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# European robins (*Erithacus rubecula*) lack an increase in testosterone during simulated territorial intrusions

Madeleine F. Scriba · Wolfgang Goymann

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**Abstract** The challenge hypothesis (Wingfield et al. in *Am Nat* 136:829–846, 1990) predicts that circulating testosterone increases when socially monogamous male birds are challenged during breeding. Although the challenge hypothesis has been confirmed in large-scale interspecific comparisons of seasonal hormone profiles, experimental tests of the challenge hypothesis are still uncommon and the results equivocal. We tested one of the predictions of the challenge hypothesis by investigating the behavioural and hormonal response of free-living European robins during simulated territorial intrusions (STIs) in the breeding season. We conducted STIs by placing a stuffed decoy in a territory and playing robin song. After the behaviour of the focal male had been recorded for at least 10 min, it was captured and a blood sample was taken immediately. Controls were caught within 10 min of the first response of the territory owner. Hormone concentrations were measured by radioimmunoassay. Although previous studies have shown that testosterone has an impact on aggression, European robins do not respond to STIs by increasing circulating levels of testosterone.

**Keywords** Aggression · Territorial behaviour · Challenge hypothesis · Testosterone · Dihydrotestosterone · Corticosterone · Progesterone · Simulated territorial intrusion · Decoy · European robin · *Erithacus rubecula*

## Introduction

Hormones can modulate the expression of behaviour, and behaviour may feed back to hormone secretion (e.g. Wingfield et al. 1999; Wingfield 2005). Testosterone, in particular, is known to activate and influence reproductive behaviours and aggression in many species of birds and in various contexts (e.g. Silver et al. 1979; Harding 1981, 1983; Balthazart 1983; Wingfield and Ramenofsky 1985; Wingfield et al. 1987, 2001; Schwabl 1992; Soma et al. 1999; Canoine and Gwinner 2002). Aggression occurs over limited resources, such as territories and mates, and a modulatory role of testosterone has been demonstrated, mainly in the context of reproductive aggression (Wingfield et al. 2006; but see Ros et al. 2002 for a role of testosterone in a non-reproductive context). The administration of testosterone to castrated or intact individuals enhances the frequency (e.g. in quail, Adkins and Pniewski 1978) and intensity (e.g. in red-winged blackbird, Searcy and Wingfield 1980) of aggressive displays, and castration decreases the levels of aggression in many species (Arnold 1975; Cheng and Lehrman 1975; Tsutsui and Ishii 1981; but see Wingfield 1994). Similarly, aggressive interactions with conspecifics may feed back to testosterone secretion (Wingfield et al. 1990).

High levels of testosterone for prolonged periods of time may be costly, as testosterone has been suggested to mediate a trade-off between investing in reproduction and

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self-maintenance. In particular, high levels of testosterone may increase the risk of injury and exposure to predators, its anabolic properties may reduce fat stores, and it may impair the function of the immune system (Duffy 1989; Ketterson et al. 1992, 1996; Folstad and Karter 1992; Wedekind and Folstad 1994; Hillgarth and Wingfield 1997; Duffy et al. 2000; Wingfield et al. 2001). Furthermore, testosterone has been reported to reduce male parental care in various bird species (Wingfield et al. 1990), although this does not seem to be the case in species for which paternal care is essential (Lynn et al. 2002, 2005; but see Lynn et al. 2009).

For species with a socially monogamous mating system and biparental care, the challenge hypothesis predicts that circulating levels of testosterone should remain close to the breeding baseline, during the reproductive season, and increase only during social challenges (Wingfield et al. 1990). This rise in plasma levels of testosterone is considered to be linked with an increase in the intensity and persistence of aggressive behaviour (Wingfield 1994; Oyegbile and Marler 2005). Evidence to support the challenge hypothesis has been found in large-scale interspecific comparisons of seasonal hormone profiles (Wingfield et al. 1990, 2000; Hirschenhauser et al. 2003; Hirschenhauser and Oliveira 2006). However, experimental studies have shown inconsistent results (reviewed in Landys et al. 2007; Goymann et al. 2007; Goymann 2009). Some socially monogamous bird species show an increase in testosterone during simulated territorial intrusions (Wingfield 1984a, b, 1985; Wingfield and Hahn 1994; Wingfield and Wada 1989), whereas other species display no rise in testosterone concentrations during such encounters (e.g. Landys et al. 2007; Lynn and Wingfield 2008; Moore et al. 2004a, b; Van Duyse et al. 2004; Wingfield and Hunt 2002).

Here, we describe the hormonal response to simulated territorial intrusions in male European robins (*Erithacus rubecula*, hereafter referred to as *robins*). Previous studies have demonstrated that testosterone is involved in the modulation of breeding season territoriality in male robins: territorial aggression declined when the action of testosterone was blocked with the anti-androgen flutamide (Schwabl and Kriner 1991). Thus, during the breeding season, testosterone appears to facilitate territorial aggression in this species. However, whether testosterone increases during territorial challenges in this species and thus may be involved in the modulation of the persistence of aggression is not known. We therefore conducted simulated territorial intrusion (STI) experiments with robins. We expected a strong response and an increase in testosterone during STIs. In addition, we also investigated other hormones, i.e. plasma levels of corticosterone, progesterone, oestradiol and dihydrotestosterone, in response to

these STIs. These hormones have received less attention than testosterone but may be of potential importance.

## Animals and methods

### Study population and study site

We studied robins during the early breeding season (between 10 April and 13 June 2006) in the vicinity of the Max Planck Institute for Ornithology in Andechs (48°58' N, 11°11' E), Germany.

The European robin is a socially monogamous, monomorphic songbird that lives in open, deciduous, and scrubby woodlands (Hoelzel 1989). Our population of robins consisted of short-distance migrants that had arrived from their Mediterranean winter quarters in April and had laid their first clutches at the end of April or beginning of May (Schwabl 1992). Males defend breeding territories, while females incubate the eggs and brood the young (Lack 1965; Hoelzel 1986). Males feed females at the nest during incubation and for at least 2–3 days after the eggs have hatched (Pätzold 1995; Grajetzky 2000). Robins breed two to three times per season until August. After their moult, in late September, the birds move back to their winter areas (Schwabl 1992).

### Experimental protocol

To simulate the intrusion of a foreign male into the territory of a focal male, we placed a stuffed male robin (mounted in an upright position on a perch, with the orange chest patch clearly visible) under an inconspicuous net-cage, to avoid damage to the decoy, at a distance of 1–2 m from a mist-net in the territory of the focal male. Because it has been shown that male robins behave more aggressively when visual and acoustical signals are combined (Chantrey and Workman 1984), we also played robin song (recordings were obtained from the British Library Sound Archive, London). Two different stuffed robins and two tapes with different songs were used to minimise pseudoreplication (Hurlbert 1984). We randomly selected one robin and song for each trial. After starting the playback, we observed the behaviour of the territory owner, with binoculars, from a distance of approximately 50 m and recorded the following agonistic behaviours: number of threats (upright position close to the decoy, presenting the red chest), wing flaps, movements around the decoy, swaying (slow rhythmic swaying of the body from one side to the other with the feet on the ground), tail up, attacks and flights over the intruder.

Wingfield and Wada (1989) have shown that the minimum time for a testosterone response to STIs is 10 min.

Hence, to exclude other factors that might have caused a difference in testosterone concentration, we considered robins that were caught within 10 min of the onset of the playback and positioning of the decoy as controls. For the experimental birds we opened the mist-net only 10 min after the first response of the resident bird and continued the STI until the bird was finally caught in the mist-net. For most birds we decided in advance whether they should represent controls or experimental males. In five cases, however, we set up the mist-net at the beginning of the experiment but caught birds much later than 10 min and assigned them to the experimental group. With the latter procedure we might have accidentally selected for less aggressive males (with potentially lower testosterone concentrations). Therefore, we analysed the data including and excluding these five birds.

We chose this experimental setup to minimise the potential impact of different capture procedures on testosterone concentrations. Control birds ( $n = 20$ ) experienced a short STI of, at most, 10 min duration [mean  $\pm$  standard error (SE)  $3.6 \pm 0.6$  min], whereas experimental birds ( $n = 12$ ) were challenged for at least 10 min ( $31.6 \pm 5.1$  min).

Within  $5.7 \pm 0.5$  (mean  $\pm$  SE) min of the birds' capture, we collected 100–200  $\mu$ l of blood by puncturing the alar wing vein with a 25-gauge needle. We measured the birds' body masses, wing lengths and tarsus lengths, ringed the birds (size B, Vogelwarte Radolfzell, Germany) and released them on their territory within 10–35 min after their capture. All robins were caught between 6:00 h and 12:00 h, so that we would avoid diurnal variation in hormone concentrations (Balthazart and Hendrick 1979; Romero and Remage-Healey 2000). The blood samples were kept on ice until they were returned to the laboratory (<6 h), where the plasma and blood cells were separated by centrifugation, and the plasma was frozen at  $-40^{\circ}\text{C}$  until required for further analysis. Red blood cells were stored in Queen's lysis buffer for genetic sex determination (Griffiths et al. 1998), which confirmed that all the robins used in the study were males.

### Hormone assays

Plasma testosterone, dihydrotestosterone (DHT), progesterone, oestradiol and corticosterone concentrations were determined by radio-immunoassay after column chromatographic separation of the hormones using a modification (Goymann et al. 2008) of the protocol by Wingfield and Farner (1975). Mean ( $\pm$ SE) extraction efficiencies were  $83 \pm 0.9\%$  for testosterone,  $84 \pm 1.1\%$  for DHT,  $66 \pm 1.2\%$  for progesterone,  $80 \pm 1.5\%$  for oestradiol, and  $43 \pm 1.1\%$  for corticosterone. The intra-assay coefficients of variation, as determined from the assay controls, were 7.7% for testosterone, 8.8% for DHT,

8.5% for progesterone, 28.4% for oestradiol and 12.2% for corticosterone. All samples were analysed in one assay per hormone. The standard curves and concentrations were calculated with ImmunoFit (Beckmann Inc., Fullerton, CA, USA). The detection limits were 0.7 pg/tube for testosterone, 12 pg/ml for DHT, 5.9 pg/tube for progesterone, 0.5 pg/tube for oestradiol, and 7.7 pg/tube for corticosterone. Samples with undetectable levels of hormone were set to their individual adjusted detection limits (corrected for recoveries and plasma volume) for statistical analysis.

### Statistical analyses

The statistical analyses were conducted with Systat 11.01 (Systat Software GmbH, Erkrath, Germany). Data were tested using general linear models, including the variables Julian day, time between capture and blood sampling, STI duration and aggression score. To calculate the aggression score we combined the five agonistic parameters taken (frequency of threats, wing flaps, swaying, tail up, attacks) with a principal components factor analysis into one aggression score which explained 60% of the total variance.

We tested the residuals of each model for normality using Wilk–Shapiro tests. To meet this criterion, testosterone and corticosterone data were log-transformed and DHT data were square-root transformed. Progesterone data could not be normalised. We therefore investigated the relationship between progesterone and STI duration using a Spearman rank correlation. Oestradiol data were not analysed, as only three samples contained oestradiol levels above the detection limit. Unless indicated otherwise, all reported values represent means and standard errors of the mean (sem). Transformed summary data were backtransformed for graphical illustration, resulting in asymmetrical standard errors.

## Results

Robins responded with song to the simulated territorial intrusion. Some, but not all, birds behaved aggressively towards the decoy.

Testosterone concentrations of STI birds did not differ from those of control birds (Table 1; Fig. 1a). However, testosterone increased significantly with Julian day, when all birds were included (Table 1; Fig. 1b), but not when the five STI birds were excluded for which the nets had already been opened from the beginning of the trial (see Methods; Table 1). The aggression score did not explain a significant proportion of the variance in testosterone concentrations (Table 1).

**Table 1** General linear models for testosterone, dihydrotestosterone (DHT) and corticosterone concentrations in European robins, including all birds

<i>n</i> = 32	Testosterone $R^2 = 0.24$		DHT $R^2 = 0.34$		Corticosterone $R^2 = 0.45$	
	$F_{1,30}$	<i>P</i>	$F_{1,30}$	<i>P</i>	$F_{1,30}$	<i>P</i>
Independent variables						
Time between capture and blood sampling	0.06	0.81	2.60	0.12	<b>18.15</b>	<b>&lt;0.001</b>
Aggression score	1.17	0.29	<b>4.67</b>	<b>0.04</b>	0.29	0.60
Julian day	<b>4.41</b>	<b>0.045</b>	<b>4.64</b>	<b>0.04</b>	<0.001	0.99
STI versus control	0.22	0.64	0.05	0.83	0.50	0.49
Backtransformed mean (–sem; +sem) hormone concentrations (ng/ml)						
Hormone levels after STI	2.5 (0.5; 0.7)		0.3 (0.07; 0.09)		12.2 (2.4; 3.0)	
Hormone levels of controls	3.9 (0.7; 0.8)		0.37 (0.05; 0.06)		15.8 (2.1; 2.4)	

At the bottom, the backtransformed means of hormone concentrations of STI and control birds are shown. Significant effects are printed in bold type. When the five STI birds for which the mist-net was open from the beginning of the trial (see [Methods](#)) were excluded, the only significant result was a relationship between corticosterone concentrations and time between capture and blood sampling

DHT concentrations of STI and control birds also did not differ significantly from each other (Table 1), but DHT levels increased with Julian day and was higher in birds with a lower aggression score (Fig. 2). The relationship between DHT and aggression score was mainly driven by one outlier. When the five STI birds for which the nets had been opened from the beginning of the trial were excluded, there was no relationship between Julian day, aggression score and DHT.

Corticosterone concentrations of STI and control birds did not differ from each other (Table 1). The only factor influencing corticosterone concentrations was the time between capture and blood sampling (Table 1).

Progesterone concentrations of control birds [median (lower quartile; upper quartile) = 0.97 (0.60; 1.69) ng/ml] and STI birds [0.89 (0.62; 1.37) ng/ml] did not differ significantly (Mann–Whitney U test,  $U = 129$ ,  $P = 0.73$ ) and were not related to the other variables (Spearman rank correlations with time between capture and blood sampling  $r = 32$ ,  $P > 0.05$ ; aggression score  $r = 0.08$ ,  $P > 0.1$ ; Julian day  $r = 0.23$ ,  $P > 0.1$ ).

## Discussion

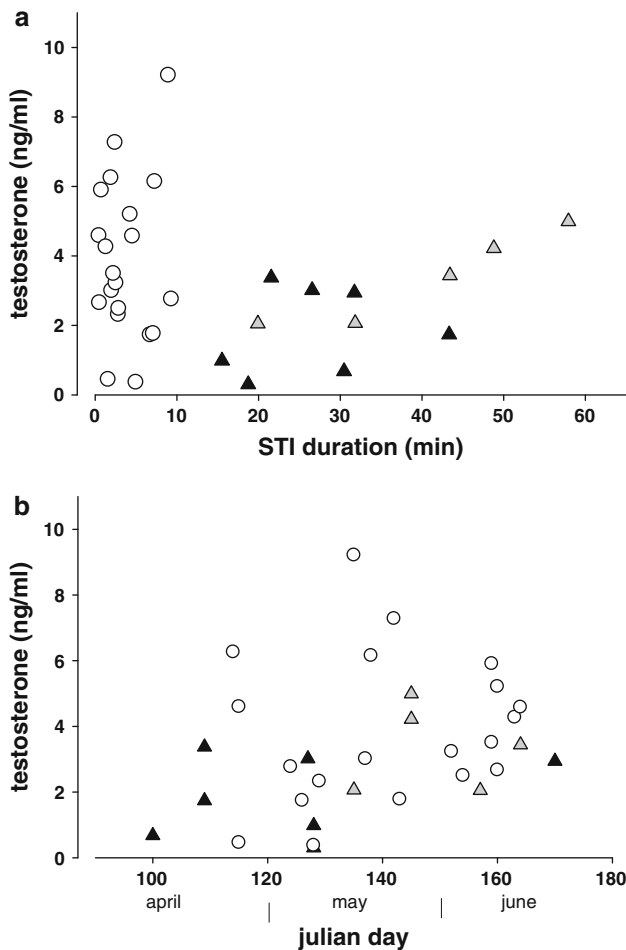
In this study we tested one of the predictions of the challenge hypothesis, which states that during a territorial challenge, plasma levels of testosterone should increase in socially monogamous and bi-parental males (Wingfield et al. 1990). Schwabl and Kriner (1991) showed that treatment of free-ranging male European robins with the anti-androgen flutamide led to an increase in the latency of approach to a decoy during STIs. Although Schwabl and Kriner (1991) did not find an effect on other behavioural parameters, their results suggested that testosterone is involved in the expression of aggressive behaviours during

male–male encounters in the breeding season. Contrary to our expectation, testosterone did not increase in male robins during STIs. Similarly, there was no STI-related change in dihydrotestosterone, corticosterone, and progesterone levels. Interestingly, there was a negative relationship between the aggression score and DHT concentrations, a fact for which we have no good explanation at hand.

The predictions of the challenge hypothesis have been tested and confirmed mainly based on interspecific comparisons of seasonal hormone profiles. However, recent studies have demonstrated that such seasonal testosterone patterns do not necessarily reflect the testosterone patterns during male–male interactions (Landys et al. 2007; Goymann et al. 2007; Goymann 2009). The seasonal androgen response  $R_{\text{season}}$  is extrapolated from seasonal patterns of maximum and baseline testosterone levels (Wingfield et al. 1990; Hirschenhauser et al. 2003), whereas the androgen responsiveness to direct male–male competition  $R_{\text{male–male}}$  is derived from acute changes in testosterone during STIs (Goymann et al. 2007). These different measures of androgen responsiveness are not necessarily correlated (Goymann et al. 2007; Goymann 2009).

Comparative evidence suggests that birds with short breeding seasons or single-brooded species (i.e. species that raise only one brood per season) are less likely to show an increase in testosterone levels during STIs than species with longer breeding seasons or with multiple broods per season (Wingfield and Hunt 2002; Landys et al. 2007; Goymann et al. 2007; Goymann 2009). Instead, corticosterone concentrations are more likely to rise during STIs in single-brooded species than in multiple-brooded species (Landys et al. 2007).

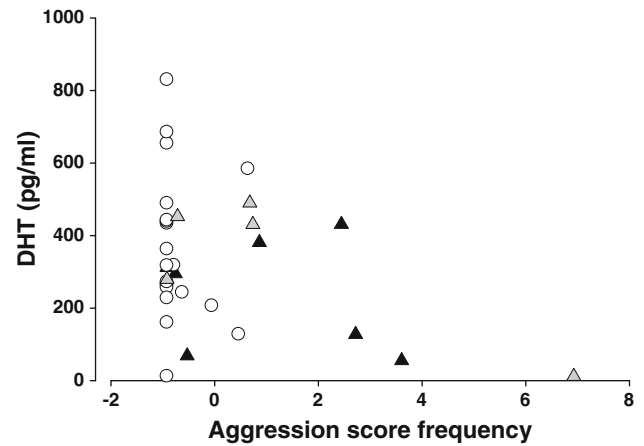
Another set of research led to the formulation of the essential paternal care hypothesis (Lynn et al. 2002, 2005). According to this hypothesis, males become insensitive to



**Fig. 1** **a** Relationship between testosterone levels and duration of simulated territorial intrusion (STI) in experimental birds (caught more than 10 min after onset of the STI, *black triangles*) and control birds (caught within 10 min of onset of the STI, *circles*). Additional data (see [Methods](#)) are presented as *grey symbols*. Testosterone concentrations did not change with STI duration (**a** for  $n = 32$ ,  $P = 0.639$ ). **b** Correlation between testosterone concentration (*black symbols*) and Julian day in experimental birds (caught more than 10 min after onset of the STI, *triangles*) and control birds (caught within 10 min of onset of the STI, *circles*). Additional data (see [Methods](#)) are included as *grey symbols*. Testosterone levels show an increase with Julian day in both situations (**b** for  $n = 32$ ,  $P = 0.045$ )

testosterone in species in which reduced parental assistance by males may lead to a significant reduction in reproductive success. As a consequence, males of such species may also not respond with an increase in testosterone during territorial encounters (Lynn et al. 2007; Lynn 2008).

Robins raise two or more broods during a single breeding season and, therefore, as a multiple-brooded species, they should show an increase in testosterone levels during male–male challenges (Landys et al. 2007; Goymann et al. 2007). However, this was not the case. To our knowledge, there are three further multiple-brooded bird species that do not show an increase in testosterone during



**Fig. 2** Relationship between dihydrotestosterone (DHT) and aggression score in experimental birds (caught more than 10 min after onset of the STI, *black triangles*) and control birds (caught within 10 min of onset of the STI, *circles*). Additional data (see [Methods](#)) are presented as *grey symbols*. DHT levels are higher in robins with a lower aggression score, but this result is mainly driven by one outlier (for  $n = 32$ ,  $P = 0.03$ ; for  $n = 27$ ,  $P = 0.10$ )

STIs: the tropical rufous-collared sparrow (*Zonotrichia capensis*; Moore et al. 2004a, b), the mountain white-crowned sparrow (*Zonotrichia leucophrys oriantha*; Lynn et al. 2007), and the black redstart (*Phoenicurus ochruros*; Apfelbeck and Goymann, unpublished data). Although it remains unclear why rufous-collared sparrows and black redstarts do not show an increase in testosterone during STIs, the pattern of testosterone secretion during STIs in mountain white-crowned sparrows is in accordance with predictions of the ‘short-season’ hypothesis (Wingfield and Hunt 2002; Lynn et al. 2007; Goymann 2009).

Although it is unknown whether male parental care is essential in robins, males do provision incubating females at the nest. Furthermore, the male feeds both the female and young for at least 2–3 days after hatching (Pätzold 1995; Grajatzky 2000). Thus, it is possible that robins belong to the set of species in which male parental care is essential, and the lack of an increase in testosterone during STIs is consistent with the essential paternal care hypothesis (Hunt et al. 1999; Lynn et al. 2002, 2005; Lynn 2008; but see Lynn et al. 2009).

Originally, the essential paternal care hypothesis was formulated to explain why experimental elevation of testosterone does not affect paternal care in some species (Lynn et al. 2002). The notion that some species in which testosterone does not affect paternal care also do not show an increase in testosterone during STIs came only later (Lynn 2008). But also here results seem to be inconsistent. The song sparrow, for example, shows a clear testosterone response to STIs (e.g. Wingfield and Wada 1989), despite the fact that paternal care seems to be essential in this species (Smith et al. 1982).

Another interesting possibility is that corticosterone binding globulin (CBG), which also binds other steroids such as testosterone and progesterone in birds, might have changed during agonistic encounters. This happened in white-crowned sparrows (Charlier et al. 2009), but the amount of free CBG binding sites made it unlikely that the fraction of free testosterone changed in white-crowned sparrow. Similarly, another study did not find a significant effect of CBG on free testosterone (Landys et al. 2007). Thus, although we cannot exclude a potential effect of CBG, we consider it unlikely that changes in CBG may have mediated an effect of testosterone.

Apparently, an increase in testosterone levels is not necessary for male robins to maintain aggressive behaviour during STIs. However, when the androgen receptor is blocked, territorial aggression is reduced (Schwabl and Kriner 1991).

Robins did not show an increase in corticosterone levels during STIs. Corticosterone levels were in the range of 12–15 ng/ml (Table 1), similar to those found by Scriba and Goymann (2008) for a different set of robins challenged with a stuffed decoy. Because robins challenged with a live intruder expressed lower levels of corticosterone than those challenged with a stuffed decoy (Scriba and Goymann 2008) corticosterone concentrations most likely increase at the very beginning of a STI with a stuffed decoy.

## Conclusions

The results of this study suggest that simulated territorial intrusions in robins during the breeding season do not lead to an increase in testosterone. Together with rufous-collared sparrows, black redstarts, and mountain white-crowned sparrows, robins represents the fourth multiple-brooded species investigated so far that does not show an increase in testosterone concentrations during STIs. Whereas a short breeding season may be responsible for the absence of an increase of testosterone during male–male interactions in mountain white-crowned sparrows, and little is known regarding rufous-collared sparrows or black redstarts, the essential paternal care hypothesis may explain the absence of a testosterone response to STIs in European robins.

## Zusammenfassung

Rotkehlchen zeigen keinen Testosteronanstieg während simulierter Revierkonflikte

Die sogenannte „Challenge Hypothese“ sagt für Männchen sozial monogamer Vogelarten voraus, dass die

Testosteronkonzentration im Blutplasma während Auseinandersetzungen um ihr Revier ansteigen (Wingfield et al. 1990). Im Rahmen von zwischenartlichen Vergleichen saisonaler Hormonprofile wurde die „Challenge Hypothese“ im großen Umfang bestätigt. Experimentell wurde die „Challenge Hypothese“ selten getestet und die Ergebnisse situativer Tests sind widersprüchlich. Wir untersuchten die hormonelle Reaktion und das Verhalten freilebender Rotkehlchen auf simulierte Revierübergriffe während der Brutzeit. Dazu stellten wir einen ausgestopften Lockvogel in das Revier von Rotkehlchenmännchen und spielten Rotkehlchengesang ab. Wir zeichneten dazu zunächst das Verhalten des Reviereigners für mindestens 10 Minuten auf, um ihn im Anschluss mit einem Japannetz zu fangen und eine Blutprobe zu nehmen. Da der mögliche Anstieg von Testosteron mindestens 10 Minuten dauert, wurden diese Hormonwerte mit denen von Kontrollvögeln verglichen, die in weit weniger als 10 Minuten nach Beginn des Tests gefangen wurden. Obwohl Rotkehlchen aggressiv auf simulierte Revierübergriffe reagierten und frühere Studien gezeigt haben, dass Testosteron einen Einfluss auf das Revierverhalten hat, reagierten Rotkehlchen nicht mit einem Anstieg der Plasmatestosteronkonzentrationen auf einen simulierten Revierübergriff und wir konnten die Gültigkeit der „Challenge Hypothese“ bei Rotkehlchen daher nicht bestätigen.

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