females in dyadic, intersexual mating trials in new plastic containers lined with plaster holding a square *G. tsugae* resource. Following procedures of the competition trials, I recorded the mating trials in the dark using infrared lighting. To capture all potential mating behavior, I recorded mating videos for 12 hours. Our aim was to observe the onset of mating behavior rather than the outcome, so I assessed the behaviors expressed during the first four hours of mating trials to allow comparisons across contexts. Specific details on the video recording set up and scoring procedure is described in Chapter 1 and 2.

I observed and scored the same behaviors in mating trials as in competition trials (touching, bumping, head-to-head, mounting, grappling, chasing, and flipping), as well as three mating specific behaviors: courting, attempted copulation, and guarding. Courting occurred when a male mounted a female posterior-to-anterior and rubbed its abdomen on top of the female's head, likely releasing a chemical signal. Attempted copulation then followed in which a male adjusted its position posterior-to-posterior and lowered its abdomen into position to insert its aedeagus. I scored courting as any time I observed a male stationary on top of a female partner in the posterior-to-anterior position. Attempted copulation was scored any time I observed a male on top of a female, posterior-to-posterior-to-posterior with his abdomen dipping towards the female's underside. Because of the camera angle and video resolution, I could not assess whether the attempted copulation was successful. The final mating behavior, guarding, occurred anytime a male mounted a female posterior-to-posterior and remained on her back for an extended period of time

but was not rocked back in a copulation attempt. I observed no instances of a female courting, attempting copulation, or guarding a male partner.

Chapter 1 and 2 classified behaviors initiated during male-male and femalefemale competition in the form of ethograms. Following Chapter 1, I created ethograms of statistically significant transitions among behaviors in our mating trials. Our ethograms described the probability of transitions between behaviors initiated by females, males, or either individual. To create these ethograms, I first constructed matrices of transitions among behaviors that either individual (male or female) initiated in the first set of mating context trials. I then combined the matrices for each trial into one matrix containing the total number of transitions among behaviors for every beetle's first mating trial. I used contingency analysis to determine which transitions in our final matrix occurred at a greater likelihood than expected by random chance, then constructed a visual ethogram containing the probabilities for each transition between behaviors using Markov chain analysis (R package: markovchain (Spedicato 2017). After constructing the mating ethogram with behaviors initiated by both sexes, I constructed individual sex ethograms that included only behaviors initiated by either males or females. I used the same procedure for constructing ethograms from individual sex mating trial matrices as I did for the both sex matrices.

Once ethograms were constructed, I assigned behaviors into three classifications – aggressive, non-aggressive, and sexual using the rationale defined in Chapter 1. Non-aggressive behaviors were more likely to lead to another behavior, aggressive behavior most frequently resulted in the ending of an interaction, and sexual behaviors was most frequently followed by courting (Chapter 1 and 2).

Statistical analysis

Mating context repeatability: I calculated intraclass correlation coefficients (ICCs) of individual behaviors using variance and covariance components obtained from univariate linear mixed models to quantify within-individual repeatability in behaviors initiated during mating contexts. I implemented our univariate mixed models in a Bayesian framework using the 'MCMCglmm' package in R (Hadfield 2010). Our models included each individual behavior (bump, mount, grapple, and court) as the dependent variables and beetle ID as random effects. I ran separate models for male and female behaviors. MCMC model parameters included 1 x 10⁶ iterations with a 100,000 burn-in rate and 100 thinning intervals. I used non-informative priors with a Poisson error model.

Phenotypic (P) variance matrix construction: I calculated among- and withinindividual (co)variances and associated correlations among behaviors both within and across contexts using generalized linear mixed models implemented in a Bayesian framework. I ran our models using the 'MCMCglmm' package in R (Hadfield 2010). Our models estimated variance and covariance of the following behaviors - bump, mount, grapple, and court within the mating and competition contexts. I included elytra length as a proxy for body size as a fixed effect and beetle ID as a random effect in our models. Our covariances were calculated separately for males and females using the posterior distribution of 1 x 10^6 MCMC chains with a 100,000 burn-in rate and thinning intervals of 100. Statistical significance of our (co)variance values are represented as 95% highest posterior density (HPD) intervals. To maximize the power of our analysis, I limited our model to four behaviors that were well represented in both contexts and that I considered biologically meaningful. I chose the bumping and mounting behaviors because they were the most frequently initiated behaviors in both contexts and previously described as independent from one another in competition trials (Chapter 1). I chose grappling and courting as two behaviors that were socially important within specific contexts (grappling for competition and courting for mating). I did not observe any instances of males or females attempting to court same-sex partners in competition trials, so I did not include courting within the competition context in those models.

Results

Intersexual mating behaviors

Overall, beetles spent 3.2% (range: 0% - 47.8%) of the observed 4-hour trial time interacting with their partners. Male-initiated mounting and grappling were highly repeatable but no female-initiated behaviors were repeatable (Table 1). Courting comprised the majority of time spent interacting (84%, range 0% - 99.8%). Males initiated mostly touch (mean: 5.14 initiations per trial, range: 0-46) and courting (mean: 3.27 initiations per trial, range: 0-15) behaviors towards their female partners, whereas females initiated mostly touch (mean: initiations per trial 3.90, range: 0-59) and bump (mean: initiations per trial 0.30, range: 0-5) behaviors. Males mounted their female partners an average of 1.98 initiations (range: 0-26) during the 4-hour trial period, whereas females mounted their male partners an average of 0.68 initiations (range: 0-14).

Only five females initiated any grappling behavior during any of their three mating trials. Many males initiated grappling towards female partners with an average of 0.80 initiations per trial (range: 0-13).

Females initiated far fewer behaviors in the mating context compared to males. Males initiated an average of 14.26 (range: 0-92) behaviors, whereas females initiated an average of 5.05 (range: 0-78) behaviors. Female interactions towards males were also less complex in their transitions compared to male interactions towards females (Figure 3A). For both males and females, behaviors in mating trials were classified as either nonaggressive or sexual, with no behaviors emerging as aggressive (Figure 3A-B). Although grapple and flip appear as aggressive in male competition trials, these behaviors were more likely to lead to courting by males in mating trials and were minimally observed or absent in females (Figure 3B). Chasing was similarly determined to be aggressive in male-male competition, but in a mating context this behavior is non-aggressive in either sex (Figure 3). Similar to female competition, females in the mating context initiated bouts of touching to bumping and touching to mounting. Female-initiated mounts resulted in end of an interaction if it did not lead back to touching (Figure 3A). Males also initiated similar bouts; though in males, bouts of touching to mounting often led to courting behaviors (Figure 3B).

Correlations among behaviors in competition and mating contexts

I report our phenotypic matrices results in terms of correlations. Unstandardized (co)variances are reported in Supplemental Figures 1 and 2. Both sexes exhibited strong within-individual correlations among behaviors within contexts, but no significant

correlations among behaviors across contexts (Figures 4-5). Behaviors initiated by males were all strongly positively correlated within-individuals in the competition context (Figure 4; top panel). Grapple and court were the only behaviors in the mating context that were significantly correlated within-individuals (Figure 4; top panel). Withinindividual correlations for behaviors initiated by males across contexts were not statistically significant (Figure 4; top panel).

Behaviors of males show signatures of a within-context syndrome in that amongindividual correlations between bump, mount, and grapple in the competition context were positive and significant (or nearly so) (Figure 4; bottom panel). In the mating context, among-individual correlations for court and mount and grapple and court were nearly significant (Supplemental figure 1).

For females, levels of bump and mount were significantly correlated withinindividuals in both the competition and mating context (Figure 5; top panel). No other within-individual correlations were statistically significant for female-initiated behaviors either within or across contexts. No among-individual correlations for female-initiated behaviors were statistically significant (Figure 5; bottom panel).

Discussion

Despite observing mating behaviors in competitive contexts, and vice versa, *B. cornutus* showed no indication of personalities across contexts for either sex. Competition and mating behaviors expressed by both sexes of *Bolitotherus cornutus* were highly plastic across contexts. Male *B. cornutus* showed a strong signal of a withincontext competition syndrome. However, no other indication of behavioral syndromes or personalities was found for either sex or context. Analogous behaviors likely serve different purposes in competition versus mating contexts and individuals could pursue different behavioral strategies in response to social partner sex (Sih et al. 2004a; Mathot et al. 2012).

Intersexual mating behaviors

Analogous behaviors were determined to have different elicited outcomes in mating and competition trials, indicating that these behaviors had different functions depending on the context. Aggressive behaviors are defined as those that result in social partners fleeing an interaction (Holekamp and Strauss 2016; Chapter 1). Aggression in competition often leads to winning individuals gaining access to resources (Berglund et al. 1996; Holekamp and Strauss 2016). However, aggression in an intersexual mating context can function as a signal for unwanted partners to leave the interaction (Hews et al. 2004; Huffard et al. 2010; Kralj-Fišer et al. 2012). Aggression in mating is often initiated by females fending off unwanted courters (Hews et al. 2004; Kralj-Fišer et al. 2012; Hohmann and Fruth 2015), but males have been observed aggressing females to increase social distance or gain access to resources (Bercovitch et al. 1987; Huffard et al. 2010). The lack of observed aggression in our trials indicates that individuals were receptive to mating, ignoring mating opportunities, or utilizing different strategies to avoid conflict.

I observed no instances of aggression in our mating trials. Male initiated grapples and flips were determined to be sexual (related to courtship) in mating trials, and may have functioned as a means of sexual coercion in which females were forced into courtship (Clutton-Brock and Parker 1995; Wey et al. 2014; Cassini 2020; Sentenská et al. 2020). Sexual coercion often results in mating success for males and may be a viable strategy if female partners were not responsive to courtship (Clutton-Brock and Parker 1995; Fox et al. 2019; Cassini 2020).

Mating was either always accepted by females or difficult for females to avoid. Females in the wild do not appear to control courtship interactions (Brown et al. 1985; Conner 1988). Females may benefit from accepting courtship and blocking copulation from unwanted males by keeping their anal sternite closed instead of engaging in energetically costly aggressive behaviors to fend off male courtship. Females may use non-aggressive and mounting behaviors as a way to gain information about social partners and determine if the partner is an acceptable mate (Chapter 4).

Plasticity and personality in competition and mating contexts

Both male and female beetles responded plastically to mating partners. The observed plasticity may be due differences in receptibility caused by previous experience (Jennions and Petrie 1997; Lynch et al. 2005; Billman et al. 2014). Male competition trails may have induced chemicals cues that females detect and responded to according to their own preferences (Johansson and Jones 2007). Previous studies determined that females preferred to associate with the chemical cues of losing males in the absence of competition and winning males immediately following competition (Vilella-Pacheco et al. 2021). Those cues may then diminish as males participate in mating trials which could have caused the observed plastic responses in both sexes' behavior (Johansson and Jones 2007). However, this scenario is unlikely to explain our results because male competition

trials occurred 10 days before mating trials, and there was no signal of winner-loser effects among competition trials (pers. obs.). Alternatively, differences in female experience may explain the observed differences in behaviors initiated during mating trials. Males may have initiated less sexual coercion if female partners had previously mated, were not ready to mate, or were not preferred by males (Lynch et al. 2005; Swierk et al. 2013; Formica et al. 2016; Schlupp 2018).

While grappling, mounting, and bumping behaviors were highly plastic, courting and mounting behaviors initiated by males in mating trials indicated the presence of two, separate within-context syndromes. Males were likely to initiate similar amounts of courting and mounting behaviors in response to different female partners. Courting and mounting, however, were not correlated. Mounting may be used as an exploration strategy to discern phenotypes of potential mates, whereas courting is used to persuade females before copulation attempts (Réale et al. 2007). The mate exploration interpretation aligns with our observations of mounting in competition trials. Mounting, though not correlated across contexts, may be used for exploration of social partners of either sex (Réale et al. 2007).

Previous studies determined that male *B. cornutus* prefer larger females (Formica et al. 2016), but males in our study initiated repeatable quantities of courting towards females of different body sizes. Mating is likely beneficial even when males cannot interact with their preferred mates (Jennions and Petrie 1997), which would explain why courtship was observed in almost every mating trial. Male quality instead of male preference may then explain the observed differences in courtship behaviors among males (Sih et al. 2015). Physiology and energy stores may affect the ability to initiate

costly courting behaviors causing healthier males to initiate more courtship (Sih et al. 2015; Näslund and Johnsson 2016).

Individual males expressed consistent and correlated suites of competitive behaviors that, at the same time, differed substantially from other males. I identified a suite of aggressive grappling, non-aggressive bumping, and mounting behaviors as a within-competition syndrome that defines meaningful differences in fighting ability among male *B. cornutus*. Differences in within-competition syndromes may reflect statedependent differences in male quality similar to our within-mating courting syndrome (Sih et al. 2015). Higher quality males may invest more in costly behaviors during competition, win competitive interactions, and ultimately gain access to mates (Vitousek et al. 2014; Sih et al. 2015). Under this scenario, competitive interactions in the wild should select for initiation of more aggressive behaviors (grappling, flip, and chase behaviors) because they increase the probability of winning contests (Chapter 1). This advantage could in turn generate indirect selection non-aggressive behaviors including bumping and mounting whose expression is correlated within competitive contexts (Lande and Arnold 1983).

Female behaviors showed no indication of within context syndromes or across context personality. Females were highly plastic in response to social partner sex across contexts and more discrete changes in social partner phenotypes within contexts. Although females in our study were likely to initiate correlated bump and mount behaviors within mating and competition trials, these behaviors were not repeatable in either context (Chapter 2; Table 1), rendering the importance of the correlation questionable. High behavioral plasticity may be a beneficial strategy for females who already invest a lot of energy in costly gamete production (Yanagi and Miyatake 2003; Clutton-Brock 2007). Females may respond only as necessary depending on the cues of social partners and regulate the expression of potentially unnecessary behaviors (Clutton-Brock 2007; Careau and Garland 2012).

Conclusions

Behaviors observed in multiple contexts are expected to have some level of correlated responses across those contexts (Sih et al. 2004b; Dingemanse et al. 2010b). Stimuli in each context are assumed to be similar enough to cause consistent and correlated expression of the same behaviors (Careau and Garland 2012). A remaining portion may be plastic to changing cues, but most published research on personalities and behavioral syndromes note that behaviors expressed in multiple contexts have correlated responses across those contexts. An individual's strategy is expected to be constrained, in part, by intrinsic personality (Stamps and Groothuis 2010). I, however, found no correlation in the responses of any behavior expressed in either competition or mating across contexts. Personalities across mating and competition contexts were not explored in past literature, which may explain our lack of observed personality. Sex as the provided cue may be different enough to trigger different expression of the same behavior and allow individuals to maintain different strategies in mating and competition contexts (Careau and Garland 2012; Bailey et al. 2013; Sih et al. 2015). Alternatively, publication bias may also skew towards studies that report significant personalities over lack of personalities.

Understanding the extent to which competitive and mating behaviors correlate within and across contexts provides a holistic view of the ability of animals to respond to cues of social partner in important contexts related to sexual selection (Dall et al. 2012; Wolf and Weissing 2012). Our results highlighted differences in behavioral strategies between the sexes in competition and mating contexts. I show that behavior can be both plastic and repeatable, with both mating and competition behaviors in *B. cornutus* exhibiting high plasticity across social contexts, but high repeatability within contexts. Individual behavioral strategies in competition and mating contexts were not constrained by intrinsic personalities.

Recent work in the field of animal personalities has focused on determining the genetic underpinnings of repeatable and correlated behaviors (White and Wilson 2018; Kasper et al. 2019; Rudin et al. 2019; Royauté et al. 2020). These studies demonstrate that the genetic architecture of personalities can differ among populations (Kasper et al. 2019; Royauté et al. 2020), and evolution of personalities can, therefore, differ accordingly. Identifying the genetics of male within-competition syndromes and withinmating syndromes in *B. cornutus* will be an important next step for determining how populations can respond to selection on these behaviors.

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Figures and Tables

Table 1: Repeatability (intraclass correlation coefficients) for behaviors initiated by either males or females during mating trials. Bracketed values are lower and upper 95% confidence intervals.

Behavior	Description	Male ICC	Female ICC
Bump	Head of one beetle comes into contact with any part of the body of the second beetle	0.00 [0.00, 0.89]	0.00 [0.00, 0.37]
Mount	One beetle crawls onto the back of the second beetle	0.76 [0.45, 0.98]	0.00 [0.00, 0.26]
Grapple	Both beetles are in full contact and tumbling over each other	0.00 [0.00, 0.46]	N/A
Court	One beetle is stationary on the back of the second beetle faced posterior-to- anterior	0.88 [0.60, 1.00]	N/A

Within-individual correlations (plasticity)

A) High plasticity in response to sex of social partner







Figure 1: Interpretations of within-individual correlations (plasticity) in mating and competition behaviors. The matrix of pairwise correlations can be used to determine levels of plasticity and personality. Using competition and mating interactions in *B. cornutus* as an example, here is how patterns of significant correlation (+ significant positive correlation, - significant negative correlation) are interpreted. **A. High plasticity in response to sex of social partner sex.** Individuals respond differently to partners depending on their sex, but respond similarly to social partners of the same sex. The absence of within-individual correlations among behaviors across contexts indicates high plasticity in response to differences in social partner sex. The presence of positive within-individual correlations within contexts indicates low plasticity in responses to differences in social partner set of sex of social partner. Individuals have similar behavioral responses to all male and female partners. The presence of positive within-individual correlations across contexts indicates low plasticity in response to social partner sex and is likely associated with positive within-individual correlations across.

Among-individual correlations (personality)



Figure 2: Interpretations of among-individual correlations (personality and behavioral syndromes) in mating and competition behaviors. The matrix of pairwise correlations can be used to determine levels of plasticity and personality. Using competition and mating interactions in *B. cornutus* as an example, here is how patterns of significant correlation (+ significant positive correlation, - significant negative correlation) are interpreted. A. Within-context syndromes. Individuals differ by their behavioral responses in either competition or mating, but responses do not carryover across contexts. The presence of positive or negative among-individual correlations within context but absence of amongindividual correlations across contexts indicates within context syndromes. **B. Single** behavior personality. Individuals differ by their initiation of either aggressive or mating behaviors in both contexts. The presence of positive among-individual correlations across contexts in the same behavior (aggression or mating) indicates a personality for a single behavior. C. Multiple behavior personality. Individuals differ by their correlated responses of aggressive and mating behaviors in both social contexts. The presence of positive or negative among-individual correlations of different behaviors within and across contexts indicates a multiple behavior personality.



Figure 3: Ethograms for female-male courtship interactions. A. behaviors and transitions initiated by females, B. behaviors and transitions initiated by males, C. behaviors and transitions initiated by either sex. Arrow width indicates the probability of one behavior transitioning to the next connected behavior. Node size indicates the number of times that behavior was initiated among all trials. Blue = non-aggressive behaviors, purple = courting/mating behaviors, gray = end states.



Among-individual correlations (personality)



Figure 4: Within-individual (top) and among-individual (bottom) phenotypic correlation matrices for behaviors expressed by males. Asterisks indicate significant correlations based on 95% HPD intervals. Correlations are heat-mapped based on strength of correlation; deep-red indicates stronger positive correlations and deep-blue indicates stronger negative correlations.



Among-individual correlations (personality)



Figure 5: Within-individual (top) and among-individual (bottom) phenotypic correlation matrices for behaviors expressed by females. Asterisks indicate significant correlations based on 95% HPD intervals. Correlations are heat-mapped based on strength of correlation; deep-red indicates stronger positive correlations and deep-blue indicates stronger negative correlations.



Among-individual (co)variance (personality)



Supplemental figure 1: Male within-individual (top) and among-individual (bottom) phenotypic matrices. 95% HPD intervals in brackets below (co)variance. Asterisks indicate significant (co)variance based on 95% HPD intervals.



Supplemental figure 2: Female within-individual (top) and among-individual (bottom) phenotypic matrices. 95% HPD intervals in brackets below (co)variance. Asterisks indicate significant (co)variance based on 95% HPD intervals.

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CHAPTER FOUR:

Male competition reverses female preference for male chemical cues⁴

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Abstract

Females must choose among potential mates with different phenotypes in a variety of social contexts. Many male traits are inherent and unchanging, but others are labile to social context. Competition, for example, can cause physiological changes that reflect recent wins and losses that fluctuate throughout time. I may expect females to respond differently to males depending on the outcome of their most recent fight. In *Bolitotherus cornutus* (forked fungus beetles), males compete for access to females, but copulation requires female cooperation. In this study I use behavioral trials to determine if females use chemical cues to differentiate between males and if the outcome of recent male competition alters female preference. I measured female association time with chemical cues of two size-matched males both before and after male-male competition. Females in our study preferred to associate with future losers before males interacted, but changed their preference for realized winners following male competitive interactions. Our study provides the first evidence of change in female preference based solely on the outcome of male-male competition.

Introduction

Sexual selection operates through a dynamic interplay between intra- and intersexual selection (Hunt et al. 2009). In systems where males gain access to potential mates via intrasexual competition, females tend to be the choosier sex and often assess mate quality by assessing specific male characteristics associated with success in competition (Candolin 1999; Wong and Candolin 2005; Hunt et al. 2009; Filice and Dukas 2019). Competitive interactions can increase a male's opportunity to mate with females by excluding or outcompeting other, less dominant, males (Andersson et al. 2002). Although traits associated with competition success are usually a good indication of male quality (Zahavi 1975; Ditchkoff et al. 2001; Rantala and Kortet 2004), dominance may be accompanied by aggressive behaviors or deceptive signals that can negatively affect female fitness (Moore et al. 2003; Sih et al. 2014; Kiyose et al. 2015). These fitness effects associated with male competitive traits may consequently alter female preference (Moore et al. 2001; Sih et al. 2014). The outcomes of female choice, however, can be difficult to determine in systems where males are highly aggressive and exclude other males (Wong and Candolin 2005), nevertheless, the role of female preference in sexual selection can be determined in systems where females – either through pre or post-copulatory mechanisms – control male mating success (Firman et al. 2017; Wong & Candolin, 2005).

One way females can detect and assess quality of potential mates is through pheromones (Johansson and Jones 2007), and cuticular hydrocarbons in insects (Roux et al. 2002, Ivy et al. 2005, Thomas and Simmons 2009). Pheromones and cuticular hydrocarbons can be used as indicators of mate quality and social status, and aid in the assessment of direct or indirect benefits related to the mating process (Johansson and Jones 2007; Harari et al. 2011; Steiger and Stökl 2014; Baruffaldi and Andrade 2015). For example, male *Nauphoeta cinerea* competition status is associated differences in pheromone and cuticular hydrocarbon profile (Moore er al. 2001, Roux et al. 2002), and females use these olfactory signals to identify subordinate males, which they prefer to mate with over violent, dominant males (Moore et al. 2001; Moore et al. 2003). Chemical cues sometimes provide information independently of direct interaction when individuals deposit cues on substrates or release into air (Larsson 2003).

Signal traits assessed during mate choice are often condition-dependent. Some male traits, such as body size, remain static throughout the breeding season whereas social status and other sexual signals can fluctuate through time and depends on the outcome of recent interactions. Specifically, frequent winners are more likely to engage in and win aggressive encounters, whereas losers tend to be more submissive and avoid aggressive interactions (Hsu and Wolf 1999; Hsu et al. 2006). Experimental studies show that changes in the internal state of an organism – including changes in nutritional state and hormone levels – lead to changes in the expression of sexual signals (Reviewed in: Vitousek et al. 2014). Furthermore, both the internal state and the behavior of individuals can be affected by social context. Aggressive encounters can alter the endocrine state of individuals (Earley & Hsu, 2008); bursts of stress-induced hormones, for example, can intensify aggression, while prolonged high levels of these hormone have the opposite effect (Mikics et al. 2004; Øverli et al. 2002). Male competition thereby has the potential

to alter chemical signals (Candolin 1999; Setchell and Dixson 2001; Rhodes and Schlupp 2012), which are often dually used for competition and female choice (Johnstone 1995; Tarof et al. 2005; Martín and López 2009). The question remains, are males chosen because of their inherent and unchanging phenotype, or can female choice be influenced by temporally fluctuating social status?

In this study I examined the behavior of *Bolitotherus cornutus* (forked fungus beetles) to ask: 1) Do females perceive males through substrate born chemical cues?, 2) Do females choose to associate with the chemical cues of winning males?, and 3) Do female choose to associate with male chemical cues based on their interactivity? *Bolitotherus cornutus* are a tenebrionid beetle in which males are distinguished by the presence of elaborate horns, which they use in competition for access to females (Conner 1988; Conner 1989). Usually the larger, more aggressive males with long horns win opportunities to mate and typically choose to court bigger females (Conner 1988; Conner 1989; Formica et al. 2016). Males are observed in male-male combat both in the presence and absence of females (Conner 1988). Females appear to have little control over which males court her, yet they do control the ultimate decision of who to copulate with (Brown et al. 1985). Females have an anal sternite (a hard covering on the ventral surface of the terminal abdominal segment) that acts as a lock system giving the female control over copulation (Conner 1988), and may use chemical cues during courtship to determine if copulation should proceed. In this system, larger males have been considered to be of higher quality with more access to females (Conner 1988; Formica et al. 2016), but previous work done in controlled experiments suggests that females do not necessarily

prefer larger males (Brown et al. 1985; Brown and Bartalon 1986). These studies, however, took place in the absence of male competition. The outcome of competition may affect who females prefer to associate with.

Despite rising interest in the mechanisms underlying chemical signaling, surprisingly little is known about chemical communication in *B. cornutus*. *B. cornutus* produce a defensive chemical secretion when disturbed, and these chemical secretions are diet-based (Conner et al. 1985; Holliday et al. 2009; Tschinkel 1975), but no previous studies determine how pheromones or other chemical signals, if any, aid in communication among *B. cornutus* in competition and mating interactions. I conducted a sequence of two female choice trials to determine if B. cornutus females prefer to associate with the chemical signals of winning males. First, I performed female choice trials to test whether female beetles use substrate born chemical cues to detect and distinguish between males. Following the first female choice assays, I used the same male beetles from which I collected scent to conduct male interaction trials and determine which males were winners in male competition. Lastly, I performed a second set of female choice trials using the same male pairs to test whether females altered their preference (i.e. female choice, given that female preference is restricted to our experimental conditions) for winning or losing males.

Methods

Study species

I collected a total of 75 (50 male and 25 female) beetles from a large metapopulation near Butt Mountain, Virginia in June 2019. I housed subjects individually

in small, plastic containers (5cm X 2.5cm X 5cm) under natural light conditions (14.5:9.5 h light:dark cycle ±17 minutes) and room temperature (22 ± 3°C) at the Mountain Lake Biological Station (Salt Pond Mountain, Giles County, Virginia, USA). Containers included a small amount of mulch over a layer of plaster of Paris to help maintain a humid environment and mimic natural substrate. I added a small piece of *Ganoderma tsugae* (a polypore fungus) fruiting body as a food source and provisioned water *ad libitum*. Beetles remained in isolation in their containers for 12 days before trials began. All individuals were assigned a unique ID by adding colored dots to their elytra using non-toxic Testors[®] Enamel paint. I recorded elytra length to the nearest 0.01 mm from images taken with a flatbed scanner (Epson Perfection V600 Photo) in ImageJ (Abramoff et al. 2004, Formica et al. 2012). Age and reproductive status are impossible to determine from wild-caught *B. cornutus*, so I did not control for the age or experience of our individuals.

Video recording

I assayed behaviors in a dark, temperature-controlled room at 19 ± 2°C using infrared lights to enhance visibility in video recordings. Female choice trials were video recorded for two hours and male competition trials were recorded for four hours. I placed a Canon PowerShot G1 X digital camera approximately 1m above the arena to record behaviors by taking a photograph every five seconds for the duration of the trial using a Neewer© LCD digital shutter-release remote control. I processed these images into a five-minute time lapse video using FFpeg software (Version beld1d234). I then scored the initiation and duration of behaviors using Inqscribe[®] transcription software (version 2.2.4).

Overview of experimental design

I conducted two female choice trials to assess if preference changes following male competition. I performed female choice and male competition assays in the following order (Figure 1): first, I provided females with the chemical cues of two sizematched males prior to competition. Then, I placed those same males in a competition trial to determine the winner and loser of the pair and collected male chemical cues immediately post-trial. Finally, I performed a second set of female choice trials where females were exposed to the post-trial cues of the same male pair. This testing sequence was dictated by the goal of detecting whether females perceive differences in chemical cues resulting from male contests. Males could not be allowed to interact first and subsequently be treated as presenting an interaction-free cue. Unfortunately, this constraint of the treatment did not allow us to control for potential order effects. The details for each set of behavioral trials are specified further in the corresponding sections.

Chemical cue collection

I collected chemical signals from males by placing individuals in small petri dishes containing four equal, triangular pieces of a clean filter paper disc (50mm diameter) for a period of 24 hrs (Figure 1A). To prevent signal deterioration, pieces of filter paper remained inside petri dishes and were used within 2hrs after removing males. I modified this method from Kortet & Hedrick (2005). The colored ID was not visible in infrared video recordings; therefore, I added an additional white dot to the elytra of a randomly chosen male 24 hrs prior to male scent collection.

Pre-competition female choice assays

Female choice trials were recorded for a period of 2 hrs. I collected fresh male chemical cues before each female choice trial. I then presented females with the chemical cues of two size-matched males and two control (i.e., clean filter paper) cues and allowed her to associate freely with the cues of either male or unexposed controls. Female choice arenas consisted of a plastic box (17cm L × 15cm W, 11.14cm H) with a layer of white gridded paper. I taped filter paper cues to the grid paper equidistantly around the center of the arena using clear tape. I placed paper containing different male cues diagonally across from each other in every trial. Control cues were placed in between male cues and diagonally across from each other, treatments were placed 4.5 cm apart and at least 2cm away from the edges of the arena (Figure 1B). Female beetles were released in the center of the arena after an acclimation period of at least 4 minutes under a plastic vial. I recorded the number of times females initiated contact with each filter paper and the duration of time females were in physical contact with each filter paper. I used total time a female spent physically touching the different pieces of filter paper relative to her total time spent active as a proxy for female choice (Brown et al. 1985, Kortet and Hedrick 2005). Further, filter paper covered only approximately 8% of the area in the arena, thus,

the total time spent on chemical signals did not necessarily equal the total time of the trial. I modified this method from (Kortet and Hedrick 2005).

Male interaction trials

The purpose of our male competition trials was to induce winning and losing status in size-matched male pairs. I paired size-matched males in dyadic trials to determine winners and losers of intrasexual competition. Size-matching males within 0.05 mm allowed us to assess the effects of winning status independent of differences in male size, which is known to correlate with competition status (Chapter 1). I embedded a 5cm x 5cm square piece of Ganoderma tsugae in the center of the arena (described above) as a resource for the beetles to compete over. Trials began with each male placed on an opposite side of the G. tsugae square and ended after 4 hrs of recording. Competition duration in *B. cornutus* has been previously documented as lasting from a few seconds to about half an hour (Brown 1980, Chapter 1), so our 4-hour trial period allowed us to observe the totality of competition from onset to completion. The 4-hour period also allowed for reinforcement of winning and losing statuses due to repeated post-competition encounters during the trial time. Male competition did not isolate winning status as the only differences between males. Males may also differ in expression of behaviors. I recorded the initiation and duration of the aggressive and nonaggressive behaviors using procedures described in Chapter 1. I also recorded the number of times each male ended an interaction, defined as leaving the area before its competitor. The beetle who ended more interactions was assigned "loser" status. No beetles died during experimental trials.

Post-competition female choice trials

Following male competition trials, I conducted a second female choice assay where I presented females with the new chemical cues of the same two males to determine if her preference changed post-competition. The second female choice trials began 26 hours after completion of the male competition trials. I immediately placed males on new filter paper to collect post-competition chemical cues for 24 hours. After 24 hours of cue collection, I constructed new female choice arenas, a process that took two hours. Female choice trials then proceeded the same as the first round.

Statistical analyses

I first asked if females spent more time on filter paper than expected by chance. Filter papers occupied 8% of our total arena spaces, so our null expectation was females spend 8% of their 2hr trial time (9.8 minutes) on filter paper. I tested this assumption using a one-sample T-test comparing the duration of time females spent on any filter paper (male cue and control) to the null expectation of 9.8 minutes. Next, to test if females differentiated between male cues and control filter paper, I assessed the effects of male cue versus control cue on female choice using a generalized linear mixed model with a Poisson distribution where duration of time and counts of contact with cues were our controls in two separate models. I included the counts and durations for females in both before and after male competition trials for this model. I included filter paper treatment (male cue versus control), trial date (before or after male-male competition trials), and their interaction as fixed effects. I included female ID as a random effect.

To determine if females distinguished between males and demonstrated a preference for winning or losing males, I grouped competitors into 'future winners' and 'future losers' before interaction and 'winners' and 'losers' after interaction. I assessed the effect of male competition status (both pre- and post-competition) on the duration of time spent on each filter paper using a zero-inflated generalized linear mixed model with a Poisson distribution. I included cue type (winner, loser, or control), trial experience (precompetition and post-competition trials), and their interaction as fixed effects. I needed to standardize the proportion of time spent on each cue type because I presented females with one winner, one loser, and two control cues. To account for this difference in numbers of each type of cue, I pooled time on both control papers and divided that total by two. I included female ID as a random effect. I used pairwise comparisons of estimated marginal means to test for differences among winner, loser, and control filter papers.

Finally, I asked if females preferred cues of males based on their overall interactivity levels, specifically asking if females preferred more interactive males. I used counts of initiated behaviors with male competitors as our behavioral phenotype for each male. Number of initiated interactions is a repeatable trait (Chapter 1), and likely indicative of true differences in interactive behaviors among males. I used a zero-inflated generalized linear mixed model with identical fixed and random effects from the winner versus loser model. Here, I used the number of initiated behaviors in male competition trials, trial experience, and their interactions as fixed effects. Two females were excluded from all data analysis because they crawled under the experimental arena substrate during their trial, and one additional female was excluded because she remained inactive during the entire 2hr trial.

All statistical analyses were carried out using R v.3.6.0. I used the 'lmer' package in R for our generalized linear mixed models (Bates et al. 2015), and the 'glmmTMB' package for our zero-inflated generalized linear mixed models (Brooks et al. 2017). I assessed the significance of our models with type 3 Walds χ^2 test using the 'car' package in R (Weisberg 2019). I compared estimated marginal means using the 'emmeans' package (Lenth et al. 2020). I tested out model uniformity, zero inflation, and dispersion using the DHARMa package in R (Hartig 2016).

Results

Females spent an average of 10.35 minutes of the 2hr trial physically touching filter papers in both pre- and post-competition trials. Compared to our null assumption, females interacted with any filter paper in a proportion that was expected by chance (t = 0.29, df = 48, p = 0.77). Females did not differ in the number of touches they initiated for either control or male filter paper ($\chi^2 = 0.09$, df= 1, p = 0.77; Fig. 2A). However, females, did spend significantly more time on the filter paper of male cues compared to control cues ($\chi^2 = 14.17$, df= 1, p < 0.001; Fig. 2B). The average number of touches to any filter paper (male cue or control) was slightly greater in the post-competition trials ($\chi^2 = 3.84$, df = 1, p = 0.05) in which females performed an average of 3.92 touches in the postcompetition female choice trials compared to 1.92 touches in the pre-competition trials. Females did not differ in their duration of association time with any filter paper before (Figure 1B) or after (Figure 1E) male competition trials ($\chi^2 = 0.02$, df = 1, p = 0.88).

Females changed how much time they associated with the cues of winning and losing males after male-male competition trials (male status by trial experience interaction: χ^2 (male status X pre- vs post-competition) = 31.0513, df = 2, p < 0.001; Figure. 3). Females did not spend more time interacting with future losers compared to future winners (estimate = -0.40, SE = 0.247, p = 0.59) but did spend more time interacting with future losers compared to control papers (estimate = -0.74, SE = 0.226, p = 0.02) before male-male competition. Females did not differ in their association time with future winner cues compared to control papers (estimate = -0.37, SE = 0.267, p = 0.79). When exposed to chemical cues collected after male-male competition trials, however, females spent significantly more time on the filter paper of winning males compared to loser males (estimate = 1.51, SE = 0.243, p = <0.001). Females did not differ in their association time between loser males and control papers after male competition trials (estimate = 0.04, SE = 0.283, p = 1.00). Females did not prefer to associate with males based on how much they initiated interactions before male competition but changed their preference for more initiative males, who are more likely to win, after competition ($\chi^2_{(male})$ behavior X pre- vs post-competition) = 8.06, df = 2, p = 0.02; Figure 4).

Discussion

The outcome of intrasexual competition can alter the way males are perceived by their potential mates. Females in our study preferred chemical cues of males based on their status following competition. Females spent more time interacting with future loser chemical cues compared to future winner and control cues, but time spent the difference between future loser and winner cues was not statistically significant. Females then switched their preference to cues of realized winners after competition. The change in preference following male-male competition indicates that competitive outcomes themselves may be an important driver for female choice.

Certain male features can directly influence both the outcomes of male competition and female choice including body size, armament size, and level of interactivity. I size matched males in our study to remove the opportunity for size differences to drive female choice, but male pairs may have differed in other morphology and behaviors. First, males differed in horn length, which is strongly, but not perfectly, correlated with body size (Conner 1988). Our findings, however, suggest that females do not choose male chemical cues associated with horn length. Females chose between the same pair of males before and after competition; horn size did not change from trial to trial, but female preference did. Males also differ in their intrinsic behaviors. Winning males were frequently the more interactive males, or individuals who initiated more behaviors with their competitors. Females in our study preferred to associate with overall interactive males only following male competition, meaning females chose based on competition outcomes and not the inherent interactive phenotypes of males. Females in our study may recognize the chemical cues of aggressive and nonaggressive behavioral types and show a slight, non-significant preference to associate with the non-aggressive, subordinate male cues before male competition occurs. Chemical cues associated with male quality may be difficult to assess before competition, leading to a lack of observed female preference between future winner and loser chemical cues before competition. Females, however, preferred future losers over control cues, which I interpret as a signal for potential preference in future losing males. Then, competition may induce changes in chemical cue composition that alter the way winners and losers are perceived by females. Females may benefit from selecting males that maximizing fecundity and offspring viability (Milinski and Bakker 1990; Moore et al. 2003; Byers and Waits 2006; Kiyose et al. 2015). Behavioral dominance is often associated with high immunocompetence and production of offspring with 'good genes' (Hill 1991; Hill and Montgomerie 1994; Penn and Potts 1998; López et al. 2002).

Three possible scenarios could alter male chemical signals following competition. First, either winning or losing males may produce different chemical cues following competition. Under this scenario, females are selecting for males based on new chemical phenotypes after competition compared to those before. Competition may stimulate the production of new or enhanced chemical components in winning males that are attractive to females or less attractive, possible stress-induced, chemical components in losing males (Salvador and Costa 2009). While no previous work documents change in chemical cues following competition, a similar phenomenon in display coloration has been noted in sticklebacks (Candolin 1999). Brighter coloration of mating displays following competition is associated with winning male competitions, and winning males are preferred by females (Candolin 1999). Competition may induce a stress response in losing males that makes them less attractive to females (Leary and Baugh 2020). Chronic and acute stress responses reduce fitness and male attractiveness (Creel 2001; Breuner et al. 2008). For example, increased level of the stress hormone corticosterone in male green treefrogs during male-male competition compromises male attractiveness by relocating energy from courtship behavior to survival (Leary and Crocker-Buta 2018). To test if chemical cues are changed or enhanced following competition, I suggest analyzing individual male chemical cues before and after male competition to determine the specific pheromonal changes occurring during competition.

Alternatively, chemical cues may be transferred between males during male competition. Losing males may transfer a cue to winning males during combat, therefore explaining why winning males are preferred in post-competition trials. However, given this scenario, I would expect losing and winning males to be equally preferred by females in post-competition trials. Winning males may also apply an unattractive cue to losing males that masks their previously attractive cues. Given this explanation, I would predict females to avoid interactions with losing males. However, if winning males would apply an unattractive cue it is likely that remnants of the cue would still be perceived on themselves making them unattractive as well. Instead, our females interacted at a random frequency with losing males and preferred association with winning males after the competition (Figure 3). Countermarking occurs when individuals compete via chemical signals to ensure that their own scent masks any previous males' scent marks and may influence female preference in favor of the male who conceals their competitor's cue (Rich and Hurst 1998; Rich and Hurst 1999; Fisher et al. 2003). However, previous studies on countermarking examine urine-based chemical cues for territory determination in mammals (Rich and Hurst 1998; Rich and Hurst 1999; Fisher et al. 2003). There are no previously documented instances of countermarking or the application of unattractive chemical cues directly to opponents via bodily contact. To test if countermarking explains changes in female preference, I suggest a combination of visual and chemical choice trials. First, allow females to interact freely with two males, then pair males in competition trials and cross-transplant their chemical cues. Finally, allow females to reassess male visual and chemical cues.

Lastly, the switched preference for realized winners may be because females recognize odors of fungal resources collected by winning males. Winning males may gain more access to resources and therefore collect more fungus scent. While this scenario is possible, territoriality is not documented in *B. cornutus* and unlikely to affect female choice in wild populations (Brown 1980, Conner 1989). *B. cornutus* live, feed, and reproduce on the fruiting bodies of wood-decaying shelf fungi (Lile 1956). Multiple males are often observed in close proximity while not engaging in aggressive behaviors the same fruiting body (Brown 1980, Formica et al. 2012). All males to some degree have access to the same fungal resources on a given log making it unlikely that female choice is based on fungal odors (Conner 1988). Nevertheless, the possibility of female preference based on intensity of fungal odor should be explored in the future and can be tested by placing males in competition trials that lack fungus resource. The necessity of a fixed order in our experimental design means that I cannot completely rule out the possibility of order effects. Females could have haphazardly chosen to associate with one male in their first trials, then reversed their choice and associated with the opposite male in the post-competition trials. A switch in preference might be expected if females respond to a lack of positive feedback (i.e., courtship) from their initial choice and so direct their attention to alternative male. The potential for such order effects to alter our main interpretations should be further explored. One way the potential order effects can be explored is to conduct a parallel experiment where females choose between the chemical cues of two size-matched males at two different time points with no male competition trials. Results of female preference can then be compared between females given males with and without a competition context.

Social context extends beyond the immediate surrounding behavioral phenotypes to include the past experiences of social partners (Hsu and Wolf 1999; Oliveira et al. 2009; Vedenina and Shestakov 2018; Filice and Dukas 2019). Previous interactions can perpetuate dominant/subordinate relationships, and ultimately affect who has access to mates (Hsu and Wolf 1999; Oliveira et al. 2009; Oliveira et al. 2011). Our results indicate that chemical cues play a role in determining which males are preferred by females and suggest that preference for certain males may change based on past male experience. A male who has recently lost a competitive interaction will be assessed differently than that same male before competition, possibly resulting in opposite outcomes depending on the context. Winner-loser effects, where male competition outcome is dependent on previous competition experience, have been documented in a variety of species (Hsu and Wolf 1999; Mesterton-Gibbons et al. 2016), and these effects have been associated with hormonal changes in males (Oliveira et al. 2009). I might expect female choice to follow the direction of male chemical composition changes following competition to also be widespread across taxa.

Experienced-based chemical communication may have major implications for the use of space, information transfer, and social organization. Our experiment shows that physical substrate can carry chemical cues with important social information in the absence of the individuals producing those cues. Male chemical marks may provide a record, and reliable indicator, of competitive ability (Rich and Hurst 1999). Such chemical displays can then serve as advertisement for the attraction of potential mates as well as a challenge for sexual competitors (Johansson and Jones 2007). I show that females can differentiate between winning and losing males. If females can also differentiate between males in nature, then I might expect females to associate with preferred winning males (Kodric-brown and Nicoletto 2001). Male sexual or competitive displays may also attract or repel competing males, resulting in agonistic encounters that can affect female choice and selection for male traits (Gosling and Roberts 2001). The resulting space use and social organization within a population is then, in part, determined by the chemical phenotypes of the surrounding individuals.

Our results offer a potential explanation for why traits associated with losing males are maintained in wild populations. Traits associated with winning males should persist and increase within a population due to directional selection but directional selection on male competitive traits is not always observed in the wild (Moore and Moore 1999; Hunt et al. 2009). Differences in female preferences across social contexts provides one explanation for why selection does not always favor these winning males. Males with traits that beget a tendency to lose battles may receive access to mates via female preference when competition is absent; whereas, males with the tendency to win are preferred by mates immediately following competition. Frequency of competition is rarely constant across populations and can be spatially variable (Hunt et al. 2009). This fact means losing males may have the opportunity to attract mates in contexts where competition is low or absent. The resulting balancing selection could then lead to maintenance of traits associated with losing males.

The timing and outcome of competition is an important determinant of female choice. Male-male interactions change chemical composition in a way that reverses female preference and the timing could impact social organization and maintenance of multiple male phenotypes within a population. Future work is needed to determine if preference for chemical cues aligns with actual female mate choice in *B. cornutus*. Though controlling for order effects was not possible with this experimental design, measuring female preference both before and after male competitive interactions allowed us to demonstrate that competition can alter chemical cues in a way that changes female preference for one male over another. While our study did not determine the specific chemical changes that alter female preference, identifying the source of this information is an important next step. I also recommend additional studies that examine the potential for effects of testing order and chemical transfer between males during combat on female choice.

To our knowledge, I are the first to document change in female chemical preference for chemical cues following male competition. Previous studies assess female preference only following male competition (Rich and Hurst 1999; Moore et al. 2001; Darragh et al. 2017), or in the absence of competition (Moore and Moore 1999; Kortet and Hedrick 2005). I show that it is not only the inherent phenotypes of individuals that determines female preference, but the process of competition itself that influences the outcome of potential mating decisions. Context and past experiences will influence an individual's potential for reproductive success. Overall, I would expect the interplay between experience of male competition and female choice to allow for the persistence of multiple male phenotypes within a population.

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Figures and Tables



Figure 2: Scheme of experimental design. A) First, I collected the chemical cues of two size-matched males in petri dishes lined with filter paper. B) Second, I exposed females to the chemical cues of both males and two control filter papers simultaneously and recorded her movements and exploratory behaviors. C) Third, I placed the same male pairs in male-male competition trials to determine winners (W) and losers (L). D) Fourth, I immediately collected chemical cues from the same males following competition trials and E) fifth, I performed a second set of female choice trials with the new post-trial male cues.



Figure 2. A. Mean (+/- SE) count of initiated touches to control filter paper and male chemical cue filter paper combined from both before and after male competition trials. B. Mean (+/- SE) duration in minutes females spent on control filter paper versus male chemical cue filter paper combined from both before and after male competition trials.



Figure 3. Mean (+/- SE) minutes females spent on filter paper of future winning vs. future losing male chemical cues before male-male competition trials (left panel) and realized winning and losing males after male-male competition trials (right panel). Future winner/loser and behavior status was retroactively assigned after analysis of male-male competition videos. Drawings by Miles Bensky.



Figure 4: Mean (+/- SE) minutes females spent on filter paper of males who initiated more interactions in male-male competition trials vs. males who initiated less interactions for before male-male competition trials (left panel) and after male-male competition trials (right panel). Number of initiated behaviors for both males within each trial was retroactively assigned after analysis of male-male competition videos.

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CHAPTER FIVE:

Grumpy Old Beetles: Older males initiate more aggressive behaviors in forked fungus

beetles (*Bolitotherus cornutus*)⁵

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Abstract

Males in many species must fight for access to reproductive opportunities and the ability to perform in these competitive interactions varies among individuals with different internal states. Aggressive behaviors are energetically costly and therefore likely to correlate with age-dependent differences in internal state. In this study, I ask if aggressive behaviors in male-male competition vary across age groups in a cross-sectional study of a long-lived, iteroparous beetle species, *Bolitotherus cornutus*. I collected males of known ages from the wild and observed them in dyadic competition trials in the laboratory. Older males initiated, on average, more aggressive behaviors than younger males. These patterns may result from older males having more experience in fighting, investing more in aggression as survival probability diminishes with age, or disproportionally higher survival in aggressive males.

Introduction

Age-dependent changes in internal state can affect how individuals perform in energetically costly social situations (Williams 1966; Lemaître et al. 2015; Sih et al. 2015; Salzman et al. 2018). Life history theory suggests that current reproductive output comes at the expense of future reproductive output and survival (Pianka and Parker 1975; Stearns 1976). Individuals can invest in costly reproduction at a young age, or reserve reproductive effort for a time when the probability of surviving to the next breeding season is low (Stearns 1976). Life history theory has recently been expanded to include predictions about physiologically costly behaviors (Wolf et al. 2007). Aggression is especially likely to vary with age because it is energetically expensive and can cause physical damage that lowers future survival (Bennett and Houck 1983; Gerber et al. 2010; Guo and Dukas 2020).

Individuals of some species must fight for access to potential mates or nest and egg-laying sites (Berglund et al. 1996; Hunt et al. 2009; Rubenstein 2012; Stockley and Campbell 2013), and the ability to perform in these competitive interactions is likely to vary among different age groups as energy reserves differ (Hendry and Berg 1999; Fattorini et al. 2018; Guo and Dukas 2020). Two hypotheses highlight the potential ways internal state can affect reproductive effort and expression of social behaviors. First, the terminal investment hypothesis posits that older individuals should invest more in reproductive effort in an "all-or-nothing" manner as chances to survive to the next breeding season are low (Williams 1966; Clutton-Brock 1984; Isaac and Johnson 2005; Oro et al. 2014). In species in which winning competition results in more access to potential mates, older males should act more aggressive towards male competitors under

the terminal investment hypothesis (Trumbo 2012). Alternatively, expression of behaviors may be restrained by internal energy stores (McNamara et al. 2009; Jehan et al. 2021). Under the restraint hypothesis, younger males with more energy are predicted to initiate more aggressive behaviors in male competition (Quinn and McPhee 1998; Hendry and Berg 1999; Class and Brommer 2016).

Studies that correlate aggression with age require species that are easily observed, marked and recaptured in the wild making it difficult to assess age-related differences in aggression in invertebrate systems (but see: Trumbo 2012; Baxter and Dukas 2017). Forked fungus beetles (*Bolitotherus cornutus*), however, provide an ideal invertebrate system to test age-related differences in behavior because they are relatively long-lived, iteroparous, and easily marked and recaptured (Liles 1956; Pace 1967; Formica et al. 2012, 2021). Moreover, *B. cornutus* likely experience age-related changes in internal state because older individuals have higher mortality (unpublished data from Vince Formica and Vitor Dos Anjos).

In our cross-sectional study, I ask if age predicts aggression in male forked fungus beetles (*Bolitotherus cornutus*). *Bolitotherus cornutus* are sexually-dimorphic tenebrionid beetles that live on the fungal fruiting bodies that grow on decaying logs in forested areas throughout eastern North America (Liles 1956). *B. cornutus* are relatively long-lived (Liles 1956; Pace 1967). Beetles mate multiple times throughout their reproductive season and overwinter between seasons (Liles 1956; Pace 1967). Beetles can live up to 8 years in the lab and 5 years in the wild (unpublished data). Male *B. cornutus* frequently engage in competition over access to females where they use their two sets of horns (clypeal and thoracic) to dislodge competing males from the backs of females and

displace them from the surrounding area (Brown 1980; Conner 1988). Larger, more aggressive males are more likely to win competitive interactions and aggression in male competition is highly repeatable (Chapter 1). Variability in aggression among male *B*. *corntus* is not correlated with body size (Chapter 1), but may be attributable to agerelated differences in internal state.

Methods

Beetle collection and observation

I collected 149 male beetles from a large metapopulation of *B. cornutus* near Mountain Lake Biological Station in Pembroke, Virginia in 2019. Every summer, focal subpopulations were searched for newly emerged beetles. New beetles were collected, tagged, and released back at their subpopulation. Subpopulations were then surveyed three times a day between late May to late July. Previous studies determined that adults were unlikely to move among subpopulations during the breeding season (Ludwig 2008), but moved within a less than a 50m radius when they did (Starzomski and Bondrup-Nielsen 2002). All peripheral subpopulations within a 100m radius of our focal subpopulations were also searched three times a year to ensure all newly emerged beetles were marked. Our exhaustive surveying method had at least an 80% detection probability for all individuals within focal subpopulations (unpublished data from Vince Formica and Edmund D. Brodie III).

During 2019 field surveys, I collected all beetles, varying in age, from 5 focal subpopulations for behavioral assessment. Beetles were originally captured from 13

different subpopulations between 2015 and 2019. Beetle larvae eclose approximately one year after being laid (Wood et al. 2018), so I characterized newly captured adult beetles in a given year as one year old. In 2019, I categorized newly emerged beetles as age 1, and determined the age of any previously tagged beetles by their first collection date.

Beetles were imaged, tagged, and placed in male-male competition trials within 12-hours of collection. I measured elytra length to the nearest 0.01 mm from images taken with a flatbed scanner (Epson Perfection V600 Photo) in ImageJ (Abramoff et al. 2004). Because beetles do not change size as adults, morphological measurements were taken during each beetle's first collection year. Newly emerged, unlabeled beetles were assigned a unique ID printed on fluorescent paper and applied on each side of the elytra using a light-cured acrylic (TuffleyeTM Wet-A-Hook Technologies)(Figure 1A). Beetles were then kept in plastic zip bags until their male-male competition trials within 24 hours of collection.

Male-male competition trials

Following methods from Chapter 1, I performed dyadic, male-male competition trials with wild-caught beetles paired with lab-kept opponent beetles. I used lab-kept males as opponents to control for any potential previous experiences among wild-caught beetles from the same metapopulation. A total of 30 lab-kept opponent males were randomly paired with each focal, wild-caught male as they were collected throughout the summer. Most of the lab-kept males were collected from a nearby metapopulation on Butt Mountain in Pembroke, Virginia between 2016 and 2018 (n = 21), while nine lab-kept beetles were born in lab as the result of a 2018 breeding experiment. Lab-kept

beetles were assigned a unique ID by adding colored dots to their elytra using non-toxic Testors[®] Enamel paint. All lab-kept males were housed in isolated containers after their collection (described in: Chapter 1).

Male-male competition trials consisted of two beetles interacting freely in a 10 x 10 cm plastic container filled with approximately 2 cm of plaster and an embedding 5 x 5 cm piece of *Ganoderma tsugae* fungus (hereafter referred to as arenas). Because *B. cornutus* are most active at night, all trials were conducted between 18:30 - 00:30 at night in a dark, temperature-controlled room held at 19 ± 2 °C. I video-recorded male interactions for four hours by taking snapshot images every 5 seconds using a Canon PowerShot G1 X digital camera on infrared setting placed 1 meter above the arenas. Labkept opponent beetles were immediately returned to their housing containers after competition and given at least two days before pairing in another competition trial. To prepare for behavioral scoring, I then stitched still images from each trial into time-lapse videos using FFmpeg software (version b = 1d324). I scored the initiation and duration of the aggressive behaviors (chase, grapple, flip) using Inqscribe® transcription software (version 2.2.4)(ethogram described in Chapter 1).

Statistical analysis

To determine if age influenced number of initiated aggressive behaviors in wildcaught male beetles, I used generalized linear mixed models with age as a categorical predictor. Male beetles were categorized as either age 1, 2, or 3+ years old based on when they were first collected from the wild (age 1 originally collected in 2019, 2 in 2018, etc.). I clumped beetles aged 3-5 years into one category because of low sample size for older beetles and because I could not determine the exact age of beetles collected in 2015. The number of initiated aggressive behaviors by opponent males, collection date of opponent males, and the difference in body size between focal and opponent male were included as fixed effects. I included IDs of lab-kept opponent male and focal individual capture populations as random effects. Our model was run using the glmmTMB function in R (Brooks et al. 2017). I assessed the significance of our model with type 3 Walds χ^2 test using the 'car' package in R (Weisberg 2019) and conducted post hoc pairwise comparisons using estimated marginal means with the 'emmeans' package (Lenth et al. 2020). Finally, I tested our model uniformity, zero inflation, and dispersion using the DHARMa package in R (Hartig 2016). Our model passed the assumptions. All statistical analyses were carried out using R v.3.6.0.

Results

Age predicted the number of aggressive behaviors males initiated towards their competitors ($\chi^2 = 11.26$, df = 2, p = 0.004; Figure 1B). Newly emerged males (age 1) initiated fewer behaviors compared to two-year-old (estimate =-1.112, SE = 0.393, df = 117, p = 0.02) and 3+ year old males (estimate = -1.485, SE = 0.613, df = 118, p = 0.04). Two and 3+ year old males did not differ in the number of aggressive behaviors they initiated towards lab-kept opponents (estimate = -0.373, SE = 0.645, df = 118, p = 0.83). Difference in body size ($\chi^2 = 0.694$, df = 1, p = 0.40), opponent's number of initiated aggressive behaviors ($\chi^2 = 0.983$, df = 1, p = 0.32), and competitor's duration in lab

housing ($\chi^2 = 2.60$, df = 2, p = 0.27) did not predict the number of aggression initiated by wild-caught males.

Discussion

Older male *B. cornutus* were more aggressive than younger males in competition trials. Our results provide potential support for the terminal investment hypothesis. Older males experience higher mortality in the wild (unpublished data from Vince Formica and Vitor Dos Anjos), and may have pursued an "all-or-nothing" strategy late in life. Greater somatic energy stores in younger individuals often lead to more expression of social behaviors compared to older individuals. However, the physical damage and physiological costs associated with male competition may make aggression a less ideal strategy for younger, less experienced males (Hendry and Berg 1999; Guo and Dukas 2020). Aggression is likely a more optimal strategy for older males who have gained experience in male competition with age and have a lower probability of surviving to the next breeding season (Mauck et al. 2012; Billman et al. 2014).

Behaviors often correlate with life history traits in ways that distinguish individuals based on their reproductive strategies (Bielby et al. 2007; Biro and Stamps 2008; Réale et al. 2010). Individuals may sacrifice longevity by expressing risky resource and mate acquisition behaviors and investing in quality or quantity of gametes early in life (Wolf et al. 2007; Rubach et al. 2016). Alternatively, individuals may perform less risky behaviors for greater chance of survival (Bielby et al. 2007). Interpretations of these reproductive strategies operate under the assumption that life expectancy differs between risk-taking and careful individuals (Wolf et al. 2007). Without knowing the ages of individuals, differences in behaviors could be due to age-related reproductive strategies or differences in individual quality (Sih et al. 2015; Näslund and Johnsson 2016). Along with higher aggression, older male *B. cornutus* spend more time courting and receive more successful inseminations (unpublished data). If these behaviors respond plastically according to age, high aggression, courtship, and copulation may represent an agespecific reproductive strategy. Otherwise, higher quality may allow some individuals to initiate more aggressive and mating behaviors making those individuals more likely to survive to older ages.

Behaviors do not always change through time (Sih et al. 2004). Aggression may not increase with age but instead reflect survival differences among beetles that express different levels of aggression (Stearns 1976). The selection hypothesis posits that observed differences in distribution of phenotypes among age groups is due to directional selection filtering out phenotypes that experience higher mortality (Stearns 1976; Cam and Monnat 2000; Mauck et al. 2004). Selection may cause differential survival of consistent behaviors, termed personalities, ultimately resulting in different distributions of personalities among age groups (Yang et al. 1998; Réale and Festa-Bianchet 2003). Longitudinal data that measures aggression and mortality annually will clarify whether selection caused the observed differences in expression of aggression among age groups.

Aggression is energetically and physically costly and therefore likely to influence senescence (Hendry and Berg 1999; Sih et al. 2015; Guo and Dukas 2020). Here, I show that older males were more aggressive in a long-lived, iteroparous beetle species. Our study is one of the first to document age-related differences in aggression in an invertebrate species. Future longitudinal studies tracking changes in individual aggression with age will determine if these differences are due to age-related internal state changes or selection.

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Figures



Figure 1: A. Male *B. cornutus* labeled with a fluorescent tag. B. Number of aggressive interactions initiated by males of different ages ranging from 1 year old (n=99), 2 years old (n=41), and 3+ years old (n=9). Males in the 3+ category were either 3 years old (n=4), 4 years old (n=1), or 5 years old (n=4). Horizontal red lines indicate group means. Letters above treatments denote significant differences based on post-hoc pairwise comparisons of estimated marginal means.

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