BEHAVIOURAL AND HORMONAL ASPECTS OF THE BREEDING ECOLOGY IN SEX-ROLE REVERSED POLYANDROUS BIRDS

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Ich versichere hiermit ehrenwörtlich, dass die von mir vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt ist.

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Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist, und dass ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

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Author Contributions

Chapter One: Paternity in the classical polyandrous black coucal (*Centropus grillii*) - a cuckoo accepting cuckoldry?

C.M. contributed to the fieldwork, data analysis, and writing of the manuscript.
B.K. conducted the parentage analysis and contributed to the writing of the manuscript.
S.K. conducted the genotyping.
M.V. conducted the home range analysis.
W.G. designed the study and contributed to the fieldwork and to writing of the manuscript.

Chapter Two: Testosterone does not directly modulate aggression but increases mate guarding in sex-role reversed female barred buttonquails

C.M. contributed to the study concept and design, practical work (simulated territorial intrusion experiments, blood sampling), data analysis, and writing of the manuscript.
W.G. contributed to the study concept and design, practical work (blood sampling), and discussion and writing of the manuscript.

Chapter Three: Hormones mirror personality – a mirror image stimulation test in male stonechats

C.M. contributed to the study concept and design, practical work (simulated territorial intrusion experiments, blood sampling and hormone analysis), data analysis, and writing of the manuscript.
W.G. contributed to the study concept, and discussion and writing of the manuscript.

Chapter Four: Throat patch size and darkness co-varies with testosterone in females of a sex-role reversed species

C.M. contributed to the study concept and design, practical work, plumage scoring, data analyses, and writing of the manuscript.

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Summary

In most vertebrate species, intra-sexual competition over resources and mates is higher among males than females. The strong mate-competition among males drives sexual selection to the development of male secondary sexual traits such as sexual ornaments and weaponry, a larger body size, and increased aggression, while females are the more choosy sex and take over parental care.

These conventional sex-roles are reversed in a small number of land vertebrate species. In such species, females typically establish, and vigorously defend, breeding and/or feeding territories and show a stronger intra-sexual competition than males. Males, on the contrary, incubate and take care for the young, often without any help by the female. In birds, exclusive male parental care is mostly associated with sequential or simultaneous polyandry.

The proximate mechanisms behind sex-role reversal and a polyandrous mating system remain an open question. Nevertheless, there are indications that steroid hormones such as testosterone, which is closely linked to the expression of ‘male’ characteristics in conventional sex-roles, may be involved in the reversal of behavioural, physiological and morphological characteristics in classically polyandrous species.

In my present thesis I, investigated three aspects that are characteristic for sex-role reversed polyandrous species, or may even be involved in the evolution of this mating system. These aspects were mate fidelity (i.e. extra-pair paternity rates), female aggression, and female secondary sexual traits (plumage colouration). I concentrated on the occurrence of these aspects, and the potential involvement of

testosterone in their expression, in my study species. Furthermore, I explored the role of testosterone in male aggression and the potential connection of the birds' personality to their behavioural and hormonal response during a territorial challenge, i.e. to their male typical behaviour.

In **Chapter One** I showed that promiscuity occurred at astonishingly high levels in a classically polyandrous species, the African black coucal. These results contradict theoretical models of the relationship between paternity and parental care, which imply low levels of extra-pair paternities for males to take over most of the parental duties. The high extra-pair paternity rates in black coucals seem to result from a low mate guarding ability of males, which is due to the dense habitat they live in, and due to the onset of incubation before clutch completion. Classical polyandry therefore may develop despite disadvantageous breeding conditions for the male.

In **Chapter Two** I found that experimentally elevated testosterone levels did not trigger an aggressive response during a territorial challenge in sex-role reversed polyandrous female barred buttonquails. Instead, high levels of testosterone affected female mate-guarding behaviour, which can be seen as a part of territorial defence, as well. In this species, circulating testosterone levels may therefore regulate behaviour involved in female-female competition. Moreover, considering the male-typical correlation of testosterone and mate-guarding intensity, testosterone may indeed be involved in the reversal of the sex-roles in barred buttonquails.

In **Chapter Three** I demonstrated that monogamous male stonechats responded agitated and/or aggressive towards their own mirror reflection and also towards a decoy, but not to a control glass panel. Yet none of these three challenges elicited a testosterone response related to the observed aggression. Interestingly, males showed one of two distinct patterns of aggression towards their own reflection in the mirror, and these patterns were related to hormone levels. Individuals which showed strong and physical aggression towards the mirror had characteristically higher testosterone and lower corticosterone levels, and males which did not exhibit aggression but only agitation behavior towards the mirror were characterized by relatively lower testosterone and higher corticosterone levels. Individual differences in hormone levels were consistent between the three experimental tests. From these results I hypothesize that in captive male stonechats the behavioral and hormonal response during competitive fights is not provoked by the stimulus alone but mainly modulated by the birds' personality. Similarly, personality may be play a role in the development of sex-role reversed behaviour in polyandrous species – an hypothesis that has scarcely been investigated.

In **Chapter Four** I describe a highly significant correlation between testosterone and the expression of a melanin-based throat patch in classically polyandrous female barred buttonquails. In males, no such relationship was apparent despite comparable testosterone levels between the sexes, suggesting that females may have an enhanced sensitivity for testosterone or its metabolites. These results are unique as they reveal that even un-manipulated testosterone levels may trigger 'male' characteristics in polyandrous females, suggesting that a hormonal mechanism may be involved in sex-role reversal in barred buttonquails.

Despite the variety of subjects and species investigated in my thesis, the results of all four chapters demonstrate that the development of sex-role reversed characters and behaviour in polyandrous species is dependent on a variety of ecological and physiological aspects which may differ between species. While testosterone may indeed play a role in sex-role reversal, its expression may depend on habitat structure, food availability, mate quality and abundance, and even on personality traits of the individual bird.

Zusammenfassung

Bei den meisten Wirbeltieren ist die intra-sexuelle Konkurrenz über Resourcen und Paarungsmöglichkeiten stärker bei den Männchen als bei den Weibchen ausgeprägt. Durch sexuelle Selektion führt dieser starke Konkurrenzdruck unter den Männchen zu der Entwicklung von sekundären Geschlechtsmerkmalen, die typisch für die Männchen und ihre Rolle in einer Paarbindung sind. So weisen Männchen oft Ornamente und Waffen auf, haben einen kräftigeren Körperbau, und verhalten sich aggressiver als Weibchen. Diese wiederum sind wählerischer als Männchen und übernehmen meist die Aufzucht der Jungen.

Diese konventionelle Verteilung der Geschlechterrollen ist bei einigen wenigen Landwirbeltieren vertauscht. Bei diesen Arten erkämpfen und verteidigen Weibchen die Brut- und Futterhabitate, und auch die intra-sexuelle Konkurrenz um Partner ist bei den Weibchen stärker als bei den Männchen. Die Männchen hingegen brüten und kümmern sich um die Nachkommen, oft ohne jegliche Hilfe des Weibchens. Bei Vögeln ist diese extreme Form von väterlicher Brutpflege meist mit einem polyandrischen Paarungssystem verbunden.

Die unmittelbaren Mechanismen von Geschlechterollentausch und polyandrischem Paarungssystem ist noch weitgehend ungeklärt. Es gibt jedoch Anzeichen dafür, dass Steroide eine gewisse Rolle bei der Umkehr geschlechtstypischen Verhaltens und Merkmale in klassisch polyandrischen Arten spielen. Das Hormon Testosteron zum Beispiel ist sehr nah mit der Ausprägung von “männlichen” Charakteristika in konventionellen Geschlechterrollen verbunden, und beeinflusst möglicherweise auch „männliche“ Züge bei Weibchen.

In meiner Dissertation habe ich drei Aspekte untersucht, die charakteristisch für Geschlechterrollentausch in polyandrischen Arten sind, oder sogar bei der Evolution dieses Paarungssystems eine Rolle spielen. Diese Aspekte sind Promiskuität (Häufigkeit von ausser-ehelichen Vaterschaften), Aggressionsverhalten der Weibchen (Verteidigung des Territoriums), und die Ausprägung von sekundären Geschlechtsmerkmalen bei Weibchen (Gefiederfärbung). Dabei liegt der Schwerpunkt zum einen im Auftreten dieser Aspekte und zum anderen in deren möglicher Testosteronabhängikeit bei den von mir untersuchten Arten. Zusätzlich habe ich getestet, inwieweit Testosteronwerte durch territoriale Auseinandersetzungen zwischen Männchen beeinflusst werden und eventuel von deren Persönlichkeit abhängt.

Im **Ersten Kapitel** konnte ich zeigen, dass Promiskuität ein erstaunlich oft auftretendes Phänomen bei einer klassisch polyandrischen Vogelart, dem Afrikanischen Grillkuckuck, ist. Diese Ergebnisse stehen im Kontrast zu theoretischen Modellen über das Verhältnis von Vaterschaft und väterlicher Fürsorge für die Nachkommen. Diese Modelle setzen eine sehr niedrige Häufigkeit von ausserehelichen Vaterschaften als Bedingung für die Entwicklung von ausgiebiger Brutpflege der Männchen voraus. Die jedoch relativ hohe Rate an ausserehelichen Vaterschaften beim Grillkuckuck scheint darauf zu beruhen, dass die Männchen nur sehr schwer ihre Partnerinnen bewachen und vor anderen Männchen beschützen können. Dies wiederum liegt einerseits an der sehr dichten und hohen Vegetation des Bruthabitats, andererseits daran, dass die Männchen mit dem Brüten beginnen, sobald das erste Ei im Nest liegt, und die Weibchen somit freien Spielraum haben. Klassische Polyandrie kann demnach auch dann entstehen, wenn die Brutbedingungen für die Männchen unvorteilhaft sind.

Das **Zweite Kapitel** dokumentiert, dass bei klassisch polyandrischen Bindenlaufhühnchen-Weibchen experimentell erhöhte Testosteronwerte keine aggressive Reaktion während territorialer Auseinandersetzungen hervorrufen. Stattdessen intensivieren die hohen Testosteronwerte bei den Weibchen das Bewachen/Beschützen der Partner, was auch eine Art von Territoriumsverteidigung sein kann. Daher scheint bei Bindenlaufhühnchen Testosteron tatsächlich das Konkurrenzverhalten zwischen Weibchen zu beeinflussen oder gar zu regulieren. Wenn man bedenkt, dass eine Korrelation zwischen Testosteron und Partner-bewachendem Verhalten eher bei Männchen bekannt ist, könnten diese Ergebnisse ein Hinweis darauf sein, dass Testosteron eine Rolle beim Geschlechterrollentausch der Bindenlaufhühnchen spielt.

Das **Dritte Kapitel** beschreibt die Reaktion von männlichen, monogamen Schwarzkehlchen auf ihr eigenes Spiegelbild oder auf einen ausgestopften Lockvogel. Beide Reize simulieren eine territoriale Bedrohung und lösten beunruhigtes oder aggressives Verhalten bei den Testvögeln hervor. Eine gleichzeitige Erhöhung der Testosteronwerte blieb jedoch aus. Interessanterweise verhielten sich die Männchen während der Spiegelbilddemonstration auffällig nach zwei sehr verschiedenen doch eindeutigen Verhaltensmustern, und diese Verhaltensmuster entsprachen der Höhe der Testosteronwerte. Inidividuen, die sehr aggressiv auf ihr eigenes Spiegelbild reagierten und es physisch angriffen, hatten bezeichnend hohe Testosteron- und niedrige Corticosteronwerte. Individuen, die keine Aggression zeigten, sondern eher unruhig oder verängstigt reagierten, hatten niedrige Testosteron- und hohe Corticosteronwerte. Diese individuell unterschiedlichen Hormonwerte waren während beider experimenteller Tests und auch wahrend der Kontrollsituation konstant. Aus diesen Ergebnissen schliesse ich, dass sowohl das Verhalten als auch die hormonelle Reaktion von Schwarzkehlchenmännchen (in Gefangenschaft lebend) in einer Konkurrenzsituation nicht allein vom Reiz abhängt, sondern auch von der Persönlichkeit der Vögel. Auf eine ähnliche Weise könnte die Persönlichkeit von Individuen bei der Entwicklung von vertauschten Geschlechterrollen bei polyandrischen Arten eine Rolle spielen – eine Hypothese die noch kaum erforscht wurde.

Im **Vierten Kapitel** beschreibe ich eine höchst signifikante Korrelation zwischen dem Testosteronspiegel und der Ausprägung einer auf Melanin basierenden Kehlgefiederfärbung bei weiblichen polyandrischen Bindenlaufhühnchen. Bei den Männchen trat keine ähnliche Korrelation auf, obwohl sie gleich hohe Testosteronwerte hatten, was darauf hindeutet, dass Weibchen eine erhöhte Sensibilität für Testosteron oder dessen Zwischenabbauprodukte haben. Diese Ergebnisse sind bisher einmalig und zeigen dass auch unmanipulierte Testosteronwerte “mannliche” Merkmale bei polyandrischen Weibchen hervorrufen können. Dies könnte ein Hinweis darauf sein, dass bei Bindenlaufhühnchen der Geschlechterrollentausch auf einem hormonellen Mechanismus basiert.

Trotz der unterschiedlichen Themen und Vogelarten, die ich in meiner Dissertation behandelt habe, zeigen die Ergebnisse aller vier Kapitel zusammen, dass die Entwicklung von Geschlechterrollentausch bei polyandrischen Arten von einer Vielfalt an ökologischen und physiologischen Aspekten abhängt, und diese von Art zu Art differieren können. Während Testosteron tatsächlich eine Rolle beim Geschlechterrollentausch spielen könnte, ist dessen Ausprägung weiterhin vom Habitat, Nahrungsquellen, Qualität und Erreichbarkeit von Paarungspartnern, und sogar von der Persönlichkeit der einzelnen Vögel abhängig.

General Introduction

Sex-roles and mating systems

In most vertebrate species, sex-roles are behaviourally and morphologically based on the degree of sexual selection, that is, on the bias of intra-sexual competition over mates towards one sex (Shuster and Wade 2003). The conventional sex roles are characterised through competitive males and caring females (Clutton-Brock 1991, Black 1996, Kokko and Jennions 2008). A common explanation for this classification of sex-roles derives from differential investment into progeny of males and females, and from the availability of mates (Trivers 1972, Emlen and Oring 1977). In general, females invest more into the production of gametes than males, both in terms of energy and time. This anisogamy has been suggested to promote further divergence in the investment of offspring (Shuster and Wade 2003). As females have higher costs through gamete production, it is hypothesised that females stay with their offspring rather than seeking new mating opportunities, while the relatively low costs of sperm production promotes males to abandon their offspring and mate with a new partner (Trivers 1972). The larger pre- and post-mating parental investment of females leads to a less frequent accessibility of females for reproduction compared to males (Trivers 1972). Hence, females are a scarce resource for males, and the male-biased operational sex-ratio (i.e. the ratio of fertilizable females to sexually active males in a population at a given time) promotes male-male competition and drives the bias of sexual selection towards males (Emlen and Oring 1977). Strong intra-sexual competition over mates is the mechanism of sexual selection to drive the development of secondary sexual traits (Clutton-Brock and Vincent 1991, Clutton-Brock 1991). Such traits compass sexual ornaments and weaponry, a larger body size, and increased aggression among individuals of the more competitive sex, i.e. generally the male (Shuster and Wade 2003, Barlow 2005), while females are the more choosy sex and take over parental care (Eens and Pinxton 2000).

However, there are various vertebrate species in which males are heavily involved into parental care (Clutton-Brock 1991), and females show strong competition over resources such as mates and territories (Clutton-Brock 2009). In these cases, the general explanation of parental investment and mate availability may not be satisfactory. First of all, the operational sex-ratio may not only depend on the accessibility of individual mates but factually on the actual adult sex ratio of a population (Kokko and Jennions 2008). If the adult sex ration is even between males and females, males cannot reproduce faster than females, hence intra-sexual competition over mates may not be higher in males than in females. If the adult sex ratio is biased towards females, mate competition may actually be stronger in females than in males (Kokko and Jennions 2008).

A further factor that influences intra-sexual mate-competition (and hence the characteristics of the sex-roles) is sex differences in mate quality (Owens and Thompson 1994). If there is more variation in parental quality in one sex, a strong competition over the access to the high quality mates will arise in the opposite sex. Mate quality and mate choice may drive intra-sexual competition towards one sex (which may not necessarily be the more abundant or more caring sex), and hence may be reflected in the development of sex-role characteristics in some species (Owens and Thompson 1994, Kokko and Jennions 2008).

One factor that is often neglected in the context of sex-roles is the rate of extra-pair paternity (Black 1996, Kokko and Jennions 2008). Extra-pair paternities are very common and decrease the value of the offspring for the social (caring) males (Kokko and Jennions 2008). Therefore, the risk of extra-pair paternity may favour the development of classical sex-roles, i.e. females staying with the offspring, males leaving the offspring for new mating opportunities (Ligon 1999, Kokko and Jennions 2008). On the other hand, the development of strategies to ensure paternity may actually support the occurrence of paternal care, especially if the latter in turn keeps females from engaging in extra-pair matings, i.e. if a high levels of paternal care is a countermeasure against extra-pair paternities itself (Ligon 1999).

Finally, resource availability, predation risk and sex-differences in mortality may affect sexual selection and thus characteristics of sex-roles in some species (Candolin 2003, Andersson 2005, Kokko and Jennions 2008; see also paragraph 1.2. *The evolution of paternal care*). For example, food availability or predation risk may favour bi-parental care to ensure offspring survival. Bi-parental care in turn may affect the operational sex ratio and thus intra-sexual competition (Clutton-Brock 1999). Furthermore, adult mortality divergence between the sexes affects the adult sex ratio, which in turn has an impact on the operational sex ratio (Kokko and Jennions 2008). And, as mentioned above, the operational sex ratio again influences the evolution of parental investment by each sex (Shuster and Wade 2003, Kokko and Jennions 2008).

Whatever the reason for a bias in mate competition in one sex – mate abundance, mate quality, parental investment, or resource abundance – the defined sex-roles and the potential frequency of how often males and females mate within a breeding season provide a framework for the definition of the mating system (Shuster and Wade 2003). Accordingly, in socially monogamous species, males and females mate once per lifetime or per (breeding) season with only one (social) partner, the male aggressively defends his mate and the territory, and does or does not take part in incubation and offspring rearing, the main duties of the female (Ligon 1999, Shuster and Wade 2003). In polygynous species, the conventional sex-roles are established as in monogamous species, with the difference that the male mates with more than one female per lifetime/season, either sequentially or simultaneously (Ligon 1999, Shuster and Wade 2003). In the rather unusual breeding system of social (classical) polyandry, it is the female that mates with several males, also either sequentially or simultaneously. Moreover, in classically polyandrous species, the sex-roles are, with only a few exceptions (e.g. in humans, Hill and Hurtado 1996, Beckerman et al. 1998, Mesoudi and Laland 2007), reversed (Clutton-Brock 1991, Ligon 1999).

Sex-role reversal and the evolution of paternal care

Sex-role reversed polyandrous species are very rare among vertebrates. This mating system only occurs in some fish and avian species, and its recognizable exception provides unique possibilities to test hypotheses about evolutionary theories on mating systems, parental care and sexual selection (Clutton-Brock 1991, Ligon 1999, Owens 2002, Andersson 2004). However, despite numerous comparative and experimental studies on sex-role reversal, its evolutionary significance is not well understood and remains very much debated (Clutton-Brock 1991, Andersson 1995, Owens 2002).

In sex-role reversed species, females typically establish, and vigorously defend, breeding and/or feeding territories and show a stronger intra-sexual competition for mates than males (Clutton-Brock and Vincent 1991, Andersson

2004). Males, on the contrary, take care for the young, often without any help by the female (Clutton-Brock 1991, Clutton-Brock and Vincent 1991). The predominant male care leads to a lower potential reproductive rate in males compared to females (Clutton-Brock and Vincent 1991), whereas the variance in mating success is supposedly higher in females (Eens and Pinxton 2000). Sex-role reversed species may also develop more pronounced secondary sex traits and mate-attracting displays in females, leaving males with a more critical choice of mates (Eens and Pinxton 2000, Shuster and Wade 2003).

In birds, exclusive male parental care is mostly associated with sequential or simultaneous polyandry (Ligon 1999). This mating system has been discovered in only 11 bird taxa (Table 1). Two classical polyandrous species, the African black coucal (*Centropus grillii*) and the barred buttonquail (*Turnix suscitator*), are subject of my present thesis, wherein I investigated mating success (extra-pair paternity), secondary sexual traits (plumage colouration), and intra-sexual competition (territorial aggression) in these birds. In addition, I included one study on a socially monogamous species, the European stonechat (*Saxicola torquata rubicola*), to invest further details on intra-sexual competition, this time, however, in males.

Table 1

Families of birds with parental care exclusively by males and in which classical polyandry is known or thought to occur (modified from Ligon 1999).

Family			
Common name	**Scientific name**	**Order**	**Comments on species**
Tinamous	Tinamidae	Tinamiformes	Females of some species may lay eggs in the nest of several males
Rheas	Rheidae	Rheiformes	Males accept the eggs of several females into their nests; females may lay eggs in the nests of more than one male
Emu (only member of genus Dromaius)	Dromaiidae	Casuariiformes	Emus are thought to be primarily monogamous, with male care of eggs and chicks.
Cassowaries	Casuariidae	Casuariiformes	
Buttonquails	Turnicidae	Taxonomic position debated [*]	Classical polyandry prevalent
Jacanas	Jacanidae	Charadriiformes	Classical polyandry is thought to occur in 7 of the 8 living species.
Painted-snipes	Rostratulidae	Charadriiformes	Classical polyandry is thought to occur in 1 of the 2 living species.
Plains-wanderer	Pedionomidae	Charadriiformes	Classical polyandry is thought to occur in the only living species.
Sandpipers and phalaropes	Scolopacidae	Charadriiformes	Classical polyandry occurs commonly in the spotted sandpiper and less commonly in phalaropes.
Plovers and lapwings	Charadriidae	Charadriiformes	Classical polyandry occurs in the Eurasian dotterel.
Coucals	Cuculidae	Cuculiformes	Parental care conducted primarily by male in most species. Classical polyandry occurs in at least one species, the black coucal.

[*] e.g.*Chraradriiformes*, Hackett et al. 2008; *Gruiformes*, Rotthowe and Starck 2008

The evolution of classical polyandry still remains an open question. Nevertheless, various hypotheses have been proposed to explain the development of exclusive paternal care, which is probably a major precondition for the evolution of polyandry (Figure 1, Ligon 1999, Andersson 2005).

Paternal care may be an ancestral trait in these families, or has derived secondarily from biparental care (Ligon 1999). Both theoretical ways are supported by some phylogenetic studies but are rejected by others (Ligon 1999). The question of the source of paternal care (ancestral or derived from biparental care), however, may play only a minor role in the evolutional pathway of classical polyandry.

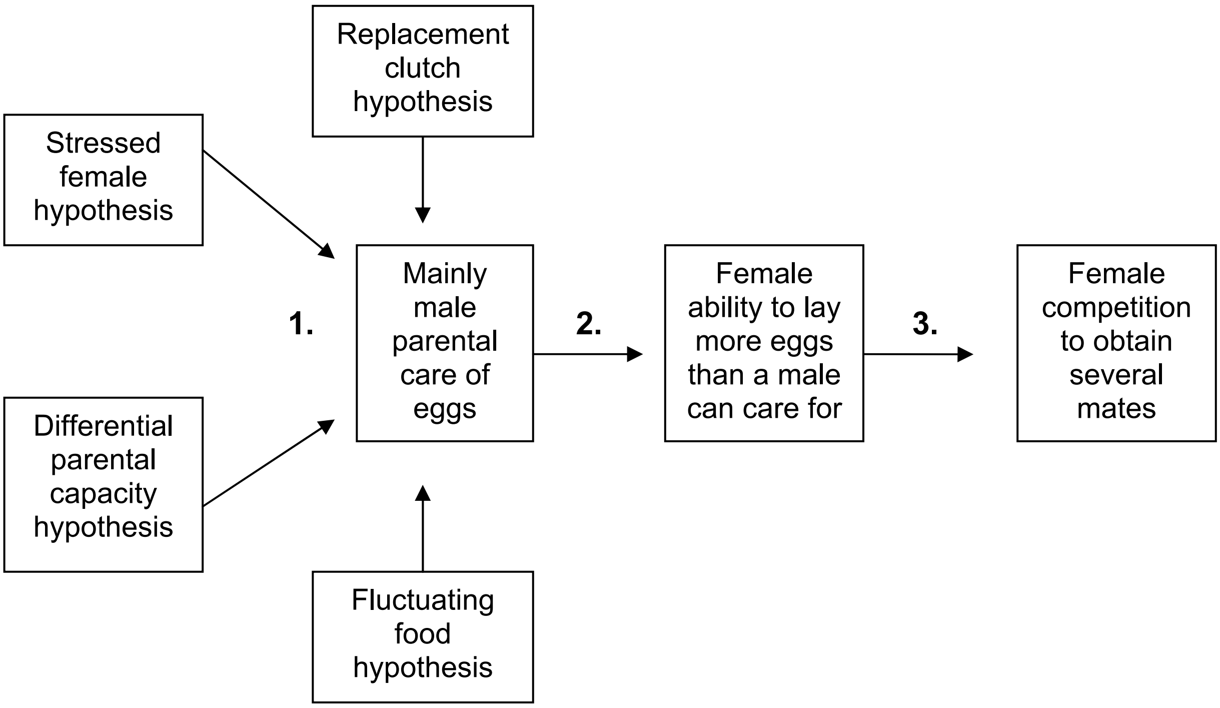
Paternal care has been suggested to have evolved via four different ecological pathways. (Figure 1, Erckmann 1983, Ligon 1999, Andersson 2005). These pathways are all based on the idea that paternal care reduces the costs of offspring care for females so that the female in turn can produce more eggs, and therefore, increases the reproductive success of both males and females. The *hypothesis of food-stressed females* suggests that in the case of scarce food resources, females may have to reduce the egg production due to increased investment into foraging (Graul et al. 1977). If males (at least partly) take over incubation and offspring care, egg production may again be enhanced, and consequently the reproductive success of the pair increased, too. The *replacement clutch hypothesis* predicts that repeated nest failure increases the male’s share in incubation, so that the female’s resources are saved, if necessary, for a rapid production of a replacement clutch (Emlen and Oring 1977, Butchart 2000, Goymann et al. 2004, Schamel et al. 2004). The *hypothesis of fluctuating food resources* explains paternal care as an evolutionary response to annual variation in food availability (Graul 1974, Parmalee and Payne 1973). In the case of low food abundance, females may be able to lay only one clutch. However, in the eventuality of high food abundance, and with the support through male incubation and male offspring care, the female may lay multiple clutches, which is beneficial for the male, too. And finally, the *differential parental capacity hypothesis* suggests that females – after the energy demanding production of eggs – are less able to ensure incubation and further offspring care compared to males, especially if one sex deserts the nest (Graul 1973, Maynard-Smith 1977).

Neither the hypotheses of stressed females, nor the hypothesis of fluctuating food resources, did get much support from empirical studies on polyandrous shorebirds (Erckmann 1983). In contrast, food scarcity has been shown to select for bi-parental care (Erckmann 1983) rather than polyandry or polygyny. And females do not require a special abundant food to lay successive broods (Erckmann 1983, Lank et al. 1985). Apparently, none of the four hypotheses constitute a general model, but 'several are plausible for some species' (Erckmann 1983, Ligon 1999). Whatever hypothesis may lead to the development of mainly or only paternal care, the result is that females become able to produce multiple clutches. And this ability encourages females to lay clutches for several males as to increase their own reproductive success.

One potential - and actually occurring - consequence from female multiple matings within one breeding season is sperm competition, resulting in extra-pair fertilizations (Birkhead 1998). Independent of whether these extra-pair fertilizations are intended by the female or whether they are a mere byproduct of multiple matings, extra-pair paternity diminishes the value of the offspring for the social male and hence – at least in theory - counteracts the development of paternal care (Kokko and Jennions 2008).

Figure 1

Evolutionary steps to paternal care and classical polyandry (modified from Ligon 1999 and Andersson 2005).



1. Mainly or only male parental care has evolved from one of four ecological hypotheses (for more details see text).

2. Females are able to lay more eggs than a male can handle.

3. Females compete to produce clutches for several males and obtain higher reproductive success, spreading classical polyandry and its secondary sex differences.

Sex-role reversal and extra-pair paternity

As mentioned above, the risk of paternity loss supports the development of the conventional sex-roles, i.e. competitive males and caring females (Kokko and Jennins 2008). Extra-pair paternity is a widespread phenomenon in birds, reaching up to 65% of offspring in socially monogamous species (Brooker et al. 1990) and up to 72% of offspring in cooperatively breeding species (Mulder et al. 1994). The uncertainty of fatherhood therefore lowers the value of the offspring for the male, hence, may cause selection on males to leave their offspring with the mother and seek additional mating opportunities in order to maximise their reproductive success. However, theoretical models predict that high levels of paternal care could only evolve, and reach an evolutionary stable strategy, if extra-pair paternity is prevented or at least is kept at very low levels (e.g. reviewed by Schwagmeyer et al. 1999, Mauck et al. 1999, Møller and Cuervo 2000, Griffith et al. 2002, Sheldon 2002). Males should invest less in broods in which their paternity is uncertain (Whittingham et al. 1992, Westneat and Sherman 1993, Houston 1995, Kempenaers and Sheldon 1997, Houston and McNamara 2002), while females should not seek or accept extra-pair copulations when their reproductive success depends on the help of their mates (Trivers 1972, Hoi-Leitner et al. 1999, Whittingham and Dunn 2001, Sheldon 2002, Arnqvist and Kirkpatrick 2005).

Therefore, in sex-role reversed polyandrous species with male-only care, the rate of extra-pair paternity is expected to be relatively low. Indeed, the proportion of extra-pair offspring ranges from 0% in the Wilson's phalarope (*Phalaropus tricolor*, Dale et al. 1999) to only 8.6% in the spotted sandpiper (*Actitis macularia*. Oring et al. 1992), and female Wilson’s phalaropes actively prevent extra-pair paternity by rejecting copulation attempts from extra-pair males (Delehanty et al. 1998).

On the other hand, a high degree of paternal care may give females extra opportunities to pursue extra-pair copulations while their social mates are occupied with nest-building, incubation or feeding. In sequential or simultaneous polyandrous species, the occurrence of extra-pair paternity may also be a simple consequence of females copulating with multiple (social) males during one breeding cycle. Furthermore, paternal care may actually be a trait selected for through sexual selection, i.e. female mate choice may be based on cues that predict paternal behavior and it may therefore not be adaptive for males to reduce paternal investment even when they face extreme uncertainty of paternity (Ketterson and Nolan 1994, Kokko and Jennions 2008).

In **Chapter One**, we investigated extra-pair paternity rates and potentially associated supporting or inhibiting behavioural and environmental factors in the sex-role reversed, socially polyandrous African black coucal.

African black coucal, fotograf by Wolfgang Goynann

The African black coucal belongs to the genus *Centropus* within the cuckoo family *Cuculidae* (Payne 1997). The genus is non-parasitic and mainly found in Africa and Asia, where they live and breed semi-terrestrial in dense vegetation and feed on large insects and small vertebrates (Payne 1997). Cuckoos have a remarkable variance in social behaviour and parental care, and they are most closely related to species of the Gruiformes family, e.g. cranes, rails and bustards (Payne 1997, Hacket et al. 2008). The African black coucal is found widely spread throughout sub-Saharan Africa, where it is found in wet grasslands and marshes (Andersson 1995, Payne 1997). In south-western Tanzania, the breeding season lasts from January to July, and males can raise up to 3 successive broods per season. Females have multiple males simultaneously (up to 3 males), with each having their own nests, therefore females can have a maximum breeding success of 9 broods per season (Goymann et al. 2004). The African black coucal is the only coucal with a sex-role reversed, socially polyandrous mating system, even though a high level of paternal care is very common in this bird group (Andersson 1995). Males and females express the same plumage colouration, however, there is a pronounced sexually reversed size-dimorphism with the females being 69% heavier and 39% larger than males (Goymann *et al.* 2004). Females aggressively and vocally defend large territories, while males defend only relatively small areas around the nest site within the female territory (Goymann et al. 1994). Males incubate and care for the young with no help by the female (Goymann et al. 2004, 2005). Astonishingly, the African black coucal is the only classically polyandrous species with altricial young, hence, the investment into offspring care is exceptionally high for the male even within the group of polyandrous species. Therefore, African black coucals combine two traits that should either promote (sperm competition favored by simultaneous polyandry) or inhibit (exceptionally high male investment into offspring care) extrapair copulations, and hence offers a unique opportunity to study extrapair paternity in sex-role reversed species.

Hormonal mechanisms of sex-role reversal

The proximate mechanisms responsible for sex-role reversal, both in physiological and organizational aspects, remain an open question (Andersson 1995, Owens 2002). One hypothesis is an associated reversal of the sex-hormone levels. In male birds of non-sex-role reversed species, testosterone rises during the breeding season and is an important initiator and modulator of aggressive behaviour, the development of secondary sexual traits, and status in a dominance hierarchy (Wingfield et al. 1990, Andersson 1994, Gonzalez 2002, Adkins-Regan 2005, Bókony et al. 2008). Females have generally much lower concentrations of testosterone (Wingfield and Farner 1993, Wingfield et al. 2001, Møller et al. 2005). Nevertheless, a comparable relationship between testosterone and aggression has also been proposed for females (Staub and DeBeer 1997, Ketterson et al. 2001, 2005). Therefore, reversed testosterone concentrations (females high levels, males low levels) seem a plausible and maybe most obvious hormonal mechanism behind sex-role reversal.

In all sex-role reversed polyandrous species studied so far, however, males had higher levels of testosterone than females. In fact, the levels of males were even comparable to those of non-sex-role reversed species (e.g. Rissman and Wingfield 1984, Fivizzani and Oring 1986, Schlinger et al. 1989, Goymann and Wingfield 2004). Nevertheless, testosterone may still be involved in sex-role reversed behaviour or characteristics, albeit at a different level than acute circulating levels of the hormone (e.g. Schlinger et al. 1989, Voigt and Goymann 2007, Ball and Balthazart 2008, Clutton-Brock 2009). Low testosterone levels may act via an increased sensibility towards the hormone, i.e. through an increased receptor density (Canoine et al. 2006, Voigt and Goymann 2007) or an increased enzyme activity (Schlinger et al. 1989, Ball and Balthazart 2004, Scordalakes and Rissman 2004). Alternatively, testosterone may act in combination with other hormones (Landys et al. 2007, Goymann et al. 2008) or via the concentration of corticosterone-binding globulin, a carrier protein for both testosterone and corticosterone (reviewed in Breuner and Orchinik 2002) that can affect the reservoir of hormones in the circulation (Bright 1995). However, these hormonal mechanisms have mainly been investigated in species with classical sex-roles. There are only a few studies that analyzed hormones of sex-role reversed polyandrous species, and these studies concentrated mainly on the description of circulating hormone levels. Therefore, this field of study has only been started out and still offers hypotheses for proximate mechanisms behind sex-role reversal.

Aggression in sex-role reversed females

In many bird species, male aggressive behaviour is interlinked with the steroid hormone testosterone (reviewed e.g. in Wingfield 2005, Soma 2006, Kempenaers et al. 2007). In these species, administration of testosterone increases aggression, while aggressive interactions in turn influence testosterone levels (Oliveira 2004, Wingfield et al. 1987, 1990, 2001, Hirschenhauser and Oliveira 2006, Goymann et al. 2007). In other species, however, testosterone levels are not affected by aggressive behaviour (e.g. Meddle et al. 2002, Moore et al. 2004a, 2004b) or even decrease during aggressive interactions (e.g. Van Duyse et al. 2004, Peters et al.

2006, Landys et al. 2007, for a review on all studies in birds see Goymann et al. 2007, Goymann 2009). And other studies in turn showed that male aggressive behaviour does not relate to testosterone alone but may depend on a combination of hormone levels, social context, environmental input, and the birds' experience (Gill et al. 2007, Hirschenhauser et al. 2008).

In female birds, the relationship between testosterone and aggression has been studied much less. Nevertheless, there is evidence that testosterone affects aggressive behaviour in this sex as well, depending on the species and the context (see also Gill et al. 2007). A standard procedure to investigate the effect of agonistic interactions on testosterone and future aggression is an experimentally simulated territorial intrusion - by placing a con-specific decoy into the centre of the territory and playing back con-specific song – and to analyse the behavioural and hormonal response of the focal birds. Just as in males (reviewed in Goymann et al. 2007 and Landys et al. 2007), testosterone levels of females sampled after such territorial intrusion experiments with female decoys either increased (Desjardins et al. 2006, Gill et al. 2007) or were unaffected (Elekonich and Wingfield 2000, Davis and Marler 2003, Jawor et al. 2006, see also review in Voigt and Goymann 2007). Furthermore, experimentally increased testosterone levels may enhance song and aggression in female birds, too (reviewed in Staub and de Beer 1997, Fusani et al. 2003, Ketterson et al. 2005).

Like studies on steroid hormones, studies on female aggression in sex-role reversed species are very scarce. And in fact, there is only one study that investigated the effect of territorial aggression on hormone levels experimentally (Goymann et al. 2008), while only one other study analysed the expression of androgen receptors in the brain of females and males of a sex-role reversed species

(Voigt and Goymann 2007). These two studies, however, both suggest that acute circulating testosterone levels do not modulate aggressive behaviour in females of the sex-role reversed study species but rather an increased receptor activity in the brain and circulating concentrations of progesterone.

In **Chapter Two**, we investigated the role of testosterone in aggressive behaviour of sex-role reversed, socially polyandrous barred buttonquail (*Turnix suscitator*). This species belongs to the family Turnicidae (buttonquails), a group of small, quail-like terrestrial birds, which are found in Africa, Eurasia and Australasia (Debus 1996). Buttonquails generally live and breed in dense, grassy or low bushy habitats, which offers good opportunities to hide from predators but makes it difficult to observe these birds in the wild, too (Debus 1996).


Barred buttonquail, fotograf by Conny Voigt

Female barred buttonquails aggressively defend territories and vocalize to deter rivals and to attract males, while the latter are mainly involved in incubation and raising the offspring (Trollope 1970, Ridley 1978, Debus 1996). They have a marked reversed sexual size dimorphism (Starck 1991, Debus 1996), and only females express a black throat patch throughout the year (Sutter 1955, Starck 1991). Furthermore, only females have an enlarged trachea which allows the female to produce a loud, low-frequency booming call important for mate attraction (Debus 1996).

We experimentally increased testosterone levels in caged female barred buttonquails by implanting testosterone pellets and tested the behavioural and hormonal response of these females during a simulated territorial challenge to test whether females with increased testosterone levels would react more aggressively towards an intruder than control females with un-manipulated hormone levels. Results of such experiments could give deeper insight into the role of testosterone in female aggression in barred buttonquails in particular, and in sex-role reversed species in general.

Individual variation in aggression

The hormonal response after such simulated territorial intrusion experiments may differ not only between species but also between individuals of one population. These between-individual differences may depend on an individuals' perception of the given situation (e.g. Hirschenhauser et al. 2008). Furthermore, the outcome of an acute fight or of previous fights may influence behavioural and hormonal reactions during a territorial challenge (Oliveira 2004, Kempenaers et al. 2007, Goymann et al.

2007, Goymann 2009, Pryke 2009). For instance, losers may show a reduction in testosterone synthesis (Oliveira et al. 1996, Oliveira 2004), while winners keep high fighting-induced testosterone levels (Harding 1981, Ramenofsky 1984, 1985), or show an acute testosterone response after repeated winning experiences (Oyegbile and Marler 2005).

Using simulated territorial intrusion experiment with a mirror image as an opponent, the self-assessment and the assessment of the opponent's strength, as well as the perception of the outcome of the interaction are blurred and may lead to a different hormonal response compared to fights with real (alive) opponents (Hirschenhauser et al. 2004, 2008, but see Desjardins and Fernald 2010). Moreover, the perception of the own and the opponent's fighting ability, as well as the corresponding behavioural and hormonal response during an agonistic interaction, may not only depend on an individual's experience or the actual relative fighting ability, but may be determined by individual differences in conflict behaviour (Armitage and Van Vuren 2003, Kempenaers et al. 2007, Hirschenhauser et al. 2008).

In many vertebrates, individual differences in behaviour and physiology are consistent across contexts and stable over time. Such consistent individual differences are referred to as temperament or personality, and may have a genetic component (Réale et al. 2007, Sih and Bell 2008, Dingemanse et al. 2010). Differences in personality may be reflected in neuroendocrine parameters (Koolhaas et al. 1999, Carere et al. 2003, Kralj-Fišer et al. 2007), and during aggressive interactions (Verbeek et al. 1996, Carere et al. 2003, Fuxjager et al. 2010) including territory defence (Amy et al. 2010). Hormonal responses during stressful or aggressive interactions may therefore be determined by an individual's personality in addition to the (perceived) agonistic situation itself.

In **Chapter Three** we investigated the behavioural and the hormonal response of European stonechats during a simulated territorial challenge using either a stuffed decoy or a mirror. Based on previous mirror image stimulation studies on cichlid fish and Japanese quail (Oliveira et al. 2005, Hirschenhauser et al. 2008, Desjardins and Fernald 2010), our study aimed at testing the effect of reduced information about the fighting ability of the opponent (i.e. likely outcome of a contest) on the hormonal and behavioural response of challenged males in a passerine bird species. We intended to determine hormonal correlates of personality traits in the behavioural response during aggressive conflicts. Consistent individual personality traits during agonistic situations could - in addition to the effect of information on the likely outcome of a contest - further explain the variation in baseline and induced hormone levels.

We studied a partially migratory population of the European stonechat, the Irish stonechat (Passeriformes, Muscicapidae, Turdinae). This monogamous species is found widespread in the southern Palaeartic region, where they generally breed bushy grassland (Collar 2005). Paired males and females defend a territory year-round, i.e. during the breeding and the non-breeding season, and they can raise multiple broods per season (Collar 2005). Females incubate alone, but males participate in nestling care (Collar 2005). Seasonal plasma hormone profiles and behavioral responses during agonistic interactions have been well described for free-living and captive males of this species, showing that males react aggressively to intruding opponents, and blockage of testosterone reduces aggressive behavior during the breeding season (Gwinner et al. 1994, Canoine 2001, Canoine and Gwinner 2002, Schwabl et al. 2005, Helm et al. 2009).

Stonechat, fotograf by Eberhard Gwinner

Secondary sexual traits in sex-role reversed females

Secondary sexual traits are mainly the result of intra-sexual competition over mates. Sexual selection drives the development of these often exaggerated traits, which may reflect the superiority of some individuals over others (Shuster and Wade 2003, Clutton-Brock 2007). In birds, for example, plumage coloration and ornaments often function as signals for status, strength, health, and/or parental quality, and their expression are important during mate search and intra-sexual competition (Andersson 1994, Berglund et al. 1996, Hill 2006, Kraaijeveld et al. 2007, Møller 1988, Riters et al. 2004). Due to the generally higher competition for mates among males, it is usually the male bird that develops colourful and exaggerated feather formations, while the female wears a rather dull plumage (Andersson 1994, Kraaijeveld et al. 2007, Clutton-Brock 2009). There are species with also bright female plumage ornamentation, though, which may be a mere by-product of sexual selection for ornaments in males (Lande 1980, Kraaijeveld et al. 2007) or could have evolved under sexual selection through direct male choice or competition between females (Kraaijeveld et al. 2007, Clutton-Brock 2009).

There is a large literature regarding the modulation of secondary sexual traits in general, and plumage colouration in birds in particular, by sex steroids such as estrogen (Kimball and Ligon 1999) and testosterone (Buchanan et al. 2001, Strasser and Schwabl 2004, Adkins-Regan 1998, reviewed in Candolin 2003, Blas et al. 2006). Such hormonal dependency may support the honesty of such traits as signals for genetic quality or the current condition (Jawor and Breitwisch 2003). High levels of testosterone may bear costs to the individual as it impairs immune function and parental care (Wingfield et al. 1990, Ketterson et al. 1992, Nolan et al. 1992, Ketterson et al. 1995). Therefore, theory predicts that only individuals of good quality and condition are able to deal with these detrimental costs of high testosterone levels and can express the hormone-dependent trait (Jawor and Breitwisch 2003, Bokony et al. 2008).

In this context, sex-role reversed dichromatism in sex-role reversed bird species (for example the red-necked phalarope *Phalaropus lobatus*, Wilson's phalarope *Phalaropus tricolor*, or Eurasian Dotterel *Charadrius morinellus*), may be under hormonal control, too, just as in non-sex-role reversed females (Ketterson et al. 2005). For instance, an increased sensitivity to testosterone in the skin in females would allow the development of testosterone-dependent plumage colouration in this sex without a reversal of the circulating testosterone levels (Schlinger et al. 1989).

In **Chapter Four**, we describe and discuss the relationship between circulating testosterone levels, the birds' condition and the expression of a melanin-based plumage patch in female barred buttonquails. This correlational study was done to investigate potential hormonal mechanism behind plumage colouration in a sex-role reversed species. The barred buttonquail shows a marked sexually reversed dichromatism with females expressing a melanin-based black throat patch and a partly melanised breast patch. Males, on the contrary, have a white throat patch with sometimes melanised feather tips (Starck 1991, for more details on barred buttonquails see Chapter Two and Four). The plumage coloration of this species therefore offers an ideal opportunity to examine the co-variation in melanin plumage areas, condition and testosterone in female birds. Furthermore, results of this study may give us a more precise insight of the hormonal mechanism behind sex-role reversed dichromatism.

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Chapter One

Paternity in the classical polyandrous black coucal (*Centropus grillii*) - a cuckoo accepting cuckoldry?

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Abstract

In birds, a high degree of paternal care is expected to correlate with a relatively low frequency of extrapair paternity. If females seek extrapair copulations, they may lose fitness because their partners may reduce paternal care. Conversely, a high degree of paternal care might give females extra opportunities to pursue extrapair copulations, whereas their mates are occupied with offspring care. The aim of this study was to investigate paternity in the classically polyandrous African black coucal. In this sex-role reversed species, males intensively invest in offspring care of altricial young. However, the likelihood of extrapair fertilizations through stored sperm or extrapair copulations seems very high because females lay up to 3 clutches for up to 3 males each. Using 9 novel, highly polymorphic microsatellite loci developed for black coucals, we determined paternity of 127 chicks from 35 nests. Of these, 18 chicks (14.2%) from 13 broods (37.1%) were not fathered by the social male. Black coucals thus have the highest average rate of extrapair paternity reported for any classically polyandrous species. Extrapair young were the result from both extrapair copulations with males outside the female's group of social mates and stored sperm from previous mates. We suggest that males were unable to effectively guard their

females to prevent extrapair paternity due to 1) the dense habitat they live in and 2) the onset of incubation before clutch completion.

Introduction

Extrapair paternity is a widespread phenomenon in birds. Its frequency varies tremendously between species (ranging from 0 to 65% of offspring in socially monogamous species (Brooker et al. 1990) and up to 72% of offspring in cooperatively breeding species (Mulder et al. 1994) and even between populations within a species (e.g., Gyllensten et al. 1990; Fridolfsson et al. 1997). A large body of literature discusses the ecological and behavioral factors, which might promote or inhibit extrapair paternity, and the potential costs and benefits of extrapair fertilizations to males and females. However, the causes underlying the variation in the level of extrapair paternity remain controversial or are still merely theoretical (e.g., reviewed by Petrie and Kempenaers 1998; Griffith et al. 2002; Neff and Sherman 2002; Westneat and Stewart 2003; Griffith 2007).

One major issue is the relationship between paternity and paternal care (e.g., reviewed by Schwagmeyer et al. 1999; Mauck et al. 1999; Møller and Cuervo 2000; Griffith et al. 2002; Sheldon 2002). In species with a high degree of paternal care, extrapair paternity is expected to be relatively low. Parental effort is costly in terms of reduced survival and breeding or mating opportunities (Clutton-Brock 1991). Therefore, theoretical studies predict that - at least under certain circumstances - males should invest less in broods in which their paternity is uncertain (Whittingham et al. 1992; Westneat and Sherman 1993; Houston 1995; Kempenaers and Sheldon 1997; Houston and McNamara 2002). Male birds may not be able to measure their paternity directly, but they may use cues such as the behavior of their mates and other males to judge about paternity and adjust their investment into the brood accordingly (Kempenaers and Sheldon 1997; Lifjeld et al. 1998; Neff and Sherman 2002).

When females depend on the help of their mates, they should not seek or accept extrapair copulations, if this results in a loss of fitness through their partners reduced paternal care (Trivers 1972; Hoi-Leitner et al. 1999; Whittingham and Dunn 2001; Sheldon 2002; Arnqvist and Kirkpatrick 2005). Thus, in sex-role reversed classically polyandrous species, in which each male of a polyandrous female incubates and cares for his own clutch with no or only little help from the female partner (Emlen and Oring 1977), the rate of extrapair paternity should be relatively low. Indeed, the proportion of extrapair offspring ranges from 0% in the Wilson’s phalarope (*Phalaropus tricolor*; Dale et al. 1999) to 8.6% in the spotted sandpiper (Actitis macularia; Oring et al. 1992), and female Wilson’s phalaropes actively prevent extrapair paternity by rejecting copulation attempts from extrapair males (Delehanty et al. 1998; see Table 1).

Alternatively, a high degree of paternal care might give females extra opportunities to pursue extrapair copulations or seek additional mates, whereas their social mates are occupied with nest building, incubation, or feeding (Anderson 2005). For example, in the red-necked phalarope (*Phalaropus lobatus*), a male’s solo nest building gives females the chance to engage in extrapair copulations close to the time of egg laying (Schamel et al. 2004). In sequential or simultaneous classically polyandrous species (females mating with more than one male within one breeding season consecutively or at the same time, respectively), the occurrence of extrapair paternity may also be a simple consequence of females copulating with multiple (social) males during one breeding cycle. This may lead to sperm management (sensu Zuk 2002) if the first mate's spermatozoa survive until the eggs from the second male's clutch are fertilized (period of viable sperm storage in birds: 6–45 days; Birkhead and Møller 1992) or if the female continues to copulate with the first social mate during clutch fertilization of the second mate. Indeed, in 4 of 7 polyandrous species for which paternity data are available, storage of sperm from the first male can explain the occurrence of extrapair young (Table 1).

We studied paternity in the African black coucal, a bird species within the cuckoo family, with a simultaneous classically polyandrous mating system (Vernon 1971; Goymann et al. 2004). Females aggressively and vocally defend territories against other females, whereas males build nests, incubate, and care for the young in general without help from the female (Goymann et al. 2004, 2005). Furthermore, the sexes are size dimorphic with females being 69% heavier than males (Goymann et al. 2004). Black coucals rarely fly and spend most of their time hidden in high and dense vegetation (Vernon 1971; Goymann et al. 2004). The African black coucal combines 2 traits that should either promote or inhibit extrapair copulations. On the one hand, the black coucal is one of a few classically polyandrous species that raises altricial young (with the exception of some woodpeckers with rare incidents of classical polyandry, reviewed in Anderson 2005). Hence, paternal investment is likely to be more costly compared with other classically polyandrous species with precocial young. This suggests that male black coucals may have evolved strategies to prevent loss of paternity or to keep females from engaging in extrapair copulations. Within one breeding season, females may lay up to 3 clutches for up to 3 males each, with a mean interval of just 14 days (Goymann et al. 2004). Thus, the likelihood of extrapair fertilizations through stored sperm and the potential for females to copulate with their other social mates or engage in extrapair copulations seems very high. These 2 opposing features make the black coucal an interesting species to study the occurrence of extrapair paternity.

The general aim of this paper was to determine extrapair paternity rates and their potential correlates in the African black coucal. First, we tested whether the frequency of extrapair paternity depended on female condition or territory size and on the number of social mates. Females with larger territories or females in better condition may have more opportunities to engage in extrapair copulations, or they may have more social mates, which in turn may lead to extrapair paternity through sperm storage or copulations with other social mates. Second, we investigated whether male condition explained variation in the frequency of extrapair paternity. Females paired to males with a low body condition may be more likely to pursue extrapair copulations (Double and Cockburn 2000; Chiver et al. 2008). Finally, we compared the growth rates of within-pair and extrapair chicks. One can expect that extrapair chicks will grow better than withinpair chicks if extrapair chicks are of higher genetic quality (e.g., Schmoll et al. 2009). Alternatively, extrapair chicks may grow less well if males distinguish and invest more in their own genetic offspring (Johnstone and Hinde 2006) or broods with extrapair chicks grow more poorly (Boncoraglio and Saino 2008).

Table 1

Rates of extra-pair paternity and life-history information for classically polyandrous species.

Species	% EPY	Sample size (No. of broods)	Young	Habitat	Mate guarding	Cause of extra-pair paternity	Reference
Eurasian dotterel (*Charadrius morinellus*)	4.6	22	Precocial	Flat, open (montane -zone plateau)	Pair bond and mate guarding during pre-laying period	RMS genetic father of EPY not known	Owens et al. 1995
Wattled jacana (*Jacana jacana*)	7.5	74	Precocial	Flat, open (riverbank)	No	High copulation rate genetic father: co-mate	Emlen et al. 1998
Wilson's phalarope (*Phalaropus tricolor*)	0	17	Precocial	Flat, open (wetlands)	Pair bond throughout laying period		Delehanty et al. 1998
Red phalarope (*Phalaropus fulicarius*)	6.5	18	Precocial	Flat, open (beach, marshes)	Pair bond until clutch completion	stored sperm genetic father of EPY not known	Dale et al. 1999
Spotted sandpiper (*Actitis macularia*)	8.6	34	Precocial	Flat, open (lake shoreline)	Mate-guarding before and during laying period	Stored sperm genetic father of EPY: former mate	Oring et al. 1992
Red-necked phalarope (*Phalaropus lobatus*)	1.7	63	Precocial	Flat, open (tundra)	Pair bond throughout laying period	stored sperm genetic father of EPY not known	Schamel et al. 2004
Comb-crested jacana (*Irediparra gallinacea*)	2.8	10	Precocial	Flat, open (river-, pondbanks)	No data	No data	Haig et al. 2003
African black coucals (*Centropus grillii*)	14.2	35	Altricial	High, dense (grassy floodplains)	Pre-breeding, until first egg is laid	RMS, stored sperm genetic father of EPY: co-mates	This study

RMS = rapid mate switching, EPY = extra-pair young

Methods

Study site and species

We conducted fieldwork in the Usangu wetlands of the Mbeya region, South-West Tanzania (8°41’S, 34°5’E) from mid January until the end of May in 2001, 2002 and 2005. The habitat was characterized mainly by grassy floodplains with very dense and high vegetation (>2m), and occasional small thorn bush thickets (see Goymann & Wingfield 2004, Goymann *et al.* 2004, Goymann *et al.* 2005).

Adult black coucals were caught either with nontoxic adhesive (Kollant S.P.A., Padova, Italy) attached to artificial perches with or without playback song (for more details see Goymann & Wingfield 2004, Goymann *et al.* 2004), or by using mistnets and playback song with or without a decoy. Each bird was individually colour-banded, and a 200 µl blood sample was taken from the alar wing vein and stored on ice until return from the field each day when the plasma was separated and frozen in liquid nitrogen for hormone studies. A small amount of the remaining red blood cells was stored in Queen’s lysis buffer for DNA analysis and sexing (Seutin *et al.* 1991). We measured tarsus length to the nearest 0.1mm and weight to the nearest 1.0g, and calculated body condition from body mass and body size using PCA. The first component of this analysis describes 71.4% (females) and 61.04% (males) of the variance of the two measurements representing condition. In total, we captured 42 female and 37 male black coucals. Of those, 37 females and 24 males were additionally equipped with a radio transmitter (Holohil, BD-2; for more details see Goymann *et al.* 2004). All birds were released close to the place where they were caught.

Radiotracking and estimation of home-range size

Every 1-3 days each radio-tagged bird was located using a TRX 1000S receiver (Wildlife Materials), and its position recorded with a GPS device (Garmin GPS II and Garmin GPS 76^{TM}). In addition, we recorded the position of birds when they were observed in an exposed position (e.g. perched in a bush, or in the open field on the ground) and thus could be identified by their color bands.

We calculated the home range size for each bird for which a minimum of five positions had been recorded (females: n = 33, males: n = 21), as individual utilization distribution areas (bivariate normal kernels; Worton 1989). We used the 50% isopleths as an estimate of home range boundaries (Börger *et al.* 2006). To compute home range size we used the software R (version 2.6.0) with the add-on package adehabitat (Calenge C. 2006, R Developing core Team 2007). In addition, we present the home range boundaries calculated with the minimum convex polygon (MCP) method to allow direct comparison with territory sizes of black coucals reported by Goymann *et al*. (2004). Both estimates of home range size were strongly correlated (Pearson correlation, males: r = 0.90, N = 21, p < 0.001, females: r = 0. 75, N = 33, p < 0.001).

Breeding data

Each year, we searched an approx. 200ha area for nests by following males equipped with a radio transmitter or by observing males feeding nestlings. The identity of the male that fed the offspring was recorded so that the social father could be unequivocally assigned to a nest. Females were assigned as social mothers when they had been seen copulating with the social father of a nest and/or when the nest was situated within their home-range and they defended both the nest and the corresponding male against intruders.

Nests found early were checked every second day during laying and eggs were numbered according to the laying sequence. Shortly before fledging, chicks were banded with individually numbered aluminum rings (size G, Vogelwarte Radolfzell, Germany), and a small blood sample (≤50 µl) was taken either from the alar wing vein or from the tip of the wing, and stored in Queen's lysis buffer (Seutin *et al.* 1991).

Most nests were discovered only after at least part of the chicks had hatched. We then estimated nestling age based on a linear regression of tarsus length with age (using data from chicks of known age: tarsus length = 7.7052 + age*1.684, see Goymann *et al.* 2005). We calculated the date of clutch completion as 14 days prior to hatch date (Vernon 1971).

As described for adult birds, we measured tarsus length and weight of the chicks every other day until fledging. We estimated the growth rate (size and mass) of each chick as the slope of the regression of tarsus length or body mass with age, during the period of linear growth (between age 4-11days). Only chicks that were measured at least 3 times were included.

In total we caught 42 female black coucals, but only 22 produced clutches we were able to find and sample for paternity analysis. We collected blood samples from a total of 47 nests (15 in 2001, 16 in 2002, and 16 in 2005). In 26 of these nests, both parents were sampled, in 11 only the social father, in 8 only the social mother, and in 2 none of the social parents were sampled. All 10 nests where the social male was not sampled were excluded from further analysis. We also excluded two nests where only one chick (out of four eggs) was sampled. In 17 of the remaining 35 nests we sampled all offspring, except for 11 unhatched eggs (1 or 2 eggs in 9 nests each). In 4 nests some offspring were known to have left the nest before a blood sample had been taken. Fourteen nests were found when the young were already between 3-14 days post-hatch. In these nests, the oldest chicks may have left the nest before we found it. This is not unlikely, because black coucals build cryptic dome-shaped nests 15–50 cm above ground with an additional “emergency exit’’ on the opposite side of the entrance, which older chicks use to escape when potential predators approach the nest (Vernon 1971, Goymann *et al.* 2005). Furthermore, males may have discarded chicks that died soon after hatching. Thus, the true frequency of extra-pair paternity may have been underestimated due to incomplete sampling.

Molecular sexing

All individuals were genetically sexed by polymerase chain reaction (PCR) with the P2/P8 primer pair (Griffiths et al. 1998). Coucal genomic DNA was extracted from blood samples stored in Queen’s lysis buffer using the GFX Genomic Blood DNA Purification Kit (GE Healthcare Europe, Freiburg, Germany). The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used to isolate DNA samples from unhatched embryos stored in ethanol (n = 5). The PCR was performed with 20– 100 ng DNA, 1.25 U Taq DNA Polymerase (MBI Fermentas, St. Leon-Rod, Germany), 13 PCR buffer with (NH4)2SO4 (supplied with MBI Fermentas Taq), 2.0 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate, 0.5 lM of primer P2, and 0.5 lM or the fluorescently labeled primer P8 (6-FAM). PCRs were carried out on a GeneAmp 2700 PCR System (Applied Biosystems, Darmstadt, Germany) with the following cycling conditions: 3 min initial denaturation at 94 ºC, 28 cycles of 30 s denaturation at 94 ºC, 30 s annealing at 50 ºC, and 45 s extension at 72 ºC, followed by one 45 s completing final extension step at 72 ºC. A total of 1.5 ll of the PCR product was mixed with formamide containing the GeneScan 500 LIZ size standard, heat denatured, and resolved in POP4 polymer on an ABI 3100 Genetic Analyzer (all Applied Biosystems). Raw data were analyzed with GeneMapper 3.7.

Parentage Analysis

Microsatellite markers

We isolated and characterized novel microsatellite loci for the black coucal. Enriched genomic library construction and microsatellite isolation were performed by Ecogenics GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al. 1994). The library was enriched by magnetic bead selection with biotin-labeled (CA)13 and (ACAG)7 oligonucleotide repeats (Gautschi, Tenzer, et al. 2000; Gautschi, Widmer, and Koella 2000). Of 384 recombinant colonies screened, 58 gave a positive signal after hybridization. Plasmids from all positive clones were sequenced, and primers were designed for 19 microsatellite inserts. Of these, 17 were tested for polymorphism and 11 markers were used for further analysis (Table 2). All 11 microsatellite loci were highly polymorphic: The number of alleles per locus ranged from 9 to 42 with a mean of 21.7 alleles per locus (Table 2). Expected and observed heterozygosities were calculated using Cervus v3.0 (Kalinowski et al. 2007).

Two markers (Cgr5 and Cgr11) showed a high number of null alleles (Table 2) and were not used further. Thus, for parentage analysis, we used a set of 9 microsatellite markers with a combined nonexclusion probability of P = 0.00011 for the first parent and P = 1.6 x 10^{-6} for the second parent (one parent known). For GenBank accession numbers of the primers, please see Table 2.

Parentage analysis

Paternity was analyzed for a total of 127 chicks from 35 nests. All offspring were successfully genotyped at 9 microsatellite markers.

Microsatellite amplifications were performed in multiplexed PCRs using the Qiagen Multiplex PCR kit (Qiagen) and primer mixes containing 3–4 primer pairs (multiplex mix no. 1–3, Table 2). Forward primers were labeled at their 5# end with fluorescent dyes from Applied Biosystems. Differences in amplification efficiency and dye strength of the primers were accommodated by adapting the primer concentrations in these mixes (details given in Table 2). Each 10 ll multiplex PCR contained 25–120 ng DNA, 5 ll of the 23 Qiagen Multiplex PCR Master Mix, and 1 ll of one primer mix. PCRs were carried out on a GeneAmp 2700 PCR System (Applied Biosystems) with the following cycling conditions: 15 min initial denaturation at 95 ºC, 24–30 cycles of 30 s denaturation at 94 ºC, 30 s annealing at 60 ºC for mixes 1 and 2 or 63 ºC for mix 3, and 1 min extension at 72 ºC, followed by a 30 min completing final extension at 60 ºC. PCR products were analyzed on an ABI 3100 Genetic Analyzer as described above.

We used Cervus v3.0 (Kalinowski et al. 2007) with the following simulation parameters: proportion of candidate parents sampled: 80% (based on fieldwork estimates), proportion of loci typed: 100%, and proportion of loci mistyped: 1% (basing the analysis on 40% of the candidate parents sampled did not change the results). First, we conducted a maternity analysis with all known females as potential mothers (N = 26 broods for which the social mother was known). In all but one brood, Cervus assigned the social female as the most likely parent (with 95% confidence level). There was either no mismatch of the alleles (N = 76) or one mismatch (N = 13) with the offspring. In one brood with 2 young, the candidate social mother was clearly not the biological mother (.4 mismatches).

In a second step, we conducted a paternity analysis with the identified mother (if any) as the known parent and with all known males as potential fathers. Offspring were assigned to the social male if and only if 1) they showed only 0 (N = 99), 1 (N = 7), or 2 (N = 1) mismatches with the offspring; 2) Cervus assigned the social male as the true parent with high confidence (95%); and 3) in case of 2 mismatches, the social male sired other offspring in the brood. The remaining 18 offspring showed at least 4 mismatches with the social male and were therefore considered extrapair. Of these, a candidate extrapair male was assigned as the true sire if and only if 1) there was either no (N = 6) or a single mismatch (N = 1) with the offspring; 2) Cervus assigned the male as the true parent with high confidence (95%); and 3) in the case of the single mismatch, the male sired other offspring in the same brood (without mismatch). For the remaining 11 extrapair offspring, Cervus did not assign a candidate male with confidence, and the number of mismatches was at least 3 in all cases.

Table 2

Characterization of 11 microsatellite loci for Centropus grillii.

Locus	Genbank accession number	Repeat motif	Primer sequences (5' - 3')	*C* (μM)	*Multiplex Mix No*	N_{ind}	N_A	Size range (bp)	H_O	H_E	H_E - H_O
Cgr2	EU795679	$(CA)_{38}$	F: VIC-GCTATTACAGAGCTGAAGGAAGG R: GATCCCGCTGCAGTTAGATATG	0.18 0.18	1	130	25	125-191	0.88	0.91	0.03
Cgr5	EU795680	$(CA)_{17}$	F: 6FAM-ACCTGAGTGCCAAGACCACT R: CTGTTTCTTTTTATTACCTCTCATTGT	1.6 1.6	2	129	22	90-140	0.35	0.92	0.57
Cgr6	EU795681	$(CAGA)_{13}(TAGA)_9$	F: 6FAM-GCAGCACTATGCAGAGCTAGG R: GCTTAACCCTTGGTACACTTGG	0.24 0.42	1	130	42	209-417	0.94	0.96	0.02
Cgr7	EU795682	$(GACA)_{10}$	F: NED-GCAGAAGGAAAACCACTGCT R: GAGTGGCGTGCAGCTGAG	0.6 0.6	3	129	28	139-267	0.89	0.88	-0.01
Cgr9	EU795683	$(CA)_{31}$	F: 6FAM-AAAAGCAGCTGTTTGTACTGTG R: CATAAAAGGAATGTAAGCGTTCG	0.32 0.48	3	130	20	135-179	0.88	0.88	0.00
Cgr11	EU795684	$(CA)_{16}$	F: PET-CCAGTTCAAAACCCCGACAC R: ATTTTAGAACCAGTTGGGTAAAGC	0.72 0.72	1	130	24	115-177	0.51	0.93	0.42
Cgr13	EU795685	$(GT)_{13}GCGTGC(GT)_4$	F: VIC-CCCCGTTTCTAATCATGTCTCT R: GAGACTGCAGCTGTGAAAATCC	0.12 0.16	2	130	13	200-224	0.72	0.80	0.08
Cgr15	EU795686	$(GT)_{16}(GC)_4$	F: VIC-CTTGCTAGTGCGGACTTGGT R: TTACTACAAAGCATGGGCTCTC	0.36 0.6	3	130	14	91-123	0.68	0.83	0.15
Cgr16	EU795687	$(GT)_{17}$	F: PET-AAGTGAAAACAATTTATTATTCCAG R: CAGTGTCTGGTTTTATGAGTCTGC	0.6 0.8	2	130	14	140-166	0.88	0.89	0.01
Cgr17	EU795688	$(CA)_6(CG)_3(CA)_{18}$	F: PET-AAGCACCACGTGGCTCAG R: GTAGAACCACGGACGGGATG	0.9 0.9	3	130	28	184-248	0.93	0.93	0.00
Cgr19	EU795689	$(CA)_{12}....(CA)_5$	F: NED-TTTGTGTGCTCGCGTGTAAT R: CTGCTTGCTGCTGGAGTATG	0.4 1	1	130	9	195-211	0.74	0.77	0.03

In total, 130 presumably unrelated adults (65 males and 65 females) were genotyped. Primer sequences include information on fluorescence labels used. *C*, primer concentration in multiplex primer mix; Ni_{nd}, number of individuals successfully scored; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

Results

Statistical analysis

All data were analyzed using SPSS 15.0 for Windows. We tested the assumptions for the use of parametric tests (normal distribution, homogeneity of variance) and if these assumptions were not met, we used the appropriate non-parametric test. Throughout the paper, values are expressed as mean ± SE and the statistical significance was set at $\alpha \leq 0.05$ (2-tailed).

In a few cases, biometric data of the birds are missing because some individuals escaped before all measurements were taken. Hence, the sample size of some statistical tests differs from the original sample size and is presented for each test separately.

The number of extrapair young per brood was expressed as a proportion of the brood’s total number of chicks and was arcsine transformed for statistical analysis. A total of 95% confidence limits for the percentage of extrapair young were calculated according to Griffith et al. (2002).

Estimates of chick growth (slope for weight and size) were compared between extrapair and within-pair young using a general linear mixed model with sex as a factor, brood size as a covariate, and brood ID as a random factor.

We compared home range size (log transformed) between the sexes using 1-way analysis of variance (ANOVA). We tested for correlations between the proportion of extrapair young and male and female home range size (all broods for an individual were pooled for this analysis). We also correlated the proportion of extrapair young with male and female body mass, size (tarsus length), and condition using Pearson correlations. Furthermore, we tested correlations between the number of mates per female per season and female home range size; proportion of extrapair young (all female broods combined); and female size, mass, and condition, using Spearman rank correlations. Sperm management due to stored sperm from another male is more likely in females which have more social mates. Therefore, we also tested whether females which had more social mates were more likely to have extrapair young in their brood using a logistic regression. Finally, we investigated whether the occurrence of extrapair young was season dependent by correlating the proportion of extrapair young in a brood with laying date, using Spearman rank correlations.

Social Polyandry

Of a total of 22 females for which nesting data were available, 2 had only one mate (9.1%), 12 had 2 mates (54.6%), 5 had 3 mates (22.7%), 1 had 4 mates (4.6%), and 2 females had at least one mate, but observations were not sufficient to exclude additional mates (9.1%). Altogether, at least 86.4% of all females were polyandrous.

Parentage

Eighteen of 127 chicks (14.2%; 95% confidence interval: 8.1– 20.2%) were not fathered by the social male. These extrapair chicks came from 13 of 35 broods (37.1%; 95% confidence interval: 21.1–53.2%). In one nest with 2 offspring, the social female was clearly not the genetic mother of the chicks (see Methods). It remains unclear whether this is a case of intraspecific brood parasitism or whether the identified social female had taken over the territory from another female and had ‘‘usurped’’ the male with his brood. Note, however, that the male at the nest was the father of both offspring (‘‘quasi-parasitism’’; Griffith et al. 2004).

The fathers of 7 extrapair young from 5 broods could be identified (Table 3). Two of these broods were from the same social pair (laying interval 23 days), and in both broods, the father of the extrapair young was another social mate of the female. This male sired 1 of 4 offspring in each of these broods. His own clutch was produced 14 days prior to the other male’s first nest and 37 days prior to the second nest and did not contain extrapair offspring (sampled completely). The extrapair young of the other 3 nests were fathered by 4 different males that nested with other females on territories adjacent to the one of the cuckolded male. Their social female produced clutches later in the season (onset of laying 7–19 days after the onset in the focal nest). All 3 broods of the extrapair fathers also contained extrapair young, but the genetic fathers could not be identified.

One female produced a first brood (200206; Table 3) with a known male, which contained one extrapair young sired by an unknown male. Six days later she started laying a second clutch (200205) for another, unidentified male (hence, paternity analysis for this second nest was not possible). One of the 4 offspring in this second brood was sired by the known first social male.

Table 3

Paternity data of 35 black coucal broods.

Brood ID	Male ID	Female ID	No. of chicks in nest	No. of extrapair chicks	Extrapair sire
200101	GN 41706	not caught	5	0	
200102	GN 41707	GN 41703	4	0	
200103	GN 41720	GN 41717	3	0	
200106	GN 41713	GN 41703	4	0	
200109	**GN 41708**	**GNRH2001**	**4**	**2**	**GN 41704/GN 41768 (EGM)**
200110	GN 41734	GN 41743	3	0	
200111	GN 41752	GN 41701	5	0	
200113	GN 41751	GNRH/200	5	0	
200116	GN 41762	not caught	3	0	
200118	GN 41768	not caught	3	1	unknown
200121	**GN 41707**	**GN 41703**	**4**	**1**	**GN 41713 (comate)**
200122	**GN 41707**	**GN 41703**	**4**	**1**	**GN 41713 (comate)**
200202	GN 41798	GN 41796	2	0	
200205	*Not caught*	GN 41795	4	1	unknown
200206	GN 41794	GN 41795	2	1	unknown
200208	GN 44352	not caught	4	0	
200209	**GN 44357**	**not caught**	**3**	**1**	**GN 44368 (EGM)**
200211	GN 41797	GN 41796	3	0	
200213	GN 44395	GN 44373	2	1	unknown
200214	GN 44380	GN 41776	4	0	
200215	GN 44368	not caught	5	1	unknown
200216	GN 44376	GN 41781	4	0	
200219	GN 41775	not caught	5	0	
200220	**GN 44393**	**not caught**	**5**	**2**	**GN 41775 (EGM)**
200501	GN 44451	GN 44448	4	1	unknown
200502	GN 44480	GN 44431	3	0	
200504	GN 44456	GN 44458	4	0	
200509	GN 44470	GN 44472	3	0	.
200510	GN 44493	GN 44444	3	2	unknown
200512	GN 44478	GN 44437	4	2	unknown
200517	GN 44491	GN 52369	4	0	
200519	GN 52355	GN 44443	4	0	
200520	GN 44478	GN 44437	3	0	
200522	GN 44471	not caught	3	0	
200524	GN 44493	GN 44444	3	2	unknown
200526	GN 52380	GN 44428	3	0	

The genetic father of broods marked in bold could be identified. The brood highlighted in italic was not included into the analysis (see text).
EGM = extra group male.

Chick growth

During the period of linear growth, female chicks grew faster than males in body mass (Table 4; general linear mixed model, $F_{1,63} = 21.47$, $P < 0.001$). There was no significant effect of sex on tarsus growth ($F_{1,52.98} = 0.81$, $P = 0.37$). Growth rates of tarsus length and body mass did not differ significantly between broods of different sizes, and there was no significant brood ID effect (Wald Z, $X^2 = 0.34$, $P = 0.73$, $X^2 = 0.61$, $P = 0.54$, respectively).

Table 4

Growth rates (mean 6 standard error) of female and male black coucal chicks during the period of linear growth (age of 4–11 days).

Offspring	Brood size	Tarsus growth	Mass growth
Female EP	3.7 ± 0.3 chicks (6)	1.51 ± 0.11 (6)	5.10 ± 0.60 (6)
Female WP	4.0 ± 0.2 chicks (37)	1.65 ± 0.06 (34)	5.33 ± 0.34 (33)
Male EP	3.4 ± 0.4 chicks (8)	1.64 ± 0.07 (8)	5.48 ± 0.62 (8)
Male WP	4.0 ± 0.2 chicks (26)	1.47 ± 0.07 (25)	3.86 ± 0.44 (25)

Data were compared between extrapair (EP) young and within-pair (WP) young. Growth rates were calculated from regression analysis of mass over age and tarsus length over age. Sample sizes are given in brackets.

Finally, we did not find a significant difference in the growth rates between extrapair and withinpair offspring (body mass: $F_{1,57.5} = 0.08$, $P = 0.78$; tarsus length: $F_{1,54.9} = 1.42$, $P = 0.24$).

Home range size

Average female home range size (kernel: 4.54 6 0.67 ha; MCP: 3.10 6 0.47 ha) was significantly larger than average male home range size (kernel: 2.77 6 0.70 ha;

MCP: 2.45 6 0.86 ha; 1-way ANOVA for kernel data: $F_{1,52} = 5.56$, $P = 0.02$). Male black coucals restricted their home ranges to an area around their nests, whereas females expanded their territories and in most cases comprised more than one nest. However, female home range size did not correlate with the number of social mates (Spearman rank correlation, $r_s = 20.17$, $P = 0.34$).

Correlates of extra-pair paternity

Of the 35 nests included in the paternity analysis (see above), 19females and 33 males were identified as genetic mothers andfathers (both within-pair and extrapair sires). Paternity loss(proportion of extrapair young) was not significantly correlatedwith male condition ($r = 0.003$, $n = 32$, $P = 0.99$), body mass($r = 0.24$, $n = 32$, $P = 0.19$), tarsus length ($r = 0.06$, $n = 33$, $P = 0.74$), or home range size ($r = 20.23$, $n = 18$, $P = 0.36$).

Female condition, body mass, or tarsus length did not correlate significantly with either the number of mates or the proportion of her young that were extrapair ($n = 18$, all $P > 0.13$; details not shown). Similarly, female home range size did not correlate with the proportion of extrapair young in her broods ($r = 0.15$, $n = 15$, $P = 0.59$).

Females that had more social mates were not more likely to have extrapair young in their brood (Wald statistic, $R^2 = 0.012$, $n = 19$, degree of freedom = 1, $X^2 = 0.16$, $P = 0.69$) nor did they have broods with a higher proportion of extrapair young ($r_s = 20.05$, $P = 0.84$, $n = 22$).

We did not find evidence that later broods had more extrapair young than early broods (correlation of proportion of extrapair young with laying date: $r_s = 0.18$, $P = 0.3$, $n = 35$; Figure 1).

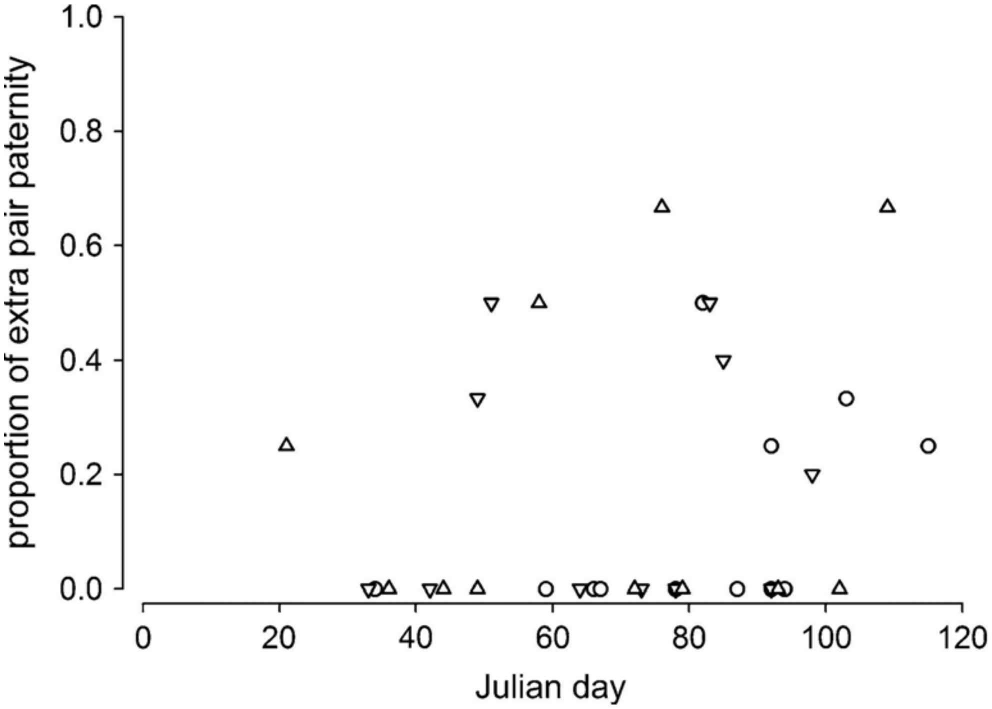

Figure 1

Correlation between laying date (Julian day) and proportion of extrapair paternity in 35 nests of African black coucals (Spearman rank correlation r = 0.18, P > 0.3; dots refer to broods in 2001, downward triangles to broods in 2002, and upward triangles to broods in 2005).

Discussion

In our study population of African black coucals, 14% of the offspring were fathered by an extrapair male, and extrapair paternity was found in 37% of all broods. Among the classically polyandrous species investigated so far, black coucals thus have the highest population rate of extrapair paternity (Table 1).

Females in classical polyandrous mating systems may use stored sperm from their previous mate to fertilize eggs of subsequent broods (Oring et al. 1992; Owens et al. 1995; Dale et al. 1999). Alternatively, males may lose their paternity to males outside the polyandrous group and/or to other males simultaneously paired to the female (Emlen et al. 1998). In both cases, mixed sperm from multiple mates may lead to mixed paternity within a brood. The outcome of the sperm management may be influenced by female choice and depends on the male’s genetic quality (compatibility), timing and frequency of copulations, and the amount of spermatozoa transferred by the male (Birkhead 1998) or retained by the female.

Overall, about 33% of black coucal males lost paternity. This is astonishing given that male black coucals invest so heavily in parental care including nest building, incubation, and feeding young (Goymann et al. 2004, 2005). Even though paternal care is probably more costly than in other classically polyandrous species with precocial young, males frequently care for unrelated offspring (in one case, a new incoming male even adopted the offspring of another male who had abandoned his nest; W.G., personal observations). Theory predicts that genetic relatedness is an important factor for parents to decide about how much care to provide to their offspring (Whittingham et al. 1992; Westneat and Sherman 1993; Houston 1995; Kempenaers and Sheldon 1997; Houston and McNamara 2002; Neff and Sherman

2002; Neff 2003). So why did black coucal males fail to ensure their paternity, and why did they continue to invest so heavily in parental care?

Whether male black coucals use cues to judge their paternity has not been studied. Our observations at least suggest that males closely guard their mate during the prelaying period. Hence, males may be able to assess the risk of paternity loss by the female's absence or by observed extrapair copulations (Sheldon et al. 1997; Ewen and Armstrong 2000). However, if males would respond to these cues by reducing their amount of care, it will most likely also affect their own genetic offspring (Westneat and Sargent 1996; Whittingham and Dunn 1998), unless they can recognize their offspring. Extrapair and within-pair chicks did not grow differently in terms of size and weight gain (Table 3), suggesting that male black coucals did not differentiate between extrapair offspring and their own. Because black coucal females in general do not participate in offspring care (Goymann et al. 2004), any reduction in male investment would thus affect all offspring.

In this context, it may be elucidating to see how the mating system of black coucals may have evolved and how this relates to paternity insurance. In most coucals investigated so far, the male seems to take a greater share in parental duties than the female, suggesting that predominant male parental care - in particular male incubation - is the ancestral condition in this group of birds (Andersson 1995). Male incubation may be advantageous if females are resource limited in egg laying (Andersson 1995) or if the nest predation rate is high (Goymann 2004). In such circumstances, male incubation may be favored because it frees females to gather additional resources for egg production (Andersson 1995; Goymann 2004). Thus, she may be able to more quickly finish the first clutch (Andersson 1995) or produce a replacement clutch in case the first nesting attempt fails (Goymann 2004). The black coucal differs from other coucals in that it has specialized on feeding grasshoppers, mantises, and frogs, which represent an overabundant food resource during the rainy season (Goymann et al. 2004, 2005). This may facilitate egg production over an extended period (Andersson 1995) and may make it easy for a male to raise the young alone (Ligon 1999; Anderson 2005) and for a female to control a territory with enough food for several nesting males (Goymann et al. 2004). Thus, insurance of genetic paternity may have played a minor role during the evolution of uniparental care, or it may have been important for the initial evolutionary origin of male uniparental care but not for its maintenance as suggested for wattled jacanas (Emlen et al. 1998). Classical polyandry in black coucals most likely is the result of the evolutionary history and specific ecological features of the habitat black coucals live in (Ligon 1999). On this background, male coucals may have to accept some extrapair young so that they do not lose the entire brood including their own genetic offspring.

To improve the genetic quality of their offspring, females paired with a poor-quality male may seek extrapair copulations more frequently than females paired with a high-quality male (Petrie and Lipsitch 1994; Petrie and Kempenaers 1998; Jennions and Petrie 2000). Furthermore, low-quality or inexperienced (young) males may be less effective in preventing females from engaging in extrapair copulations (Gowaty and Bridges 1991; Wagner et al. 1996) or may be less effective in avoiding extrapair paternity through sperm storage from previous males. However, we found no correlation between the proportion of extrapair young and the condition, body mass, or size of the brood’s social father.

When females benefit greatly from male incubation and offspring feeding, female mate choice should be based on cues that predict paternal behavior

(Ketterson and Nolan 1994). Male black coucals regularly (during 30% of copulations, N = 50) offer females a food item as a mating gift when they initiate copulations (Vernon 1971; W.G., unpublished data). This courtship feeding may act as a signal for the male's parental qualities, which then may be the target of female choice (Mills 1994; Pandolfi and Dastore 1994; Freeman-Gallant 1996). If female black coucals choose mates for the direct benefit of paternal care, it may not be adaptive for males to reduce paternal investment even when they face extreme uncertainty of paternity.

We found that at least 16.7% of the extrapair young were sired by another social male of the female. This could be either from copulations with other social mates during the time when a female lays a clutch or from sperm of the previous social partner being stored in the female reproductive tract. The reproductive tract of female black coucals contains sperm storage tubules that could serve this function (Frey and Goymann 2009). If sperm is stored from previous mates, males which pair early in the season may be more likely to gain additional offspring if their female produces subsequent clutches with other males, as reported for spotted sandpipers (Oring et al. 1992) and red-necked phalaropes (Schamel et al. 2004). If extrapair paternity through sperm storage or copulations with other social mates is common, one would expect that the risk of paternity loss is higher for males breeding later in the breeding season and for males paired to (highly) polyandrous females (see also Emlen et al. 1998). Although our sample size is currently too small to be conclusive, we found no correlation between the proportion of extrapair young in the brood and either laying date or the number of mates a female had within one season.

Our data suggest that in the majority of cases, extrapair young were fathered by males outside the social group of the female. In the 3 nests where the genetic father could be determined, they were males breeding in a territory directly adjacent to the focal territory. This suggests that females engaged in extrapair copulations with these males. Interestingly, all 4 extrapair sires were mated to later-laying females and were cuckolded themselves.

A striking difference between the 7 other classically polyandrous species for which paternity data are available and the black coucal lies in the characteristics of the breeding habitat (Table 1). All other species are shorebirds (Charadriiformes) and breed on predominantly flat, open habitats with relatively short vegetation such as riverbanks (Emlen et al. 1998; Haig et al. 2003), marshes (Dale et al. 1999), montane zone plateaus (Owens et al. 1995), and tundra (Schamel et al. 2004). In these habitats, it might be reasonably easy for males to monitor the behavior of their mate and to notice the presence of extrapair males, potentially explaining the lower levels of extrapair paternity in these species (see also Pierce and Lifjeld 1998). Three of the polyandrous species form a pair bond until clutch completion, and in 5 species, females are guarded intensively during the prelaying phase and beyond (Table 1). In contrast, black coucals breed in very dense, high grassland (often > 2 m). This vegetation provides excellent cover and limits both visibility and mobility and might therefore aid females seeking additional males and extrapair copulations. A similar hypothesis was suggested for the common sandpiper, which also often breeds in habitats with tall vegetation and has a comparably high rate of extrapair paternity (15.7% of offspring) despite the predominant male parental care (Mee et al. 2004).

A further limiting factor to paternity insurance of male black coucals is the onset of incubation. Males already start incubation once the first egg of a clutch is laid, which strongly reduces the time spent close to the female (Vernon 1971; C.M.

and W.G., personal observations). A similar situation has been described for the closely related pheasant coucal (*Centropus phasianinus*; Maurer 2007). Also, coucals do not seem to use other behavioral strategies such as cloaca pecking, as observed in dunnocks (Prunella modularis; Davies 1992), to prevent extrapair paternity.

In one nest, the social female was not the genetic mother of the young, whereas the caring male was the genetic father. The genetic mother could have been a sneaker female that secretly mated with the male and dumped the eggs in his nest within the territory of the social mother. Such a strategy may exist in black coucals as we identified nonterritorial females (Goymann et al. 2008). Alternatively, the genetic mother of the nest may have been the previous holder of the territory who may have been replaced by the social mother that defended the territory and the male when we found the nest. Taking over a male from another female and adopting his young might ensure that he stays in the territory and will breed again with her. However, there is circumstantial evidence that female black coucals do not always accept the offspring of previous territory owners. During the breeding season 2001 and 2005, 4 of our nests were partly destroyed and we found the broken eggs (1 case) or the chicks lying dead (3 cases) near the nest. In all 4 cases, this coincided with the disappearance of the respective territorial female and the appearance of a new female who had taken over the territory (W.G. and C.M., personal observations). Possibly, the new female territory owner may have committed infanticide to be able to immediately start a new clutch with the male (Blumstein 2000; Digby 2000; Veiga 2004).

The results of this study show that extrapair paternity in African black coucals may result from both extrapair copulations with males outside the female's group of social mates and copulations or stored sperm from one of the female's other social mates. Among classically polyandrous species studied so far, black coucals expressed the highest population rate of extrapair paternity. We suggest that males are unable to effectively guard their mates due to 1) the dense vegetation in their habitat and 2) the onset of incubation before clutch completion. Classical polyandry in black coucals most likely is a result of the evolutionary history and the species' ecology. Against this background, females may select nesting partners based on their parental qualities and males may accept solo incubation and offspring care without paternity insurance because reducing care without maternal compensation would jeopardize the entire brood. Thus, males of this cuckoo may be better off by accepting a certain degree of cuckoldry.

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Chapter Two

Testosterone does not directly modulate aggression but increases mate guarding in sex-role reversed female barred buttonquails

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unpublished manuscript

Abstract

In sex-role reversed species, females are the more competitive sex that defends territories and the access to mates, while males take care for the young, often without any help by the female. In male birds, testosterone levels generally rise during the breeding season to modulate territorial and aggressive behaviour. As some female birds show a similar relation of testosterone and aggression, testosterone may also have a modulating role in aggressive behaviour in females of sex-role reversed species, and may even play a role in the evolution of sex-role reversal. To test this hypothesis, we experimentally increased testosterone levels in sex-role reversed polyandrous female barred buttonquails by implanting testosterone pellets, and tested the behavioural and hormonal response of these females during a simulated territorial challenge. Testosterone treatment did not lead to an aggressive response, hence, the hormone did not directly modulate aggression in female barred buttonquails. However, it increased female mate-guarding intensity, which can be seen as a part of territorial defence, as well. We therefore conclude that circulating testosterone levels do regulate behaviour involved in female-female competition in this species. Moreover, considering the male-typical correlation of testosterone and

mate-guarding intensity, testosterone may indeed be involved in the reversal of the sex-roles in this species.

Introduction

Sex-role reversal belongs to the most intriguing phenomena in evolutionary ecology (Andersson 1995, Owens 2002). It occurs only in a small fraction of vertebrate species, and despite numerous comparative and experimental studies on sex-role reversed species, its evolutionary significance is not well understood and remains very much debated (Andersson 1995, Owens 2002). In sex-role reversed species, females typically establish, and vigorously defend, breeding and/or feeding territories and show a stronger intra-sexual competition for mates than males (Clutton-Brock and Vincent 1991). Males, on the contrary, take care for the young, often without any help by the female (Clutton-Brock and Vincent 1991).

Also the mechanisms that stand behind sex-role reversal, both in physiological and organizational aspects, remain an open question (Andersson 1995, Owens 2002). The mechanistic hypothesis that sex-role reversal is associated with a reversal in sex-hormone levels has been an auspicious possibility because sex hormones are involved in the modulation of sex-role-associated behaviours in species with ‘traditional’ sex-roles (Wingfield et al. 1990). However, this hypothesis did not get much support from hitherto existing studies.

The hormone investigated in most detail in this context is the androgen testosterone. In many male birds of non-sex-role reversed species, testosterone levels rise during the breeding season to initiate and modulate territorial and aggressive behaviour (Wingfield et al. 1990). Some female birds show a similar relation of testosterone and aggression (Staub and DeBeer 1997, Wingfield et al.

2000, Ketterson et al. 2005), indicating the potential of a modulating role of testosterone in this sex, too. However, in most sex-role reversed classically polyandrous species studied so far males and females had levels of testosterone comparable to males and females of species with conventional sex roles. (e.g. Rissman and Wingfield 1984, Fivizzani and Oring 1986; Schlinger et al. 1989, Goymann and Wingfield 2004). Therefore, reversed circulating testosterone concentrations (i.e. females high levels, males low levels of testosterone) in sex-role reversed species could not be demonstrated, hence, do not seem to be the important elicitor of sex-role reversal. Nevertheless, there is some evidence that hormonal mechanisms other than circulating testosterone concentrations could be at work, such as an increased sensitivity to testosterone or its metabolites in the brain or in specific tissues such as the skin (Schlinger et al. 1989, Goymann et al. 2004, Muck and Goymann in press). Alternatively, in sex-role reversed females aggressive behaviour during resource defence may be regulated by an interaction of testosterone with other hormones, for example with progesterone (Goymann et al. 2008).

Diverse neuroendocrine interrelations with “male-typical” behaviour have likewise been described for males of non-sex-role reversed species. Experimentally induced territorial aggression - elicited by placing a decoy into the centre of a territory and playing back song - has been shown to be accompanied by increased levels of plasma testosterone only in some birds (e.g. Wingfield et al. 1985, Wikelski et al. 1999). Other studies found that testosterone levels were either not affected (e.g. Meddle et al. 2002; Moore et al. 2004a, b) or even decreased during simulated territorial intrusion experiments (e.g. Van Duyse et al. 2004, Peters et al. 2006, Landys et al. 2007; for a complete list of simulated territorial intrusion studies in birds see reviews in Goymann et al. 2007, Goymann 2009). And other studies in turn showed that male aggressive behaviour does not relate to testosterone levels alone but may depend on a combination of hormone levels, social context, environmental input, and the birds' experience (Gill et al. 2007, Hirschenhauser et al. 2008). Similarly, experimentally elevated testosterone levels in males may or may not increase territorial behaviour such as song or aggression (Moore 1984, Collis and Borgia 1992, Hunt et al. 1997, De Ridder et al. 2000, Foerster and Kempenaers 2005). Therefore, alternative hormonal mechanisms may also be at work in sex-role reversed species, and testosterone may indeed be physiological relevant and involved in sex-role reversed behaviour in some species despite low levels of circulating testosterone.

The aim of this study was to investigate the role of testosterone in the behaviour of sex-role reversed, socially polyandrous barred buttonquail (*Turnix suscitator*). In this species, females aggressively defend territories and vocalize to deter rivals and attract males, while the latter are mainly involved in incubation and offspring care (Trollope 1970, Ridley 1978, Debus 1996). They have a marked reversed sexual size dimorphism (Starck 1991, Debus 1996), and only females express a black throat patch throughout the year (Sutter 1955, Starck 1991). In captivity, circulating levels of testosterone in barred buttonquail are relatively low throughout the year and females and males express comparable levels, an exception in sex-role reversed species (Muck and Goymann, in press). Moreover, the black throat patch of females is strongly correlated to circulating testosterone levels. Given that testosterone levels are similar between the sexes, this sex-role reversed dichromatism may be due to a higher sensitivity of females to testosterone in this species (Muck and Goymann in press). Thus, there is already evidence for an involvement of testosterone in sex-role reversal in barred buttonquails.

We experimentally increased testosterone levels in captive female barred buttonquails by implanting testosterone pellets and tested the behavioural and hormonal response of these females during a simulated territorial challenge. Exogenous testosterone, as well as a territorial challenge, may lead to an increase in corticosterone (Astheimer et al. 2000; Van Duyse et al. 2004, Landys et al. 2007, Scriba and Goymann 2008). We therefore determined - in addition to testosterone concentrations – the levels of the stress hormone corticosterone in focal females. We recorded the frequency of aggressive behaviour, as well as behaviours indicating fear, and measured both testosterone and corticosterone levels after the territorial challenge to look for corresponding changes in hormone levels. We hypothesized that females with increased testosterone levels would react more aggressively towards an intruder than control females with un-manipulated hormone levels. These results would confirm our hypothesis of a modulating role of testosterone in aggression in female barred buttonquails. Moreover, these results would further approve that the sex-role reversal in this species is accompanied by a reversal in testosterone sensibility not only in the expression of a secondary sexual trait but also in the behavioural reaction during a territorial challenge.

Methods

Study species and housing

We investigated the behaviour and corresponding hormone levels of 12 female barred buttonquails during a simulated territorial challenge, between 2007 and 2009. The birds were kept in female-male pairs in indoor boxes made from opaque polypropylene (1.0m x 1.0m x 0.7m) at a light-dark schedule of 13:11hrs, mimicking conditions close to their equatorial origin. The boxes were equipped with grass tussocks, artificial plants and little haystacks to offer hiding and nesting areas, and food and water were provided *ad libitum.* The top of the boxes was covered with a net so that the birds could not escape and did not injure themselves when jumping inside the boxes. All birds were naïve to the experimental procedures and have not been used for other experimental studies beforehand.

Experimental setup

Females and their male partners were introduced into their cages at least six weeks prior to the experiment to let the birds adjust to both the environment and their social mate. Cages were accommodated in two separate rooms with six cages per room. Adjacent pairs could hear but not see each other. Furthermore, the birds could only hear but not see the person preparing and conducting the experiment.

The twelve females were randomly divided into two groups. Six females got a testosterone implant (see below), while the other six females got a sham control implant. We took a first blood sample of all birds 1-3 weeks before implantation to determine testosterone baseline levels. Furthermore, birds were weighed to the nearest 0.1g. Two days after implantation, we took another blood sample to determine the actual increase in testosterone elicited by the implant, and to measure corticosterone baseline levels of the females. Four days after implantation we simulated a territorial intrusion, after which we immediately caught the birds for a final blood sample to determine again levels of testosterone and corticosterone after the 20-minute-challenge.

For the territorial challenge, we placed a stuffed female decoy in the centre of the cage (for each focal female the same decoy) and played back barred buttonquail song (20 seconds of booming repeated every minute) with a loudspeaker situated outside the box right next to its side wall. For each female we played back the same song, which was a deep resonant booming call females produce to attract males and to deter other females (Debus 1996). Furthermore, females respond aggressively to another female booming in or near her territory (Debus 1996). The behaviour of the focal bird was recorded during the 20 minutes while the decoy was present in the cage using a Sony DCR-TRV6E digital camera, which was fixed above the cages.

We tested 2-3 birds per day, while females that were experimentally challenged on the same day were not of neighbouring cages, and for successive tests we altered between rooms as to prevent disturbances affecting the outcome of the experiment. All tests were done in the morning between 9:00 and 12:00am. Time of the day and the order in which females were challenged (i.e. first, second or third on the same day) did not significantly affect the behaviour or hormone levels of the birds (Kruskal-Wallis-Test, df = 2, approach: $X^2 = 3.90$, $p = 0.35$; jump: $X^2 = 0.31$, $p = 0.92$; hide: $X^2 = 0.73$, $p = 0.74$; pace: $X^2 = 5.31$, $p = 0.06$; freeze: $X^2 = 0.44$, $p = 0.83$; latency: $X^2 = 1.05$, $p = 0.63$; corticosterone: $X^2 = 2.46$, $p = 0.35$; testosterone: $X^2 = 2.31$, $p = 0.35$).

Hormone implantation

Females were implanted with time release pellets received from Innovative Research of America (Sarasota, FL). Testosterone pellets had a dosage of 1mg/pellet and were designed to release the hormone at 0.05mg/day, hence keeping a constant elevation of testosterone for 21 days. Control birds received placebo pellets of the same size but without testosterone. The pellets were implanted subcutaneously on the birds' back between the scapulas by inserting them through a small incision in the skin. The incision was then sealed with tissue glue (Histoacryl, Braun Surgical; GmbH). The whole procedure took about 2 minutes, and the birds were released into their boxes after the incision was closed and the birds were alert.

Behavioral measures

Videos were analysed using the program JWatcher^{TM} 0.9 (2000). During video analysis the observer was blind regarding whether the focal female belonged to the control- or testosterone implanted group. We recorded seven distinct behaviours shown by the focal bird to analyse the reaction towards the decoy during the simulated territorial challenge: 1) latency until the first reaction towards the decoy (latency); 2) how long the bird stood still (freezing behaviour); 3) how long the bird kept hiding behind a grass tussock (hiding); 4) how long the bird repeatedly meandered back and forth along the cage wall (pacing); 5) how often the bird approached the decoy to a distance of at least 20cm (approach); 6) how often the bird physically attacked the decoy (attack); and 7) how often the bird jumped vertically with sometimes simultaneously rapid wing movements (flight-jump). There are only very little data on behaviour of Turnix species (Ridley 1978, Debus 1996), however, studies on Japanese quails (*Coturnix japonica*) and domestic chicken (*Gallus domesticus*) describe pacing, freezing, and jumping as typical behaviours associated with fear and escape in these species, which are similar to buttonquail with respect to being “terrestrial” birds (Jones and Harvey 1987, Richard et al. 2008, Schweitzer et al. 2010). On the other hand, we assumed that approaching and attacking the decoy were aggressive behaviours.

Finally, we recorded how often the female and how often her paired male stopped with one of the above listed behaviours and changed position within the cage to start a new behaviour, and whether the partner followed this behaviour within 5 seconds or remained in its former activity/position. The proportion of how often a bird did follow its mate's initiated behaviour/position and how often it did not follow the initiated behaviour/position was taken as a measure of mate-guarding.

Blood samples

Blood samples (150-200 µl) were taken from the alar wing vein within 2.4 ± 0.06 minutes after opening the cage to capture the birds. We used fine needles (0.6 mm diameter) to pierce the vein and collected the blood using heparinized capillaries. The plasma was separated immediately (centrifuged at 10000 rpm for 15 min) and stored at -80°C until hormone assays were completed.

Hormone analysis

Testosterone and corticosterone were analyzed using a radioimmunoassay as described in Goymann et al. (2006). For testosterone, we conducted 4 separate assays to analyse all samples. Mean extraction efficiency was 90 ± 4%, mean intra- and inter-assay coefficients of variation were 8.8% and 8.4%, respectively, and the detection limit (mean ± SD) was 0.40 ± 0.08 pg/tube. Because the testosterone antibody used showed some cross-reactivity (44%) with dihydrotestosterone, our measurements may include a minor fraction of this other potent androgen. For corticosterone, we conducted only one assay, which had a mean ± SD extraction efficiency of 85 ± 4 %, a mean intra-assay coefficient of variation of 6.8%, and a detection limit of 3.6 pg/tube.

Statistical analysis

Statistical analyses were conducted using SPSS 15.0. Throughout the paper, values are expressed as mean ± SEM and the statistical significance was set at $\alpha \leq 0.05$ (2-tailed).

Testosterone and corticosterone data were log-transformed to obtain a better model fit. We used a general linear mixed model to compare testosterone and corticosterone levels, both measured before and immediately after the experimental challenge, between control-females and testosterone-implanted females. This model comprised hormone concentration as the dependent variable, experimental group and time of measurement (i.e. before or after the experiment) as fixed factors, test order (the order the birds were tested on one day) and bird ID as random factors, body mass as a covariate, and the interactions between experimental group and time of measurement, and between experimental group and test order.

Behavioural data were not normally distributed, thus differences in behaviour between control females and testosterone-implanted females were analysed non-parametrically using Mann-Whitney U-tests. No female attacked the decoy, therefore, this behaviour was not included in the analysis.

Results

Plasma testosterone and corticosterone

Testosterone implants increased the hormone level 10 to 30-fold in experimental birds compared to testosterone baseline levels of all females (Fig. 1). Accordingly, there was a significant difference in testosterone levels between experimental groups, and also between baseline levels and post-challenge levels of testosterone (general linear mixed model, $F_{1,7.43} = 31.36$, $p = 0.001$, $F_{1,9.72} = 33.82$, $p < 0.001$, respectively). The significant interaction between experimental group and time of measurement ($F_{1,9.72} = 80.78$, $p < 0.001$) points out that the testosterone levels of testosterone-implanted females significantly increased after implantation, while testosterone levels of control females remained the same (Fig. 1). Bird body mass and test order did not significantly affect testosterone levels (body mass: $F_{1,4.75}$ = 0.21, p = 0.67; test order: $F_{2,4.3}$ = 0.92, p = 0.46), and there was no significant interaction between experimental group and test order ($F_{2,4.43}$ = 0.56, p = 0.61).

Corticosterone levels did not differ between testosterone-implanted females and control females, neither before nor after the experiment (Fig. 2, general linear mixed model, exp.group: $F_{1,4.46}$ = 0.05, p = 0.0.83; time of measurement: $F_{1,8.17}$ = 2.75, p = 0.14; experimental group*time of measurement: $F_{1,8.17}$ = 0.09, p = 0.77). Furthermore, there was no significant effect of bird mass, test order, or the interaction experimental group * test order on corticosterone levels (bird mass: $F_{1,4.09}$ = 0.48, p = 0.53, test order: $F_{2, 3.76}$ = 0.79, p = 0.52, exp.group*test order: $F_{1,3.94}$ = 1.21, p = 0.34).

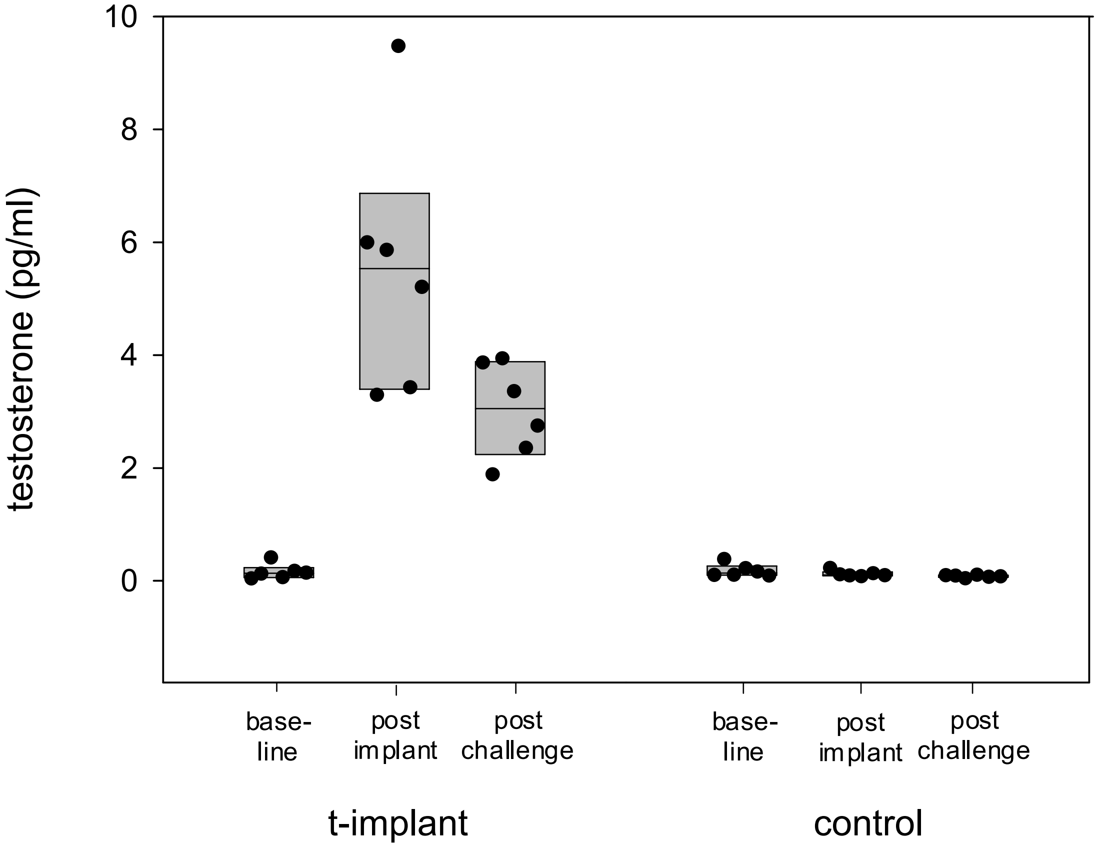

Figure 1

Testosterone concentration of female barred buttonquails before implantation (baseline), after implantation, and after the 20-minute territorial challenge compared between testosterone-implanted females (N=6) and control females (N=6). Testosterone-implanted females and control females had similar baseline levels, however, testosterone-implanted females had significantly higher testosterone levels after implantation than control females (general linear mixed model, exp.group: $F_{1,7.43} = 31.36$, $p = 0.001$; baseline-challenged: $F_{1,9.72} = 33.82$, $p < 0.001$; interaction: $F_{1,9.72} = 80.78$, $p < 0.001$). Box-plots show the median and the 25th and 75th percentiles. Black circles show the actual testosterone values of the single birds.

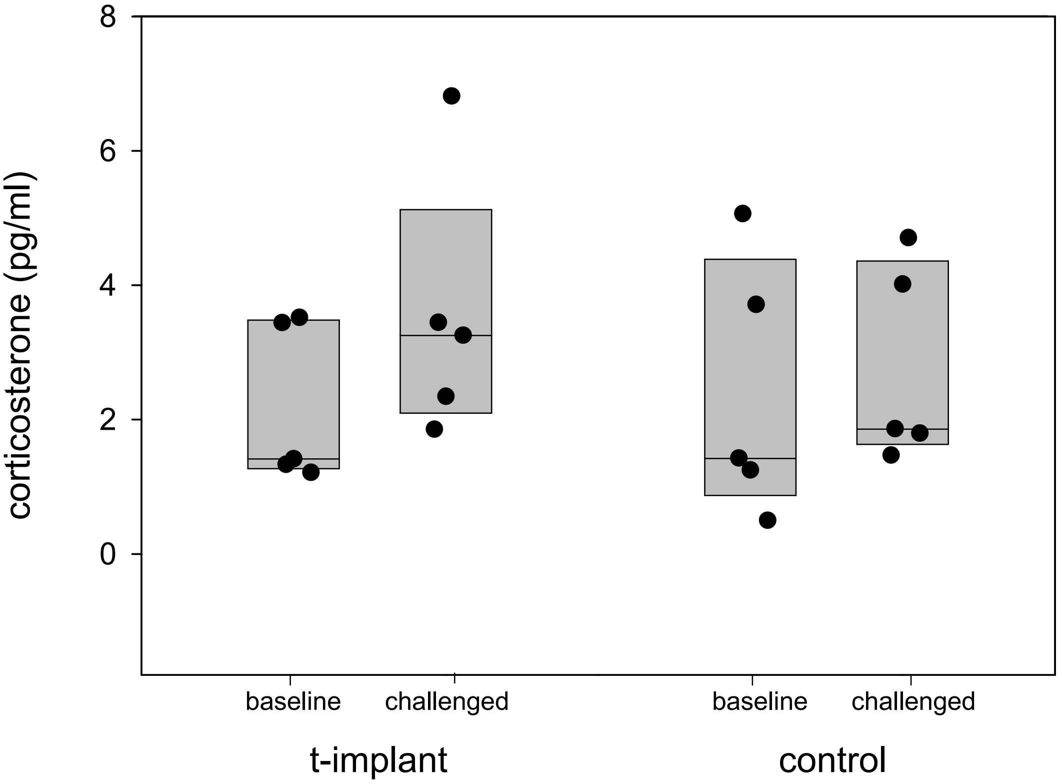

Figure 2

Corticosterone baseline levels (A) and corticosterone levels after 20 minutes of a territorial challenge (B) did not differ significantly compared both within experimental groups (baseline and challenged, $F_{1,8.17} = 2.75$, $p = 0.14$*) and between experimental groups (control females N=5, t-implanted females N=5;* $F_{1,4.46} = 0.05$, $p = 0.0.83$*). Box-plots show the median and the 25th and 75th percentiles; black circles show the actual change in corticosterone levels of individual females.*

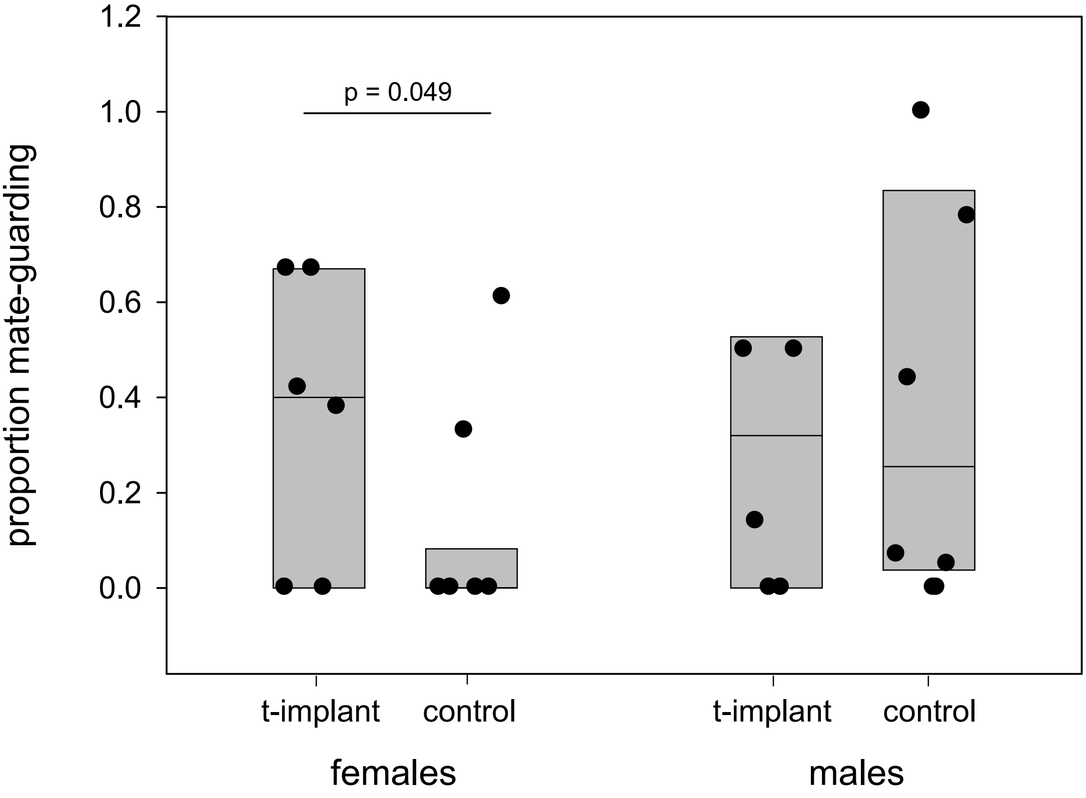

Figure 3

Mate-guarding behaviour shown by male and female barred buttonquails during a 20-minute territorial challenge. T-implanted females guarded their mates significantly more often than control females (Mann-Whitney U-test, Z = -1.97, p = 0.049). Males of t-implanted females and of control females did not differ in their mate-guarding behaviour (Z = -0.32, p = 0.75). Box-plots show the median and the 25th and 75th percentiles; black circles show the actual change in corticosterone levels of individual females.

Behaviour

Latency until the first behavioural reaction after the start of the territorial challenge did not differ significantly between control females and t-implanted females (Table 1, Mann-Whitney U-test, Z = -0.64, p = 0.59). Similarly, testosterone-implanted females and control females did not differ significantly in the frequencies of flight-jumps (Z = -1.44, p = 0.20), freezing (Z = -0.64, p = 0.59), pacing (Z = -0.16, p = 0.94), hiding (Z = -0.64, p = 0.59) and approaching the decoy (Z = -0.66, p = 0.56). However, females with testosterone implants followed their mates significantly more often than control females (Fig. 3, Mann-Whitney U-test, Z = -1.97, p = 0.049). Males did not follow their females more often when they were paired to a testosterone-implanted female or a control female (Z = -0.32, p = 0.75).

Table 1

Behaviours shown by testosterone-implanted females and control females towards a simulated intruder during a 20-minute-challenge.

	mean ± SE		**Mann-Whitney U-test**	
behaviour	**control (N=6)**	**t-implant (N=6)**	**Z**	**p**
latency (min.)	2.6 ± 1.67	0.6 ± 0.47	-0.64	0.59
freezing (min.)	5.4 ± 2.72	5.6 ± 2.46	-0.16	0.94
hiding (min.)	6.8 ± 2.93	8.9 ± 3.16	-0.64	0.59
pacing (min.)	5.3 ± 2.23	4.3 ± 1.41	-0.16	0.94
approaching (frequency)	2.5 ± 1.93	2.2 ± 1.05	-0.66	0.56
flight-jump (frequency)	0.3 ± 0.33	3.0 ± 2.41	-1.44	0.20

Table 2

Effect sizes of experimental studies investigating the effect of exogenous testosterone on aggressive behaviour in female birds.

Species	**Measures**	***p***	**control (N)**	**exp.group (N)**	**effect size (δ)**	**References**
barred buttonquail *Turnix suscitator*	aggression/fear	> 0.50	6	6	1.58*	This study
red-winged blackbird *Agelaius phoenicus*	aggressive songs	< 0.01	8	7	1.67	Searcy 1988
European starlings *Sturnus vulgaris*	proportion of time spent near cage/decoy	0.027	10	10	1.79	Sandell 2007
dark-eyed juncos *Junco hyemalis carolinensis*	number of aggressive displays	< 0.05	10	15	0.88	Zysling et al. 2006
zebra finches *Taeniopygia guttata*	chasing (aggression)	0.036	9	10	0.81	Adkins-Regan 1999

*measurable effect size estimated from a sensitivity analysis based on our sample size.

Unfortunately, the results are based on very small sample sizes, which may limit their overall validity. Hence we conducted a sensitivity analysis, demonstrating that the minimal effect size that we could have demonstrated with our sample was 1.53 (see Table 2). The few other studies that investigated the effect of exogenous testosterone on aggressive behaviour in female birds indeed produced effects in that range (with one exception; Table 2). Hence, if testosterone would have had a similar effect in buttonquails as in these other species we should have been able to at least observe a trend in our dataset.

Discussion

In sex-role reversed female barred buttonquails experimentally elevated testosterone levels did not affect the aggressive response during a simulated territorial challenge. These results are in contrast to our prediction that circulating testosterone levels mediate female aggression in this species. Also, corticosterone levels were unaffected by the simulated territorial challenge (and testosterone implantation). However, testosterone-implanted females followed their mates at a higher rate than control females, possibly suggesting that testosterone may be involved in mate-guarding behaviour of females in this species.

Females implanted with testosterone and control females showed similar behaviours during a 20-minute-presentation of a female decoy. These reactions comprised behaviours reflecting fear rather than aggression. Most females approached the decoy. None of them, however, actually attacked the decoy physically or showed other high levels of aggression such as calls or aggressive postures. High levels of testosterone therefore did not appear to directly affect aggression in barred buttonquails.

The lack of an aggressive response directed towards the decoy in barred buttonquails may be due to various reasons. First, circulating levels of testosterone may not be the key mechanism in the regulation of aggression. Instead, other hormones such as progesterone, dehydroepiandrosterone, or estrogens could be involved (Hau et al. 2004, Goymann et al. 2008, Pärn et al. 2008). One argument for the evolution of aggression independent of circulating testosterone levels in female birds are detrimental effects of high testosterone levels in this sex, such as interference with normal ovulation (Searcy 1988, DeRidder et al. 2002, Clotfelder et al. 2004, Veiga et al. 2004, Zysling et al. 2006, Veiga and Polo 2008), impaired immune function (Duffy et al. 2000, Eens et al. 2000, Peters et al. 2002, Mougeot et al. 2004), and maternal effects detrimental for the offspring (e.g. delay of hatching, Navara et al. 2005; reduced egg hatchability, Sockman and Schwabl 2000). Apart from the impaired immune function, these negative effects of testosterone only affect female reproduction. Thus, a role for testosterone in the regulation of aggression may have only evolved in males (Ketterson et al. 2005).

A second reason for the lack of an aggressive response in our study could be that female barred buttonquails experienced the setup not as a territorial intrusion and thus did not respond in an aggressive way towards the decoy. Instead, females may have expressed a neophobic reaction because they perceived the decoy as an unknown object rather than an intruder (Scriba and Goymann 2008). The fact that females responded with behaviours that indicated fear rather than aggression supports this idea. On the other hand, corticosterone levels were unaffected by the simulated challenge. This contradicts the threatening effect of the decoy, because corticosterone - as a “stress-hormone” that assists animals in coping with increased energetic demand (reviewed in Landys et al. 2006) – should have risen if the experimental situation was stressful.

Thirdly, the treatment surprisingly resulted in supra-physiological levels of testosterone in testosterone-treated females, i.e. the manipulation resulted in testosterone levels that these females would have never experienced under natural conditions, which may be due to an uneven release of the hormone by the implanted pellets (see also Fusani 2008). It therefore remains speculative whether these hormone levels could actually trigger a behavioural response (see also Fusani 2008, Edler et al. 2011). Along those lines, exogenous testosterone did indeed have an effect on female behaviour in our study. Testosterone-implanted females guarded their mates during the presence of a female decoy more often than control females. Mate guarding is mostly seen in male birds, including males of sex-role reversed polyandrous species (Eurasian dotterel *Charadrius morinellus*, Owens et al. 1995; African black coucal *Centropus grillii*, Goyman et al. 2004; red-necked phalaropes *Phalaropus lobatus*, Schamel et al. 2004). In control broods, female barred buttonquails also showed lower mate guarding frequencies than males. Hence, intensive mate guarding as a response to exogenous testosterone could indeed be an artefact, i.e. females displayed male behaviour that does not belong to their natural repertoire of behaviour. A comparable effect is known in female canaries (*Serinus canaria*), which develop a male-like song after testosterone treatment (Leonard 1939, Shoemaker 1939, Nottebohm 1980). In this case, exogenous testosterone induces structural changes in the female brain, which in turn leads to the expression of a new, male-like song structure (Nottebohm 1980, Gahr and

Garcia-Segura 1996). Therefore, testosterone did not enhance (i.e. modulate) a female behaviour but evoked a mechanism for the expression of a male behaviour.

In male birds, mate guarding prevents or reduces the occurrence of extra-pair copulations, thus prevents a decrease of the potential breeding success (e.g. Sorenson 1994, Komdeur et al. 1999, Blomqvist et al. 2002, Davis 2002). In some species, male mate guarding may also be correlated with testosterone levels (white-crowned sparrows *Zonotrichia leucophrys*, Moore 1984; barn swallows *Hirundo rustica*, Saino and Møller 1995; mallards *Anas platyrhynchos,* Davis 2002), which suggests a modulating role of testosterone in mate-guarding as a selected trait that ensures breeding success of the male (Birkhead 1998). The advantage of mate guarding in females is slightly different than that of males. The risk of their male fertilizing the eggs of another female may reduce the breeding success of the female, however, not in terms of loss of genetically own offspring but by a potential reduction in paternal care (Trivers 1972; Hoi-Leitner et al. 1999; Whittingham and Dunn 2001; Sheldon 2002; Arnqvist and Kirkpatrick 2005). Because in sex-role reversed species the male does most of the incubation and chick rearing, females may loose some offspring or even the whole brood if males put effort into the upbringing of another clutch for another female. The male may even abandon its former female for another female of better quality (Ens et al. 1993).

Furthermore, female mate guarding may also protect the breeding male from harmful effects of (forced) extra-pair copulations, such as injuries, infections, disruption of feeding, or disruption of incubation (Jenkins et al. 1965, Lumpkin 1981, Sheldon 1993). Comparable to mallards, female barred buttonquails typically grab the head or neck feathers of a male during courtship, and even deliver pecks to head, neck and back (Hoesch 1959, Debus 1996). Injured males may be prone to infections or may reduce the care for the offspring, and therefore the breeding success may be at risk. An even greater risk induced by multiple matings are sexually transmitted diseases, which may cause a reduction in weight, fertility and egg production, and could even lead to the death of the infected bird (Sheldon 1993).

If mate-guarding ability indeed depends on testosterone in female barred buttonquails, and increases female breeding success through securing offspring care and averting disease transmission, mate-guarding would indeed be a selected trait in these birds. Females also express a black breast patch, which is testosterone-dependent and may be a signal for condition (Muck and Goymann, in press). This secondary sexual trait may further reflect female breeding success through its testosterone-dependency, and given the polyandrous mating system of barred buttonquails, may therefore attract multiple males (Redpath et al. 2006).

The results of our study showed that experimentally elevated testosterone levels did not directly affect territorial aggression in female barred buttonquails, However, it increased female mate-guarding intensity, which can be seen as a part of territorial defence, as well. We therefore conclude that circulating testosterone levels do regulate behaviour involved in female-female competition in this species. Moreover, considering the male-typical correlation of testosterone and mate-guarding intensity, testosterone may indeed be involved in the reversal of the sex-roles in this species.

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Chapter Three

Hormones mirror personality – a mirror image stimulation test in male stonechats

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unpublished manuscript

Abstract

In male birds, the hormonal response following experimentally induced territorial aggression differs between species and even within populations. The direction of both testosterone and corticosterone responses after a competitive fight may depend on the relative fighting ability of males, the responsiveness of the opponent, and/or the outcome of the fight. These factors indicate individual differences in the hormone synthesis during agonistic interactions. We investigated behavioral and hormonal responses of captive Irish stonechats (*Saxicola torquata hibernans*) during simulated territorial intrusion tests in captivity using a mirror, a decoy, and a control glass panel. We found that both a mirror and a decoy, but not the control glass, provoked an agitated and/or aggressive response in tested males, yet none of these conditions elicited a testosterone response related to the observed aggression. The behavioral response towards a decoy was accompanied by an increase in corticosterone. Furthermore, males showed one of two distinct patterns of aggression towards their own reflection in the mirror, and these patterns were related to hormone levels. Individuals which showed strong and physical aggression towards the mirror had characteristically higher testosterone and lower corticosterone levels, and males which did not exhibit aggression but only agitation behavior towards the mirror were characterized by relatively lower testosterone and higher corticosterone levels. Individual differences in hormone levels were consistent between the three experimental tests. From these results we hypothesize that in captive male stonechats the behavioral and hormonal response during competitive fights is not provoked by the stimulus alone but mainly modulated by the birds' personality.

Introduction

The steroid hormone testosterone plays a fundamental role in the neuroendocrine modulation of aggressive behavior in male birds (reviewed e.g. in Wingfield, 2005; Soma, 2006; Kempenaers et al., 2007). Administration of testosterone may increase aggression, while aggressive interactions in turn may influence testosterone levels (Oliveira, 2004; Wingfield et al., 1987, 1990, 2001; Hirschenhauser and Oliveira, 2006; Goymann et al., 2007). For example, experimentally induced territorial aggression - elicited by placing a decoy into the centre of a bird's territory and playing back song - has been shown to be accompanied by increased levels of plasma testosterone in some species (e.g. Wingfield, 1985; Wikelski et al., 1999). Other studies, however, found that testosterone levels were either not affected (e.g. Meddle et al., 2002; Moore et al. 2004a, 2004b) or even decreased during such simulated territorial intrusion experiments (e.g. Van Duyse et al., 2004; Peters et al., 2006; Landys et al., 2007; for a review on all studies in birds see Goymann et al., 2007; Goymann 2009).

Between-species differences in the testosterone responsiveness to male-male competition in birds have been linked to a variety of life-history traits (e.g. paternal care: Wingfield et al., 1990; Oliveira, 2004; Lynn, 2008; the length of the breeding season or the number of broods: Landys et al., 2007; Goymann et al., 2007; Goymann, 2009; phylogeny: Oliveira, 2004; methodological issues: Kempenaers et al., 2007; Goymann et al., 2007; Goymann, 2009). For example, the blue tit (*Cyanistes caeruleus*), a monogamous, temperate-zone species which raises one brood per season, responds to a territorial challenge with a decline in circulating testosterone levels (Peters et al. 2006; Landys et al., 2007). In contrast, the monogamous song sparrow (*Melospiza melodia*), a temperate-zone species which raises multiple broods per season, shows an increase in circulating testosterone levels after simulated territorial intrusions (Wingfield, 1985).

Species that lack a testosterone response during simulated territorial intrusions often show an increase in glucocorticoids such as corticosterone (Van Duyse et al., 2004; Landys et al., 2007). Rising levels of corticosterone have been suggested to support the bird by maintaining high activity levels and increasing the metabolic rate during agonistic encounters (Van Duyse et al., 2004; Landys, 2007). Moreover, increasing levels of corticosterone may be responsible for static or decreasing levels of testosterone following a simulated territorial intrusion in some species (Landys et al., 2007). In mammals, corticosteroids have been suggested to even directly influence aggression (Mikics et al., 2004).

Differences in hormone responses during male-male competition may also occur between individuals of one population. These between-individual differences may depend on an individuals' perception of the progress or the outcome of the acute fight or of previous fights (Oliveira, 2004; Kempenaers et al., 2007; Goymann et al., 2007; Goymann, 2009; Pryke, 2009). For instance, losers may show a reduction in testosterone synthesis (Oliveira et al., 1996; Oliveira, 2004), while winners keep high fighting-induced testosterone levels (Harding, 1981; Ramenofsky, 1984, 1985), or show an acute testosterone response after repeated winning experiences (Oyegbile and Marler, 2005). Moreover, agonistic interactions may lead to high levels of corticosterone in losers compared to winners (e.g. Knapp and Moore, 1996; Von Holst, 1998; Pryke, 2009).

Based on this ‘winner-loser effect’ (Oliveira, 2004), the relative fighting ability of males and the responsiveness of the opponent may further influence the hormone response during aggressive interactions (Oliveira et al., 2005; Hsu et al., 2006; Hirschenhauser et al., 2008). Relative fighting ability and the responsiveness of opponents have both been investigated in mirror image stimulation tests (a simulated territorial intrusion experiment with a mirror image as an opponent). During such tests the self-assessment and the assessment of the opponent’s strength, as well as the perception of the outcome of the interaction are blurred (Oliveira et al., 2005; Hirschenhauser et al., 2008; Desjardins and Fernald, 2010). Both in the cichlid fish *Oreochromis mossambicus* (Oliveira et al., 2005) and the Japanese quail *Cortunix japonica*, (Hirschenhauser et al., 2008) males vigorously attacked their reflection in a mirror, but testosterone levels remained low. In contrast, comparable fights with real (alive) opponents increased testosterone in both species (Hirschenhauser et al., 2004, 2008; but see Desjardins and Fernald, 2010). The authors conclude that neither the act of fighting nor the actual conflict outcome may trigger a testosterone response. Instead, the testosterone response may depend on an individual’s perception of its own and the opponent’s fighting ability, i.e. on information regarding the likely outcome of a contest. Interestingly, Hirschenhauser et al. (2008) found that only about half of their birds (Japanese quails) responded aggressively to the mirror, while the others refrained from aggressive interactions with the mirror image. In contrast to the behavioral difference, testosterone levels remained low in all birds, fighters and non-fighters (Hirschenhauser et al., 2008). These data suggest marked individual differences in conflict behavior.

In many vertebrates, individual differences in behavior and physiology are consistent across contexts and stable over time. Such consistent individual differences are referred to as temperament or personality, and may have a genetic component (Réale et al., 2007; Sih and Bell, 2008; Dingemanse et al., 2010). Differences in personality may be reflected in neuroendocrine parameters (Koolhaas et al., 1999; Carere et al., 2003; Kralj-Fišer et al., 2007), and during aggressive interactions (Verbeek et al., 1996; Carere et al., 2003; Fuxjager et al., 2010) including territory defense (Amy et al., 2010). For example, in great tits (*Parus major*), individuals differing in coping strategies with new environments (fast and slow explorer) expressed their individual character also during confrontation with an aggressive resident male (Carere et al., 2003). Fast explorers were more aggressive and had lower corticosterone metabolite concentrations following the aggressive encounter, slow explorers on the other hand were less aggressive and expressed higher corticosterone metabolite concentrations (Carere et al., 2003; see also Koolhaas et al., 1999). Hormonal responses during stressful or aggressive interactions may therefore be determined by an individual's personality in addition to the (perceived) agonistic situation itself.

In this study, we investigated the behavioral and the hormonal response of a partially migratory population of the European stonechat, the Irish stonechat (*Saxicola torquata hibernans*) to three different stimuli. Seasonal plasma hormone profiles and behavioral responses during agonistic interactions have been well described for free-living and captive males of the continental population of this species (Gwinner et al., 1994; Canoine, 2001; Canoine and Gwinner, 2002; Schwabl et al., 2005; Helm et al., 2009). Stonechats react aggressively to intruding opponents, and blockage of testosterone reduces aggressive behavior during the breeding season (Canoine and Gwinner, 2002). We presented captive males either with a mirror or a stuffed decoy, and compared their responses to these stimuli with a control situation, during which they were confronted with a glass panel only. Based on previous mirror image stimulation studies on cichlid fish and Japanese quail (Oliveira et al., 2005; Hirschenhauser et al., 2008; Desjardins and Fernald, 2010), our study aimed at testing the effect of reduced information on the fighting ability of the opponent (i.e. likely outcome of a contest) on the hormonal and behavioral response of challenged males in a passerine bird species. We decided to present a decoy rather than a life bird during our experiments to reduce the risk of major injuries during potentially arousing fights. However, decoy and mirror image represent different stimuli in terms of movements and three-dimensionality. Furthermore, we investigated whether the birds differed individually in their behavioral and hormonal reactions and whether these differences were consistent between experiments. This aim of our study is based on the work of Hirschenhauser et al. (2008), who found significant differences in individual responses during simulated mirror image stimulation tests. We intended to determine hormonal correlates of personality traits in the behavioral response during aggressive conflicts. Consistent individual personality traits during agonistic situations could - in addition to the effect of information on the likely outcome of a contest - further explain the variation in baseline and induced hormone levels.

Methods

Study species

We conducted our experiments on 15 male Irish stonechats, which were bred and hand-raised at the Max Planck Institute for Ornithology (MPIO), Andechs/Germany (47°58’N, 11°10’E) between 2002-2004. The experiments were carried out in March 2006, when gonads of this population were maximal and seasonal levels of testosterone reached their peak (Rödl et al., 2004; Helm et al., 2009; Helm, 2009). The birds were kept indoors at room temperature in individual 75cm x 40cm x 40cm cages, where they could hear but not see each other. Artificial lighting simulated the natural photoperiodic changes at 47.5°N (for more details see Helm et al., 2009). Light intensity was set to 300-400 lux during daytime and 0.01 lux during the night. Food and water was provided *ad libitum*. All birds were introduced into the experimental housing 2 weeks prior to the experiments to allow them to adjust to the new environment.

We collected data from further eight male Irish stonechats, which were raised and kept under the same conditions as described above in Andechs/Germany in 2000. These birds were not subjected to any experimental procedures but were bled in the beginning of April to obtain testosterone baseline levels serving as additional control data for our study.

Experimental procedures

We tested aggression in male stonechats by simulating an encounter with a rival male. Each focal male was tested under three different experimental conditions in randomized order with an interval of two to three days between tests. For the three experimental conditions we either placed a mirror (40 x 40cm), a stuffed decoy of a stonechat (which was the same for all birds), or a glass panel (40 x 40cm) as a control for the effect of a novel object into the left side of the cage. The behavior of the focal bird was recorded using a Sony DCR-TRV6E digital camera for 5 minutes before the experiment and during the 20 minutes while the glass panel, the mirror or the decoy were present in the cage. Immediately after the recording, we caught the bird to take a blood sample. All samples were taken within 3 minutes after approaching the cage. For some individuals after some experimental tests we did not get enough blood for hormone analyses (testosterone: N = 4 out of 45 tests, corticosterone N = 15 out of 45 tests). Thus, sample sizes for hormone data vary between tests.

Each bird was weighed to the nearest 0.01g one to two days prior to the experiments. Body mass of the birds was not correlated with corticosterone or testosterone concentrations (Spearman Rank Correlation, corticosterone, mirror (n = 12): r = 0.16, p = 0.62, decoy (n = `9), r = 0.52, p = 0.15, glass (n = 9), r = -0.35, p = 0.36; testosterone, mirror (n = 12): r = 0.21, p = 0.51, decoy (n = 15), r = 0.11, p = 0.70, glass (n = 14), r = 0.05, p =0.88). Furthermore, sequence of treatment (i.e. whether the glass, the mirror or the decoy was introduced into the cage at first) did not affect the hormone levels of the birds (oneway ANOVA, corticosterone, mirror: $F_{2,11}$ = 0.02, p = 0.99, decoy: $F_{2,8}$ = 0.36, p = 0.71, glass: $F_{2,8}$ = 2.60, p = 0.15; testosterone, mirror: $F_{2,11}$ = 0.24, p = 0.79, decoy: $F_{2,14}$ = 0.67, p = 0.53, glass: $F_{2,13}$ = 2.27, p = 0.15). Hence, we did not consider these variables in the further statistical analysis of the data.

Behavioral measures

Videos were analyzed using the program JWatcher^{TM} 0.9 (2000). Based on the method to quantify behavioral responses in stonechats described by Gwinner et al. (1994) and Schwabl et al. (2005), we recorded the frequency or duration of seven behaviors during each experimental test (Table 1). These behaviors comprised information about aggression (threat display and attacks), agitation (hopping, tails flicks, wing-tail flicks, approaching), and positioning in the cage. During the mirror experiment, we observed that five individuals frequently exhibited a very conspicuous threat posture towards their own mirror image, while the ten other individuals did not show this behavior. Four of the five birds which showed the threat posture also attacked the mirror physically, a behavior shown by only one of the remaining birds. We included this classification (mirror-fighter, mirror-non-fighters) into the analysis (see below).

Hormone analysis

Immediately after collection, blood samples were transferred to the laboratory to separate the plasma, which was stored at -40˚C until hormone analysis.

We analyzed the androgen testosterone and the glucocorticoid corticosterone using radioimmunoassays as described in Goymann et al. (2006). Plasma samples of the eight control birds were analyzed for testosterone in a separate assay. All other samples were analyzed in one assay for testosterone and one assay for corticosterone, respectively. For the control birds, we used 74.7 ± 17.9 µl (mean ± SD); extraction efficiencies were 78 ± 2 % (mean ± SD), intra-assay coefficient of variation (CV) was 7.8 %, and the detection limit was 0.64 pg/tube. For the experimental birds, we used 43 ± 9.4 µl plasma (mean ± SD) for the analysis of testosterone and 24 ± 11.6 µl plasma for the analysis of corticosterone. Mean ± SD extraction efficiencies were 94 ± 2 % for testosterone and 93 ± 3 % for corticosterone. The intra-assay CVs were 4.2 % for testosterone and 5.3 % for corticosterone. The detection limits were 1.02 pg/tube for testosterone and 6.46 pg/tube for corticosterone. Because the testosterone antibody used shows significant cross-reactions with 5a-dihydrotestosterone (44%) our testosterone measurement may include a fraction of 5a-DHT.

Statistical analysis

For all tests, we used the statistical software package SPSS 15.0 and Systat 13. Data are presented as mean ± SEM, and statistical significance was considered at $P \leq 0.05$.

We used a non-parametric Friedman test and post-hoc multiple comparisons implemented in Systat to assess whether individual behaviors of the birds varied between treatments. We also looked for within-individual correlations between the behavioral response during the mirror presentation and the behavioral response during the decoy presentations using a Spearman rank correlation.

Testosterone data were $1/\sqrt{}^2$-transformed to satisfy the assumption of a normal distribution (Lamprecht, 1992). To analyze the hormonal response of the birds we used a linear mixed model with post-hoc Bonferroni adjustments for multiple pairwise comparisons. The model included treatment (glass, mirror, decoy) and mirror-response (mirror-fighter, mirror-non-fighter) as fixed factors, and bird ID as a random factor, as well as the interaction between mirror-response and treatment. Testosterone data of the eight control birds were compared to testosterone data from the experimental birds using a t-test. Furthermore, we used

Spearman rank correlations to investigate the relationship between hormone concentrations and the behavioral reaction of the birds.

Results

1) Behavioral response

The Friedman test showed significant differences between behavioral responses towards the glass panel, the mirror and the decoy (Table 1). Agitation behaviors were similar during the mirror and decoy tests, and significantly higher compared to the control situation with a glass panel (Table 1). Aggression occurred significantly more often during the mirror presentation than during the presentation of a decoy and the control glass panel (Fig. 1A). Comparing the behavioral response between mirror and decoy experiments within individuals, we found significant correlations in the frequency of hopping (Spearman correlation coefficient $r_s = 0.63$, $p = 0.01$) and in the frequency of tail flicking (frequency: $r_s = 0.63$, $p = 0.01$, Fig. 2).

Table 1. *Behaviors recorded during simulated territorial intrusion experiments using a mirror, a decoy and a control glass panel. Agitation behavior (approach, hopping, tail flicking, wing tail flicking) mainly occurred during the experimental tests with a mirror or a decoy, while aggression mainly occurred during the mirror test, only. Significant differences are indicated by different letters and additionally highlighted in bold.*

Behaviour	**Description**	**Glass**	**Mirror**	**Decoy**	**Friedman's ANOVA**	**post-hoc test**
attacking (frequency per 20 min, mean ± SEM)	bird attacks the intruder with bodily contact	0.0 ± 0.0 ^{A}	0.9 ± 0.4 ^{B}	0.2 ± 0.2 ^{A}	$\chi^2 = 8.85$ $p = 0.012$	**glass - mirror: 3.20, p = 0.003** glass - decoy: 0.53, p = 0.60 **mirror - decoy: 2.66, p = 0.01**
threat display (duration per 20 min, mean ± SEM)	flattened back, neck extended with head and bill pointed at intruder, wings held at side and spread, open beak ,occasionally calling	0.01 ± 0.006 ^{A}	0.4 ± 0.2 ^{B}	0.001 ± 0.001 ^{A}	$\chi^2 = 6.10$ $p = 0.05$	**glass - mirror: 2.22, p = 0.03** glass - decoy: 0.17, p = 0.87 **mirror - decoy: 2.40, p = 0.02**
approach (frequency per 20 min, mean ± SEM)	bird directly approaches the stimulus	3.4 ± 1.5 ^{A}	1.1 ± 0.6 ^{AB}	0.4 ± 0.4 ^{B}	$\chi^2 = 5.88$ $p = 0.05$	glass - mirror: 1.31, p = 0.20 **glass - decoy: 2.61, p = 0.01** mirror - decoy: 1.31, p = 0.20
hopping (frequency per 20 min, mean ± SEM)	bird hops conspicuously upwards from its present position	5.13 ± 1.51 ^{A}	56.13 ± 20.67 ^{B}	84.53 ± 26.03 ^{B}	$\chi^2 = 9.59$ $p = 0.008$	**glass - mirror: 2.70, p = 0.01** **glass - decoy: 3.45, p = 0.002** mirror - decoy: 0.75, p = 0.46
tail flicking (frequency per 20 min, mean ± SEM)	conspicuous tail flicking while sitting	39.5 ± 12.6 ^{A}	106.0 ± 14.7 ^{B}	132.8 ± 32.9 ^{B}	$\chi^2 = 6.66$ $p = 0.04$	**glass - mirror: 2.34, p = 0.02** **glass - decoy: 2.54, p = 0.02** mirror - decoy: 0.20, p = 0.84
wing-tail flicking (frequency per 20 min, mean ± SEM)	conspicuous wing and tail flicking while sitting	11.5 ± 5.2 ^{A}	62.2 ± 15.5 ^{B}	61.7 ± 18.8 ^{B}	$\chi^2 = 9.19$ $p = 0.01$	**glass - mirror: 3.10, p = 0.004** **glass - decoy: 3.00, p = 0.006** mirror - decoy: 0.11, p = 0.92
being on left perch (duration, mean ± SEM)	bird sits on the left perch, right in front of the introduced object (6cm)	6.4 ± 1.3 ^{A}	2.1 ± 0.8 ^{B}	1.6 ± 0.8 ^{B}	$\chi^2 = 14.93$ $p < 0.001$	**glass - mirror: 3.98, p < 0.001** **glass - decoy: 4.98, p < 0.001** mirror - decoy: 1.00, p = 0.33

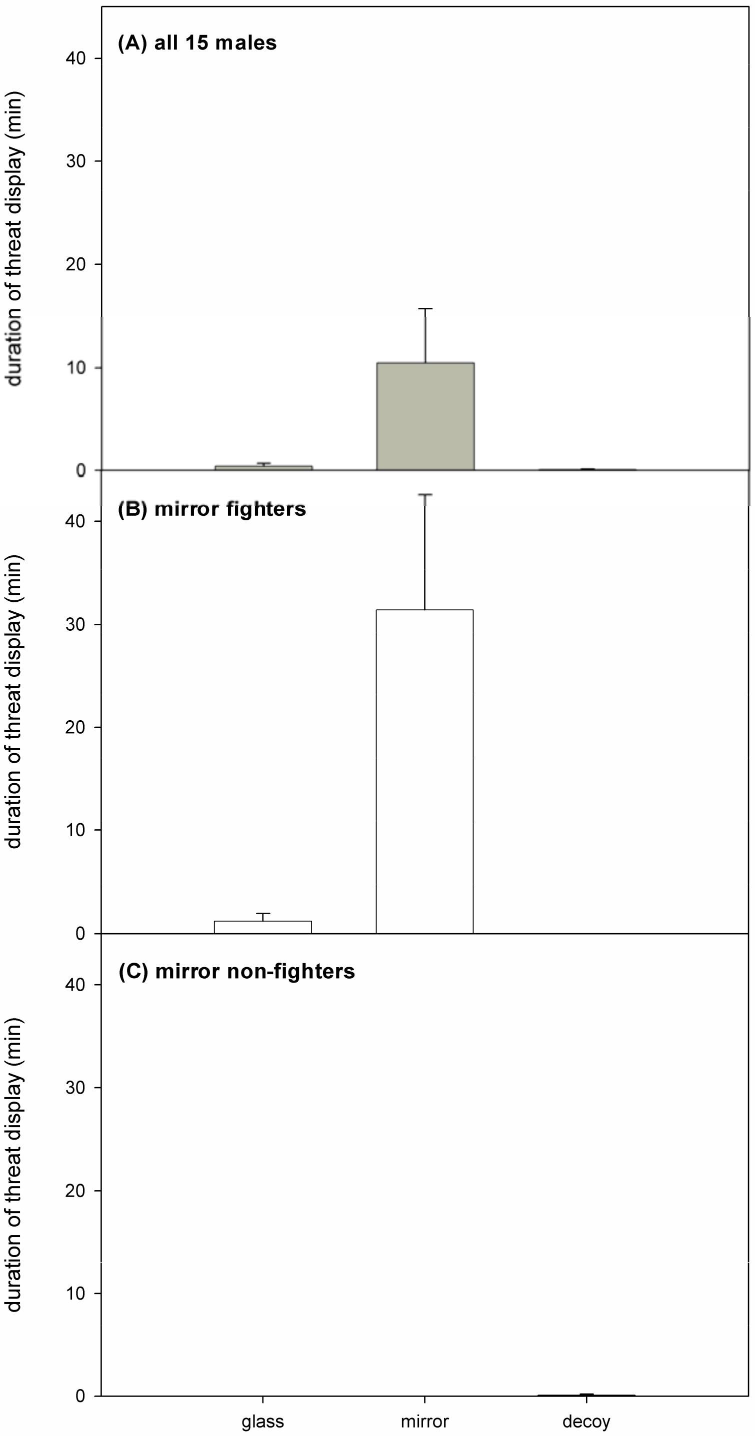

Figure 1

Duration (min) of threat posture displayed by male stonechats during a glass panel, a mirror and a decoy presentation (N = 15). (A) Males showed the threat posture significantly longer towards their mirror image than towards the glass panel or the decoy (for statistics see table 1). Five males threatened their own mirror image but refrained from attacking the decoy (B). The other ten males showed hardly any threat display towards glass panel, mirror or decoy (C).

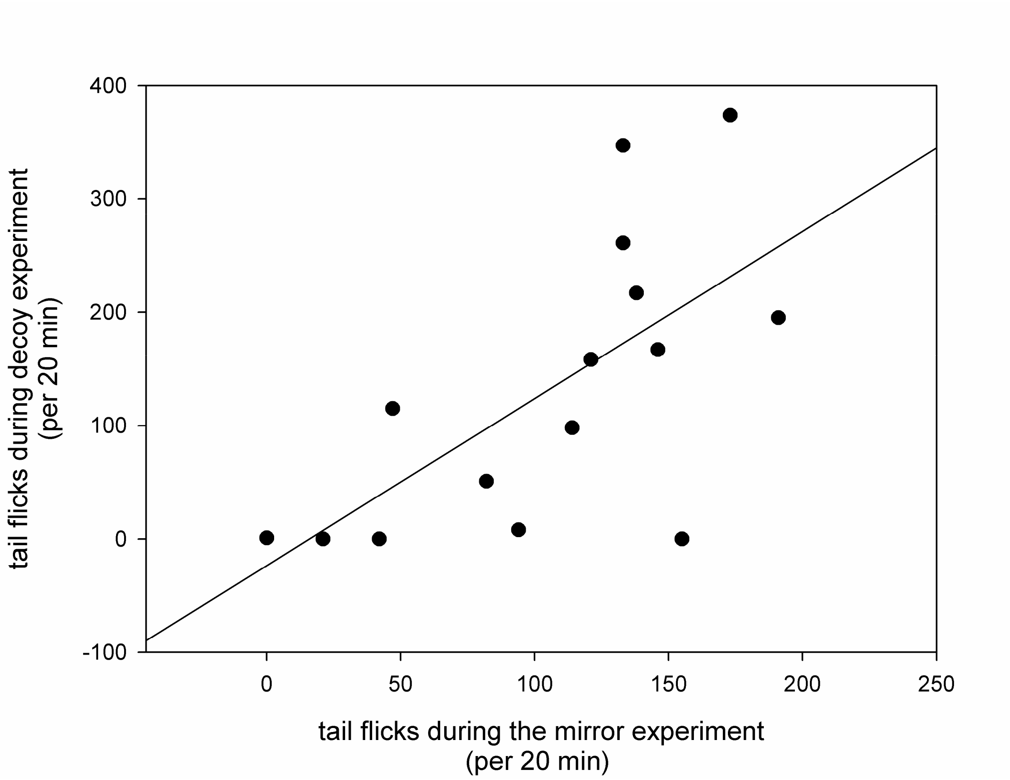

Figure 2

Individual frequency of tail flicking was positively correlated between the experiment with a decoy and the experiment with a mirror in male stonechats (r_s = 0.63, p = 0.01).

2) Hormonal response

A 20min presentation of a glass panel, a mirror or a stuffed decoy did not elicit different testosterone responses in male stonechats (general linear mixed model, table 2, $F_{2,15} = 0.37$, p = 0.70). However, mirror-fighting males expressed significantly higher levels of testosterone than males not responding aggressively towards the mirror ($F_{1,15} = 6.19$, p = 0.03, Fig. 3A). This effect was independent of the type of experiment, as there was no significant interaction between treatment and mirror-responsiveness ($F_{2,15} = 0.96$, p = 0.40; Fig. 3A). Testosterone concentrations of control birds not subjected to any experimental treatment were not significantly different from those of experimental birds measured after the glass panel test (df = 20, t = -1.73, p = 0.10).

Corticosterone levels of male stonechats differed after a 20min presentation of different stimuli ($F_{2,15}$ = 4.25, p = 0.03). The presentation of a decoy elicited a significantly higher corticosterone response than the presentation of a mirror (Fig. 3B, p = 0.04). Furthermore, mirror-fighters (N = 5) expressed significantly lower levels of corticosterone than mirror-non-fighters (N = 10; $F_{1,15}$ = 5.14, p = 0.04), independent of the trial (non-significant interaction between treatment and mirror response: $F_{2,15}$=0.49, p = 0.62; Fig. 3B). Within experimental tests, testosterone and corticosterone concentrations were not significantly correlated with any behavior, or with each other (all p > 0.10).

Table 2

Results of the linear mixed model for testosterone and corticosterone concentrations in male stonechats challenged by a glass panel, a decoy and a control glass panel.

independent variables	Testosterone		Corticosterone	
	test statistic	P	test statistic	P
treatment (glass, mirror, decoy)	$F_{2,15}$ = 0.37	0.70	**$F_{2,15}$ = 4.25**	**0.03**
mirror-response (fighter, non-fighter)	**$F_{1,15}$ = 6.19**	**0.03**	**$F_{1,15}$ = 5.14**	**0.04**
treatment x mirror-response	$F_{2,15}$ = 0.96	0.40	$F_{2,15}$ = 0.49	0.62

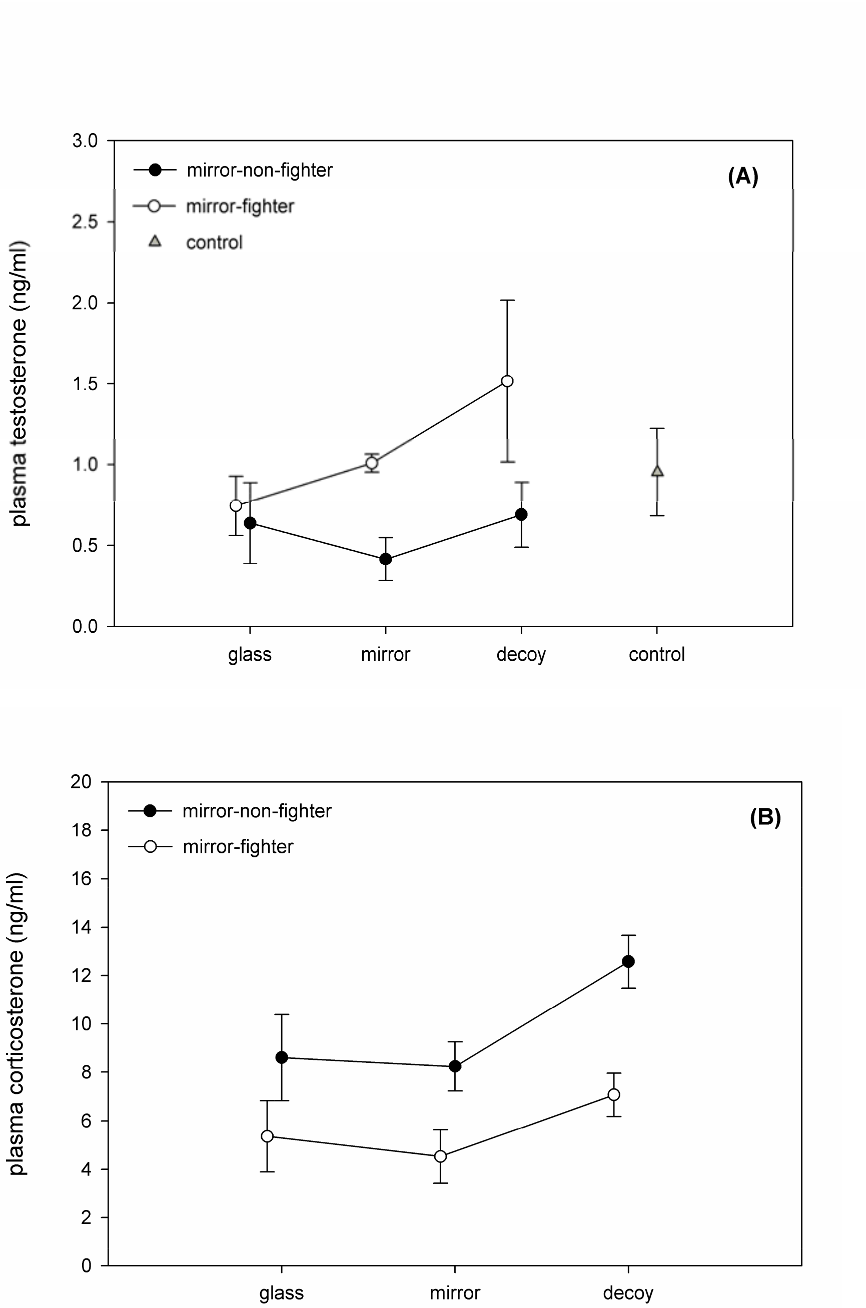

Figure 3

Hormone concentration (mean ± SE) compared between a control group (testosterone only), the glass panel test, and the mirror and decoy experiments. (A) Testosterone concentration did not differ significantly between the three experimental groups (for statistics see table 2), or between the glass panel group and the control group (df = 20, t = -1.73, p = 0.10). However, mirror-fighter had significantly higher testosterone levels than mirror-non-fighter. (B) Corticosterone concentrations were significantly higher after the decoy experiment than after the mirror experiment. Furthermore, mirror-non-fighter had higher corticosterone concentrations than mirror-fighter.

Discussion

This experimental study on European stonechats revealed that both a mirror and a decoy provoked an increase in agitation behavior in all males. In contrast to the behavioral response, none of the stimuli elicited a change in plasma testosterone concentrations. Instead, males responded with a rise in corticosterone levels when presented with a decoy, but not when confronted with their own mirror image. In addition to the increase in agitation behavior, the birds responded to their own reflection in the mirror in two distinct ways. Some birds showed strong and physical aggression towards the mirror. These birds were characterized by high testosterone and low corticosterone levels irrespective of the test situation. The remaining birds did not exhibit aggression towards the mirror and had relatively low testosterone and high corticosterone levels regardless of the situation. From these results we hypothesize that the behavioral response towards a mirror or a decoy was not provoked by the stimulus alone but in addition influenced by the birds' personality, which was reflected in the hormonal profile.

All male stonechats reacted to the presence of a mirror and a decoy with an increase in agitation behavior such as hopping and wing-tail flicking. However, aggression comprising physical attacks and threat displays was only observed in some individuals and mainly during the mirror experiment. Differences in the degree of aggression towards decoy and mirror have also been described in the cichlid *Aequidens pulcher* (Munro and Pitcher, 1985). These fish showed consistently more aggression towards mirrors than towards model fish. The authors suggested that the observed differences may be due to differential feedback of these two stimuli. The opponent in a mirror gives unusual immediate responses and approaches the focal fish to an atypically close distance (Pitcher, 1979). The unfamiliar reactions of the mirror opponent may induce fear in tested animals. Indeed, in cichlids of the species *Astatotilapia burtoni* presentation of a mirror led to immediate early gene expression in brain areas associated with fear and fear conditioning (Desjardins and Fernald, 2010). Oliveira et al. (2005) describe fights against mirror reflections in the cichlid fish *Oreochromis mossambicus* as escalating, indicating an excessively strong aggressive response towards the mirror image while the fish try to resolve the conflict. In our study, male stonechats were exposed to a similarly unnatural situation, in which the opponent reacts in an immediate and unfamiliar way, which may explain the stronger aggression towards a mirror than towards a decoy in some of the birds.

The behavioral response towards mirror and decoy - both agitation and aggression – did not lead to a testosterone response in male stonechats. Experimentally induced agitation and aggression was therefore not related to testosterone concentrations in general. These results confirm the findings on mirror image stimulation tests in cichlid fish (Oliveira et al., 2005; but see Desjardins and Fernald, 2010) and Japanese quails (Hirschenhauser et al., 2008). In both species aggression towards the own image in a mirror did not induce a testosterone response. This led to the assumption that the individual assessment of the antagonistic situation, such as strength of the opponent and possible outcome of the conflict, is important to induce a hormone response during aggression. Cichlids of the species *O. mossambicus* and Japanese quail both expressed a testosterone response when fighting a real opponent, i.e. when the correct assessment of the antagonistic situation was given (Hirschenhauser et al., 2004, 2008). Unfortunately, directly comparable data with a real opponent in European stonechats are not available. Simulated territorial intrusion experiments with a decoy and playback song

– an experimental setup comparable to ours – did not induce a testosterone response in captive European stonechats (Canoine, 2001). A stuffed decoy does not show a behavioral response towards the behavior of the focal male. Thus, similar to the mirror confrontation, this situation precludes the individual assessment of the contest. A decoy may even represent a more threatening opponent than a mirror since it may remain in a dominant position throughout the (escalated) conflict (Scriba and Goymann, 2008). Thus, our data are not directly comparable to those of Hirschenhauser et al. (2004, 2008) because a decoy and a live bird may not affect hormone concentrations in a similar way.

In contrast to the absence of a testosterone response, the presentation of a decoy led to an increase in corticosterone concentrations in focal males, whereas the presentation of a mirror did not. Because all challenged males responded with a rise in corticosterone and aggression was virtually absent during the decoy presentation, we suggest that the rise in corticosterone may indicate that the decoy was indeed perceived as a more stressful stimulus than a mirror image (Scriba and Goymann, 2008).

The behavioral and hormonal response during the presentation of a decoy was similar in all males. However, the behavioral response towards the mirror strikingly divided our focal birds into two groups. While all fifteen males reacted with increased agitation towards the stimulus, only five individuals actually attacked the mirror and displayed a conspicuous threat display towards their own reflection. In a similar test in Japanese quails, about half of the birds attacked a mirror, while the remaining birds responded rather submissively (Hirschenhauser et al., 2008). Mirror-image stimulation tests have previously been used for personality studies in mammals (yellow-bellied marmots *Marmota flaviventris*, Armitage, 1986; Armitage and Van Vuren, 2003). The authors determined behavioral phenotypes of the animals according to their reaction towards their own mirror-image. Comparable to our study, there were two groups of individuals. Marmots which approached the mirror and had physical contact with it were defined as *bold*, and marmots which had no or only little contact with the mirror, but withdrew from the simulated intruder were defined as *shy*. These experimentally defined phenotypes were consistent during other behavioral situations in the field such as play, dispersal, and territoriality (Armitage and Van Vuren, 2003). Consistency of behavioral traits across time and context are a precondition for the definition of animal personalities (e.g. Sih et al., 2004; Réale et al., 2007). In our study agitation behavior (hopping, tail flicking) displayed during the presentation of mirror and decoy was positively correlated between the experiments. Individuals that showed a high frequency of hopping and tail flicking towards their mirror image also responded with a high frequency of hops and tail flicks towards the presented decoy. The distinct difference in behavior between fighters and non-fighters during the mirror experiment was not eminent during the decoy experiment, though. However, mirror-fighters expressed higher testosterone and lower corticosterone concentrations than non-fighters during all three experimental tests. Personality studies have shown that generally passive individuals respond to stressors with a stronger increase in plasma glucocorticoid levels when compared to active individuals (Koolhaas et al., 1999; Wingfield, 2003). Both in fish (rainbow trout *Oncorhynchus mykiss*, Øverli et al., 2002; Schjolden et al. 2005) and in birds (zebra finch *Taeniopygia guttata*, Martins et al., 2007) artificially selected breeding lines for the adrenocortical response to mild stress differed in their exploratory and risk taking behavior. Individuals with a high corticosterone response reacted rather passively and behaved less risky in new environments. In contrast, individuals with low corticosterone response showed a proactive way in coping with the new environment. Similarly, in greylag geese levels of corticosterone (and partly of testosterone) and aggressiveness have been shown to be repeatable traits and may therefore be essential determinants of personality (Kralj-Fišer et al., 2007). A correlation between aggression and corticosterone concentrations (and explorative behavior) has also been found in great tits (Carere et al., 2003).

Simulated territorial intrusion experiments with a decoy or a caged bird placed in the centre of a territory and playing back conspecific song have been used as a standard method to examine the hormonal responsiveness to territorial aggression (Wingfield, 1985). The duration of the simulated challenge, and the time it takes to catch the focal bird after the experiment, are important factors to incorporate into the interpretation of the data, because hormone levels of challenged birds may change over time (e.g. Moore et al., 2004a; Peters et al., 2006; Landys et al., 2007, Apfelbeck and Goymann, submitted). However, hormone levels of individuals certainly differ before the onset of such an experiment. Our study suggests that such initial differences in testosterone and corticosterone levels may predict differences in the behavioral response towards an intruder, which has consequences for the interpretation of hormone data. For example, individuals with a bold personality and high levels of testosterone may approach the decoy faster than individuals with low levels of testosterone. As a consequence, birds with a bold personality (and high levels of testosterone) may be caught more easily and faster than shy birds (with low levels of testosterone). Consequently, early caught birds may express higher levels of testosterone than later caught birds. Thus, for the interpretation of data from male-male competition it would be helpful to know more about the personality of an individual bird before testing it for aggression and its hormonal response during simulated territorial intrusions (see also Kempenaers et al., 2007).

In male Irish stonechats the overall aggressive response towards a decoy and a mirror did not directly relate to a testosterone response. Individual differences in aggression, on the other hand, were related to individual hormone levels. From these results we hypothesize that in male stonechats the behavioral response during competitive fights is not provoked by the stimulus alone, but is in addition affected by the birds' personality. Furthermore, individual hormone profiles may be modulated not by induced behavior but by the bird's personality alone.

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Chapter Four

Throat patch size and darkness covaries with testosterone in females of a sex-role reversed species

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Abstract

In male birds, size and blackness of melanin-based ornaments may signal competitive ability and quality. Testosterone is an important physiological mediator of these signals, as these ornaments are testosterone-dependent. Experimental manipulation of circulating testosterone in females suggests that similar mechanisms are at work in plumage ornaments of females. However, hormone manipulations typically result in testosterone levels that are far higher than physiological concentrations experienced by females. Thus, it remains unclear whether natural variation of testosterone levels would be sufficient to generate variation in female plumage coloration. A signaling value of plumage ornamentation is particularly important in females of sex-role reversed species, in which females are the more competitive sex. We examined the relationship between plumage coloration, body condition and circulating testosterone in female barred buttonquails (*Turnix suscitator*), a classically polyandrous species. Even though female testosterone levels were low, they were strongly positively related with body condition and the size and blackness of the melanin throat patch. In males, no such relationships were apparent, suggesting that females may have an enhanced sensitivity for

testosterone or its metabolites. These unique results reveal that a hormonal mechanism may be involved in sex-role reversal in this species.

Introduction

In birds, plumage coloration and ornaments often function as signals for status and/or quality (Andersson 1994, Berglund et al. 1996, Hill 2006, Kraaijeveld et al. 2007). Individuals may be able to judge the strength and health of potential competitors and may use this information to avoid the costs of fighting (Maynard Smith and Harper 2003). In most species, the potential for variation in reproductive success is higher between males than between females, resulting in stronger competition over resources (i.e. territories and mates) among males. As a consequence of this stronger sexual selection in males, secondary sexual characteristics – such as plumage coloration and ornamentation in birds – are typically stronger expressed in males than in females (Andersson 1994, Kraaijeveld et al. 2007, Clutton-Brock 2009). Like many other secondary sexual traits, plumage coloration and ornaments may be under genetic control (Norris 1993, Roulin et al. 1998, Kimball and Ligon 1999, Roulin and Dijkstra 2003) and modulated by sex steroids such as estrogen (Kimball and Ligon 1999) and testosterone (Buchanan et al. 2001, Strasser and Schwabl 2004, Adkins-Regan 1998, reviewed in Candolin 2003, Blas et al. 2006). In males of some bird species, testosterone mediates sexual and aggressive behaviors (Wingfield et al. 2001) and controls the degree of plumage melanization (reviewed in Hill and McGraw 2003, Jawor and Breitwisch 2003, Kimball 2006, Bókony et al. 2008). Therefore, melanin ornaments may signal competitive ability related to testosterone (testosterone–regulation hypothesis, reviewed in Jawor and Breitwisch 2003). For example, the expression of the black throat patch of male house sparrows (*Passer domesticus*) is correlated and influenced by testosterone (Evans et al. 2000, Gonzalez et al. 2001, Buchanan et al. 2001, Laucht et al. 2011). Concurrently, patch size correlates with the position in the dominance hierarchy (e.g. Gonzalez et al. 2002, see also Rohwer and Rohwer 1978) and is an honest indicator of condition (Veiga and Puerta 1996, Gonzalez et al. 1999, Buchanan et al. 2001), which females may use for mate selection (Møller 1988, Riters et al. 2004, but see Griggio and Hoi 2010).

Furthermore, testosterone is involved in the trade-off between self-maintenance and reproduction (reviewed in Hau 2007). There are indications that elevated levels of testosterone may impair immune function (reviewed in Roberts et al. 2004) or resistance to oxidative stress (reviewed in Alonso-Alvarez et al. 2007). On the other hand, there is strong evidence that an impaired immune system lowers testosterone levels (Boonekamp et al. 2008). Several studies have found that the resistance to pathogens and parasites relates to sexual signaling (Potti and Merino 1996, Verhulst et al. 1999, Blas et al. 2006), including melanized plumage (Roulin et al. 2001, Fitze and Richner 2002, Garvin et al. 2008). These studies suggest that melanin ornaments may not only signal competitive abilities but also quality through condition-dependency, and therefore may represent an honest signal during mate choice (Bókony et al. 2008).

Secondary sexual traits have evolved in females as well as males, including brightly colored plumage and ornamentation in some bird species (Clutton-Brock 2009). However, proximate and ultimate factors underlying female plumage coloration and ornamentation are less well known than those of males. Female ornamentation has been suggested to be a mere by-product of sexual selection for ornaments in males (Lande 1980, Kraaijeveld et al. 2007) or could have evolved under sexual selection through direct male choice or competition between females (Kraaijeveld et al. 2007, Clutton-Brock 2009). Accordingly, plumage ornamentation in females may indicate competitive ability, status and/or parasite resistance, too (Potti and Merino 1996, Roulin et al. 2001, Morales et al. 2007, Murphy et al. 2009, Griggio et al. 2010).

In females, levels of testosterone are generally lower than in males (Wingfield and Farner 1993, Wingfield et al. 2001, Møller et al. 2005), but testosterone may still influence behavioral and morphological traits in this sex (Staub and De Beer 1997, Lank et al. 1999, Ketterson et al. 2005, Peters 2007, Voigt and Goymann 2007, Jawor and Winters 2010). For example, treatment with testosterone induces the growth of nuptial feathers in female Wilson's phalaropes (*Phalaropus tricolor*) and red-necked phalaropes (*Phalaropus lobatus*; Johns 1964), initiates the development and influences the brightness of the red frontal shield in female moorhens (*Gallinus chloropus;* Eens et al. 2000), or affects feather growth and bill coloration in female starlings (*Sturnus vulgaris*; De Ridder et al. 2002). Furthermore, female ruffs (*Philomachus pugnax*) show male mating behavior and develop a male-type breeding plumage after testosterone treatment (Van Oordt and Jung 1934, Lank et al. 1999). While these studies indicate the potential for testosterone to influence secondary sexual traits including plumage coloration in female birds, the evidence these studies generate come with a drawback: all these manipulations resulted in testosterone levels that these females would have never experienced under natural conditions. Thus, it remains completely unclear whether variation in physiological levels of testosterone could play a role in the expression and variation of secondary sexual characteristics in female birds. Only if such naturally occurring variation of testosterone has an effect on secondary sexual characteristics of females, can natural and sexual selection act on these traits (Adkins-Regan 2005). The manipulation of hormone levels within a physiological range is difficult even in males, because they typically induce a supraphysiological peak after implantation (e.g. Fusani 2008, Edler et al. 2011). Thus, especially for females, correlational studies relating circulating levels of testosterone with secondary sexual traits and body condition are required. As long as such correlational relationships are not established, it remains open whether and how testosterone could play a role in the expression of secondary sexual traits in females (Owens and Short 1995, Potti and Merino 1996). In an extensive literature search we could only find one study suggesting a correlation between circulating testosterone and plumage coloration in female northern cardinals (Jawor et al. 2010).

In so called sex-role reversed, classically polyandrous bird species, females compete for males or resources and are typically more aggressive than males (Andersson 2004). Some of these species such as the red-necked phalarope, Wilson’s phalarope, or Eurasian Dotterel (*Charadrius morinellus*), show sex-role reversed dichromatism with females expressing moderately or even highly brighter coloration of the plumage than males (Wingfield et al. 2000, Ketterson et al. 2005). Females of these species may therefore use the coloration of their plumage to signal competitive ability and condition during courtship or resource defense. However, females of all sex-role reversed species investigated so far have lower testosterone levels than males (e.g. Rissman and Wingfield 1984, Fivizzani and Oring 1986; Schlinger et al. 1989, Goymann and Wingfield 2004). If such low levels of testosterone should mediate secondary sexual traits such as plumage ornaments and coloration in these females, the hormone needs to act locally in target tissues that have a high sensitivity for low levels of the hormone or its metabolites (e.g.

Schlinger et al. 1989, Voigt and Goymann 2007, Ball and Balthazart 2008, Clutton-Brock 2009).

The barred buttonquail (*Turnix suscitator*) is a classically polyandrous species in which females aggressively defend territories and vocalize to deter rivals and attract males (Ridley 1978, Debus 1996). They have a marked reversed sexual dichromatism (Starck 1991, Debus 1996) with females expressing a black throat patch and a partly melanized breast patch throughout the year (Starck 1991). The plumage coloration of this species offers an ideal opportunity to examine the co-variation in melanin plumage areas, condition and testosterone in female birds. We compared circulating testosterone levels between female and male barred buttonquails throughout the course of one year to look for seasonal changes of hormone concentration and for potentially sex-role reversed hormone levels in this classically polyandrous species. Furthermore, we measured size and blackness of throat patches and breast patches of females to look for differences between individual birds and relate these differences with testosterone levels and condition. If circulating testosterone correlates with the expression of melanin-based plumage in females and with their condition, this would be a strong indicator that testosterone may mediate differences in the expression of secondary sexual traits in females of a sex-role reversed bird species.

Methods

Study species

We investigated size and blackness of breast and throat patches of 13 female and 14 male barred buttonquails, between 2007 and 2009. The birds were kept in pairs in cages of 1.0 x 1.0 x 0.7 m at a light-dark schedule of 13:11hrs, mimicking conditions close to their tropical origin. Grass tussocks, artificial plants and little haystacks in the cages offered hiding and nesting areas, and food and water were provided *ad libitum.*

Plumage measurements

We took three pictures of each bird’s throat and breast. The pictures were taken in succession on the same day in December 2007, except for one female which was included into the study and photographed in March 2009. To take the potential biases of month and year on hormone levels and plumage melanization into account we included the date into our analysis (see statistical analysis). In females, the throat patch was defined as the area covered by completely melanized feathers at the throat of the birds (Fig. 1A and B). Males grow white throat patches, whose feathers have melanized tips only in some individuals, and which are enclosed by an area with rufous- and black- melanized feathers (Fig. 1C). White plumage patches and ornaments may have a signaling function as well (Galvan and Sanz 2009, Griggio et al. 2011, Török et al. 2003). We therefore measured this throat patch of white feathers in males as an equivalent to the black throat patch in females for the comparison of throat patch patterns between the sexes. The breast patch area confined all feathers at the throat and the breast which were at least partly melanized, and which was clearly separated from the surrounding rufous plumage feathers without any black melanization (Fig. 1). Birds were held by one person fixing legs, wings and bill with the hands, and were presented ventrally with a stretched neck towards the camera at a standard distance of 37cm. Birds were re-arranged in the hand for each picture to ensure random positioning of the feathers. We used a flashlight to have equal light conditions for all birds. To measure throat patch and breast patch size we followed the procedure described in Laucht et al.

(2010). In brief, breast patch and throat patch area were encircled and the areas' pixel number was measured using Adobe Photoshop CS2. For standardization, we divided this area by a standard area present in each photograph and measured in the same way and then converted the result into square centimeters. Furthermore, the blackness of the plumage area was expressed through gray tone values (= luminance) computed from RGB using Adobe Photoshop CS2. The lower the gray tone values, the more intense the blackness. For the analyses, we used the average of all three pictures for each bird. The measurements were highly repeatable within individuals (females (N=13), patch size: $R = 0.98 \pm 0.01$, $F_{12,26} = 134.62$, $p < 0.001$; patch blackness: $R = 0.89 \pm 0.05$, $F_{12,26} = 26.04$, $p < 0.001$, breast size: $R = 0.89 \pm 0.05$, $F_{12,26} = 26.56$, $p < 0.001$; males (N=14), patch size: $R = 0.79 \pm 0.08$, $F_{13,28} = 12.45$, $p < 0.001$, patch blackness: $R = 0.87 \pm 0.06$, $F_{13,28} = 20.66$, $p < 0.001$, breast size: $R = 0.95 \pm 0.02$, $F_{13,28} = 60.27$, $p < 0.001$; estimated according to Becker 1984, Lessells and Boag 1987). We also measured the body mass to the nearest 1.0g and tarsus length to the nearest 0.1mm either on the day the pictures were taken or within a week afterwards. We conducted a regression analysis of body mass on tarsus length (for both sexes separately), and used the residuals of this analysis as a measure of the birds' condition.

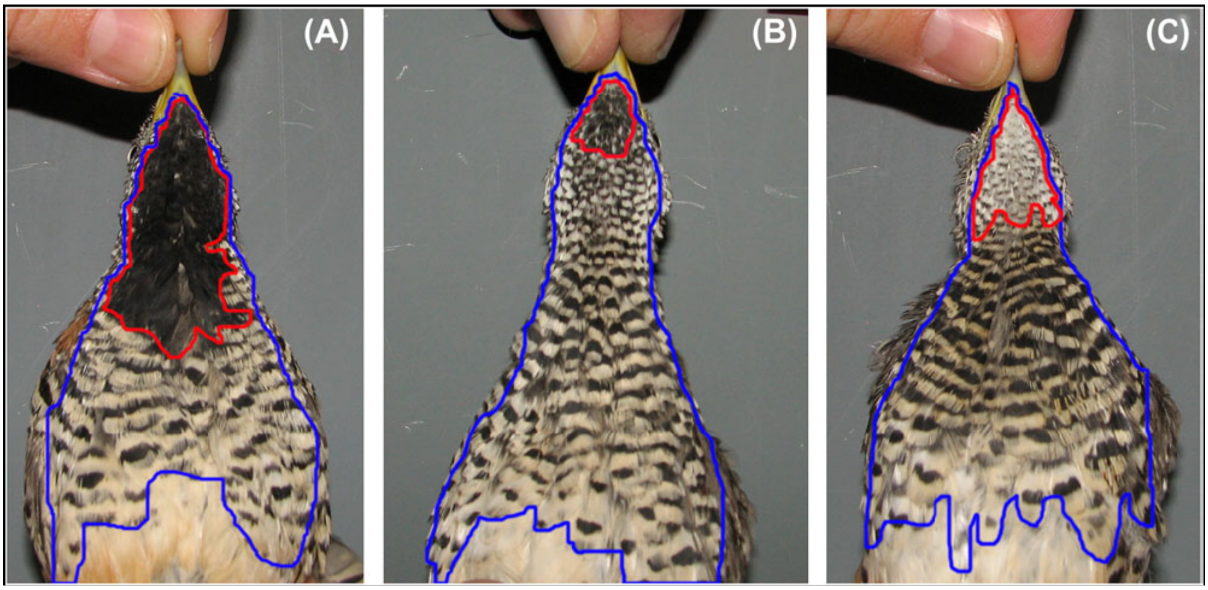

Figure 1

Breast patches (blue line) and throat patches (red line) of barred buttonquails. Throat patches of females are melanin-based and vary conspicuously between individuals (A and B). Throat patches of males are white and only some feathers may show melanized tips (C).

Blood samples

Throughout the course of 11 months (Jan. to Nov. 2008) we took monthly blood samples and measured the body mass of each bird between 9:00 and 12:00h in the morning. Blood samples (150-200 µl) were taken within 2.4 ± 0.06 minutes after opening the cage to capture the birds. The plasma was separated immediately (centrifuged at 10000 rpm for 10 min) and stored at -80°C until hormone analysis.

Ethical note

The measurements and sampling procedures were carried out according to the German laws for experimentation with animals. Breeding and housing of the buttonquails, as well as blood sampling, were conducted with permission of the Regierung von Oberbayern, Germany, and followed legal permits. After the study all birds remained in the laboratory stock at the Max Planck Institute for Ornithology, Germany.

Hormone analysis

Testosterone was analyzed using a radioimmunoassay as described in Goymann et al. (2006). We conducted 6 separate assays to analyse all samples. Mean ± SD extraction efficiency was 91 ± 5 %. Mean intra- and inter-assay coefficients of variation were 6.3% and 11.5%, respectively. The detection limit (mean ± SD) was 0.50 ± 0.17 pg/tube. Because the testosterone antibody used showed some cross-reactivity (44%) with DHT, our measurements may include a minor fraction of this other potent androgen.

Statistical analysis

Statistical analyses were conducted using SPSS 15.0. Throughout the paper, values are expressed as mean ± SEM and the statistical significance was set at $\alpha \leq 0.05$ (2-tailed).

Testosterone data were square-root transformed to get a normal distribution. We used a general linear mixed model to compare testosterone concentrations of each month between males and females. The model comprised month and sex as fixed factors, bird ID as a random factor, body mass as a covariate, and the interactions between month and sex, and between weight and sex. We further conducted multiple pairwise comparisons with post-hoc Bonferroni adjustments. We calculated the coefficient of variation (CV) for monthly testosterone levels for each bird, and compared the mean CV of males and females to test whether one sex has a greater variation in testosterone levels compared between months using a t-test.

We tested for correlations between testosterone concentration and condition of the birds, body mass, throat patch size, throat patch blackness, and breast patch size in females and males separately using a Pearson correlation. Because barred buttonquails express the sexually dimorphic plumage with a black throat patch in females throughout the year (Sutter 1955, Starck 1991), breed throughout the year (Ridley 1978, Starck 1991) and do not necessarily have a time-restricted molt pattern (Sutter 1955, Debus 1996, personal observations), we used the mean annual testosterone concentration for each bird. Sample sizes may differ between statistical tests due to missing data points for single individuals (natural death, small plasma volume).

Results

Testosterone concentration of males and females did not differ significantly between the sexes (Fig. 2, linear mixed model, $F_{1,64.87} = 0.95$, $p = 0.33$) or between months ($F_{8,19.39} = 1.94$, $p = 0.11$). However, there was a significant interaction between sex and body mass ($F_{2,58.12} = 7.22$, $p = 0.002$), because females with larger body mass had significantly higher levels of testosterone ($r_p = 0.60$, $p = 0.04$), while males showed no significant correlation between body mass and testosterone levels ($r_p = -0.36$, $p = 0.31$).

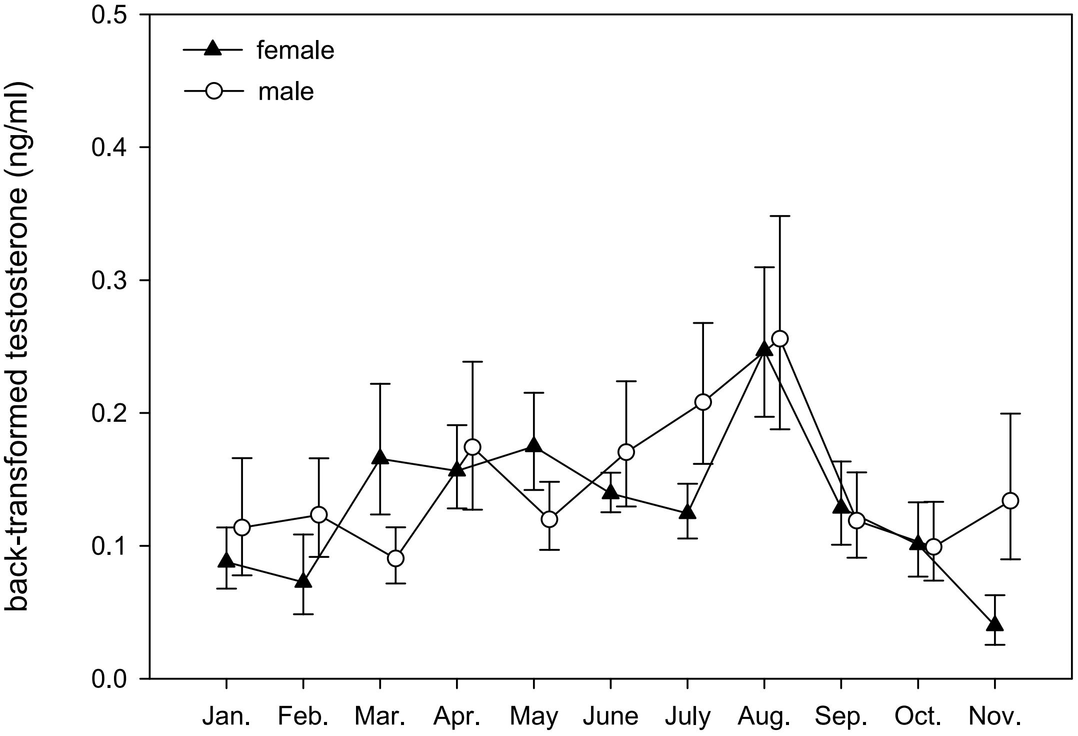

Figure 2

Annual pattern of plasma testosterone concentrations (back-transformed means ± SEM) of male and female barred buttonquails. Testosterone concentrations did not differ significantly between the sexes (linear mixed model, $F_{1,64.87} = 0.95$, $p = 0.33$) or between months ($F_{8,19.39} = 1.94$, $p = 0.11$) throughout the course of eleven months. However, there was a significant interaction between month and sex ($F_{8,19.39} = 2.94$, $p = 0.03$).

Furthermore, there was a significant interaction between month and sex because in some months females had higher levels than males and in other months males had higher levels than females (Fig. 2, $F_{8,19.39} = 2.94$, $p = 0.03$). There was also a significant difference between subjects (=ID) in testosterone levels over the course of the study period (Wald Z = 1.93, p = 0.05), because some individuals showed a greater variance between months than others (Fig. 3). To further explore the similarity of testosterone levels in males and females we calculated the coefficient of variation (CV) for each individual, which was similar in females and males over the period of 11 months (females: 34.14 ± 4.72, males: 32.71 ± 5.84, t-test, df = 25, t = 0.03, p = 0.98). This suggests that not only were the mean levels of testosterone similar between the sexes, but also the magnitude in change of testosterone over the year.

In females, testosterone significantly correlated with condition (Pearson correlation $r_p = 0.67$, $p = 0.03$), throat patch size ($r_p = 0.60$, $p = 0.03$) and throat patch darkness ($r_p = -0.69$, $p = 0.009$; Figs. 4). Breast patch size did not correlate with testosterone concentration ($r_p = 0.05$, $p = 0.86$). Furthermore, in females condition significantly correlated with the size ($r_p = 0.68$, $p = 0.02$) and darkness of the throat patch (Fig 5; $r_p = -0.78$, $p = 0.005$), and the size of the melanized breast patch area ($r_p = -0.80$, $p = 0.003$). In males, condition or testosterone did not correlate with any plumage measurements (condition-testosterone: $r_p = -0.35$, $p = 0.32$, throat patch size – testosterone: $r_p = -0.11$, $p = 0.71$, breast size – testosterone, $r_p = 0.56$, $p = 0.07$, condition – throat patch size: $r_p = 0.02$, $p = 0.96$, condition – breast size: $r_p = -0.38$, $p = 0.29$).

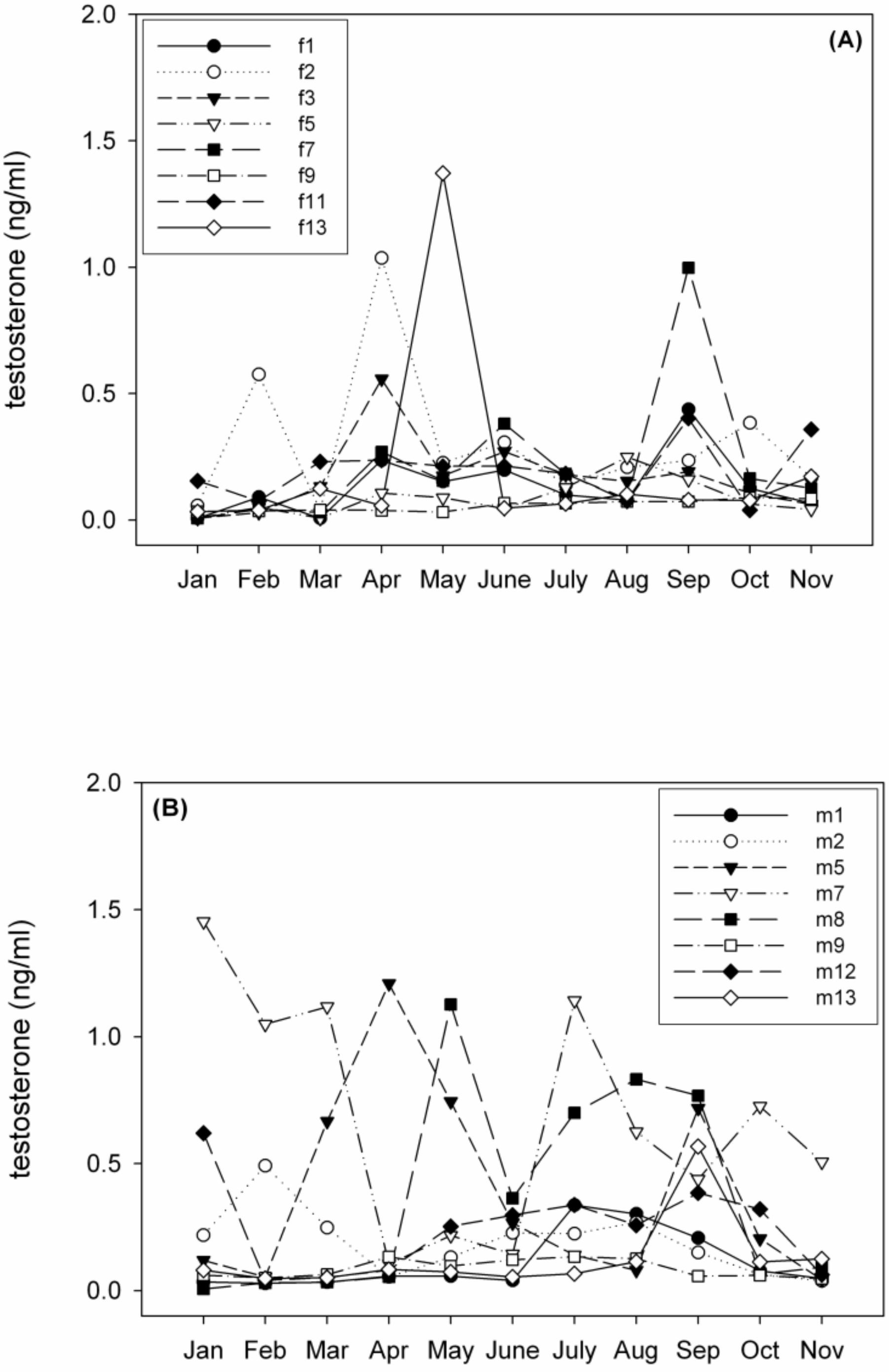

Figure 3

Testosterone levels of individual female (A) and male (B) barred buttonquails over the course of 11 months.

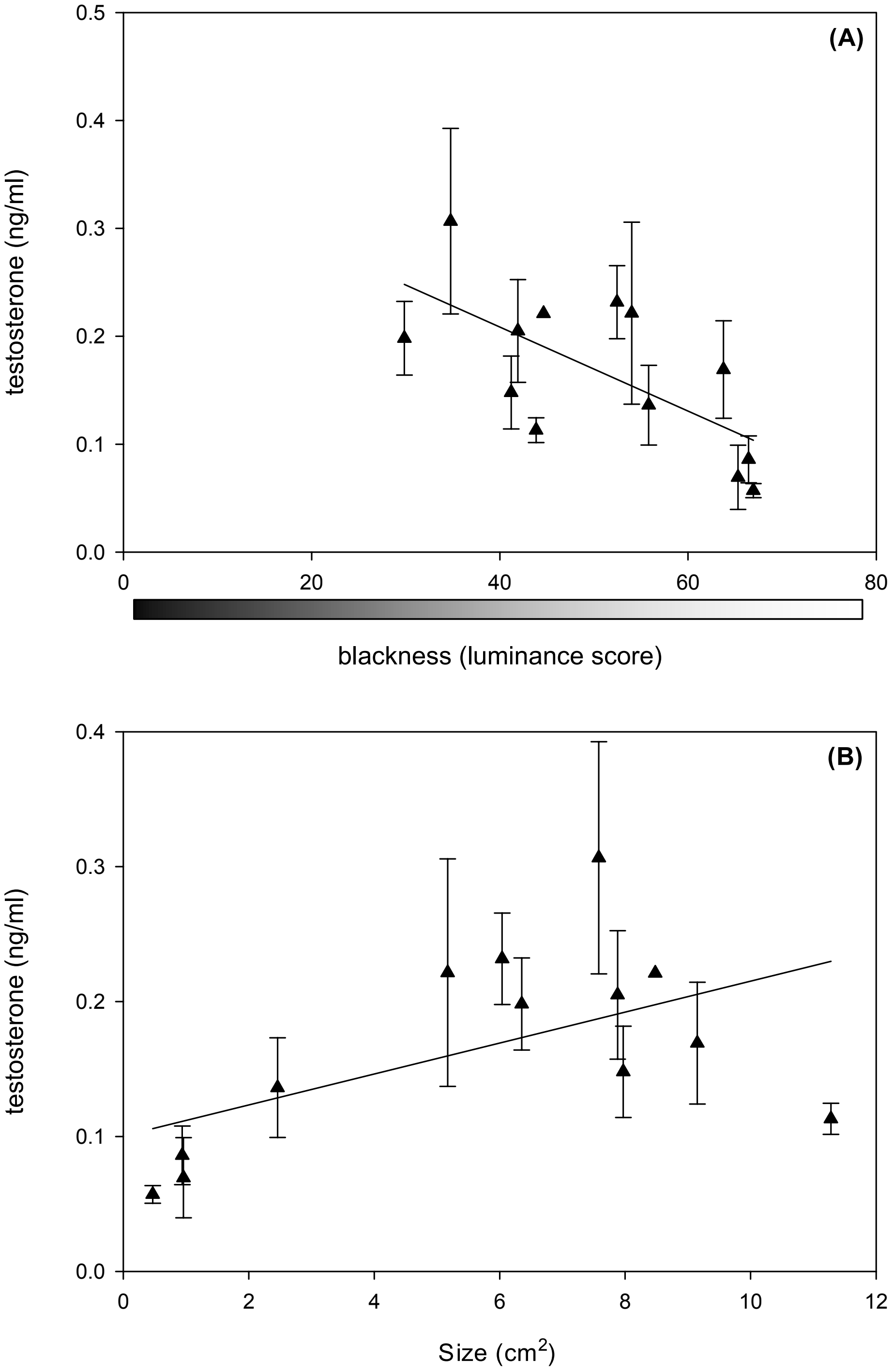

Figure 4

Annual mean testosterone concentration (mean ± SE) significantly correlated with (A) blackness (r_p = -0.69, p = 0.009) and (B) size of the throat patch (r_p = 0.60, p = 0.03) in female barred buttonquails.

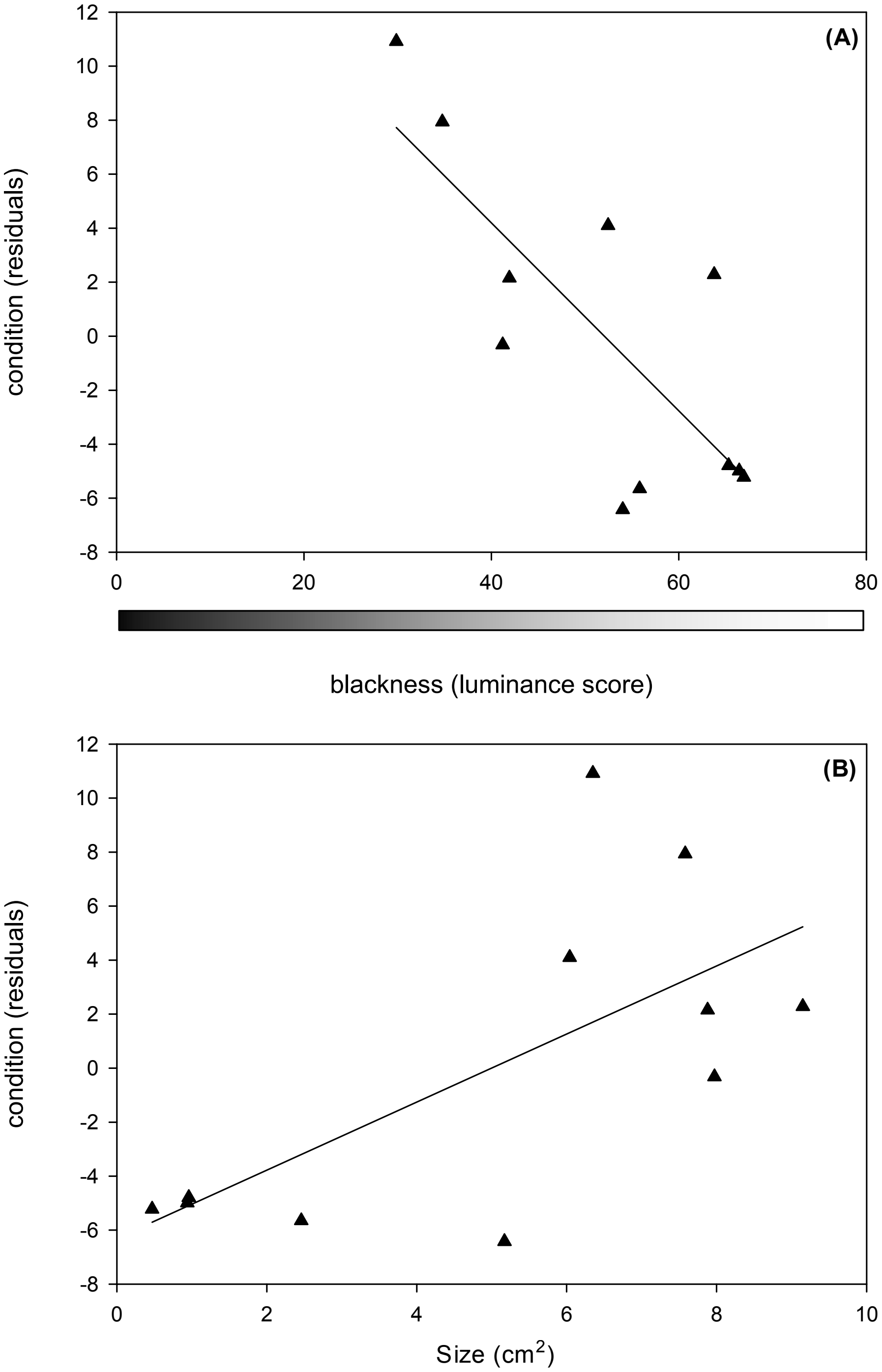

Figure 5

The condition of female buttonquails significantly correlated (A) with blackness of the throat patch (r_p = -0.78, p = 0.005), and (B) with size of the throat patch (r_p = 0.68, p = 0.02).

Discussion

Female barred buttonquails with larger and darker throat patches expressed higher circulating levels of testosterone and were in better condition than females with smaller and paler throat patches. These results suggest that low circulating levels of testosterone could mediate variation in plumage coloration in female birds. A comparable relationship did not exist in male buttonquails, even though overall levels of testosterone were similar between the sexes. Therefore, in barred buttonquails the sex-role reversed mating system may be accompanied by a reversal of testosterone-dependency in the expression of a secondary sexual trait.

Circulating testosterone levels of male and female barred buttonquails remained very low throughout the whole study, with only a minor peak during August in both sexes. Low levels of circulating testosterone are common in tropical birds with a low degree of seasonality (Goymann et al. 2004). Nevertheless, testosterone may still mediate behavioral and physiological traits - such as aggression and competition over resources - in these species (Wikelski et al. 1999, Goymann et al. 2006).

Interestingly, female barred buttonquails had similar mean levels of testosterone than males and also the variation in testosterone was similar in both sexes. This is in contrast to all other sex-role reversed bird species studied up to date, in which males expressed significantly higher levels of testosterone than females (reviewed in Eens et al. 2000, Goymann and Wingfield 2004). Indeed, in some months testosterone levels of female barred buttonquails were higher than those of males, while in other in months it was the other way around. We could not identify an environmental or physiological reason for the changing differences between females and males. Furthermore, the variation in testosterone levels between the months did not differ between the sexes. Hence, the significant interaction of month and sex in testosterone levels may be merely due to individual differences in testosterone levels and the low sample size. The overall similarity of testosterone levels in female and male barred buttonquail is exceptional among sex-role reversed birds.

The plumage of female barred buttonquails is more brightly colored than that of males, and includes the year-round expression of a melanin-based black throat patch (Starck 1991). There are a few indications that melanin-based ornaments may serve as signals for quality in female birds similar to those that have been described for males (Amundsen 2000). For example, in barn owls (*Tyto alba*) the amount and size of melanized spots on breast, belly, flanks and underside of females reflect parasite resistance (Roulin et al. 2001) and offspring immunocompetence (Roulin et al. 2000). Similarly, in female great tits (*Parus major*) the size of a melanin-based breast-stripe may indicate resistance against ectoparasites (Fitze and Richner 2002) and social dominance (Wilson 1992). However, there is no evidence that these melanin-based signals in females are mediated by testosterone.

In female barred buttonquails, the variation in size and blackness of the throat patch and the breast patch co-varied with mean annual circulating testosterone concentrations of individual birds. Such a correlation has not been described for females of any classical polyandrous species, and also for other species there is little evidence. Jawor et al. (2010) describe the relationship between testosterone levels during molt and darkness of a melanin-based face mask in female northern cardinals (*Cardinalis cardinalis*). In this species, testosterone concentrations increased during aggressive female-female interactions. Hence, the face mask of female cardinals may be a signal of aggressiveness or status just as in male northern cardinals (Jawor et al. 2004).

In many bird species, circulating levels of testosterone show a strong seasonal variation (e.g. Wingfield et al. 1990). The relationship between testosterone and plumage melanization should be most eminent when testosterone is measured during feather formation, that is during molt (e.g. Evans et al. 2000, Buchanan et al. 2001, Gonzalez et al. 2001, Bókony et al. 2008). However, correlations between plumage melanization and testosterone are found also during other life-cycle stages (e.g. Laucht et al. 2011), suggesting that testosterone concentrations during molt may be indicative of testosterone levels during other times of the year. Similar to many other tropical birds with a low seasonality (Tallman and Tallman 1997) barred buttonquails may not have a regular or time-restricted molt pattern (Sutter 1955, personal observations), or molt may be interrupted due to seasonal conditions (Starck 1991) and hence may occur over a longer period of time. Furthermore, compared to other species investigated so far, levels of testosterone showed little variation throughout the year. As a consequence, we are confident that the mean annual level of testosterone per individual represents a good and conservative estimate of testosterone levels during the extended period of molt in this species.

In male birds, melanin based ornaments may signal condition, quality, status or parental ability to females and competing males (Jawor and Breitwisch 2003, Clutton-Brock 2009). If the expression of melanin based ornaments depends on testosterone and only healthy individuals in good condition can maintain high levels of testosterone (or melanin production), the honesty of this signal can be ensured (Jawor and Breitwisch 2003). In sex-role reversed barred buttonquails, females compete more strongly for resources and mates than males. Thus, the evolution of secondary sexual characters that signal competitive ability should come with no surprise. Furthermore, in species with substantial paternal investment female plumage may provide males with information about condition, fecundity and parental qualities (Møller 1993, Blount et al. 2002, Jawor et al. 2004). There is evidence that males commonly prefer brighter and more ornamented females (reviewed in Clutton-Brock 2009). Because male barred buttonquails substantially invest in parental care, they may use plumage cues from females when selecting a mate.

Nutritional condition and parasite infection, especially during molt, both may affect the expression of melanin ornaments (Fitze and Richner 2002, Jawor and Breitwisch 2003, Roulin et al. 2008). In our study all birds were kept under 'benign laboratory conditions', i.e. they received food *ad libitum* and were not exposed to the large assortment of parasites or predators they may experience in their natural environment. Thus, the standardized laboratory environment and the optimal feeding conditions may have limited the range of phenotypic variability in throat patch morphology that is associated with condition compared to a natural situation in a more variable and challenging environment. Given our preliminary evidence in the laboratory, studies in free-living populations of barred buttonquails would be highly desirable to further explore the relationship between condition, testosterone, and plumage melanization in this species.

In contrast to non-sex-role reversed species with sexually dimorphic traits expressed only by the male (the sex which shows generally higher testosterone concentrations), a graded dose-response relationship between circulating hormone levels and the morphological trait (Ball and Balthazart 2008) is unlikely in barred buttonquail. Because female and male barred buttonquails express similar levels of testosterone, one expects other endocrine mechanisms that restrict the expression of a black throat patch to females, if indeed testosterone should play a modulating role in plumage melanization. For example, a sex-specific and locally enhanced sensitivity for low levels of the hormone or its metabolites may be at work (e.g. Schlinger et al. 1989, Voigt and Goymann 2007, Ball and Balthazart 2008, Clutton-Brock 2009). Follow-up studies on the sensitivity of the skin and feather follicles to testosterone and its metabolites in female barred buttonquails could provide an answer to this possibility.

Our study on melanin based plumage coloration in barred buttonquails demonstrate for the first time that variation in natural levels of testosterone could mediate variation in plumage coloration in females of a sex-role reversed bird species, and that this variation may be condition-dependent. Hence, plumage melanization in barred buttonquail may function as a quality signal during inter- and intra-sexual competition. The lack of any correlations with testosterone levels in males despite similar levels of plasma testosterone in both sexes, suggests that females may have an enhanced sensitivity for low levels of the hormone or its metabolites. Therefore, in barred buttonquails the reversed sex roles may go along with a reversal of testosterone-dependency in sexual signaling. These results hence reveal that a hormonal mechanism may be involved in sex-role reversal in this species.

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General Discussion

Evolution of paternal care and extra-pair paternities

Females of sex-role reversed species have developed the capacity to lay more eggs than one male can handle (Andersson 2005). They have therefore the option to lay multiple clutches for multiple males, either sequentially or simultaneously (Andersson 2005). This mating system seems advantageous for females only, as their potential reproductive rate exceeds by far the one of males (Clutton-Brock and Vincent 1991, Goymann et al. 2004). However, considering the hypotheses of the evolutionary pathway of sex-role reversal and polyandry (see Introduction), this mating system may actually have evolved to increase male reproductive success, as well. One proposed pre-condition for the evolution of paternal care is a situation which favours bi-parental care as to secure the breeding success of both the male and the female. Such a situation may be, for example, scarce or fluctuating food resources, high predation risk, bad weather conditions, or a generally low parental capacity of the female (Figure 1, Ligon 1999, Andersson 2005). An alternative situation may be limited re-mating opportunities for males (e.g. low availability of breeding territories or sexually active females), thus they do better if they stay with the female and share parental duties, which may be beneficial for the offspring and therefore increases the breeding success (Owens 2002, Andersson 2005). With males participating in offspring care, females can save energy for future egg production, which may exceed the number of eggs the pair could raise together, thereby giving the female the chance to lay an additional clutch, i.e. increasing the breeding success for both herself and her male (Ligon 1999, Andersson 2005). However, females may exploit this situation to engage in multiple matings. In this case, the potential reproductive rate of males, of course, decreases compared to females.

Furthermore, polyandry bears a high potential for extra-pair fertilisations, either through sperm competition as a consequence of sequentially multiple matings or through indeed actively induced extra-pair copulations (Birkhead 1998). Males of sex-role reversed polyandrous species therefore do face a potentially high risk of a further reduction of their breeding success through paternity loss. Evolutionary theoretical models therefore predict that a high level of paternal care could only evolve, and be an evolutionary stable strategy, if extra-pair paternity is prevented or at least kept at very low levels (e.g. reviewed by Schwagmeyer et al. 1999, Mauck et al. 1999, Møller and Cuervo 2000, Griffith et al. 2002, Sheldon 2002). Extra-pair paternity may reach up to 65% of offspring in monogamous species (Brooker et al. 1990). Similar rates of extra-pair offspring in sex-role reversed species would confront males with an extreme reduction in breeding success considering their intense parental investment. And indeed, field data of extra-pair paternity rates in species with high levels of male parental care, including polyandrous species, are relatively low (0% – 8.6% in polyandrous species, Oring et al. 1992, Dale et al. 1999). Female Wilson’s phalaropes even actively prevent extra-pair paternity by rejecting copulation attempts from extra-pair males (Delehanty et al. 1998).

Paternal care – a sexually selected trait?

Regarding extra-pair paternity rates, the African black coucal therefore seems to be an exception within the classically polyandrous species, as we discovered 14% of the offspring were fathered by a male different from the one raising the young, and extra-pair paternity was found in 37% of all broods (Chapter 1). Among the classically polyandrous species investigated so far, black coucals thus have the highest rate of extra-pair paternity.

Overall, a minimum of 33% of black coucal males lost paternity. This is astonishing given that black coucals are the only polyandrous species with altricial young, hence males invest more into parental care than males of other polyandrous species (Goymann et al. 2004, 2005). In the course of our paternity study we suggested that the main reason why black coucals have the highest rate of extra-pair paternity compared to other species with a polyandrous mating system, is the incapability of males to effectively guard their mates (see discussion Chapter One). Mate-guarding is supposedly the most effective strategy to prevent extra-pair paternities, as females do not engage in extra-pair copulations while their partner is present (Birkhead 1998). The low ability of male black coucals to perform mate guarding successfully may be partly due to the dense vegetation in their habitat which limits both visibility and mobility and might therefore aid females in seeking additional males and extra-pair copulations (Mee et al. 2004). Other classically polyandrous species whose paternity has been studied are shorebirds (Charadriiformes), which breed on predominantly flat, open habitats with relatively short vegetation where it might be reasonably easy for males to monitor the behaviour of their mate and to notice the presence of extra-pair males. This may explain the low levels of extra-pair paternity in these species, even in the absence of close mate guarding (see also Pierce & Lifjeld 1998).

A further disadvantage that may prevent males from effectively guarding their mates is the fact that black coucals start with incubation once the first egg has been laid (Vernon 1971; C.M. and W.G., personal observations). As the loss of eggs and chicks through predation is relatively high compared to other coucal species

(Goymann et al. 2005), males obviously decide to stay with their prodigy from the first moment onwards in the trade-off between extensive mate-guarding and incubation during the fertile period of the female.

When females benefit greatly from male incubation and offspring feeding, female mate choice should be based on cues that predict paternal behaviour (Ketterson & Nolan 1994). Courtship feeding in African black coucals may act as a signal for the male’s parental qualities, which then may be the target of female choice (Mills 1994, Pandolfi and Dastore 1994, Freeman-Gallant 1996). If female black coucals choose mates for the direct benefit of paternal care, it may not be adaptive for males to reduce paternal investment even when they face extreme uncertainty of paternity. Thus, paternal care as a sexually selected trait may prevent the occurrence of the conventional sex-roles and may support the evolution of sex-role reversal (Kokko and Jennions 2008). In that case, classically polyandrous males may be in an “evolutionary trap” and may have to accept solo incubation and offspring care without paternity assurance, because reducing care without maternal compensation would negatively affect the entire brood.

Competitive females and choosy males in sex-role reversed species: melanin-based plumage colouration as a sexually selected trait in barred buttonquails

Sex-role reversed polyandrous females may select males for their paternal abilities as to ensure their reproductive success without participating in offspring care (Ketterson and Nolan 1994). Males in sex-role reversed species, however, have theoretically the more critical choice of mates than females (Eens and Pinxton 2000, Shuster and Wade 2003). As females compete highly over monopolization of mates or other resources, they have a potentially higher variance in mating success which leads to the development of more pronounced secondary sex traits and mate-attracting displays (Eens and Pinxton 2000, Shuster and Wade 2003). Such sexually selected traits and displays may be important cues for males during mate selection, as well as for females during intra-sexual competition as indicators of condition or status (Kraaijeveld et al. 2007, Clutton-Brock 2009).

In the barred buttonquail, we discovered a positive correlation between female body condition and the size and blackness of their melanin-based throat patch. The black plumage colouration at throat and breast may therefore be a sexually selected trait which informs both males and females about the condition of individual females (Potti and Merino 1996, Roulin et al. 2001, Morales et al. 2007, Murphy et al. 2009, Griggio et al. 2010).

A very good example for the signalling value of a melanin throat patch comes from the male house sparrow (*Passer domesticus*). In this species patch size correlates with the position in the dominance hierarchy (e.g. Gonzalez et al. 2002, see also Rohwer and Rohwer 1978) and is an honest indicator of condition (Veiga and Puerta 1996, Gonzalez et al. 1999, Buchanan et al. 2001), which females may use for mate selection (Møller 1988, Riters et al. 2004, but see Griggio and Hoi 2010). Moreover, the expression of the black throat patch of male house sparrows is correlated and influenced by testosterone (Evans et al. 2000, Gonzalez et al. 2001, Buchanan et al. 2001, Laucht et al. 2011). Testosterone-dependency of the sexually selected trait forms a potential link between plumage colouration and other testosterone-dependent characters, for example aggression, and may additionally support the honesty of the sexually selected plumage trait through impaired immune functions, which only individuals of high quality/good condition can withstand (testosterone–regulation hypothesis, reviewed in Jawor and Breitwisch 2003).

Similar to house sparrows, throat patch size and blackness in female barred buttonquails of our study were correlated to circulating testosterone levels. There has been experimental evidence for a potential influence of testosterone on plumage characteristics in female birds (Van Oordt and Jung 1934, Johns 1964, Lank et al. 1999, Eens et al. 2000, De Ridder et al. 2002). However, we showed for the first time that naturally occurring testosterone levels of females could have a regulatory effect on the expression of a secondary sexual trait. Moreover, the fact that only female barred buttonquails develop a black throat patch despite similar levels of circulating testosterone levels of males and females suggests that females may have an enhanced sensitivity for low levels of the hormone or its metabolites. Therefore, in barred buttonquails the reversed sex roles may go along with a reversal of testosterone-dependency in sexual signalling. These results hence reveal that the same hormonal mechanism that operates in males may be involved in sex-role reversal in this species.

Hormonal mechanisms behind sex-role reversal

The significant correlation of throat patch size and blackness with testosterone levels in female barred buttonquails is one of only a few studies that suggest a hormonal mechanism behind sex-role reversed traits and behaviour (Schlinger et al. 1989, Voigt and Goymann 2007, Goymann et al. 2008). In species with conventional sex roles, behaviours and traits involved in mating and reproduction are often initiated and/or modulated by sex steroids (Wingfield et al. 1990, Andersson 1994, Gonzalez et al. 2002, Adkins-Regan 2005, Bókony et al. 2008). Hence, a simple reversal of sex-hormone concentrations in males and females had been one of the first and obvious implications to explain the reversal of such behaviours and traits in sex-role reversed species (Ligon 1999). This hypothesis did not find much support as sex-role reversed species show comparable circulating hormone levels as non-sex-role reversed species (e.g. Rissman and Wingfield 1984, Fivizzani and Oring 1986; Schlinger et al. 1989, Goymann and Wingfield 2004). Relatively recent evidence from studies on the African black coucal offers two alternative hormonal mechanisms (Voigt and Goymann 2007, Goymann et al. 2008). Sex-role reversed females may have an increased androgen receptor activity in the brain, whereby sex-role reversed behaviour may be regulated through androgens such as testosterone without a necessarily high level of the hormone (Voigt and Goymann 2007). Such a mechanism has been described for males of non-sex-role reversed species, as well (e.g. Canoine et al. 2006, Sperry et al. 2010), and seems a promising mechanism for the reversal of sex-role characteristics, as reversal of androgen receptor sensitivity rather than of circulating hormone levels would avoid detrimental effects of high testosterone levels. Our present study further supported this potential mechanism in barred buttonquails (see also Schlinger et al. 1989). Another alternative to a simple reversal of circulating sex-hormones would be the involvement of different hormones in the behavioural feedback mechanisms for male and female birds (Wingfield et al. 1990). Accordingly, Goymann et al. (2008) showed that aggressive behaviour in female black coucals was modulated by progesterone, potentially in interaction with testosterone, while in male birds aggression is mainly modulated by testosterone or its metabolites (Soma 2006, Hau 2007).

We did not find a direct interaction between aggressive behaviour and testosterone in experimentally challenged female barred buttonquails (Chapter 2). While the sex-role reversed dichromatism in this species may indeed be modulated by circulating testosterone, sex-role reversed territorial aggression seems to be subject of a different (hormonal) mechanism or is not appropriately expressed in captivity. In our study we found a significant effect of exogenous testosterone on mate-guarding behaviour during a territorial challenge, though. While these results should be regarded with caution, as manipulated (i.e. unnaturally high) hormone levels may trigger behavioural artefacts (Leonard 1939, Shoemaker 1939, Nottebohm 1980), our results nevertheless demonstrated that the hormone-behaviour mechanism in mate-guarding behaviour described for male birds (Moore 1984, Saino and Møller 1995, Davis 2002) could potentially work in females, too.

Mate-guarding as a response to territorial intrusion

Mate guarding behaviour in male birds mainly reduces the risk of extra-pair copulations (e.g. Sorenson 1994, Komdeur et al. 1999, Blomqvist et al. 2002, Davis 2002). It has further been suggested as a means to protect the female from harmful effects of (forced) extra-pair copulations, such as injuries, disruption of feeding, or disruption of incubation (Jenkins et al. 1965, Lumpkin 1981, Dahlgren 1990, Davis 2002). Female barred buttonquails, however, would probably benefit from mate-guarding in a different manner. In the context of a territorial intrusion, the male may mate with the extra-pair female, and the resident female then may face the risk of reduced offspring care or even brood abundance if the new female is of better quality, has a higher status, or provides other advantages to the male (Trivers 1972, Hoi-Leitner et al. 1999, Whittingham and Dunn 2001, Sheldon 2002, Arnqvist and Kirkpatrick 2005). In this context, it has been reported for polyandrous species that intruding females may actually mate with a male of a resident female and destroy its nest and the brood as to bind the male to their own prodigy (Muck et al. 2009).

Furthermore, mate-guarding may diminish the risk of sexually transmitted diseases (Sheldon 1993). The risk of getting infected by sexually transmitted diseases in females increases with the number of successful copulations. Hence, females in polyandrous mating systems may be particularly prone to such infections (Sheldon 1993). As sexually transmitted diseases may cause a reduction in body mass, fertility and egg production, and could even lead to the death of the infected bird, it is very likely that strategies evolved to evade an increased exposure to such diseases. Mate guarding by female barred buttonquails could indeed diminish the exposure to sexually transmitted diseases for both herself and her breeding male.

Therefore, we suggest that mate-guarding may indeed be an advantageous and (naturally) selected trait in female barred buttonquails. If mate-guarding ability in female barred buttonquails is indeed dependent on testosterone, females with higher testosterone levels may have a relatively higher breeding success compared to females with low testosterone levels by the means of a reduced risk of pathogens and a reduced risk of offspring loss through extra-pair matings. Moreover, sexually transmitted diseases, like parasites, may play a role in the evolution of secondary sexual characteristics (Sheldon 1993, Spencer et al. 2005), which are, at least in males, often modulated by testosterone (Wingfield et al. 1990, Andersson 1994, Gonzalez et al. 2002, Adkins-Regan 2005, Bókony et al. 2008).

Personality, mate choice, and sexual selection

Secondary sexual traits may not only reflect an individual’s condition and parasitic load (Hamilton and Zuk 1982) but may also reveal the personality of the individual (Garamszegi et al. 2008). Personality is defined through consistent behavioural responses displayed in different situations, i.e. describes how an individual generally copes with challenges in its physical and social environment (Réale et al. 2007, Sih and Bell 2008, Dingemanse et al. 2010). As different coping styles may differently affect breeding success, traits revealing the personality may be subjected in mate choice, hence be subject to sexual selection (Garamszegi et al. 2008). Moreover, differences in personality may be reflected in neuroendocrine parameters (Koolhaas et al. 1999, Carere et al. 2003, Kralj-Fišer et al. 2007). The results of our study on male European stonechats (Chapter Four) suggested that personality may play a role in the behavioural and hormonal response during a territorial challenge, such as individuals with generally higher testosterone levels demonstrate a rather proactive character. It would therefore be of great interest to integrate personality traits such as individual hormone levels and individual aggressiveness into the research of hormonal modulation of territorial aggression.

Territorial aggression is a very obvious behaviour of sex-role reversed females, and since its hormonal mechanisms are not yet fully understood, it may be fascinating to investigate personality traits in sex-role reversed aggression. Moreover, as personality may determine aggressiveness (Verbeek et al.1996, Carere et al. 2003, Amy et al. 2010, Fuxjager et al. 2010), and concurrently may play a role in mate choice (Garamszegi et al. 2008) - hence may affect sexual selection which in turn affects the evolution of sex-roles (Ligon 1999) – personality could theoretically play a role in the evolution of sex-role reversal. It would therefore be of great interest to investigate personality aspects of sex-role reversed, polyandrous males and females in general.

Despite the variety of subjects and species investigated in my thesis, the results of all four chapters demonstrate that the development of sex-role reversed characters and behaviour in polyandrous species is dependent on a variety of ecological and physiological aspects which may differ between species. While testosterone may indeed play a role in sex-role reversal, its expression may further depend on habitat structure, food availability, mate quality and abundance, and even on personality traits of the individual bird.

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