

# Intra- and interspecific challenges modulate cortisol but not androgen levels in a year-round territorial damselfish

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## ABSTRACT

Interactions between individuals of different species are commonplace in animal communities. Some behaviors displayed during these interspecific social interactions may be very similar to those displayed during intraspecific social interactions. However, whether functional analogies between intra- and interspecific behaviors translate at the proximate level into an overlap in their underlying endocrine mechanisms remains largely unknown. Because steroids both mediate social behaviors and respond to them, we approached this question by comparing the behavioral and steroid response of free-living dusky gregories (*Stegastes nigricans*) to standardized territorial intrusions (sTI) of either conspecific or heterospecific food competitors. *Stegastes nigricans* is a year-round territorial fish that 'cultivates' the algae on which it feeds and is highly aggressive to both intra- and interspecific intruders. Behavioral differences between intra- and interspecific aggressive responses to sTI were marginal, and sTI tests caused an increase in cortisol levels that was positively related with the levels of aggression. In contrast, androgen levels did not increase in response to sTI, yet they showed a positive relationship with agonistic behavior. These results parallel a pattern that was first described for year-round territorial bird species. Furthermore, they suggest that changes in endocrine-hormone levels during territoriality might be independent of the species that induces the territorial response.

**KEY WORDS:** Teleost, Testosterone, 11-ketotestosterone, Cortisol, Territorial aggression, Interspecific behavior

## INTRODUCTION

Animals live in competitive social environments in which each behavioral response may have important fitness costs or benefits. To manipulate their social environment, individuals frequently use competitive agonistic behaviors including overt aggression (Wingfield et al., 2006; Székely et al., 2010). These aggressive behaviors can be classified based on the type of tactic the individual is using (e.g. reactive versus proactive situations) and on the context (e.g. spatial, sexual, parental, antipredator) in which they are expressed (Koolhaas et al., 1999; Wingfield et al., 2006). The context of aggression has been postulated to have important influences on the evolution of the underlying hormone-behavior relationships as in the commonly found associations between gonadal hormones such as testosterone and estradiol and aggressive behaviors during sexual competition, and between stress hormones such as cortisol and defensive aggression in response to threats

(Wingfield et al., 2006). These relationships can act both ways in that social challenges modulate the release of sex or stress steroid hormones, while the release of these hormones in turn facilitates different behavioral responses [i.e. the reciprocal model or challenge hypothesis (see Wingfield et al., 1990; Hirschenhauser and Oliveira, 2006; Oliveira and Gonçalves, 2008)]. Although these relationships may be species-specific and dependent on age and social context (Blumstein et al., 2010; Koolhaas et al., 2010), the degree of variation in sex and stress steroid hormone concentrations has been used to extract information about the responsiveness of individuals to changes in their social environment (Wingfield et al., 1990; Kotschal et al., 1998; Hirschenhauser et al., 2010).

Periods of stress, for example during attacks by potential predators (mostly interspecific), and periods of social instability, for example during reproductive competition (mostly intraspecific), are amongst the most acute situations in which animals modulate their levels of steroid hormones (Wingfield et al., 1990; Goymann, 2004; Goymann, 2009). This social modulation is adaptive because steroid hormones facilitate fight or flight responses by regulating allocation of energy resources and by priming of steroid-hormone-sensitive brain areas; such effects of steroid hormones potentially trade off with other traits such as immunity and brood care; and social modulation therefore reduces such potentially costly effects by restricting elevated steroid levels to the periods that are most challenging (Folstad and Karter, 1992; Wingfield et al., 2001; Ros et al., 2004; Oliveira, 2005; Earley and Hsu, 2008). Suppressing the effect of steroid hormones through the use of hormone blockers, castration or adrenalectomy may result in subtle to major failures to give appropriate behavioral responses during challenging periods (Sapolsky et al., 2000; Adkins-Regan, 2005). Social modulation of steroid-hormone production by endocrine organs is regulated by tropic hormones (adrenocorticotropin, gonadotropin) that are released by the hypothalamus-pituitary axis into the blood. In turn, steroid hormones assert most of their behavioral effects after binding to receptors in specific brain areas, which in turn modulate neuropeptide production (Adkins-Regan, 2005). Such genomic pathways to modulate behavior are relatively slow, acting on a time frame of several minutes to hours, and they are therefore thought to play a more important role in the behavior and physiology shown in the period following behavioral challenges rather than during social challenges (Ros et al., 2002; Oliveira et al., 2009; Schlinger and Remage-Healey, 2012).

In comparison with what is known about the role of steroid hormones in intraspecific interactions, there is little known regarding the role of steroid hormones in interspecific interactions. Based on Huntingford (Huntingford, 1976), Vulliou et al. (Vulliou et al., 2013) postulated that in shaping the relationships between steroid hormones and behavior, the context in which a behavior is shown plays a more important role than the species to which these behaviors are directed. This prediction is in line with the recently proposed 'finite state machine' theory, which emphasizes that

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hormonal regulation of behavior varies with life history state and the context in which a behavior is shown (Wingfield et al., 2006). In a pioneering study, Owen-Ashley and Butler (Owen-Ashley and Butler, 2004) showed that testosterone might be involved in interspecific conflicts in birds. Vulliouid et al. (Vulliouid et al., 2013) tested this possible effect of interspecific conflicts on androgens in the dusky gregory, *Stegastes nigricans* (Lacépède 1802). This sexually monomorphic damselfish holds solitary year-round territories in small colonies within coral reefs (Karino and Kuwamura, 1997). They live from the algae growing in their territory, which both sexes fiercely defend against any species that passes (Ceccarelli, 2004; Hata and Umezawa, 2011). As long as animals are not reproductively active (females spawn in male territories), the territory serves similar functions in males and females, and both intra- and interspecific intrusions may convey a similar threat. In the context of this non-sexual territoriality, Vulliouid et al. (Vulliouid et al., 2013) demonstrated that simulated territorial intrusion of both inter- and intraspecific individuals did not modulate the concentration of testosterone and 11-ketotestosterone, the two principal androgens in teleosts (Borg, 1994; Knapp, 2003; Oliveira and Gonçalves, 2008). However, a blocker of androgen receptors (flutamide) suppressed aggressive behavior in males, and in agreement with their hypothesis, this effect was independent of the species that was used as the experimental intruder (Vulliouid et al., 2013).

As interspecific interactions may include competitive and therefore stressful aspects, they may be associated with steroid-hormone modulation. For example, Soares et al. (Soares et al., 2012) showed that the stress response (measured by the production of cortisol) of cleaning gobies was higher in individuals interacting with potential predators than in individuals that were interacting with non-predatory species. Stress hormones might also play a role in territoriality, although all studies that we are aware of measured this intraspecifically (Emerson and Hess, 2001; van Duyse et al., 2004; Wingfield et al., 2006; Landys et al., 2010). Finally, stress hormones can act to either enhance (Schoech et al., 1999; Emerson and Hess, 2001; Castranova et al., 2005) or suppress (Foo and Lam, 1993; Consten et al., 2002) androgens. Therefore, to obtain a more complete picture of the relationship between steroid hormones and intra- and interspecific territoriality, we measured both androgens and cortisol in the present study in free-living animals. We first examined territorial behavior of *S. nigricans* and then experimentally tested whether intra- and interspecific intrusions affect plasma levels of these steroid hormones in the natural habitat of this tropical year-round territorial teleost species.

## RESULTS

### Description of baseline territorial interactions

Both intra- and interspecific interactions were frequently observed during baseline observations (i.e. fishes passing the territory boundaries). All but one observed individual ( $n=38$ ) interacted with neighboring conspecifics with on average  $8.7\pm 1$  interactions per 15 min. A total of  $52.7\pm 4.5\%$  of all aggressive interactions were directed towards interspecific intruders, with a mean of  $2.7\pm 0.2$  different intruding species per 15 min observation. Most of the interspecific interactions were directed towards *Dascyllus aruanus*, *Thalassoma klunzingeri* and *Gomphosus caeruleus* (in 76%, 71% and 58%, respectively, of the observations the focal individuals interacted with these species). Other species with which the focal individuals interacted were: *Acanthurus nigrofuscus*, *Canthigaster margaritata*, *Chaetodon auriga*, *Chaetodon austriacus*, *Chaetodon fasciatus*, *Chaetodon trifascialis*, *Chrysiptera unimaculata*,

*Labroides dimidiatus*, *Neoglyphidodon melas*, *Ostracion cyanurus*, *Plectroglyphidodon leucozonus*, *Pomacentrus sulfureus*, *Pterois miles*, *Siganus luridus* and *Pomacentrus aquilus* (all in less than 10% of the observations).

Intraspecific interactions were more diverse in behavioral responses than interspecific interactions, as the former often had an agonistic display or courtship component, whereas behavior in interspecific interactions was predominantly aggressive (incidence of aggression:  $55\%\pm 4\%$  versus  $98\%\pm 1\%$ ; Wilcoxon paired sample test:  $W=8$ ,  $n=29$ ,  $P<0.001$ ). Although fish responded to all intruding fish species with aggression, we found a significant negative relationship between trophic level and aggressive responses (Spearman:  $\rho=-0.55$ ,  $n=18$ ,  $P=0.018$ ), indicating that focal individuals were more aggressive to herbivores than to mesopredators.

### Effect of intruder simulations on steroid-hormone levels

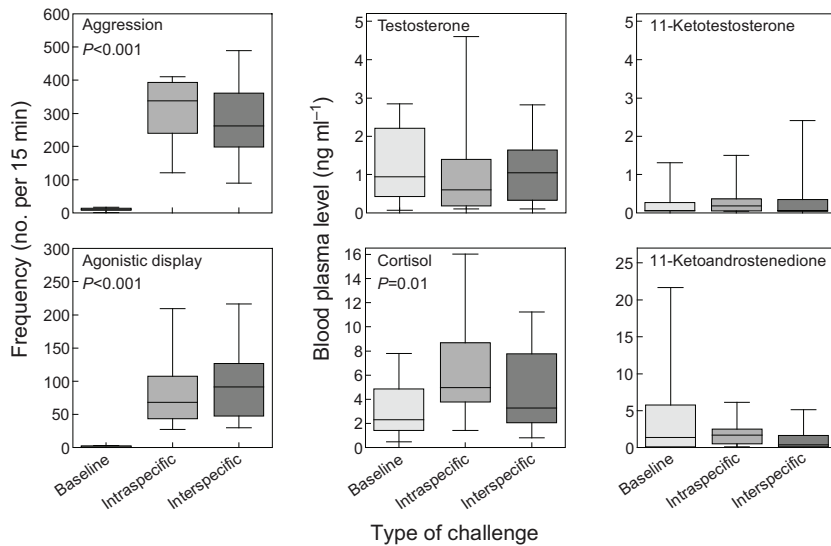
For the analyses, we only considered cases where the mass spectrometry results had a high signal-to-noise ratio. Both testosterone and cortisol noise levels were low and we could dose 78 out of 79 samples. For 11-ketotestosterone, measurement noise levels were relatively high and we could dose 46 out of 79 values for the analyses. The plasma levels of testosterone showed a significant positive relationship with the levels of 11-ketotestosterone [Spearman:  $\rho=0.60$ ,  $n=45$  (one outlier),  $P<0.001$ ], and thus results for testosterone were likely valid for 11-ketotestosterone as well.

Baseline blood samples showed no significant relationships between steroid-hormone levels (androgen or cortisol) and social behavior (total agonistic and courtship displays) (Spearman:  $n=23$ , all correlations:  $P>0.44$ ). Furthermore, levels of testosterone and 11-ketotestosterone did not show significant relationships with cortisol levels [Spearman: testosterone,  $\rho=0.16$ ,  $n=78$ ,  $P=0.15$ ; 11-ketotestosterone,  $\rho=0.11$ ,  $n=45$  (one outlier),  $P=0.47$ ].

During intra- and interspecific standardized territorial intrusion (sTI) tests, focal aggressive behavior increased more than 30-fold over the level of aggression that was observed during baseline observations [sTI:  $385.5\pm 21.1$  versus baseline:  $12.4\pm 1.1$  (means  $\pm$  s.e.m.); Mann–Whitney  $U$ -test:  $U=0$ ,  $n_1=46$ ,  $n_2=38$ ,  $P<0.001$ ; Fig. 1). Aggressive behavior did not differ between sTI tests of intra- or interspecific fish [ $399.5\pm 26.1$  versus  $372.6\pm 32.9$  (means  $\pm$  s.e.m.); Mann–Whitney  $U$ -test:  $U=226$ ,  $n_1=22$ ,  $n_2=24$ ,  $P=0.41$ ; Fig. 1].

Testosterone and 11-ketotestosterone levels of the focal individuals after the sTI tests did not differ from the steroid-hormone levels measured after baseline observations (Kruskal–Wallis test: testosterone,  $\chi^2=1.19$ , d.f.=2,  $P=0.55$ ; 11-ketotestosterone,  $\chi^2=1.62$ , d.f.=2,  $P=0.45$ ; Fig. 1). However, cortisol levels were higher after sTI tests in comparison with cortisol levels after baseline territorial interactions ( $\chi^2=9.13$ , d.f.=2,  $P=0.010$ ; Fig. 1). More specifically, intraspecific sTI tests significantly increased cortisol levels in comparison with baseline levels (Mann–Whitney  $U$ -test:  $U=191$ ,  $n_1=31$ ,  $n_2=23$ ,  $P=0.003$ ; Fig. 1). Cortisol levels following interspecific sTI tests tended to be higher than after baseline territorial interactions, but were not statistically different from levels following intraspecific sTI tests (Mann–Whitney  $U$ -test: baseline versus interspecific sTI,  $U=284.5$ ,  $n_1=31$ ,  $n_2=23$ ,  $P=0.14$ ; intra- versus interspecific sTI,  $U=196$ ,  $n_1=24$ ,  $n_2=23$ ,  $P=0.091$ ; Fig. 1).

Although testosterone levels did not differ significantly between individuals used for baseline sampling and individuals sampled following sTI tests, the levels of testosterone were positively related to the frequencies of aggression and agonistic display (Spearman:



**Fig. 1. Boxplots of territorial behaviors (aggression and agonistic display) and steroid-hormone levels during baseline and simulated territorial intrusion tests.** Tests consisted of 15 min baseline observations (light gray bars) or 15 min exposure to a tube with an intraspecific (medium gray bars) or an interspecific intruder (dark gray bars). Each box depicts the median  $\pm$ 25% quartiles; the whiskers indicate the minimum to maximum range of the data.

aggression,  $\rho=0.43$ ,  $n=45$ , adjusted  $P=0.010$ ; agonistic display,  $\rho=0.60$ ,  $n=45$ , adjusted  $P<0.001$ ; Fig. 2). No such relationship was found for 11-ketotestosterone (Spearman:  $\rho<0.23$ ,  $n=28$ , adjusted  $P>0.23$ ; Fig. 2). In contrast, cortisol levels showed a highly significant relationship with aggression and a nearly significant relationship with agonistic display following sTI tests (Spearman: aggression,  $\rho=0.55$ ,  $n=45$ , adjusted  $P<0.001$ ; agonistic display,  $\rho=0.33$ ,  $n=45$ , adjusted  $P=0.053$ ).

An ANCOVA testing the relationship between testosterone and all behavior showed that testosterone levels were primarily related to agonistic display shown during sTI tests ( $F_{1,41}=18.53$ ,  $P<0.001$ ), whereas the frequency of aggression and the type of sTI test (intra- or interspecific) did not show a significant relationship ( $F_{1,41}<1.05$ , d.f.=1,41,  $P>0.31$ ). For cortisol, this analysis showed a significant relationship with aggression (aggression,  $F_{1,41}=5.66$ ,  $P=0.022$ ), but

not between cortisol levels and the type of sTI test (agonistic display and type of intrusion,  $F_{1,41}=1.11$ ,  $P>0.15$ ).

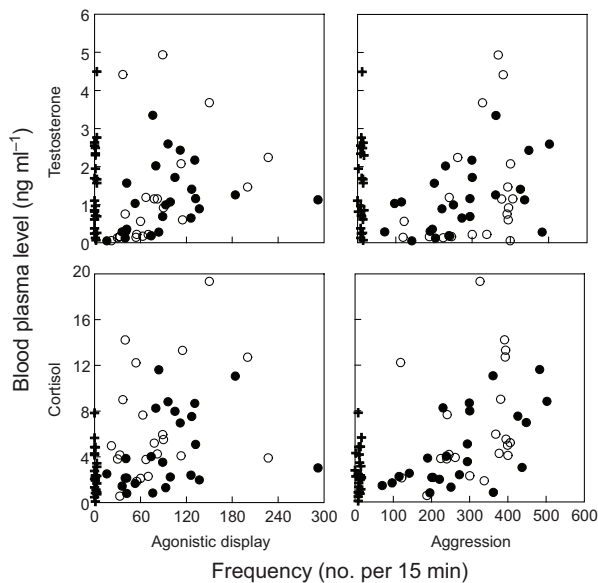
## DISCUSSION

Free-living *S. nigricans* responded aggressively to both intra- and interspecific simulated territorial intrusions. Experimental simulation of these intrusions (sTI tests) did not modulate plasma levels of testosterone and 11-ketotestosterone. However, cortisol levels were significantly higher following simulated intrusions than following baseline sampling, and were significantly correlated with the level of aggression displayed during sTI. No significant differences in cortisol levels were found between intra- and interspecific sTI. These results suggest that cortisol plays a role in territorial aggression of year-round territorial teleost species.

### Steroid-hormone levels and non-reproductive territorial aggression

Few studies have tested the impact of social challenges in teleost species, and most of these studies found a positive relationship between these challenges and androgen levels (reviewed in Oliveira and Gonçalves, 2008). Social challenges have been described to result in increased levels of both testosterone (Hannes, 1985; Hay and Pankhurst, 2005; Desjardins et al., 2006) and 11-ketotestosterone (Hirschenhauser et al., 2004; Desjardins et al., 2006), although the response of both hormones is not always identical (Hirschenhauser et al., 2004; Hay and Pankhurst, 2005). Furthermore, the outcome of interactions appears to play an important modulatory role on androgen levels (Neat and Mayer, 1999; Earley and Hsu, 2008; Oliveira et al., 2005). Thus we were interested in the responses to sTI in both testosterone and 11-ketotestosterone. Unfortunately, because of a low signal-to-noise ratio in the 11-ketotestosterone analyses using UHPLC-MS/MS, we obtained a low sample size for this steroid hormone (we now know that changing to solid phase extraction and partitioning of the extract with ammonia increases the sensitivity of the analysis; G. Glauser and A.F.H.R., unpublished results). However, because within our samples both 11-ketotestosterone and testosterone showed a significant positive relationship, the results obtained for testosterone levels might be indicative for 11-ketotestosterone as well.

Testosterone and 11-ketotestosterone levels did not increase in response to sTI tests in the field. This result is consistent with results of Vullioud et al. (Vullioud et al., 2013), who performed sTI-like



**Fig. 2. Scatterplots of significant relationships between territorial behaviors (aggression and agonistic display) and steroid-hormone levels (cortisol and testosterone) during baseline and simulated territorial intrusion tests.** Tests consisted of 15 min baseline observations (crosses) or 15 min exposure to a tube with an intraspecific (open symbols) or an interspecific intruder (closed symbols).

tests on *S. nigricans* in a controlled laboratory setting. Interestingly, they showed that an androgen antagonist (flutamide) suppresses aggressive responsiveness of male resident fish. Our current results now demonstrate that individual variation in testosterone levels showed a positive relationship with territorial behavior during sTI tests, particularly with displays, in free-living *S. nigricans*. Although a single resident intruder interaction is not sufficient to modulate testosterone levels, some elevation in baseline androgen levels might be involved in facilitating territorial aggression. Further, it would be interesting to study whether androgens that are of non-gonadal sources would play a role in non-reproductive territorial behavior, such as production of androgens in the brain, as has been found for bird species (Schlinger and Remage-Healey, 2012).

Although we found a positive relationship between testosterone levels and aggression during sTI tests, the relationship was non-significant during baseline interactions. This might be explained by the difference in duration between the responses displayed under normal situations (i.e. baseline) and during sTI. Baseline territorial interactions involve quick aggressive chases that keep most intruders at bay (Jan et al., 2003). Moreover, many such intra-specific interactions in our observations were with neighboring individuals. In vertebrates in general, neighbors are known to evoke less aggression than strangers, and this has been described as the ‘dear enemy’ effect (e.g. Temeles, 1994). In the dusky gregory, however, neighbors provoke more aggression than stranger conspecifics when presented in a territorial intrusion test (Jan et al., 2003). Nevertheless, such different challenges (neighbor versus stranger) might constitute different threat levels and thereby affect hormonal systems in different ways. Until now, not much was known about the hormonal correlates of such neighbor effects (Oliveira, 2005). In contrast to most baseline interactions, sTI tests expose territory holders to a persistent challenger because the intruder cannot leave. Although a situation in which a challenger persists is rare in nature for gregories (Jan et al., 2003), such a situation might occasionally occur, for example, during territory establishment. A result of a persistent challenger is that aggression may escalate, and measures of persistence or vigor in fighting are expressed more clearly (e.g. Payne, 1998; Ros et al., 2004; Wingfield et al., 2006). As such, the frequency of aggression may measure fighting ability and motivation during escalated fighting in sTI tests, but during short baseline interactions such a measure would yield too little variation.

The results we obtained for androgen levels in *S. nigricans* are consistent with the patterns described for year-round territorial birds (Wingfield and Monk, 1994; Canoine and Gwinner, 2002; Hau et al., 2004; Hau et al., 2008). Clearly, more details about the relationship between territorial aggression and endocrine factors for a larger group of vertebrates are necessary (see Wingfield et al., 2006). Recently, researchers have started to test the effects of social challenges on more detailed and diversified neuromodulators or chemical switches, such as steroid hormone receptors, neurotransmitters and steroid hormones synthesized in the brain (Soma et al., 2008; Schlinger and Remage-Healey, 2012). This has important consequences on our understanding of how organisms adjust the expression of social behaviors to the social environment. These new approaches move away from the idea of simple pleiotropic regulation of multiple traits in which a singular mechanism (i.e. a blood-born steroid hormone) coordinates both peripheral and central adaptations to a stimulus. Alternatively, one might reverse this argument and propose that system-wide regulation of energy allocation and behavioral activation is only favorable in situations of extreme fluctuation in social competition, as occurs at the start of the reproductive season.

### **Cortisol and non-seasonal territorial responses**

In contrast to androgen levels, cortisol levels increased after intra- and interspecific challenges, and we found a strong positive relationship between cortisol concentration and aggression. This suggests that cortisol plays a role in non-reproductive territorial aggression in our study species. The increase in cortisol levels might be explained within the ‘allostatic framework’ (McEwen and Wingfield, 2003; Creel et al., 2013), in which it is postulated that glucocorticosteroids are produced when external challenges are too demanding to sustain general homeostasis. These increased levels of glucocorticosteroids may alter physiology and behavior in order to reach a new state of homeostasis (Donaldson, 1981; McEwen and Wingfield, 2003). Higher levels of territorial activity demand higher energy turnover and thus increase allostatic load. Indeed, cortisol in fishes has been shown to modulate energy metabolism and general activity (Arends, 1999; Barton, 2002; Leong et al., 2009; Vijayan et al., 1997). This homeostasis-through-change framework may facilitate switches in energy allocation and thereby support the escalated territorial behavior induced by sTI tests.

Here we found a positive correlation between cortisol levels and aggression towards the intruder challenge. The relationship between aggression and glucocorticoids is variable between species, and a recent review has emphasized the role of predictability of the social environment on variation in glucocorticoid levels (Creel et al., 2013). In animals with year-round territories, aggressive responses to intruders are part of the daily routine. In such situations, testosterone responses might be suppressed whereas escalated territorial challenges might still provoke stress-hormone responses, as was shown by Landys et al. (Landys et al., 2010) for nuthatches (*Sitta europaea*). Munro and Pitcher (Munro and Pitcher, 1985) showed that cichlid fish (*Aequidens pulcher*) treated with cortisol were more aggressive to a model intruder. This suggests that cortisol modulation caused by a territorial dispute may increase the responsiveness of a territory holder to future social challenges. It remains to be studied whether such effects of increased stress-hormone levels are specific for aggressive behavior or whether these have general effects on activity. Either way, social modulation of cortisol levels might play an additional role next to the effects of social modulation of androgen levels as described in the challenge hypothesis.

### **Mechanisms for interspecific territorial behavior**

One of our main questions is whether intra- and interspecific challenges have similar effects on the release of steroid hormones (see Vulllioud et al., 2013). By studying year-round territoriality in *S. nigricans* during the non-reproductive season, we excluded the confounding effect of sexual motivation on the behavioral responses. Resident *S. nigricans* in this study were aggressive to most species that came close to their territory, and even attacked novel objects (P.V. and A.F.H.R., unpublished observations). This pattern differs from related gardening species that are more selective in their aggressive responses, attacking mainly herbivorous fishes (Di Paola et al., 2012; Ebersole, 1977; Myrberg and Thresher, 1974). Still, in relative terms they were most aggressive to herbivores, which are the main competitors for the algal turf they are defending.

Vulllioud et al. (Vulllioud et al., 2013) compared the territorial responses to conspecifics and to a related herbivorous species (jewel damselfish, *Plectrogllyphidodon lacrymatus*) and found no significant differences in androgen-dependent territoriality towards standardized simulated intrusions to the different species. Here, we found that the cortisol modulation to an intra- and interspecific territorial intrusion did not differ. Thus both the androgen-hormone

regulation (Vulllioud et al., 2013) and the social modulation of steroid-hormone levels (present study) of territorial responses in the non-reproductive period seem to be similar across intra- and interspecific situations. This result supports the proposition that steroid-hormone behavior relationships are dependent on context (e.g. territoriality), rather than on the species against which the behavior is directed (intra- or interspecific intruders).

## MATERIALS AND METHODS

The study was carried out from August to November 2011 in the Gulf of Aqaba, Dahab, Egypt. All fish were studied by SCUBA diving on a shallow coral reef flat south of Dahab (Napoleon reef). We selected territorial *S. nigricans* from within 12 different colonies, and switched colonies after each observation (corals containing colonies were approximately 5 m apart). Individuals were never tested twice. In total, we collected 87 focal observations, each lasting 15 min [baseline observations:  $n=38$ ; intraspecific territorial intrusion tests:  $n=25$  (see below for details); interspecific specific territorial intrusion tests:  $n=24$ ]. Colonies consisted of ~20 individuals each and we sampled approximately one-third of the individuals of each colony. We used a diameter of 2 m as the area in which the focal territory holders could respond to trespassing species during baseline observations and the intruder tests (see below). Each individual passing that area was considered in interaction with the nest-holder. The 2 m diameter was chosen because *S. nigricans* are known to attack intruders at a distance around the center of the territory of 0.6 to 2.8 m (Jan et al., 2003), and this area generally did not overlap with territories of other individuals.

### Baseline territorial interactions

Observations were performed while SCUBA diving at approximately 3 m from the center of the focal individual's territory. The fish were allowed to habituate to the presence of the observer for 5 min. Subsequently, for 15 min we recorded all species that passed within 1 m of the center of the territory and all the behavioral responses of the focal individual to these intruding fish (Di Paola et al., 2012). FishBase (Froese and Pauly, 2011) was consulted as an independent source for collecting data on trophic levels of the different species (Pauly and Palomares, 2000). Here we used trophic level as an estimate of whether a species might compete for food with the herbivorous *S. nigricans*. The following behavioral responses of the focal individuals were recorded: aggression (the sum of mouth contact, chases and accelerated swimming directed towards the intruder); agonistic display [the sum of threat displays (postures with opercular spread) and tail beats directed towards the intruder]; and courtship display (the sum of quivering and nest-oriented behavior) (Myrberg and Thresher, 1974; Karino, 1993; Karino, 1995; Di Paola et al., 2012; Vulllioud et al., 2013). Overt sexual behavior was not observed during the study period.

### Simulated territorial intrusion tests

We measured the effect of standardized intra- and interspecific territorial intruder tests (sTI) on steroid-hormone levels and behavior of territory holders as follows. First, we captured one territorial *S. nigricans* for an intraspecific sTI, or a territorial *Plectroglyphidodon lacrymatus* for an interspecific sTI. Then we selected a territory holder out of sight of the capture event for the sTI test. Sizes of these territory holders showed little variation and overlapped between treatments (range 9.4–11.8 cm total length,  $P>0.10$ ). Sizes of intruders were not measured, but we took steps to ensure that these sizes represented the normal variation by selecting median-sized individuals: total lengths were approximately 10 cm for *S. nigricans* and approximately 8 cm for *P. lacrymatus*.

As during baseline territorial intrusions, the territory holder was habituated for 5 min to the presence of the observer 3 m away from the center of the territory. Subsequently, the intruder was placed in a Plexiglas tube in the center of the territory, and the observer returned to their former position. The Plexiglas tube was transparent and perforated to allow the exchange of both visual and chemical cues. The observation started directly after the first behavioral response of the focal individual towards the intruder. All behavioral responses of the focal individual were recorded for a period of 15 min after the start of the observation. After the observation

period, the tube was removed, and we waited 3 min before capturing the focal individual to draw a blood sample. This delay was included because positive information on a contest outcome (i.e. the retreat of the intruder) might be required to trigger an increase in androgen levels (Oliveira et al., 2005) and is identical to a previous study (Vulllioud et al., 2013). In the present study, we additionally aimed to measure variation in cortisol levels. Cortisol release from interrenal cells in fishes has a lag time of several minutes (Barton, 2002). In fishes living in tropical waters, cortisol levels increase in response to exposure to stressors after a lag time of 2.5 to 10 min (Pankhurst, 2011). Blood samples were taken within 2 min of capture and thus capture stress is expected to have no effect on the circulating cortisol levels. Because blood samples were taken within 5 min of the end of the observation period, and because all handling of animals was standardized after the observation period, we expected cortisol levels to represent levels related to the experimental procedures during the 15 min observation period.

### Blood sampling and analysis of androgen levels

All handling of fish was carried out underwater. Each fish was placed in a plastic zip lock bag containing seawater and anesthesia at a concentration of  $0.5 \text{ ml l}^{-1}$  2-phenoxyethanol (Koi Med Sleep, Schönbach Apotheke, ABlar, Germany). Reaching the first signs of anesthesia (weak to no response to touch but still breathing) took approximately 1 min and the depth of anesthesia was controlled by changing the 2-phenoxyethanol concentration by adding water. While under anesthesia, a small blood sample (0.2 ml) was drawn from the caudal vasculature using a heparinized 25-gauge needle and syringe. Before recovering the fish by repeatedly refreshing the water in the zip lock bag, the fork size of the fish was measured from the tip of the mouth to the fork of the tail to the nearest millimeter. For sex determination of these fish, microscopic inspection of the genital papillae is needed, and thus sex could not be established reliably in the field (Vulllioud et al., 2013).

Blood was directly transported to the laboratory, where it was centrifuged at 500 g for 5 min. Plasma was collected and stored at  $-20^{\circ}\text{C}$  until analysis. The free steroid-hormone fraction was extracted from the plasma using a double liquid-liquid extraction of 100  $\mu\text{l}$  plasma (with addition of 400  $\mu\text{l}$  ultrapure  $\text{H}_2\text{O}$ ) and 5 ml 75% di-ethylether and 25% hexane (HPLC grade). The extracts were evaporated under vacuum, and the steroid-hormone residues were suspended in 80  $\mu\text{l}$  of 50% methanol/50%  $\text{H}_2\text{O}$  (HPLC grade/ultrapure) and kept at  $-20^{\circ}\text{C}$  until analyzed.

The analyses were optimized by ultra-high pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (Ultimate 3000 RS-Dionex, Thermo Fisher Scientific coupled to 4000 QTrap-ABSciex, MA, USA). Using the multiple reaction monitoring mode, transition specific for testosterone, 11-ketotestosterone and 11-ketoandrostenedione can be monitored, which greatly increases the sensitivity of the mass spectrometer by decreasing the background noise. The steroid-hormone fraction was reconstituted in 80  $\mu\text{l}$  50% methanol 50%milliQ  $\text{H}_2\text{O}$  and 10  $\mu\text{l}$  was injected in an Acquity BEH C18 column (1.7  $\mu\text{m}$  particle size, 2.1 $\times$ 50 mm, Waters, Milford, MA, USA) in a gradient mode with acetonitrile- $\text{H}_2\text{O}$  as an eluent and a flow rate of  $0.4 \text{ ml min}^{-1}$ . Samples that we spiked with a known quantity of testosterone or cortisol gave a 93% recovery after extraction.

### Data management and statistical analysis

As all individuals were only tested once and as we took care not to test animals that were near each other during the same day, we treat each individual as an independent sample in the statistical analysis. From the 18 different species that were seen near the territories of *S. nigricans*, seven were seen trespassing on three or more territories, and these cases were used to estimate the effect of independent ecological factors on territorial behavior. The variables we obtained were not normally distributed (Kolmogorov–Smirnov test,  $P<0.05$ ) and we carried out non-parametric tests to analyze main effects (type of sTI test) and pairwise correlations (paired sample Wilcoxon tests, Kruskal–Wallis test, and Spearman correlations using the R2.15 package) (R Development Core Team, 2012). As a *post hoc* test, to evaluate spurious effects in correlative relationships between steroid hormones and behaviors, we included the effect of aggression, agonistic display and courtship display as covariables, and type of sTI test as a fixed factor, using a parametric ANCOVA model (log–log

transformed linear model). Throughout the article all averages are shown as means with standard errors of measurement.

### Ethical issues

The reefs at the study site are protected areas with strict regulations. Therefore, we took special care to select procedures that allowed us to collect the samples with minimal impact on these reef ecosystems. We adhered to the regulations set by the Egyptian Environmental Affairs Agency (EEAA, Cairo, Egypt). We used anesthesia to reduce the potential stress of treatment, handling and sampling the fish. Following anesthesia, the fish were allowed to recover in an isolated and well-aerated plastic bag, and upon recovery they were immediately released in their own territory. Intruder fish were used for only one sTI test and were released after this test in their original territory. Before release, we chased off potential intruders, and all fish successfully reestablished themselves on their original territory. Research permits for Dahab were granted by the Suez Canal University (affiliated with NCS/EEAA, Egypt), which kept close contact with the former DMRC research center in Dahab.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

All of the authors have made significant contributions to the different phases of the study.

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