

A Proximate Perspective on the Cooperative Behavior of a Lekking Passerine

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## ABSTRACT

Elucidating the mechanisms responsible for driving individual variation in behavior is a foundational question in organismal biology. Answering these types of questions is necessary for understanding how tradeoffs are mediated as well as potential constraints on evolutionary responses to selection. In Chapter I, I synthesize the evidence suggesting that testosterone plays a central role in driving individual variation in cooperative reproductive behaviors and mediating the tradeoff between cooperation and competition. The subsequent chapters of my dissertation then focus on understanding the mechanistic sources of individual variation in the cooperative courtship behaviors of male wire-tailed manakins (*Pipra filicauda*), a Neotropical lekking songbird. Wire-tailed manakins exhibit cooperative courtship display behaviors wherein both floater and territory-holding males perform coordinated courtship displays. Territory-holding males sire essentially all offspring and, among territory-holders, those that are more cooperative exhibit higher reproductive success. Cooperation also benefits floater males in that more cooperative floater males have a higher probability of becoming a territory-holder. In Chapter II, I detail the difficulties associated with measuring circulating testosterone in free-living animals and develop a new field technique that can improve our ability to accurately account for the effects that the stress of capture has on circulating testosterone levels in birds. In Chapter III, I quantify individual variation in the cooperative courtship behaviors of territory-holding male wire-tailed manakins using video cameras. I then use both observational and experimental approaches to show that among territory holders, high testosterone has antagonistic effects on a male's cooperative behavior. Chapter IV focuses on quantifying how an individual's cooperative behavior relates to their telomere length. Telomeres are the segments of repetitive DNA found at the end of chromosomes. Telomeres shorten in response to both physiological and environmental perturbations, are predictive of an individual's mortality risk and, because of these characteristics, are thought to reflect an individual's biological age (as opposed to chronological age). My results show that short telomeres are associated with increased cooperative behaviors and, given that a male's cooperative behavior is reflective of their reproductive investment, suggest that males with lower future reproductive potential (i.e., shorter telomeres) invest more in reproduction. My last chapter (Chapter V) focuses on synthesizing these results and suggests that future research on cooperative behaviors will need to integrate biomarkers of an individual's condition with mechanisms that reflect an individual's social competence to further understand the sources of individual variation in cooperation.

# A Proximate Perspective on the Cooperative Behavior of a Lekking Passerine

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## GENERAL AUDIENCE ABSTRACT

Cooperative reproductive behaviors occur when multiple individuals coordinate their reproductive efforts to gain an advantage over other individuals or those individuals that attempt to reproduce in the absence of cooperation. In this context, some individuals have been found to consistently act more or less cooperative, and outstanding questions regarding the evolution of cooperative reproductive behaviors focus on attempting to understand the intrinsic differences among individuals that consistently vary in their cooperative tendencies. In this dissertation, I focus on measuring individual variation in the cooperative reproductive behavior of male wire-tailed manakins, a passerine found in the Amazon Rainforest, as well as the sources of this individual variation. Male wire-tailed manakins perform acrobatic courtship displays for females. Wire-tailed manakins are especially interesting in that multiple males will perform coordinated courtship displays, but only one male within the group typically reproduces. Individual variation in this cooperative behavior also influences how likely an individual is to reproduce. Chapter I synthesizes the evidence across species to suggest that testosterone, a hormone known for influencing competitive reproductive behaviors, influences an individual's likelihood of acting in a cooperative or competitive manner. Chapter II describes the difficulties associated with measuring circulating testosterone in wild animals and describes techniques that can be used to overcome some of the difficulties associated with measuring circulating testosterone in free-living birds. Chapter III focuses on understanding the relationship between testosterone and individual differences in the cooperative reproductive behaviors of the wire-tailed manakin. I find that those males with higher testosterone or those with experimentally increased testosterone are less cooperative. I discuss my results in the context of understanding how this relationship could influence the evolution of circulating testosterone levels more broadly. Chapter IV examines the relationship between the cooperative reproductive behavior of male wire-tailed manakins and the repetitive segments of DNA found at the end of chromosomes called telomeres. Telomeres shorten as individuals age and the length of an individual's telomeres has been found to be predictive of their lifespan and mortality risk. My research shows that those individuals with shorter telomeres (or a higher mortality risk) are more cooperative. Given that an individual's cooperative behavior is predictive of their probability of successfully reproducing, my research suggests that those individuals that are more likely to perish invest more time and energy in attempting to reproduce. In Chapter V, I conclude by summarizing my results and suggesting future research that can further our understanding of the sources of consistent individual differences in cooperative behavior and the evolution of cooperation more broadly.

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## ATTRIBUTION

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**CHAPTER I.** Testosterone as a mediator of the tradeoff between cooperation and competition in the context of cooperative reproductive behaviors

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### **ABSTRACT**

Behavioral tradeoffs occur when the expression of one fitness-related behavior conflicts with the expression of another. Determining how individual variation in behaviors relates to variation in mechanisms proposed to underlie those behaviors, such as hormones, is necessary for understanding how tradeoffs are mediated as well as potential constraints on evolutionary responses to selection. Here, we describe the tradeoff between cooperation and competition faced by species that exhibit cooperative reproductive behaviors and propose that testosterone is a key hormonal mediator of the tradeoff. Cooperative reproductive behaviors occur when potential reproductive competitors coordinate their efforts to gain a reproductive advantage over other individuals or those individuals attempting to reproduce in absence of cooperation. We propose that testosterone, a sex steroid known to mediate a number of physiological and behavioral processes associated with reproduction (including competition), is involved in mediating the tradeoff between cooperation and competition. To support this proposition, we first describe the importance of individual variation in behavior to the evolution of cooperative behaviors. We then describe how proximate mechanisms represent a prominent source of individual variation in social behaviors and highlight evidence suggesting testosterone mediates variation in cooperative behaviors. Two case studies in which the relationship between testosterone and cooperative

behaviors have been investigated in detail are then summarized followed by suggestions for future studies. Throughout we highlight the importance of studying individual variation to understand the mechanistic basis of behaviors, behavioral tradeoffs, and the evolution of cooperative reproductive behaviors more broadly.

## **INTRODUCTION**

Social interactions often occur in aggressive and/or competitive contexts (Alexander 1974). Individuals frequently compete for social status or access to resources such as food or mating opportunities. Therefore, the outcomes of these interactions can have direct fitness consequences. These fitness consequences are also due to the behavioral tradeoffs that occur when the expression of one fitness-related behavior detracts from the expression of another (Stearns 1992). Measuring behavioral tradeoffs, and the mechanisms underlying them, is essential as tradeoffs and their proximate underpinnings can influence evolutionary responses to selection (Sinervo and Svensson 1998, Zera and Harshman 2001, McGlothlin and Ketterson 2008). A recognized but understudied behavioral tradeoff is between cooperative reproductive behaviors and competitive behaviors as they are often considered to be at opposite ends of a continuum and the expression of both, simultaneously, does not occur (Young et al. 2005, Shen et al. 2014, Radford et al. 2016, Ridley and Nelson-Flower, *in press*).

Cooperative reproductive behaviors occur when individuals coordinate their reproductive efforts to gain advantage over reproductive competitors, including those individuals attempting to reproduce in absence of cooperation (Díaz-Muñoz et al. 2014). Examples of cooperative reproductive behaviors among vertebrates include: 1) cooperative breeding behaviors wherein three or more individuals collectively raise young in a single reproductive attempt (Koenig and Dickinson 2016); 2) cooperative courtship behaviors wherein multiple individuals perform

coordinated courtship displays but only one individual in the group reproduces (Krakauer and Duval 2012); and 3) cooperative coalition formations wherein multiple individuals work together to aggressively fight with same-sex conspecifics for access to potential mates (Olson and Blumstein 2009).

Within species that exhibit cooperative reproductive behaviors there often exists a tradeoff between cooperation and competition. That is, individuals can either act in a manner that contributes to the success of another individual (e.g., alloparent care behaviors, affiliative behaviors that promote group cohesion, or defending mates from reproductive competitors) or they can invest in behaviors that promote their own success (e.g., status-seeking behaviors, dispersal behaviors, reproduction, or acquiring resources from themselves as opposed to offspring). For example, successful reproduction among meerkats (*Suricata suricatta*) is dependent upon contributions (e.g., pup-feeding or predator detection during group foraging events) from either related or unrelated subordinate individuals (Clutton-Brock and Manser 2016). Subordinate males can only breed by visiting other social groups and, when they engage in this roving behavior, subordinate males provide less care to the young in their own social group (Young et al. 2005). Additionally, when subordinate female meerkats attempt to breed themselves, they spend less time babysitting pups of the dominant pair and the young of subordinates subsist off resources that would otherwise be provided to the young of the dominants (Clutton-Brock et al. 2004). Individuals among either sex vary in their propensity to reproduce or cooperate and this individual variation is likely a result of differences in hormonal mechanisms (Bergmuller et al. 2010, Soares et al. 2010). Therefore, our understanding of the tradeoff between cooperation and competition is dependent upon our knowledge of potential hormonal mediators of this tradeoff.

Testosterone influences the expression of male secondary sexual characteristics and promotes the expression of various behaviors related to reproduction including courtship, aggressive, and status-seeking behaviors (Wingfield et al. 2005, Fusani 2008, Eisenegger et al. 2011). In general, males with high testosterone have a competitive advantage over those with lower testosterone (Hau 2007). However, while there are advantages to having high testosterone (e.g., greater expression of secondary sexual signals, increased rates of extra-pair young; Ketterson et al. 1992, Hau 2007), the fact that not all individuals maintain high levels of testosterone suggests there must also be some costs that balance the benefits of high testosterone. Indeed, studies have documented a number of fitness-related costs that arise including reduced immune function (Roberts et al. 2004, Foo et al. 2017), suppressed parental care (Ketterson et al. 1999, Cain and Ketterson 2013), decreased self-maintenance behaviors, as well as increased mortality rates (Marler and Moore 1991, Wingfield et al. 2001). Due to testosterone's permissive and antagonistic effects on a variety of fitness-related behaviors and traits, it is thought to mediate both physiological and behavioral tradeoffs associated with reproduction (Hau and Wingfield 2008, Ketterson et al. 2009, Mills et al. 2009).

Here, we propose that testosterone plays a central role in mediating the tradeoff between cooperation and competition in species that exhibit cooperative reproductive behaviors. This paper was structured under the premise that measuring individual variation in fitness-related traits and the mechanisms underlying those traits is key to understanding both behavioral tradeoffs and the mechanisms mediating them (Williams 2008). We first highlight the importance of individual variation for the evolution of cooperative reproductive behaviors to substantiate the idea that behavioral variation at the level of the individual is prominent among vertebrates that exhibit cooperative reproductive behaviors. We then describe the role of

proximate mechanisms in driving individual variation in social behaviors and summarize the evidence suggesting testosterone is involved in mediating and modulating both cooperative and competitive behaviors as well as the tradeoff between the two. Two case studies, in which the relationship between testosterone and cooperative behaviors have been thoroughly studied, are then described and discussed in the context of the tradeoff between competition and cooperation. Importantly, these case studies represent two different types of cooperative reproductive behaviors and thus broadly demonstrate testosterone's role in mediating this tradeoff. We conclude by describing future steps that should be taken to further our understanding of testosterone's role in mediating the tradeoff between cooperation and competition, and the importance of this tradeoff to the evolution of cooperative behaviors.

## **INDIVIDUAL VARIATION AND THE EVOLUTION OF COOPERATIVE REPRODUCTIVE BEHAVIOR**

For cooperative reproductive behaviors to evolve the benefits of cooperating must outweigh the costs (Hamilton 1964, Nowak 2006). Additionally, cooperative behaviors are more likely to evolve when individuals non-randomly sort into those that are more likely to cooperate and those that are less likely to cooperate, as this increases the chances of reciprocation and reduces the likelihood of cheaters being successful (Fletcher and Doebeli 2009). An essential assumption underlying both of these assertions is that individuals consistently vary in their cooperative behaviors, a tenet that has been underscored by theoretical models (McNamara et al. 2010, Barta 2016). As predicted, results from empirical studies have documented consistent individual differences in cooperative behavior (Bergmüller et al. 2010). For instance, alloparental Seychelles warblers (*Acrocephalus sechellensis*) consistently differ in their propensity to help at

the nest (Komdeur and Edelaar 2001). Similarly, among lions (*Panthera leo*), some individuals consistently lead the approach to simulated territorial intrusions by other male lions whereas other males in the group consistently lag behind (Heinsohn and Packer 1995). Among wire-tailed manakins (*Pipra filicauda*), males also vary in how many cooperative courtship display partners they have as well as the frequency with which they interact with courtship display partners (Dakin and Ryder 2018). Given that the neuroendocrine system is a prominent source of among-individual variation in social behaviors, components of the neuroendocrine system, such as circulating hormones, are likely to underlie this consistent individual variation in cooperative behaviors (Adkins-Regan 2005, Bergmuller et al. 2010, Soares et al. 2010).

## **THE PROXIMATE BASIS OF COOPERATIVE REPRODUCTIVE BEHAVIORS**

Consistent among-individual differences in behavior as well as within-individual behavioral plasticity are at least partly due to differences in state variables (Wolf and Weissing 2010). State variables can broadly be defined as the factors (e.g., morphological, physiological, or environmental) that determine whether or not the consequences of an individual's behavior are costly or beneficial (Houston and McNamara 1999, Wolf and Weissing 2010). For example, performing courtship behaviors may entail fitness benefits in the form of mating opportunities, but may also be costly if an individual exists in a predator-dense environment or has low energy reserves. Here, we focus on the role of an individual's intrinsic state, specifically circulating testosterone, in driving individual differences in cooperative behaviors. Circulating testosterone levels have been found to exhibit significant among-individual variation (Holtmann et al. 2017) and this hormone is thought to mediate both behavioral plasticity and consistent individual differences in behavior (Taff and Vitousek 2016, Kelly and Vitousek 2017).

Testosterone's role in modulating both competitive and aggressive behaviors that are expressed in the context of reproduction (Wingfield et al. 2005, Rosvall and Peterson 2014, Kelly and Wilson 2019) make it a potential mediator of the tradeoffs associated with cooperative reproductive behaviors. This proposition is supported by the fact that in some species that exhibit cooperative reproductive behaviors, breeding individuals exhibit significantly higher levels of testosterone than subordinate, nonbreeding individuals (e.g., Ryder et al. 2011b, Lutermann et al. 2013). This status-specific variation in testosterone levels is thought to support the intrasexual behaviors (e.g., aggression) that prevent nonbreeding individuals from breeding (Schoech 2004, Adkins-Regan 2005). Further support for the role of testosterone in mediating intra-sexual competition for breeding opportunities comes from the fact that circulating testosterone levels of subordinate individuals predict their social rise. For instance, among females in cooperatively breeding naked mole-rats (*Heterocephalus glaber*), those with higher testosterone prior to the death of the breeding female are more likely to ascend to breeder status (Faulkes and Abbott 1997). Similarly, among cooperatively displaying wire-tailed manakins, testosterone levels of subordinate males are also related to their subsequent social ascension (Ryder et al. *in press*). At the level of the individual, however, relatively less is known about the role of testosterone in mediating individual differences in cooperative reproductive behaviors as few studies have measured individual variation in both testosterone and cooperative behaviors.

## **CASE STUDIES**

### **Testosterone and the Cooperative Breeding Behavior of the Meerkat**

Long-term monitoring efforts, in combination with both observational and experimental studies, have provided a detailed understanding of the causes and consequences of individual variation in

cooperative behaviors among meerkats (reviewed in Clutton-Brock and Manser 2016). Meerkats live in stable groups ranging from 3 to 50 individuals that contain a dominant breeding male and female and usually several helpers that can be either related or unrelated to the breeders (Kutusukake and Clutton-Brock 2008). The dominant pair produce the majority of the offspring and both dominant and subordinate individuals cooperate to help raise the offspring. Dominant female meerkats attempt to actively suppress the reproduction of subordinate females by means of overt aggression, infanticide and resource competition (Clutton-Brock et al., 1998, Clutton-Brock et al. 2001). This behavioral reproductive suppression is thought to function to increase the amount of resources available to their own litter (Clutton-Brock and Manser 2016) and this overt aggression is facilitated by the morphological and physiological changes that occur when a female ascends in status (Russel et al. 2004, Kutusukake and Clutton-Brock 2006, Davies et al. 2016). Cooperative behaviors of subordinate individuals include babysitting young, allolactation, pup feeding, teaching young to forage or hunt, burrow maintenance, and group defense against conspecifics (Clutton-Brock and Manser 2016). Subordinates occasionally reproduce in a sex specific manner. Subordinate females will mate with either unrelated group members or males from another group and subordinate males will mate with females while visiting neighboring groups (Clutton-Brock and Manser 2016). Importantly, when subordinates attempt to breed it detracts from their investment in cooperative reproductive behaviors and negatively effects the reproductive success of the dominant breeders (Young et al. 2005, Clutton-Brock and Manser 2016, Cram et al. 2017).

The tradeoff between cooperation and competition is most well-studied in subordinate male meerkats. Subordinate males are physiologically capable of reproducing as levels of testosterone, luteinizing hormone, estradiol, and androstenedione do not differ between males of

different status classes (O'Riain et al., 2000; Carlson et al., 2004; Davies et al. 2016). The reproduction of subordinate males is, however, suppressed via behavioral mechanisms (i.e., mate guarding by the dominant male within a group) and subordinate males only reproduce via roving behaviors (i.e., visiting neighboring groups to mate; Young et al. 2007, Clutton-Brock and Manser 2016). Subordinate males that invest more in roving behaviors spend less time caring for the pups of their group (Young et al. 2005). Subordinate males, then, face a tradeoff between caring for their group's young (a cooperative behavior) and investing in reproducing independently of their owl social group (Young et al. 2005). Testosterone's role in mediating this tradeoff is supported by the fact that males that engage in roving behaviors exhibit higher circulating testosterone than subordinate males that did not engage in roving behavior (Young et al. 2005). Furthermore, in another study in which androgen receptors were experimentally blocked in subordinate males, treated individuals initiated fewer aggression interactions, received more aggression from group members, engaged in more prosocial behaviors, and, during play events, treated subordinate males were less frequently the dominant actor (delBarco-Trillo et al. 2016). This experimental evidence suggests that testosterone is involved in mediating aggressive interactions, within-group conflict, and social hierarchies and thus promotes aggressive behaviors at the cost of behaviors that are important for reinforcing social bonds and a stable social environment among group-living species (i.e., affiliative behaviors; delBarco-Trillo et al. 2016). Viewed cumulatively, these studies suggest that androgens promote the expression of aggressive and reproductive behaviors (i.e., visiting other groups to reproduce) in subordinate males and that testosterone has antagonistic effects on affiliative and cooperative behaviors (Davies et al. 2016, delBarco-Trillo et al. 2016).

In contrast to male meerkats, circulating hormone levels do exhibit status-specific patterns among females: dominant females exhibit significantly higher values of androstenedione, estradiol, and testosterone levels than subordinate individuals (Davies et al. 2016). These status-specific differences may contribute to the reduced cooperative behavior shown by dominant females (Clutton-Brock and Manser 2016), but have previously been proposed be associated with the increased aggressive behaviors exhibited by dominant females. The functional significance of the heightened aggression appears to center around preventing subordinates from breeding as aggression from dominant females has not been found to cause subordinates to increase their cooperative behaviors, even when faced with experimentally simulated increases in reproductive demands (Santema and Clutton-Brock 2012, Dantzer et al. 2017). Status-specific hormone levels among females are also thought to be associated with the adaptive morphological growth females undergo following dominant status acquisition (Clutton-Brock et al. 2006, Russel et al. 2004). Overall, the current evidence suggests androgens and other sex steroids are associated with the status-specific differences in behavior and morphology among females. However, essential questions to address are: (1) how does individual variation in aggressive behaviors directed at subordinates relate to differences in the cooperative behavior of dominant females and (2) does testosterone influence a female's probability of acting in an aggressive or cooperative manner? Addressing these questions and following up results with hormone manipulations will provide strong contributions to our understanding of the role testosterone plays in mediating the tradeoff between cooperative and competitive behaviors.

### **Testosterone and the Cooperative Display Behavior of Wire-tailed Manakins**

The Neotropical manakins are a family of passerine birds that are known for their acrobatic courtship displays that males perform at leks (Kirwan and Green 2011). In some species of manakins, males form cooperative display coalitions whereby multiple males will perform coordinated courtship displays for females but only one male within the coalition will mate (Díaz-Muñoz et al. 2014). In these cooperative species, males exhibit two social classes that differ in their reproductive opportunities: males that lack a territory rarely sire offspring (i.e., floater males), while those males that maintain a territory in the forest to perform courtship displays alone or with visiting males sire the vast majority of offspring (i.e., territory-holding males; McDonald and Potts 1994, Duval 2007, Ryder et al 2009). Although cooperative display behaviors have evolved under kin-selection in another species of bird (Krakauer 2005), studies on multiple species of manakins show that coalition partners are not more closely related than expected by chance (McDonald 2007, Duval 2007, Ryder et al. 2011a). The benefits of cooperation in manakins appear to be related to social ascension and reproductive success: floater males that are more cooperative have a higher probability of gaining a territory (McDonald and Potts 1994, Duval 2007, Ryder et al. 2008) and, among territory-holders, those that are more cooperative exhibit greater reproductive success (Ryder et al. 2009).

Multiple studies on manakins have described the role of testosterone in activating and coordinating the expression of courtship behaviors and, in some cases, studies on cooperatively displaying species have also compared circulating testosterone levels between floater and territory-holding males. Patterns of circulating testosterone are largely what is predicted from studies of higher latitude species despite this group being completely tropical (Moore et al. 2019). Specifically, testosterone levels of male golden-collared manakins (*Manacus vitellinus*) are elevated during the breeding season (i.e., when courtship behaviors are predominantly

expressed) and basal during the times of the year when males do not perform courtship displays (Day et al. 2007, Schlinger et al. 2013). These males also exhibit high densities of androgen receptors in both neural tissues and skeletal muscles and androgen receptors are thought to be essential for coordinating the expression of the acrobatic courtship displays males perform (reviewed in Schlinger et al. 2013). In those species that exhibit cooperative courtship display behaviors, social status has also been found to be related to testosterone levels. Specifically, in both lance-tailed manakins (*Chiroxiphia lanceolata*) and wire-tailed manakins, territory-holding males exhibit significantly higher testosterone levels than floater males (Duval and Goymann 2011, Ryder et al. 2011b). These results suggest that elevated testosterone could be associated with obtaining and maintaining an individual's social status.

Beyond differences in testosterone with social status, studies on the cooperatively displaying wire-tailed manakins have detailed the relationship between individual variation in testosterone and cooperative courtship display behaviors. Male wire-tailed manakins have been found to exhibit significant among-individual variation in cooperative behaviors (Dakin and Ryder, 2018) and circulating testosterone levels (Ryder et al. *in press*). Moreover, Ryder et al. (*in press*) found that the relationship between an individual's social behavior and their circulating testosterone levels is status specific. Among floater males, those with higher testosterone levels spent more time within another male's territory and more frequently interacted with other males (Ryder et al., *in press*). That is, floater males exhibit a positive relationship between testosterone and cooperative behavior. However, territory-holders with higher endogenous levels of testosterone or those with experimentally increased testosterone levels, interacted with other males less frequently and tended to have fewer courtship display partners (Ryder et al., *in press*). Additional work showed that territory-holders with high testosterone or those with

experimentally increased testosterone perform relatively fewer cooperative courtship displays (Vernasco et al., in review). That is, territory-holders showed a negative relationship between testosterone and cooperative behavior in both observation and experimental studies. It is worth noting that the relationship between testosterone and cooperative behaviors is not an overall effect of the hormone on courtship behavior. That is, testosterone and the expression of courtship display behaviors are not related in a dose-dependent manner. However, testosterone may indirectly mediate a male's courtship behavior by modulating the frequency that males perform cooperative displays (Vernasco et al., in review).

Overall, studies on the wire-tailed manakin demonstrate that high testosterone promotes status-seeking behaviors among floater males and, among territory-holders who exhibit higher endogenous testosterone levels than floaters, high testosterone has antagonistic effects on cooperative behaviors. Therefore, the above studies suggest testosterone promotes competitive behaviors at the cost of cooperative behaviors among territory-holders as those exhibiting high testosterone levels and those with experimentally increased testosterone levels less frequently interacted with other males and performed fewer cooperative courtship displays (Ryder et al. *in press*; Vernasco et al., *in review*). In floater males, however, testosterone may be beneficial in that it promotes the expression of the behaviors important for social ascension (Ryder et al. 2008).

## **FUTURE DIRECTIONS**

Hormonally mediated tradeoffs have been the focus of much recent attention and these tradeoffs are a result of the pleiotropic nature of hormones (Ketterson et al. 1992). Our understanding of hormonal pleiotropy is partly dependent on our ability to explain how circulating hormones

relate to individual variation in the behaviors involved in a tradeoff (Williams 2008). With the exception of the case studies highlighted above, relatively few studies have focused on quantifying relationships between individual variation in both circulating testosterone and cooperative behaviors. The majority of studies have instead focused on measuring status-specific patterns of hormones and a few studies on species that exhibit cooperative reproductive behaviors have also measured behavioral responses to experimental hormone manipulations (Schoech 2004). Given that cooperative behaviors exhibit significant among-individual variation (Bergmüller et al. 2010), broadening our understanding of the sources of this variation and how cooperative behaviors relate to competitive behaviors will require examining patterns of variation at the individual level as well as experimental hormone manipulations (Williams 2008).

Among the diversity of behaviors included under the umbrella term “cooperative reproductive behaviors”, reproductive competition is more prominent in some species and less prominent in others. The factors that can influence the potential for reproductive competition include: relatedness between cooperating individuals (West et al. 2002); how energetically costly reproduction is (Creel and Creel 1991); the amount of size dimorphism between dominant and subordinate individuals (Hamilton et al. 2005, Clutton-Brock and Manser 2016); and environmental factors such as resource availability or climatic conditions (Bateman et al. 2013, Rauber et al. 2019). In the absence of reproductive competition among cooperating individuals, testosterone is predicted to play a limited role in mediating cooperative behaviors (Adkins-Regan 2005). Furthermore, testosterone has also been proposed to play a limited role in mediating cooperative behaviors when individuals exhibit cooperative reproductive behaviors prior to puberty (reviewed in Schradin et al. 2018). In those cases, however, testosterone may play a role in mediating the cooperative behavior of adults as well as influencing the extent to which adults

tolerate other individuals in their social group. Environmental factors such as habitat quality or resource availability can influence within-individual variation in cooperative behavior as well as the social dynamics of groups (Bateman et al. 2013, Shen et al. 2014). More broadly, environmental factors can also determine whether or not tradeoffs arise (Stearns 1992). Circulating hormones function as integrators of environmental factors, therefore the hormone-behavior relationship underlying cooperative reproductive behaviors likely depend on factors related to both the physical and social environment (Hau and Wingfield 2008). Future studies examining the tradeoff between cooperation and competition should therefore consider how the factors described above influence the potential for reproductive competition as well as how environmental factors may influence the prominence of this tradeoff.

In addition to testosterone, multiple other neuroendocrine mechanisms are associated with the expression of cooperative behaviors (Soares et al. 2010). For instance, while the studies on meerkats do suggest testosterone is involved in mediating tradeoffs that include cooperative, aggressive, or reproductive behaviors, additional studies on meerkats have also identified the roles of glucocorticoids, prolactin, and oxytocin in mediating cooperative behaviors (Carlson et al. 2006, Madden et al. 2011, Dantzer et al. 2017). Future studies should therefore prioritize measuring multiple hormones as opposed to circulating levels of a single hormone. Moreover, it is widely acknowledged among behavioral endocrinologists that studies should also prioritize measuring additional components (e.g., receptor densities) or properties (e.g., responsiveness to GnRH challenges) of hormonal cascades (Ball and Balthazart 2008, Hau and Goymann 2015). The responsiveness of hormonal cascades to upstream releasing hormones or negative feedback signals, for instance, is hypothesized to be related to an individual's ability to adjust to environmental change (Taff and Vitousek 2016). In the context of cooperative behaviors, the

responsiveness of the hypothalamic-pituitary-gonad axis to experimental stimulation via upstream hormones (i.e., releasing hormones sent from the hypothalamus to the anterior pituitary, Nelson 2011) may explain with-individual variation in cooperative behavior or reflect an individual's social competence (Taborsky and Oliveria 2012). Importantly, previous work has linked the expression of competitive behaviors with the responsiveness of the hypothalamic-pituitary-gonad axis to upstream hormones that stimulate the production of gonadal steroids (e.g., McGlothlin et al. 2007). Studies should also prioritize focusing on endocrine traits that exhibit high repeatability, as among-individual variation in behavioral traits are thought to have co-evolved with variation in state variables that exhibit significant repeatability (Holtmann et al. 2017, Niemelä and Dingemanse 2018).

The influence of hormones on evolutionary responses to selection depend upon whether or not they promote behaviors that are costly or beneficial (Sinervo and Svensson 1998, Zera and Harshman 2001, McGlothlin and Ketterson 2008). In the context of this review, the costs or benefits of engaging in cooperative or aggressive behaviors depend upon the time scale they are being analyzed. For instance, while these costs or benefits may be apparent and/or measurable over short time scales, one study suggests that the costs of immediate cooperating incurred by subordinates do not persist later in life (Russel et al. 2003). Therefore, to understand the how hormones may influence evolutionary responses to selection, future works need to prioritize measuring long term costs or benefits of individual variation in cooperative behaviors and consider that these fitness-outcomes may only be significant for one particular status class (e.g., the breeding social class). Studies that have detailed pedigrees for populations of free-living animals will also broaden our understanding of the heritability of both cooperative behaviors and the proximate mechanisms underlying cooperative behaviors using quantitative genetics

(McGlothlin and Ketterson 2008, Williams 2008). In sum, advancing our understanding of the long-term costs of cooperative or competitive behaviors and their proximate underpinnings will require biologists to track the reproductive success of individuals over the course of their entire life.

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## **CHAPTER II. Sampling baseline androgens in free-living passerines: methodological considerations and solutions.**

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### **ABSTRACT**

Obtaining baseline hormone samples can be challenging because circulating hormone levels often change rapidly due to the acute stress of capture. Although field protocols are established for accurately sampling baseline glucocorticoid concentrations, fewer studies have examined how common sampling techniques affect androgens levels. Indeed, many studies focused on understanding the functional significance of baseline androgen levels use sampling methods known to activate the endocrine responses to stress. To understand how different field sampling protocols affect plasma androgen levels, we measured the androgen response to two types of capture stressors in a free-living tropical bird, the wire-tailed manakin (*Pipra filicauda*). First, we subjected males to a standardized capture and restraint protocol lasting either 15 or 30 minutes. Second, males were passively captured in nets that were filmed (to establish exact duration of time between capture and blood sampling) and checked every 30 minutes. The first study showed that circulating plasma androgen levels decreased significantly following both 15 and 30 minutes of restraint in a cloth bag with a trend for the 30min samples to be lower than the 15min samples. Further, the change in androgen levels was dependent on an individual's initial

androgen levels with the individuals with the highest initial levels registering the largest declines. The results of the second study suggest that hanging in a mist net for extended periods of time also leads to a decrease in circulating androgen levels, but this effect was weaker than that of capture and restraint in a cloth bag. Our findings demonstrate that, overall, circulating androgen levels decrease in response to common sampling techniques, a finding that has important implications for studies measuring baseline androgen levels in free-living birds. Future studies should prioritize sampling individuals immediately upon removal from the mist net, as handling and restraint have a strong negative effect on circulating androgen levels. When constant monitoring of the mist net is not possible, investigators should use video cameras to record the amount of time an individual spends in the net prior to blood sampling and then statistically control for the effect of this variable in analyses.

## **INTRODUCTION**

The ability to accurately quantify individual baseline levels of circulating hormones in vertebrate field studies is essential to understanding how the endocrine system mediates phenotypic differences and, ultimately, the fitness trade-offs that constrain life history strategies (Ketterson and Nolan, 1999; Williams, 2008; Zera and Harshman, 2001). Because the endocrine system is highly responsive to environmental cues, a number of extrinsic factors, including perturbation, can induce transient changes in circulating hormone levels (McEwen and Wingfield, 2003; Silverin, 1998; Wingfield et al., 1998). Although these acute changes in hormone levels facilitate temporary adjustments in physiology and behavior to cope with environmental challenges (Sapolsky, 2000; Wingfield et al., 1985), they can also introduce noise into datasets that may obscure meaningful variation in baseline hormone levels (Small et al.,

2017). The ability to accurately quantify individual variation in baseline hormone levels therefore requires a working knowledge of how perturbation, including routine capture and sampling events, affects circulating levels of the hormone of interest. Indeed, accurately quantifying baseline hormone levels in free-living vertebrates is challenging because the acute stress of capture and handling is known to cause rapid changes in plasma hormone levels (e.g. Deviche et al., 2010; Krause et al., 2014; Romero and Romero 2002). Further consideration of how routine capture and sampling methods can alter circulating levels of various hormones across a range of species will: 1) increase our ability to recognize and account for sources of variation in measures of baseline hormone levels, 2) inform useful modifications to field sampling protocols, and 3) broaden our understanding of adaptive variation in vertebrate stress responses.

Avian field studies have been integral in advancing our knowledge of how the vertebrate endocrine system responds to perturbation (Wingfield et al., 1998; Wingfield 2012). Studies of endocrine stress responses in birds typically employ a capture and restraint protocol in which individuals are blood sampled immediately after capture to obtain a baseline hormone sample, held in a cloth bag, and resampled at designated time points to measure stress-induced changes in hormone levels (Wingfield et al., 1992). Most of these studies have focused on the responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis, and in nearly all cases, capture and restraint induces a robust increase in plasma corticosterone concentrations (Breuner 2008; Cockrem and Silverin 2002; McEwen and Wingfield 2003; Silverin 1998; Wingfield et al., 1992). However, mounting evidence suggests that capture stress can also alter the circulating levels of multiple other hormones including testosterone, dihydrotestosterone, progesterone, and prolactin (Angelier et al., 2007; Chastel et al., 2005; Deviche et al., 2010; Gratto-Trevor et al.,

1991; Krause et al., 2014; Verreault et al., 2008; Wingfield et al., 1982). Given the rather pervasive effect of capture and restraint stress on circulating hormone levels, it is necessary to evaluate sampling approaches to ensure that field efforts effectively measure baseline (i.e., pre-capture) hormone levels, and whenever possible to account for variation introduced by capture stress. For example, studies of the adrenocortical response in birds frequently operate under the assumption that a sample acquired within 3min of capture effectively measures pre-capture corticosterone levels (Romero and Reed 2005). Yet, a recent study on Florida scrub jays (*Aphelocoma coerulescens*) found that plasma corticosterone levels may increase in as little as 2min after capture (Small et al., 2017), a result similar to a previous study on European starlings (*Sturnus vulgaris*, Dawson and Hower 1983). Other studies have revealed that the timing and magnitude of capture-induced changes in corticosterone levels can vary among individuals according to capture method (e.g., using a potter trap versus a mist net), sampling regime (e.g., how long a bird is left in a trap or net before blood sampling), and prior experience with capture and handling (Angelier et al., 2010; Carroll et al., 2016; Romero and Romero 2002). These studies of the corticosterone response to capture stress underscore the need for further scrutiny of how capture and sampling efforts affect circulating levels of other hormones.

Numerous studies on free-living male birds have demonstrated that capture and restraint can cause circulating levels of androgens to decrease (Chiver et al., 2014; Davies et al., 2016; Deviche et al., 2012, 2014, 2016, 2017; Gratto-Trevor et al., 1991; Li et al., 2012, 2017; Moore et al., 2002; Silverin, 1998; Wingfield et al., 1982). Although an increase in androgen levels following capture and restraint was reported in one field study by Grotto-Trevor et al. (1991), this finding was based on very small effect sizes (androgen increases of 8-93 pg/ml in n = 3 males) and provides limited evidence that the directionality of the androgen response to stress in

free-living male birds is variable. Whether and how strongly male androgen levels respond to capture stress, however, has been found to be context dependent. More specifically, capture and restraint was shown to have no effect on androgen levels in non-breeding male birds (i.e., when the hypothalamic-pituitary-gonadal (HPG) axis is inactive, Deviche et al., 2016) and, during the breeding season, male birds with higher initial androgen levels exhibited a greater decrease in androgen levels in response to capture and restraint than those with lower initial levels (Deviche et al., 2012, 2014). Further study is ultimately needed to identify the intrinsic and extrinsic factors that influence variation in the responsiveness of the avian HPG axis to perturbation.

For decades, studies of androgens in free-living birds have regarded samples collected within 10min of capture as representative of baseline androgen levels, an approach often based on the findings of Wingfield and Farner (1976). Given that only a few additional studies have measured the effect of capture stress on androgens in birds, it is possible that the 10-minute rule is inappropriate for some species. For example, significant changes in androgen levels occurred within 10min of capture in rufous-winged sparrows (*Peucaea carpalis*, Deviche et al., 2012). More time-stringent sampling regimes may indeed reduce artificial variation in baseline hormone data. Furthermore, no study has yet evaluated whether different capture methods and sampling regimes have variable effects on circulating androgens. Finally, additional studies from species inhabiting diverse environments and with variable life history strategies are needed to broaden our understanding of how selection pressures have shaped responsiveness of the avian HPG axis to perturbation.

In this study, we examined changes in circulating androgen levels in response to acute stress in free-living wire-tailed manakins (*Pipra filicauda*). First, we measured the effect of a standardized capture and restraint protocol on circulating androgen levels in male manakins.

Then, to determine the influence of a common capture technique (i.e., capture in a mist net) on androgen levels, we passively captured males and examined whether the amount of time that they were in a mist net before blood sampling (hereafter ‘net time’) influenced individual variation in plasma androgen levels. To quantify net time, we filmed mist nets with GoPro video cameras and calculated the difference between the exact capture time and sampling time from videos of each sampling event. This novel approach is an effective method for accurately documenting capture events and is broadly applicable to other hormone studies where passive capture without constant net monitoring is necessary. Net time can subsequently be used in an attempt to statistically control for variation in hormone data induced by differences in net time or be used as a justification for excluding individuals with long capture times that are more likely to exhibit stress-induced (as opposed to baseline) androgen levels from statistical analyses.

## **MATERIALS AND METHODS**

### *Study Species, Location, and Field Methodology*

We conducted our research on wire-tailed manakins (*Pipra filicauda*) at the Tiputini Biodiversity Station, Orellana province, Eastern Ecuador (0° 83’ S, 76°08’ W, 190-270 m elevation). The wire-tailed manakin is a lekking, Neotropical sub-oscine bird that is found in lowland tropical rainforests throughout the northwestern Amazon Basin (Schwartz and Snow 1978). In this study, males were passively captured using mist nets that were set up in territories the day before the experiment and opened before dawn. All individuals were banded with both a numbered aluminum leg band and a unique combination of two or three colored leg bands. Birds were sampled during the population’s peak breeding period (November to March) over the course of three field seasons (2014-2015, 2015-2016, 2016-2017). Males wire-tailed manakins exhibit delayed plumage maturation and do not obtain their definitive plumage until their third

year of life (Ryder et al. 2008). Additionally, males do not obtain territories until they molt into their definitive plumage, although the age of obtaining a territory varies from 3 to 7 years (Ryder et al. unpublished data). Using both plumage characteristics and behavioral observations (following Ryder et al. 2008), we grouped males into four status classes (territory-holder, definitive floater, pre-definitive floater, and formative floater) that have previously been found to differ in age, behavior, plasma androgen levels, and, among floaters, plumage characteristics (Ryder et al., 2008, 2011a, 2011b).

#### *Measuring the Androgen Response to a Capture and Restraint Protocol*

During the 2014-2015 field season, we measured the androgen response to capture and restraint following the protocol described in Wingfield et al. (1995). First, nets were watched from ~8-10m away and, upon capture, a blood sample of approximately 75uL was collected within 3min of capture using heparinized capillary tubes following brachial venipuncture with a 27-gauge needle. Birds were then placed in a cloth bag and randomly selected to be restrained in the bag for either 15min or 30min. Following restraint, a second blood sample of equal volume was collected. Capillary tubes were capped with Critoseal® and stored in a cooler with an ice pack (2-3hrs) until centrifuged for 5min, after which the plasma was removed and its volume measured to the nearest 0.25ul using a Hamilton syringe. Plasma was then stored in ~100% EtOH following Goymann et al. (2007b).

#### *Measuring the Androgen Response to Capture in a Mist Net*

During the 2015-16 and 2016-17 field seasons, we assessed how the time a captured bird spent suspended in a mist net influenced circulating androgens by sampling birds that spent variable amounts of time in a mist net before the blood sample was collected. First, 10 to 15 mist nets were set up within a lek, and on each net a GoPro HERO4 Silver camera was attached to the

base of one of the supporting poles using the GoPro Jaws: Flex Clamp. The wide-angle setting of the GoPro camera allows for the entirety of the net to be seen in the field of view so that exact capture times could be determined from video footage. After opening the net and turning on the cameras, we stated the time into the camera and left the area. Nets were subsequently checked every 30 mins. All birds captured were sampled within 3 mins of extraction from the net, and the exact time at which blood samples were acquired was stated into the camera. We determined the capture time for each bird by adding the amount of recording time elapsed between the time the net was opened and the time the bird was captured. We then quantified “net time” by calculating the difference between the time an individual was captured and the time the blood sample was acquired. The majority of individuals ( $n = 320$ ) were sampled between 06:00 and 10:00 hours, but some ( $n = 34$ ) were sampled between 11:00 and 16:00 hours. Sample storage in the field and processing followed the procedures described in Section 2.2.

### *Hormone Assay*

After each field season, we quantified total plasma androgen levels following double extraction with dichloromethane (average extraction efficiency for 2014-2015, 2015-2016, and 2016-2017: 68%, 63%, and 72% respectively) using a direct radioimmunoassay (following Moore et al. 2002, Wingfield et al., 1991). The average plasma volume was  $32.97 \pm 0.44$  (mean  $\pm$  SEM) and we ran samples in singlets to increase the detection probability. Androgen concentrations were adjusted for individual extraction efficiency. For each assay, samples from the same individual were grouped together, but the order of individuals was random. We calculated the intra-assay coefficient of variation among standards within an assay and the inter-assay coefficient of variation among these standards across the three assays. The intra-assay coefficient of variation was 13.8%, 6.6%, 11.6% for each field season (2014-2015, 2015-2016, 2016-2017,

respectively), and the inter-assay coefficient of variation was 8.9%. The average detection limit for the assays was ~0.22 ng/mL, and all measured samples that fell below the assay's detection limit were assigned the detection limit for that assay.

### *Statistical Analyses*

For the capture and restraint experiment, androgen data were analyzed using a linear mixed model with sample type (baseline, 15min, or 30min) as a categorical fixed effect and individual as a random effect using the *lme4* package in the program R (R Core Team 2015). (Bates et al., 2015). Tukey's post-hoc comparisons were used to determine if average androgen levels significantly differed between each group (i.e., baseline, 15 min, or 30min). All androgen levels were log-transformed to meet model assumptions prior to statistical analyses.

To analyze how net time affected androgen levels, we built linear mixed models with both additive and interactive effects of the time of day and net time. Time of day and net time were modeled as a fixed effect and, to account for repeated measures across two different field seasons, individual and year (i.e., 2015-2016 or 2016-2017) were modeled as random effects (individual with 2 captures  $n = 45$ , 3 captures  $n = 32$ , 4 captures  $n = 18$ , 5 captures  $n = 2$ ). All models, including the null model, contained male status class as a fixed predictor variable, as previous work has documented a significant effect of a male's social status on androgen levels (Ryder et al., 2011b). Candidate model sets were symmetrical with respect to all predictor variables (Doherty et al., 2012). We ranked models using Akaike's Information Criterion (corrected for small sample size, AICc) and determined the relative likelihood of each model using model weights ( $w_i$ ; Burnham & Anderson 2002). Model effects are presented as standardized  $\beta$  parameter estimates and 95% confidence intervals. As above, all androgen levels

were log-transformed prior to statistical analyses and fitted values were back-transformed prior to graphing in Program R (R Core Team 2015).

## RESULTS

### *Capture and Restraint Experiment*

We collected baseline and stress-induced samples from 24 males, of which 13 were restrained for 30min and 11 were restrained for 15min. Stress-induced androgen levels following restraint were significantly lower than baseline levels at both the 15min and 30min time points ( $\beta_{15} = -0.472$ ,  $t_{25} = -2.332$ ,  $p = 0.028$ ;  $\beta_{30} = -1.054$ ,  $t_{25} = -5.623$ ,  $p < 0.0001$ ). Additionally, there was a near-significant decrease in androgen levels between the 15min and 30min groups ( $\beta_{PM15-PM30} = -0.5817$ ,  $z = -2.194$ ,  $p = 0.069$ , Figure 1). To further examine whether the magnitude of stress-induced changes in androgen levels depended on initial androgen levels or the duration of restraint, we built a linear model with the change in androgen levels as the response variable and an interaction term between initial androgen levels and restraint time as predictors. The magnitude of the change in androgen levels was positively associated with initial androgen levels ( $\beta_{initial\ androgens} = -0.50$ , 95% CIs = -0.27, -0.72,  $p < 0.001$ ), but this effect was found to be dependent on the duration of restraint ( $\beta_{initial\ androgens*duration} = -0.39$ , 95% CIs = -0.13, -0.64,  $p < 0.01$ ; Figure 2).

### *The Androgen Response to Capture in a Mist Net*

We measured the net time and obtained blood samples for 354 individuals (mean net time  $\pm$  SEM = 17.4min  $\pm$  0.93, range: 1 to 72 min). The top statistical model explaining variation in androgen levels included the factors status, year, and net time ( $w_i = 0.75$ , Table 1). This model suggests that net time negatively influences circulating androgen levels (Figure 3,  $\beta_{tElap} = -0.02$ ,

95% CIs = -0.039, -0.01) and that territory-holding males had higher testosterone levels than the three floater classes ( $\beta_{formative\ floater} = -1.46$ , 95% CIs = -1.81, -1.11;  $\beta_{pre-definitive\ floater} = -1.16$ , 95% CIs = -1.48, -0.84;  $\beta_{definitive\ floater} = -0.27$ , 95% CIs = -0.56, 0.03). Our results also suggest that the androgen response to capture does not depend on an individual's status, as the model with the interaction between status and net time was the lowest ranked model. Furthermore, we found no support for a time of day effect on androgen levels (Table 1). The effect of net time on androgen levels appeared to be driven by a relatively small number of samples ( $n = 7$ ) collected  $> 40$ min after capture. Although androgen levels do decline linearly with net time, the effect is not supported after those samples from captures with long net times are removed from the analysis (Table 2).

## DISCUSSION

In this study, we measured the androgen response to two different acute stressors, a standardized capture and restraint protocol and capture in a mist net. Male wire-tailed manakins exhibited significant decreases in plasma androgen levels following capture and restraint (Figure 1). Androgen levels also responded negatively, but more slowly, to capture and prolonged entanglement in a mist net (Table 2, Figure 3). Overall, our results suggest that the effect of acute stress on androgen levels depends on the type and duration of the stressor. Moreover, these findings highlight how common methods used in avian field sampling protocols, including periodically checking nets and holding birds in bags, can themselves cause changes in circulating androgen levels and, ultimately, limit our ability to understand the functional significance of natural variation in baseline androgen levels.

For future studies to obtain accurate measures of baseline androgen levels, it is imperative that sampling protocols are designed to minimize and, when necessary, control for the influence of capture and restraint as well as net time on circulating hormone levels. Many avian field studies focused on measuring androgens use passive netting to capture birds, whereby nets are left unmonitored for extended periods of time. Often, such sampling regimes prioritize obtaining a blood sample within 10min of extracting the bird from the net, but do not measure (or at least account for) the amount of time the bird spent in the net before being extracted and sampled (e.g., Day et al., 2007; Duval et al., 2011; Peters et al., 2001). When using such methods, sampling-induced variation in androgen levels may obscure meaningful relationships between phenotypic traits of interest and baseline hormone levels. For example, in a study by McGlothlin et al. (2007) on breeding dark-eyed juncos (*Junco hyemalis*), males were captured, placed in a cloth bag, and transported to another location before blood sampling. The authors reported a negative effect of handling and restraint time (range: 14 to 217 min) on circulating testosterone levels and also found no relationships between initial testosterone levels and male reproductive behaviors (parental care and aggression; McGlothlin et al., 2007), even though these behaviors have previously been described as being regulated by testosterone in this species (Ketterson et al., 1992; Schoech et al., 1998).

Future endocrine studies interested in measuring baseline androgen levels should use methods designed to avoid or control for potential sampling-induced changes in androgens. Foremost, we recommend taking blood samples immediately after removing an animal from a net or trap, as many studies on free-living birds, including ours, have shown that handling and restraint cause androgens to decrease (Chiver et al., 2014; Davies et al., 2016; Deviche et al., 2012, 2014, 2016, 2017; Li et al., 2012, 2017). For studies using passive, unmonitored netting

protocols to capture birds, we recommend quantifying the amount of time between capture and blood sampling. The amount of time that an individual spends in the net or trap can then be included as a covariate in statistical models to help control for capture-induced variation in androgen levels. The methodology presented in this paper (i.e., filming the nets) provides a novel and simple way to measure the time between capture and sampling, especially when passive, unmonitored netting is preferred, as in cases where human presence or frequently checking nets to modify behaviors of interest (e.g., social interactions) and/or decreases capture rates. Moreover, using video cameras to quantify capture times presents an opportunity to increase daily capture rates by allowing for more nets or traps to be open simultaneously. This technique could also be applied to other trapping techniques in addition to mist nets, and to non-avian studies.

Although our results suggest that, for male wire-tailed manakins, being tangled in a mist net has a reduced effect on androgen levels compared to that of handling and restraint, and that this effect is most evident in birds with long net times, there are some important caveats to consider. Previous research has documented among-individual plasticity in endocrine responses to acute stressors (Cockrem and Silverin 2002; Lendvai et al., 2014; Small et al., 2017), suggesting the rate and magnitude of a change in endocrine levels in response to perturbations varies among individuals. Indeed, the magnitude of a change in androgen levels following capture and restraint has been shown to depend upon an individual's initial androgen levels (Deviche et al., 2012). That is, individuals with higher initial androgen levels exhibit greater decreases in circulating levels than do individuals with lower levels (Deviche et al., 2012). In our study of how net time affects androgen levels, it was impossible to assess whether the observed effect of net time on androgen levels depended on initial hormone concentrations. However, in

our capture and restraint experiment, the magnitude of the decline in androgen levels did depend on initial hormone levels (Figure 2). It is therefore likely that the effect of net time on androgen levels consistently varies among individuals in manakins and in other species. Thus, although including net times (e.g., as acquired from video) as a covariate in statistical analyses can help control broadly for capture-induced variation in androgen levels, it cannot control for individual variation in the androgen response to capture stress. As such, watching nets and sampling birds immediately after capture is the most effective approach to reduce capture-induced variation in baseline androgen levels. Passive netting supplemented with video to record capture times provides an alternative only when the former method is not a tractable option. Additional capture and restraint studies at shorter time intervals (i.e., 5 and 10 minutes) would be useful to further our understanding of the sensitivity of androgen levels to acute stress and fine-tune methodologies for sampling baseline androgens.

Endocrine responses to acute stress are known to vary within and across species according to life history stage and life history strategy (Ricklefs and Wilkelski 2002; Wingfield et al., 1998). For example, individuals that invest more heavily in current reproduction at the expense of survival and future reproductive opportunities have been found to exhibit reduced glucocorticoid responses to capture stress in some cases (Wingfield et al., 1995; Bókony et al., 2009). It is therefore plausible that androgen responses to stress may vary across and within species according to selection pressures and life history constraints. On the one hand, our study focused on a species that has a relatively low value of current reproduction (i.e., it is long-lived, has a long breeding season, and has low reproductive investment per attempt) and, as predicted, these male manakins exhibited a robust decrease in androgen levels in response to one type of stressor (i.e., capture, handling, and restraint). On the other hand, males exhibited a relatively

attenuated androgen response to another form of stressor, entrapment in a mist net for a period of time. Currently, the degree to which variation in the strength of the androgen response to perturbation reflects differences in life history strategy is poorly understood. Similarly, the extent to which variation in the responsiveness of the HPG axis to acute stress is driven by individual differences in the reactivity of the hypothalamic-pituitary-adrenal axis is also poorly understood. Indeed, previous research has shown that androgen levels decrease as glucocorticoid levels increase (Moore et al. 1991, Moore et al. 2000, Deviche et al. 2010) and that multiple components of the HPG axis are sensitive to glucocorticoids (Orr and Mann 1992, Dong et al. 2004, Deviche et al. 2010). A lack of a correlation in the magnitude of change in circulating levels of both hormones following acute stress (Deviche et al. 2014, Davies et al. 2016), however, suggests the exact relationship is complex and may rely on multiple other mechanisms in addition to circulating glucocorticoids (Deviche et al. 2010, Deviche et al. 2017). Comparative studies aimed at addressing the influence of life history on endocrine responses to acute stress and mechanistic studies addressing the interactions between the HPA and HPG axes are promising directions for future research.

## **CONCLUSIONS**

Measuring individual variation in baseline hormone levels is essential for environmental endocrine studies and our understanding of the evolution of life-history strategies (Ketterson and Nolan 1999; Ricklefs and Wilkelski 2002; Williams 2008; Zera and Harshman 2001). Here, we showed that circulating androgen levels in male wire-tailed manakins decline in response to capture and restraint, a pattern consistent with other studies on free-living male organisms. We also demonstrated that prolonged entanglement in a mist can also induce decreases in androgen

levels. Thus, sampling protocols that do not properly account for the potential effects of capture and sampling on androgen levels risk a reduced ability to detect meaningful relationships between traits and androgens (e.g., McGlothlin et al., 2007, Day et al., 2007, Duval and Goymann 2011). Moving forward, studies constrained to using sampling regimes with unmonitored, passive netting should incorporate both field and statistical methods that can help reduce and account for capture-induced variation in baseline androgen levels. The use of video described in this study provides a novel method for measuring the amount of time between capture and sampling, and thus the ability to account for artificial variation in androgen levels induced by the acute stress of capture and sampling.

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## TABLES AND FIGURES

Table 1. AICc rankings for models examining the effect of net time (tElap), a male's status (Status), time of day (ToD), and year on androgen levels of wire-tailed manakins.  $K$  is the number of parameters in each model,  $\Delta\text{AICc}_i$  is the difference in AICc score between each model and the best-fitting model, and  $w_i$  is the model weight.

	<b>Model</b>	<b>K</b>	<b><math>\Delta\text{AICc}_i</math></b>	<b><math>w_i</math></b>
1	~tElap + Status + Year	8	0.00	0.75
2	~Status + Year	7	2.67	0.20
3	~tElap + ToD + Status + Year	9	5.64	0.04
4	~ToD + Status + Year	8	7.48	0.02
5	~TOD * Status + Year	11	19.1	0.00
6	~tElap * Status + Year	11	23.40	0.00
7	~Status	6	34.38	0.00
8	~tElap + Status	7	35.61	0.00
9	~ToD + Status	7	41.11	0.00
10	~tElap + ToD + Status	8	42.66	0.00
11	~TOD * Status	10	54.11	0.00
12	~tElap * Status	10	59.24	0.00

$_{1}\text{AIC} = 1063.18$  for the top ranked model

Table 2. AICc rankings for models examining the effect of net time (tElap), a male's status (Status), time of day (ToD), and year on androgen levels of wire-tailed manakins. In this analysis, 7 individuals with capture times great than 40min were excluded from the dataset.  $K$  is the number of parameters in each model,  $\Delta AICc_i$  is the difference in AIC score between each model and the best-fitting model, and  $w_i$  is the model weight.

	<b>Model</b>	<b>K</b>	<b><math>\Delta AICc_i</math></b>	<b><math>w_i</math></b>
1	~Status + Year	7	0.00	0.75
2	~tElap + Status + Year	8	3.04	0.16
3	~ToD + Status + Year	8	4.77	0.07
4	~tElap + ToD + Status + Year	9	8.45	0.01
5	~TOD * Status + Year	11	16.46	0.00
6	~tElap * Status + Year	11	24.65	0.00
7	~Status	6	34.55	0.00
8	~tElap + Status	7	38.96	0.00
9	~ToD + Status	7	41.27	0.00
10	~tElap + ToD + Status	8	45.95	0.00
11	~TOD * Status	10	54.27	0.00
12	~tElap * Status	10	61.24	0.00

$\Delta AICc = 1042.32$  for the top-ranked model

Figure 1. Comparisons of androgen levels (untransformed) in male wire-tailed manakins sampled within 3min of capture (baseline) and again after 15min or 30min of restraint in a cloth bag. A measurable decrease in circulating androgens occurred after 15min; this decline was more pronounced after 30min. Different letters above points denote significance differences ( $p < 0.05$ ).

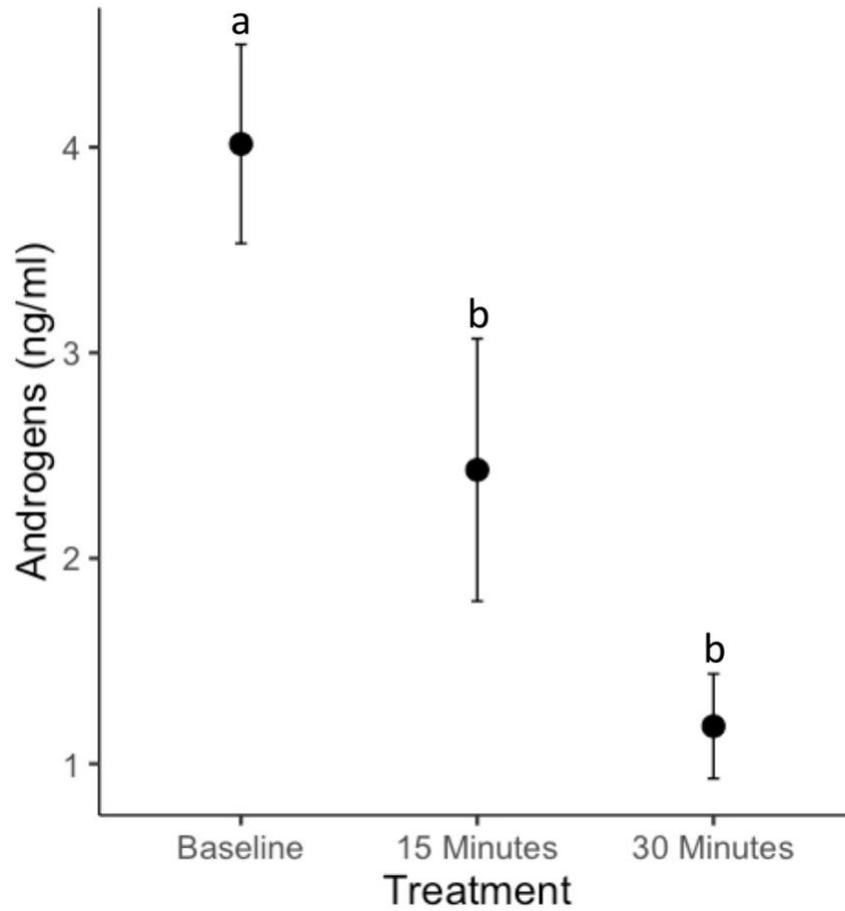


Figure 2. The magnitude of the change in androgen levels (untransformed) following capture and either 15 or 30 minutes of restraint in relation to an individual's initial androgen levels. The change in androgen levels was negatively related to initial androgen levels ( $\beta_{initial\ androgens} = -0.50$ , 95% CIs = -0.27, -0.72), but this effect was dependent on the duration of the restraint ( $\beta_{initial\ androgens*duration} = -0.39$ , 95% CIs = -0.13, -0.64).

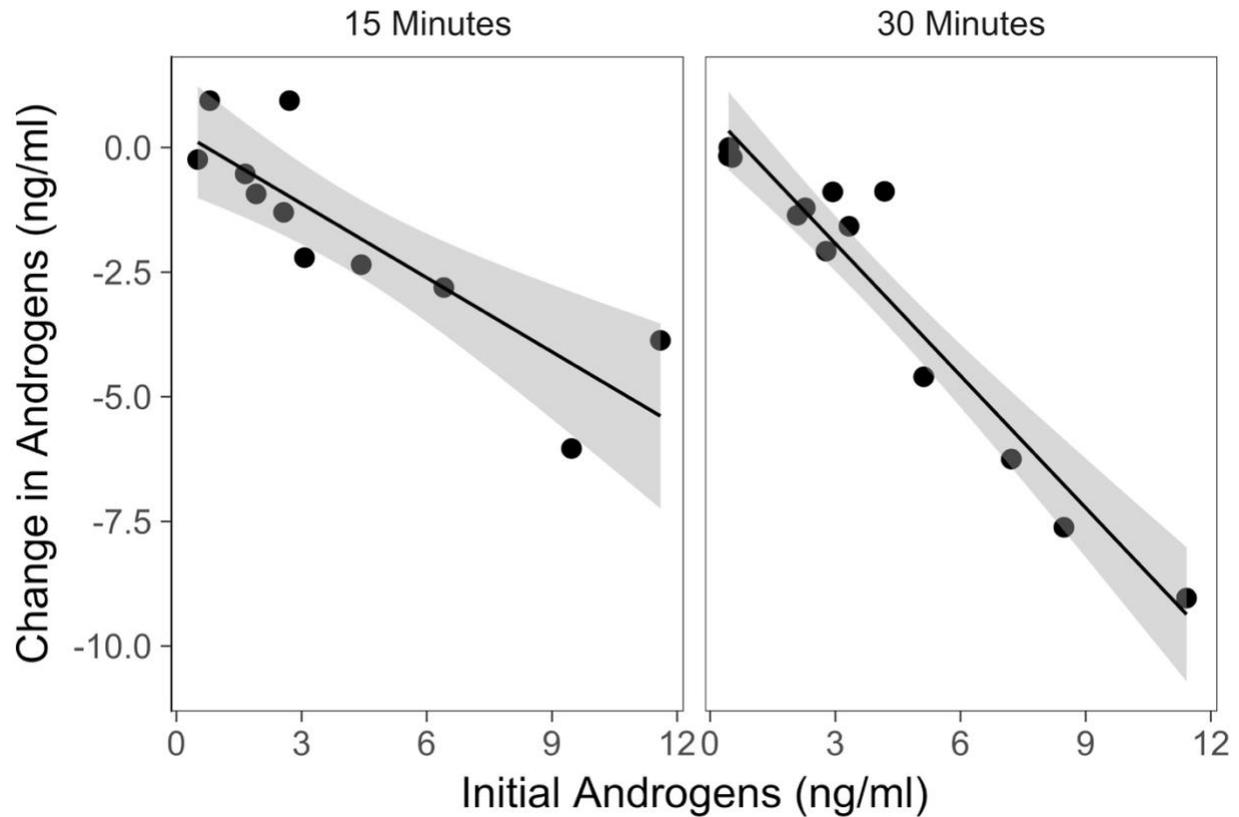
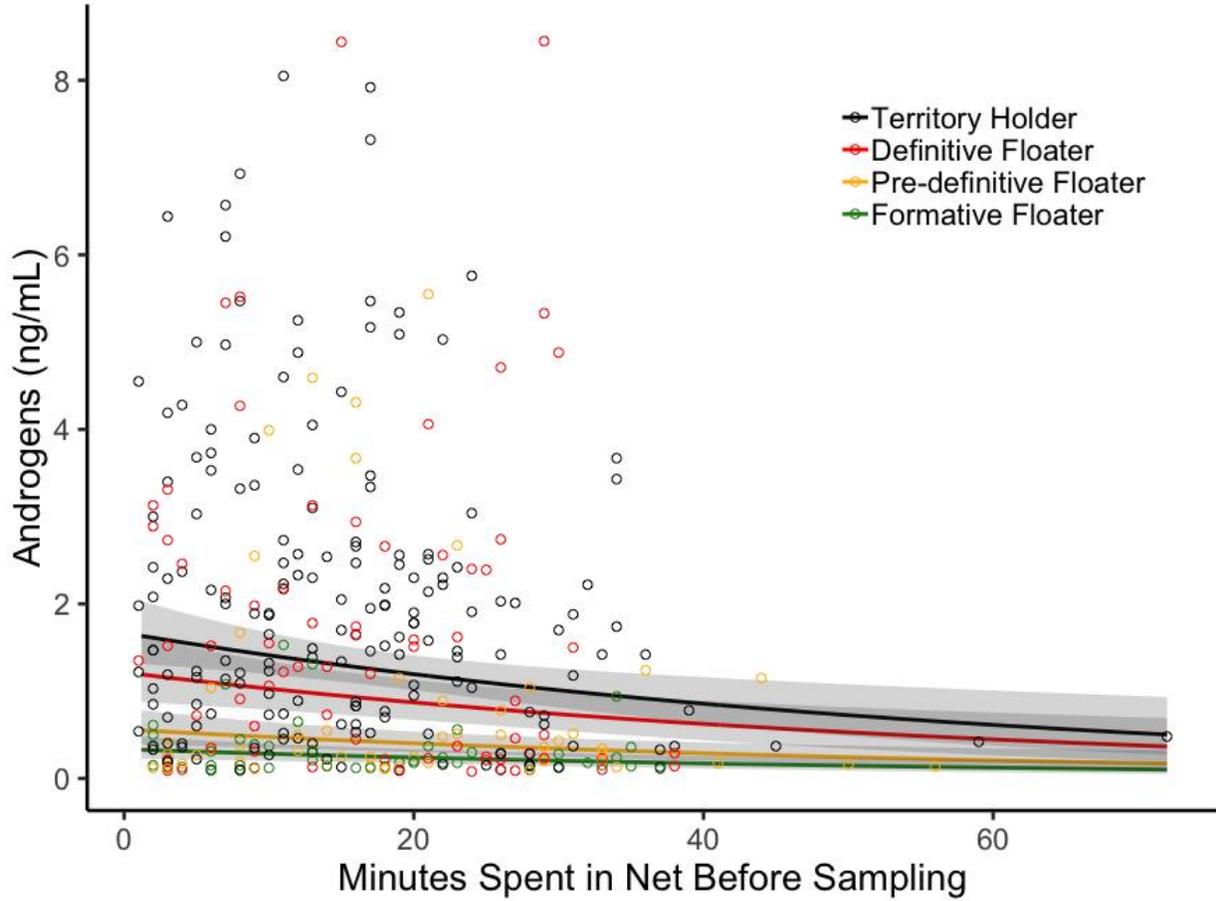


Figure 3. A negative relationship exists between androgen levels and the time elapsed between capture and sample collection ( $\beta_{tElap} = -0.02$ , 95% CIs = -0.036, -0.011). The color of each point and each line represents one of the four status classes of male wire-tailed manakins that are known to differ in mean levels of androgens. Fitted values and their confidence intervals were back-transformed prior to graphing.



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**CHAPTER III.** Reduced cooperative behavior as a cost of high testosterone in a lekking passerine

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### **LAY SUMMARY**

Here, we show that reduced cooperative behavior is a cost of high testosterone among male wire-tailed manakins. Reduced cooperative behavior is costly because more cooperative males have higher reproductive success. Males that were more cooperative spent more time displaying, but testosterone was not related to how much time a male spent displaying nor their display rate. Our results suggest testosterone may however indirectly mediate a male's courtship display effort by influencing their cooperative behavior.

### **ABSTRACT**

Many studies have identified the reproductive benefits of cooperative behaviors, yet few have identified the mechanisms that underlie these behaviors. Mechanistic studies can inform our understanding of why some individuals are more or less cooperative as well as identify the physiological constraints imposed upon the evolution of reproductive traits. Male wire-tailed manakins exhibit cooperative courtship behaviors and more cooperative territory-holders have been shown to exhibit higher reproductive success. To begin to understand the proximate basis

of cooperative display behaviors, we conducted both an observational study and an experimental study. Additionally, because coordinated courtship displays underlie this form of cooperation, our study also examined both the hormonal and social drivers of individual variation in courtship behavior more broadly (e.g., display rates). Our observational study revealed that males with higher testosterone levels performed fewer cooperative display bouts. Furthermore, in our experimental implant study, males that received a testosterone-implant significantly decreased the proportion of their displays that were cooperative. We found no relationship between an individual's courtship display effort (i.e., display rate and time spent performing courtship displays) and circulating testosterone in either study. However, more cooperative males spent a greater proportion of time performing courtship displays than did less cooperative males, suggesting that testosterone may indirectly mediate courtship display behaviors by influencing a territory holder's cooperative behavior. Overall, both our observational and experimental results suggest that reduced cooperative behavior is a cost of maintaining high levels of testosterone.

## **1. INTRODUCTION**

In some species, a male's reproductive success is dependent upon not only competing but also cooperating with other males (reviewed in Díaz-Muñoz et al. 2014). One particular form of male-male cooperation, cooperative display coalitions, occurs when two or more males perform courtship displays together to attract females but only one male within the display coalition has the chance to mate with females (Díaz-Muñoz et al. 2014). Studies focused on cooperative display coalitions have found that non-breeding individuals who are more cooperative are quicker to obtain breeding status and, among breeding males, more cooperative males have higher reproductive success (Duval 2007, McDonald 2007, Ryder et al. 2009). Therefore,

essential to a male's reproductive success is his ability to form and maintain cooperative partnerships with other males. While many studies have investigated the ultimate aspects of cooperative display coalitions, few have investigated the mechanisms mediating or modulating a male's cooperative behavior. By quantifying the relationship between individual variation in cooperative behaviors and the mechanisms proposed to underlie variation in male reproductive traits, we can begin to understand the physiological drivers of cooperation (Williams 2008). Furthermore, because courtship displays form the basis of cooperative display coalitions, this approach can also inform our understanding of how an individual's social environment and physiology interact to shape variation in courtship behavior more broadly.

Both neural and endocrine mechanisms are hypothesized to underlie cooperative behaviors (Soares et al., 2010). However, given that male-male interactions form the basis of cooperative display coalitions, testosterone is likely to be a prominent modulator. Testosterone is known to have permissive effects on some male reproductive behaviors (e.g., territorial behaviors) and antagonistic effects on others (e.g., paternal care; Nelson 2011). Testosterone is also involved in coordinating a male's response to its social environment, as circulating levels can respond to both territorial challenges and the presence of receptive mates (Wingfield et al. 1990, Adkins-Regan 2005, Goymann 2009, Goymann et al. 2019). Furthermore, due to its role in promoting aggressive behaviors and social dominance (Wingfield et al. 2005), testosterone is predicted to be antagonistic to cooperative behaviors. Few studies have, however, examined the relationship between testosterone and individual variation in cooperative behaviors (but see Mehta et al. 2009).

While the permissive effects of maintaining high levels of testosterone can be beneficial, the simultaneous suppression of other behaviors important to an individual's fitness can entail

costs (Wingfield et al. 2001). For example, if an individual experiences multiple social challenges and testosterone levels increase, then that individual may become more vulnerable to predation due to the behavioral changes (e.g., reduced vigilance or increased territorial behaviors) that elevated levels of testosterone promote (e.g., Marler et al. 1991; Wingfield et al. 2001). Additionally, high levels of testosterone can be immunosuppressive and are also associated with increased parasites loads (Roberts et al. 2004, Foo et al. 2017). In species that exhibit cooperative courtship displays, high levels of testosterone may be costly if they promote the expression of behaviors that are antagonistic to stable social partnerships (e.g., aggression), yet few studies have examined this possibility. Identifying the fitness costs of testosterone is necessary to understand the potential physiological constraints imposed upon the evolution of the suite of testosterone-mediated behaviors that influence an individual's fitness (Wingfield et al. 2001, Hau 2007, Ketterson et al. 2009).

High circulating testosterone levels are often predicted to correspond with increased expression of courtship display behaviors, yet this relationship is inconsistent across studies (Adkins-Regan 2005, Fusani 2008). Testosterone is known to promote courtship behaviors because administering testosterone to castrated males rescues courtship behaviors that diminish with castration (Adkins-Regan 1981, Balthazart et al. 1990), and experimentally blocking androgen receptors decreases the expression of courtship behaviors (Fusani et al. 2007, Fuxjager et al. 2013). However, studies on free-living animals have reported both a dose-dependent relationship and no relationship between courtship behaviors and circulating testosterone levels (Borgia and Wingfield 1991; McDonald 2001; Wiley and Goldizen 2003, Day et al. 2007; reviewed in Fusani 2008). These discrepancies highlight the need for additional observational and experimental studies focused on elucidating the relationship between circulating testosterone

and individual variation in courtship behavior. This is especially pertinent to evolutionary biology, as the costs of testosterone are predicted to maintain the honesty of secondary sexual traits (e.g., courtship behaviors) in a dose-dependent fashion (Folstad and Karter 1992).

Here we use both observational and experimental approaches to determine the role of testosterone in modulating the cooperative courtship behavior of territory-holding male wire-tailed manakins. Wire-tailed manakins are lekking songbirds that exhibit male-male coordinated courtship displays (Schwartz and Snow 1978, Heindl 2002), and these courtship displays form the basis of long-term, stable cooperative coalitions (Ryder et al. 2011b). Males may display either alone or cooperatively with another male, and previous work has demonstrated that territory-holders who form more cooperative coalitions with other males have higher reproductive success (Ryder et al. 2009). In addition, the circulating testosterone levels of territory-holders were found to be significantly higher than those of floater males, but also highly variable among territory-holding males (Ryder et al. 2011a). To understand whether variation in testosterone levels is related to an individual's cooperative behavior and overall courtship display effort, we first measured individuals' behaviors and then quantified their circulating testosterone levels. We then experimentally tested these relationships by measuring an individual's behavior before and after experimentally increasing testosterone levels using hormone implants. Given that testosterone promotes aggressive behaviors in many other species (Wingfield et al. 2005), we predicted that testosterone will have antagonistic effects on an individual's cooperative behavior. Furthermore, given that Ryder et al. (2011b) found that the presence of a display partner resulted in higher display rates, we predicted that an individual's cooperative behavior will be positively related to their overall display effort. This work was conducted in tandem with another study (Ryder et al., *in press*) that measured the cooperative behavior of the same

individuals using the proximity datalogging approach described in Ryder et al. (2012) and Dakin and Ryder (2018). These two studies are separate but complimentary in that they measured different aspects of a male's social and reproductive behavior using two distinct methodologies. Specifically, the current study directly measured a male's cooperative behavior and courtship behavior more broadly using a video-based approach while Ryder et al. (*in press*) measured male social interactions by examining the spatiotemporal overlap of the detections of radio-tagged males.

## **2. METHODS**

### *2.1 Study System*

We conducted our research on free-living wire-tailed manakins (*Pipra filicauda*) at the Tiputini Biodiversity Station, Orellana province, Eastern Ecuador (0° 83' S, 76°08' W, 190-270 m elevation). This population of wire-tailed manakins has been marked and monitored since 2003. Each captured bird is marked with both a numbered aluminum leg band and a unique combination of two or three colored leg bands to facilitate future identification in the field. For the current study, males were sampled during the population's peak breeding period (November to March) over the course of three field seasons (2015-2016, 2016-2017, 2017-2018). Wire-tailed manakin leks at Tiputini Biodiversity Station can range from 5 to 13 territories, and neighboring leks range from 400 to 800 m apart. The individual territories of wire-tailed manakins within the leks are about 40 m in diameter, and the territory-holding male will use 1 to 3 branches within his territory to perform courtship displays either alone or with a partner (Schwartz and Snow 1978). These 'display perches' are typically the horizontal branches of saplings that are 1-2.5 m off the ground (Schwartz and Snow 1978).

## *2.2 Observational Study*

We located display perches by observing territories during the morning (06:30 to 11:00) and afternoon (14:00 to 16:00) periods of maximal activity. Observations were conducted by directly watching territories from a distance, and we identified display perches by determining the specific branches where males repeatedly performed courtship displays. In some cases, we confirmed suspected courtship display perches using video recordings. After locating the courtship display perches on the majority of the territories at each lek, one to two GoPro Hero 4 SLVR cameras were deployed in each territory to quantify courtship behavior. The number of cameras deployed in each territory depended on the number of display perches found (typically one camera per perch). The day before filming started, cameras were mounted 2-4m from display perches and concealed using camouflage webbing and leaves from the surrounding vegetation.

All territories at each lek were simultaneously filmed for 2.5 to 6 hours on three consecutive days, and cameras were usually turned on before or at dawn (i.e., between 0550 to 0630). After the camera began recording, the time, date, and the identity of the territory holder were immediately spoken into the camera. If it began to rain, cameras were turned off and filming was resumed 30 minutes after the rain had stopped. If it was raining at dawn, cameras were turned on 30 minutes after the rain stopped. Following the completion of the filming session, the camera batteries and memory cards were replaced and cameras were redeployed that afternoon. We attempted to passively capture and sample territory-holders after filming a territory for three consecutive days following the methodology described in section 2.4.

## *2.3 Hormone Manipulation Experiment*

During the 2016-2017 and 2017-2018 field seasons, we filmed courtship display perches (following the same methods described in section 2.2) before and after administering the territory-holding male a subcutaneous silastic implant either filled with crystalline testosterone (i.e., experimental treatment) or left empty (i.e., control). Specifically, after three days of consecutive filming, birds were captured and blood sampled (following the methods described in section 2.4), and then each individual was randomly chosen to receive either a testosterone-filled implant or an empty (i.e., control) implant. Implants were 7mm x 1.47mm (I.D.) pieces of silastic tubing sealed on both ends with ~1mm of silicone, and they were inserted under the skin on a male's flank. This size of implant elevates the circulating testosterone levels of wire-tailed manakins to levels at the upper end of their naturally occurring range 9 to 15 days after implants were administered (2.93 ng/mL [95% CIs = 1.67, 5.15]; Ryder et al. *in press*). In another study, testosterone levels were found to be stable between four- and fourteen-days after administering testosterone-filled silastic implants (Figure 3, Quispe et al. 2015). Although the circulating hormone levels observed when the males in this study were recaptured 9 to 15 days after implanting may have been higher, data from Quispe et al. (2015) suggest that the measured values are reflective of the hormone levels exhibited when the territory-holder's behaviors were measured. All implant surgeries were performed under aseptic conditions and at the perimeter of the lek. After the implants were administered, the display perches in each experimental territory were filmed for three consecutive days starting 6 to 8 days after the territory-holding male was implanted. Males were then recaptured following post-implant behavioral data collection to remove implants and measure the effect of implants on circulating testosterone levels.

#### *2.4 Capture and Sampling*

After the completion of each three-day filming session for both the observational and experimental studies, we attempted to passively capture all the territory holders that were filmed (following Vernasco et al. 2019). To reduce the amount of human disturbance within the lek on the day of capture, mist nets were set up (but left closed) within all territories the day before capture. The following day, nets were opened at dawn and subsequently checked every 30 minutes. Nets were filmed with GoPro Hero 4 SLVR cameras to document the time of capture and duration of time a bird was restrained in the net (see Vernasco et al. 2019). Upon capture, birds were immediately removed from the net and a blood sample (~ 75-100uL) was collected within 3min of removal from the net using heparinized capillary tubes following brachial venipuncture with a 27-gauge needle. Capillary tubes were capped with Critoseal® and stored in a cooler with an ice pack (2-3hrs) until centrifuged for 5min, after which the plasma was removed and its volume measured using a 50uL Hamilton syringe. Plasma was stored in ~100% EtOH following Goymann et al. (2007).

### *2.5 Behavioral data collection from video footage*

After the conclusion of the field season, video footage from each territory was transcribed to quantify a male's behavior. We use the behavioral descriptions in Schwartz and Snow (1978) to classify courtship display behaviors. A courtship display bout was considered to start at the exact time a bird began to perform a courtship display maneuver (e.g., side-to-side jumps, stationary display, etc.). The bout was considered over when a courtship display behavior was not performed for 1 minute. Given the average time between courtship display bouts across all males (mean  $\pm$  SEM = 19.84  $\pm$  0.95 minutes), we considered this 1-minute threshold to accurately represent the independence of courtship display bouts. A male-male coordinated courtship

display bout was considered to begin when a second bird actively began performing courtship behaviors with the bird on the courtship display perch and ended when no male-male courtship display interactions were observed for 1 minute.

To quantitatively describe an individual's courtship behavior, we calculated the display rate (i.e., number of independent courtship display bouts divided by number of hours sampled) and the proportion of time a bird spent performing courtship behaviors (i.e., the time spent performing courtship displays divided by sampling time). Furthermore, to quantify an individual's cooperative behavior, we calculated the proportion of a bird's courtship displays that were cooperative (i.e., the number of male-male coordinated courtship display bouts divided by the total number of courtship display bouts). We calculated daily values for each of the above behavioral variables; therefore, each individual had 3 behavioral measures for each filming session.

We did not calculate the rates at which specific courtship display maneuvers were performed because we could not reliably identify individuals on the video. However, we are confident that, in these videos, the individual performing courtship displays alone was the territory holder or that at least one of the individuals in a pair of displaying males was the territory-holder due to the potential fitness consequences of letting a male that's not the territory-holder perform courtship displays alone on a territory (e.g., Boyle and Shogren 2019). Furthermore, floater males and territory-holding males are rarely observed performing courtship displays alone on the territories of other males. However, to empirically confirm that a single male or one male in a pair was the territory holder in our videos, we filmed 22 radio-tagged territory-holding males in which an automated datalogger was simultaneously recording which males were present in the territory (for more details about datalogging system, see Ryder et al.

2012). In 81% of the 1,262 instances where a bird was in the camera's field of view, the territory holder was detected by the datalogger, and in 91% of the 348 instances when a courtship display occurred, the territory-holder was also detected. Additionally, for each period of time a bird was detected in the camera, we regressed the duration of time the bird was present in the video to the duration of time the territory holder was detected by the datalogger using a simple linear model. There was a highly significant, positive relationship between these two variables ( $\beta = 0.97$ ,  $p < 0.0001$ ), and the model explained 85% of the variance in the duration of time a male was seen in the video. As such, we are confident that the behavioral measures described above and acquired from video accurately quantify the behavior of the focal territory-holder.

## *2.6 Testosterone Assay*

After each field season, we quantified total plasma androgen levels following double extraction with dichloromethane (average extraction efficiency for 2015-2016, 2016-2017, and 2017-2018: 63%, 72%, and 65% respectively) using a direct radioimmunoassay (following Moore et al. 2002, Wingfield et al. 1991). The average plasma volume was  $32.97\text{uL} \pm 0.44$  (mean  $\pm$  SEM), and we ran samples in singlets to increase the detection probability. Testosterone concentrations were adjusted for individual extraction efficiency. For each assay, samples from the same individual were grouped together, but the order of individuals was randomized. We calculated the intra-assay coefficient of variation among standards within an assay and the inter-assay coefficient of variation among these standards across the three assays. The intra-assay coefficient of variation was 6.6%, 11.6%, 9.2% for each field season (2015-2016, 2016-2017, 2017-2018 respectively), and the inter-assay coefficient of variation was 19.5%. The average detection limit

for the assays was 0.22 ng/mL, and all measured samples that fell below the assay's detection limit were assigned the detection limit for that assay.

## *2.7 Statistical Analyses*

All statistical analyses were performed in R (R Core Team 2018). For our observational study, we quantified the relationships between an individual's overall courtship display behavior, cooperative courtship behavior, and circulating testosterone using a mixed model approach in the lme4 package (Bates et al. 2015). Prior to modeling the hormone–behavior relationships for our observational study, we corrected testosterone values for the conditions of each capture by calculating the residuals of a linear-mixed model. This model included log-transformed circulating testosterone levels as the response and Julian date, field season, and the duration of time a bird spent restrained in the mist net (see Vernasco et al. 2019) as fixed-effect predictors. Individual ID was included as a random effect to control for repeated measures. To increase the accuracy of the estimated effects of these predictors, we included data from 216 other captures of territory-holding wire-tailed manakins that occurred during the same three field seasons of this study (total sample size = 246 captures, for more information see Supplementary Table S1, Figure S1).

For our observation study, we built three different sets of models. The response of the first model set was the proportion of cooperative displays and this was modeled using a generalized linear mixed model with binomial distribution and a logit link function. For the second model set, an individual's display rate was modeled using a linear mixed model. For the third model set, the proportion of time a male spent displaying was also modeled using a linear mixed model. We tested for overdispersion and assessed model fit of the first model set (i.e., the

generalized linear mixed models) using the simulation-based approach within the DHARMA package in Program R (Hartig 2018). For the linear mixed models (i.e., the second and third model sets), we visually assessed model fits by examining the relationships between Pearson residuals, fitted values, and our explanatory variables. The response variables of the second and third model sets were log-transformed to meet the assumptions of normality.

Model selection was used to determine which predictors best explained variation in each of the response variables. We ranked models using Akaike's Information Criterion (corrected for small sample size, AICc) and determined the relative likelihood of each model using model weights ( $w_i$ ; Burnham & Anderson 2002). All candidate models within each model set, including the null models, contained territory-holder ID as a random effect. We compared models with and without circulating testosterone levels as a predictor variable and considered testosterone to be related to a particular behavior if the most supported model included testosterone levels. To further understand what influences the proportion of time a male spent displaying, we also included a male's display rate (log-transformed) and the proportion of their displays that were cooperative displays as predictors.

We analyzed the effect of the implant treatments on an individual's cooperative behavior, display rate, and time spent performing courtship displays using the same model structures as those described for our observational study. These models, however, only included the following fixed effects: timepoint (before or after birds were implanted), treatment (empty or testosterone-filled implant), and field season. Territory-holder ID was also included as a random effect. Each model included an interaction term between the timepoint and the treatment to determine if the changes in behavior significantly differed between the treatment groups. In other words, a

significant interaction between the timepoint and treatment indicates that the effects of the treatments on an individual's behavior significantly differed.

### **3. RESULTS**

#### *3.1 Observational study*

We measured the behavior and circulating testosterone levels of 28 males. Over the course of the study, two males were sampled twice (i.e., in different field seasons) for a total sample size of 30 (mean filming time  $\pm$  SEM = 12.6  $\pm$  1.0 hour, range = 7.0 to 20.8 hours). Following model selection, the top model explaining variation in a male's cooperative behavior revealed that testosterone levels were negatively related to the proportion of courtship displays that were cooperative (Tables 1 and 2; Figure 1). We found no support for testosterone explaining variation in an individual's courtship display rate and the amount of time an individual spent performing courtship displays (Table 1). The top model explaining variation in the proportion of time a male spent displaying indicated that the proportion of courtship displays that were cooperative and the number of independent courtship display bouts per hour were both positively correlated with the proportion of time a male spent displaying (Table 1 and 2; Figure 2). Display rate and cooperative behavior were uncorrelated ( $r = 0.04$ ,  $p = 0.70$ ).

To further understand the influence of a male's cooperative behavior on the proportion of time a male spent displaying, we compared the average courtship display bout length between solo courtship displays and cooperative courtship displays using a linear mixed model that included year as a fixed effect and territory ID as a random effect. On average, cooperative courtship display bouts were longer than solo courtship display bouts (mean solo bout length  $\pm$  SEM = 51.16  $\pm$  5.5 seconds, mean cooperative bout length  $\pm$  SEM = 157.5  $\pm$  7.47 seconds;  $p <$

0.0001). Display rates and average courtship display bout length were not correlated ( $r = -0.05$ ,  $p = 0.67$ ).

### *3.2 Hormone Manipulation Experiment*

A total of 12 individuals were filmed before and after receiving an implant (testosterone implant  $n = 7$  males; empty control implant  $n = 5$  males; mean filming time  $\pm$  SEM; before implant =  $17.6 \pm 0.85$ , range = 12.97 to 20.76 hours; after implant =  $19.3 \pm 0.80$ , range = 12.74 to 23.28 hours). Males that received a testosterone implant significantly decreased the proportion of cooperative courtship displays after implantation (pre-implant mean cooperative courtship displays  $\pm$  SEM =  $0.16 \pm 0.04$ , post-implant mean cooperative courtship displays  $\pm$  SEM =  $0.03 \pm 0.01$ ,  $p < 0.0001$ ; Table 3, Figure 3C). We did not observe any changes in a male's display rate nor the proportion of time a male spent displaying for either treatment group (Table 3; Figure 3A, 3B).

## **4. DISCUSSION**

In this study, we measured the relationship between circulating testosterone and the cooperative courtship behavior of territory-holding wire-tailed manakins using both an observational and experimental approach. Results from both studies suggest that reduced cooperative behavior is a cost of maintaining high levels of testosterone. Additionally, our results provide no evidence that testosterone directly influences overall courtship display effort, but suggest that an individual's cooperative behavior influences their courtship display rates because 1) males with a higher proportion of cooperative courtship displays spent more time displaying, and 2) cooperative courtship displays were, on average, longer in duration than non-cooperative courtship displays.

These results suggest that testosterone may indirectly mediate an individual's courtship behavior by influencing their ability to form and maintain cooperative partnerships.

High levels of circulating testosterone are predicted to be favored by selection for polygynous species without parental care, as multiple studies on polygynous species with no paternal care have documented positive relationships between circulating testosterone and reproductive success (Borgia and Wingfield, 1991; Beletsky et al., 1992; Alatalo et al., 1996; Vitousek et al., 2008; Smith et al., 2015). However, the costs associated with high levels of testosterone (reviewed in Wingfield et al. 2001) are also predicted to act as constraints on the evolution of testosterone levels and testosterone-mediated traits (Hau 2007; Ketterson et al. 2009). Among species that exhibit cooperative courtship displays, high testosterone levels seem to be necessary for a territory holder to attain and maintain territory-holder status, as territory-holders have been found to exhibit significantly higher testosterone levels than non-breeding males (Ryder et al. 2011a, Duval and Goymann 2011). However, the results of the current study show that high levels of testosterone are antagonistic to the cooperative behaviors of territory-holders.

This work builds upon another study conducted concurrently with the observational and experimental studies described in this manuscript. Specifically, Ryder et al. (*in press*) found that those individuals that exhibited higher mean testosterone levels or experimentally elevated testosterone also interacted with fewer males less frequently. The current study adds to our understanding of the relationship between testosterone and cooperative courtship display behaviors by showing that the negative effects of testosterone on a male's social behavior translate into changes in a male's cooperative courtship display behavior. Overall, the results of both provide complimentary evidence that reduced cooperative behavior is a novel cost of

maintaining high testosterone levels for territory-holders, and this cost may act as a physiological constraint on the evolution of testosterone-mediated traits.

The frequency with which an individual interacts with conspecifics can have profound effects on their behavior and reproductive success. For instance, in highly competitive social environments (e.g., as in lekking species), same sex conspecifics are known to elicit aggressive responses. However, few studies have addressed this response in systems where an individual's reproductive success depends upon cooperating with same sex conspecifics. Here, we show that the presence of a display partner results in longer courtship display bouts and that individuals who display more often with a partner also spend more time performing courtship displays than those who more frequently display alone. While we were unable to determine the number of display partners each territorial male had, another study on this species found a highly positive correlation (correlation coefficient = 0.87) between the frequency of male-male interactions and the number of partners a male has (Dakin and Ryder 2018; Table S2). In other words, males with more partners also spend more time interacting with other males. In the context of mate choice, more cooperative males have higher reproductive success (Ryder et al. 2009), possibly because females prefer more cooperative males because those males are able to perform courtship displays more frequently and/or vigorously (Byers et al. 2010; Ryder et al. 2011b). Alternatively, detailed analyses of an individual's courtship display performance, similar to that of Barske et al. (2011), may reveal that a male's ability to perform specific components of the courtship display (e.g., the rate at which their wings vibrate during the stationary display or their speed during a swoop-in flight; Schwartz and Snow 1978) is what drives variation in a male's cooperative behavior and female preference (Mitoyen et al. 2019). In other words, males that are exceptional

at performing courtship displays may attract more display partners and females to his territory and, ultimately, sire more offspring.

Theory predicts a dose-dependent relationship between testosterone and the rate that courtship behaviors are performed and, in combination with the costs of maintaining high levels of testosterone (Wingfield et al. 2001), this dose-dependent relationship is thought to maintain the signal honesty of courtship behaviors (Folstad and Karter 1992). In our study, however, we did not find any relationships between testosterone and courtship display effort (i.e., display rates or time spent displaying), nor did display efforts change after experimentally increasing testosterone levels. These results corroborate the findings of studies that found no effect of testosterone implants on the courtship behavior of intact, courting males of other species (Fusani and Hutchison 2003; Day et al. 2007, reviewed in Fusani 2008). The relationship between testosterone and courtship behavior, then, appears to follow either a threshold response or a multiple-threshold response relationship whereby no proportional changes in behavior are observed once testosterone levels reach a threshold (Adkins-Regan 2005). However, in the case of cooperatively displaying species, testosterone may indirectly mediate an individual's courtship behavior as high levels of testosterone decrease the frequency of a male's cooperative interactions.

Despite the agreement between both our observational and experimental results, many questions regarding the mechanistic basis of cooperative and courtship display behaviors remain unanswered. For example, other mechanisms (e.g., steroid receptor expression or steroid-dependent nonapeptide hormones; Soares et al. 2010) could be driving individual variation in courtship display efforts and cooperative behavior, as androgen and nonapeptide hormone receptor densities in the brain and muscles are thought to be involved in coordinating the

expression of courtship behaviors in another species of manakin (reviewed in Schlinger et al. 2013). Furthermore, an individual's display behavior may depend on their physiological condition. This hypothesis could be tested by examining the relationship between potential biomarkers of physiological condition (e.g., markers of oxidative stress or telomere lengths; Monaghan 2010) and display behaviors. A holistic understanding of the mechanistic basis underlying individual variation in cooperative courtship behavior requires further study of the diverse mechanisms known to be involved in mediating reproductive behaviors, including those mechanisms linked to physiological condition.

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## FIGURE CAPTIONS

**Figure 1.** The proportion of a male's displays that were cooperative (i.e., performed with another male) in relation to their circulating testosterone levels (corrected for capture conditions, see section 2.7). Individuals with higher levels of circulating testosterone performed relatively fewer cooperative courtship displays.

**Figure 2.** The proportion of time a male spent displaying was significantly and positively related to the proportion of displays that were cooperative (i.e., performed with another male, A) and the number of courtship displays performed per hour (B). Note that these two explanatory variables do not covary (see Section 3.1 for more details). Both the response variable and the number of courtship displays performed per hour are log-transformed.

**Figure 3.** Boxplots showing the behaviors of the control (i.e., blank implants) and experimental (i.e., testosterone-filled implants) groups before and after receiving implants. Overlaid on the boxplot are points depicting the raw data. The number of display bouts per hour (A) and proportion of time spent displaying (B) did not differ significantly between the two treatments before or after males were implanted. However, the proportion of courtship displays that were cooperative (C) significantly decreased from before to after males received a testosterone implant. Note the y-axes are scaled differently for each graph.

## TABLES AND TABLE CAPTIONS

**Table 1.** AICc rankings for models explaining variation in the proportion of a male’s courtship displays which were cooperative, a male’s display rate (i.e., courtship displays performed per hour), and the proportion of time a male spent displaying. The response variable of each model set is denoted in bold above the top model of each set. The null models for each group include a random effect denoting the territory holder ID. Testosterone (corrected for capture conditions, see section 2.7) was included to examine support for its importance to a male’s courtship and cooperative behaviors. For the model explaining variation in the proportion of time a male spent displaying, we also included a male’s log-transformed display rate (Display Rate) and the proportion of courtship displays that were cooperative (Prop.coop). The three columns denote (from left to right) the number of parameters, the difference between the model’s AICc and the top model’s AICc, and the model weights.

<b>Proportion Cooperative Displays</b>	Parameters	$\Delta\text{AICc}_1$	weight
Testosterone	3	0	0.95
Null model	2	5.86	0.05
<b>Display rate</b>	Parameters	$\Delta\text{AICc}_2$	weight
Null model	4	0	0.88
Testosterone	3	3.97	0.12
<b>Proportion Time Spent Displaying</b>	Parameters	$\Delta\text{AICc}_3$	weight
Display Rate + Prop.coop	5	0.00	0.87
Testosterone + Display Rate + Prop.coop	6	3.84	0.13
Display Rate	4	19.76	0.00
Testosterone + Display Rate	5	23.83	0.00
Testosterone + Prop.coop	5	52.11	0.00
Prop.coop	4	55.63	0.00
Null model	3	75.25	0.00
Testosterone	4	76.68	0.00

<sup>1</sup>AIC of top model = 287.55, <sup>2</sup>AIC of top model = 342.15, <sup>3</sup>AIC of top model = 243.49.

**Table 2.** Model summaries and coefficients for the top ranked model for each measure of display behavior in Table 1. The response variable of each model is specified in bold above each of the fixed-effect variables included in the top model. All models include a random effect denoting the territory-holder ID (Individual). The proportion of time a male spent displaying depended on the proportion of his displays that were cooperative displays and his display rate. The proportion of his displays that were cooperative displays depended on his circulating testosterone levels (corrected for capture conditions, see section 2.7; Testosterone). The top model explaining a male’s display rate was the null model, therefore, only the intercept is displayed for this model summary.

<b>Proportion Cooperative Displays</b>	Estimate	Std. Error	z value	P value	
Testosterone	-1.01	0.34	-2.95	0.003	

<b>Display rate</b>	Estimate	Std. Error	df	t value	p value
Intercept	0.31	0.18	23.66	1.71	0.10

<b>Proportion Time Spent Displaying</b>	Estimate	Std. Error	df	t value	p value
Proportion Cooperative Display	1.82	0.36	87.51	5.09	<0.00001
Display Rate	0.55	0.06	88.60	9.39	<0.00001

**Table 3.** Coefficients of mixed models quantifying the effects of the implant experiments on courtship behavior. The response variable of each model is shown in bold. All models included the following fixed effects: the timepoint (Before/After), the experimental treatment (Treatment), field season, and an interaction term between the timepoint and the experimental treatment. Each model also contains a random effect of territory-holder ID. The reference group for the variable denoting timepoint (i.e., Before/After) is the post-implant values (i.e., After) and, for the treatment variable, the reference group is the control group.

<b>Proportion Cooperative Displays</b>	Estimate	Std. Error	z value	p value	
Intercept	-0.9	0.57	-1.58	0.11	
Before/After	-0.68	0.37	-1.86	0.06	
Treatment	-2.43	0.75	-3.23	0.001	
Field Season	-0.56	0.66	-0.84	0.4	
Before/After x Treatment	2.51	0.56	4.45	< 0.00001	

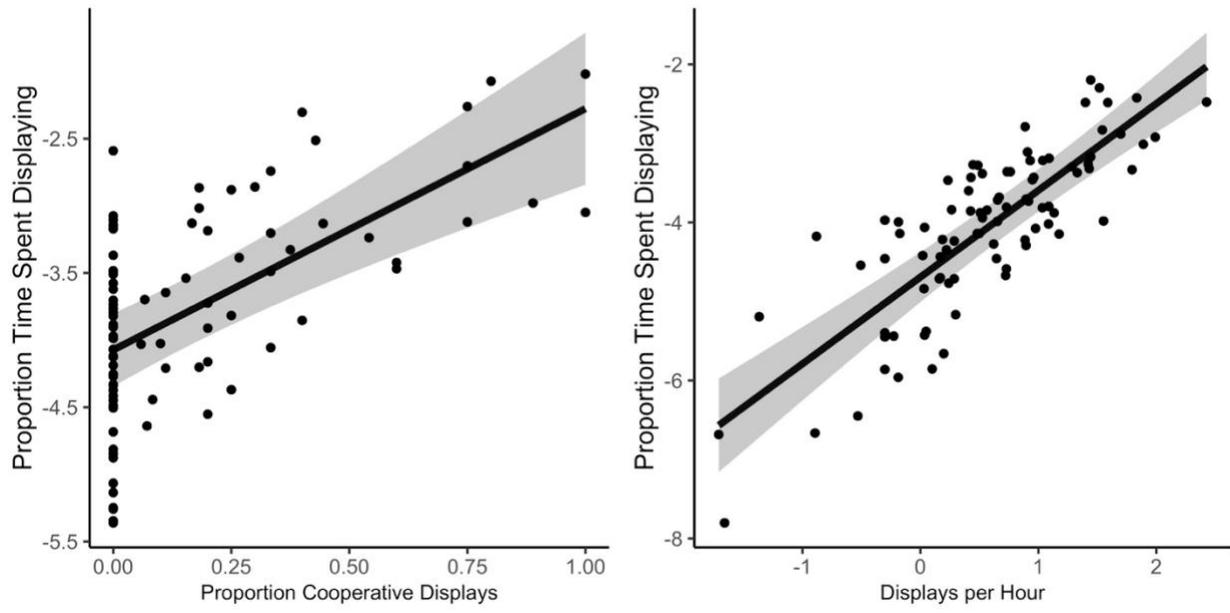
<b>Displays per hour</b>	Estimate	Std. Error	df	t value	p value
Intercept	0.03	0.54	17.76	0.06	0.95
Before/After	-0.83	0.6	60.58	-1.38	0.17
Treatment	0.6	0.68	19.38	0.89	0.39
Field Season	0.04	0.55	8.72	0.08	0.94
Before/After x Treatment	0.7	0.79	60.68	0.89	0.38

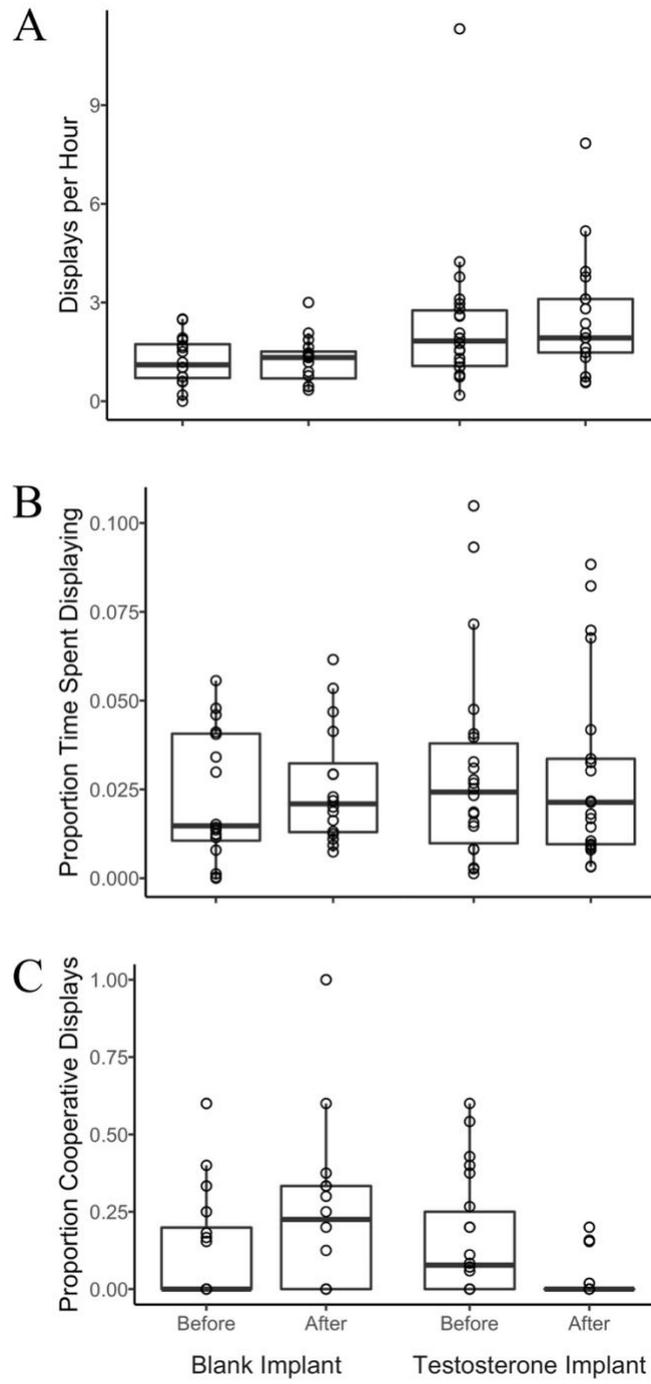
<b>Proportion time spent displaying</b>	Estimate	Std. Error	df	t value	p value
Intercept	-3.77	0.48	18.14	-7.9	< 0.00001
Before/After	-0.99	0.53	60.69	-1.88	0.07
Treatment	-0.04	0.59	19.79	-0.07	0.95
Field Season	-0.16	0.48	8.82	-0.32	0.75
Before/After x Treatment	0.88	0.7	60.79	1.26	0.21



Figure 2.



**Figure 3.**



**Chapter IV.** Telomeres shorten with age in a status-specific manner and predict the cooperative courtship behaviors of a lekking songbird.

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### **ABSTRACT**

Telomeres are useful biomarkers of aging because they are predictive of an individual's lifespan and mortality risk. As aging is predicted to influence reproductive behaviors, telomere lengths should also be related to individual variation in reproductive behaviors. However, these relationships have not been well established in free-living species. Wire-tailed manakins (*Pipra filicauda*) are a lekking songbird in which floater males must ascend a social hierarchy and become territory-holders before they can reproduce. In addition, males perform coordinated courtship displays and these displays form the basis of cooperative display coalitions. A floater's cooperative behavior is predictive of his social ascension and more cooperative territory-holding males attain higher reproductive success. Here, we tested for status-specific patterns of aging by longitudinally sampling telomere lengths from known-age floater and territory-holding male wire-tailed manakins. We also examined the relationship between cooperative reproductive behaviors and telomere length to further investigate how telomeres relate to reproductive behaviors. We did not detect significant within-individual changes in telomere lengths over the course of a four-year sampling period. Telomere lengths, however, were negatively correlated

with age among territory-holding males, but not floater males. Independent of age and social status, shorter telomeres were also associated with more frequent cooperative interactions and a more exclusive position within an individual's social network. These results are unique in that they describe how patterns of biological aging depend on social status and also suggest that short telomeres are associated with greater reproductive investments. Given that short telomeres are also associated with shorter lifespan and higher mortality risk, these results are consistent with the hypothesis that males with low future reproductive potential invest more in reproduction.

## **INTRODUCTION**

Telomeres are repetitive, non-coding strands of DNA that maintain chromosomal integrity and, upon shortening to a critical length, initiate cellular senescence and cellular apoptosis (von Zglinicki et al. 2001, Monaghan 2010). At the cellular level, telomeres decrease in length because of the limitations imposed upon the DNA replication process by linear chromosomes (i.e., the end replication problem) and telomere loss is also thought to be due to oxidative stress (Monaghan 2010, Young 2018). Moreover, a number of extrinsic and intrinsic factors that act upon higher levels of biological organization have also been found to be associated with telomere shortening. These factors include aging, noxious stimuli, environmental perturbations, habitat quality, nutrition, and disease (Angelier et al. 2013, Mizutani et al. 2013, Herborn et al. 2014, Nettle et al. 2014, Ashgar et al. 2015, Hau et al. 2015, Meillère et al. 2015, Angelier et al. 2018). Understanding the factors that contribute to telomere loss is important because longer telomeres or slower rates of telomere attrition are associated with lower mortality risk and/or longer lifespan both within and among species (Heidinger et al. 2012, Blackburn et al. 2015, Tricola et al. 2018, Wang et al. 2018, Wilbourn et al. 2018, Whittemore et al. 2019). Overall, because telomeres are sensitive to damage from physiological and environmental perturbations

as well as predictive of an individual's mortality risk, telomere lengths can be used to measure an individual's biological age (Monaghan 2010, Hau et al. 2015, Haussmann and Heidinger 2015, Young 2018). A growing number of biologists, then, are measuring telomere lengths or rates of telomere loss to further understand among individual variation in patterns of reproductive investment and the factors that contribute to the aging process more broadly.

The terminal investment hypothesis predicts that individuals will increase their reproductive investments as their remaining future reproductive potential declines (i.e., residual reproductive value; Clutton-Brock 1984). Accurately measuring an individual's residual reproductive value, however, has proven challenging as mortality risks are not consistent across individuals. Recently, biologists have hypothesized that telomere lengths are indicative of an individual's residual reproductive value, but few studies have specifically tested this hypothesis (Young 2018, Giraudeau et al. 2019). Some support for this hypothesis, however, is provided by studies on migratory birds. Specifically, among migratory birds, those individuals that arrive to the breeding grounds earlier have higher reproductive success and lower survival probabilities (Price et al. 1988, Sheldon et al. 2003). As predicted, individuals with shorter telomeres have been found to arrive to the breeding grounds earlier (Bauch et al. 2013, Bauer 2018). Therefore, individuals with lower residual reproductive value, as indicated by their short telomeres, are investing more in reproduction by arriving to the breeding grounds earlier. Support for the hypothesis that individuals with short telomeres exhibit higher reproductive investments also comes from a study that found individuals with shorter telomeres exhibit higher levels of baseline corticosterone levels (Angelier et al. 2019), a hormone known to mobilize energy stores to meet the energetic demands of reproduction (Bonier et al. 2009). To critically test this relationship, however, patterns of reproductive investment and telomere lengths must be

measured in known-age individuals (Giraudeau et al. 2019). Lek-breeding species can also provide a novel perspective on the relationship between telomeres and patterns of reproductive investment because the behavior of the sex that only contributes gametes to reproduction is directly correlated with their reproductive investment and, in some cases, reproductive success (Fiske et al. 1988).

Manakins of the family Pipridae are small-bodied, Neotropical passerines that are known for their lek-breeding behavior and the elaborate, often acrobatic, courtship displays that males perform (Kirwan and Green 2011). In some species of manakins, males engage in cooperative display coalitions wherein multiple males perform coordinated courtship displays for females, but only the dominant male (i.e., territory-holding male) within the coalition will mate with the female (Diaz-Munoz et al. 2014). Subordinate males (i.e., floater males) rarely sire offspring, however, those that are more cooperative are more likely to ascend the social hierarchy to become a territory-holder (McDonald and Potts 1994, Duval 2007, Ryder et al. 2008). Among territory-holders, those that are more cooperative also exhibit higher reproductive success (Ryder et al. 2009). Studies of the wire-tailed manakin (*Pipra filicauda*), a species in which males form cooperative display coalitions (Heindl 2002), have provided a detailed understanding of individual variation in cooperative courtship behavior. First, repeated behavioral sampling has shown that males exhibit significant among-individual variation in cooperative behavior (Dakin and Ryder 2018). In addition, more cooperative males have also been found to spend more time performing courtship displays (Vernasco et al., in review). Cooperatively displaying manakins, then, offer a novel opportunity to investigate how telomeres relate to reproductive investments because variation in cooperative courtship display behaviors is predictive of a male's courtship behavior (Vernasco et al., in review) and reproductive success (McDonald and Potts 1994, Duval

2007, and Ryder et al. 2008, Barske et al. 2011). Additionally, because two distinct social classes exist (i.e., floater males and territory-holders), the relationship between social status and patterns of biological aging can also be examined.

Within vertebrates that exhibit distinct social classes, previous work has found that costs can be incurred by either subordinate or dominant individuals depending on multiple factors including: 1) the stability of the social hierarchy, 2) the type of breeding system, 3) environmental conditions, 4) and the manner in which rank is obtained (Goymann and Wingfield 2004, Creel et al. 2013). In some cases, subordinate individuals are excluded from certain resources and this has been shown to be detrimental in terms of the health and telomere lengths of subordinates (Sapolsky 2005, Lewin et al. 2015). Dominance status among lekking species, however, has been found to be costly due to either challenges from subordinate males or because of the energetic demands of prolonged lek attendance (Andersson 1994). For example, males with the more central territories (a predictor of mating success) more frequently engage in competitive behaviors (Isvaran and Yadavendradev 2000, Hämäläinen et al. 2012), spend less time foraging (Isvaran and Yadavendradev 2000), and exhibit greater mass loss over the course of the breeding season (Lebigre et al. 2013). In the context of telomeres, however, no study has examined how social status influences telomere length or attrition in a lekking species.

In this study, we sought to address the following questions: 1) do male wire-tailed manakins exhibit significant among-individual variation in telomere lengths, 2) do telomeres vary with age and is this relationship dependent on a male's social status, and 3) are telomere lengths related to an individual's reproductive behavior (i.e., lek attendance and cooperative courtship behavior)? To address these questions, we longitudinally sampled known-age males from a long-term study population. Over the same sampling period, we measured a male's

cooperative social behavior using the automated proximity data-logging system described in Ryder et al. (2012) and Dakin and Ryder (2018). Given that the partnerships of wire-tailed manakins are relatively stable over time (Ryder et al. 2011) and social ascent is mediated by courtship display behaviors (as opposed to overt aggression, Ryder et al. 2008), status-specific costs may not be apparent in this system. Furthermore, in the context of reproductive behaviors, if males increase their reproductive effort as their residual reproductive value declines, then we predict that males with shorter telomeres will exhibit higher reproductive investments because short telomeres are associated with greater mortality risks. Overall, our study will broaden our understanding how an individual's social status influences the aging process and the nature of the relationship between telomeres and reproductive behaviors.

## **METHODS**

### *Study System*

We studied free-living wire-tailed manakins at the Tiputini Biodiversity Station, Orellana province, Eastern Ecuador (0° 83' S, 76°08' W, 190-270 m elevation). Individuals in this population have been marked and monitored since 2002. Upon initial capture, individuals are marked with a numbered aluminum leg band and a unique combination of two or three colored bands to facilitate identification in the field. For the current study, we sampled males one or two times per field season during the population's peak breeding period (November to March) over the course of five field seasons (2013-2014, 2014-2015, 2015-2016, 2016-2017, 2017-2018). A male's social status was determined using plumage characteristics and by directly observing his behavior at leks. Territory-holding males are reliably found in the same small area of the forest (i.e., their territory) whereas floater males are detected in multiple territories and, in some cases, multiple leks (Heindl 2002). Many males in this population are of known age (age range 1 to 15

years old) as a result of long-term monitoring efforts and because males exhibit age-related changes in their plumage until their 3<sup>rd</sup> molt cycle (Ryder and Durães 2005).

Birds were passively captured in mist nets (following the methodology described in Vernasco et al. 2019). Nets were opened at dawn and subsequently checked every 30 minutes. After being seen in a mist net, birds were immediately removed and a blood sample (~ 75-100uL) was collected from the brachial vein. Samples were collected using heparinized capillary tubes following brachial venipuncture with a 27-gauge needle. Capillary tubes were then capped with Critoseal® and stored with an ice pack in a cooler for 2-3hrs. Upon returning from the field, samples were centrifuged for 5min, the plasma was removed, and the red blood cells were stored at room temperature for 2 to 3 months in Longmire's Lysis Buffer, a buffer known to effectively preserve DNA quality for telomere analyses (Eastwood et al. 2018). Upon returning to the United States, samples were stored in a freezer at -20 C until DNA extraction.

#### *Behavioral Data Collection*

During three field seasons (2015-2016, 2016-2017, 2017-2018), we measured the behavior of male wire-tailed manakins using the proximity data logging system described in Ryder et al (2012) and Dakin and Ryder (2018). Briefly, upon capture, each male was outfitted with a coded nano-tag (~0.4g; NTQB-2, Lotek Wireless) that transmits a unique signal every 20 seconds. After sampling and tagging males at a given lek, proximity data-loggers (SRX-DL800, Lotek Wireless) were subsequently deployed within the territories of all tagged males. Each data-logger continuously recorded pings from all tagged males within a 30 m radius from the data-logger, a distance that corresponds to the size of a male's territory. Data-loggers collected data from 6:00 to 16:00, were deployed at a given territory for 6 days per sampling interval, and each lek was sampled 3 times per field season. Data were processed as described in Dakin and Ryder (2018).

Briefly, we quantified male-male cooperative interactions by examining the spatiotemporal overlap of tag detections in a given territory (for more information see Dakin and Ryder 2018). The resulting data were used to calculate four behavioral metrics that describe a male's daily lek attendance and male-male cooperative interactions. More specifically, we calculated each of the following behavioral variables: the number of unique data-logger pings by a focal individual per day (effort), the number of males the focal bird interacted with per day (degree), the sum of a male's edge weights within their social network per day (strength), and a measure of the exclusivity of an individual's partnerships (importance, Dakin and Ryder 2018). Importance was calculated for each male by taking the proportion of his lek partners' interactions that were with him, and then taking the weighted average of these values (weighted by the focal male's interaction frequencies). Higher values of importance indicate that the focal male's lek partners interacted more exclusively with him; lower values indicate that his partners devoted most of their interactions to other males. To analyze how behavior was related to telomere lengths, we took the average for each metric and each male using all of his behavioral recording dates that were within 30 days after the date he was captured and his blood was sampled.

#### *DNA Extraction and Telomere Analysis*

To extract DNA from samples, we used the Gentra Puregene Blood Kit (Qiagen) with the following modifications to the extraction protocol. We added ~100 uL of the sample-lysis buffer mix to 600 uL of Cell Lysis solution and 5 uL of proteinase K for digestion at 55°C overnight. Samples were then cooled on ice for 5 minutes, 200 uL of Protein Precipitation solution was mixed into the cell lysate, and the proteins were then removed following centrifugation. DNA was precipitated using 100% isopropanol and 70% ethanol washes and then resuspended in 25 uL of the DNA hydration solution. DNA purity and concentration were assessed using a

NanoDrop (ND-1000) and DNA integrity was assessed by running samples on a 1% agarose gel (100 V for 45 minutes).

To quantify relative telomere lengths, we used a qPCR method based on Criscuolo et al. (2009) and Eastwood et al. (2018). Samples were analyzed using a CFX96 Touch System (Bio-rad) on 96 well plates with a total reaction volume of 15 uL. The reaction included 7.5 uL of SsoAdvanced™ Universal SYBR® Green Supermix, 3.6 uL of Dnase-free water, 0.15 uL of forward and reverse 100uM telomere or single-copy control gene (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) primers (Integrated DNA Technologies), and 3.6 uL of template DNA (2ng/uL concentration). Each plate also included a two-fold serial dilution of a “golden sample” that containing the following concentrations: 36, 18, 9, 4.5, 2.25, 1.13, 0.56, and, for plates with the telomere primers, 0.28 ng. The “golden sample” was made up of a mix of 3 different second-year males that were not included in subsequent age or behavior analyses. The telomere primers were Tel1b 5' - CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT - 3' and Tel2b 5' - GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT - 3'. The GAPDH primers were developed from the GAPDH sequence of the *Manacus* genome, a closely related species of manakin (GAPDH Forward: 5' - GCC ACA CAG AAG ACA GTG GA – 3' and GAPDH Reverse: 5' - TTT CCC ACA GCC TTA GCA GC – 3'). We determined the optimal annealing temperature of both the telomere and GAPDH primers by examining the efficiencies of the standard curves ran at four different annealing temperatures (58.4°C, 57°C, 56°C, and 54.7°C). We used melt curve analyses to confirm the presence of a single, tight peak in the qPCR product. After optimizing the assay, reaction conditions for both sets of primers were as follows: 98°C for 10 min, followed by 40 cycles of 1 minute at 98°C and 1 minute at 56°C. A melt curve analysis was also performed at the end of the 40 cycles to check for primer

dimerization and the amplification of nontarget genes. Two controls that lacked template DNA were also included in each plate. All samples and standards were run in triplicate. To reduce the effects of inter-assay variation, all samples from the same male were analyzed on the same plate. Therefore, samples were grouped by individual and then individuals were randomly sorted into nine plates. Telomere primers and GAPDH primers were on separate plates.

*LINREGPCR* (version 11) was used to calculate individual well qPCR efficiencies and Cq values (Ruijter et al. 2009). We used standard curves made using the Cq values of the standards to calculate both assay efficiencies and coefficients of determination. All coefficients of determination were  $>0.99$  and all efficiencies ranged from 90% to 115% (mean  $\pm$  standard deviation; GAPDH =  $94.4\% \pm 3.49\%$ , Telomere =  $100.7\% \pm 5.6\%$ ). For each set of primers, we quantified assay precision by calculating the intra-assay repeatability among all standards within each assay and the inter-assay repeatability among all the standards across the assays (Eisenberg 2016). Repeatability was calculated using the *ICC* package within Program R (Wolak et al. 2012). The average intra-assay repeatability ( $\pm$  standard deviation) for the GAPDH and telomere plates was  $0.998 (\pm 0.0003)$  and  $0.997 (\pm 0.001)$  respectively. The inter-assay repeatability for the GAPDH and the telomere plates was  $0.995$  and  $0.964$  respectively. To normalize inter-assay variation, we used the value at 7.2 ng interpolated from the serial dilution linear regression line (following Eastwood et al. 2018). Relative telomere lengths (i.e., the amount of telomeric DNA relative to the single-copy gene) were then calculated following equation one in Pfaffl (2001). Specifically, individual well efficiencies were raised to the power of the interpolate control minus the well Cq value. The values from the sample replicates were then averaged and relative telomere lengths were calculated by dividing the average telomere value by the average GAPDH value. Finally, comparing telomere length estimates from qPCR to telomere lengths measured

using another technique is a necessary step for validating qPCR assays for measuring telomere lengths (Nussey et al. 2014). Therefore, we measured a subset of samples ( $n = 7$ ) using both qPCR and a telomere restriction fragment assay (following the methodology described in Haussmann and Mauck 2008) and examined their correlation. A linear model with the TRF values as the response and the relative telomere lengths as the predictor showed a significant relationship between the two measures and explained 57% of the variation ( $\beta = 4.418$ ,  $p = 0.049$ ).

### *Repeatability Analysis*

Given that males in this study were longitudinally sampled, we were able to measure the repeatability of relative telomere lengths to understand the amount of variation in telomere lengths that can be attributed to among-individual difference (i.e., the consistency of an individual's telomere lengths, Nakagawa and Schielzeth 2010). We used a linear-mixed model that included an individual's relative telomere length as the response variable and a random effect of individual ID following methodology described in Nakagawa and Schielzeth (2010). Confidence intervals were estimated using parametric bootstrapping in the lme4 package (Bates et al. 2015).

### *Telomere Length and Age Analysis*

We quantified age-based changes in relative telomere lengths using the within-individual centering technique described in van de Pol and Wright (2009). For each male, we calculated his average age from his ages at each telomere/blood sampling event. We then calculated an individual's delta-age ( $\Delta\text{Age}$ ) by quantifying the difference between a male's age at each sampling event and his average age. Importantly, this statistical technique allows us to quantify and compare both within- and among-individual variation in telomere length dynamics (van de

Pol and Wright 2009). We tested for status-specific differences in telomere lengths by including an interaction term between both age terms ( $\Delta$ Age and average age) and social status.

Additionally, a random effect denoting individual ID was also included in this model. The full model testing for age-effects on relative telomere lengths (rTL) is as follows:

$$rTL = (Average\ Age + \Delta Age) * Status + Individual$$

#### *Age, Behavior and Telomere Length Analysis*

We determined how age and telomere lengths relate to an individual's cooperative behavior and social network position using linear mixed-effects regression models the lme4 package (Bates et al. 2015). Specifically, each behavioral metric (i.e., effort, degree, strength, and importance) was used as the response variable in four different model sets, respectively. All models within each model set, including the null model, contained a categorical variable denoting field season and a random effect of individual identity. The global model of each model set included an individual's relative telomere length (rTL), age at capture, and an interaction between both age and relative telomere length with an individual's social status at capture:

$$Behavioral\ Metric = (rTL + Age) * Status + Individual$$

Competing models contained the follow variables: 1) relative telomere length, 2) age at capture, 3) relative telomere length and an interaction with status, 4) age at capture and an interaction with status, and 5) a model that included both age at capture and relative telomere length. We visually assessed model fits by examining the relationships between Pearson residuals, fitted values, and our explanatory variables. We log-transformed effort, strength, and degree to meet model assumptions. Model selection was then used to determine which predictors best explained variation in each of the behavioral response variables. More specifically, we ranked models

using Akaike's Information Criterion (corrected for small sample size, AICc) and determined the relative likelihood of each model using model weights ( $w_i$ ; Burnham and Anderson 2002).

## RESULTS

### *Telomere Repeatability and Age Analysis*

We measured the telomere lengths from 187 samples that represent 53 known-age males (average number of samples per individual  $\pm$  SD:  $3.53 \pm 1.77$ , age range: 1 to 14.22 years, average time between individual sampling events  $\pm$  SD:  $0.71 \pm 0.51$  years, range: 0.04 to 2.4 years). Telomere lengths exhibited significant among-individual variation as the estimated repeatability of relative telomere lengths was 0.73 (95% CI: 0.61-0.81). Among territory-holding males, we found a significant, negative relationship between an individual's average age and relative telomere lengths ( $p = 0.04$ , Table 1, Figure 1B). However, among floater males there was a near-significant ( $p = 0.09$ , Table 1) positive relationship between an individual's average age and relative telomere length (Table 1, Figure 1A). Neither status class exhibited significant within-individual age-related changes in relative telomere length (Table 1). To exclude the possibility that the contrasting relationships between the two status classes was due to differences in the age ranges of each status (age range of floater: 1 to 6.98 years, age range of territory-holders: 2.86 to 14.22 years), we ran the same model after filtering the data such that the age range of territory-holders matched that of floater males (i.e., 2.86 to 6.96 years). The pattern between the age and relative telomere length among territory-holders remained statistically significant after filtering the data ( $\beta_{\text{Average.Age:Territory-holder}} = -0.08$ , 95% CI: -0.13, -0.03,  $p = 0.005$ ).

### *Behavior, Age, and Telomere Length Analysis*

Of the 187 samples used above, 87 (representing 42 males) also had corresponding behavioral data collected within 30 days after blood sampling (average number of blood samples per individual  $\pm$  SD:  $2.04 \pm 0.66$ ). Following model selection, the most supported model revealed that age significantly predicts an individual's lek attendance, with older individuals having a higher frequency of attendance (Table 2A, Table 3, Figure 2A). While multiple models that included telomere length as a predictor provided a similarly good fit to the data (i.e., within 2  $\Delta$ AICc; Burnham and Anderson 2000), in no case was the telomere length predictor statistically significant ( $p \geq 0.27$  for all 4 models within 2 AICc). We therefore considered telomere length to be an uninformative parameter (Arnold 2010) and chose to focus on the most parsimonious top-ranked model (i.e., the age-only model). Furthermore, there was also some model selection uncertainty for the model set that predicted the number of partners a male had (degree, Table 2B). More specifically, while the top-ranked model was the null model, the model ranked second (i.e., the model that only included relative telomere lengths, Table 2B) had a  $\Delta$ AICc of 0.24 and the model received 46% of the weight (Table 2B). The p-value of the estimated effect of relative telomere length was also nearly significant ( $p = 0.08$ , Table 3), therefore, we concluded that there was a weak, negative relationship between degree and relative telomere lengths (Figure 2B). The top model among those predicting strength indicated that there is a significant negative relationship between relative telomere length and strength (Table 2C, Table 3, Figure 2C). Similarly, among those models that included importance as the response variable, the top-ranked model indicated that there was a significant negative relationship between importance and relative telomere length (Table 2D, Table 3, Figure 2D). Importantly, patterns between relative telomere lengths and an individual's cooperative behavior did not change when age was included in the model (Supplementary Table 1).

To further understand the broader behavioral differences between the two status classes, we built linear mixed models that included each behavioral metric as the response and a categorical fixed effect denoting an individual's social status. As above, each model included a fixed effect of field season and a random effect denoting individual. Of all the behavioral metrics, only effort significantly varied between the status classes (Supplementary Table 2). Specifically, territory-holding males exhibited significantly higher values of effort than floater males ( $\beta_{\text{social status}} = 1.59$ , 95% CI: 0.91, 2.28,  $p < 0.0001$ , Supplementary Table 2).

## DISCUSSION

This study documents significant among-individual variation in relative telomere lengths and suggests that an individual's social status influences the aging process. More specifically, our results show telomere shortening is more prominent among territory-holders as only territory-holding males exhibit a negative relationship between age and relative telomere lengths (even after filtering the data such that the age ranges of the two status-classes do not differ).

Furthermore, short telomeres are associated with greater reproductive investment independent of a male's age and social status, therefore, males with low future reproductive potential appear to increase their reproductive investment.

Individual male wire-tailed manakins with shorter telomeres more frequently interacted with another male and also maintained a more exclusive position within their social network. As an individual's courtship behavior is dependent upon his cooperative behavior, the presence of a courtship display partner corresponds with an increase in the frequency that multiple types of courtship behaviors are performed (Ryder et al. 2011) and males that more frequently perform cooperative courtship displays also spend more time displaying (Vernasco et al, in review). Work on the golden-collared manakin (*Manacus vitellinus*) has shown that manakin courtship

displays are highly metabolically demanding, however, the magnitude of these demands depends upon how frequently a male performs courtship displays (Barkse et al. 2014). Therefore, it is plausible that more cooperative male wire-tailed manakins are spending more energy because more cooperative males spend more time performing courtship displays. In combination with previous work showing more cooperative males also exhibit higher reproductive success, our work suggests that shorter telomeres are associated with greater reproductive investment. Short telomeres are also associated with reduced lifespan and a higher mortality risk in other studies (Heidinger et al. 2012, Wang et al. 2018, Wilbourn et al. 2018), therefore our results support the hypothesis that telomere lengths function to modulate an individual's reproductive effort based on its remaining future reproductive potential (Young 2018 and Giraudeau et al. 2019).

Aging also appears to be a cost of maintaining a dominant social status as we found that age was negatively related to telomere lengths only in the dominant, territory-holding class. This hypothesis is also supported by work on two other species of manakins that revealed that territory-holding males exhibit higher levels of oxidative damage (a prominent contributor to telomere shortening) than non-territory-holding males (Gomes et al. 2019). Moreover, on average, territory-holders in our study spent significantly more time on the lek than floater males, suggesting that territory-holders may be limited in the resources they have access to (i.e., those resources that are further from the lek that are otherwise accessible for floaters). Alternatively, territory-holders may be spending more energy trying to attract females to their territory either by performing courtship displays alone or using vocalizations. Territory-holders may also have to invest more time and energy into maintaining their social status because cooperative courtship displays partly function as a means of maintaining the social hierarchy (Prum 1994). In other

words, territory-holders may incur multiple costs from having to perform courtship displays with floater males regardless of their physiological state or condition.

Across species, those that live longer exhibit relatively slower rates of telomere attrition (Tricola et al. 2018). Despite the fact that older individuals exhibited shorter telomere lengths in this study, we did not detect significant telomere shortening within individuals as they aged and the majority of variation in telomere length was found to be attributed to among-individual variation (as opposed to within-individual variation, Nakagawa and Schielzeth 2010). Wire-tailed manakins are relatively long-lived for their size (adult male mass = 13 to 15 grams) and, in this study, the time elapsed between sampling events was relatively short (0.2 to 2.4 years between sampling events for a given individual). Telomere shortening is more apparent during development and adult telomeres are known to shorten relatively slowly (reviewed in Angelier et al. 2018). Therefore, one possibility is that most telomere shortening in male manakins may occur prior to the ages of reproductive maturity when we studied them. Importantly, previous work has shown that male wire-tailed manakins exhibit significant among-individual variation in their cooperative behavior (Dakin and Ryder 2018). Given that males are consistently more or less cooperative and telomeres are hypothesized to shorten in response to high metabolic demands (Casagrande et al. 2019), the observed result between territory-holder age and telomere lengths could also reflect telomere shortening that occurs over longer time periods (and that could not be detected in a 0.2 to 2.4 year sampling window).

Overall, our results suggest that short telomeres are negatively correlated with age only among the social class that spends significantly more reproductive effort and sires the vast majority of offspring (i.e., territory-holders). Furthermore, shorter telomeres are also associated with increased investments in reproductive behaviors independent of an individual's age. In

combination with the evidence suggesting that shorter telomeres are also associated with increased mortality risk and/or shorter lifespan (Heidinger et al. 2012, Blackburn et al. 2015, Wang et al. 2018, Wilbourn et al. 2018), our work supports the hypothesis that telomeres integrate information about an individual's residual reproductive value and modulate an individual's reproductive effort (Young 2018, Giraudeau et al. 2019). Future studies focusing on the relationship between telomere lengths and reproductive effort or success should prioritize using a cohort approach because individuals from a single cohort experience similar environmental conditions both during development and adulthood. Broadening our understanding of telomeres and their proximate function will also require experimental manipulations of telomere length using techniques that still require development for applications in free-living animals (Criscuolo et al. 2018).

## TABLES AND FIGURES

**Table 1.** Model summary and coefficients describing the relationship between age and relative telomere length. Given our longitudinal sampling design, we used the within-individual centering technique described in van de Pol (2009) to quantify both within- (i.e.,  $\Delta$ Age) and among-individual (Average.Age) differences in telomere lengths. We included an interaction between both age terms and status (i.e., floater vs territory-holder) to quantify status-specific patterns of telomere regulation. For this model, the reference group is the floater class.

<b>Relative Telomere Length</b>	Estimate	SE	df	t value	p value
Intercept	0.98	0.07	100.83	13.40	0.00
Average.Age	0.03	0.02	148.73	1.70	0.09
$\Delta$ Age	0.01	0.01	140.91	0.39	0.70
Territory-holder	0.21	0.11	138.67	1.94	0.05
Average.Age:Territory-holder	-0.05	0.02	151.86	-2.12	0.04
$\Delta$ Age:Territory-holder	0.00	0.02	135.85	0.21	0.83

**Table 2.** AICc rankings for models explaining variation a male's lek attendance (effort, A), number of display partners (degree, B), average frequency of cooperative interactions (strength; C), and the exclusivity of a male's partnerships (importance; D). The response variable of each model set is denoted in bold. All models include a categorical fixed effect denoting field season and a random effect denoting individual ID. The three columns denote (from left to right) the number of parameters, the difference between the model's AICc and the top model's AICc ( $\Delta$ AICc), and the model weights.

<b>A) Effort</b>	Parameters	$\Delta$ AICc <sub>1</sub>	weight	<b>B) Degree</b>	Parameters	$\Delta$ AICc <sub>2</sub>	weight
Age	6	0	0.25	Null	5	0	0.51
Age + rTL	7	0.01	0.25	rTL	6	0.24	0.45
(rTL+Age)*Status	10	0.02	0.25	rTL*Status	8	5.97	0.03
rTL*Status	8	1.1	0.14	Age	6	8.4	0.01
Age*Status	8	1.74	0.11	rTL+Age	7	8.75	0.01
rTL	6	16.28	0	Age*Status	8	18.05	0
Null	5	18.34	0	(rTL+Age)*Status	10	18.27	0
<b>C) Strength</b>	Parameters	$\Delta$ AICc <sub>3</sub>	weight	<b>D) Importance</b>	Parameters	$\Delta$ AICc <sub>4</sub>	weight
rTL	6	0	0.52	rTL	6	0	0.58
rTL*Status	8	1.04	0.31	Null	5	1.55	0.27
Null	5	3.54	0.09	Age	6	4.09	0.07
rTL + Age	7	4.81	0.05	rTL+Age	7	4.21	0.07
(rTL+Age)*Status	10	6.29	0.02	rTL*Status	8	7.86	0.01
Age	6	7.14	0.01	Age*Status	8	17.54	0
Age*Status	8	11.82	0	(rTL+Age)*Status	10	20.26	0

<sup>1</sup>AICc of the top model = 320.75, <sup>2</sup>AICc of the top model = 115.06,  
<sup>3</sup>AICc of the top model = 225.01, <sup>4</sup>AICc of the top model = -27.37

**Table 3.** Model summaries and coefficients for the top ranked model (with the exception of degree) for each behavioral measure described in Table 2. The response variable of each model is specified in bold above each of the fixed-effect variables included in the top model. All models include a categorical fixed effect denoting field season (Field.Season) and a random effect denoting the individual.

<b>Effort</b>	Estimate	SE	df	t value	p value
Intercept	2.53	0.4	82	6.32	<0.01
Age	0.31	0.06	82	5.64	<0.01
Field.Season16-17	1.27	0.38	82	3.32	<0.01
Field.Season17-18	0.93	0.39	82	2.38	0.02
<b>Degree</b>	Estimate	SE	df	t value	p value
Intercept	1.42	0.31	41.25	4.58	<0.01
rTL	-0.48	0.27	38.4	-1.77	0.08
Field.Season16-17	0.72	0.11	54.88	6.83	<0.01
Field.Season17-18	0.22	0.11	65.34	1.99	0.05
<b>Strength</b>	Estimate	SE	df	t value	p value
Intercept	2.7	0.62	50.24	4.34	<0.01
rTL	-1.3	0.54	47.75	-2.39	0.02
Field.Season16-17	1.3	0.2	57.35	6.47	<0.01
Field.Season17-18	0.61	0.22	66.32	2.81	0.01
<b>Importance</b>	Estimate	SE	df	t value	p value
Intercept	0.61	0.14	55.05	4.41	<0.01
rTL	-0.31	0.12	53.12	-2.58	0.01
Field.Season16-17	-0.05	0.04	52.52	-1.32	0.19
Field.Season17-18	0.18	0.05	61.3	4.03	<0.01

**Supplementary Table 1.** Model summaries and coefficients for the models predicting degree, strength, and importance. All models contain both age and an individual's relative telomere length (rTL). Each response variable is denoted in bold. Importantly, the patterns between an individual's relative telomere length and reproductive behavior reported in Table 1 of the main text do not change when age is included in the model. All models include a categorical fixed effect denoting field season (Field.Season) and a random effect denoting the individual.

<b>Degree</b>	Estimate	SE	df	t value	p value
Intercept	1.44	0.35	49.27	4.15	0.00
rTL	-0.49	0.28	40.31	-1.74	0.09
Age	0.00	0.02	40.49	-0.18	0.86
Field.Season16-17	0.71	0.11	48.97	6.66	0.00
Field.Season17-18	0.22	0.11	61.48	1.97	0.05

<b>Strength</b>	Estimate	SE	df	t value	p value
Intercept	2.26	0.68	57.82	3.31	0.00
rTL	-1.14	0.55	49.98	-2.07	0.04
Age	0.06	0.04	46.84	1.54	0.13
Field.Season16-17	1.35	0.20	52.98	6.69	0.00
Field.Season17-18	0.63	0.21	64.36	2.92	0.00

<b>Importance</b>	Estimate	SE	df	t value	p value
Intercept	0.46	0.15	62.45	3.16	0.00
rTL	-0.26	0.12	55.90	-2.20	0.03
Age	0.02	0.01	46.68	2.48	0.02
Field.Season16-17	-0.04	0.04	50.81	-1.01	0.32
Field.Season17-18	0.18	0.04	62.84	4.18	0.00

**Supplementary Table 2.** Model summaries and coefficients for the models comparing the behavioral differences between the two status classes. The response variable of each model is denoted in bold. All models include a categorical fixed effect denoting an individual's social status, a categorical fixed effect denoting field season (Field.Season) and a random effect denoting the individual. The reference group in each model is the floater group.

<b>Effort</b>	Estimate	Std. Error	df	t value	p value
(Intercept)	3.22	0.35	81.85	9.16	0.00
Status: Territory-holder	1.59	0.34	41.61	4.61	0.00
Field.Season16.17	1.31	0.40	58.38	3.26	0.00
Field.Season17.18	1.07	0.41	64.13	2.60	0.01

<b>Degree</b>	Estimate	Std. Error	df	t value	p value
(Intercept)	0.90	0.10	81.82	8.84	0.00
Status: Territory-holder	-0.01	0.11	54.88	-0.11	0.91
Field.Season16.17	0.72	0.11	54.69	6.69	0.00
Field.Season17.18	0.27	0.11	59.26	2.40	0.02

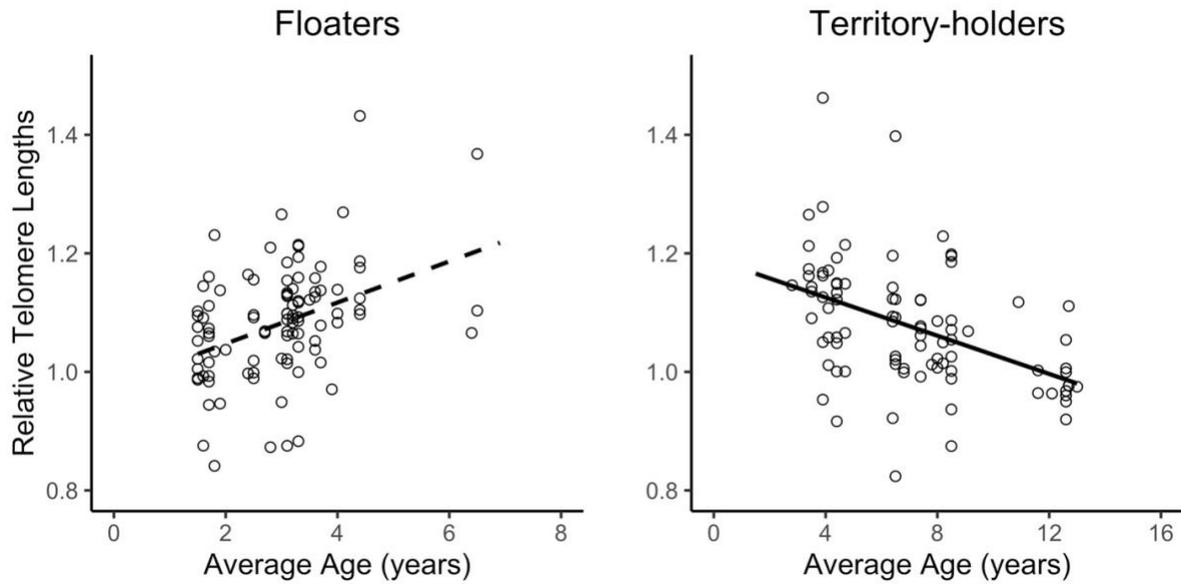
  

<b>Strength</b>	Estimate	Std. Error	df	t value	p value
(Intercept)	1.12	0.20	81.73	5.63	0.00
Status: Territory-holder	0.24	0.22	61.20	1.11	0.27
Field.Season16.17	1.38	0.21	56.23	6.71	0.00
Field.Season17.18	0.77	0.21	60.33	3.60	0.00

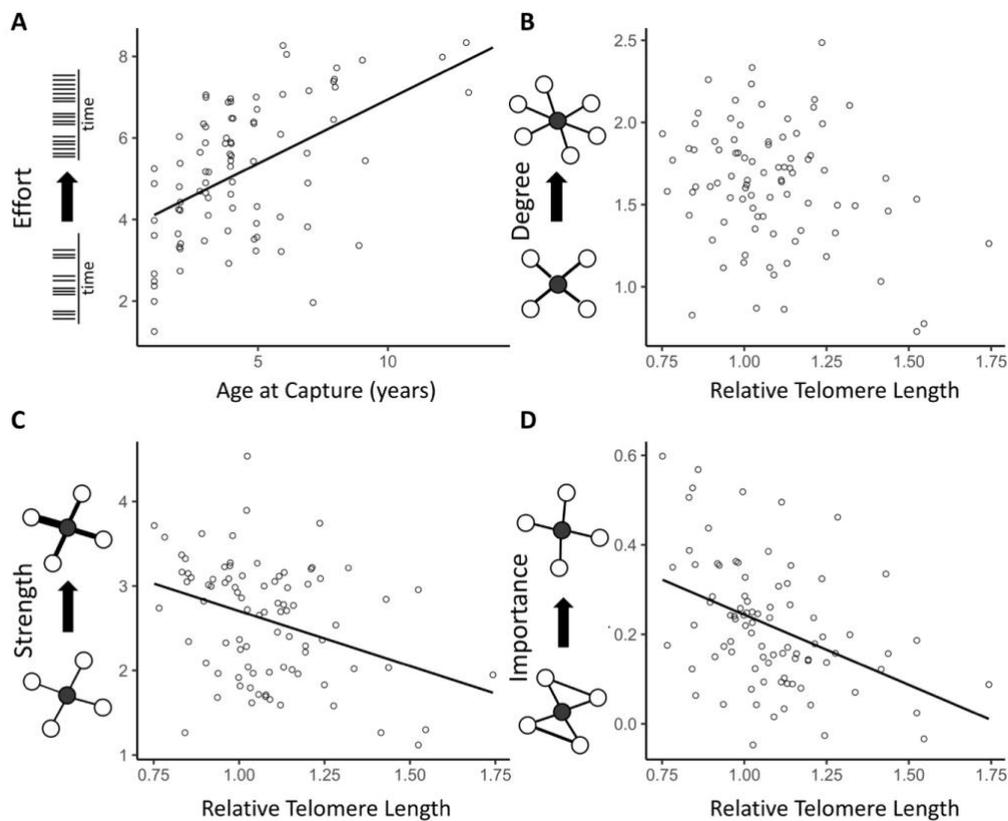
  

<b>Importance</b>	Estimate	Std. Error	df	t value	p value
(Intercept)	0.23	0.04	81.39	5.30	0.00
Status: Territory-holder	0.07	0.05	60.68	1.46	0.15
Field.Season16.17	-0.04	0.04	50.17	-0.86	0.39
Field.Season17.18	0.22	0.04	54.52	4.84	0.00

**Figure 1.** Relative telomere lengths in relation to an individual's average age (averaged across all telomere sampling events) among both floater and territory-holding male manakins. The expected negative relationship between relative telomere length and age was only observed among territory-holding males ( $p = 0.04$ ). Among floater males, there was a near-significant ( $p = 0.09$ , Table 1) positive relationship with age. Note the x-axes of each graph are scaled differently.



**Figure 2.** Social behavior in relation to relative telomere lengths. Each graph represents the relationship from the top-ranked model describing A) the number of unique pings detected at each data-logger (Effort), B) the number of display partners each male has (Degree), C) the frequency that a male interacted with another male (Strength), and D) a proportion representing the exclusivity of a male's partnerships (Importance). Effort was best explained by an individual's age while the top-ranked model for importance and strength included a significant relationship with relative telomere lengths. Although there was some model-selection uncertainty (see Table 2), the 2<sup>nd</sup> ranked model explaining the number of partners suggests there is a negative relationship with relative telomere length (Table 3).



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## CHAPTER V. Dissertation Summary and Synthesis

### **Dissertation Summary**

In Chapter I, I synthesize the evidence suggesting that testosterone mediates the tradeoff between cooperative and competitive behaviors among those organisms that exhibit cooperative reproductive behaviors. Chapter II summarizes the evidence showing the negative effects of capture and handling on circulating testosterone levels and utilizes both observational and experimental approaches to further our understanding of the relationship between capture stress and circulating testosterone. A novel field technique that allows researchers to account for some of the variation in circulating testosterone that is due to capture stress is then described. Chapter III implements the field technique detailed in Chapter II and also provides observational and experimental support for the antagonistic effects of testosterone on the cooperative courtship behavior of territory-holding male wire-tailed manakins. Chapter IV then provides a different proximate perspective on the cooperative courtship behavior of wire-tailed manakins by examining how telomere lengths (a biomarker of biological age) are related to both social status and courtship behaviors. Below, I detail future steps that should be taken to further understand the mechanistic basis of cooperative behaviors and the evolution of cooperation more broadly.

### **A Mechanistic Perspective on the Tradeoff Between Cooperation and Competition**

Among some organisms, individuals must cooperate to successfully reproduce (Diaz-Munoz et al. 2014, Koenig and Dickinson 2016). From afar, cooperative reproductive behaviors may appear as altruistic associations among multiple individuals. Closer inspection, however, often reveals that individuals within a group are more frequently competing amongst themselves for either social status or breeding opportunities. As in any other competition, there are winners and losers. In this context, determinants of success are hypothesized to be related to appropriately

timing the expression of cooperative or competitive behaviors (Taborsky and Oliveira 2012, Sih et al. 2019). Testosterone is thought to modulate behavioral plasticity in competitive behaviors (Taff and Vitousek 2016, Kelley and Vitousek 2017) and may therefore mediate the tradeoff between cooperation and competition (Chapter I). To further understand the extent of testosterone's role in mediating this individual variation in cooperative behavior or the tradeoff between the two, however, studies will need to measure both individual variation in cooperation as well as circulating testosterone (Williams 2008). Moreover, future studies will also have to compliment observational studies with experimental studies that entail either increasing circulating testosterone or blocking its receptors and/or conversion to other hormones (Fusani 2008). When attempting to measure this tradeoff, it will also be important to consider to what extent cooperating individuals can compete for reproductive opportunities. Potential factors to consider include relatedness (West et al. 2002), the extent to which cooperators differ in size (Hamilton et al. 2005, Clutton-Brock and Manser 2016), as well as the environmental factors that influence rates of competition (Bateman et al. 2013). By taking this novel perspective on the behaviors that have intrigued organismal biologists since their discovery, we can further understand why some individuals are more or less cooperative (Bergmüller et al. 2010).

### **Testosterone and Cooperative Behaviors**

Testosterone is known to have a multitude of effects on both physiological and morphological traits. The traits or processes that are sensitive to testosterone include spermatogenesis, muscle growth or function, the development or expression of secondary sexual characters, courtship behaviors, aggressive behaviors, paternal care, and, in some cases, immune function (Wingfield et al. 2001, Roberts et al. 2004, Fusani 2008, Schlinger et al. 2013, Foo et al. 2017). The ability of testosterone to influence multiple traits related to reproductive has led to the proposal that

testosterone functions as the proximate mediator of tradeoffs related to reproduction (Ketterson et al. 1992). Our understanding of these tradeoffs and the extent to which testosterone acts as a proximate mediator is, however, dependent on our understanding of the extent to which traits are sensitive to testosterone. In the context of cooperative reproductive behaviors, relatively few studies have examined testosterone's role in modulating individual variation. Quantifying the relationship between testosterone and cooperative behavior is important as some traits can act as constraints on the evolution of circulating testosterone levels (Hau 2007, Ketterson et al. 2009). In Chapter III, I show that testosterone has antagonistic effects on the cooperative behavior of territory-holding male wire-tailed manakins. The cooperative display behavior of territory-holding males has previously been shown to be predictive of their reproductive success, therefore, reduced cooperative behavior is a novel cost of maintaining high levels of circulating testosterone. In other words, for species in which it is advantageous to cooperate, maintaining high testosterone levels may be detrimental to their fitness. The cost of maintaining high testosterone for male wire-tailed manakins is, however, status-specific. Specifically, among floater males, testosterone has been shown to promote behaviors that are associated with social rise (Ryder et al., preprint). Therefore, future studies examining the relationship between testosterone and cooperative behavior may find high testosterone to be beneficial in other cooperative contexts or in specific social classes. Given that few studies have examined patterns of individual variation in both cooperative behavior and circulating testosterone, measuring this relationship using both observational and experimental approaches in other species represents an exciting avenue for future research.

### **Integrating Testosterone and Telomeres to Understand Individual Variation in Behavior**

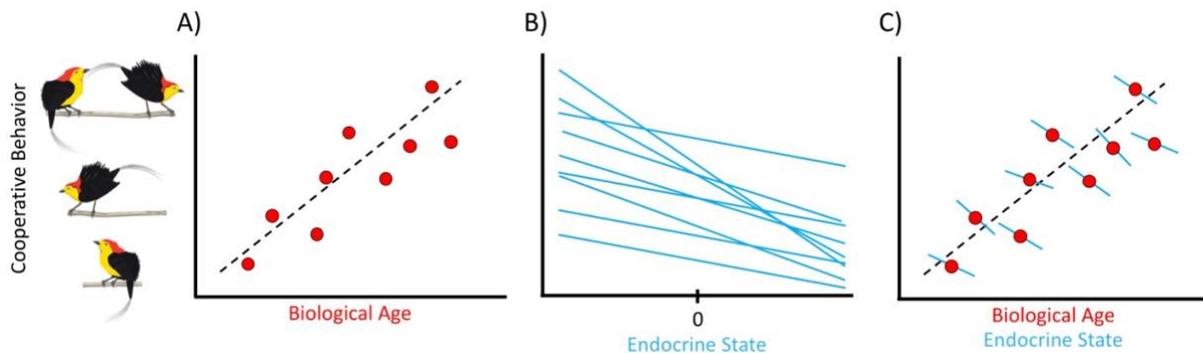
Outstanding questions regarding the evolution of cooperation, as well as animal behavior more generally, surround trying to understand the sources of this individual variation in behavior (Sih et al. 2015). State-based models represent a powerful framework that have previously been implemented to understand the sources of individual variation in behavior (Dingemanse and Wolf 2010). State can broadly be defined as any aspect of an individual or their environment that influences the costs and benefits of expressing a specific behavior (Houston and McNamara 1999). I demonstrate that an individual's endocrine state (Chapter III) as well as biological age (Chapter IV) explain individual variation in their cooperative behaviors. These results represent some of the few studies that have elucidated the mechanistic underpinnings of individual variation in cooperative behaviors and are complimentary in that the two mechanisms exhibit different levels of repeatability.

The repeatability of a given trait represents the extent to which individuals consistently vary in a trait as well as the extent to which variation in a trait can be attributed to within-individual variation (Nakagawa and Schielzeth 2010). Consistent individual variation in behavior is thought to be driven by state-variables that exhibit similar levels of repeatability, while within-individual plasticity is driven by highly labile traits (Holtmann et al. 2017). Circulating hormone levels are thought to be underlie within-individual plasticity as they exhibit fairly low repeatability and function as integrators of proximate environmental information (Holtmann et al. 2017, Kelly and Vitousek 2017, Goymann et al. 2019). Telomere lengths, on the other hand, exhibit high levels of repeatability among adults (Chapter IV), are primarily determined by an individual's developmental environment (Hausmann and Heidinger 2015, Angelier et al. 2018), and are predictive of an individual's mortality risk (Wilbourn et al. 2018). Given that an individual's residual reproductive value is partly determined by their mortality risk (Williams

1966, Wolf et al. 2007), telomeres have been proposed to relate to consistent behavioral variation that is determined by an individual's remaining reproductive potential (Young et al. 2018, Giraudeau et al. 2019).

My results support the idea that circulating hormones underlie within-individual plasticity while consistent individual variation in behavior is due to variation in an individual's residual reproductive value. Specifically, I found that circulating testosterone was related to behavioral variation that occurred over the 3 days prior to measurement, while variation in telomere length was related to more consistent individual variation in cooperative behaviors that was measured over a month. Therefore, my work can serve as an example of a new powerful framework for understanding the sources of both behavioral plasticity as well as consistent individual variation in behavior. This framework centers around measuring an individual's endocrine state and their biological age in combination with measurements of both consistent individual variation in behavior and within-individual behavioral plasticity (Figure 1). Future works on cooperative behaviors should utilize this framework to further understand the mechanistic basis of both within- and among-individual variation in cooperation and, therefore, the evolution of cooperative behavior more broadly.

**Figure 1.** Conceptual diagram describing the mechanistic basis of consistent individual variation in behavior and within-individual variation in behavioral plasticity. Consistent individual variation in behavior (A) is due to the positive relationship between biological age (as indicated by telomere lengths) and cooperative behaviors (Chapter IV). Within-individual plasticity (B) in cooperative behavior, on the other hand, is due to the negative relationship between an individual's endocrine state (i.e., circulating testosterone) and cooperative behaviors (Chapter III). Mean centering is used to represent within-individual plasticity in cooperative behavior and variation in the slopes of the lines is due to individuals varying in their sensitivity to circulating testosterone. Both consistent individual variation and within-individual plasticity are displayed in C.



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