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Social instability promotes hormone–behavior associated patterns in a cichlid fish

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ABSTRACT

Androgens are known to respond to social challenges and to control the expression of social behavior and reproductive traits, such as gonadal maturation and sperm production, expression of secondary sex characters and reproductive behaviors. According to the challenge hypothesis variation in androgen levels above a breeding baseline should be explained by the regime of social challenges faced by the individual considering the trade-offs of androgens with other traits (e.g. parental care). One prediction that can be derived from the challenge hypothesis is that androgen levels should increase in response to social instability. Moreover, considering that a tighter association of relevant traits is expected in periods of environmental instability, we also predict that in unstable environments the degree of correlations among different behaviors should increase and hormones and behavior should be associated. These predictions were tested in a polygamous cichlid fish (Mozambique tilapia, *Oreochromis mossambicus*) with exclusive maternal care. Social instability was produced by swapping dominant males among groups. Stable treatment consisted in removing and placing back dominant males in the same group, in order to control for handling stress. Cortisol levels were also measured to monitor stress levels involved in the procedure and their relation to the androgen patterns and behavior. As predicted androgen levels increased in males in response to the establishment of a social hierarchy and presence of receptive females. However, there were no further differential increases in androgen levels over the social manipulation phase between social stable and social unstable groups. As predicted behaviors were significantly more correlated among themselves in the unstable than in the stable treatment and an associated hormone–behavior pattern was only observed in the unstable treatment.

Introduction

Hormones play a key role in the physiological adaptation of organisms to their environments. On one hand hormones are physiological integrators that interact with multiple systems and with each other, therefore controlling suites of morphological and behavioral traits. On the other hand, they respond to different environmental cues, hence adjusting phenotypic form and function to predictable (e.g. seasonal) and unpredictable (e.g. stressor) environmental changes (Cohen et al., 2012). Androgens are known to regulate spermatogenesis, the expression of secondary sex characters and reproductive and territorial behavior on one hand, and to respond to predictable changes that signal the breeding season in temperate species, therefore linking the expression of reproductive traits to the appropriate environmental context (Adkins-Regan, 2005). Additionally, androgens also respond to short-term changes in the social environment (e.g. mating opportunity, territorial intrusion; Wingfield et al., 1990; Hirschenhauser and Oliveira, 2006; Goymann, 2009) and these have been interpreted as a mechanism to fine-tune androgen-dependent traits to acute and transient changes in the environment (Oliveira, 2009; Oyegbile and Marler, 2005). The challenge hypothesis (Wingfield et al., 1990) has been proposed as an explanatory framework for the observed variation in androgen responsiveness to the environment. According to this hypothesis androgen levels rise above a constitutive baseline during the breeding season triggered by environmental cues (e.g. photoperiod), and this breeding baseline is both necessary and sufficient for successful breeding (i.e. spermatogenesis, expression of secondary sex characters and reproductive behavior). Further increases in androgen levels above this breeding baseline would be explained by the degree of social stimulation that males are exposed to, and would not be required for breeding. Given the detrimental effects of androgens on male parental care behavior, this androgen responsiveness to social stimulation would also have a trade-off with paternal care (Wingfield et al., 1990, 2001). Therefore, the challenge hypothesis has predicted that androgen responsiveness to social challenges would vary with mating systems and

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parental type. Androgen responsiveness should be higher in monogamous (that are expected to face less social challenges) than in polygamous species, and in species with biparental or paternal care than in species with maternal care only (Wingfield et al., 1990). These predictions have been supported by comparative data in different vertebrate taxa (fish: Oliveira et al., 2002; Hirschenhauser et al., 2004; birds: Hirschenhauser et al., 2003; Goymann, 2009). Further hypotheses have been subsequently proposed in an attempt to explain notable cases of lack of androgen modulation by the social environment. For example, the lack of androgen responsiveness in males with short-breeding seasons or single brooded has been interpreted as an adaptation to facilitate a rapid transition from the mating to the parental care phase (Goymann, 2009; Landys et al., 2007; Wingfield and Hunt, 2002). Similarly, the exceptions to the predictions derived from the costs of androgens on parental care have been tentatively explained by discriminating species in which male parental care is critical for offspring survival (i.e. essential paternal care hypothesis, Lynn et al., 2002).

Despite the adaptive hypotheses described above for patterns of hormone–behavior association there are many cases in which hormones become dissociated from putatively hormone-dependent traits. For example, aggressiveness and androgen levels may become seasonally dissociated in species that express aggressive behavior outside the breeding season when androgen levels are low (e.g. Apfelbeck et al., 2013; Canoine and Gwinner, 2002; Dittami and Reyer, 1984; Landys et al., 2010; Logan and Wingfield, 1990; Wingfield, 1994). It has also been noted that the strength of the correlations between androgens and behavior seems to be higher at periods of social instability, such as during the establishment of territories or dominance hierarchies, the response to territorial intrusions or the competition with other males for access to mating opportunities, than during periods of social stability when behavioral output drops to baseline levels (Oliveira et al., 2002 and references therein). This socially-driven temporal variation in the association between hormones and behavior within the same species may reflect the dual role of hormones on phenotypic integration (i.e. when multiple functionally-related traits are correlated with each other) and independence (i.e. when multiple functionally-related traits are independent of each other; McGlothlin and Ketterson, 2008; Ketterson et al., 2009). As described above for androgens, the co-regulation of different traits by the same hormone gives rise to correlations among traits, which become linked in their response to the environment hence promoting phenotypic integration. However, in certain conditions the independent response of some of the correlated traits could be advantageous (e.g. need to express androgen-dependent aggression outside the breeding season). Therefore, it would be adaptive if the pleiotropic effects of hormones could vary between different life-history stages, or even within the same life-history stage depending on the regime of environmental challenge, in order to offer the best compromise given the environmental demands faced by the organism. Indeed, heterogeneous environments have been shown to favor plasticity in the correlation structures among phenotypic traits, and patterns of integration may vary across environments (Earley et al., 2012; Schlichting, 1989). Therefore, we predict that plasticity in hormone-driven phenotypic integration should vary with social stability, such that unstable social environments characterized by a high rate of social challenges that require a tighter phenotypic integration would promote associated hormone–behavior patterns, whereas in stable social environments (with low rates of social challenges) where phenotypic integration can be relaxed hormone–behavior patterns can become dissociated.

Here we tested this hypothesis in a lek-breeding African cichlid fish (Mozambique tilapia, *Oreochromis mossambicus*). In this species males establish territories in breeding aggregations to which they attract females to spawn with, and parental care is exclusively provided by the females (Baerends and Baerends-van Roon, 1950). Territorial males adopt a dark nuptial coloration which may vary in intensity and that can be turned on and off within minutes. Androgen responsiveness to social challenges has been previously reported in this species (Oliveira et al., 1996) and its magnitude in comparison to that observed in other cichlid species with different mating systems and parental care types matched the predictions of the challenge hypothesis (Hirschenhauser et al., 2004). After the establishment of mixed-sex breeding groups social manipulations created either unstable or stable social environments. For social instability dominant males were swapped among groups. For social stability the dominant male of each group was removed and placed back in its own group, in order to control for handling stress. It was predicted that group formation would trigger an androgen response and that hormone and behavior would become correlated. After the social manipulations it was predicted that the unstable treatment would promote hormone–behavior associated patterns, whereas the stable treatment would promote a dissociated hormone–behavior pattern. Apart from the males that were part of the social groups, bystander males that were visually exposed to the social groups but prevented from engaging in social interactions were also tested, in order to assess if the mere perception of social context is enough to promote the predicted responses.

Methods

Animals and housing

Adult Mozambique tilapia (*O. mossambicus*) from a stock held at ISPA - Instituto Universitário (Lisbon, Portugal) was used. Stock fish were kept in mixed-sex groups (3–4 males to 4–5 females per tank) in glass tanks (120 × 40 × 50 cm), with fine gravel substrate, a double filtering system (both sand and external biofilter, Eheim) and constant aeration. Water quality was monitored once a week for nitrites (0.2–0.5 ppm), ammonia (<0.5 ppm) (Palintest kit®) and pH (6.0–6.2). Water temperature was kept at 26 ± 2 °C and photoperiod was 12L:12D. Fish were fed ad libitum daily with commercial cichlid floating and sinking sticks.

Experimental procedures

Mixed-sex groups (2 males + 2 females) and 1 bystander male were tested in social stable or unstable social environments. Fish were observed in aquaria with two compartments, a larger one (40 cm × 75 cm × 50 cm) to receive males and females, and a smaller one (40 cm × 25 cm × 50 cm) to house the bystander male (Fig. 1A). The two compartments were divided by a one-way mirror, which allowed the bystander to observe the social group without being seen by them, and the water to circulate between the two compartments. To improve the unidirectional properties of the one-way mirror a light bulb was placed on top of the group compartment, and direct illumination of the bystander's compartment was prevented by using a black opaque plastic around it (except in the frontal wall of the tank to allow observing the fish), so that the bystander's compartment was illuminated through the unidirectional glass. The one-way mirror formed a mirror image for fish in the group compartment with which they could interact. In fact, in the first day dominant males interacted aggressively with their mirror image in the one-way-mirror. However, they subsequently habituated to the mirror and such interactions were greatly reduced in the following days, when males occasionally approached the one-way mirror without displaying any aggressive behavior.

The experimental procedure consisted of the following 4 phases (Fig. 1B):

Phase 1 Social isolation — forty five males were isolated in 40 l aquaria for 7 days to dilute previous social experience.

Phase 2 Group formation — afterwards, two size-matched males [mean ± standard deviation (SD) of standard length (SL); difference between dominant and subordinate = 0.38 + 0.26 cm; stable group: SL = 12.88 ± 2.44 cm, coefficient of variation (CV) =

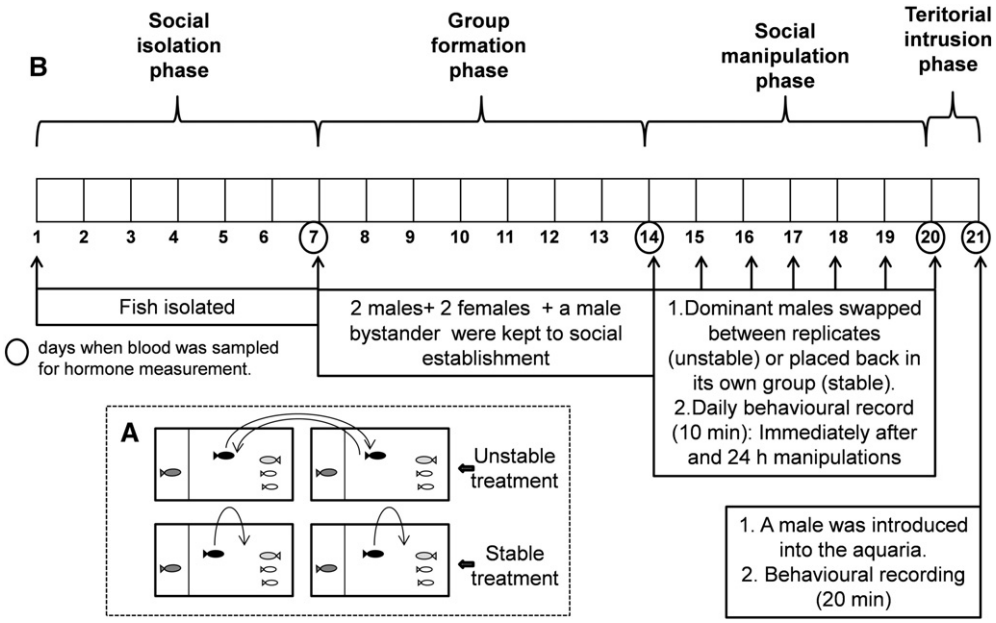


Fig. 1. Schematic representation of the experimental aquaria (A), with the compartments for the bystander (dark gray fish) and for the social group: dominant male (black fish), subordinate male (light gray fish) and females (white fish), and sequence of the procedures during the experimental protocol (B), in which the timing of blood sampling and behavioral recording is indicated.

18.96%; unstable group SL = 12.49 + 4.77 cm; CV = 38.17%] were placed at the same time in the group compartment of the set-up where two females (SL: 11.8 ± 1.19 cm) had been previously introduced. At this time a bystander male was also placed in the small compartment (stable group bystander SL = 15.00 ± 1.86 cm; unstable group bystander SL = 14.13 ± 1.18 cm). Fish remained in these conditions for 7 days to allow for the establishment of male territories and dominance relationships (n = 15 replicates).

Phase 3 Social manipulations — after this group formation period, groups were manipulated according to two different treatments: (1) a social instability treatment (n = 8 replicates), where dominant males (1 per group) were swapped between groups for 5 consecutive days; and (2) a social stability treatment (n = 7 replicates), where group composition remained the same, but dominant males were removed and placed back on theirs own groups to control for handling stress (Fig. 1A).

Phase 4 Territorial intrusion — the day after the end of the social manipulations phase a small glass container (18 cm × 30 cm × 15 cm) was placed inside the group compartment of the experimental aquarium, and the next day a naïve male was placed inside the container to mimic a territorial intrusion.

Behavior sampling

The ethogram of *O. mossambicus* provided by Baerends and Baerends-van Roon (1950) was used to identify the relevant behavioral patterns. Aggressive behavior (attack, fighting, chasing, lateral and frontal displays) and courtship were recorded, using a behavioral sampling with continuous recording method (Martin and Bateson, 2007), at the following sampling points:

1) Group formation phase — daily behavioral observations of 10 min per group;
2) Social manipulation phase — two daily behavioral observations of 10 min each per group were performed immediately after (IA) and 24 h after (24 h) swapping the dominant fish;
3) Territorial intrusion phase — behavioral observation of 20 min immediately after the introduction of the intruder male in the experimental tank.

The frequency of dominant and subordinate male aggressive behavior towards the other male and the females in the group formation and social manipulation phases, and towards the intruder in the territorial intrusion phase was estimated by summing up all the occurrences of the different aggressive behavior patterns in the observation period.

Nuptial coloration and bower-digging were also recorded using a scan sampling with instantaneous sampling recording method (Martin and Bateson, 2007). Behavioral observations of 10 min with a sample interval of 10 s were performed in parallel with the behavioral sampling for aggressive behavior described above and for the same sampling points, except for the territorial intrusion phase. Nest presence was also annotated after each behavioral observation. All dominant and bystander males had built a bower by the end of the group formation phase.

A computer-based multi-event recorder (The Observer v. 5, Noldus) was used for behavioral quantification.

Blood sampling and steroid radioimmunoassay

Blood samples were collected from dominant, subordinate and bystander fish after each phase of the experiment (i.e. social isolation, group formation, social manipulation and territorial intrusion, Fig. 1B). Fish were quickly anaesthetized (MS-222, Pharmaq; 300–400 ppm) and blood was collected from the caudal vein using 1 ml syringes with 25G/16 mm needles. Blood sampling always took less than 4 min since the start of the procedure, thus preventing possible effects of handling on cortisol levels (Foo and Lam, 1993). Blood was centrifuged (at 600 *g* for 10 min) and the obtained plasma stored at −20 °C.

Testosterone (T), 11-keto-testosterone (KT) and cortisol were extracted from plasma by adding diethyl-ether to the samples, centrifuging the mix (800 *g* for 10 min at 4 °C) and subsequently freezing it (10 min, −80 °C) in order to separate the ether fraction (containing the steroids) from the aqueous one, which was subsequently evaporated (speed vac, Savant instruments). The extracted steroids were resuspended in phosphate buffer.

Steroid concentrations were measured by radioimmunoassay, using commercially available antibodies and marked hormones for cortisol (rabbit anti-cortisol cortisol-32, ref. 20-CR50, Interchim, Fitzgerald; 1,2,6,7-3H Cortisol, Amersham Biosciences, ref. TRK407-250uCi) and T (rabbit anti-testosterone, Research Diagnostics Inc, ref. WLI-T3003; 1,2,6,7-3H Testosterone, Amersham Biosciences, ref. TRK402-250mCi), and a custom made antibody for KT kindly donated by D.E. Kime (specificity table for this antibody has been published in Kime and Manning, 1982) and titrated KT produced in-house from marked cortisol (see ref. above). Inter-assay variability and intra-assay variability were respectively: 23% and 5.2% for cortisol; 14.4% and 4.0% for T; and 13.3% and 4.8% for KT. Samples from different treatments were distributed across different batches to avoid assay biases.

Data analysis

Parametric assumptions were checked by using the Shapiro–Wilk and the Jarque–Bera adjusted multiplier to test for normality and both Levene and Bartlett tests to check for the homogeneity of variances. When needed data were transformed (see below) in order to meet the parametric criteria.

The effects of social manipulation (unstable vs. stable) on behavioral variables were assessed using either ANOVA or linear mixed models, depending on the nature of the variables (i.e. repeated vs. non-repeated measures), and taking also in consideration the social status of the individuals.

The effects of social manipulation (unstable vs. stable) and social status (dominant vs. subordinate) on aggressive behavior were tested using a linear mixed model analysis. The average of the frequency of aggressive behavior directed by dominant and subordinate males towards the other male or towards females at each sampling point [i.e. immediately after (IA) and 24 h after the manipulation] across the 5 days of the social manipulation phase was computed for each treatment (i.e. stable and unstable) and used as dependent variable (random effect: aggressive behavior IA vs. aggressive behavior at 24 h). Data were square root transformed to fit parametric analysis. Contrast effect tests were used to test for the following planned comparisons that were established a priori: (1) overall (i.e. dominants and subordinates pooled together) temporal variation (IA vs. 24 h) for each treatment (stable, unstable); (2) temporal variation within each social status: IA vs. 24 h for dominant and for subordinate for each treatment (stable, unstable); and (3) contrast between treatments (stable vs. unstable) within each social status (dominant, subordinate) at each sampling point (IA, 24 h). Aggressive behavior directed towards the intruder by both dominant and subordinate males of the two treatments was compared using a two-way ANOVA type II sum of squares (categorical variables: treatment and social status) followed by the same planned comparisons to contrast dominant and subordinate males within the same treatment and dominant and subordinate males between the two treatments. Data were log transformed to fit the parametric assumptions. The effects of treatment (stable vs. unstable) and social status (dominant vs. subordinate vs. bystander) on courtship behavior and bower-digging were assessed using a two-way ANOVA type II sum of squares. In order to meet the parametric assumptions the proportion of time spent bower-digging was arcsin transformed, and the frequency of courtship was log transformed. The data for nuptial coloration did not pass the homogeneity of variance tests and therefore a logistic model was used. Contrast effect tests were also performed for the following a priori planned comparisons: (1) contrast between treatments (stable vs. unstable) within each social status (dominant, subordinate, bystander) and (2) effect of social status (dominant vs. subordinate vs. bystander) within each treatment (stable, unstable).

The effects of group formation, social manipulation and territorial intrusion on hormone levels were assessed using linear mixed model analyses. Cortisol data were square root transformed to fit the parametric assumptions. To test for the effect of group formation on hormone levels we considered social status (dominant vs. subordinate vs. bystander) as a categorical variable and hormone level at the end of group formation (d_{14}) and the level at the end of social isolation (d_7) as a random effect. Contrast effect tests were used to test for temporal variation within each social status (d_7 vs. d_{14} for each social status), and to contrast between social statuses (dominant vs. subordinate vs. bystander) at each sampling point (d_7, d_{14}). To test for the effects of social manipulation and of territorial intrusion a single linear mixed model analysis was used in which social status (dominant vs. subordinate vs. bystander) and social manipulation (stable vs. unstable treatment) were used as categorical variables and the hormone levels at the end of group formation (d_{14}), at the end of the social manipulation period (d_{20}), and at the end of the territorial intrusion phase (d_{21}) were used as a random effect. In this model the effect of social manipulation was assessed by comparing pre- (d_{14}) with post-manipulation (d_{20}) hormone levels for each treatment (stable, unstable). Similarly the effect of territorial intrusion was assessed by comparing pre- (d_{20}) with post-intrusion (d_{21}) hormone levels for each treatment (stable, unstable). The specific effect of the social manipulation on individuals of different social statuses was also assessed by using the same planned comparisons as above for each status (dominant, subordinate, bystander). Finally, the effect of social status for each of these two phases (social manipulation and territorial intrusion) was also assessed using planned comparisons (dominant vs. subordinate vs. bystander) for each sampling point (d_{14}, d_{20}, d_{21}) within each treatment (stable, unstable).

Pearson correlations were computed to test for hormone–behavior associations in the group formation, the social manipulation, and the territorial intrusion phases of the experiment. To control for multiple comparisons a false discovery rate adjustment following the Benjamini–Hochberg procedure was used. For each treatment phase correlations were computed between the observed behavior in that phase and the hormone levels before and after (i.e. d_7 and d_{14} for group formation; d_{14} and d_{20} for social manipulation; and d_{20} and d_{21} for territorial intrusion). This temporal cross-correlation approach (i.e. before vs. after) may allow to infer the most parsimonious relationship between the two variables. For example, if hormone levels before the behavioral interaction (at t_1) are correlated with subsequent behavior (at t_2), and there are no correlations between behavior observed during the interaction and subsequent hormone levels (at t_3) the most parsimonious explanation is that hormone levels at t_1 are driving behavior (at t_2). If in contrast hormone levels at t_1 are not correlated with behavior at t_2, but behavior at t_2 is correlated with hormone levels at t_3, then the most parsimonious explanation is that behavior during the interaction is leading after-match hormone levels (at t_3). The same data transformations used for the ANOVA/linear mixed models described above were also used to compute the correlations, except for the variable "nuptial coloration" for which an arcsin transformation was used here, whereas raw data were used in the logistic model. The proportion of significant correlations was compared between treatments using the test for differences between proportions.

Sample sizes varied due either to technical problems (e.g. one fish remained motionless for the whole observation during the territorial intrusion, and video camera showed a technical problem during another) or to outlier values (considered as mean $\pm$ 3 $\times$ standard deviation) which removed from all the statistical analyses. All statistical tests were performed on R (R D.C.T., 2008), using the following packages: Hmisc (correlations), car (ANOVA type II SS), fbasics (Jarque–Bera test), nlme (linear mixed models), and multcomp (multiple comparisons). All tests were two-tailed and significance level used was $p < 0.05$.

Ethical note

The experimental procedures used in the studies presented here were in compliance with the regulations on animal experimentation in Portugal and were approved by a permit (ref. 0420/2007) from The

Portuguese Veterinary Authorities (Direcção Geral de Veterinária, Portugal).

Results

Effect of social manipulation on behavioral variables

Effect of social manipulation on aggressive behavior

The effects of social manipulation (stable vs. unstable) on aggressive behavior were assessed by comparing the behavior expressed immediately after and 24 h after the manipulation, using a linear mixed model, followed by planned comparisons, that also took into consideration the social status of the animals (dominant vs. subordinate). There were significant effects of social status (dominant vs. subordinate male) and of observation period (IA vs. 24 h), but not of treatment (stable vs. unstable) on male–male aggressive behavior (Table 1, Fig. 2A). Twenty four hours after the manipulation (24 h), there was a significant reduction of aggressive behavior in stable groups (planned comparison, dominant + subordinate IA vs. dominant vs. subordinate 24 h: z-value = −2.831, $p < 0.01$; Fig. 2A) but not in unstable groups (planned comparison, dominant + subordinate IA vs. dominant vs. subordinate 24 h: z-value = −1.509, $p = 0.13$; Fig. 2A). There was a significant decrease in male–male aggression for both dominant and subordinate males between IA and 24 h in the stable treatment (planned comparisons: dominant: z-value = −2.635, $p < 0.01$; subordinate: z-value = −2.013, $p < 0.05$; Fig. 2A), whereas in the unstable treatment male aggressive behavior was maintained between IA and 24 h in dominants (planned comparison: z-value = 0.913, $p = 0.36$; Fig. 2A) but decreased in subordinates (planned comparison: z-value = −3.318, $p < 0.001$; Fig. 2A). Planned comparisons of male–male aggression between the two treatments for each social status at each sampling point only revealed a significant difference for dominant males at 24 h (stable vs. unstable: dominant, IA, z-value = −0.378, $p = 0.71$; subordinate, IA, z-value = 1.417, $p = 0.16$; dominant, 24 h, z-value = 2.568, $p < 0.05$; subordinate, 24 h, z-value = 0.482, $p = 0.63$; Fig. 2A).

There were no main effects of any of the independent variables (treatment: stable vs. unstable; social status: dominant vs. subordinate male; observation period: IA vs. 24 h) on male–female aggressive

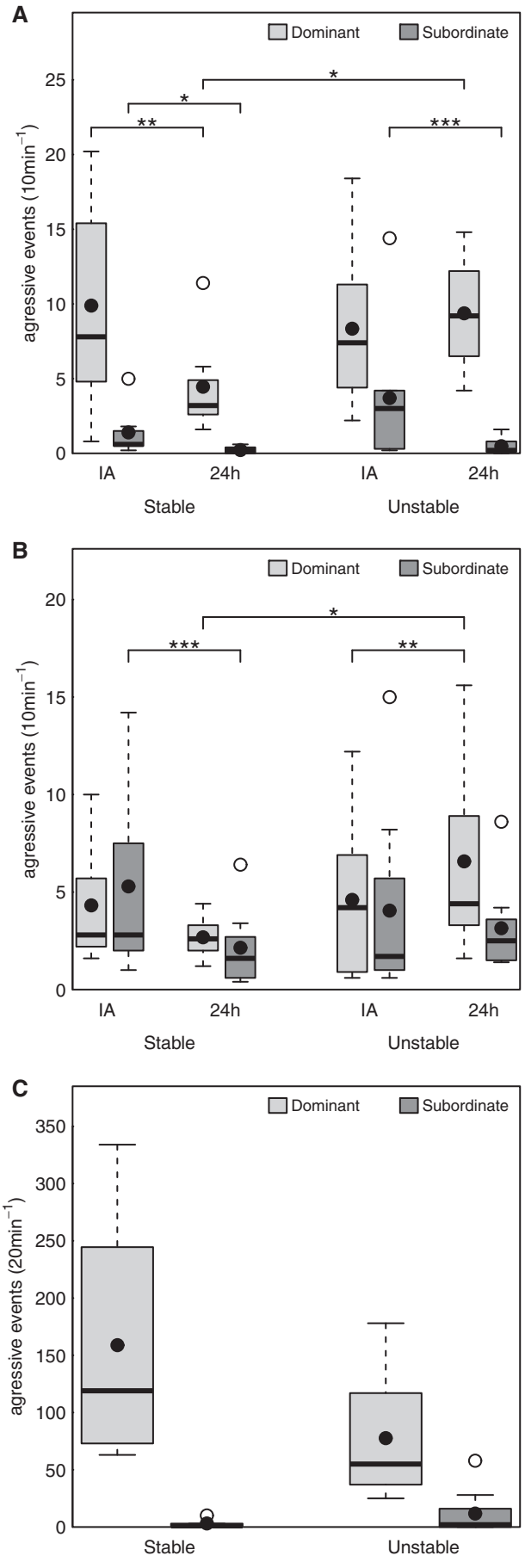
Table 1
ANOVA analyses for male aggressive and reproductive behaviors in the social manipulation and territorial intrusion phases using as categorical variables (in this order): period of observation (immediately after and 24 h after the social manipulation; only in aggression towards males and females); treatment (stable and unstable); and social status (dominant, subordinate and bystander). Main effects and interaction are from either a linear mixed model, a two-way ANOVA type II SS or a logistic model as appropriate. Results from planned comparisons are also presented.

	Aggressive behavior						Reproductive behavior					
	Male–male		Male–female		Male–intruder		Coloration		Digging[a]		Courtship[b]	
	F	p-Value	F	p-Value	F	p-Value	Chi-sq	p-Value	F	p-Value	F	p-Value
Period	11.86	0.002	1.40	0.247	–	–	–	–	–	–	–	–
Treatment	2.32	0.140	0.51	0.481	0.09	0.770	3.94	0.047	0.18	0.675	3.44	0.075
Status	51.97	<0.001	1.08	0.308	45.54	<0.001	81.30	<0.001	2.10	0.161	65.17	<0.001
Period × Treatment	1.15	0.294	14.54	<0.001	–	–	–	–	–	–	–	–
Period × Status	2.89	0.102	6.25	0.019	–	–	–	–	–	–	–	–
Treatment × Status	0.01	0.910	0.61	0.443	0.92	0.348	0.63	0.731	0.46	0.502	0.47	0.500
Period × Treatment × Status	4.81	0.038	0.40	0.531	–	–	–	–	–	–	–	–
Planned comparisons I (Period × Treatment)												
	z-Value	p-Value	z-Value	p-Value								
St: 24 h-IA	−2.83	0.005	−3.35	<0.001	–	–	–	–	–	–	–	–
Un: 24 h-IA	−1.51	0.131	1.65	0.100	–	–	–	–	–	–	–	–
Planned comparisons II (Period × Treatment × Time)												
	z-Value	p-Value	z-Value	p-Value								
St; D: 24 h-IA	−2.64	0.008	−1.73	0.084	–	–	–	–	–	–	–	–
St; S: 24 h-IA	−2.01	0.044	−3.68	<0.001	–	–	–	–	–	–	–	–
Un; D: 24 h-IA	0.91	0.361	3.10	0.002	–	–	–	–	–	–	–	–
Un; S: 24 h-IA	−3.32	0.001	−0.24	0.813	–	–	–	–	–	–	–	–
IA; D: Un-St	−0.38	0.705	−0.10	0.920	–	–	–	–	–	–	–	–
IA; S: Un-St	1.42	0.156	−0.88	0.380	–	–	–	–	–	–	–	–
24 h; D: Un-St	2.57	0.010	2.33	0.020	–	–	–	–	–	–	–	–
24 h; S: Un-St	0.48	0.630	0.89	0.372	–	–	–	–	–	–	–	–
Planned comparisons III (Status × Treatment)												
					t-value	p-Value	z-Value	p-Value	t-value	p-Value	t-value	p-Value
D: Un-St	–	–	–	–	−0.91	0.376	1.97	0.049	−0.72	0.474	−0.83	0.4157
S: Un-St	–	–	–	–	0.44	0.666	−0.28	0.783	–	–	−1.80	0.0843
B: Un-St	–	–	–	–	–	–	1.73	0.083	0.23	0.820	–	–
St: S-D	–	–	–	–	–	–	−2.88	0.004	–	–	−5.02	<0.001
St: B-D	–	–	–	–	–	–	−2.84	0.005	−1.43	0.152	–	–
St: B-S	–	–	–	–	–	–	1.65	0.098	–	–	–	–
Un: S-D	–	–	–	–	–	–	−3.16	0.002	–	–	–6.36	<0.001
Un: B-D	–	–	–	–	–	–	−3.22	0.001	−0.60	0.549	–	–
Un: B-S	–	–	–	–	–	–	2.19	0.029	–	–	–	–

Abbreviations: Period, period of observation; status, social status; male–male, male aggressiveness towards males; male–female, male aggressiveness towards females; male–intruder, male aggressiveness towards intruders; coloration, percentage of time males presented nuptial coloration; digging, percentage of time males spent digging nests; courtship, male courtship towards females; St, stable treatment; Un, unstable treatment; 24 h, observation 24 h after social manipulation; IA, observation immediately after social manipulation; D, dominant status; S, subordinate status; B, bystander status.

[a] Subordinates did not dig a nest.

[b] Bystanders did not court females.



behavior (Table 1; Fig. 2B). However, there were also significant interactions for male–female aggressive behavior between observation period and treatment and between observation period and social status (Table 1). Similarly to what was described above for male–male aggression a reduction in male–female aggression was also observed 24 h after the manipulation in stable (planned comparison, dominant + subordinate IA vs. dominant vs. subordinate 24 h: z-value = −3.346, $p < 0.001$; Fig. 2B) but not in unstable groups (planned comparison, dominant + subordinate IA vs. dominant vs. subordinate 24 h: z-value = 1.646, $p = 0.10$; Fig. 2B). The frequency of aggressive behavior towards females was not significantly different across observation periods for dominants in the stable treatment (planned comparison: z-value = −1.728, $p = 0.08$, Fig. 2B), whereas in the unstable treatment, the frequency of dominant male aggressive behavior directed towards females increased from IA to 24 h (planned comparison: z-value = 3.098, $p < 0.01$, Fig. 2B). Therefore, the frequency of male–female aggressive behavior in dominant males was significantly higher in the unstable than in the stable treatment at 24 h (planned comparison: z-value = 2.325, $p < 0.05$, Fig. 2B), but not at IA (planned comparison: z-value = −0.100, $p = 0.92$, Fig. 2B). Subordinate male aggressive behavior directed towards females in the stable treatment was higher at IA than at 24 h (planned comparison: z-value = −3.680, $p < 0.001$, Fig. 2B), but not in the unstable treatment (planned comparison: z-value = −0.237, $p = 0.81$, Fig. 2B). Furthermore, there were no significant differences in subordinate male aggression directed towards females between the stable and the unstable treatments at each sampling point (planned comparisons: IA; z-value = −0.878, $p = 0.38$; 24 h: z-value = 0.893, $p = 0.37$, Fig. 2B).

There was a main effect of social status in the aggressive response towards intruders (dominants > subordinates, Table 1, Fig. 2C). However, there were no significant effects either of treatment (Table 1, Fig. 2C) or of the interaction between social status and treatment on the aggression directed towards the intruder (Table 1).

Effect of social manipulation on reproductive behaviors

The effects of social manipulation (stable vs. unstable) on reproductive behaviors were assessed by comparing the behaviors expressed 24 h after the social manipulation, using either ANOVA or logistic models depending on the nature of the variables. Social status of the animals was also considered in the analyses. There were main effects of treatment and of social status (Table 1, Fig. 3A) on the percentage of time that males exhibited the body nuptial coloration, whereas the interaction between treatment and social status was not significant (Table 1). Time spent exhibiting the nuptial coloration was higher in the unstable than in the stable treatment for dominant males (planned comparison: z-value = −1.973, $p < 0.05$), whereas no differences were found either for subordinates (planned comparison: z-value = −0.275, $p = 0.78$) or for bystander males (planned comparison: z-value = 1.732, $p = 0.08$) (Fig. 3A). In stable groups the amount of time exhibiting the nuptial coloration was significantly higher in dominant than in either subordinate (planned comparison: z-value = −2.884, $p < 0.01$) or bystander (planned comparison: z-value = −2.840, $p < 0.01$) males, with no significant differences between the latter two male types (planned comparison: z-value = 1.654, $p = 0.10$) (Fig. 3A). In unstable groups differences in time exhibiting the nuptial coloration were significant

Fig. 2. Effect of social manipulation on aggressive behavior: (A) male–male agonistic interactions; (B) male–female agonistic interactions; and (C) dominant male–intruder male interactions. In A and B, the effects of treatment (stable and unstable), social status (dominant and subordinate) and observation period (immediately after, IA vs. 24 h after) were tested using a linear mixed model. In C, the effects of treatment (stable and unstable) and social status (dominant and subordinate) were tested using a two-way ANOVA type II SS. Significant relevant a priori planned comparisons are presented. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) between treatments (stable vs. unstable) and between observation periods (IA vs. 24 h) for each male type. A standard boxplot is presented with a dark dot representing the mean value.

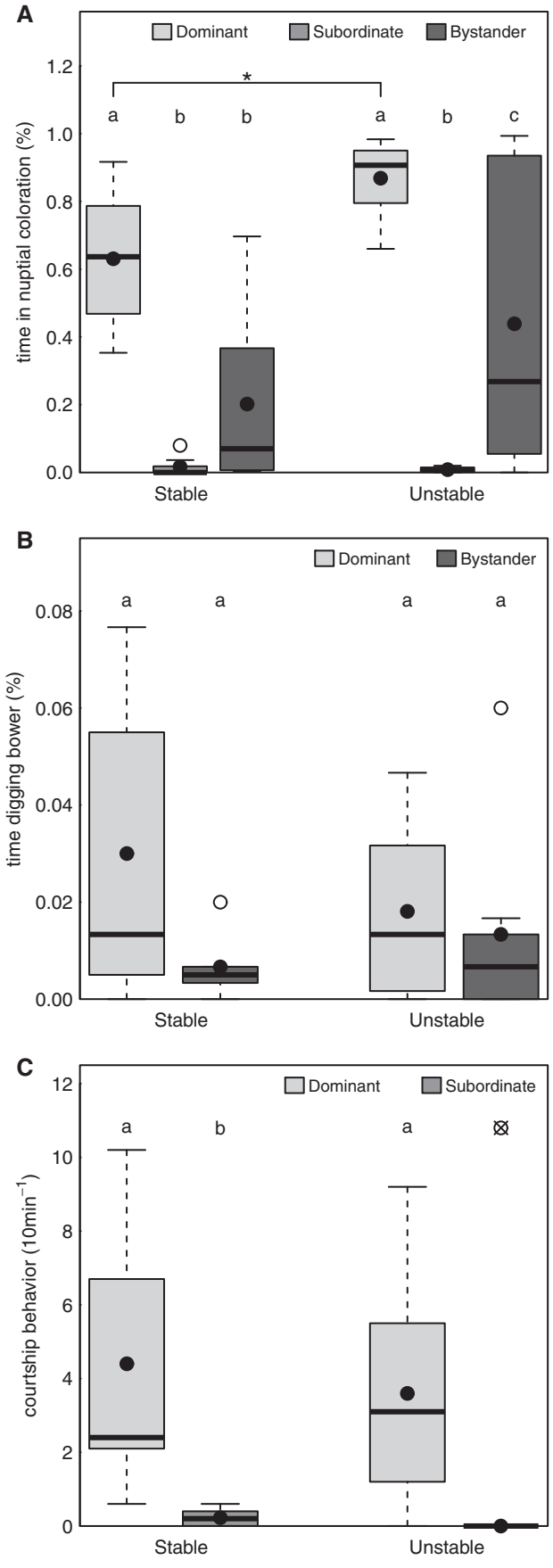


Table 2
ANOVA analyses for hormonal level in the group formation phase using as categorical variables (in this order): sampling time (before, day 7, and after, day 14, the group formation phase); and social status (dominant, subordinate and bystander). Main effects and interaction are from a linear mixed model. Results from planned comparisons are also presented.

	T		11KT		Cortisol	
	F	p-Value	F	p-Value	F	p-Value
Time	0.48	0.492	0.69	0.412	1.91	0.175
Status	3.27	0.048	3.54	0.038	2.03	0.145
Time × Status	1.45	0.247	7.89	0.001	2.04	0.144
Planned comparisons						
	z-Value	p-Value	z-Value	p-Value	z-Value	p-Value
D: 14-7	1.59	0.112	2.67	0.008	−0.53	0.594
S: 14-7	−0.93	0.352	−2.79	0.005	0.49	0.625
B: 14-7	0.63	0.527	1.73	0.084	−2.45	0.014
7: S-D	−0.90	0.369	0.06	0.951	−0.49	0.628
7: B-D	−0.26	0.798	−0.82	0.410	−0.48	0.629
7: B-S	0.64	0.522	−0.86	0.390	0.01	0.991
14: S-D	−3.14	0.002	−4.43	<0.001	0.46	0.647
14: B-D	−1.01	0.310	−1.55	0.121	−2.29	0.022
14: B-S	2.04	0.042	2.85	0.004	−2.70	0.007

Abbreviations: T, testosterone level; 11KT, keto-testosterone level; cortisol, cortisol level; time, sampling time; status, social status; D, dominant status; S, subordinate status; B, bystander status; 7, sampling at day 7; 14, sampling at day 14.

across all male types (planned comparisons: dominant vs. subordinate: z-value = −3.158, $p < 0.01$; dominant vs. bystander: z-value = −3.218, $p < 0.01$; subordinate vs. bystander: z-value = 2.188, $p < 0.05$) (Fig. 3A).

All dominants and bystander males dug a bower during the baseline period and continued to express bower-digging behavior during the manipulation phase (i.e. stable vs. unstable treatments). Subordinate males did not express this behavior and were removed from the statistical analysis. The percentage of time spent bower digging did not differ either with treatment or with social status (Table 1, Fig. 3B). Furthermore, the interaction between treatment and social status was also not significant (Table 1).

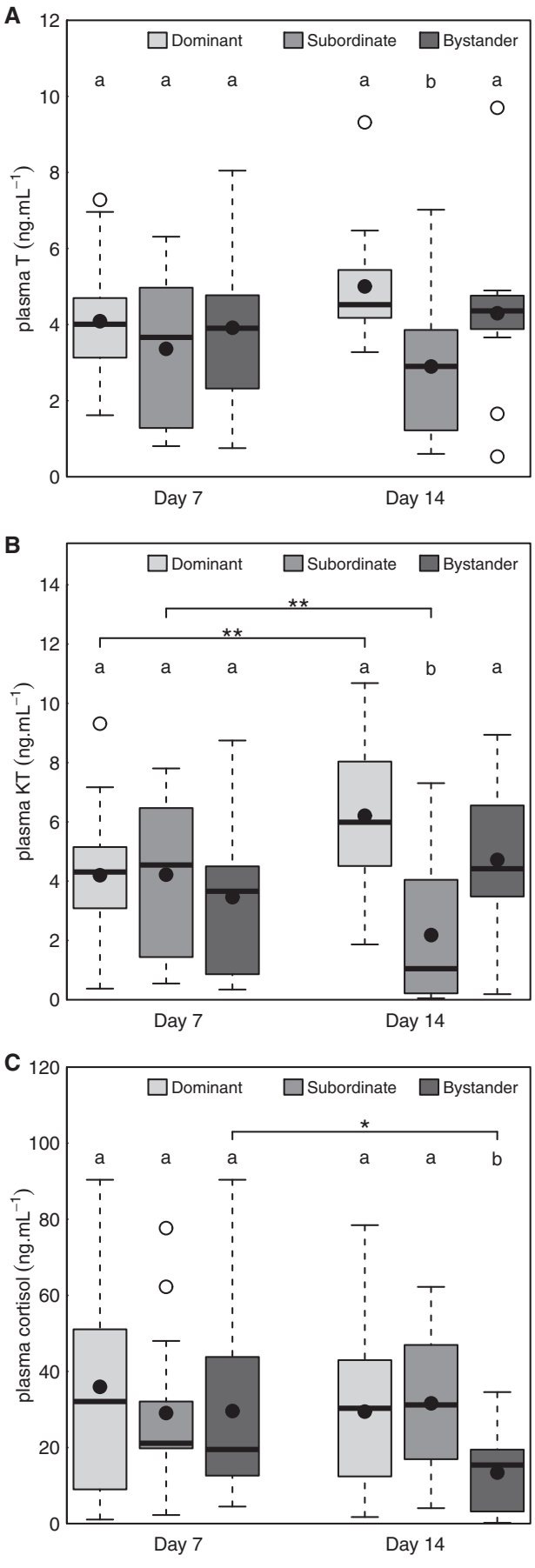
Treatment did not affect the frequency of courtship behavior but there was a significant effect of social status (Table 1, Fig. 3C) due to the fact that subordinates very rarely expressed courtship (Fig. 3C). The interaction between treatment and social status was not significant (Table 1).

Effect of social manipulations on hormonal variables

Effects of group formation on steroid hormone levels

The effects of initial group formation on steroid hormones (T, KT, cortisol) were assessed by comparing the hormone levels before (d_7) and after (d_{14}) group formation, using a linear mixed model, followed by planned comparisons, that also took into consideration the social status of the animals. There was a main effect of social status (dominant vs. subordinate vs. bystander) on T and KT but not on cortisol levels (Table 2), whereas sampling period (d_7 vs. d_{14}) was not significant for

Fig. 3. Effect of social manipulation on male reproductive behavior: (A) time spent (%) with nuptial coloration; (B) time spent (%) digging a nest; and (C) frequency of courtship. In A, the effects of treatment (stable and unstable) and social status (dominant, subordinate and bystander) were tested using a logistic model. In B, the effects of treatment (stable and unstable) and social status (dominant and bystander) were tested using a two-way ANOVA type II SS. In C, the effects of treatment (stable and unstable) and social status (dominant and subordinate) were tested using a two-way ANOVA type II SS. Significant relevant a priori planned comparisons are presented. Asterisks indicate significant differences (*, $p < 0.05$) between treatments (stable vs. unstable) for each male type, letters indicate significant difference between male types within each setting ($p < 0.05$). A standard boxplot is presented with a dark dot representing the mean value. Note that, in C, no variation was found in subordinates in stable treatment.



any of the steroids measured. There was a significant interaction between social status and sampling period for KT but not for the other two steroids (Table 2). Planned comparisons showed no significant differences between males of different social statuses for any of the measured steroids before group formation (d_7). In contrast, after group formation dominants had similar androgen levels (T and KT) to those of bystanders, and both had significantly higher androgen levels than those of subordinates (Table 2, Fig. 4). After group formation cortisol levels were lower in bystanders than in either dominants or subordinates (Table 2, Fig. 4). Planned comparisons for sampling time (d_7 vs. d_{14}) showed a significant increase in KT in dominant males and a significant decrease in KT in subordinates following group formation (Table 2, Fig. 4). Similarly there was a significant decrease in cortisol with group formation (d_7 vs. d_{14}) in bystanders (Table 2, Fig. 4). All other planned comparisons for sampling period were non-significant (Table 2, Fig. 4).

Effects of social manipulation on steroid hormone levels

The effects of social manipulation (stable vs. unstable) on steroid hormones (T, KT, cortisol) were assessed by comparing the hormone levels before and after the manipulation, using a linear mixed model, followed by planned comparisons, that also took into consideration the social status of the animals. During the social manipulation and territorial phases there was a main effect of social status on KT and cortisol levels, and a main effect of sampling time on cortisol levels (Table 3). A significant interaction was also observed between social status and sampling time for KT levels (Table 3). All other interactions among independent variables were not significant (Table 3).

Planned comparisons were computed between pre- (d_{14}) and post-manipulation (d_{20}) levels for each treatment (stable, unstable) irrespective of social status. Only T levels increased from pre- (d_{14}) to post-manipulation (d_{20}) in the unstable but not in the stable treatment (Table 3). For KT and cortisol there were no significant differences between pre- (d_{14}) and post-manipulation (d_{20}) levels for any of the treatments (stable, unstable) (Table 3).

To further assess the relative roles of social status (dominant vs. subordinate vs. bystander) and sampling time (before manipulation = d_{14} vs. after manipulation = d_{20}) other planned comparisons were also computed between the relevant pairs of variables (see Table 3). Significant differences between the stable and the unstable treatments for any social status (dominant, subordinate, bystander) were only observed at d_{20} where T levels were lower in subordinate males of the unstable group than the ones of the stable group (Table 3, Fig. 5A). Significant differences in hormone levels between males of different social statuses were observed at d_{14} between the following pairs: T: dominant > subordinate in unstable groups; KT: dominant = bystander > subordinate in unstable groups and dominant > subordinate in stable groups; and cortisol: dominant = subordinate > bystander in unstable groups (Table 3, Fig. 5). At d_{20} significant differences were observed between the following pairs: T: dominant > subordinate in unstable groups and KT: dominant > bystander in stable groups (Table 3, Fig. 5). The variation between hormone levels before (d_{14}) and after (d_{20}) the social manipulation was only significant for androgens in subordinate males, where an increase of T was observed in the stable treatment, and an increase of KT was observed in the unstable treatment (Table 3, Fig. 5A, B).

Effects of territorial intrusion on steroid hormone levels

To assess the effects of a simulated territorial intrusion on hormone levels (T, KT, cortisol) planned comparisons were computed between

Fig. 4. Effect of group formation on hormonal levels: (A) testosterone; (B) ketotestosterone; and (C) cortisol. In A, B and C the effects of social status (dominant, subordinate and bystander) and sampling time (day 7 and day 14) were tested using a linear mixed model. Significant relevant a priori planned comparisons are presented. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$) between sampling times (day 7 vs. day 14) for each male type, letters indicate significant difference between male types within each setting ($p < 0.05$). A standard boxplot is presented with a dark dot representing the mean value.

Table 3
ANOVA analyses for hormonal level in the social manipulation phase using as categorical variables (in this order): sampling time (before, day 14, and after, day 20, the social manipulation and after territorial intrusion phase, day 21); treatment (stable and unstable); and social status (dominant, subordinate and bystander). Main effects and interaction are from a linear mixed model. Results from planned comparisons are also presented.

	T		11KT		cortisol	
	F	p-Value	F	p-Value	F	p-Value
Time	2.17	0.121	0.25	0.783	6.66	0.002
Treatment	2.77	0.105	0.75	0.391	0.11	0.742
Status	2.28	0.116	6.72	0.003	3.55	0.039
Time × Treatment	1.21	0.305	0.32	0.730	1.83	0.167
Time × Status	1.99	0.105	3.27	0.016	2.07	0.094
Treatment × Status	0.59	0.562	0.23	0.795	0.23	0.798
Time × Treatment × Status	0.82	0.518	1.03	0.399	0.36	0.833
Planned comparisons I (Time × Treatment)						
	z-Value	p-Value	z-Value	p-Value	z-Value	p-Value
St: 20-14	2.47	0.014	0.11	0.909	−0.24	0.812
Un: 20-14	0.52	0.605	−0.10	0.921	−0.63	0.528
St: 21-20	−0.70	0.487	0.80	0.426	3.50	<0.001
Un: 21-20	−0.24	0.815	0.06	0.956	1.51	0.130
Planned comparisons II (Time × Treatment × Status)						
	z-Value	p-Value	z-Value	p-Value	z-Value	p-Value
14; St: D-S	−1.14	0.256	−2.22	0.027	−0.02	0.981
14; St: B-D	0.03	0.976	−1.23	0.219	−1.67	0.095
14; St: B-S	1.12	0.262	0.90	0.367	−1.59	0.112
14; Un: S-D	−2.95	0.003	−4.09	<0.001	0.73	0.467
14; Un: B-D	−1.49	0.135	−1.27	0.203	−2.35	0.019
14; Un: B-S	1.40	0.161	2.87	0.004	−3.07	0.002
20; St: S-D	−0.47	0.639	−1.68	0.093	1.00	0.319
20; St: B-D	−1.35	0.179	−2.41	0.016	−0.88	0.381
20; St: B-S	−0.89	0.371	−0.80	0.425	−1.83	0.067
20; Un: S-D	−2.20	0.028	−1.89	0.059	1.51	0.131
20; Un: B-D	−1.45	0.147	−1.83	0.067	0.30	0.765
20; Un: B-S	0.75	0.456	0.06	0.951	−1.21	0.225
21; St: S-D	−0.33	0.744	−1.62	0.105	0.13	0.893
21; St: B-D	0.87	0.386	−1.17	0.243	0.34	0.733
21; St: B-S	1.18	0.238	0.39	0.695	0.21	0.832
21; Un: S-D	−1.19	0.234	−2.28	0.023	1.16	0.246
21; Un: B-D	−0.59	0.555	−2.38	0.018	0.34	0.734
21; Un: B-S	0.60	0.548	−0.10	0.924	−0.82	0.413
14; D: Un-St	0.69	0.492	0.29	0.770	0.64	0.523
14; S: Un-St	−0.94	0.346	−1.45	0.148	1.32	0.188
14; B: Un-St	−0.77	0.442	0.35	0.730	0.16	0.873
20; D: Un-St	−0.67	0.506	−0.53	0.595	−0.01	0.989
20; S: Un-St	−2.30	0.021	−0.67	0.502	0.42	0.676
20; B: Un-St	−0.60	0.551	0.24	0.813	1.17	0.243
21; D: Un-St	−0.30	0.765	−0.17	0.866	−1.05	0.296
21; S: Un-St	−1.11	0.266	−0.70	0.487	−0.06	0.949
21; B: Un-St	−1.73	0.084	−1.16	0.247	−1.04	0.299
St; D: 20-14	1.94	0.052	0.37	0.713	−0.86	0.390
St; S: 20-14	2.86	0.004	1.23	0.221	0.29	0.774
St; B: 20-14	−0.27	0.789	−1.49	0.137	0.07	0.942
Un; D: 20-14	−0.11	0.914	−0.94	0.350	−1.69	0.092
Un; S: 20-14	1.07	0.284	2.59	0.009	−0.80	0.427
Un; B: 20-14	0.00	0.997	−1.91	0.056	1.32	0.187
St; D: 21-20	−1.60	0.110	−0.13	0.895	2.11	0.034
St; S: 21-20	−1.37	0.169	−0.04	0.970	1.14	0.257
St; B: 21-20	1.84	0.065	1.80	0.072	3.29	0.001
Un; D: 21-20	−1.12	0.264	0.45	0.651	1.05	0.295
Un; S: 21-20	0.45	0.651	−0.08	0.937	0.65	0.518
Un; B: 21-20	0.23	0.821	−0.33	0.740	1.09	0.274

Abbreviations: T, testosterone level; 11KT, keto-testosterone level; cortisol, cortisol level; time, sampling time; status, social status; St, stable treatment; Un, unstable treatment; D, dominant status; S, subordinate status; B, bystander status; 14, sampling at day 14; 20, sampling at day 20; 21, sampling at day 21.

pre- (d_{20}) and post-territorial intrusion (d_{21}) levels for each treatment (stable, unstable) irrespective of social status.

Only cortisol levels increased from pre- (d_{20}) to post-intrusion (d_{21}) and this occurred in the stable but not in the unstable treatment (Table 3). There was no observable androgen response to simulated territorial intrusion [i.e. there were no significant differences between pre- (d_{20}) and post-intrusion (d_{21}) T or KT levels for any of the treatments (stable, unstable); Table 3].

To further assess the relative roles of social status (dominant vs. subordinate vs. bystander) and sampling time (before intrusion = d_{20} vs. after intrusion = d_{21}) planned comparisons were also computed between the relevant pairs of variables (see Table 3).

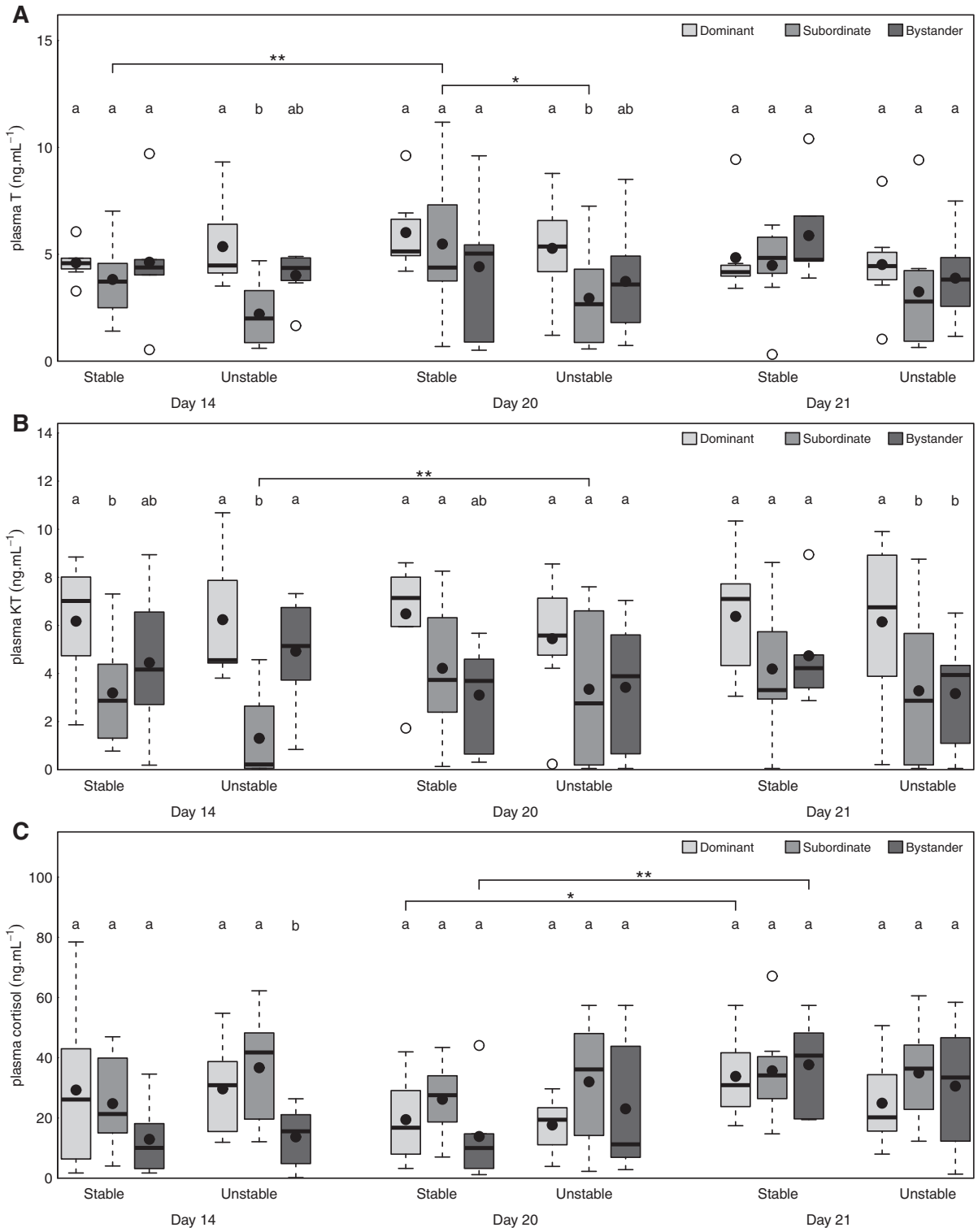


Fig. 5. Effect of social manipulation and territorial intrusion on hormonal levels: (A) testosterone; (B) keto-testosterone; and (C) cortisol. In A, B and C the effects of treatment (stable and unstable), social status (dominant, subordinate and bystander) and sampling time (day 14, day 20 and day 21) were tested using a linear mixed model. Significant relevant a priori planned comparisons are presented. Asterisks indicate significant differences (*, $p < 0.05$; **, $p < 0.01$) between sampling times (day 14 vs. day 20 and day 20 vs. day 21) and between treatments (stable vs. unstable) for each male type, letters indicate significant difference between male types within each setting ($p < 0.05$). A standard boxplot is presented with a dark dot representing the mean value.

After the territorial intrusion (d_{21}) there were no significant differences between the stable and the unstable treatments for any social status (dominant, subordinate, bystander) (Table 3, Fig. 5A). After the territorial intrusion (d_{21}) significant differences in hormone levels between males of different social statuses were only observed for KT in unstable groups (dominant > bystander = subordinate; Table 3, Fig. 5). The variation between hormone levels before (d_{20}) and after (d_{21}) the territorial intrusion was only significant for cortisol in both dominants and bystanders in the stable treatment, in which an increase in cortisol levels was observed (Table 3, Fig. 5C).

Association patterns of hormones, behavior and hormone–behavior

Effects of group formation on hormone–behavior associations

The effects of group formation on the association patterns between hormones and behavior were assessed using Pearson correlations. Before group formation (d_7) there were no significant correlations between hormonal and behavioral variables (Fig. 6A). In contrast, after group formation (d_{14}) 9 out of 15 (60%) hormone–behavior correlations became significant. KT became positively correlated with all behavioral variables, T also became positively correlated with all behaviors except courtship and there was also a marginally non-significant correlation between cortisol and male–male aggression (Fig. 6B). Therefore, group formation significantly increased the proportion of significant correlations (test for difference between two proportions: z-value $= -3.586$, $N = 15$, $p < 0.001$).

Effects of social manipulation on hormone–behavior associations

The effects of social manipulation (unstable vs. stable) on the association patterns between hormones and behavior were assessed using Pearson correlations. Hormone levels before social manipulations (d_{14}) were correlated with the majority (11 out of 21, 52.4%) of the behavioral variables, observed during the manipulation period, for the unstable but not for the stable treatments (Figs. 6C, D). KT levels before the social manipulation were positively correlated with all subsequently observed behaviors except for male–female aggression at IA, and T levels were also positively correlated with all observed behaviors except for male–female aggression both at IA and at 24 h. In contrast, at the end of the period of social manipulation (d_{20}), there was only 1 significant correlation (4.8%) between hormonal and behavioral variables (T vs. nuptial coloration), and it occurred in the unstable treatment (Figs. 6E, F). Therefore, the proportion of significant correlations significantly decreased after the social manipulations in the unstable (test for difference between two proportions: z-value $= 3.416$, $N = 21$, $p < 0.001$) but not in the stable group.

Effects of territorial intrusions on hormone–behavior associations

The effects of simulated territorial intrusions on the association patterns between hormones and behavior were assessed using Pearson correlations. Androgen levels measured before the territorial intrusion (d_{20}) were good predictors of the subsequent aggressive response towards the intruder. However, in the unstable treatment it was T that was significantly correlated with aggressive behavior (Fig. 6G) whereas in the stable treatment it was KT (Fig. 6H). After the simulated territorial intrusion (d_{21}) only KT levels in the unstable group were significantly correlated with the observed aggressive response towards the intruder.

Patterns of association between behaviors

To assess the effects of social manipulation (unstable vs. stable) on the association patterns between behaviors Pearson correlations were used. The proportion of significant correlations among behavioral variables was similar between the unstable (15 out of 28, 53.6%) and the stable treatments (13 out of 28, 46.4%) (test for difference between two proportions: z-value $= -0.535$, $N = 28$, $p = 0.60$; see Supplementary material Fig. S1). However, in an exploratory perspective we noted that the effect sizes (i.e. r-values) of the associations in the unstable treatment were generally higher than those in the stable treatment. Using conventional reference values for effect sizes of association measures (small: $r = 0.1$; medium: $r = 0.3$; large $r = 0.5$; Cohen, 1988) the stable treatment had 60.7% associations with medium or large effect sizes (i.e. $r \geq 0.3$), whereas the unstable treatment had 89.2%. This observation suggests a higher behavioral integration in the latter treatment.

Patterns of association between steroid hormones

To assess the effects of group formation and social manipulation (unstable vs. stable) on the association patterns between steroid hormones Pearson correlations were used. The two measured androgens (T and KT) were positively correlated with each other, but not with cortisol, in all sampling points (d_7, d_{14}, d_{20}, d_{21}; see Supplementary material Fig. S2). During group formation there were no significant correlations between pre- (d_7) and post-group formation (d_{14}) levels for any of the measured steroids. However, during social manipulation (d_{14} vs. d_{20}) and during territorial intrusion (d_{20} vs. d_{21}) pre- and post-manipulation hormone levels for each hormone tended to be correlated both in the unstable and in the stable treatment (see Supplementary material Fig. S2). KT was the steroid that presented the highest temporal consistency with significant positive correlations between d_{14} and d_{20} and between d_{20} and d_{21} for both treatments (unstable, stable; see Supplementary material Fig. S2). T levels were correlated across both pairs of sampling points in the unstable treatment but only between d_{20} and d_{21} (i.e. territorial intrusion phase) in the stable treatment (see Supplementary material Fig. S2). Finally, cortisol was the steroid with the highest temporal inconsistency, with a significant temporal correlation only present in the unstable treatment between d_{20} and d_{21} (i.e. territorial intrusion). The proportion of significant temporal correlations for steroid levels was not different between treatments (unstable treatment: 5 out of 6, 83.3%; stable treatment: 3 out of 6, 50%; test for difference between two proportions: z-value $= 1.225$, $N = 6$, $p = 0.22$).

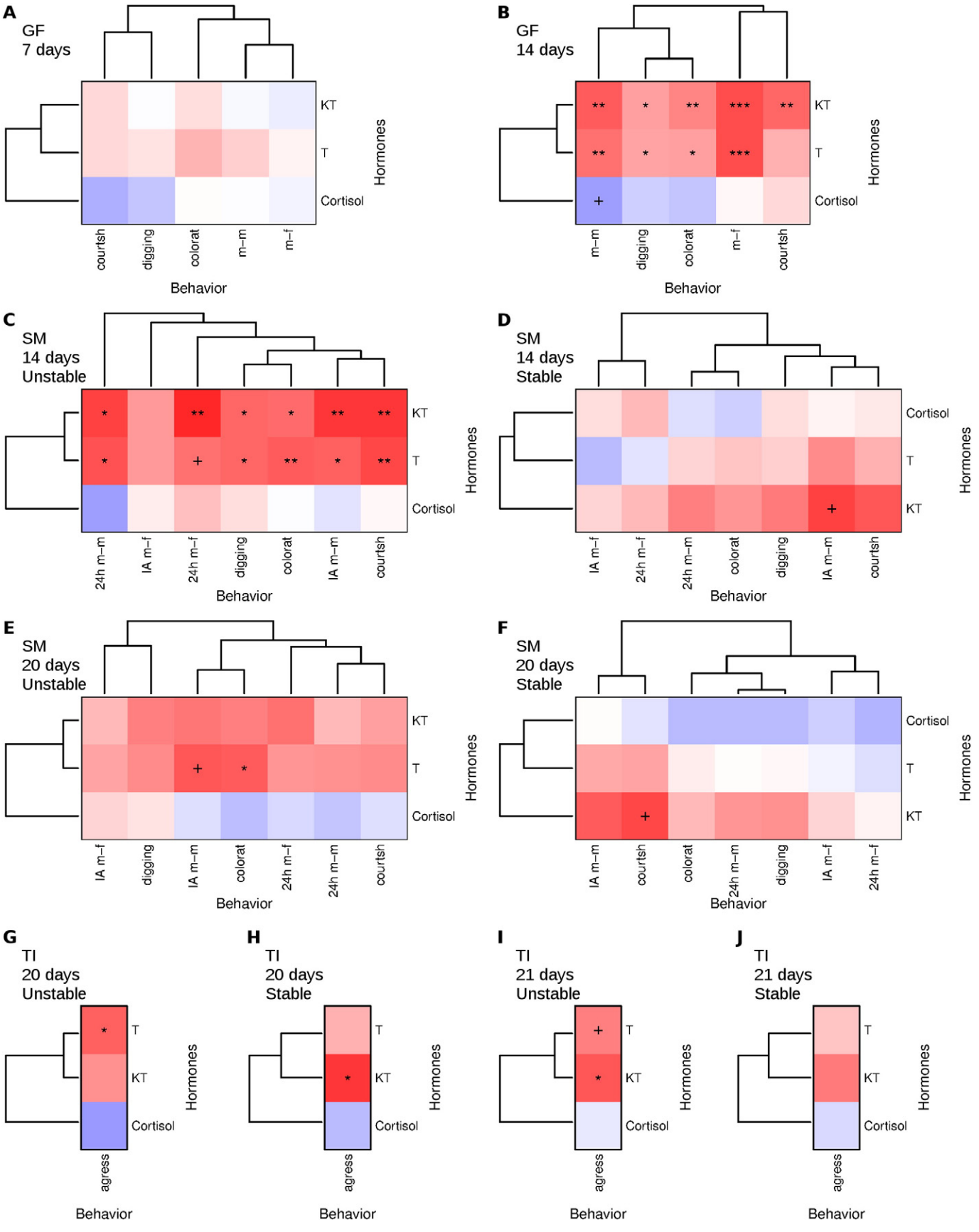
Discussion

During the group formation phase males established a dominance hierarchy and the dominant male successfully established breeding territories, dug nests and courted females. Interestingly, despite being physically separated from the group and without behavioral feedback from group members due to the one-way mirror, bystander males also build a bower and adopted the nuptial coloration. These behavioral data confirm previous studies in this species that indicate that one week is enough for the establishment of dominance hierarchies and breeding territories (Oliveira and Almada, 1998).

In response to group formation KT increased in dominant males and decreased in subordinates. In contrast, the response of cortisol to group formation was only significant in bystanders where a decrease was observed. Finally, there were no correlations between any of the steroid hormones measured before group formation (d_7) and the behavior expressed during group formation, but androgen (both KT and T) and not cortisol levels measured after group formation (d_{14}) was positively correlated with most of the behavioral variables. This pattern of androgen response to group formation in dominants and subordinates confirms previously results on this species (Oliveira et al., 1996) that found that androgen levels before group formation were not good predictors of social status achieved after group formation, but that social status achieved (dominant vs. subordinate) would predict androgen levels after group formation, with dominants having higher levels than subordinates. It is worth nothing that the similarity in these results was obtained using two different methodologies, since here we have assayed steroids directly from the plasma and in the previous study (Oliveira et al., 1996) steroids were measured from urine. Thus, urinary steroid levels in this species seem to reflect accurately the responses of circulating levels to social challenges. This pattern of androgen response to group formation also matches the prediction of the challenge hypothesis (Wingfield et al., 1990, Wingfield et al., 2001) that in response to social challenges, posed both by the presence of reproductive females and male competitors, androgens should increase from a constitutive breeding baseline, mimicked here in lab conditions by the right temperature and photoperiod, to an heightened level, hypothetically close to the physiological maximum.

Given that the rise in androgen levels above a constitutive breeding baseline is supposed to be driven by social challenges (Wingfield et al., 1990, Wingfield et al., 2001) we hypothesized that different regimes of social challenge would elicit different androgen responses. Therefore,

it was predicted that in the unstable groups, where there is a new dominant male each day that faces the challenge of re-establishing its status and creates a social opportunity for subordinate males to gain status, androgen levels would increase from pre- to post-manipulation, whereas in the stable groups, where dominant males are removed but placed back on the group, therefore not disrupting the social hierarchy,



androgen levels were not predicted to vary with manipulation. According to the same rationale it was also hypothesized that a territorial intrusion after the social manipulation phase would drive androgen levels very close to their physiological maximum and therefore the androgen responsiveness to the intrusion should be smaller in the unstable group where androgen levels would already be closer their physiological maximum, than in stable groups in which the scope for responses would still be larger.

In contrast to our first prediction, only T levels significantly changed from the pre- (d_{14}) to the post-manipulation (d_{20}) sampling point and it was in the stable, rather than in the unstable, treatment. Despite these overall negative results for the hormonal response to the social manipulation, the success of the social manipulation treatment used to create socially stable vs. socially unstable groups was confirmed by the differences in aggressive behavior observed between the two treatments. Interestingly these differences were not observed immediately after swapping the dominant males among the groups, but rather 24 h after the manipulation. This suggests that immediately after an event that promotes social instability there is a period of mutual assessment of competitive abilities by the males, only after which the asymmetries in aggressive behavior are expressed. Thus, the behavioral data indicate that animals perceived the two treatments differently, hence the lack in androgen responsiveness to the social manipulation was not due to a lack of discrimination between the two treatments. An explanation for this lack of androgen response could be that maximum physiological levels have already been achieved by the social challenges involved in group formation, and thus there was no further scope of response for the subsequent social challenges. However, a closer look at Fig. 5 suggests that overall androgen levels tend to increase from the end of group formation to the end of the social manipulation period, but that this increase is present in both the stable and the unstable treatments. An alternative explanation is that above a certain threshold of social challenge fish no longer adjust the androgen response to the level of perceived aggressiveness, and that this threshold has been achieved in both treatments. Future research, using a behavioral reaction norm approach (sensu Dingemanse et al., 2010) in which individuals are exposed to a gradient of social challenges, is needed to clarify this point.

In contrast to our second prediction, the territorial intrusion also failed to elicit an androgen response in both treatments (stable, unstable). However, an effect of the treatment was detected on the cortisol response to the territorial intrusion, with males from socially stable groups displaying a higher response. The lack of androgen response to the territorial intrusion treatment by the dominant males observed in this study contrasts with previous results in this species where socially isolated males or males kept in the presence of gravid females increased their androgen levels in response to a territorial intrusion (Hirschenhauser et al., 2004) or even to the observation of a fight between third parties (Oliveira et al., 2001), and is compatible with the social challenge threshold hypothesis proposed above according to which above a certain threshold of social challenge (achieved in this study by the group formation followed by the social manipulations both in the stable and the unstable treatments) no further scope of androgen response is available. In a reassessment of the challenge hypothesis, Goymann et al. (2007) noted that the original predictions of the challenge hypothesis referred to variation in the seasonal androgen responsiveness and that this differs from the androgen responsiveness to a social challenge alone, in a number of species. Moreover, it was noted that among birds, males from multiple brooded species tend to exhibit an androgen response to social challenges whereas males from single-brooded species lack this response despite the high seasonal androgen responsiveness. Thus, apparently in some species males may become irresponsive to social challenges, even if they still have a physiological scope of response (e.g. Apfelbeck and Goymann, 2011). As mentioned above previous studies in the Mozambique tilapia have documented male androgen responsiveness to different social stimuli, namely territorial intrusions, gravid females alone, or a territorial intrusion in males already in the presence of gravid females (Hirschenhauser et al., 2004; Oliveira et al., 2001). Taken, together these results support this hypothesis that males may become irresponsive after a certain threshold of social stimulation has been achieved.

The main focus of this study was to test if variation in the stability of the social environment could induce changes in the patterns of hormone–behavior associations. Heterogeneous environments are known to promote both phenotypic plasticity (Schlichting, 2002) and flexible correlation structures among phenotypic traits (i.e. plasticity of integration, Schlichting, 1989; Earley et al., 2012). Thus, the heterogeneity that typically characterizes the social environment, which may offer periods of social stability alternating with periods of social instability, could be viewed as promoting variation both in the degree and pattern of correlations among behavioral traits and between these and the hormones responsible for their phenotypic integration. Phenotypic integration (as expressed by the correlations among traits and between them and the physiological integrator) was predicted to increase in periods of social instability, when behavioral traits need to be co-expressed in face of the social challenges/opportunities that the individuals are face with. In contrast, in periods of social stability, characterized by the lack of social challenges/opportunities it was predicted that the correlations among behavioral traits and between them and the underlying physiological integrator (e.g. hormone) could become relaxed. The results of this study fully confirm these predictions. In the unstable treatment there was a tighter association among behaviors and between hormones and behavior as revealed by the higher proportion of significant correlations (see Fig. 6 and Supplementary material Fig. S1). In particular it was the hormone levels measured before the manipulation that were correlated with subsequent behavior and only in the unstable treatment. This result suggests that in periods of social instability steroid hormones act as phenotypic integrators that blend together a set of behaviors (aggressive and reproductive behaviors), which are more free to vary in response to various environmental factors in periods of social stability, where behavior becomes dissociated from hormones. In this respect it is also interesting to note the lack of hormone–behavior associations at the end of the social isolation period (d_7), and its appearance after the group formation phase (d_{14}), when sets of behaviors have to be co-expressed. A similar pattern of a socially-driven shift between associated and dissociated hormone–behavior association patterns has been recently described for another cichlid fish (Maruska and Fernald, 2010). In *Astatotilapia burtoni* when subordinate males are given the opportunity to establish a territory they rapidly change their behavior, expressing territorial and reproductive behaviors and increase the circulating levels of KT. However, once social stability is achieved after social ascent, aggressive behavior becomes dissociated from KT (Maruska and Fernald, 2010). Thus, the results of these two studies are highly concordant and supportive of the proposed hypothesis.

Finally, the pattern of association among steroid hormones shows overall positive correlations between the 2 measured androgens

Fig. 6. Pearson correlations between hormone levels (T, testosterone; 11KT, keto-testosterone; and cortisol) and aggressive (m–m, male aggressiveness towards males; m–f, male aggressiveness towards females; agress, male aggressiveness towards intruders) and reproductive (colorant, time in nuptial coloration; digging, time digging; courtsh, courtship towards females) behaviors: (A) behavior during group formation (GF) and hormone levels at day 7; (B) behavior during group formation (GF) and hormone levels at day 14; (C) behavior during social manipulation (SM) and hormone levels at day 14 in unstable treatment; (D) behavior during social manipulation (SM) and hormone levels at day 14 in stable treatment; (E) behavior during social manipulation (SM) and hormone levels at day 20 in unstable treatment; (F) behavior during social manipulation (SM) and hormone levels at day 20 in stable treatment; (G) behavior during territorial intrusion (TI) and hormone levels at day 20 in unstable treatment; (H) behavior during territorial intrusion (TI) and hormone levels at day 20 in stable treatment; (I) behavior during territorial intrusion (TI) and hormone levels at day 21 in unstable treatment; and (J) behavior during territorial intrusion (TI) and hormone levels at day 21 in stable treatment. Color scheme represents correlation values from −1 (blue) to 1 (red). Asterisks indicate significant correlations after p-value adjustment (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

(T and KT) but not between these and cortisol, suggesting an independent functioning between the reproductive and stress axis.

In summary, the results presented here support the proposed hypothesis that the role of androgens on phenotypic integration and independence may vary with the stability of the social environment. Future research should explore the mechanisms that allow this temporal variation in the hormone–behavior association patterns, such as differential expression of steroid receptors in relevant brain regions underlying aggressive and reproductive behaviors that may allow the compartmentalization of steroid effects.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2014.05.007.

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