SPECIAL ISSUE: MATING DISPLAYS

Animal Behaviour 184 (2022) 185–195

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Special Issue: Mating Displays

Physiological innovation and the evolutionary elaboration of courtship behaviour

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ARTICLE INFO

Article history:
Received 19 September 2020
Initial acceptance 27 November 2020
Final acceptance 13 January 2021
Available online 14 May 2021
MS. number: SI-20-00704

Keywords:
courtship behaviour
elaborate courtship display
golden-collared manakin
hormonal signalling
mate choice

Biologists have long been fascinated by the elaborate courtship displays performed by diverse organisms throughout the animal kingdom. The evolution of courtship behaviour often requires specializations of neural, sensory and motor systems. In addition, physically impressive displays may also require optimized metabolic, respiratory and cardiovascular systems to sustain the neuromuscular demands. Hormonal signalling can reach all of these tissues simultaneously to prepare them for use in courtship. Studies of male golden-collared manakins, *Manacus vitellinus*, a small bird of the Neotropics with a physically intense and noisy courtship display, have uncovered numerous androgen-dependent neuromuscular and metabolic specializations that enable not only the performance of elaborate courtship routines, but also their evolutionary exaggeration. However, physiological specializations for one function can create limits on their use for other purposes. Such trade-offs may influence the way courtship develops but may also provide information used by females for mate choice. We review this body of work with an eye towards expanding our appreciation of the evolution of widespread tissue hormone sensitivity and hormone action as the system through which elaborate courtship behaviours evolve.

Courtship is a long-studied phenomenon in the field of animal behaviour, in which one individual of a given species (often males, but not always) attempts to obtain copulations from another (Bastock, 2018). Iconic examples of courtship include the elaborate mating dance of many tropical birds, like the birds of paradise (Miles & Fuxjager, 2018), or the raucous vocal choruses of frogs that call into the night (Gerhardt & Klump, 1988). Evidence of courtship behaviour exists throughout the entire animal kingdom in a wide range of diverse invertebrates and vertebrates (Mitoyen, Quigley, & Fusani, 2019), occurring even in species like lampreys (Buchinger et al., 2017). Many of the display routines used to mediate courtship are highly complex behavioural programmes, which invariably involve multiple constituent components that simultaneously stimulate a variety of sensory modalities in the receiver (Hebets & Papaj, 2005; Mitoyen et al., 2019). Of course, the sheer diversity of courtship behaviour among species is its most spectacular feature, with courtship displays evolving in a seemingly endless number of ways (Mitoyen et al., 2019). With these considerations in mind, it is perhaps unsurprising that a major goal of animal behaviour research is to figure out the function and evolution of courtship behaviour in the natural world.

One way that biologists have attempted to address this objective is by studying morphological, physiological and neurobiological bases of courtship (Clifton, Hedrick, & Biewener, 2015; Husak & Lovern, 2014; Leininger & Kelley, 2013; Losos, 1990; Remage-Healey & Bass, 2006; Remage-Healey, Coleman, Oyama, & Schlinger, 2010; Schuppe et al., 2017). Indeed, this issue represents a fascinating biological puzzle. For example, the evolution of an elaborate display occurs by way of selection acting on a signaller (Guilford & Dawkins, 1991; Hebets & Papaj, 2005), which drives changes to the neural programmes that underlie behavioural production and the organ systems that support this process (e.g. skeletal muscular system, cardiovascular system, respiratory system, etc.). At the same time, this evolution takes place in a landscape defined in part by the physiology of the receiver (Guilford & Dawkins, 1991). This individual observes and responds (or not) to the courtship display, and its own sensory biases,

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https://doi.org/10.1016/j.anbehav.2021.03.017
0003-3472/

cognitive capacity and physiological abilities help define the effects of selection on the signaller (Endler, 1992; Rosenthal, 2018; Ryan & Keddy-Hector, 1992). Despite the decades-long recognition of these intricate evolutionary relationships, it often seems like we are still in the infancy of understanding how this all works to produce the remarkable array of courtship phenotypes we see.

Our research groups have focused on these issues largely through the lens of the signaller, and thus the physiological basis of display performance. In this way, our thinking boils down to understanding the anatomical and functional specializations of the signaller's neuromuscular and sensory systems. This is because these systems generate the behaviour that receivers detect to make their mate choice decisions. The developmental and functional limits of these systems therefore stand to constrain the production of a successful display, and thus its evolutionary trajectory. For example, selection can only favour display speeds that fall within the physiological realm of neuromuscular possibilities for the signaller. However, elaborate courtship displays may also require optimized metabolic, respiratory and cardiovascular systems to sustain those neuromuscular demands. Modification to these systems may therefore be necessary to indirectly support the motor control of courtship.

Here, we explore these topics by reviewing the body of research on the physiology and evolution of elaborate courtship displays in male golden-collared manakins, *Manacus vitellinus*, a tropical bird that inhabits the rainforests of Central America and Colombia. Our aim is to crystalize a framework that fosters thinking about how existing physiological substrates that support vital functions in animals are evolutionarily repurposed to underlie sexual selection for courtship behaviour. In this way, we highlight the complexities of behavioural design in the natural world, whereby complex selection regimes influence the traits, tactics and strategies an animal uses to navigate its social and physical environment.

ELABORATE COURTSHIP BEHAVIOUR IN GOLDEN-COLLARED MANAKINS

Golden-collared manakins are part of a larger family of suboscine passerines (Pipridae) that emerged from Amazonia. The clade's history is marked by strong sexual selection, such that males of most species have bright, colourful plumage, adopt lek mating systems and court females by performing acrobatic and elaborate dance displays (Prum, 1990, 1994). Golden-collared manakins exemplify these features (Chapman, 1935; Lowe, 1942). Females are drab green, whereas males have deep black wings and a bright golden collar made of elongated feathers that can be erected into a beard (Stein & Uy, 2006). Males form leks of ~3–15 individuals in which each bird makes its own courtship arena by clearing a small (1 m) circle on the forest floor of all leaf litter (Chiver & Schlinger, 2017a; Fusani, Giordano, Day, & Schlinger, 2007; Schlinger, Day, & Fusani, 2008). Males perform their courtship dance by leaping among saplings that surround the display arena, snapping their wings together above their backs in mid-air. This creates a loud and conspicuous firecracker-like pop that penetrates the forest (Bodony et al., 2016; Fusani, Giordano, et al., 2007). Females watch the display from above; if they decide to engage the male further, they join the dance by flying (not jumping) among the saplings (Barske, Schlinger, & Fusani, 2015). One idea is that the females participate in this manner to assess male condition by challenging his motor abilities (Barske et al., 2011, 2015; Fusani, Barske, Day, Fuxjager, & Schlinger, 2014). Males establish consistent dance choreographies that appear to result from motor learning (Janisch, Perinot, & Fusani, 2020), suggesting that precision achieved through fine neuromuscular control is also subject to assessment during mate choice.

The so-called *jump-snap* display described above is not the only display routine that males produce. For example, they can also generate *wing-snaps* away from the arena to either attract females they see in the forest to their court or compete with other males from within and outside the lek (Fusani, Barske, et al., 2014; Schlinger, Barske, Day, Fusani, & Fuxjager, 2013; Schlinger et al., 2008). Perhaps one of the most interesting manoeuvres is a permutation of the wing-snap, in which males produce multiple snaps in rapid succession. This display is called a *roll-snap*, and it sounds like a loud mechanical buzz. From a physiological perspective, roll-snaps are especially intriguing because they are the product of wing beat movements above the back at speeds of nearly 60 Hz (snaps per second) (Fuxjager, Longpre, Chew, Fusani, & Schlinger, 2013; Miles, Goller, & Fuxjager, 2018). This is roughly two times the maximum wing beat frequency that the size of a golden-collared manakin would use to power flight (Donovan et al., 2013); presumably, the muscle tissues that actuate these displays are modified to perform accordingly.

On the receiver side, mate choice studies suggest that females prefer to mate with males that perform these displays more frequently and at fraction-of-a-second faster speeds (Barske et al., 2011). On one hand, this behavioural work confirms that the golden-collared manakin's exaggerated display repertoire likely evolves through forces of strong sexual selection by female choice. On the other hand, these findings also make a more intriguing point – namely, that display exaggeration occurs through selection for extraordinary vigour and motor skill. We can think of vigour as the frequency with which individuals perform a display, such that greater vigour underlies more frequent production (Beyers, Hebets, & Podos, 2010). By contrast, skill refers to how well a given display is generated (Beyers et al., 2010). This attribute is more nebulous to conceptualize, but it likely involves display performance with greater speed, agility, coordination, etc. (Manica, Macedo, Graves, & Podos, 2016; Schuppe & Fuxjager, 2018).

Display vigour and skill are in many ways emergent properties of motor functioning. Vigour, for instance, might be related to properties of endurance, or resistance to fatigue. Meanwhile, skill might be related to any number of performance attributes associated with motor control, such as how fast individuals produce a display manoeuvre or the precision with which the behaviour is executed. Equally important to recognize is that motor skill may be tied to the simultaneous production of multiple performance attributes. In other words, a skillful display might be one that is executed with both great speed and great precision, which itself is challenging to accomplish (Batmaz, de Mathelin, & Dresp-Langley, 2016). Indeed, increasing the speed at which individuals perform exacting behaviour can lead to greater motor mistakes (Amir Abdul Nasir, Clemente, Wynn, & Wilson, 2017; Wheatley, Niehaus, Fisher, & Wilson, 2018).

Bringing these ideas back to manakins and courtship, we expect that selection for elaborate display behavioural occurs through the concomitant evolution of the bird's motor system (Fusani, Barske, et al., 2014; Fuxjager & Schlinger, 2015). This means that physiological evolution likely influences the process by which behavioural evolution can (or cannot) unfold. Evidence for such effects is likely present in the neural and muscular systems that support wing and leg movement, as well as in the various acoustic, visual and proprioceptive sensory systems. Such modification would underlie the production of jump-snaps, wing-snaps and roll-snaps, which engage a variety of cognitive and motor pathways. Moreover, when a display is performed, afferent and efferent motor and somatosensory pathways must exert exceptionally fine control over contractile states of a vast number of the manakin's skeletal muscles. Of course, just how all of this behaviour is orchestrated remains a mystery, but our research groups have explored these physiological specializations and how they might arise over time. The remaining sections of this review will therefore describe much of this work, with an eye toward addressing principles that guide evolutionary coordination between physiology and behaviour.

ANDROGENIC HORMONES AND COURTSHIP BEHAVIOUR

Hormone systems regulate target tissues throughout the animal body, and thus integrate organ system functioning in response to a variety of intrinsic and/or extrinsic stimuli (Adkins-Regan, 2005). Differential evolution of the machinery that mediates such signalling across the body therefore alters how hormonal integration of physiology unfolds and impacts behavioural output (Cox, 2020; Fuxjager & Schuppe, 2018; Hau, 2007; Ketterson, Atwell, & McGlothlin, 2009). These principles are especially relevant to the nervous and muscular systems, including those mentioned above that regulate sexual behaviour (Canoine, Fusani, Schlinger, & Hau, 2007; Johnson, Kircher, & Castro, 2018; Rosvall et al., 2012; Voigt & Goymann, 2007).

Selection for reproductive behaviour, including courtship, often takes place through the evolution of sex steroid systems (e.g. pathways of androgenic and oestrogenic action) (Crews et al., 1984; Crews & Moore, 1986). Such effects are most commonly studied in males, focusing on the (adaptive) modification of the molecular pathways by which androgens are produced and detected throughout the body. This includes research of species differences in the expression patterns of enzymes that make testosterone (T) and/or dihydrotestosterone (DHT) in the gonads and other parts of the body (Schmidt et al., 2008; Soma, Scotti, Newman, Charlier, & Demas, 2008), as well as androgen receptor (AR) and its signalling cascade in target tissues throughout the brain, spinal cord and skeletal muscles (Brantley, Marchaterre, & Bass, 1993; Mangiamele et al., 2016; Rand & Breedlove, 1992, 1995; Sassoon, Gray, & Kelley, 1987; Schuppe & Fuxjager, 2019; Veney & Wade, 2004). By altering the location and abundance of these proteins' expression, evolution can reshape the capacity of androgenic hormones to influence the probability of behaviour (Cox, 2020; Fuxjager & Schuppe, 2018; Hau, 2007). However, there is a major point to keep in mind about the evolution of the androgenic system — T and/or DHT action are often thought to impose evolutionary ‘costs’ to an organism (Folstad & Karter, 1992; Roberts, Buchanan, Hasselquist, & Evans, 2007). For instance, T can be immunosuppressive or change behaviour in a manner that dramatically reduces viability (Ezenwa, Ekernas, & Creel, 2012; Fuxjager, Foufopoulos, Diaz-Uriarte, & Marler, 2011; Marler & Moore, 1988; Owen-Ashley, Hasselquist, & Wingfield, 2004). The mechanisms underlying these effects are not always abundantly clear and seem to differ among taxa; nevertheless, the ‘costs’ of androgenic action mean that evolution must balance these potentially negative effects with the advantageous ones associated with behavioural innovation.

Our research suggests that evolution of the androgenic system — as described above — underlies the adaptive elaboration of courtship in golden-collared manakins. At the onset of the dry season in Panama and Colombia, males begin to gather in leks, with each male establishing its display court. Relative to the wet season, when courtship is minimal, T levels in the blood quickly rise (Day et al., 2007). Behavioural studies indicate that this androgen activates numerous elements of the male's courtship display and vocal repertoire (Chiver & Schlinger, 2017b, 2019; Day et al., 2006, 2007). For example, within a week after being treated with slow-release capsules of T and released into an aviary surrounded by rainforest, juvenile males (who maintain low T without such implants) will aggressively claim display courts and perform their full range of behaviours that include keeping the courts cleared of dried leaves, performing jump-snap displays and producing wing-snaps as well as roll-snaps (Chiver & Schlinger, 2017a, 2017b, 2019). Once elevated, however, it is not clear if the amount of T in blood is at all relevant to the amount or quality of the male's courtship display (Fusani, Day, et al., 2007). Moreover, we also have evidence that, across species of manakins, absolute levels of testosterone in blood in sexually mature males bear little on the complexity of a species' courtship display (Fuxjager, Miles, & Schlinger, 2018).

What does appear to bear more closely on male courtship involves the absolute levels of androgen receptor (AR) in various tissues. In the brain, AR is widely expressed across the arcopallium (Fusani, Donaldson, London, Fuxjager, & Schlinger, 2014), a region that is known to house premotor circuits and that usually expresses AR only in its more medial part (Metzdorf, Gahr, & Fusani, 1999). We also find AR in nucleus intercollicularis (Fusani, Donaldson, et al., 2014), a brain region known to innervate the tracheosyringeal nucleus, which contains the motoneurons that innervate the syrinx (avian vocal organ). In the spinal cord, AR is also widely expressed in motoneurons, especially in those motoneurons innervating muscles used in male courtship (Fuxjager, Schultz, et al., 2012; Schultz & Schlinger, 1999). In addition, AR is expressed abundantly in somas of afferent sensory neurons housed in dorsal root ganglia (Fuxjager, Schultz, et al., 2012). Lastly, AR is expressed widely and abundantly in much of the skeletal muscle tissue, including the muscular and cartilaginous tissues of the syrinx (Feng, Katz, Day, Barske, & Schlinger, 2010).

Comparative research creates a richer evolutionary framework to appreciate the link between AR expression and the golden-collared manakin's courtship behaviour. For instance, relative to a variety of passerine species, male golden-collared manakins express higher levels of AR in their wing musculature (Fig. 1; Fuxjager et al., 2015). Importantly, this analysis includes closely related manakin species, such as the blue-crowned manakin, *Lepidothrix coronate*, and the lance-tailed manakin, *Chiroxiphia lanceolata*, which do not perform rapid wing displays in their courtship routines. One interpretation of these results is that elevated AR is a key molecular tool associated with muscular actuation of rapid display behaviour. Indeed, compelling support for this idea comes from additional analyses that uncovered a strong positive predictive

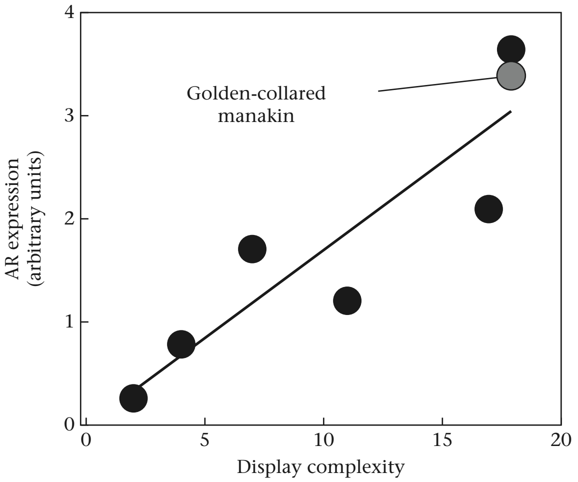

Figure 1. Positive relationship between androgen receptor (AR) expression in the wing musculature and species differences in the overall complexity of their behavioural display routines. The correlation is statistically significant ($P < 0.05$; black line represents best-fit line), with each dot representing a different passerine species. The grey dot represents the golden-collared manakin. The statistical model accounted for the shared phylogenetic history among the different species. Note that complexity was measured as the total number of discrete movements incorporated into a singular display routine (Fuxjager et al., 2015; Lindsay, Giuliano, Houck, & Day, 2015). Data from Fuxjager et al. (2015).

relationship between species variation in courtship dance motor complexity and wing muscle AR expression levels (Fuxjager et al., 2015). This connection between motor intricacy of courtship and putative muscular androgen action persists despite the shared ancestry among the taxa included in the analysis, suggesting coevolution between these variables. Interestingly, this relationship does not exist in the spinal cord, and there is no association between species differences in display complexity and oestrogen receptor (ER) expression in either wing muscle or spinal cord. Thus, for golden-collared manakins, these findings are consistent with the hypothesis that selection for an exaggerated and complex courtship routines the evolution of wing muscle AR.

In addition to the expression of AR, many of these target tissues express the enzyme 5α-reductase, converting T to its more potent androgenic metabolite 5αDHT (Fuxjager, Schuppe, et al., 2016). This is a key part of androgenic signalling, particularly in birds, because androgenic effects may very well occur via 5αDHT, rather than by T itself (Jalabert, Ma, & Soma, 2020). Thus, the enzymatic machinery necessary to boost androgen signalling in the motor system appears to be upregulated in the golden-collared manakin.

ANDROGENIC ACTIVATION OF COURTSHIP

If the idea described above is true, then one would predict that androgenic activation of muscular AR helps males generate a more effective signal. Multiple studies have addressed this idea. The first showed that administration of the widely used AR antagonist flutamide reduces courtship in free-living adult males, reducing the frequency in which individuals perform wing-snaps, roll-snaps and jump-snap displays (Fusani, Day, et al., 2007). Flutamide blocks AR throughout the entire body, and thus the effects of AR on display behaviour could not be pinpointed to any particular target tissue or set of target tissues. As such, because AR expression is especially high in the golden-collared manakin musculature, a second study tested how the peripherally selective AR inhibitory called bicalutamide (also called Casodex) influences male courtship in the wild. The results of these experiments were quite striking – after only a few days of treatment, birds given bicalutamide showed reduced performance in the number of wing-snaps, roll-snaps and jump-snap displays males produced (Fuxjager et al., 2013). Furthermore, treated birds decreased the total number of snaps they included in a roll-snap, while also reducing the speed at which they generated this display. These latter effects were seemingly subtle, with treated males slowing roll-snap speeds by 3–4 ms. Males given bicalutamide did not abandon their display courts, which suggest that they remained at least somewhat sexually motivated. From this work, we posit that androgenic activation of peripheral AR mediates the fine motor control of display performance, whereas central AR may activate sexual motivation. Of course, it is also possible that some sexual motivation in manakins is activated by oestradiol (E_2) binding to ER, as both aromatase (which catalyses the conversion of T to E_2) and ER are expressed in the manakin hypothalamus (Fusani, Donaldson, et al., 2014; Saldanha, Schultz, London, & Schlinger, 2000).

Finally, a separate study suggests that bicalutamide treatment also modifies male vocal output (Fuxjager, Heston, & Schlinger, 2014). We found that the male's dominant courtship vocalization, the *chee-poo* call, showed an increase in duration (chee note) and a decrease in the fundamental frequency (poo note). These effects are presumably the results of altered androgenic modulation of musculature and/or cartilaginous tissue of the manakin syrinx. Importantly, the repetition rate of chee-poo calls remained unchanged by bicalutamide, suggesting that the motivation to call was unchanged by peripheral AR. Additional studies in females show that T activates chee-poo production, although the acoustic parameters do not appear to match those of an adult male. Similarly, if juvenile males are given T, changes to call duration and frequency are apparent; however, not in the direction we would expect from the adult male data described above (Chiver & Schlinger, 2019). This suggests clear androgen-dependent control of vocal behaviour, but a complex relationship whereby androgenic regulation varies according to factors related to ontogeny, sex differences and location of hormone action within the bird.

FUNCTIONAL EFFECTS OF ANDROGENS ON COURTSHIP DISPLAY

The work described above suggests that elevated expression of AR – particularly in the wing muscles – is an adaptive trait that enhances display performance by increasing its speed and frequency. But, how does AR trigger such effects? This question is as compelling as it is straightforward, because it provides insight into why the androgenic systems provides a conduit in which selection can drive the evolution of courtship behaviour to an extreme. The golden-collared manakin provides an ideal system to address this issue, because functional effects of androgenic action on muscle are more easily assessed than functional effects of androgen action on discrete brain nuclei, at least with respect to the way these functional effects impact behavioural production. In this spirit, we have performed a variety of experiments to elucidate how AR acts via the skeletal muscular systems to regulate display production.

A good starting point to this topic is a brief primer about how vertebrate striated muscle works (also see Syme & Josephson, 2002 for another excellent description of how muscle works). This tissue is made up of long, parallel fibres, or cells (myocytes), that are filled with two main contractile filaments, which run parallel to each other. This includes thick filaments that are made from myosin proteins, as well as thin filaments that are made from actin proteins. Striated muscle fibres also contain machinery that mediates the flow of calcium ions (Ca^{2+}) from storage centres called sarcoplasmic reticulum to the myocytic cytoplasm (and then back again). Muscle contraction is a complex process that hinges on an ATP-dependent cycle, in which myosin repeatedly binds to, pulls on, and detaches from actin. This causes both myosin and actin filaments to slide past each other, and thus shortens the entire fibre. Calcium regulates these events – high concentrations of Ca^{2+} in the fibre cytoplasm facilitate the myosin and actin interactions that shorten the fibre, whereas low concentrations of Ca^{2+} in the fibre prevent this process from occurring. The nervous system activates a muscle (causes it to contract) because action potentials from motoneurons trigger the release of Ca^{2+} from the sarcoplasmic reticulum, which consequently floods the cytoplasm and induces fibre shortening. In the absence of neural stimulation, Ca^{2+} is pumped from the cytoplasm into the sarcoplasmic reticulum, where it does not interact with the contractile filaments.

With these principles in mind, AR can modify many aspects of this machinery to change how muscles work. This is because AR is a ligand-activated transcription factor; thus, when T or DHT binds to AR, the complex translocates to the cell nucleus to regulate gene expression (Chang, 2002). Because all the machinery described above is little more than an amalgam of different proteins encoded by various genes, AR has the capacity to regulate the muscular apparatus (Wyce, Bai, Nagpal, & Thompson, 2010; Yoshioka, Boivin, Bolduc, & St-Amand, 2007). For instance, AR can alter muscles in a way that enhances their strength or their ability to generate force (Chambon et al., 2010; Oki, Wiseman, Breedlove, & Jordan, 2013). Similarly, by increasing the expression of proteins that mediate Ca^{2+} flow through the fibre, AR can enhance the speed of contraction (see below). Many of these parameters are especially important for the golden-collared manakin, in which display performance likely hinges on a muscle's ability to quickly contract and relax several times in a row. Likewise, the muscles may need to quickly contract to catapult males through the air, as they perform their incredible jump-snap displays around their courtship arena.

We have conducted a number of studies to investigate these possibilities. For example, through a series of pharmacological manipulations and RNA-seq analyses, we showed that AR upregulates a host of genes related to muscle contractile dynamics (Fuxjager, Lee, et al., 2016). For example, T increased the expression of genes that encode proteins for shuttling calcium through the muscle cell, as well as a variety of novel myosin isoforms. Likewise, T also enhanced the expression of genes that promotes lipid transport out of the myocyte, which in turn reduces triglyceride accumulation in the muscle to potentially help attenuate insulin resistance (Fuxjager et al., 2018). The functional result may be to enhance glucose uptake during bursts of activity that underlie the demanding 'exercise' that makes up the courtship display.

From a comparative standpoint, this transcriptomic work also shows that higher levels of AR in the manakin wing muscle confer a more robust transcriptional response to androgenic hormones (Fuxjager, Lee, et al., 2016). In other words, T upregulates more genes, compared to other species with less AR in their wing muscles. The magnitude of upregulation in this context is also greater in the manakin, meaning that T causes the levels of expression to increase more than they otherwise would if AR levels were lower. Altogether, these data suggest that abundant myocytic AR provides a mechanism for a specialized cellular response to sex steroid action.

In a second set of studies, we investigated how AR influences wing muscle performance itself. This work focused on a dorsal muscle called the scapulohumeralis caudalis (SH), which originates along the scapula and inserts on the humerus (Fig. 2a). During flight, the SH is thought to mediate wing retraction and rotation. However, in golden-collared manakins, studies using radiotelemetric electromyography (EMG) suggest that the SH is the main muscle that underlies the snapping movements of the wing-snap and roll-snap (Fuxjager, Fusani, et al., 2017). As a result, this muscle is the likely target of selection for fast display. High-speed videography combined with computational modelling showed that the loud snaps are produced by the modified wing bones colliding at very high speed (Bodony et al., 2016; Friscia et al., 2016). Further work assessing SH performance revealed that its twitch speeds (the speed at which the muscle can repeatedly contract and

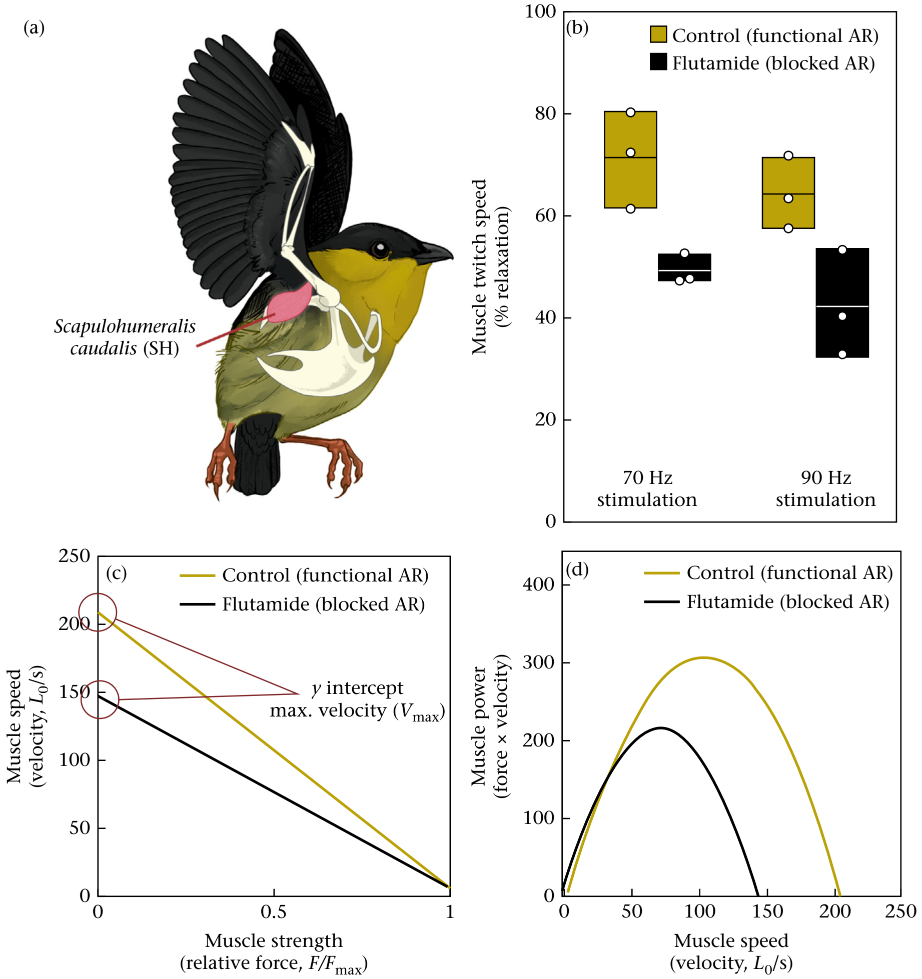

Figure 2. (a) Illustration of the scapulohumeralis caudalis (SH) muscle in the golden-collared manakin. Modified from Miles et al. (2018) through Creative Commons by 4.0 license. (b) Muscle twitch speed (as measured by average % relaxation during stimulation train) of the SH in adult male golden-collared manakins treated with either blank implants or flutamide implants to block androgen receptor (AR). Flutamide treatment significantly ($P < 0.05$) decreases twitch speeds. (c) Force–velocity relationship of SH in birds from the same experiment (control versus flutamide treatment). Flutamide flattens the slope of the regression line, suggesting that speed is decreased with respect to the production of the same amount of relative strength (force). *Y* intercepts represent estimates of the maximum velocity (V_{max}) for the muscle, with flutamide decreasing V_{max} estimates. (d) Power curve of the SH in birds from the same experiment (control versus flutamide treatment). Flutamide decreases estimates of power output by the muscle, which in turn suggests that androgenic action increases SH speed and boosts SH power. Panels (b–d) are modified from Fuxjager, Miles, et al. (2017).

relax without fusing) are extraordinarily fast, generate discernible twitches at ≈100 Hz (body temperature ≈ 33–40 °C) (Fuxjager, Goller, Dirkse, Sanin, & Garcia, 2016). This is far faster than the twitch times of flight muscles in other birds, including hummingbirds, which stop showing discernible twitches around 50 Hz (body temperature ≈ 40 °C) (Hagiwara, Chichibu, & Simpson, 1968). In fact, given the twitch speed of the manakin SH, this tissue could be classified as a 'superfast' limb muscle. Other superfast muscles include those of the toadfish swimbladder, avian syrinx, bat larynx and rattlesnake tailshaker (Elemans et al., 2008, 2011; Rome et al., 1999). Of these tissues, the manakin SH is perhaps most similar in terms of speed with the rattlesnake tailshaker, which can drive tail rattling at ≈ 85 Hz at 35 °C (Schaeffer, Conley, & Lindstedt, 1996); nevertheless, both of these tissues are extraordinarily fast, and thus likely sit next to each other at the lower end of the superfast category.

From an endocrine standpoint, we have shown that androgenic hormones mediate the superfast contractile properties of the SH. If, for example, adult males are given flutamide, then SH twitch speeds decrease substantially (Fig. 2b; Fuxjager, Miles, Goller, Petersen, & Yancey, 2017). This means that the muscle begins to fuse (reach a tetanic state) at a lower stimulation frequency, which suggests that it is not capable of producing distinct contractions at high rates necessary for the roll-snap. At the same time, this work shows that AR boosts estimates of $V_{\max}$, or maximal estimated contraction velocity (Fig. 2c). This value is proportional to the cross-bridge detachment rate (Huxley, 1957), which sets the speed of sarcomere shortening during muscular contractions. These studies therefore dovetail with molecular work by showing that AR-mediated expression of genes related to calcium trafficking and myofilament composition/dynamics likely supports dramatic changes to two properties of extreme speed – twitch times and shortening velocity (Fuxjager, Barske, Du, Day, Schlinger, 2012; Fuxjager, Lee, et al., 2016). In this way, we can conceptualize why selection for rapid display manoeuvring likely favours increased expression of AR in the musculature that actuates this behaviour.

DISPLAY EVOLUTION THROUGH A TRADE-OFF LANDSCAPE

Adaptive changes to animal performance often occur along an 'evolutionary tightrope', given that so many performance attributes are mutually exclusive. Thus, if selection favours the emergence of a behavioural trait that emphasizes one aspect of performance, then other traits associated with opposing performance skills might be blocked from arising (Garland, 2014; Heinen-Kay et al., 2015; Levinton & Allen, 2005). Speed is a perfect example of this phenomenon – it comes at a cost to both strength and endurance, particularly when extreme speed arises to support facets of behaviour (Rome et al., 1999; Rome & Lindstedt, 1998; Vanhooydonck, Van Damme, & Aerts, 2001; Wilson, James, & Van Damme, 2002). It is therefore no surprise that the fastest muscles are often those with singular functions, because these tissues are liberated from the constraints imposed by intrinsic performance trade-offs (Mead et al., 2017). The muscles of the syrinx are a perfect example, as they function primarily to regulate airflow through the trachea to generate sound and are not constrained by other quotidian functions and/or processes (Goller & Riede, 2013; note, for example, that birds also have a larynx that protects their airways). However, for species that display by engaging the musculature of their limbs and trunk, performance trade-offs can pose a major evolutionary hurdle because these tissues also need to control other 'normal' behaviours, like locomotion.

Speed versus Force

The golden-collared manakin is an intriguing system to explore how performance trade-offs might influence display evolution. If, as we describe above, selection drives the evolution of display speed, then it might do so by favouring the evolution of faster skeletal muscles. Rapid roll-snaps should be accompanied by an especially rapid SH muscle. However, increasing the speed of the SH muscle might simultaneously hamper its ability to generate force, which in theory could alter the muscle's function in flight. One way around this problem would be to recoup muscular strength through other modifications to the tissue. Indeed, we suspect this is the case with the SH, given that this tissue is markedly hypertrophied in male golden-collared manakins (Schultz, Hertel, Bauch, & Schlinger, 2001). Thus, we suspect that the evolutionary push to superfast contractile speeds was compensated for by a simultaneous push toward increased strength by enhancing muscle fibre diameter and number. Building on this idea is work showing that T acts on the SH to increase the expression of IGF-1 (insulin-like growth factor 1), a growth factor known to trigger muscle hypertrophy (Fuxjager, Barske, et al., 2012). Other work shows that AR mediates the SH in a manner that preserves its ability to generate force, despite a concomitant increase in $V_{\max}$ (Fig. 2c; Fuxjager, Miles, et al., 2017). The result of this effect is an SH that can generate power, which is the product of force and velocity (Fig. 2d). In this way, androgens likely provide a mechanism to bypass constraints imposed by the intrinsic trade-off between speed and strength.

Still, the suite of traits that endow the SH with both speed and strength can, and likely do, impose their own set of evolutionary costs. Studies show, for example, that especially large muscles can impose above-average energetic demands, and even potentially suppress native immune function (Lassek & Gaulin, 2009). Likewise, small limb muscles are associated with enhanced locomotor activity in certain contexts (Garland et al., 2002). Thus, for the manakin, one might expect that a large SH imposes similar costs that influence an individual's survival and time budget. At the same time, we recognize that a large SH might hinder the kinematics of sexual display behaviour, even including the production of the roll-snap itself. The latter possibility is intriguing, because it implies that sexual selection for an effective display favours the evolution of a fast, smaller SH, whereas natural selection for efficient locomotion would favour a larger SH to ensure that the tissue's power is preserved. Future studies are certainly needed to explore these ideas; for example, it would be particularly interesting to test whether a large SH muscle encumbers aspects of flight, such as manoeuvrability and/or endurance.

Speed versus Endurance

Endurance is another performance attribute that can trade off with speed (Schaeffer et al., 1996; Vanhooydonck et al., 2001; Wilson et al., 2002), and studies in golden-collared manakins have thoroughly explored this phenomenon with respect to the SH (Miles et al., 2018). As we describe above, this muscle shows incredible fast twitch kinetics – it relaxes around 50% in response to stimulation frequencies of 100 Hz. Wing movement can even be observed when the muscle is stimulated at this speed, suggesting that the tissue is capable of generating positive work in this context (Fuxjager, Goller, et al., 2016). However, when we examine twitch dynamics more closely, we see that the % relaxation changes over the course of a given high-speed stimulation train. For example, the SH fully relaxes in response to the first few stimulations in a 100 Hz train, but the % relaxation quickly begins to decline as the train progresses (Miles et al., 2018). By the end of the stimulation train, the muscle is nearly completely fused. However, at lower-frequency stimulation traits, such as 50 Hz, these effects are absent – rather, the SH fully relaxes across the entire stimulation train. The SH muscle therefore shows signs of fatigue only when it is repeatedly stimulated by the nervous system at exceptionally high frequencies.

How might this speed–endurance dynamic impact display behaviour, particularly a move like the roll-snap since it requires rapid, repetitive contractions of the SH? To address this question, we first assessed how the % relaxation changes across a stimulation train at a variety of different frequencies. Decline in the % relaxation is proportional to an increase in muscle fusion; thus, during high-frequency stimulations, we detect negative relationships between % relaxation and stimulation number within a given train (Fig. 3a). The slope of this line reflects the severity of the trade-off, with more negative slopes reflecting a steep drop-off in the % relaxation as a stimulation train unfolds.

Next, we plotted the various slopes from these analyses as a function of stimulation train frequency. This analysis produced its own negative relationship, whereby trade-off severity (slope, see above) decreased (becomes more negative) as stimulation frequency increased (Miles et al., 2018). The result itself makes intuitive sense: the SH begins to show signs of fusing only during high-frequency stimulation trains (>70 Hz; Fig. 3a and b). Notably, the best-fit line for this association intersects with the zero (0) slope value near the stimulation frequency that corresponds to the bird's average roll-snap speed (≈60 Hz). Thus, this finding suggests that birds roll-snap at speeds where the SH operates as fast as it can, without incurring costs to endurance. Presumably, these endurance costs manifest in terms of roll-snap length (total number of snaps), since the muscle would be unable to fully relax in a manner that would allow for a subsequent contraction. Behavioural data confirm this notion, showing a strong negative relationship between roll-snap speed and length across the entire population of golden-collared manakins throughout Panama and Colombia. We hypothesize that sexual selection has driven the evolution of roll-snap speed to its maximum, hitting the limits of SH performance because of a performance limit created by the trade-off with endurance. Further exaggeration of speed would require mechanistic innovations that help preserve full muscle contraction–relaxation cycles to further preserve signal length.

A closer examination of the muscle physiology data reveals what might be occurring at the level of the contractile machinery. SH relaxation times do not change during the course of a high-speed stimulation train, including stimulation trains that exceed 70 Hz (Miles et al., 2018). However, as we describe above, measures of % relaxation do in fact decrease during these stimulation trains. One possible explanation for this effect is that the calcium cannot be cleared from the myoplasm fast enough to sustain superfast speeds, even if the kinetics of actin and myosin contractile filaments can. If so, then there appears to be an evolutionary discord between the two sets of protein machinery that either mediate calcium transients or directly shorten the sarcomere. In line with our reasoning above, innovations necessary to enhance roll-snap speed would likely require a modification to the former.

Building on these insights, we have also found that AR influences the trade-off between speed and endurance in the SH. Specifically, activation of AR increases SH twitch speed in a manner that triggers the actual trade-off with endurance (Tobiansky, Miles, Goller, & Fuxjager, 2020). Thus, if AR is blocked in adult males, then SH twitch times are slowed, and we do not see any evidence of reduced % relaxation across the duration of a stimulation train. In this way, adaptive changes to the androgenic system appear to ‘imperfectly alter’ the performance landscape, in that they mediate beneficial shifts in display output that become stymied by a new suite of physiological constraints.

Effects of androgens on the speed–endurance dynamics in the golden-collared manakin display may have broader consequences for the species' evolution (Miles et al., 2018). Golden-collared manakins are part of a small genus with three other species, all of which are referred to as bearded manakins (*Manacus* manakins). This complex evolved roughly 300 000 years ago, emerging from Amazonia and moving north through the Isthmus of Panama. Phylogeographic modelling of ancestral ranges for the four different *Manacus* taxa suggests that the species showed a large degree of overlap during the speciation process, creating a biogeographical context for speciation with some degree of gene flow (or at least periods in which gene flow may have occurred during divergence, depending on other geological or climactic factors). In such a case, species differences in display behaviour may be a key factor contributing to assortative mating, which in turn helps enforce reproductive isolation between diverging populations. Support for this notion comes from comparative work in all *Manacus* showing that species differ in terms of roll-snap speed, but not length. Golden-collared manakins, for example, produce the fastest roll-snaps; thus, physiological innovations that boost speed may have augmented the ability for these signals to diverge

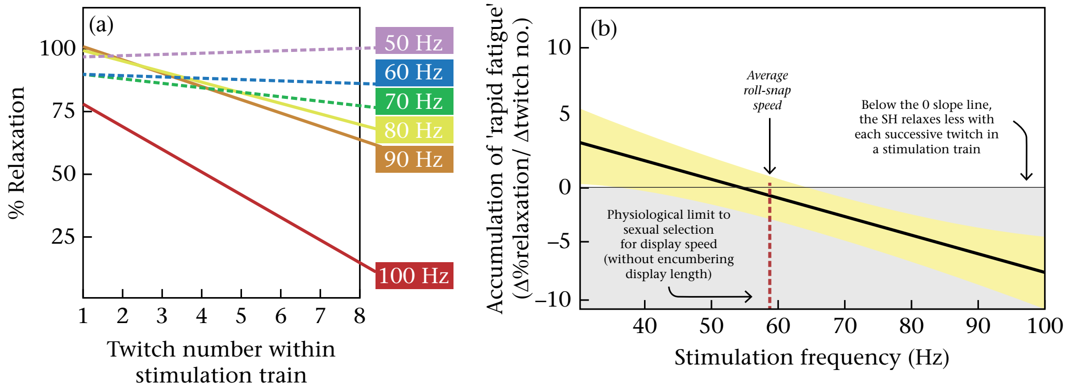

Figure 3. (a) Muscle twitch speeds (as measured by % relaxation) of the SH in adult golden-collared manakins during a single stimulation train. The horizontal axis depicts the twitch number within a stimulation train, which consists of eight separate 1 ms stimulations given at different frequencies (Hz; depicted through different colours indicated on the right of the graph). Solid lines represent statistically significant ($P < 0.05$) negative correlations between twitch number in a train and % relaxation. Note that lines become statistically significant at 80–100 Hz, which indicates when ‘rapid fatigue’ begins to appear during a stimulation train. (b) Relationship between stimulation frequency and the slope of the lines from panel (a), which indicate the severity of ‘rapid fatigue’ (greater propensity for fatigue during a stimulation train is reflected by a more negative slope, i.e. stimulations given at 80–100 Hz). The yellow shaded area on either side of the best-fit line represent the 95% confidence intervals (CI). Note that the best-fit line and upper CI intersect the zero (0) slope threshold (point below which suggests the presence of rapid fatigue) intersect near the species average roll-snap speed. This suggests that birds display as fast as they can, without incurring significant costs of ‘rapid fatigue’. Both panels are modified with permission from Miles et al. (2018) through Creative Commons by 4.0 license.

in a way that fosters speciation. Additional work is needed to explore this hypothesis, as few studies have looked at the physiological basis of traits that enable (or prevent) one species from splitting into two. Certainly, a host of other factors were similarly involved in the speciation within the *Manacus* complex (Bennett, Lim, & Braun, 2021), but our data imply that AR and muscle performance may have played a part in their own right.

BEYOND MOTOR SYSTEMS: ORGAN SPECIALIZATIONS THAT SUPPORT ELABORATE COURTSHIP

Although manakins clearly use numerous neuromuscular systems to vocalize and produce displays, our evidence suggests that males are not metabolically challenged by these activities (Barske et al., 2014). Using miniature telemetry on wild courting males, we first found that when males performed wing-snap displays, as well as roll-snaps, their heart rates accelerated briefly to 1000 beats/min or greater, as high as seen in some hummingbirds in flight (Barske et al., 2011). Interestingly, we also saw that heart rates of courting males accelerated rapidly, and then rapidly returned to baseline. Such a heart rate pattern suggests that courtship did not produce an oxygen deficit, as might be expected from a bout of intense physical activity (Fagard, 2003). Perhaps male manakins have optimized their metabolic physiology to enable courtship?

To further explore metabolic properties of male golden-collared manakins, we calibrated heart rate to O_2 consumption in captive males placed in a respirometer. Using heart rates collected from a number of courting males over continuous 24 h periods, we could then estimate their daily energy expenditure (DEE). Again, to our surprise, we found that the golden-collared manakin had a DEE similar to that of other similar small tropical birds, including the spotted antbird, *Hylophylax naevioides*, a bird with minimal physical courtship behaviour (Barske et al., 2014). Thus, although individual male manakins can perform more than 100 displays and several hundred roll-snaps per day, which would appear to greatly increase energy expenditure, those males do not seem to be metabolically taxed (Barske et al., 2014). One interpretation of these results is that the oxygen supply to peripheral muscles is sufficient during male courtship.

One way to increase O_2 availability is to increase cardiac output, and one way to increase cardiac output is to increase the size of the heart itself. Manakins do indeed have larger hearts relative to other small tropical birds, averaging approximately 15% larger than birds of similar size (Hartman, 1955). These larger hearts likely reflect greater mass of the myocardium to increase the force of contractions for ejecting blood systemically. Compared to male zebra finches, *Taeniopygia guttata*, both the left ventricular wall and the septum separating the left and right ventricles are significantly larger in golden-collared manakins (Fig. 4; Barske, Eghbali, Kosarussavadi, Choi, & Schlinger, 2019). Because contraction of the left ventricular myocardium is responsible for ejecting blood to the periphery, the enhanced thickness of these walls could account for the greater mass of manakin hearts relative to other birds and might function to increase cardiac output. By evolving a more powerful left ventricle, manakins may more readily supply O_2 to their peripheral musculature during active seasons of courtship and lessen their overall metabolic demands.

As manakin courtship is AR dependent and AR is expressed at relatively high levels in many golden-collared manakin tissues, we were curious if AR is expressed in the manakin heart and whether expression of cardiac genes is AR dependent, especially genes that promote strong and rapid contractions. When compared to the hearts of zebra finches, we found both ER (α subtype) and AR were expressed in heart, but only AR was expressed at significantly higher levels in the manakin heart (Barske et al., 2019). Moreover, following T treatment of nonbreeding males, we found increased expression of SERCA, a sarcoplasmic/endoplasmic reticulum calcium ATPase required for removal of intracellular calcium ions (Ca^{2+}). Cardiomyocyte contraction and relaxation involves rapid release and removal of Ca^{2+} available to intracellular contractile proteins. Thus, elevated levels of SERCA could promote the rapid cardiomyocyte relaxation required before a new contraction can be initiated. These results hint at an important role for androgens in optimizing cardiac output in an animal with physically challenging courtship displays. More work is required to understand how sex steroids may promote cardiac function under other energy-demanding conditions.

FUTURE DIRECTIONS AND CONCLUSIONS

Courtship signals an animal's readiness to find a mate for copulation; members of the opposite sex use these signals to identify potential mates and likely assess the signals to select some individuals for mating. Elaborate male courtship displays have the capacity to send a great deal of information to females, providing numerous cues upon which females can base their choice. These

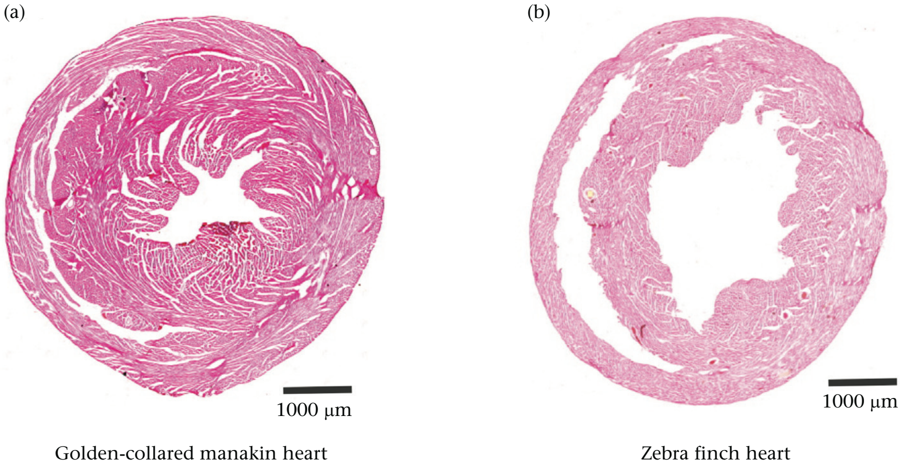

Figure 4. Cross sections through the hearts of (a) a male golden-collared manakin and (b) a male zebra finch. Note the thickened cardiac muscle surrounding the lumens of the ventricles in the manakin relative to the zebra finch. Data are modified with permission from Barske et al. (2019).

rich physical displays potentially reveal to females a vast number of underlying physiological specializations crucial for display performance. T reaches, optimizes and activates most or all of these deep physiological systems to enable complex courtship. These actions may also limit the optimal function of some physiological systems, and such trade-offs may drive how the evolution of courtship display can or cannot unfold.

Our studies of male golden-collared manakins provide evidence to support these conclusions, as this animal model represents one of the most in-depth explorations of behavioural evolution and its physiological underpinnings. Yet, many 'big picture' questions remain unanswered, and in many ways our studies merely scratch the surface of a much more complex process with respect to the relationship between hormones, physiology and courtship behaviour. Below, we highlight a few interesting future directions that we feel are pressing.

(1) Do elaborate dance displays, like the golden-collared manakin's display, evolve as a means to showcase motor skill and ability (sensu Beyers et al., 2010)? Our research groups expect that they do, likely because the courtship dance pushes a male's neuromuscular systems to its performance limits (see above). If, as many suspect, neuromuscular skill is tied to past or current health and condition, then the dance itself may have the capacity to provide females (and competing males) with honest information about the signaller. In this way, lower-quality males may not be able to perform the display as fast or with as much agility as higher-quality males. This idea needs direct testing not only in golden-collared manakins, but also in the many other taxa that perform extraordinary gestural displays to court mates and compete with rivals. These displays — and the signals that might arise from the observation of movement in and of itself — are poorly understood in such an evolution and functional context.

(2) From the endocrine perspective, we might also ask, how often does sexual selection for innovation in reproductive behaviour occur through the evolutionary modification of the androgenic system? We suspect that sexual selection frequently capitalizes on AR signalling to drive behavioural evolution, especially when motor skills need to be revamped to accommodate novel forms of performance during sexual interactions (be they courtship, competition or copulation). Biologists are beginning to address this issue in species other than manakins; for instance, increased AR expression in select muscles is associated with the emergence of drumming displays in woodpeckers, pushup displays in *Anolis* lizards and foot-flagging displays in tropical frogs (Johnson et al., 2018; Mangiamele et al., 2016; Schuppe & Fuxjager, 2019). In this latter group, studies have even suggested that increases in muscular AR represent a 'path of least resistance' for selection to drive the emergence of behavioural displays that require specialized motor control (Anderson et al., in press). With this in mind, we might also ask why might androgen–muscle interactions represent a common pathway for sexual selection to modify animal performance? Surely the answer involves the ability of testicular androgens to coordinate the numerous anatomical, physiological and behavioural facets of reproduction that are tied to fecundation. But, there is also likely more involved, and future studies should address this topic.

(3) Another important question is, how do changes in the androgenic modulation of a target tissue support the emergence of new behavioural traits? The heart of this question rests in the functional effects of AR, and how they might vary across target tissues and in response to high or low levels of AR expression. Molecular endocrinologists have investigated how AR can change the transcriptional milieu of a tissue, but significantly less work relates these changes to tractable differences in behavioural outflow. Our work in manakins has begun to approach this topic, but there is certainly ample room for additional studies in a wide range of vertebrate taxa. The time to address this gap is likely now, as molecular approaches are becoming increasing accessible to researchers who study the endocrinology of behaviour in non-model species. We expect that such work represents the modern frontier in the fields of behavioural and evolutionary neuroendocrinology.

(4) Perhaps the biggest question of all is, how exactly does the androgenic system evolve to support behavioural diversification? We are not the first to discuss this issue, as many have described models for such change to occur (Adkins-Regan, 2008; Cox, 2020; Fuxjager et al., 2018; Fuxjager & Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; Rosvall et al., 2012, 2016). Our work in manakins points to AR as a lynchpin for the evolution of elaborate physical courtship behaviour, but androgenic signalling systems are highly complex molecular cascades that involve numerous intracellular processes. Any components of this signalling machinery may, in theory, be a target of selection to change behaviour. For example, co-factors that mediate transcriptional strength of AR could be a target of selection for androgen action on a specific target tissue. Arguably, selection that alters properties of these co-factors could create undue cellular problems because these same co-factors might serve other signalling systems in addition to those mediated by AR (Schuppe & Fuxjager, 2019). Clearly, these issues merit further investigation to disentangle the process of adaptive endocrine system evolution in manakins and other species.

With these points in mind, we close our review by acknowledging that more work remains. Indeed, an organismal approach to broader evolutionary, behavioural and physiological questions — like the one we have taken by studying the biology of courtship in golden-collared manakins — has the power to address long-standing topics in biological research. We encourage others to similarly think in an integrative way to further probe how animal life thrives, how it works and how it changes over time.

Acknowledgments

We thank Jeff Podos for organizing the Presidential Symposium for the Animal Behavior Society Meeting. We also thank the Smithsonian Tropical Research Institute (STRI) and its amazing staff for their continued support of our research on golden-collared manakins and other species over the years. Funding for this research and paper was from U.S. National Science Foundation (NSF) grant IOS-1947472 (to M.J.F.), NSF grant OISE-1952542 (to M.J.F.), NSF grant IBN-021319 (to B.A.S.), and by an AHA predoctoral fellowship 12PRE11890059 (to Julie Barske and B.A.S.).

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