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How research on female vertebrates contributes to an expanded challenge hypothesis

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Abstract

The bi-directional links between hormones and behavior have been a rich area of research for decades. Theory on the evolution of testosterone (T) was greatly advanced by the challenge hypothesis, which presented a framework for understanding interspecific, seasonal, and social variation in T levels in males, and how they are shaped by the competing demands of parental care and male-male competition. *Female* competition is also widespread in nature, although it is less clear whether or how the challenge hypothesis applies to females. Here, we evaluate this issue in four parts: (1) We summarize and update prior analyses of seasonal plasticity and interspecific variation in T in females. (2) We evaluate experimental links between T and female aggression on shorter timescales, asking how T manipulations affect aggression and conversely, how social manipulations affect T levels in female mammals, birds, lizards, and fishes. (3) We examine alternative mechanisms that may link aggression to the social environment independently of T levels in circulation. (4) We present a case study, including new data analyses, in an aggressive female bird (the tree swallow, *Tachycineta bicolor*) to explore how variation in tissue-level processing of T may bridge the gap between circulating T and variation in behavior that is visible to natural selection. We close by connecting these multivariate levels of sex steroid signaling systems alongside different temporal scales (social, seasonal, and evolutionary) to generate broadly applicable insights into how animals respond to their social environment, regardless of whether they are male or female.

Keywords

androgen receptor; neural sensitivity; evolutionary endocrinology; 5-alpha-reductase; aromatase; amygdala; pectoralis

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Introduction

“Females are generally regarded as not aggressive and testosterone is regarded as the ‘male sex hormone.’ These views are incorrect...” – Wingfield and colleagues (2000)

The sex steroid hormone testosterone (T) is thought to regulate phenotypic responses to dynamic social environments in male vertebrates. For instance, males typically produce more T during breeding stages that involve mating competition and less T during parental breeding stages (Ketterson and Nolan, 1992; Wingfield, 1984). Likewise, males may temporarily elevate T levels in response to a prospective rival or mate (Gleason et al., 2009; Wingfield, 1985). As a result, T levels may exceed what is necessary for reproduction, instead promoting behaviors that are adaptive during competition (Wingfield et al., 1990). Similar patterns may apply over evolutionary time, with higher T in species with heightened competition for territories or mates (Hirschenhauser and Oliveira, 2006; Hirschenhauser et al., 2003; Oliveira et al., 2002). Patterns of T secretion across three timescales – social, seasonal, and evolutionary – form the crux of the challenge hypothesis, which broadly suggests that variation in T secretion within and among species stems from natural selection related to trade-offs between mating effort and parental effort (Wingfield, 2012; Wingfield et al., 1990; Wingfield et al., 2001).

This framework has been largely supported in males across many taxa (Archer, 2006; Hirschenhauser and Oliveira, 2006; Hirschenhauser et al., 2003; Oliveira et al., 2002). However, we still have a relatively limited understanding of whether and how the challenge hypothesis applies to females. Our primary goal is to synthesize current data on the challenge hypothesis in females. In addition, we evaluate hypotheses that broaden the challenge hypothesis beyond its initial focus on T, asking how other hormones and tissue-level processes may modulate phenotypic responses to social competition. We begin by overviewing relationships between T and female behavior, including prior analyses of seasonal and interspecific variation in T. We then provide a novel synthesis of 80 previously published studies across vertebrate taxa to evaluate two key aspects of the challenge hypothesis: (H1) Elevated T promotes female aggression, and (H2) females respond to competition by elevating T. Next, we broaden our focus and begin to evaluate two related hypotheses: (H3) Other components of sex steroid signaling systems and (H4) non-steroidal mechanisms may influence aggression, independently of T levels in circulation, promoting the adaptive expression of aggression without necessitating elevated T per se. Finally, we describe a case study in an avian species with strong female-female competition to explore how tissue-level variation in sex steroid signaling systems may interact with seasonal patterning of T and aggression. We close by connecting these different mechanistic levels and temporal scales (social, seasonal, and evolutionary) to generate broad insights on the challenge hypothesis that are applicable not only to females but to both sexes.

Functional role of T in females

Despite its reputation as the ‘male hormone,’ T is a natural and important part of female physiology. Arguments to the contrary may stem from observations that in many species,

females have lower levels of T in circulation than males, and female gonads convert more T to estrogenic metabolites, such as 17β -estradiol (E_2). In addition to the costs of T incurred by males (Wingfield et al., 2001), females with high T also may experience reduced maternal care, disrupted mate choice, or delayed breeding (Clotfelter et al., 2004; Gerlach and Ketterson, 2013; McGlothlin et al., 2004; Rosvall, 2013a), meaning there are potentially fitness costs to the activational effects of T in females. Comparative studies also report correlations between female and male T levels in birds (Goymann and Wingfield, 2014; Ketterson et al., 2005; Møller et al., 2005) and fishes (Mank, 2007), suggesting that T levels in females may simply be non-functional byproducts of selection shaping T secretion in males.

However, several observations instead suggest that T *is* functionally relevant for females. T is critically involved in many aspects of female reproduction (Drummond, 2006). Leading up to ovulation, females experience a surge in T (Johnson and Van Tienhoven, 1980; Kobayashi et al., 1989; Rush and Blake, 1982), which can directly mediate ovulation or fertility via binding to androgen receptors (AR) (Hu et al., 2004; Rangel et al., 2006). Females can allocate T to offspring, and this maternal effect varies in relation to life history (Bentz et al., 2016; Mousseau and Fox, 1998), suggesting it may be shaped by selection. T produced from the ovary is also released into circulation, and, critically, females have all of the cellular and molecular machinery to respond to T in tissues that regulate a variety of morphological, physiological, and behavioral traits (Staub and DeBeer, 1997). Indeed, T has been linked with many adaptive female traits, including parenting, aggression, and muscle functioning (Cain and Ketterson, 2012; Cain and Ketterson, 2013; Dent et al., 2012). Like males, some of these effects may stem from T's estrogenic metabolites acting on the brain or other target tissues (Soma et al., 2008), and the challenge hypothesis does not inherently distinguish between androgenic or estrogenic effects of T or its metabolites. We carry this perspective forward here, although we note that the activational effects of T are not necessarily identical to those of E_2 (Duque-Wilckens and Trainor, 2017; Floody, 1983; French et al., 2013), a point we return to below. In sum, T is naturally produced at certain times of the female reproductive cycle, and there are many opportunities for natural selection to influence T levels via effects on organismal traits.

Seasonal and life history patterns of T secretion in females

One key component of the challenge hypothesis is that temporal patterns of circulating T are shaped by trade-offs between competition and parental care, via activational effects of T that enhance aggression and impede parental care. It is well known that, on average in many species, females provide more parental care than males (Trivers, 1972), but only recently has the adaptive function and ubiquity of female-female competition become clear. Females compete with one another in a variety of contexts across nearly all species studied to date (Clutton-Brock, 2009; Rosvall, 2011; Stockley and Bro-Jørgensen, 2011). In some cases, female competition determines access to mates, similar to how sexual selection operates in males (Eens and Pinxten, 2000; Hare and Simmons, 2019). Even when all females obtain a male, some females outcompete others for *better* mates, territories, nesting sites, or exclusive paternal support (Rosvall, 2011; Slagsvold and Lifjeld, 1994; Tobias et al., 2012).

Considering that this competition often occurs during breeding and pre-breeding periods when females can produce T but are not yet parenting, T could serve to promote competitive traits, like aggression, without deleterious effects on parenting. Later in the breeding season, lower T levels or less socially responsive T levels may be advantageous if elevated T also interferes with maternal care. Earlier analyses on female birds support this seasonal component of the challenge hypothesis, that T levels tend to peak during periods of territorial establishment or mate acquisition, and decline as parental demands increase (Goymann and Wingfield, 2014; Ketterson et al., 2005). We used the new resource HormoneBase (Vitousek et al., 2018) to analyze variation in female T levels in other vertebrate taxa and found similar patterns (Figure 1, see SI methods), albeit with limited taxonomic sampling in mammals and reptiles. These data show that, across vertebrates, T is elevated beyond its role in reproduction. Further, at least in species in which we have substantive data, seasonal variation in female T largely tracks expected shifts between competition and care, consistent with past work on the challenge hypothesis in males.

How and why these patterns of T secretion scale over evolutionary time is less certain. Møller et al. (2005) reported higher T levels in females in colonial species of birds, and Ketterson et al. (2005) found higher T in females from socially monogamous bird species. A more recent analysis included more species alongside improved phylogenetic and meta-analytical methods (Goymann and Wingfield, 2014), but results were decidedly null: maximum female T levels and seasonal plasticity in female T were unrelated to any variable studied, including mating system, coloniality, and degree of dimorphism (Goymann and Wingfield, 2014, see also Garamszegi 2014). These analyses have some limitations (Groothuis et al., 2014; Ketterson, 2014), but one implication is that the selective pressures shaping female-female competition and aggression are not related to classic indices of male-male competition and sexual selection (sensu Tobias et al., 2012). Resolving whether female T levels are shaped by the relative importance of female competition vs. care, as expected by the challenge hypothesis, will require that we re-imagine how to measure the strength of female-female competition outside of a framework derived for studying males. Echoing some of the earliest analyses on the challenge hypothesis in females (Wingfield, 1994), we urge future work that evaluates how variation in T relates to known drivers of *female* competition, such as competition for nesting sites, high quality males, or the direct/indirect benefits they provide. We also must systemically evaluate the organism in which this hormone acts because, ultimately, the evolutionary process depends upon how T interacts with other traits that are visible to selection, including aggression and parental care. In sum, it is not yet clear how selection generates interspecific variation in female T secretion, but it is clear that females modulate T across different breeding stages, and many species have elevated T during periods of breeding and pre-breeding social competition.

Evaluating bidirectional links between T and female aggression

Progress towards understanding how T is shaped by selection acting on females will require broad synthesis of the bidirectional links between T and female aggression, particularly on shorter time-scales where selection acts (i.e. minutes, hours, days). This relationship has been examined in several ways, including correlative studies on T and aggression and more experimental approaches in which T or social environments are manipulated. We conducted

a systematic search of the literature that yielded 80 studies (n=97 cases) across mammals, birds, lizards, and fishes in which overt conspecific aggression in females was measured in relation to T (see SI methods and SI Figure S1), summarized below.

H1: Females respond to competition by increasing T

We located only 11 cases testing this hypothesis using simulated territorial intrusions (or resident-intruder tests) in which a live or stuffed decoy was presented to a focal female, aggressive response was quantified, and blood T levels were measured shortly thereafter and compared to unchallenged controls (Figure 2A; Table S1). In all cases, females responded to female intruders with robust aggression. However, we identified only 2 cases (18%) in which females exposed to territorial intrusion had higher T levels than controls (Davis and Marler, 2003; Gill et al., 2007). In all other cases, intrusions did not affect female T levels in circulation (Canoine and Gwinner, 2005; Davis and Marler, 2003; DeVries et al., 2015; Gill et al., 2007; Goymann et al., 2008; Jawor et al., 2006) or T levels were lower in challenged females compared to controls (Elekonich and Wingfield, 2000; Navara et al., 2006). Correlative studies generally mirror this same pattern, with a few reporting positive (Woodley and Moore, 1999) or negative (Cantarero et al., 2015) relationships between T and female aggression, and most reporting no such pattern (Moreno et al., 2016; Ross and French, 2011; Rosvall et al., 2012; Schwabl et al., 2005). Thus, social elevation of T is not a common female response to an acute social challenge.

The challenge hypothesis emphasizes that T levels may be more constrained during parental breeding stages, a constraint that may be stronger for females due to extended parental responsibilities. Consistent with this view, the two cases reporting increased T following a territorial intrusion occurred during pre-breeding stages (Davis and Marler, 2003; Gill et al., 2007), but these studies also repeated the challenge during later (parental) stages and found that females did *not* elevate T to intrusions. In addition, females in some species decrease T following social competition (Elekonich and Wingfield, 2000; Navara et al., 2006), hinting at other hormonal processes that ‘use up’ T during social competition. Existing data are too limited to statistically test the effect of breeding stage (see Table S1); however, all but one case reviewed here was conducted during various pre-breeding/breeding stages, suggesting that stage alone cannot account for these patterns.

Another possibility is that a single conspecific intrusion does not sufficiently mimic hormonal responses to real competition, a point that has been discussed in research on males (Oliveira et al., 2005; Scriba and Goymann, 2008). We therefore investigated two related analyses that may speak to the cumulative effects of repeated competitive interactions in females (Figure 2A; Table S1). First, we examined studies correlating dominance ranks with T, and we found that 46% (6/13 cases) show a positive relationship between T and female dominance (Batty et al., 1986; Beehner et al., 2005; Clarke and Faulkes, 1997; Davies et al., 2016; Desjardins et al., 2008; Renn et al., 2012). Second, looking at studies that either manipulate the social environment (e.g., by increasing breeding density) or measure natural variation in breeding density or intrusion rates, we found that 50% (5/10 cases) report higher female T levels in more competitive environments (Cantarero et al., 2015; Chapman et al., 1998; Gavasa et al., 2012; Langmore et al., 2002; Polo et al., 2010). These more persistent or

naturalistic manipulations are far more consistent with the challenge hypothesis than the relatively meager support found with a single territorial intrusion (i.e. 18%), suggesting that we have work yet to do to understand how females hormonally respond to real competition in nature, echoing some emerging findings in males (Goymann et al., 2019).

H2: Elevated T promotes female aggression

The activational effects of T on female aggression have been well known for some time (see Svare, 2013; Wingfield et al., 2000; Wingfield, 1994). Our literature review found that T implants increase female aggression in 72% of cases (3/43), while T injections increase aggression in 45% of cases (9/20; see Figure 2B; Table S2). Among the subset of studies that clearly used female-appropriate doses, most reported increased female aggression (78%; 7/9 cases). Thus, support for the hypothesis that T enhances female aggression is unlikely to be driven by supraphysiological dosing generating unnatural responses (Ketterson, 2014; Ketterson et al., 2005, see also Fusani, 2008; Goymann and Wingfield, 2014).

Our finding that T implants are more likely to increase female aggression than injections suggests that prolonged T manipulations are more behaviorally salient. Notably, only 31% of T injection studies on *ovariectomized* females report increased aggression (4/13 cases), whereas 71% of T injection studies on *intact* females report positive effects on aggression (5/7 cases). Implant experiments seem generally less influenced by this effect, with 68% (17/25) of ovariectomized vs. 78% (14/18) of intact studies reporting elevated aggression. Thus, a transient surge in T may be more likely to enhance aggression if other aspects of sex steroid signaling have primed the organism to respond. Stated another way, T does not activate aggression *on its own*; other co-regulated parts of the system may need to be in place before even brief elevations of T will promote aggression.

Related to this point, genetic, developmental, and physiological factors may influence the degree to which T affects aggression, via effects on other organismal and systemwide processes. For example, Sandnabba et al. (1994) found that female mice from lines bred for high aggression showed more overt aggression in response to T injections, but those from low aggression lines did not. Similarly, phylogenetic history almost surely influences the behavioral salience of T, although taxonomic biases make this challenging to quantify at this time (Table S2). Developmental effects, such as the temperature experienced embryonically, have also been linked with the effectiveness of T implants in affecting aggression in a gecko species (Crews et al., 1996; Flores and Crews, 1995). This effect was attributed to developmental differences in brain morphology and metabolic capacity impacting the behavioral salience of later T exposure. Finally, just as breeding stage may alter the effects of competition on T (see H1), stage also may alter the effects of T on behavior. Clarke et al. (1976) reported such a case in ewes, in which T implants increased aggression but only during early pregnancy – this effect was abolished during late pregnancy and attributed to increased metabolic clearance of T.

In sum, T *does* increase female aggression, especially when T is present at sufficient background levels as seen with T implants and T injections into intact females (Figure 2B, SI Table S2), mirroring the findings in H1 that more prolonged, cumulative social interactions are more likely to elevate T (Figure 2A; SI Table S1; SI Figure S2). Because this

behavioral effect occurs using a fraction of the T needed to elicit a similar response in males, females may in some ways be considered more sensitive to T than males. Alternatively, females may achieve these behavioral effects with lower T because T is converted to E₂. E₂ undoubtedly plays a role in aggression in females much like it does in males (summarized above), but it appears unlikely that E₂ alone drives the findings summarized here. Critically, a subset of the cases in which T treatment increased female aggression *also* contained a separate E₂ treatment. However, E₂ elicited increased (Albert et al., 1990; Greene et al., 1978), decreased (DeBold and Miczek, 1981) or no effect on aggression (Archawaranon and Wiley, 1988; Barkley and Goldman, 1978; Compaan et al., 1993; Simon et al., 1985; Winkler and Wade, 1998), despite T increasing aggression in all of these cases. Taken together, these findings suggest that our understanding of T's effect on behavior will be improved by consideration of the rest of the system in which this hormone operates.

Many roads lead to aggression: extensions of the challenge hypothesis beyond T in circulation

If the challenge hypothesis provides a framework for linking T and aggression over different timescales and contexts, then a natural extension is that other endocrine-molecular mechanisms should influence the strength of those patterns. Indeed, we know that T does not act in a vacuum (Adkins-Regan, 2005; Fuxjager and Schuppe, 2018). Once produced, gonadally-derived T is transported through the bloodstream to multiple target tissues, including brain, muscle, and immune cells. There, T can bind to AR, which interacts with co-factors and response elements to alter gene expression (Peterson et al., 2013; Peterson et al., 2014; van Nas et al., 2009). T can have downstream effects after 5 α -reductase (5 α R) converts T to dihydrotestosterone (DHT) which binds AR with higher affinity than T itself. T can also be converted to E₂ via aromatase (AROM), which is present in many tissues, including the brain and ovary. E₂ can bind to estrogen receptors (ER) or initiate rapid effects via secondary messenger systems (Remage-Healey et al., 2008). The effectiveness of steroids can also be modulated by liver metabolic processes (Mueller et al., 2015) or binding globulins (Deviche et al., 2001; Swett and Breuner, 2008) that essentially lower the availability of bioactive steroids. Furthermore, many components of the androgenic signaling system interact with other behaviorally active signaling molecules, including glucocorticoids, nonapeptides, catecholamines, and more, all of which can modulate T's effect on behavior or influence aggression (Filby et al., 2010; Nelson and Trainor, 2007; Soma et al., 2008). In short, T levels in circulation barely scratch the surface of the full suite of endocrine-molecular processes that may influence variation in aggressive behavior.

Critically, natural selection does not differentiate among these diverse endocrine-molecular mechanisms. Rather, it is their *consequence* for organismal traits that matter in the eyes of selection: differences in behavior, morphology, and physiology that generate variation in survival and reproductive success. We therefore envision two interacting phenotypic landscapes that can guide our thinking on how trade-offs between competition and care may influence the evolution of T-aggression relationships (Figure 3). The first landscape (Figure 3, top) predicts variation in intrasexual aggression, which should be highest when competition is most intense and parental demands are low, and vice versa. Animals may shift

along the phenotypic landscape with varying social or seasonal cues, and species may be restricted to certain areas of the landscape via evolutionary change. The second landscape (Figure 3, bottom) predicts variation in T secretion in an analogous way, with one key difference: selection does not assay T; it only shapes T *indirectly* via its consequence for the behavioral landscape and presumably other inter-related phenotypic landscapes not shown here (e.g. immune function). The original challenge hypothesis primarily dealt with individuals or species in the upper left or bottom right quadrants, and the degree to which T and aggression are bi-directionally linked. For females and other animals that experience co-occurring pressures of competition and care (upper right quadrant), alongside pleiotropic costs of T that may affect breeding and parenting, we should expect other mechanisms to support aggression (Rosvall, 2013b), thereby decoupling the aggression landscape and the T landscape to some degree. With this in mind, we envision two extensions of the challenge hypothesis that move beyond the initial focus on T in circulation (H3 and H4, below).

H3: Other components of sex steroid signaling systems promote aggression when T is low

Recent analyses suggest that organisms can modulate tissue-level sensitivity to T independently from one tissue to the next, and independently of T levels in circulation (Hunter et al., 2018; Lipshutz et al., accepted). Consequently, it is reasonable to expect that selection might shape the specific constellation of tissues with high vs. low androgen sensitivity to adaptively suit the competing demands of social competition vs. care (Hau, 2007; Ketterson et al., 2009). By extension, aggression in females may be modified via changes in how efficiently T is transported, how rapidly it is converted to other steroids in specific tissues, and how many steroid receptors are available to receive that signal within specific tissues (Rosvall 2013b).

Indeed, there is good evidence that variation in T sensitivity, conversion, or metabolism within specific areas of the brain can predict female aggression even when circulating T levels cannot. For instance, in female dark-eyed juncos (*Junco hyemalis*), neural expression of AR, ER α , and AROM predicts individual variation in response to simulated territorial intrusions, whereas T levels do not (Rosvall et al., 2012). Likewise, in the white-throated sparrow (*Zonotrichia albicollis*), females of the more aggressive white-striped morph differ from the less aggressive tan-striped morph in ER α expression in key nodes of the social behavior network (Horton et al., 2014), and behavioral differences between morphs persist even when sex steroid levels are experimentally equalized (Maney et al., 2009; Merritt et al., 2018). Sex-role reversed species in which females are the more aggressive sex further suggest that enhanced sensitivity to T in the brain may promote aggression in the face of low T (Voigt, 2016; Voigt and Goymann, 2007). These comparative and correlative results are bolstered by experimental work linking AR, ER α , and AROM to aggression (Duque-Wilckens and Trainor, 2017), lending support to the hypothesis that female aggression relates to T and E₂ processing in the brain.

Other steroids like progesterone (PROG) and dehydroepiandrosterone (DHEA) also can be converted to T or E₂ in the brain to influence aggression. In Siberian hamsters (*Phodopus sungorus*), for example, females are especially aggressive during the non-breeding season when gonadal sex steroid secretion is low, but adrenal DHEA production is high (Rendon et

al., 2015). These females also exhibit greater ER α expression in brain regions mediating aggression (Rendon et al., 2017). In addition, social challenges reduce levels of DHEA in circulation (Rendon and Demas, 2016), suggesting that DHEA may be ‘used up’ during competition, similar to the competition-induced decreases in T described above. Several other studies also point to interactions between PROG and female aggression (Davis and Marler, 2003; Goymann et al., 2008; Rubenstein and Wikelski, 2005), with some indication that the enzyme 5 α R may influence aggression via its joint effects on concentrations of PROG, T, or the ratio between the two, which has been linked with aggression as well (Pibiri et al., 2006; Pinna et al., 2005). Together, these studies highlight the potential for other sex steroids and prohormones to mediate aggression at times when circulating T levels are low – a hormonal state that often applies to females.

To our knowledge, no studies have addressed whether social challenges induce local changes to the female brain in a manner that parallels elevations in circulating T predicted by the challenge hypothesis. However, evidence from males suggests that challenges may influence neurosteroidogenesis (Charlier et al., 2011), conversion of steroids into more active forms (Pradhan et al., 2010; Soma et al., 2014; Soma et al., 2008), and AR expression (Fuxjager et al., 2010) in ways that promote aggression. These mechanisms may be particularly relevant when T levels are low, including male aggression during the non-breeding season (Hau et al., 2004; Soma et al., 2014). We envision that similar mechanisms occur in females, perhaps explaining why T levels sometimes decrease following competitive interactions (see H1). Future work should explore whether these putatively adaptive responses to the social environment occur in females, and whether seasonal or evolutionary changes in these mechanisms predict female aggression, even when T levels do not.

H4: Other signaling systems regulate female responses to the social environment

A related but non-mutually exclusive hypothesis is that female aggression depends less on T and more on other signaling systems, such as nonapeptides, catecholamines, or glucocorticoids (Filby et al., 2010; Nelson and Trainor, 2007). Many of these ‘alternative’ mechanisms are still regulated to some extent by T. For instance, experimental treatment with T alters neural gene expression of monoamine oxidase A (an enzyme involved in catecholamine signaling) in female dark-eyed juncos (Peterson et al., 2013). T and E₂ also regulate signaling of vasopressin, oxytocin, and their taxon-specific homologs (e.g. Auger et al., 2011; Kabelik et al., 2008; Nugent et al., in press), and variation in these nonapeptides predicts differences in aggression among individuals, sexes, sex roles, seasons, and species (e.g. Aubin-Horth et al., 2007; Goodson et al., 2012; Renn et al., 2008; Schumer et al., 2011, reviewed in Kelly and Vitousek, 2017). Like T levels in circulation, these mechanisms vary flexibly in response to changing social environments (Hsu et al., 2006; Kelly and Vitousek, 2017; O’Connell and Hofmann, 2011), and these mechanisms may also influence aggression independently of T. Thus, it is clear that we should look beyond levels of T in circulation as we continue to explore how hormone-behavior relationships relate to female competition and vary over social, seasonal, and evolutionary timescales.

Testing and expanding the challenge hypothesis in females: a case study

We have begun to integrate these extensions of the challenge hypothesis in a set of studies on the North American tree swallow (*Tachycineta bicolor*), a bird that is ideal for studying ecologically relevant female-female competition. Tree swallows need access to a cavity in order to breed, but they cannot excavate one themselves, meaning that a female without a nesting cavity will have zero reproductive success (Winkler et al., 2011). More aggressive females are more likely to obtain these limited resources, suggesting that female aggression is adaptive during pre-breeding competition for nesting sites (Rosvall, 2008). Female floaters are abundant in this system, with some estimates that one in four females do not breed, only because they do not obtain a nesting site (Stutchbury and Robertson, 1985). Floaters frequently intrude at territories, and they can evict or even kill other females, their eggs, and/or their offspring (Leffelaar and Robertson, 1985; Lombardo, 1986; Stutchbury and Robertson, 1987).

Early in the breeding season prior to egg laying, females respond to simulated territorial intrusions with robust aggression, including physical attacks, toward a female decoy placed on or near their nestbox (Bentz et al., 2019b; Rosvall, 2008). The density of nestboxes also predicts the frequency of female aggression (Bentz et al., 2013; Whittingham and Schwabl, 2002), which can escalate to displacements, acrobatic chases, and grappling in the air (pers. obs). Naturally occurring aggressive interactions may be more common during territorial establishment (George and Rosvall, 2018; Robertson et al., 1986); however, floater intrusions can persist later in the breeding season (Harris, 1979; Stutchbury and Robertson, 1987), potentially selecting for females that retain the ability to respond aggressively long after territory-establishment. Consistent with this view, female aggressiveness toward a simulated intruder remains relatively high through incubation. It is not until chicks hatch that we see a precipitous decline in female aggressiveness (Bentz et al., 2019b). Females therefore mount strong behavioral responses to social challenges, although the magnitude of these responses declines during the busy period of provisioning offspring (Figure 4a), consistent with the behavioral component of the challenge hypothesis.

T plays a role in modulating aggression in this system as well. Implants with female-appropriate doses of T significantly increased aggression in incubating females (Rosvall, 2013a), consistent with H2. Females are physiologically capable of elevating T in response to a pulse of GnRH, at least during early breeding stages (George and Rosvall, 2018), suggesting they may be capable of elevating T in response to social challenges. Yet, when we challenged pre-breeding females with a simulated territorial intrusion or a more naturalistic manipulation in nestbox availability, we did not find evidence of elevated T (George et al., *in prep*). Therefore, evidence for bidirectional links between T and aggression in this system largely echoes the taxa-wide findings for H1 and H2, described above.

Seasonal patterns of T secretion largely follow expectations of the challenge hypothesis (Figure 4a). During early breeding stages (prior to egg laying), female tree swallows have significantly higher baseline T than they do during parental stages (George and Rosvall, 2018). Females sampled during territorial establishment (i.e. before the fertile period) also expressed high levels of ovarian 5 α R mRNA, which encodes the enzyme producing the

potent androgen DHT (Bentz et al., 2019a), consistent with the idea that androgen production may serve another (behavioral) purpose beyond its role in fertility. T levels dramatically decline during incubation, a period when experimental T treatment suppresses maternal care (Rosvall, 2013a). This seasonal decline in T coincides with other organismal and tissue-level changes (H3), including a roughly 2× reduction in gene expression for other ovarian steroidogenic enzymes and a roughly 0.5× decrease in steroid-metabolizing enzyme gene expression in the liver (Bentz et al., 2019a), demonstrating that patterns of T secretion co-occur with a host of other organismal and tissue-specific processes that may affect behavioral phenotypes (Figure 4b).

Notably, seasonal patterns of T secretion do not completely mirror those of female aggression (Figure 4a), despite experimental links connecting the two. During incubation, this mismatch is especially striking because baseline T levels were quite low (George and Rosvall, 2018), but average aggression levels had not significantly declined below those measured during territorial establishment or nest building (Bentz et al., 2019b). We hypothesized that tissue-level metabolism and binding of T may bridge this gap between seasonal changes in circulating T and aggression, which we address here with two previously unpublished datasets (see SI for full methods, SI Table S3). Using quantitative PCR, we measured gene expression for AR, 5αR, ERα, and AROM in n=5 females collected in each of two breeding stages (territorial establishment or incubation). First, we measured AR, 5αR, ERα, and AROM mRNA in the ventromedial telencephalon and hypothalamus, for their role in regulating aggression. Second, we quantified expression of these genes in the pectoral muscle because almost all aggressive interactions among tree swallows occur in flight, and androgen signaling in skeletal muscle has been linked with behavioral performance in other systems (Fuxjager and Schuppe, 2018). We reasoned that local steroid sensitivity and conversion may allow for continued aggression after T levels decline (i.e. during parental phases), with the prediction that incubating females would show enhanced or at least sustained sex steroid-related gene expression in both brain and muscle.

Expression of AR, 5αR, ERα, and AROM was detected in all tissues, but almost none changed in abundance as females transitioned from territory establishment to incubation (SI Figure S3). When considered in tandem with the seasonal decline in gonadal T secretion, this means that females have a higher receptor:hormone or enzyme:hormone ratio during incubation. Studies using receptor antagonists and enzyme inhibitors effectively alter this ratio and subsequent behavior (Adkins-Regan, 2005), suggesting that changes in the ratio of hormone receptor or enzyme to hormone levels themselves may be functionally relevant. Viewed this way, 5αR in the hypothalamus, AROM in the ventromedial telencephalon, and AR in the pectoral muscle all showed near-significant increases in the ratio of gene expression to T during incubation (Figure 5), potentially indicating that incubating females are more sensitive to what little T is still in circulation (H3). We are currently taking a genome-wide approach to explore how aggression-related genes in non-steroidal pathways change across the breeding season to promote aggression when T is low (Bentz et al., submitted).

In sum, our work on the tree swallow suggests that T mediates female aggression in some way (H2). However, differences in seasonal patterning of T and aggression, paired with the

lack of support for socially-induced T elevations (H1), suggests that mechanisms beyond the scope of the original challenge hypothesis (i.e. H3, H4) may be involved in regulating female aggression at both social and seasonal timescales.

Conclusions: lessons learned from females and how to apply them towards an expanded challenge hypothesis

The challenge hypothesis has been extremely influential for several reasons, not least of which is that this framework applied the successes of early behavioral ecology and ecological determinism to understanding variation in T within and among individuals, over a lifetime, and over evolutionary time (Wingfield et al., 1990). Here we have demonstrated that many key tenets of this framework *do* find support in females. Females produce T outside of the fertile period, and patterns of T secretion vary within and among species. One striking pattern we revealed is that the predicted competition-induced elevation in T (H1) finds greater support when social interactions are more natural or cumulative (e.g. dominance hierarchies) as opposed to singular or acute (e.g. a simulated territorial intrusion), just as the predicted T-induced elevation in aggression (H2) also finds more support in more prolonged manipulations (implants) or naturalistic treatments (injections in intact animals). Thus, T may be more likely to affect aggression when T has already primed the animal to respond to its activational effects, and the reverse causal link is more robust when examined in contexts that may reflect the cumulative effect of many social interactions on the organism as a whole. Related to this issue, it cannot be ignored that H1 is also meeting its own challenges in males, particularly in birds in which most species largely do *not* elevate T in response to simulated territorial intrusions (Goymann et al., 2019). Other components of sex steroid signaling systems (H3) or other signaling systems altogether (H4) may explain variation in aggression independently of circulating T, and these alternative mechanisms may be especially salient during periods of parental care, when costs of elevated T may be high. As a consequence, females – who often bear additional parental demands beyond provisioning or defense – are particularly well suited to testing these alternative hypotheses. Our ongoing work in the tree swallow system brings together these ideas, highlighting how organismal (multi-tissue) and system-wide (AR:T ratios, metabolism, conversion) perspectives in females may extend the challenge hypothesis beyond its initial focus on T levels alone.

While the original challenge hypothesis generated a framework for asking why T levels vary within and among species, our journey through the bidirectional links between T and aggression in females highlights that today's challenge hypothesis encompasses much more. The question is not simply whether T secretion tracks the social environment in a uniform or predictable way within and among species, but moreover, how *do* hormones flexibly link behavior to the social environment and how *should* selection shape those patterns? Freed from the expectation that T alone will provide this solution, we can more broadly explore how selection influences hormone-behavior interactions. Given the almost infinite combination of endocrine-molecular parameters that could respond to the social environment and influence aggression, it would be remarkable for T alone to hold a special place in generating social, seasonal, and evolutionary variation in aggression. Indeed, there

is no gene for T and artificial selection on aggression involves changes in hundreds of genes, many of which are not repeatable across independent replications (Edwards et al., 2006; Kukekova et al., 2011). These observations, paired with mixed support for some tenets of the challenge hypothesis summarized above, highlight that the next generation of research on evolutionary endocrinology may benefit from integrating these diverse molecular and endocrine responses. Females present an opportunity to systematically identify and evaluate general principles about how animals respond to their social environment that will hopefully prompt new hypotheses in both males and females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- This review applies the challenge hypothesis (CH) to females
- Links between T and aggression are stronger in more persistent manipulations
- Other steroidal and non-steroidal mechanisms may promote aggression when T is low
- Novel data from the field shows how steroid sensitivity may influence T-behavior links
- Extended CH must include molecular perspectives and tissue level effects of T

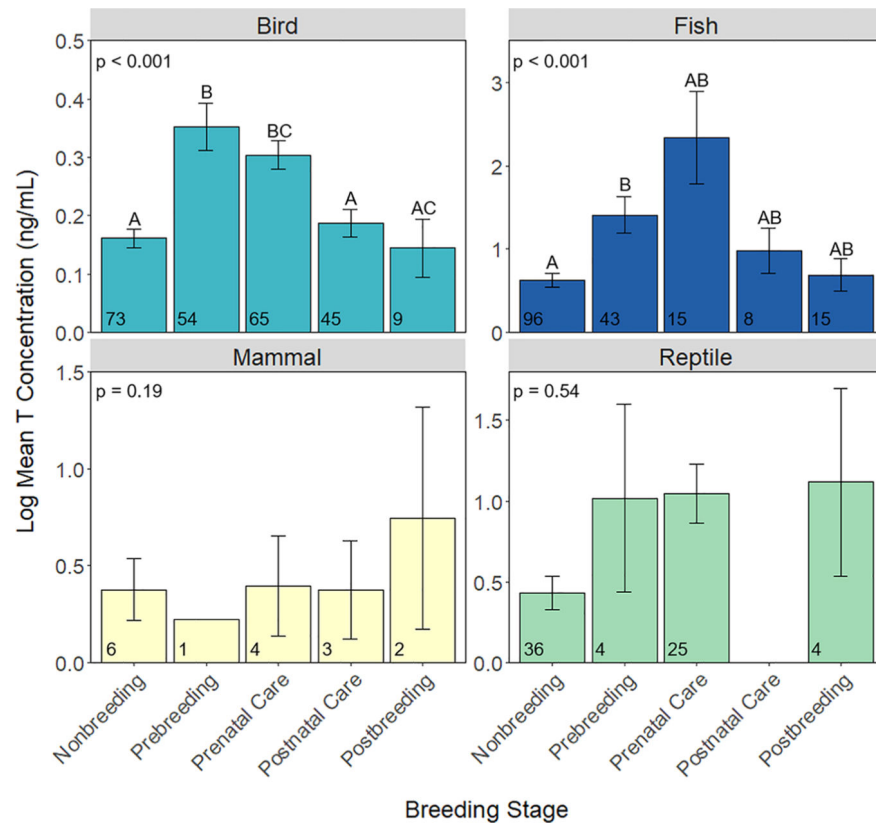


Figure 1. Mean circulating T levels in female birds, fishes, mammals, and reptiles across life stages, obtained from HormoneBase (see SI methods). Prenatal care includes egg laying, incubation, and pregnancy; postnatal care includes lactation and other care of young. T significantly varied across life stages in birds and fishes. Sample sizes correspond to # of records. Error bars are SE, and letters denote significant pairwise stage differences ($p < 0.05$), controlling for species.

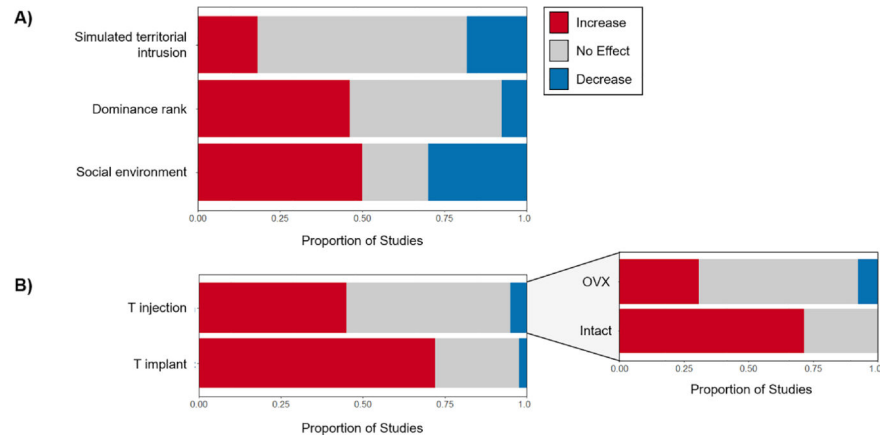


Figure 2. (A) Studies testing the effect of acute (simulated territorial intrusions) or persistent social factors (dominance rank and social environment) on female T. (B) Studies testing the effect of T on aggression (injections, implants). Injection studies are sub-divided by intact vs. ovariectomized (OVX). Color denotes proportion of studies showing that T or aggression increases (red), decreases (blue), or stays the same (no effect; grey).

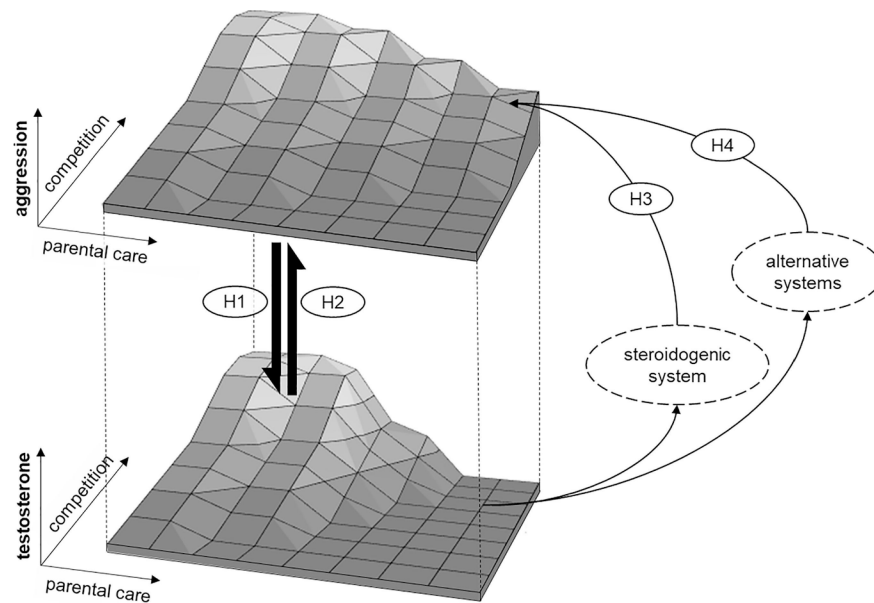


Figure 3.

Natural selection acts on the aggression landscape (upper panel), whereas variation in the testosterone landscape (below) is only linked with the behavioral landscape in some contexts. The basic tenets of the challenge hypothesis (e.g. H1 and H2, which bidirectionally link T and aggression) operate in the upper left quadrant of either surface. In the upper right quadrant, however, other mechanisms (e.g. H3, H4) may be more adaptive to promote aggression that co-occurs with parental care. The exact shapes of these surfaces are heuristic, and we imagine that individuals, sexes, and species move along these surfaces across social, seasonal, and evolutionary time.

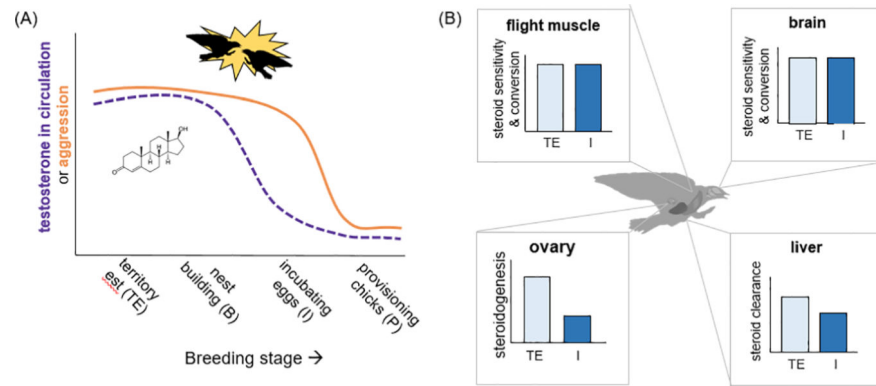


Figure 4. Mismatches between T and aggression, such as what we observed in female tree swallows (A) may be resolved by considering how T is processed elsewhere in the brain and body (B). Data not to scale. See text for details.

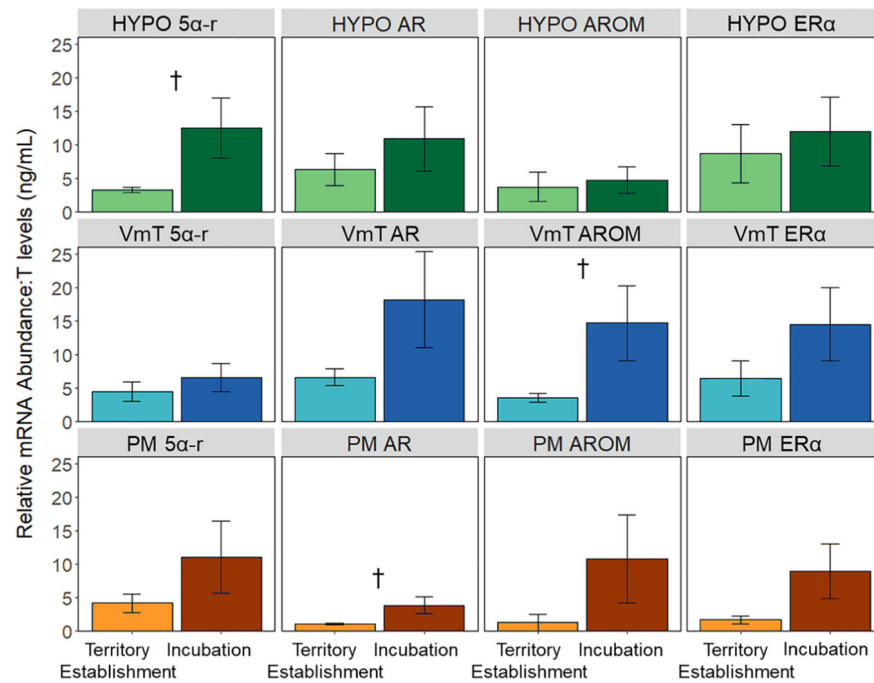


Figure 5.

Ratio of mRNA abundance of androgen receptor (AR), 5 α -reductase (5 α R), estrogen receptor α (ER α), and aromatase (AROM) in the hypothalamus (HYPO), ventromedial telencephalon (VmT), and pectoral muscle (PM) to circulating levels of T (ng/mL) during territory establishment (n=5) and incubation (n=5). T data come from Bentz et al. 2019a; neural and muscular gene expression data from these same individuals have not been previously published. Error bars represent SE and † denotes p<0.10 without correction for multiple comparisons. For full statistical analyses, see SI Table S4.