Natural endocrine profiles of the group-living skunk anemonefish *Amphiprion akallopisos* in relation to their size-based dominance hierarchy

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Group-living animals commonly display differences in behaviour, physiology and endocrine profiles between conspecifics within the group, which are tightly linked to reproduction. Teleosts exhibit a variety of social systems, where social status, as well as sex, has been linked to different androgen and oestrogen profiles. Levels of gonadal androgen and oestrogen were investigated as a function of sex and position in a social hierarchy in free-living individuals of the skunk anemonefish *Amphiprion akallopisos*, a protandrous pomacentrid fish with a size-based dominance hierarchical social system. Plasma levels of 11-ketotestosterone (11-KT), testosterone (T) and 17β-oestradiol (E2), as well as conversion ratios from T, were measured by ELISA from 111 individuals along a linear hierarchy from 38 social groups in the wild. Blood plasma levels of 11-KT and E2 showed sex differences, being higher in males and females respectively as expected based on their role as the major androgen and oestrogen in fish reproduction. However, no sex differences were found for T, which may represent its role in territorial defence or simply as a precursor for the synthesis of 11-KT and E2. In terms of the hierarchical social system within males, 11-KT levels decline as the hierarchy is descended, which may represent their decreasing reproductive opportunity, as well as the decreasing levels of aggression towards males lower in the hierarchy. In summary, the size-based dominance hierarchy is associated with distinct steroid levels of 11-KT and E2 between individual free-living *A. akallopisos* that closely resemble those of species in which breeding individuals suppress reproduction of conspecifics lower in the hierarchy.

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INTRODUCTION

Social hierarchies are widespread in nature, occurring across the Animal Kingdom in many group-living species (Drews, 1993). Social status has significant implications for fitness via access to food and reproductive opportunity, and numerous studies have been conducted to determine the behavioural and endocrinological factors mediating
differences in social status of mammals (Jarvis, 1981; Albert et al., 1986; Ziegler, 2000; Clutton-Brock et al., 2001; Mills et al., 2009), birds (Schoech et al., 1991; Khan et al., 2001; Duckworth et al., 2004), lizards (Sinervo et al., 2000; Mills et al., 2008) and fish (Taborsky & Limberger, 1981; Oliveira et al., 2002; Oliveira et al., 2003). Differences in the expression of behaviours related with dominance, as well as in secondary sexual characters and maturation of the gonads and corresponding endocrine profiles, particularly the steroids, gonadal androgens and oestrogens, are expected in social groups when reproduction is suppressed in individuals lower in the hierarchy, whereas endocrine profiles are not expected to differ in groups in which all individuals share in reproduction, such as in cooperative breeders (Creel et al., 1997).

Teleosts exhibit a variety of social systems (Sloman & Armstrong, 2002) and social status, as well as sex, has been linked to different androgen and oestrogen profiles (Ramallo et al., 2015). The fish-specific androgen, 11-ketotestosterone (11-KT), is thought to be the primary male androgen in fishes, responsible for spermatogenesis and is typically higher in breeding males compared with non-breeding males or females (Borg, 1994). 17β-oestradiol (E2), the major oestrogen in fish is vital for vitellogenesis and is higher in females (Fostier et al., 1983; Ng & Idler, 1983). Testosterone (T) levels may also be higher in reproducing compared with non-reproducing males (Kroon & Liley, 2000; Pavlidis et al., 2000), but often T levels do not differ between the sexes (Nakamura et al., 1989; Lone et al., 2001; Bhandari et al., 2003; Kroon et al., 2003). The lack of sex difference in T may be associated with the synthetic pathway of E2 as females of most gonochoristic species require T to synthesise E2 in the ovarian follicles via the aromatisation of T using P450 aromatase (CYP19) (Lee et al., 2006). However, the E2 synthetic pathway has been found to be different in some hermaphrodite species, whereby ovarian follicles synthesise E2 not from T, but from oestrone (Ohta et al., 2012). In addition, 11-KT is biosynthesised primarily via the metabolism of T using 11b–hydroxytestosterone in the testes (Kime, 1987). As both E2 and 11-KT can be synthesised from T, the conversion ratios of T to 11-KT and E2 indicate the physiological rate at which T is converted into each hormone and may provide further indications into the endocrine control of social status and sex differences.

Many social hierarchies in fish are formed based on a size-hierarchy of dominance (Brown, 1946) and the relationship between variation in circulating androgen levels and social hierarchies has been studied in laboratory as well as in free-living organisms. In species with male reproduction suppression, the relationships between social status and androgen levels are generally concordant between species, as well as between laboratory and field studies. Androgen levels (T, 11-KT and their conversion ratios) are higher in reproducing compared with non-reproducing males (Cardwell & Liley, 1991a; Cardwell et al., 1996; Oliveira et al., 2001a; Bender et al., 2008; Gonçalves et al., 2008). The only exception are the similar levels of T in both dominant and subordinate brook trout Salvelinus fontinalis (Mitchill 1814) in the wild (Cardwell et al., 1996). However, different trends in androgen levels have been found between laboratory and field studies for species where all males have the opportunity to reproduce either in cooperatively breeding species [e.g. Neolamprologus pulcher (Trewavas & Poll 1952), Astatotilapia burtoni (Günther 1894)] or in species with alternative reproductive strategies [e.g. Salaria pavo (Risso 1810), Lepomis macrochirus Rafinesque 1819]. Laboratory studies show overlapping androgen levels (11-KT, T and conversion ratios) between different males (Oliveira et al., 2003; Bender et al., 2006, 2008),
whereas field studies found higher levels of 11-KT in breeder males (Knapp & Neff, 2007; Desjardins et al., 2008; Gonçalves et al., 2008) and T levels representing all possible scenarios including overlapping levels (Desjardins et al., 2008), higher levels in parental males (Gonçalves et al., 2008) and even higher levels in satellite and sneaker males (Knapp & Neff, 2007).

This paper describes the circulating androgen and oestrogen levels of wild individuals within the size-based dominance hierarchy of anemonefish Amphiprion Bloch & Schneider 1801 (Allen, 1972; Fautin, 1992; Fautin & Allen, 1992) which has also been described as a female-control protandrous hermaphroditism (Ross, 1990). Groups of Amphiprion spp. form obligate associations with sea anemones (Actiniaria) that provide the fish with oviposition sites and protection from predators (Allen, 1972). Groups inhabiting anemones consist of a mated adult pair (female and male-functioning individuals) and typically a variable number of immature and non-reproductive individuals depending on the species, but ranging from 0–4 (Fricke & Fricke, 1977; Ross, 1978a, 1978b; Fricke, 1979; Fautin, 1992; Fautin & Allen, 1992; Godwin & Thomas, 1993; Buston, 2003a). Within each group there is a size-based dominance hierarchy; the female is largest, the male is second largest and the non-breeders get progressively smaller as the hierarchy is descended (Fricke, 1979; Buston, 2003b). Non-breeders were reported not to have functional gonads (Fricke, 1979), but they accrue direct benefits in the future along a strict queue as individuals always ascend in rank as those ahead of them are lost from the hierarchy and eventually inherit the territory within which they reside (Fricke, 1979; Ochi, 1989; Hattori, 1994; Buston, 2004b; but see Mitchell, 2005). Non-breeding male Amphiprion spp. were hypothesised to be helpers as they defend the host anemone jointly with a breeding pair (Fricke, 1979), but breeders do not accrue any fitness benefits in terms of survival, growth, reproduction or rapid mate replacement from the presence of non-breeders (Buston, 2004a).

This study investigated if the endocrine profiles in this hierarchy matched those of cooperative breeders or social groups with reproductively suppressed males. Plasma steroid profiles were examined in individuals from a wild population of the protandrous pomacentrid skunk anemonefish Amphiprion akallopisos Bleeker 1853 living in the magnificent sea anemone, Heteractis magnifica in the Indian Ocean. Hormone levels (T, 11-KT, E2 and their conversion ratios from T) were measured in 111 individuals from 38 wild anemone clusters each holding a hierarchical group of A. akallopisos. Endocrine profiles of the fish were compared as a function of sex and their position within the size-based hierarchy and also compared with those of cooperative breeders and social groups with reproductively suppressed males. The biosynthetic pathways of E2 in this species were also determined.

**MATERIALS AND METHODS**

One-hundred-and-eleven A. akallopisos from 38 anemone clusters were caught by two scuba divers using barrier and hand nets from four different sites. Each anemone cluster contained either two individuals (hereafter referred to as a pair) or a group of more than two individuals (hereafter referred to as group). Samples were taken from three of the Îles Eparses (the scattered islands): Île de Europa (Fig. 1; 22° 22.213′ S; 40° 24.140′ E; 11–24 m depth; 4 pairs and 10 groups), Île de Juan de Nova Island (17° 02.783′ S; 42° 44.551′ E; 7–15 m depth; 5 pairs and 6 groups) and Archipel des Glorieuses (11° 33.880′ S; 47° 17.562′ E; 6–18 m depth; 3 pairs and 2 groups) and off the coast of Madagascar, near Ifaty (23° 8.718′ S; 43° 35.461′ E; 3–12 m...
depth, 3 pairs and 5 groups). Blood samples of approximately 0.1 ml per fish were collected on the boat, laterally from the caudal vein held out of water using heparinised 1 ml syringes fitted with a 30 gauge needle and kept on ice until processing (Mills et al., 2010). Individual blood samples were centrifuged (Sigma Centrifuge 1–14; www.sigma-zentrifugen.de) at 10 000 g for 5 min. The supernatant, a yellow plasma layer, was collected without disturbing the white-buffy layer or the blood cells. Total length ($L_T$, ± 0.1 mm) of each fish was measured using calipers. In total, blood samples were collected from 15 breeding pairs and 23 groups (>2 individuals) of *A. akallopisos*, although hormone measures from some pairs and groups are not complete either due to the inability to catch focal fish or there being not enough plasma for all hormonal dilutions, so sample sizes differ with the hormone measured.

**HORMONE MEASUREMENTS**

Plasma testosterone (T), 11-ketotestosterone (11-KT) and $17\beta$-oestradiol ($E_2$) were measured using EIA kits (T EIA Kit, No. 582701; 11-KT EIA Kit, No. 582751; $17\beta$-oestradiol EIA Kit, No. 582251; Cayman Chemicals; www.caymanchem.com) and a Beckman Coulter AD 340
Spectrophotometer (www.beckman.com) at 405 nm as described in Mills et al., (2010) after validation with parallel displacement of serially diluted plasma to the standard curve and determination of intra and inter-assay variabilities (Supporting Information). Five dilution set ratios were used for validation of T and E₂ ranging from 1:2.5, 1:6, 1:17, 1:44 to 1:115 and six dilution set ratios were used for validation of 11-KT ranging from 1:3.3, 1:6, 1:11, 1:19, 1:34 to 1:60 which were screened with 7, 8 and 4 dilutions of the T, 11-KT and E₂ kit standards, respectively. The curves using dilutions of pooled plasma were found to run parallel to those obtained using standards provided with the T, 11-KT and E₂ kits (Supporting Information). Regression analysis found that 1:7.5, 1:3.2 and 1:3.5 were the appropriate dilution factors for 50% of antibody bound for the T, 11-KT and E₂ kits respectively (Supporting Information). A.akallopisos tested with the T, 11-KT and E₂ kits showed high precision determined from intra-assay variability (5.6, 17.5 and 19.5% respectively; Supporting Information). Inter-assay variability was only calculated for 11-KT as to date only one plate has been carried out for T and E₂. 11-KT showed a lower precision determined from inter-assay variability, which this is likely due to low sample size tested (22.9%; Supporting Information).

Hierarchical Group Structure or Social Status

Each A. akallopisos in this study was designated a position in the hierarchy of their group within the anemone in which it was collected based on their size relative to the other individuals in the anemone. Individuals in a group consisting of two individuals, i.e. a pair, were classified as rank 1 (the largest and most likely the breeding female, a female waiting for a male, or an ascendant female) and rank 2 (the breeding male or ascendant male). The largest two individuals in a group of individuals (n > 2) were classified as rank 1 and rank 2 as before and the third largest individual as rank 3 (the largest non-breeding male) and all remaining fish as rank 4 (the smallest non-breeding males). Breeding status was confirmed by the presence of eggs at the anemone cluster when present, but the egg stage was not determined. The ratio of \( L_T \) of individuals adjacent in rank within each groups were calculated from \( (L_T \text{ rank } N)(L_T \text{ rank } N+1)^{-1} \).

Statistical Tests

Levels of hormones were log-transformed to attain normality. The comparison of A. akallopisos length and hormone levels as a function of their rank within the groups was nested within their anemone cluster to control for inter-group variation and nested within their collection site to control for inter-site variation in hormone levels using the ANOVA model within SPSS (IBM; www.ibm.com). Post-hoc tests were carried out with a Bonferroni correction for multiple-comparisons. Hormone correlations were carried out using Spearman rank correlation on log-transformed values.

Results

The 111 individuals showed significant differences in mean total length based on their hierarchical position within each group \( [F_{3,42} = 41.63, P < 0.001; \text{Fig. 2(a)}] \). Rank 1 individuals were larger than all other individuals (Bonferroni post-hoc tests; \( P < 0.001 \)), rank 2 were larger than rank 3 and 4 (\( P < 0.001 \)), but there was no significant difference between the lengths of ranks 3 and 4 (\( P > 0.05 \)). However, when the lengths of rank 3 and rank 4 individuals were compared in groups of at least 4 individuals (i.e. excluding groups of only 3 individuals), rank 3 individuals were significantly larger than rank 4 \( [F_{1,20} = 8.001, P < 0.05; \text{Fig. 2(a)}] \).

The four ranks differed in circulating levels of 11-ketotestosterone (11-KT) \( [F_{3,41} = 41.766, P < 0.001, n = 89; \text{Fig. 2(b)}] \) and 17\( \beta \)-oestradiol (E₂) \( [F_{3,26} = 20.265, P < 0.001, n = 48; \text{Fig. 2(c)}] \), but not testosterone (T) \( [F_{3,16} = 3.042, P > 0.05, n = 42; \).
Fig. 2. Boxplots showing mean (•), 25th–75th quartiles, range (whiskers), excluding the outliers (○), of (a) total length (L<sub>T</sub>), (b) 11-ketotestosterone, (c) 17β-oestradiol, (d) testosterone, (e) index of conversion of testosterone to 11-ketotestosterone and (f) index of conversion of testosterone to 17β-oestradiol for individual *Amphiprion aklutopisos*. □, Rank 1, breeding females; ■, rank 2, breeding males; ☐, rank 3, largest non-breeding males; ☐, rank 4, smallest non-breeding males. Horizontal bars indicate significant differences: *P* < 0·05; **P* < 0·01; ***P* < 0·001.

Fig. 2(d)]. Rank 2 individuals had on average eight times more 11-ketotestosterone (11-KT) than rank 1 (Bonferroni post-hoc, *P* < 0·001), twice the 11-KT plasma levels of rank 3 (*P* < 0·05) and three times the 11-KT plasma levels of rank 4 (*P* < 0·01). Rank 1 individuals had significantly higher circulating E<sub>2</sub> levels than all other ranks (Bonferroni post-hocs: rank 2, *P* < 0·05; rank 3, *P* < 0·05; rank 4, *P* < 0·05). Group size had no effect on any hormone levels for any of the ranks (all *P* > 0·05).

The 11-KT:(11-KT + T) ratio and the ratio of E<sub>2</sub>:(E<sub>2</sub> + T), which indicate the physiological conversion of T to 11-KT and E<sub>2</sub> respectively, were also compared. Significant differences in the 11-KT:T ratios were found between the ranks [F<sub>3,20</sub> = 29·569,
Fig. 3. Relationship between the ratio of total length ($L_T$) between ranks 1 and 2 and the conversion ratio of plasma 11-ketotestosterone from Testosterone of rank 1 individuals, $F_{1,12} = 4.701, P < 0.05$.

$P < 0.001, n = 42$; Fig. 2(e)]. Rank 1 individuals had significantly lower 11-KT:T ratios than all other ranks (Bonferroni post-hocs: rank 2, $P < 0.05$; rank 3, $P < 0.05$; rank 4, $P < 0.05$). Furthermore, there was an effect of size ratio on the 11-KT:T conversion ratios of rank 1 individuals, the closer in size between ranks 1 and 2, the greater the conversion of T to 11-KT ($y = -0.185 - 0.24 x$, $r_s = 0.257$, $F_{1,14} = 4.839$, $P < 0.05$; $n = 15$; Fig. 3), but not between ranks 2, 3 and 4.

Individuals of different ranks also differed in their E$_2$:T ratios [$F_{3,14} = 13.810$, $P < 0.001$, $n = 37$; Fig. 2(f)]. Rank 1 individuals had significantly higher E$_2$:T ratios than rank 2 (Bonferroni post-hoc: $P < 0.05$), but the E$_2$:T ratios were not different between other ranks (Bonferroni post-hocs, $P > 0.05$). Group size had no effect on any conversion ratios for any of the ranks (all $P > 0.05$).

We also explored the correlations between hormones levels. Across all ranks there was no relationship between androgen levels (T and 11-KT; Spearman $\rho = 0.194$, $P < 0.05$, $n = 39$), however a significant positive correlation was found within rank 1 individuals (T and 11-KT; Spearman $\rho = 0.537$, $P < 0.05$, $n = 14$). The conversion ratio of 11-KT from T showed a positive relationship with levels of 11-KT [Spearman $\rho = 0.725$, $P < 0.001$, $n = 39$; Fig. 4(a)] and a corresponding negative relationship with levels of T (Spearman $\rho = -0.341$, $P = 0.05$, $n = 42$), which were driven by rank 2 individuals (Spearman $\rho = 0.567$, $P < 0.01$, $n = 13$).

A significant positive relationship was found between T and E$_2$ across all ranks [Spearman $\rho = 0.411$, $P < 0.05$, $n = 37$; Fig. 4(b)], which was driven by individuals of rank 1 (T and E$_2$; Spearman $\rho = 0.560$, $P < 0.05$, $n = 14$). Similarly, a positive
Fig. 4. Correlations between log-transformed hormone levels across the ranks (a) 11-KT:T conversion ratio on 11-KT, (b) E2 on T and (c) E2 on 11-KT. ●, rank 1, breeding females; ▲, rank 2, breeding males; □, rank 3, largest non-breeding males; ○, rank 4, smallest non-breeding males.

A significant negative relationship was also found between 11-KT and E2 across all ranks [11-KT and E2; Spearman $\rho = -0.497$, $P < 0.001$, $n = 46$; Fig. 4(c)], which was no longer present when analysed for each rank separately. The relationship between 11-KT and E2:T ratios was also negative across all ranks (11-KT and E2:T ratio; Spearman $\rho = -0.755$, $P < 0.001$, $n = 35$). Finally the two conversion ratios showed a negative relationship across all ranks (11-KT:T ratio and E2:T ratio; Spearman $\rho = -0.474$, $P < 0.01$, $n = 37$).

DISCUSSION

*Amphiprion akallopisos* has a size-based dominance hierarchy with a socially induced male to female sex change and significant differences were found between individuals of different ranks (corresponding to reproductive status or position in the social hierarchy) in circulating values of 11-ketotestosterone (11-KT) and
17β-oestradiol (E2), as well as their conversion ratios from testosterone (T), but no differences in circulating levels of T.

In terms of sex differences in hormone levels, the differences between rank 1 and the other ranks revealed sex differences in E2 and 11-KT [Fig. 2(b), (c)]. This is not surprising as E2 and 11-KT are the major oestrogen and androgen in fish respectively (Ohta et al., 2012). 17β-oestradiol plays a major role in female teleost reproduction, particularly in vitellogenesis and oocyte maturation (Fostier et al., 1983; Ng & Idler, 1983; Lazier et al., 1987) and in accordance E2 levels were significantly lower in ranks 2 and lower [Fig. 2(c)]. The higher E2 levels in female compared with male A. akallopisos are in agreement with the false clownfish, Amphilirion ocellaris Cuvier 1830 (DeAngelis & Rhodes, 2016), the cinnamon clownfish, Amphilirion melanopus Bleeker 1852 (Godwin & Thomas, 1993) as well as other fish species (Cardwell & Liley, 1991b; Ramallo et al., 2015). In contrast with circulating levels of E2, however, conversion rates of T to E2 were only different between ranks 1 and 2 (females and breeding males), not for lower ranks (non-breeding males). This may simply be an artefact of low sample sizes for the smaller non-breeding males (n = 7 and 4 for rank 3 and 4, respectively) coupled with the large variation in circulating levels of female E2. Such variation is probably due to the different time points related to egg laying date on which the females were sampled as found for A. ocellaris (DeAngelis & Rhodes, 2016). As the ratio E2:(E2 + T) was elevated for females and positive correlations were found between T and E2:(E2 + T), as well as E2 and E2:(E2 + T), these suggest that the biosynthetic pathway for E2 was from T in accordance with other teleosts (Wingfield & Grimm, 1977).

The main androgen in fish, 11-KT, showed higher levels in lower ranks (males) than in higher ranked (females) A. akallopisos [Fig. 2(b)]. 11-KT is a sex-specific steroid in many teleosts with a primary spermatogenic or spermiogenic function, but is also involved in the development and maintenance of male reproductive traits, reproductive behaviour, ornamental development, aggression and territory defence (Cochran, 1987; Brantley et al., 1993; Borg, 1994; Schulz & Miura, 2002; Desjardins et al., 2008). The higher plasma levels of 11-KT observed in breeding male (rank 2) and even in the largest non-breeding male (rank 3) A. akallopisos compared with females (rank 1) are in keeping with previous findings in other Amphilirion species (Godwin & Thomas, 1993; DeAngelis & Rhodes, 2016) and other teleosts (Borg, 1994), regardless of whether the social groups are cooperative breeders (Desjardins et al., 2008; Taves et al., 2009; Ramallo et al., 2015), show alternative reproductive strategies (Oliveira et al., 2001b; Knapp & Neff, 2007; Gonçalves et al., 2008), have suppression of reproduction (Cardwell & Liley, 1991a; Cardwell et al., 1996; Filby et al., 2010) or show differences in the direction of sex change either protogynous (Kroon & Liley, 2000; Bhandari et al., 2003; Lorenzi et al., 2008), or protandrous (Guiguen et al., 1993; Lone et al., 2001). Furthermore, the index of conversion of T to 11-KT in A. akallopisos was higher in all males (lower ranks) compared with females (rank 1) [Fig. 2(e)] which is in accordance with the general trend found in Neotropical fishes (Ramallo et al., 2015) and other fishes (Borg, 1994; Oliveira, 2004; Desjardins et al., 2008).

Circulating levels of T showed a similar trend to 11-KT but did not differ significantly between the sexes in A. akallopisos [Fig. 2(d)]. T, albeit secondarily to 11-KT, is known to promote spermatogenesis, male secondary sex characteristics and reproduction in several species (Liley & Stacey, 1983; Fostier et al., 1987) and is often linked to aggression and territory defence (Desjardins et al., 2008), therefore, the lack of a sex
difference in T may seem surprising. However, T is not exclusively confined to males as adults of both sexes, across most vertebrate taxa, naturally produce T (Nelson, 2000) and T in females has been implicated in many different physiological and behavioural functions (Staub & De Beer, 1987; Ketterson et al., 2005; Möller et al., 2005). Therefore, circulating T levels are not always higher in male A. akallopisos and the gonadal production of T might be important to promote physiological and behavioural functions in both sexes and as a precursor for the synthesis of both 11-KT and E₂. Although there are some cases in fish where males have higher T than females (Kime & Hyder, 1983; Cardwell & Liley, 1991a; Kroon & Liley, 2000; Pavlidis et al., 2000) and females having higher T levels than males (Borg, 1994; Oliveira, 2004; Desjardins et al., 2008; Taves et al., 2009), in general no sex differences in T are found (Nakamura et al., 1989; Lone et al., 2001; Bhandari et al., 2003; Kroon et al., 2003).

In A. akallopisos, the female and male breeding pair behaviourally defend their host anemone and their eggs, when present, from predators (Mariscal, 1970). Furthermore, the dominant A. akallopisos pair show intraspecific agonistic behaviour towards non-breeding males and the female often chases the dominant male (Allen, 1972; Fricke, 1979). As T mediates the expression of both female dominance behaviour and aggression in other vertebrates (Lindeque & Skinner, 1982; Ketterson et al., 2005; Zysling et al., 2006) and is important for winning territories in female fish (Taves et al., 2009), T may also be responsible for promoting both dominance and territory defence in female A. akallopisos. On the other hand, T levels are on average higher, especially for female A. akallopisos, than those of 11-KT and as such T may function primarily as a precursor of 11-KT in males and of 17β-oestradiol in females, with its role in territorial defence only secondary to its aromatisation function (Scott et al., 1980).

In terms of the male size-based social hierarchy, the two main androgens, T and 11-KT were higher in breeding (ranks 1 and 2) compared with non-breeding males (ranks 3 and 4), but this trend was only significant for 11-KT. The present results show that within males, 11-KT levels decline as the hierarchy is descended [Fig. 2(b)]. Among fish, where reproduction is not shared among all males, as is the case in A. akallopisos, breeding males generally have higher levels of 11-KT than non-breeding or subordinate males (Brantley et al., 1993; Pankhurst, 1995; Oliveira et al., 1996; Parikh et al., 2006; Bender et al., 2008). In contrast, androgen levels usually do not differ in species with alternative reproductive strategies that use queuing systems in which status acquisition does not depend on aggression, or in cooperative breeders (Oliveira et al., 2003; Bender et al., 2006). The levels of 11-KT found here in A. akallopisos males confirm previous suggestions that reproduction is not shared between A. akallopisos males in the hierarchy.

Alternatively, the production of androgens is very sensitive to the social environment, especially to social challenges (Cardwell & Liley, 1991a; Oliveira et al., 1996; Wingfield et al., 2000) and differences in levels of 11-KT may be a consequence of different social stimuli experienced by different conspecific males, with A. akallopisos males higher in the hierarchy constantly defending their position and controlling males immediately lower than them in the hierarchy. It is likely that both the social induction of hormone production and the activating effects of androgens may result in a feed-back loop facilitating the physiological changes underlying the dominance positions (Hirschenhauser & Oliveira, 2006; Parikh et al., 2006; Dijkstra et al., 2007). However, we found no effect of group size on any hormone level, including 11-KT.
A study subjecting male round gobies Neogobius melanostomus (Pallas 1814) to high densities of conspecifics, elevated their cortisol responses, but had no effect on levels of 11-KT either (Sokołowska et al., 2013). The present results suggest that in this species, it is the social challenge from a fish adjacent in rank and not group size, that results in, or is caused by, differences in hormones levels. It was found that when the size ratio between rank 1 (females) and rank 2 (breeding males) decreases, the conversion of T to 11-KT of rank 1 individuals (females) increases (Fig. 3). Female A. akallopisos are aggressive to males (Fricke, 1979) and female aggression towards the dominant male may be activated when their size differences decrease, i.e. social induction of hormone production and androgen activation are highest when the size ratio is lowest. Future work is needed, however, to determine the direction of the hormone-to-behaviour relationship by studying aggression levels between females and males.

In A. akallopisos and Amphiprion spp. in general, male aggression towards the male immediately lower in the dominance hierarchy is well known (Fricke, 1979). If aggression levels decrease as the dominance hierarchy is descended, then the corresponding decreasing levels of 11-KT may represent their role in aggression. Reproductive opportunity decreases, however, as the hierarchy is descended and the levels of 11-KT may instead, or in addition to, represent the decreasing opportunity of reproduction as the hierarchy is descended. Nest-holding status leads to a higher conversion of T into 11-KT (Rodriguez et al., 2001; Oliveira et al., 2001a), but no difference was found here in the conversion ratio between males of different status in A. akallopisos [Fig. 2(e)]. Therefore, the social environment, rather than reproduction, may act on the metabolic processes involved in 11-KT biosynthesis, increasing the conversion of T to 11KT (Taves et al., 2009; Ramallo et al., 2015). The social environment, however, is not related to density, as the conversion of T to 11KT was not affected by group size. Rather the social environment refers to male aggression towards the male immediately lower in the dominance hierarchy (Fricke, 1979) and as such may explain why breeding males (rank 2) do not have higher 11KT:T ratios than lower ranked males. Male aggression to lower ranked males have widespread functional consequences. In the cichlid fish A. burtoni, subordinate males have a suppressed brain-pituitary-gonad axis, low levels of gonadotropin hormones and low circulating levels of sex steroids compared to dominant reproductively active males (Parikh et al., 2006; Fernald, 2009). Future studies should determine whether such function is similarly suppressed in non-breeding A. akallopisos of reproducing size.

The pattern of T levels within the social hierarchy is less clear. T levels show a trend to decrease as the hierarchy is descended but this is non-significant [Fig. 2(d)]. The role of T as a precursor for the synthesis of 11-KT could explain this non-significant trend, but the data are not correlated. On the other hand, T is often linked to aggression, reproduction and territorial defence (Desjardins et al., 2008). The non-significantly different T levels in male A. akallopisos, may be due to the aggressive behaviour of all individuals, breeding or non-breeding, when defending their host anemone against heterospecific intruders or predators as has been found in other fish species (Balshine-Earn et al., 1998; Balshine et al., 2001; Desjardins et al., 2006; Aubin-Horth et al., 2007) and also against egg predators.

In summary, differences were found in androgen and oestrogen production between the sexes and between social status in a coral reef fish with a size-based dominance
hierarchy. How these different endocrine profiles might affect or are affected by reproduction and behaviour within the dominance hierarchy were discussed. The direction of hormone to behaviour relationships and their role in controlling male growth and reproduction would be interesting avenues for future investigation, especially if aggressive interactions directed at the non-breeding males caused stress-induced regulation of reproduction. The main teleost stress hormone, the glucocorticoid cortisol, is known to modulate reproductive hormones (Haddy & Pankhurst, 1999), but recent cases have highlighted that cortisol does not always promote 11-KT or nest defence (Dey et al., 2010; Sokolowska et al., 2013) or does not initiate reproduction in the direction assumed (Castranova et al., 2005), therefore future work determining the role of cortisol in maintaining body size ratios and reproduction suppression in this species would be highly relevant. In this and another anemonefish species, Amphiprion chrysopterus Cuvier 1830, environmental perturbations such as elevated temperatures and host anemone bleaching have both immediate and longer-term effects on cortisol production with detrimental effects on Amphiprion spp. reproduction (Mills et al., 2015; Beldade et al., 2017), therefore more studies measuring variation in natural endocrine profiles and behaviour and their relationship with reproduction are required if we are to understand how species reproduction will be affected by future environmental changes.

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S.C.M. and R.B. designed the study; R.B., J.O’D., G.B. and SCM collected the data; S.C.M. analyzed the data and all authors contributed to writing and revising the manuscript.

Supporting Information

Supporting Information may be found in the online version of this paper:

Appendix S1. Validation of hormone kits for A. akallopisos

Appendix S2. Validation of hormone kits.

Table S1. ANCOVA on homogeneity of slopes for sample dilution v. standard dilution curves for testosterone (T), 11-ketotestosterone (11-KT) and 17β-oestradiol kits in Amphiprion akallopisos. The dilution factors (dilution) for 50% of antibody bound determined from regression analyses [Fig. 1(a)–(c)] are also given

Table SII. Intra and inter-assay variabilities (C.V.) for testosterone (T), 11-ketotestosterone (11-KT) and 17β-oestradiol in Amphiprion akallopisos

Fig. S1. Validation of hormone kits: Dose–response curves from Amphiprion akallopisos for (a) testosterone obtained using 7 kit standards and 5 pooled plasma (simple
linear regression: kit standards: \( y = -39.91 \times 4.94, R^2 = 0.98, n = 9, P < 0.001; \)
samples: \( y = -38.06 x + 16.75, R^2 = 0.95, n = 10, P < 0.001; \) (b) 11-ketotestosterone
obtained using 8 kit standards and 6 pooled plasma (kit standards: \( y = -31.31 \times 29.41, R^2 = 0.91, n = 16, P < 0.001; \) samples: \( y = -31.81 x + 33.92, R^2 = 0.95, n = 12, P < 0.001; \) (c) \( 17\beta \)-oestradiol obtained using 4 kit standards and 5 pooled
plasma (kit standards: \( y = -27.77 x - 5.58, R^2 = 0.99, n = 5, P < 0.001; \) samples: \( y = -32.36 x + 32.35, R^2 = 0.96, n = 10, P < 0.001. \) ●, pooled sample plasma; ○, kit
standards; \( \rightarrow \rightarrow \), 50% bound (see Table SI for corresponding dilution factors).

References


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