Hormones and Behavior 115 (2019) 104550

Contents lists available at ScienceDirect

Hormones and Behavior

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

Review article

Concepts derived from the Challenge Hypothesis

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ARTICLE INFO

Keywords:
State levels
Neurosteroids
Songbird
Aggression
Testosterone
Mass spectrometry
Androgens

ABSTRACT

The Challenge Hypothesis was developed to explain why and how regulatory mechanisms underlying patterns of testosterone secretion vary so much across species and populations as well as among and within individuals. The hypothesis has been tested many times over the past 30 years in all vertebrate groups as well as some invertebrates. Some experimental tests supported the hypothesis but many did not. However, the emerging concepts and methods extend and widen the Challenge Hypothesis to potentially all endocrine systems, and not only control of secretion, but also transport mechanisms and how target cells are able to adjust their responsiveness to circulating levels of hormones independently of other tissues. The latter concept may be particularly important in explaining how tissues respond differently to the same hormone concentration. Responsiveness of the hypothalamo-pituitary-gonad (HPG) axis to environmental and social cues regulating reproductive functions may all be driven by gonadotropin-releasing hormone (GnRH) or gonadotropin-inhibiting hormone (GnIH), but the question remains as to how different contexts and social interactions result in stimulation of GnRH or GnIH release. These concepts, although suspected for many decades, continue to be explored as integral components of environmental endocrinology and underlie fundamental mechanisms by which animals, including ourselves, cope with a changing environment. Emerging mass spectrometry techniques will have a tremendous impact enabling measurement of multiple steroids in specific brain regions. Such data will provide greater spatial resolution for studying how social challenges impact multiple steroids within the brain. Potentially the Challenge Hypothesis will continue to stimulate new ways to explore hormone-behavior interactions and generate future hypotheses.

1. Introduction

The original "Challenge Hypothesis" (Wingfield et al., 1990) proffered new insights into the complexity of social interactions and hormone regulatory systems (e.g. Goymann et al., 2004, 2007a, 2007b; Moore, 2007; Oliveira, 2004). Some of these insights revisited long-standing issues in behavioral endocrinology and others inspired new hypotheses about ecological and evolutionary constraints of hormone-behavior interactions. These insights have metamorphosed in many ways over the decades allowing new explorations of the complexity of hormone-behavior interactions (Adkins-Regan, 2005) and attempts to explain it. These developments have also driven the emergence of related concepts that have influenced how we think about the organism in its environment, how social interactions change over the life cycle, and how the regulatory mechanisms may have developed independently in many species and populations of species (Wingfield, 2018).

In a related vein, two hypotheses have emerged as a potential framework. One is the evolutionary constraint hypothesis, according to which mechanisms of hormone-behavior interactions are well conserved across and within (e.g. individual variation) taxa, and there may be only restricted ways in which social interactions can be regulated by endocrine systems (Hau, 2007; Ketterson et al., 2009; Hau and Wingfield, 2011). The other is the evolutionary flexibility hypothesis, according to which there are potentially many ways by which control systems for social interactions can be adjusted despite a highly conserved endocrine system. Mechanisms may vary not only across species but also within populations and even individuals (Hau, 2007; Ketterson et al., 2009; Hau and Wingfield, 2011; Wingfield, 2018) and often include the actions of non-steroidal hormones such as peptides acting in paracrine fashion at the target cell level (e.g. Oyegbile and Marler,

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https://doi.org/10.1016/j.yhbeh.2019.06.014
Received 4 April 2019; Received in revised form 28 June 2019; Accepted 28 June 2019
Available online 01 August 2019
0018-506X/

2005; Adkins-Regan, 2005; Wingfield et al., 2005). Although social interactions and hormone secretions regulating them have been investigated for many decades (see Adkins-Regan, 2005), there is still a great need to integrate social and environmental factors (e.g. Goymann and Wingfield, 2004; Goymann et al., 2004; 2007; Rubenstein, 2007). Here we outline some developing concepts, from the original Challenge Hypothesis, that illuminate the complexities of hormone-behavior interactions, but at the same time allow clear hypotheses with testable predictions. Although the findings that originally led to these new concepts have been around for many decades, their importance for understanding how an individual interacts with a changing environment, both physical and social, has only recently been fully appreciated.

2. State levels A, B and C

According to the original Challenge Hypothesis, baseline levels of a hormone (e.g. testosterone) are sufficient for the development and maintenance of morphological, physiological and behavioral traits e.g. territoriality, see also Ball and Balthazart, 2007). Superimposed on baseline patterns of hormone secretion into blood are facultative responses to dynamic behavioral interactions that can modulate hormone secretion further (e.g. glucocorticoids, Sapolsky et al., 2000). These changes in blood levels of hormones tend to be brief (seconds to hours) raising questions of why hormone secretion patterns should be so dynamic. One of the predictions stemming from the Challenge Hypothesis was that high levels of hormones such as testosterone above the baseline may be detrimental if maintained for longer periods, such as days and weeks. These realizations led to a new appreciation of "state levels" of physiology and behavior, and hormone mediators (Wingfield, 2005, 2006). Since then, the principle of state levels has also been applied to adrenocortical responses to stress (i.e. allostatic load and overload) underlying how individuals cope with environmental perturbations (McEwen and Wingfield, 2003; Romero et al., 2009; Sapolsky et al., 2000), or surges of thyroid hormone that may regulate metabolism in the face of extreme weather events in winter (e.g. Dawson et al., 1992). It is possible, indeed probable, that the concepts of state levels are applicable to all endocrine secretions and may have important implications for how organisms cope with changing environments.

Equally important is expanding evidence that the Challenge Hypothesis applies not only to male-male interactions, but also to female-female interactions. For example, in black coucals (*Centropus grilli*) in Tanzania, Goymann et al. (2008) have shown that female-female interactions decrease progesterone and treating females with progesterone decreases aggression. George and Rosvall (2018) found that GnRH-induced increases in plasma testosterone were highest when female Tree swallows (*Tachycineta bicolor*) were showing aggressive interactions with other females. Just as male-male competition does not increase testosterone in all species, species vary in the extent to which females show an increase in testosterone when competing with other females. Plasma testosterone responses to simulated territorial intrusions (STI) were measured in females of only six avian species and only one responded with an increase in testosterone (see Rosvall, 2013 for review). The non-responders included the song sparrow, in which females challenged with a conspecific decoy and playback of vocalizations had similar levels of plasma testosterone as control females (Elekonich and Wingfield, 2000).

The next step was to translate physiological state into corresponding levels of hormone mediators of homeostasis, life history stages such as reproduction etc. (Fig. 1). These state levels are presented as follows:

i) Level A represents basic homeostasis and hormone levels are baseline (Fig. 1). If secretion rates drop below level A, then dysfunction and homeostatic failure will likely occur (Norman and Litwack, 1997; Pfaff and Jöels, 2018).
ii) Level B is the range of hormone levels that regulate the day-to-day and season-to- season changes in morphology, physiology and behavior associated with the annual cycle of life history stages (Fig. 1). Within level B are physiological states that support the development of the individual and the sequence of routine life history stages for the predictable life cycle.
iii) Level C is the highest and facultative level, usually transient, relating to some form of environmental challenge whether it be a social encounter, weather perturbation, predation attempt and so on (Fig. 1). Facultative responses to environmental events (e.g. perturbations that are unpredictable) result in higher demands represented by level C. An example of physiological state in level C is the emergency life history stage, triggered by rising glucocorticoid levels, which allows the individual to abandon the current life history stage and survive the perturbation (e.g. Romero and Wingfield, 2016). Once the perturbation passes, the individual can then return to the life history stage appropriate for the time of year (Fig. 1; Landys et al., 2006; McEwen and Wingfield, 2003; Romero et al., 2009; Wingfield, 2018). Examples for testosterone would be level A (non-breeding baseline), B (breeding baseline) and C (brief surges of plasma levels above the routine, day-to-day circulating levels (Wingfield et al., 1990).

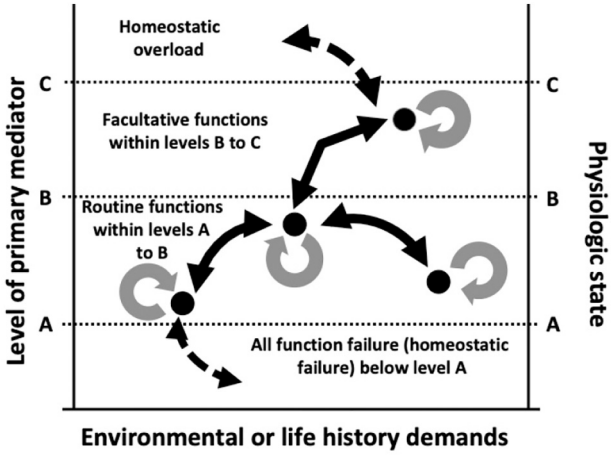

Fig. 1. Environmental and life history demands (such as reproduction, migrations, molt etc.) influence different physiological states A, B and C. Level A represents basic homeostasis. If physiological state (bottom dashed arrow) drops below the dotted line for A then failure of homeostatic functions result in dysfunction and even death. From level A to B is the reactive scope for physiological states that support the development of individuals and the sequence of routine life history stages for the predictable life cycle. The reactive scope from level A to level B can therefore be interpreted as the norm of reaction for a given life history stage. Facultative responses to environmental events (e.g. perturbations that are unpredictable) result in higher physiological demands represented by levels B to C. An example of physiological state from level B to level C is the emergency life history stage, which allows the individual to abandon the current life history stage and survive the perturbation. Once the perturbation passes, the individual can then return to the life history stage appropriate for that time of year. Therefore, demands above level B to level C represent the reaction norm for coping with perturbations of the environment both physical and social. Above level C (top dashed arrow) then problems arise from homeostatic overload (sensu Romero et al., 2009) and death may result unless demands are reduced within levels B to C. Note that levels of physiological state are matched by level (secretion) of primary mediators such as hormones. Redrawn from Landys et al. (2006, courtesy of Elsevier). See also McEwen and Wingfield, 2003; Owen-Ashley et al., 2004; Romero et al., 2009; Wingfield, 2018.

Until recently, how one hormone can have different actions at each level had not been appreciated as a fundamental concept of acclimation and adaptation of organisms to changing environments. It is important to note that functional levels A, B and C may also be regulated by

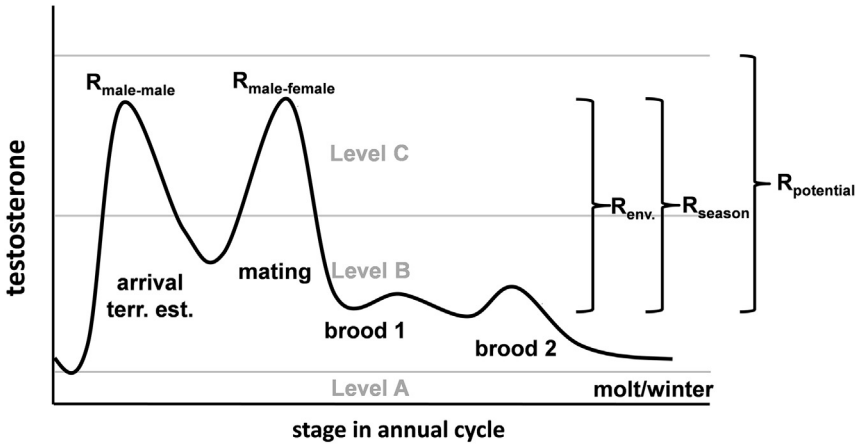

Fig. 2. Schematic view of a song sparrow's annual testosterone curve (black line) with illustrations of the different kinds of androgen responsiveness (R). After arriving in the breeding area males establish territories and compete with other males ($R_{male\text{-}male}$). When females become receptive there is another peak in males' testosterone levels ($R_{male\text{-}female}$). $R_{potential}$ indicates the maximum androgen responsiveness, i.e. the difference between breeding baseline and the maximum testosterone release after stimulation with gonadotropin releasing hormone. R_{env} indicates androgen responses to environmental stimuli such as nest boxes in starlings. R_{season} indicates the populations' seasonal androgen response, i.e. the difference between breeding baseline and the highest levels of testosterone measured during territory establishment or the mating peak. R_{season} explicitly represents a population measure, whereas $R_{potential}$ explicitly represents an individual-based measure, all the other Rs can be measured in a population. On an individual level, the magnitude of R can change depending upon the life history stage, i.e. $R_{potential}$ can be higher during territory establishment and during the mating sub-stage than the parental sub-stage.

different mediators. However, changes in sensitivity of target cells to a single mediator so that different tissues can respond in different ways as circulating levels change is an alternate scenario (Wingfield, 2018). In this case, changes in the number and distribution of receptors for a specific mediator come to mind. Transport mechanisms of hormones in blood may be another point of modulation, or expression of enzymes that metabolize mediators as they enter target cells (Ball and Balthazart, 2007; Wingfield, 2018). There is also growing evidence that local production of hormones with paracrine effects can avoid signals being broadcast to the whole body that may trigger inappropriate responses.

3. Costs of testosterone

Why are elevations of hormone levels to level C so brief? Wingfield et al., (1990) proposed that prolonged periods of level C secretion bears costs. For example, high circulating testosterone might be deleterious as are chronic high levels of glucocorticoids (Wingfield et al., 2001; Wingfield and Soma, 2002). Numerous experimental studies on the deleterious effects of high testosterone, both in the field and the laboratory, were published in the next years (e.g. reviewed in Ketterson et al., 1996; Ketterson and Nolan, 1999: Wingfield et al., 2001, 2014). These effects included suppression of the immune system, high energetic demands, injury or death from fights, reduced parental care and thus lower reproductive success. Discovery of these effects in turn led to thoughts about control of hormone secretion above level B and additional environmental and social inputs on hormone secretion. As the Challenge Hypothesis was tested further, new ideas on old problems in behavioral endocrinology began to emerge. Some of these will be discussed next.

4. Refining androgen responsiveness

Originally, the Challenge Hypothesis defined androgen responsiveness (or R) as a ratio among the different hormone levels A, B and C. Specifically, androgen responsiveness was $R = \frac{(\text{level C} - \text{level A})}{(\text{level B} - \text{level A})}$ (Wingfield et al., 1990). Responsiveness was assumed to represent the rise of testosterone mainly during social interactions among males. At that time few data regarding the change of testosterone during male-male interactions were available. It was thus assumed that seasonal peaks in testosterone (level C) reflected the testosterone peaks that occurred during such social interactions among males. Hence, the first comparative data relating the androgen responsiveness to the degree of male-male competition and male contributions to parental care (i.e. Fig. 7 in Wingfield et al., 1990) were largely based on the seasonal profiles of levels A, B and C of testosterone. Later on, two major refinements were made to this approach. The first refinement was about the statistical approach. Ratios such as the one used to calculate the androgen responsiveness R cannot incorporate the variance in the data and differences in sample size. Goymann et al. (2007a, 2007b) therefore suggested to base future calculations of the androgen responsiveness on standardized effect sizes (i.e. Cohen's d) and their 95% confidence intervals instead. Such effect sizes represent a standardized measure of the difference between groups, incorporate the variance and sample size, and are commonly used in meta-analyses (Cohen, 1988; Cumming and Finch, 2001; Nakagawa and Cuthill, 2007).

The second refinement involved the definition of androgen responsiveness. Once more, data from changes in androgen concentrations of males during experimental manipulations of male-male interactions became available, it was realized that the androgen responsiveness derived from seasonal testosterone profiles did not necessarily match the androgen responsiveness calculated from male-male interactions (Goymann et al., 2007a, 2007b, Goymann, 2009). This finding led to the distinction between the 'seasonal androgen response' (or R_{season}) and the actual androgen responsiveness to social interactions among males (or $R_{male\text{-}male}$). Such male-male interactions can be simulated with experimental territorial intrusions using decoys (Wingfield and Wada, 1989). To simplify the calculation of androgen responsiveness the seasonal androgen response (R_{season}) was defined as the standardized effect size calculated from the difference between Level C (seasonal maxima) and Level B testosterone (breeding baseline) concentrations. The androgen responsiveness to male-male interactions ($R_{male\text{-}male}$) was defined as the standardized effect size of the difference between testosterone levels of males after a STI and those of males caught under control conditions (Fig. 2). Ideally, one would wish to calculate the effect size using within-individual comparisons, i.e. control and experimental testosterone concentrations from the same individual, but in practice such data are hard to get (but see Landys et al., 2010 for a rare example). Hence, typically control and experimental testosterone concentrations come from different individuals and $R_{male\text{-}male}$ may differ depending upon the life history stage.

The Challenge Hypothesis focused on social interactions among males, but for a long time it also has been known that social interactions with females trigger changes in androgen levels of males (Harding, 1981). As a consequence one could consider the androgen responsiveness of males to interactions with females ($R_{male\text{-}female}$) as the standardized effect size of the difference between testosterone levels of males that are exposed to receptive females and those that are not (Fig. 2). In

some species, even non-social environmental cues such as the presence of nest boxes may trigger an androgen response (Gwinner et al., 2002; Carbeck et al., 2018), probably because such nest boxes are perceived as a reproductive cue. In such cases the androgen responsiveness to non-social environmental stimuli ($R_{environment}$) can be defined as the standardized effect size of the difference between testosterone concentrations of males exposed to these environmental cues and those of control males not receiving these cues (Fig. 2). Apart from nest boxes, other cues could serve as a trigger to increase androgens. For example, Schoech and Hahn (2008) suggested that food supplementation may advance breeding especially in low latitude species, which rely less on photic cues. In such species, food availability may represent a potential environmental cue influencing $R_{environment}$. It is important in this context, however, to distinguish between environmental cues that initiate the increase of testosterone levels from level A to within level B ('initial predictive cues' sensu Jacobs and Wingfield (2000)) and cues that trigger changes from level B to level C.

Generating a complete picture of androgen responsiveness includes determining the physiological capacity of an organism to produce androgens via GnRH injections. GnRH induces the release of testosterone from the gonads (Bentley, 1998) and thus can be used to determine the capacity for testosterone release. During the seasonal peak period of testosterone secretion, such GnRH-induced testosterone levels may represent a more accurate estimate of Level C concentrations than androgen concentrations obtained from seasonal hormone profiles, in particular when the seasonal profile is based on a small number of samples. From GnRH-induced testosterone concentrations we can derive the potential androgen responsiveness ($R_{potential}$) corresponding to the effect size of the difference between testosterone levels before and after GnRH injection (see also Goymann et al., 2007a, 2007b, Goymann, 2009). Similar to other measures of androgen responsiveness, the magnitude of $R_{potential}$ strongly depends on the life history stage (e.g. Jawor et al., 2006; DeVries et al., 2011; Goymann et al., 2015; Covino et al., 2018). Ideally, if the effect size R_{season} represents a good estimate of the difference between level B and level C, then it should be similar to the effect size of $R_{potential}$ at the time of the maximum testosterone responsiveness. Typically the latter occurs at the beginning of the breeding season unless the hypothalamus is not capable of producing the amount of GnRH to elicit a maximal pituitary release of luteinizing hormone (LH), which then triggers the production of testosterone. Current evidence supports the view that among individuals R_{season} is a good estimate of $R_{potential}$, as the effect sizes of these two measures did not differ (Goymann, 2009; Fig. 2).

Recent evidence suggests that many species of birds do not elevate circulating testosterone in response to challenges from other males (Goymann et al., 2019). Rather, the most consistent effect on androgen secretion in male birds seems to be related to reproductive cues such as from fertile females (i.e. $R_{male\text{-}female} > R_{male\text{-}male}$). The original Challenge Hypothesis implicitly accounted for such cues, but its main focus was interactions among males. Also, most experimental tests of the Challenge Hypothesis during the last 30 years have concentrated on male-male interactions. The main aim of the Challenge Hypothesis was to explain the large differences in circulating androgen concentrations that occur among species on the one hand and among individuals of the same species on the other hand. In the future, it will be important to determine which social or environmental stimuli trigger short peaks of androgens in the level C range. With regard to social challenges, male-female interactions may deserve more attention, even though they are harder to experimentally manipulate than male-male interactions. Also, so far most tests of the Challenge Hypothesis have been conducted in socially monogamous and biparental species. If level C testosterone bears costs mainly in terms of reducing male contributions to parental care, then further experimental work in polygynous or lekking species without male contributions to parental care should be very helpful to advance our understanding of how androgens affect behavior and vice versa (see also Goymann et al., 2019; Moore et al., 2019).

In addition, most experimental tests of the Challenge Hypothesis have concentrated on male-male interactions in the breeding season, when circulating levels of testosterone and other androgens are elevated (levels B and C). However, aggressive interactions also occur outside of the breeding season, when circulating testosterone levels are typically very low or non-detectable (level A). Accumulating evidence suggests that aggressive interactions during the non-breeding season affect steroid concentrations in the brain but not in the blood, as summarized below.

5. Neural steroid synthesis and territorial aggression

During autumn and early winter in the temperate zone, nearly all birds are in non-breeding condition, with regressed gonads and basal levels of circulating sex steroids. Often, non-breeding birds abandon territories and form flocks (e.g. Soma and Wingfield, 1999). However, in some species, individuals continue to aggressively defend territories during the non-breeding season. For example, a subspecies of song sparrow (*Melospiza melodia morphna*) is sedentary (Wingfield and Hahn, 1994). Males are territorial during the breeding season (spring and early summer), when plasma testosterone levels are high (levels B and C). Once the breeding season is finished, song sparrows molt their feathers (August–September). During the molt, plasma testosterone levels are basal (level A), gonads are regressing, and aggression is greatly reduced. Following completion of the molt, there is a resurgence of territorial behavior in October, which continues throughout the rest of the non-breeding season. Plasma testosterone remains non-detectable (level A) during the non-breeding season, and the testes are completely regressed (Wingfield and Hahn, 1994). Aggressive interactions between males do not increase circulating testosterone levels during the non-breeding season. Circulating levels of 17β-estradiol (E_2), 5α-dihydrotestosterone (DHT), androstenedione (AE), and estrone (E_1) are also basal in males during the non-breeding season (Heimovics et al., 2013). Moreover, castration does not decrease aggressive behavior in non-breeding song sparrows, suggesting that gonadal hormones do not support territorial aggression during the non-breeding season (Wingfield, 1994).

In field experiments, non-breeding male song sparrows were treated with aromatase inhibitors, with or without an androgen receptor (AR) antagonist. Aromatase catalyzes the conversion of testosterone to E_2 and regulates male aggression in reproductive contexts. First, treatment with an aromatase inhibitor (ATD) and an AR antagonist (flutamide) for 30 days decreases non-breeding aggression (Soma et al., 1999). Second, treatment with fadrozole, a more potent aromatase inhibitor than ATD, for 10 days strongly decreases non-breeding aggression (Soma et al., 2000a). Third, treatment with fadrozole for only one day reduces some aspects of non-breeding aggression (Soma et al., 2000b). These results indicate that sex steroids, particularly estrogens, support territorial aggression during the non-breeding season, even though sex steroids are non-detectable in the plasma. These data raised the hypothesis that steroids synthesized in the brain (neurosteroids; Corpechot et al., 1981; Schmidt et al., 2008; Soma et al., 2008) regulate territorial aggression in the non-breeding season.

Aromatase mRNA is highly expressed in regions of the song sparrow brain (Soma et al., 2003; Wacker et al., 2010) that regulate social behavior (Jalabert et al., 2018): preoptic area (POA), ventromedial nucleus of the hypothalamus (VMH), nucleus taeniae of the amygdala (TnA), bed nucleus of the stria terminalis (BnST), and caudomedial nidopallium (NCM). In the POA, aromatase mRNA is higher in the breeding season, compared to the non-breeding season and molt (Wacker et al., 2010). In the VMH, aromatase mRNA is high in the breeding and non-breeding seasons and reduced during molt (Wacker et al., 2010). The activity of aromatase was also measured (Soma et al., 2003). Aromatase activity in the ventromedial telencephalon (which includes TnA) is reduced during molt. Aromatase activity in the diencephalon, however, is high only during the breeding season. In contrast

to aromatase, estrogen receptorα (ERα) and ERβ mRNA do not vary seasonally in these regions (Wacker et al., 2010).

The substrate for brain aromatase in the non-breeding season was unclear, because of the basal levels of plasma testosterone and AE. Dehydroepiandrosterone (DHEA) is an androgen precursor and considered a pro-hormone that does not bind with high affinity to AR or ER (Labrie et al., 2005; Soma et al., 2015). However, DHEA can be converted into AE within tissues that express the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). In song sparrows, circulating DHEA levels were measured using radioimmunoassays (Soma and Wingfield, 2001; Goodson et al., 2005; Newman et al., 2008). Circulating DHEA levels are reduced during the molt, compared to the breeding and non-breeding seasons (Soma and Wingfield, 2001; Newman and Soma, 2009). In the non-breeding season, circulating DHEA is detectable and several-fold higher than circulating testosterone and estradiol. In the non-breeding season, circulating DHEA might originate from the adrenal glands, regressed testes, liver, or brain (Soma and Wingfield, 2001; Newman and Soma, 2009, 2011). The effects of a simulated territorial intrusion (STI) on DHEA levels in the brachial vein (in the wing) and jugular vein (exiting the brain) were examined (Newman and Soma, 2011). In both seasons, STI increases DHEA levels in the jugular vein but not in the brachial vein, suggesting that territorial challenges modulate neural DHEA synthesis and/or secretion. Treatment of wild non-breeding song sparrows with DHEA increases territorial singing (a threat behavior) but not direct attack behaviors (Soma et al., 2002) and also increases the size of the song nucleus HVC (Soma et al., 2002). However, DHEA treatment does not stimulate the growth of the cloacal protuberance, a secondary sex characteristic that is androgen-sensitive. In captive non-breeding male song sparrows, DHEA treatment increases warning behaviors in a laboratory STI (Wacker et al., 2016) and the numbers of adult-born cells and immature neurons in HVC (Newman et al., 2010; Wada et al., 2014). These effects of DHEA might be mediated by its metabolism within specific brain regions to active sex steroids.

To examine DHEA metabolism in the brain, an in vitro assay was used to measure the conversion of [^{3}H]-DHEA to [^{3}H]-AE and [^{3}H]-estrogens by the sequential activities of 3β-HSD and aromatase (Schlinger et al., 2008; Pradhan et al., 2010; Pradhan and Soma, 2012). Song sparrow brain homogenates convert DHEA to AE and estrogens, with highest levels of 3β-HSD activity in the forebrain. Brain 3β-HSD activity is generally elevated in the non-breeding season, relative to the breeding season and molt. Elevated brain 3β-HSD allows for rapid local fluctuations in active sex steroids in response to territorial challenges. During the non-breeding season, a STI rapidly increases 3β-HSD activity in regions of the telencephalon that mediate motivated behaviors, particularly when 3β-HSD activity is measured without exogenous co-factor (NAD+) (Pradhan et al., 2010). These data suggest that territorial challenges rapidly increase neurosteroids but not circulating sex steroids during the non-breeding season. Neurosteroids have rapid effects on a variety of social behaviors (Heimovics et al., 2015, 2018).

The rapid effects of steroids on aggression were studied in captive male song sparrows. Non-invasive estradiol administration rapidly (within 20 min) increases aggression in response to a laboratory STI in the non-breeding season only (Heimovics et al., 2015). Estradiol also has rapid effects on phosphorylation of signaling proteins (ERK, CREB, TH) in multiple brain regions (Heimovics et al., 2012). In other systems, these signaling proteins are rapidly regulated by estradiol binding to membrane-associated ER (Micevych et al., 2017). In both breeding and non-breeding males, DHEA, testosterone, and estradiol levels (as measured by radioimmunoassays) are higher in micro-dissected brain regions than in the circulation. In both seasons, a very short (5 min) laboratory STI modulates levels of DHEA in specific brain areas but not in the blood (Heimovics et al., 2016). These studies suggest rapid effects of steroids on aggressive behavior and neuronal signaling, as well as rapid effects of social challenges on brain steroid levels.

Taken together, these data suggest that sex steroids can be locally produced within specific brain regions to regulate aggression and that neurosteroid synthesis is up-regulated by aggressive challenges. This mechanism would reduce the exposure of peripheral tissues and other neural circuits to testosterone and estradiol. Non-breeding aggression might be dissociated from circulating testosterone because of the high costs of testosterone during the winter (Soma, 2006). Thus, neuroendocrine mechanisms may have evolved to support aggression in non-reproductive contexts, while avoiding the costs of high circulating testosterone. These insights stemmed from the original studies that gave rise to the Challenge Hypothesis.

6. Measuring effects of aggressive interactions on brain steroid levels

As summarized above, social interactions can affect steroid production in specific areas of the brain. To assess how social challenges affect local steroid levels, a powerful tool is in vivo micro-dialysis (Remage-Healey et al., 2008), but sampling is often limited to one brain region at a time. Another approach is the Palkovits punch (Palkovits, 1973), which can be used to micro-dissect multiple brain regions from a single animal (Taves et al., 2011). Samples can weigh only 1–2 mg, and sensitive techniques are needed to quantify steroids (Tobiansky et al., 2018).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an ultrasensitive technique that measures multiple analytes with great specificity (Vogeser and Parhofer, 2007). LC-MS/MS allows the measurement of 0.05 pg of some steroids, which is approximately 50× more sensitive than most immunoassays. Another advantage of LC-MS/MS is specificity. In immunoassays, structurally similar steroids in the sample can cross-react with the antibody, and therefore steroid concentrations can be overestimated (especially at low steroid concentrations) (Grebe and Singh, 2011). In LC-MS/MS, liquid chromatography first separates the steroids, and then tandem mass spectrometry monitors the formation of product ions from a precursor ion. Multiple reaction monitoring (MRM) can examine a “quantifier” product ion and a “qualifier” product ion. The qualifier product ion provides additional assurance with regard to specificity.

Using LC-MS/MS, we have measured steroids in different species and sample types (Tobiansky et al., 2018; Taves et al., 2015; Taves et al., 2016). In zebra finches, we measured progestins and androgens in plasma (Prior et al., 2016a, 2016b) and estradiol in POA (Heimovics et al., 2018). In song sparrows, a panel of 10 steroids was examined in plasma (Fokidis et al., 2019). In this study, plasma DHEA concentrations were detectable but lower than in previous studies using radioimmunoassays, which reflects the greater specificity of LC-MS/MS. Pregnenolone and progesterone are also present in the blood of non-breeding males and could serve as substrates for neurosteroidogenic enzymes (Fokidis et al., 2019).

Using LC-MS/MS, we examined testosterone in plasma from wild male song sparrows (Fig. 3). Analysis of the ion ratio of the 2 MRM transitions (between the quantifier and qualifier product ions), as well as their retention times, are used for verification of analyte identity. The concentration of plasma testosterone in the breeding subject was 6.48 ng/mL and was lower than the detection limit (< 0.01 ng/mL) in the non-breeding subject. Similar methods will be used for AE and DHT (Fig. 3). Such an approach can be used to measure a panel of steroids and to examine the effects of a challenge on systemic steroid levels in blood and local steroid levels in multiple brain regions. Estrogens are difficult to measure because of their low concentrations, but there are emerging protocols to measure several estrogens (17β-estradiol, 17α-estradiol, estrone, catecholestrogens) with increased sensitivity (Heimovics et al., 2018).

Steroid analyses can also be performed using mass spectrometry imaging (MSI) which is a cutting-edge technique to analyze the spatial distribution of small molecules directly from tissue surfaces. MSI has been used to map glucocorticoids in the mouse brain and androgens in

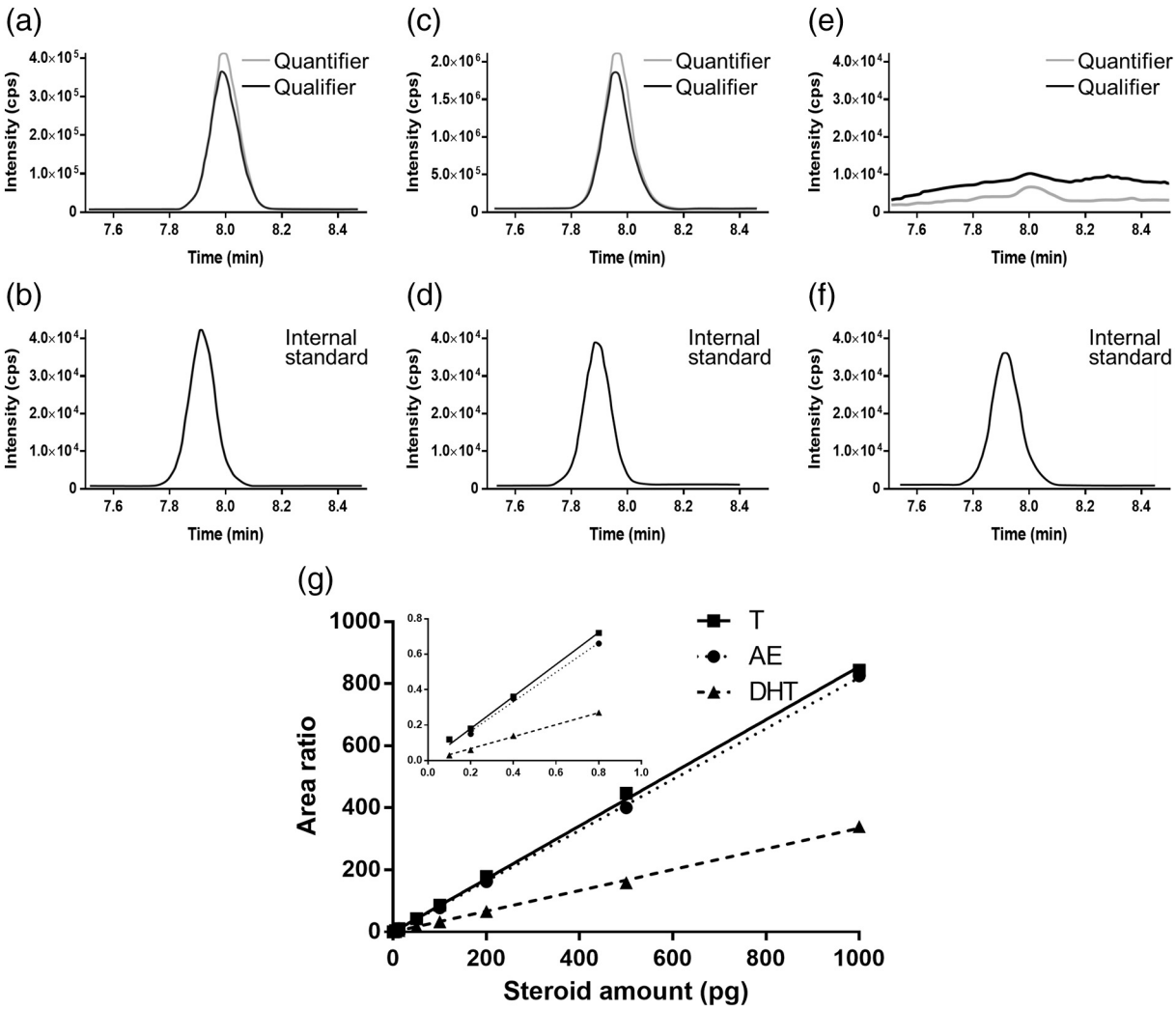

Fig. 3. Measurement of testosterone (T) and other androgens by LC-MS/MS. Representative chromatograms for the 2 multiple reaction monitoring (MRM) transitions for T (quantifier: *m/z* 289 → 97; qualifier: m/z 289 → 109) (top) and 1 MRM transition for the deuterated internal standard testosterone-d5 (T-d5) (m/z 294 → 100) (bottom). Samples were (a and b) certified reference standard (12.5 pg), (c and d) wild breeding male song sparrow plasma (10 μL), and (e and f) wild non-breeding male song sparrow plasma (10 μL). Intensity in counts per second. 50 μL of T-d5 was added to all samples to adjust for procedural losses and matrix effects. Then, 1 mL HPLC-grade acetonitrile (ACN) was added, samples were homogenized and centrifuged, and 1 mL of supernatant was transferred to a clean glass culture tube. Then 500 μL hexane was added, samples were vortexed and centrifuged. The hexane was discarded, and the ACN was dried in a vacuum centrifuge. Pellets were reconstituted with 50 μL 25% methanol. Data were acquired on a Sciex QTRAP 6500 UHPLC-MS/MS system in positive electrospray ionization mode (Tobiansky et al., 2018). The breeding male, but not the non-breeding male, had detectable T in the plasma (i.e., greater than the lowest point on the calibration curve, 0.1 pg). (g) Calibration curves for T, androstenedione (AE), and 5α-dihydrotestosterone (DHT), with inset displaying the lowest points on the calibration curves. The area ratio is calculated by dividing the analyte (quantifier transition) peak area by the internal standard peak area in the same sample. Calibration curves showed great sensitivity, as the detection range was 0.1 to 1000 pg for T and DHT and 0.2 to 1000 pg for AE. Calibration curves were linear even at the low range (< 1 pg).

the mouse testis (Cobice et al., 2013, 2015). MSI can be performed with a spatial resolution of as little as 50 μm, which is close to cellular level imaging.

7. Conclusions

Over the past few decades, investigations of hormone-behavior interrelationships in free-living animals provided new insights into what plasma levels of a hormone may mean (state levels) and how dynamic changes hint at further underlying actions and mechanisms not fully appreciated before. These include development of frameworks for understanding androgen responsiveness to different environmental cues and ultimately what neural and neuroendocrine adaptations may have evolved. New techniques that enable us to measure central production of steroids may allow us to probe the mechanisms of hormone-behavior interactions in unprecedented detail in different species with contrasting social networks. This in turn will provide new insight into their ecological bases and evolution of mechanisms. The Challenge Hypothesis will likely metamorphose even more in the next few years.

Acknowledgments

JCW is grateful for a series of grants from the National Science Foundation that span several decades. This continuity of funding was critical to develop new hypothesis and test them over the years. Wingfield is also grateful for the critical input of so many undergraduate students, graduate students, postdoctoral scholars and visiting faculty. The Challenge Hypothesis would not have flourished for so long without their creativity, criticism and boundless energy working in the field and the laboratory. This work was also supported by Canadian Institutes of Health Research Operating Grant (133606) (to KKS), Canada Foundation for Innovation Grant (to KKS), BC Knowledge

Development Fund Grant (to KKS), the Max Planck Society (to WG) and Uruguay Graduate Scholarship from the Agencia Nacional de Investigación e Innovación and UBC Zoology Graduate Fellowship (to CJ). Work on song sparrows was approved by the University of British Columbia Animal Care Committee. We thank Donna L. Maney and an anonymous reviewer for suggestions and edits that have improved the manuscript enormously.

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