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Divergent hormonal responses to social competition in closely related species of haplochromine cichlid fish

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ABSTRACT

The diverse cichlid species flocks of the East African lakes provide a classical example of adaptive radiation. Territorial aggression is thought to influence the evolution of phenotypic diversity in this system. Most vertebrates mount hormonal (androgen, glucocorticoid) responses to a territorial challenge. These hormones, in turn, influence behavior and multiple aspects of physiology and morphology. Examining variation in competition-induced hormone secretion patterns is thus fundamental to an understanding of the mechanisms of phenotypic diversification. We test here the hypothesis that diversification in male aggression has been accompanied by differentiation in steroid hormone levels. We studied two pairs of sibling species from Lake Victoria belonging to the genera *Pundamilia* and *Mbipia*. The two genera are ecologically differentiated, while sibling species pairs differ mainly in male color patterns. We found that aggression directed toward conspecific males varied between species and across genera: *Pundamilia nyererei* males were more aggressive than *Pundamilia pundamilia* males, and *Mbipia mbipi* males were more aggressive than *Mbipia lutea* males. Males of both genera exhibited comparable attack rates during acute exposure to a novel conspecific intruder, while *Mbipia* males were more aggressive than *Pundamilia* males during continuous exposure to a conspecific rival, consistent with the genus difference in feeding ecology. Variation in aggressiveness between genera, but not between sibling species, was reflected in androgen levels. We further found that *M. mbipi* displayed lower levels of cortisol than *M. lutea*. Our results suggest that concerted divergence in hormones and behavior might play an important role in the rapid speciation of cichlid fishes.

Introduction

Identifying the mechanisms that drive population differentiation and speciation has proven to be one of the most challenging problems in evolutionary biology (Fisher, 1930; Lande, 1981; Van Doorn et al., 2009). The adaptive radiations of haplochromine cichlid fishes in the East African Great Lakes provide textbook examples of rapid diversification through natural and sexual selection (Kocher, 2004; Salzburger and Meyer, 2004; Schluter, 2000). The rock-dwelling communities of these lakes comprise several species complexes or genera that are strongly differentiated in ecology. By contrast, within genera, sibling species tend to be ecologically more similar, yet strikingly different in male nuptial coloration (Seehausen, 2000). This color variation is a target of sexual selection by female mate choice and plays a central role in the evolution and maintenance of haplochromine species richness (e.g., Genner and Turner, 2005; Kocher, 2004; Maan et al., 2004; Seehausen et al., 1997). Since haplochromine males can be highly territorial, it has been proposed that interference competition among males for mating and/or foraging territories can be a source of selection (Genner et al., 1999; Seehausen and Schluter, 2004). Indeed, several studies have indicated that male–male competition can generate negative frequency-dependent selection between competing species (Dijkstra et al., 2010; Seehausen and Schluter, 2004).

Across cichlid species there is striking variation in the (intrinsic) rate of territorial aggression (Genner et al., 1999; Ribbink et al., 1983). This behavioral variation influences outcomes of competition for both mates and ecological resources, and therefore has implications for selection, patterns of gene flow and the evolution and maintenance of phenotypic diversity (Dijkstra et al., 2010; Genner et al., 1999; see also: Owen-Ashley and Butler, 2004; West-Eberhard, 1983). A clear understanding of the evolutionary consequences of aggressive behavior requires understanding the physiological causes and consequences of agonistic interactions. Variation in hormones could underlie differences in aggression between species (e.g., O'Connell and Hofmann, 2011; Oliveira, 2009). However, hormones are not only a causal factor for male social behavior, but also their excretion rates are influenced in

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turn by the social environment, in particular by interactions between conspecifics, suggesting a complex two-way relationship between hormones and behavior (Wingfield et al., 1990; reviewed in Oliveira, 2004).

In addition to their role in behavior, hormones also regulate multiple aspects of physiology and morphology. Consequently, hormones are thought to mediate trade-offs among life history traits that are important for survival and reproduction (McGlothlin and Ketterson, 2008). It follows then that ecological or social factors may select for higher rates of aggressiveness via increases in competition-induced circulating levels of androgens. Competitive challenges may also induce a stress response by activating the hypothalamic–pituitary–adrenal (HPA) axis, resulting in a rapid glucocorticoid release that helps the animal to respond appropriately to stressful stimuli. However, increased androgen and glucocorticoid secretion rates can exert negative effects on the immune system and other physiological variables (Folstad and Karter, 1992; Wendelaar Bonga, 1997). As hormones exert (antagonistic) pleiotropy over behavior and other aspects of an animal's phenotype, selection on hormone-mediated behaviors could play an important role in creating and maintaining polymorphic phenotypes (e.g. in a frequency-dependent manner) (Kitano et al., 2010; Pryke et al., 2007; for review see Zera et al., 2007). We therefore propose that studying competition-induced shifts in hormone levels may advance our understanding of the rapid evolution of the haplochromine cichlid radiation. Specifically, we ask in the present study whether interspecific variation in aggression is reflected in parallel patterns of steroid hormones in four closely related sympatric haplochromine species.

Steroid hormones, such as androgens and glucocorticoids, affect a variety of morphological, physiological and behavioral traits (reviewed by Nelson, 2005). As noted above, androgen release is modulated by the social environment, in particular through interactions with conspecifics (e.g. Cardwell and Liley, 1991, reviewed in Oliveira, 2004). Circulating androgen levels are increased in periods of social instability that constitute a challenge to the animal (Wingfield et al., 1990), preparing the animal for future competitive situations (reviewed in Oliveira, 2004). In a comparative context, the challenge hypothesis has been useful in predicting competition-induced shifts in hormone levels according to several social and life history variables, such as length of breeding season and mating system (for recent reviews see Gleason et al., 2009; Goymann, 2009; Hirschenhauser and Oliveira, 2006).

Glucocorticoids coordinate behavioral and physiological responses to acute and chronic stressors (Sapolsky et al., 2000). For example, glucocorticoids mobilize energy resources and coordinate other physiological aspects of the stress response, aiding the animal in surviving stressful situations (Romero, 2002). At least indirectly, glucocorticoids are important modulators of aggression as well (Soma et al., 2008) and, correspondingly, glucocorticoid secretion rates increase in periods of social instability (Goymann and Wingfield, 2004). Although glucocorticoid responses are essential to survival, glucocorticoid can suppress the gonadal axis (Moberg, 1985) and long term exposure of glucocorticoids can lead to a multitude of deleterious effects, including neuron death (Sapolsky, 1993). Animals must therefore strike a balance between glucocorticoid levels that help survive stressful situations while limiting (long-term) glucocorticoid secretion to prevent deleterious effects.

Within an evolutionary context, studying hormonal responses to social challenges may contribute to our understanding of the mechanism of diversification in cichlids, since steroid hormones not only regulate behavior (and vice versa), but also affect a variety of key life history traits such as sexual signaling and immune function. We test here the hypothesis that diversification in male aggression has been accompanied by differentiation in steroid hormone levels across several Lake Victoria cichlid species. We focused on two sympatric sibling species pairs of haplochromine cichlids from two different genera that have the same mating system but vary in male color, the rate of aggressiveness and foraging ecology (Fig. 1): (1) *Pundamilia pundamilia* and *Pundamilia nyererei* and (2) *Mbipia lutea* and *Mbipia mbipi* (Seehausen, 1996). Sibling species within each genus are morphologically very similar but differ markedly in male nuptial coloration and aggression (Fig. 1) with *P. nyererei* being more aggressive than *P. pundamilia* and *M. mbipi* being more aggressive than *M. lutea* (Dijkstra et al., 2010; Verzijden et al., 2008, 2009; Verzijden unpublished). Although the two genera have not been previously compared, we hypothesized that they would likewise differ in average aggressiveness (Fig. 1). *Mbipia* and *Pundamilia* spp. occupy different trophic niches and accordingly display divergent ecomorphology (Seehausen et al., 1998): *Pundamilia* prefer zooplankton and benthic insects, which are more or less uniformly distributed within the lake. *Mbipia* are more dependent on *Aufwuchs* (i.e., spatially clustered epilithic algae and associated organisms, Bouton et al., 1997; Seehausen et al., 1998), which constitutes a more defendable resource (Bouton et al., 1997; Seehausen et al., 1998). Thus, in *Mbipia* aggressive behavior has a dual function in that it enables males to compete for and attract potential mates and to defend a feeding territory. As a consequence, we predicted that *Mbipia* would exhibit higher levels of territorial aggression than *Pundamilia* (Fig. 1).

In the present study, we investigated how interspecific variation in two types of territorial challenges from a conspecific rival is reflected in variation in circulating androgen and glucocorticoid levels. To this end, we analyzed agonistic behavior patterns and subsequent hormonal responses across three experimental contexts: continuous territory defense against a familiar male; a simulated territorial intrusion challenge by an unfamiliar male; and a social isolation control. We quantified aggressive displays and attacks and measured circulating levels of testosterone (T), the teleost-specific androgen 11-ketotestosterone (11-KT) (Kime, 1993) and the glucocorticoid hormone cortisol (CORT).

We expected that the behavioral and hormonal responses toward an unfamiliar intruding rival would be stronger than toward a familiar neighbor. Further, we predicted that *Mbipia* males would exhibit higher levels of aggression, and have higher levels of circulating steroids than males of *Pundamilia* (Fig. 1). In a previous study (Dijkstra et al., 2011), we found that red and blue *Pundamilia* phenotypes differed in aggression levels, yet this phenotype difference was not reflected in circulating steroid hormone levels. Importantly, in that study red and blue males were from a location in Lake Victoria where they hybridize and behave like incipient species or color morphs (Seehausen, 2009). In the current study, in contrast, we focused on reproductively isolated

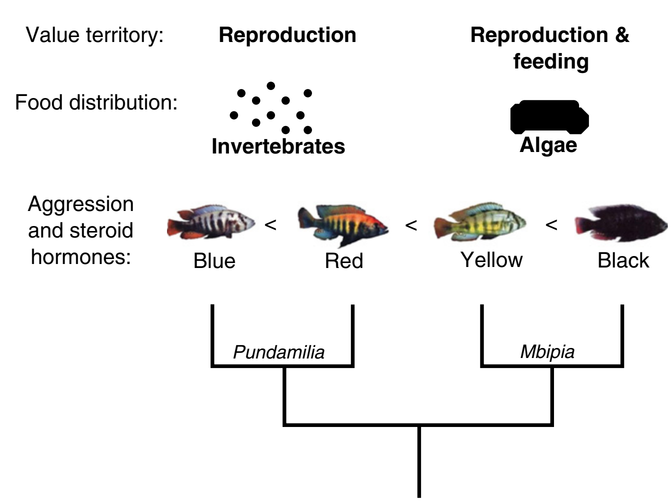

Fig. 1. Summary description of trophic ecology as well as the expected relative aggression and steroid hormone levels for the four species used in the current study, *Pundamilia pundamilia* (males are blue), *P. nyererei* (red), *Mbipia lutea* (yellow) and *M. mbipi* (black). Photos by Ole Seehausen.

sibling species from a different location, which are expected to show more pronounced phenotypic divergence (Seehausen, 2009). We therefore predicted for both genera that the more aggressive species (*P. nyererei* and *M. mbipi*, respectively) would have higher levels of circulating steroids than their less aggressive sister taxa (*P. pundamilia* and *M. lutea*, respectively).

Materials and methods

Species and subjects

The haplochromine cichlids *P. pundamilia* (Seehausen et al., 1998) and *P. nyererei* (Witte-Maas and Witte, 1985) are endemic to Lake Victoria and confined to rocky shores and islands (Seehausen, 2009). Territorial males defend territories that are essential for mating, while non-territorial males, females and juveniles school at various depths. *P. nyererei* males are yellow on their flanks and crimson in more dorsal regions, including the dorsal fin (we refer to this species as 'red'). In contrast, *P. pundamilia* males are grayish white both dorsally and on the flanks and have a blue dorsal fin (here referred to as 'blue'). Territorial red males occur at a depth of 3–8 m and are specialist plankton eaters; territorial blue males reside in water less than 3 m deep and feed predominantly on benthic insect larvae (Seehausen et al., 1998).

Mbipia spp., a haplochromine genus also endemic to Lake Victoria, is similarly confined to rocky shores and islands (Seehausen et al., 1998). Males of *M. mbipi* are black (we refer to them as 'black'), whereas males of *M. lutea* are yellow (and thus we refer to them as 'yellow' here). Territorial yellow males exclusively inhabit shallow waters (0–2 m deep) and feed predominantly on *Aufwuchs* (Seehausen et al., 1998). Territorial black, on the other hand, has its maximum territory density between 0 and 2 m but occurs up to a depth of 6 m. It also feeds on filamentous algae, although it can be best described as a partial *Aufwuchs* eater, since it also ingests other components of the algal mat such as diatoms and insect larvae (Bouton et al., 1997). All individuals used in this study were first generation offspring of fish that were collected in February 2003 at Makobe Island, located in the western Speke Gulf of Lake Victoria, where all four species are sympatric, reproductively isolated species, and likely compete with one another for resources (Seehausen, 1996). Fish were bred from 16 *P. pundamilia* pairs, 17 *P. nyererei* pairs, 10 *M. mbipi* pairs, and 9 *M. lutea* pairs. The research was carried out with an animal experiment license (DEC 3137 and DEC 4335A) from Groningen University and complied with current laws in The Netherlands.

Housing

All males were kept in compartments (size 0.5 × 0.4 × 0.6 m) within larger tanks and separated from conspecific males via perforated and transparent dividers. Each compartment contained gravel substrate and a dark gray polyvinyl chloride (PVC) tube that served as a territorial shelter. This arrangement allowed males to become territorial and interact visually and chemically while preventing physical interaction. Control males were placed in complete isolation without visual and chemical access to other fish by covering the sides and the back of the aquaria with black plastic sheets. All aquaria were connected to a central recirculating biological filter system with the water temperature at 25 ± 2 °C, and a 12:12 h light:dark cycle. The fish were fed daily with flake food (a mixture of King British Flakes and Ocean Star International Spirulina) and a mix of ground shrimp and peas.

Experimental design

Males were allowed to acclimate to the experimental tank for at least one week before data collection began. A single focal male was then observed in each experimental trial, which consisted of one of three standardized social treatments. In the *continuous social stimulation treatment* the focal male was housed next to a conspecific rival male for at least one week (mean days ± SE: 14.10 ± 0.52, range: 8–20). We then recorded the behavior (see below) of the two males for 5 min with a video recorder (Sony Handycam DCR-SR52) and collected behavioral data of one (focal) male in a given interaction. At the end of the recording session we netted the focal male and immediately (within 90 s) drew a blood sample (20- to 200-μL) from the caudal vein using a needle and a syringe. Body mass and standard length (SL) were also measured. This treatment allowed us to measure the 'social baseline' steroid levels of territorial males (Dijkstra et al., 2007).

The second treatment is referred to as the *territorial intrusion treatment*. Here, the focal male was also housed next to a conspecific rival male for at least one week (mean days ± SE: 14.13 ± 0.52, range 8–20), but we then removed the neighboring male and immediately placed an unfamiliar stimulus male enclosed in a transparent tube in the compartment of the focal male. Behavior of the focal male was recorded for the initial 15 min on video from the moment the stimulus male was introduced; the focal male was allowed to interact with the stimulus male for 45 min after which we netted the focal male and immediately took a blood sample. This treatment presumably triggers the maximum physiological steroid response (Hirschenhauser et al., 2004, but see Apfelbeck and Goymann, 2011).

Finally, in the *social isolation treatment*, we obtained blood samples from males that were kept in tanks isolated from any other fish. Males were given the same week-long acclimation period and were netted and sampled in the same way as in the other treatments. This treatment allowed us to measure physiological baseline steroid levels in the absence of social stimulation.

We used a total of 25 blue males (mean mass ± SE: 25.3 g ± 2.4; mean SL ± SE: 92.3 mm ± 2.4), 26 red males (24.6 g ± 2.1; 92.4 mm ± 2.5), 30 yellow males (40.8 g ± 2.3; 108.3 mm ± 2.0), and 20 black males (22.7 g ± 1.7; 89.5 mm ± 2.4). Some males acted both as focal and stimulus animal (i.e. in the continuous social treatment, the focal male was stimulated with a male that was later tested in the intruder treatment). We ensured that males never interacted with the same opponent twice. To increase power, we tested several males in at least two of the three treatments (in random order) with an intervening interval of at least eight days. Hormone and behavioral data were collected from, 19 blue, 21 red, 29 yellow and 20 black males. Of these, 14 blue, 16 red, 15 yellow and 11 black males were used in two different treatments; 2 blue and 1 black male were tested in all three different treatments. We detected no order effect in the analysis (data not shown). Males that were allowed to interact were approximately size-matched (SL difference as percentage of the larger male, mean ± SE: 5.61% ± 0.54, range: 0%–26.51%).

Quantification of behavior

One observer quantified behavior from the video recordings by scoring the rate that the focal male performed both display and attack behaviors toward the stimulus male (Baerends and Baerends-van Roon, 1950; Dijkstra et al., 2006; Verzijden et al., 2009). A *display* event was defined as a lateral or frontal display. During frontal displays, the focal male extends his dorsal fins, and sometimes pectoral fin and operculum as well, while facing the lateral or frontal side of the stimulus male. During a lateral display, the male extends his dorsal, anal and pelvic fins and positions himself such that his flank is in front of the head of the stimulus male. An *attack* event was defined as an individual butt or bite against the transparent screen or tube directed toward the stimulus male.

Hormone assays

We measured circulating levels of two androgens (T and the teleost-specific 11-KT) and CORT in blood plasma using enzyme immunoassays (T and CORT: Assay Design, Ann Arbor, MI; 11-KT Cayman Chemical Ann Arbor, MI) following protocols established by Kidd et al. (2010). These assay systems measure both the free and bound (to steroid binding proteins) fractions. For T and CORT, 7.2 μL of blood plasma per sample was diluted 1:30 with assay buffer and the manufacturer's instructions were followed (see Kidd et al., 2010). For 11-KT we used 3.6 μL plasma per sample. Overall, the intra-assay CV were 1.58%, 2.57% and 2.21% for T, 11-KT and CORT, respectively. The inter-assay CV were 6.56%, 4.46% and 8.85% for T, 11-KT and CORT, respectively. Cross-reactivities for T were: T 100%, 19-hydroxytestosterone 14.64%, Androstenedione 7.20%, Dehydroepiandrosterone 0.72%, Estradiol 0.40%, Dihydrotestosterone <0.001%, Estriol <0.001%, Aldosterone <0.001%, Corticosterone <0.001%, Cortisol <0.001%, Cortisone <0.001%, Estrone <0.001%, Progesterone <0.001%, Pregnenolone <0.001%. Cross-reactivities for 11-KT was: 11KT 100%, 4-Androsten-11β, 17β-diol-3-one 0.01%, Testosterone <0.01%, 5α-Androstan-17β, ol-3-one <0.01%, 5α-Androsten-3β, 17β-diol <0.01%. Cross-reactivities for CORT were: Cortisol 100%, Prednisolone 122%, Corticosterone 27.7%, 11-deoxycortisol 4.0%, Progesterone 3.64%, Prednisone 0.85%, Testosterone 0.12%, Androstenedione <0.1%, Cortisone <0.1%, Estradiol <0.1%. The detection limit for T and CORT was determined as the concentration of testosterone measured at two standard deviations from the zero along the standard curve; the detection limit for 11KT was calculated as 80% B/B_0 (sample bound/maximum bound). The detection limits were 5.67, 1.3, and 56.72 pg/ml for T, 11-KT and CORT.

Statistics

A linear mixed-model analysis of variance (LMM) was implemented in PASW Statistics (version 18) to test the influence of sibling species (red versus blue, black versus yellow), genus (*Mbipia* versus *Pundamilia*) and the treatments (fixed effects) on display rate or attack rate, with individual (referred to as fish identity) as a random effect in the model. To test whether sibling species differ in the rate of aggressiveness, we defined the explanatory factor 'species', according to the known species differences in aggressiveness (red and black are more aggressive than blue and yellow, respectively, Dijkstra et al., 2010; Verzijden unpublished). Similar LLM models were employed when comparing T, 11-KT or CORT levels. In all models concerning behavior and hormones, fish identity was never significant (Wald values < 1.042, *P* values > 0.3), unless stated otherwise. Due to significant departure from normality (Kolmogorov–Smirnov test $P<0.05$), we applied a natural log transformation [ln (X + 1)] to the behavioral data and a square root transformation to the hormone data. Backward elimination of non-significant fixed effects (using a conservative $P>0.1$) was used as a model selection criterion. Within-treatment comparisons were done using ANOVAs. All quoted probabilities are for two-tailed tests of significance.

Results

Agonistic behavior

Agonistic behavior consisted of both attack and display behavior (Fig. 2). As expected sibling species had different attack rates; red and black males were more aggressive than blue and yellow, respectively (Fig. 2, Table 1). Further, after controlling for the effect of sibling species the attack rate differed between genera, and according to the type of treatment, as indicated by a significant interaction between genus and treatment (Fig. 2, Table 1). Specifically, males of *Pundamilia* and *Mbipia* had comparable attack rates when responding to an intruder (ANOVA: $F(1,\ 55)=0.02$, $P=0.88$), but in the continuous treatment *Mbipia* males performed significantly more attacks than *Pundamilia* males (treatment: $F(1,\ 54)=13.92$, $P<0.001$). Display rates were also influenced by treatment: males displayed more frequently in the intruder treatment than in the continuous social treatment. Neither species nor genus was predictive of display rate variation (Fig. 2, Table 1).

Circulating hormone levels and the challenge response

Overall, there was a significant 'challenge response'; T and 11-KT levels were significantly higher in the social treatments (continuous social treatment and intruder treatment merged into one treatment group) compared to the isolated fish (LMM, T: $F(1,\ 146.68)=21.46$, $P<0.001$; 11-KT: $F(1,\ 142.77)=3.94$, $P=0.049$, fish identity

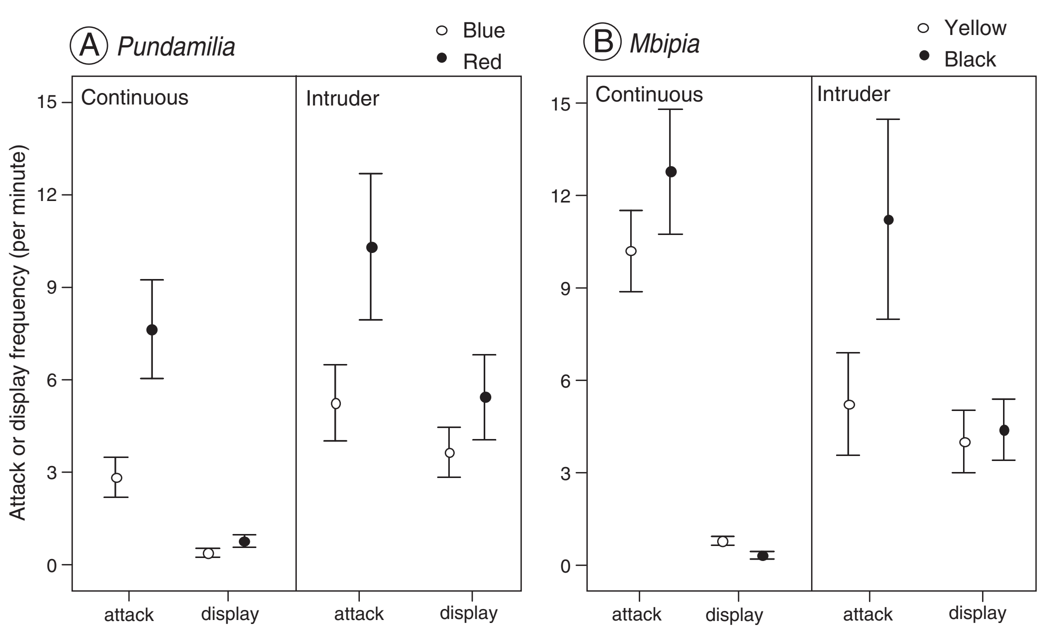

Fig. 2. Species differences in agonistic behavior in two social conditions for *Pundamilia* (A) and *Mbipia* (B). (A) Behavior of red (closed circles) and blue males (open circles). Shown are the rates per minute for attack and display behavior (mean ± SE) in the continuous social stimulation treatment and the intruder treatment. (B) Behavior of black (closed circles) and yellow males (open circles).

Table 1
Results of Linear Mixed Models examining the effects of treatment (continuous social vs. intruder), genus (*Mbipia*, *Pundamilia*) and sibling species (a significant effect means that red and black are different from blue and yellow, respectively) on attack (top) and display rates (bottom). Significance levels are reported for before factors were removed from the model during the backward elimination process and in the final model (which only retained significant terms, shown in bold). Note that if the interaction term is significant or retained, their main effects are not reported.

Factor	df	F	Significance
Attack			
Treatment × genus × species	1, 58.15	1.11	0.30
Treatment × species	1, 58.43	0.09	0.76
Genus × species	1, 59.73	0.24	0.62
Species	1, 59.70	7.22	**0.009**
Treatment × genus	1, 59.83	5.97	**0.017**
Display			
Treatment × genus × species	1, 52.02	1.38	0.25
Treatment × genus	1, 52.95	0.67	0.42
Treatment × species	1, 53.66	0.99	0.33
Genus × species	1, 57.23	1.26	0.27
Genus	1, 57.82	0.07	0.80
Species	1, 58.34	0.37	0.55
Treatment	1, 53.42	100.02	<**0.001**

Wald = 2.05, $P = 0.04$). However, there was no such overall effect for CORT (see below).

We then tested whether species, genera and treatment had an effect on the challenge responses. In contrast to the analysis of behavior, we also included isolated males in this analysis, but the statistical results were similar if only the two social treatments were included in the analysis. Contrary to our predictions, circulating T levels did not differ between sibling species, despite marked differences in the level of aggressiveness (Fig. 3, Table 2). However, an interaction between treatment and genus was predictive of levels of T (Table 2), which parallels observed differences in attack rate (Table 2). Specifically, *Mbipia* and *Pundamilia* males had similar T levels when in isolation (ANOVA: $F(1, 36) = 0.14$, $P = 0.71$) and similarly high levels when faced with an intruder ($F(1, 55) = 0.009$, $P = 0.92$). In contrast T levels in *Pundamilia* were lower during continuous exposure to a rival than those in *Mbipia*, resulting in a significant difference between the genera ($F(1, 54) = 15.17$, $P = 0.001$, see Fig. 3).

The pattern of 11-KT resembles that of T, which is unsurprising given that these two androgens are tightly correlated (Pearson's $r = 0.77$, $P < 0.001$). The analysis of 11-KT showed that treatment did not affect circulating levels, nor was there an effect of sibling species or genus, although the interactions between treatment and species and between treatment and genus were retained in the model as non-significant factors ($0.05 < P \leq 0.10$, see Table 2). Fish identity had a significant effect on variation in 11-KT (Wald = 2.273, $P = 0.023$).

In the final model for CORT, there was a significant interaction between sibling species and genus (Fig. 3, Table 2), thus species pairs differ in the degree to which they diverge in CORT response. This interaction effect is most apparent in the continuous social treatment where to our surprise *Mbipia* black males had considerably lower CORT levels compared to yellow males (ANOVA: $F(1, 26) = 11.05$, $P = 0.003$), while CORT levels did not differ between *Pundamilia* red and blue males in this treatment condition ($F(1, 26) = 1.79$, $P = 0.19$). There was no significant interaction between treatment and genus that would have explained this variation in CORT (Table 2). Thus, in contrast to T, CORT levels did not reflect genus-specific variation in agonistic behavior. Finally, as expected, after stimulation with an unfamiliar intruding rival CORT levels were much higher than during exposure to a familiar neighbor or in isolation (Table 2).

Discussion

In the present study, we investigated in closely related cichlid fish species how interspecific variation in circulating androgen and glucocorticoid levels is associated with two types of territorial challenges. We found differences in aggression between sibling species: red males attack more than blue ones (see also Dijkstra et al., 2010) and black more than yellow ones. The two genera *Mbipia* and *Pundamilia* showed different dynamics in male agonistic responses across the two types of territorial challenges. In the intruder treatment, males of both genera exhibited high attack rates. However, in the continuous treatment, attack rates of *Mbipia* males were much higher than those in *Pundamilia* males. As expected, the genus difference in agonistic behavior was reflected in the T response: in the intruder treatment, males of both genera exhibited comparably high T responses, while in the continuous social treatment *Mbipia* had higher social baseline T levels than *Pundamilia* males; thus, it appears that *Mbipia* males already exhibited high androgen levels in this social condition when faced with a familiar rival and that the introduction of an unfamiliar intruder could not increase androgen levels any further.

Variation in 11-KT levels generally followed the pattern observed in T, although the interaction term between genus and treatment in the final model was non-significant. It should be noted, however, that fish identity had a significant effect, suggesting that individual consistency in 11-KT levels may have masked treatment effects. Often, 11-KT is viewed as the more relevant androgen in fish (e.g., Hirschenhauser et al., 2004), but the evidence suggests this may not be true in haplochromine cichlids. Specifically, 11-KT levels are *ca.* 40 times lower than those of T, and at the same time 11-KT and T are also tightly correlated, consistent with observations in the model haplochromine species *Astatotilapia burtoni* (Kidd et al., 2010; Korzan et al., 2008; Parikh et al., 2006).

When exposed to an intruder challenge, males of both genera displayed higher levels of CORT and display behavior compared to the continuous social and isolation treatment. Assuming that the intruder treatment poses more of an acute challenge than the continuous social treatment, this observation is consistent with the notion that a social stressor can elevate not only androgen but also CORT levels (Romero, 2002). Despite exhibiting high levels of aggression, T and 11-KT, *Mbipia* males had relatively low CORT levels in the continuous social treatment. While short-term elevations of glucocorticoids such as CORT promote behavioral and physiological adjustments to the environment (Goymann and Wingfield, 2004), chronic elevation of glucocorticoids can have negative effects (e.g., Sapolsky, 1993), including a reduction in the expression of sexual ornaments (Bortolotti et al., 2009). It is intriguing that black males in the continuous social treatment had dramatically lower CORT levels than yellow males despite exhibiting higher rates of aggression. This inverse relationship between CORT and aggression has been previously reported in rats (Haller et al., 2004; but see Angelier et al., 2011; Mitani et al., 2002). However, we can currently only speculate why black males have lower CORT levels under continuous territorial defense, which can be viewed as a more long-term stressor. Given the fact that black males most likely express more melanin than yellow males, these findings hint at a possible role of the melanocortin system linking pigmentation, aggression and the stress axis (Ducrest et al., 2008).

Contrary to our expectation, sibling species differed in aggressive behavior, but not in androgen profiles. This result is consistent with our previous study in which red and blue *Pundamilia* phenotypes from a location where they hybridize also differed in aggression level, but not in hormone profile (Dijkstra et al., 2011). However,

Fig. 3. Circulating levels of (A) testosterone (T), (B) 11-ketotestosterone (11-KT) and (C) cortisol (CORT) in the three different treatments (isolation, continuous social and intruder) for *Pundamilia* and *Mbipia*. Shown are means ± SE. Different letters between 2 groups indicate that post-hoc test (2-sample t-tests and LMM with FDR control) was significant between those groups.

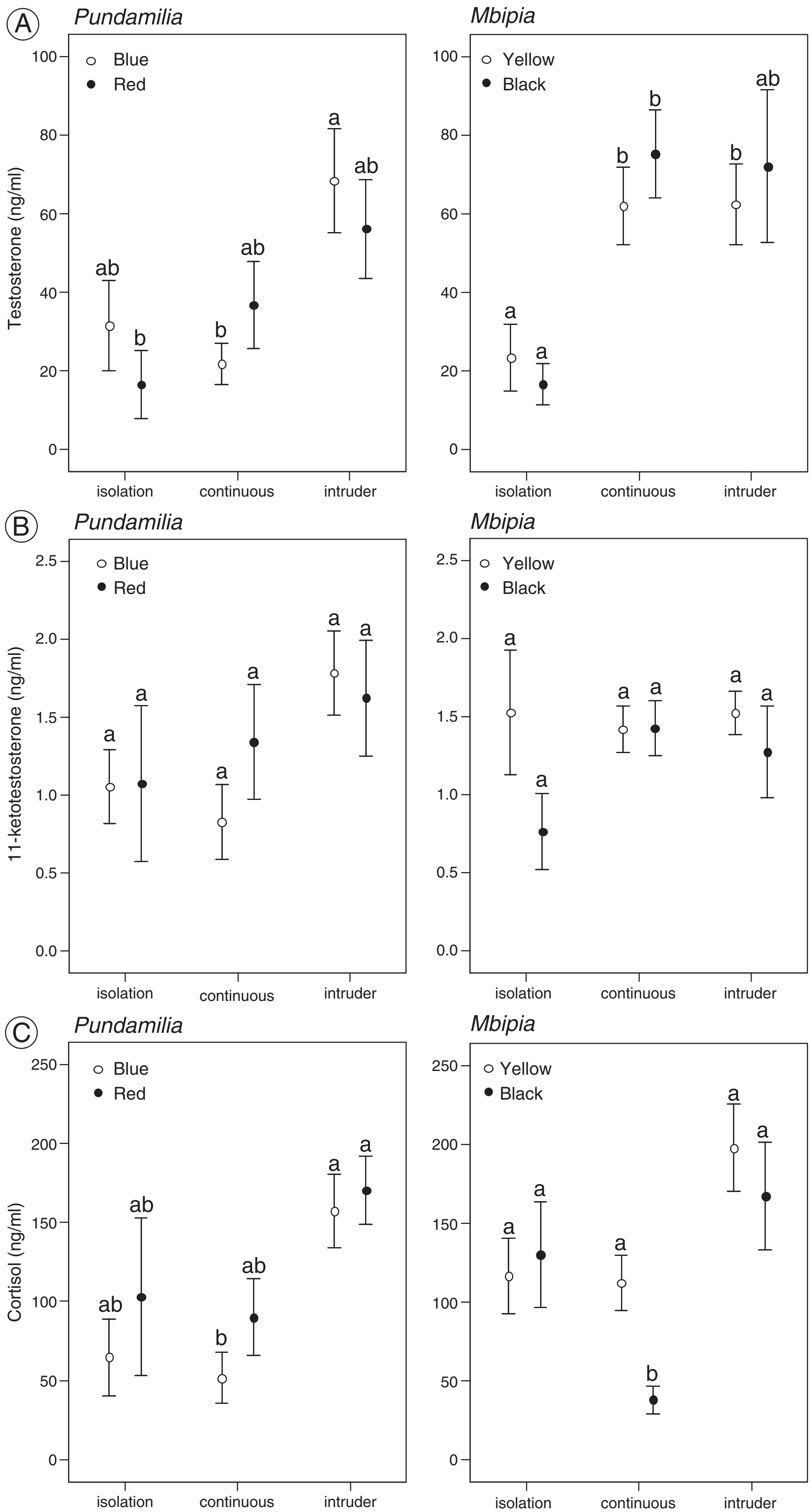
A
Pundamilia
Blue
Red
Testosterone (ng/ml)
Mbipia
Yellow
Black
isolation
continuous
intruder
B
Pundamilia
Blue
Red
11-ketotestosterone (ng/ml)
Mbipia
Yellow
Black
isolation
continuous
intruder
C
Pundamilia
Blue
Red
Cortisol (ng/ml)
Mbipia
Yellow
Black
isolation
continuous
intruder

Table 2
Results of Linear Mixed Models examining the effects of treatment (isolation, continuous social, intruder), genus (*Mbipia*, *Pundamilia*) and sibling species (a significant effect means that red and black are different from blue and yellow, respectively) on circulating levels of testosterone, 11-ketotestosterone and cortisol. Significance levels are reported for before factors were removed from the model during the backward elimination process and in the final model (which only retained significant terms, shown in bold). Note that if the interaction term is significant or retained, their main effects are not reported.

Factor	df	*F*	Significance
Testosterone			
Treatment × genus × species	2, 103.86	0.05	0.95
Genus × species	1, 82.39	0.50	0.48
Species × treatment	2, 105.23	1.68	0.19
Species	1, 83.73	0.04	0.84
Genus × treatment	2, 110.12	4.33	**0.016**
11-Ketotestosterone			
Treatment × genus × species	2, 92.81	0.30	0.74
Genus × species	1, 79.26	0.57	0.45
Treatment × species	2, 95.17	2.77	0.07
Genus × treatment	2, 95.40	2.44	0.09
Cortisol			
Treatment × genus × species	2, 105.06	1.40	0.25
Treatment × species	2, 106.72	0.56	0.57
Genus × treatment	2, 109.40	0.59	0.56
Genus × species	1, 79.80	5.38	**0.02**
Treatment	2, 110.73	20.15	<**0.001**

our results are in contrast to findings in another haplochromine cichlid, *A. burtoni*, where two distinct color morphs display morph-specific steroid and behavioral profiles (Korzan et al., 2008). Although high levels of circulating androgens can increase aggressive behavior given the appropriate social stimuli, androgens do not cause aggression to occur per se (Wingfield et al., 1987). In addition, there is a complex bidirectional relationship between androgens and behavior in that agonistic interactions themselves modulate the hormonal state of the animal as well. (Wingfield et al., 1990; reviewed in Oliveira, 2004). This complex interrelationship as well as (non)-androgenic effects on aggression could easily obscure hormone-behavior associations (Wingfield, 1994). For example, in our study, species differences in aggressiveness within the same genus might be explained by the amount of available steroids (as determined by steroid binding proteins, Jennings et al., 2000) and/or differences throughout the brain in the abundance and/or distribution of androgen receptors, aromatase activity (which converts T into Estradiol) and/or expression of estrogen receptors, all of which are known to regulate aggression (O'Connell and Hofmann, 2011; Soma et al., 2008; Trainor et al. 2006). Of course, other neuroendocrine pathways, such as those involving neuropeptide hormones (Goodson and Bass, 2001; Greenwood et al., 2008) and biogenic amines (Haller and Kruk, 2003), can also mediate behavioral and physiological differences across vertebrates. Future studies should shed more light on these different pathways that underpin the expression of aggression.

Our observation that variation in androgen profiles reflected behavioral diversification between genera, but not between sibling species is difficult to explain, though this pattern could be related to the nature of the difference in aggression. Between genera, the behavioral difference was dependent on the type of competition: males of *Mbipia* and *Pundamilia* differed in attack rate and androgen levels during continuous social defense only. By contrast, behavioral differences between sibling species occurred within treatments. Although speculative at this point, we suggest that variation in androgens might be most strongly associated with fundamental differences in how territorial defense is conducted with respect to chronic and acute social challenges and/or with respect to rival familiarity (in the continuous social treatment males were exposed to the same male for at 8 days, while the intruder male was a novel, unfamiliar rival). The way genera respond to these different types of social challenges could have been shaped by divergent ecological selection. How general these patterns are remains an exciting question for future studies.

We compared the challenge response in four cichlid species that have the same mating system, but vary in male color and aggressiveness. We hypothesized that genus differences in foraging ecology would be associated with a difference in aggression, which in turn would be mirrored in different androgen levels. We indeed found that *Mbipia* males were more aggressive than *Pundamilia* males in the continuous social treatment, consistent with previous studies suggesting that *Aufwuchs* eaters tend to be more aggressive than non-*Aufwuchs* feeders (Ribbink et al., 1983). This contrast in behavior was reflected in distinct androgen profiles, which is consistent with the idea that ecological factors exert selection on the rate of aggression as well as androgen secretion rates (Hau et al., 2008; Wingfield et al., 1990). We note, however that our study is one of the first that implicates a role for the degree of competition for food resources in the challenge hypothesis, which typically deals with territorial aggression in a reproductive context alone (Wingfield et al., 1990; but see Ros et al., 2002). One future challenge will be to tease apart the effect of (foraging) ecology from effects of phylogeny that set species apart (for a phylogenetic approach toward understanding evolutionary shifts in physiological processes, see Emerson (1996)).

The cichlid species flocks in East Africa are textbook examples for how sexual and natural selection can drive speciation and the evolution of phenotypic diversity. Our study supports the idea that ecological (here trophic) selection can affect traits that are also sexually selected (such as male–male aggression), or vice versa. This interaction between natural and sexual selection would be consistent with recent studies suggesting that disruptive or divergent sexual selection does not operate in isolation but in conjunction with ecological selection (Seehausen et al., 2008; Van Doorn et al., 2009; for reviews see Bolnick and Fitzpatrick, 2007; Salzburger, 2009). Examining the hormonal correlates of differences between species (and genera) in territorial and morphological traits can help explain how natural and sexual selection both may drive the diversification of these traits, yet only few studies have addressed this question. We have shown here that diversification in traits relevant to sexual selection (male coloration and agonistic behavior) has been accompanied by a corresponding differentiation in glucocorticoid regulation in the *Mbipia* sibling species pair. At the same time, diversification in foraging ecology, ecomorphology and, most importantly, agonistic behavior between two sympatric genera has been accompanied by a corresponding differentiation in competition-induced shifts in androgen levels. Because endocrine pathways impinge on a multitude of physiological processes and life history trade-offs, the analysis of hormonal function across closely related species is fundamental to our understanding of the processes that generate diversity (Zera et al., 2007).

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