

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 (E_2), 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 E_2 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 E_2 . 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 E_2 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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Key words: 11-ketotestosterone; coral reefs; hierarchy; oestradiol; social status; testosterone.

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

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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 (E_2), 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 E_2 as females of most gonochoristic species require T to synthesize E_2 in the ovarian follicles *via* the aromatisation of T using P450 aromatase (CYP19) (Lee *et al.*, 2006). However, the E_2 synthetic pathway has been found to be different in some hermaphrodite species, whereby ovarian follicles synthesize E_2 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 E_2 and 11-KT can be synthesised from T, the conversion ratios of T to 11-KT and E_2 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.* *Salarias 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

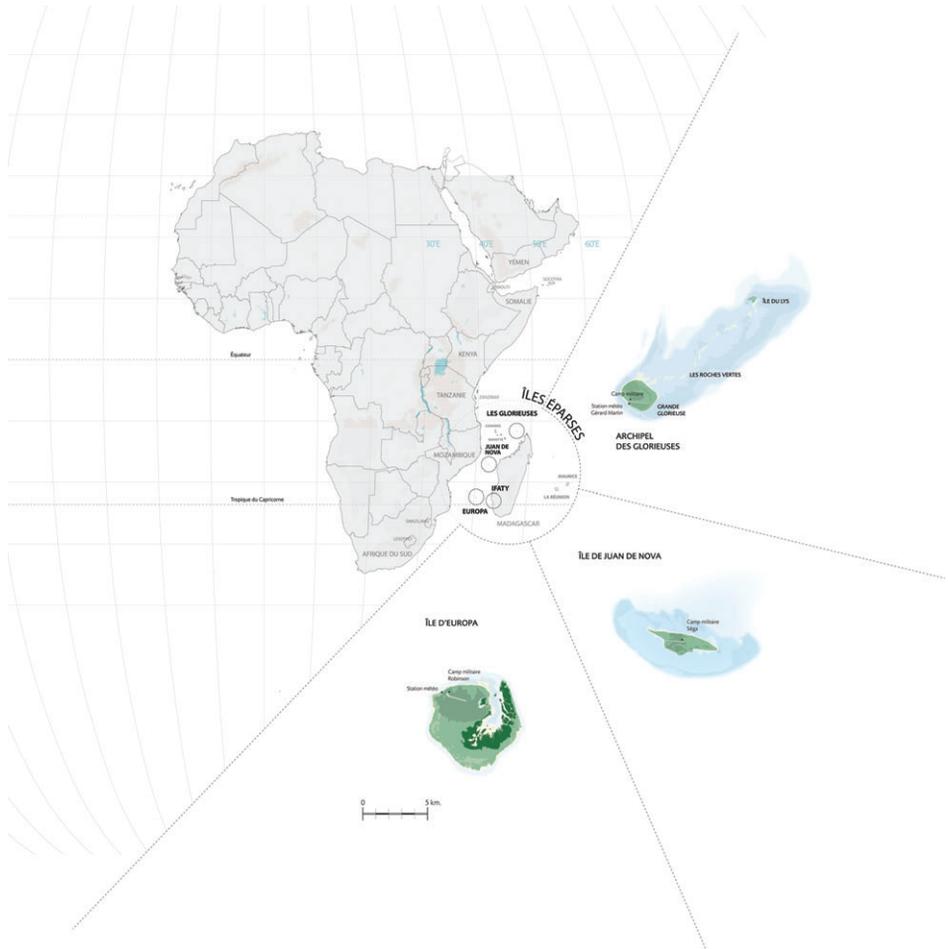


FIG. 1. The Îles Éparses within the Mozambique Channel, Indian Ocean and Madagascar. ○, *Amphiprion akallopisos* sampling sites.

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 17β -oestradiol (E_2) were measured using EIA kits (T EIA Kit, No. 582701; 11-KT EIA Kit, No. 582751; 17β -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 [$F_{3,42} = 41.63$, $P < 0.001$; 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 [$F_{1,20} = 8.001$, $P < 0.05$; Fig. 2(a)].

The four ranks differed in circulating levels of 11-ketotestosterone (11-KT) [$F_{3,41} = 41.766$, $P < 0.001$, $n = 89$; Fig. 2(b)] and 17 β -oestradiol (E₂) [$F_{3,26} = 20.265$, $P < 0.001$, $n = 48$; Fig. 2(c)], but not testosterone (T) [$F_{3,16} = 3.042$, $P > 0.05$, $n = 42$;

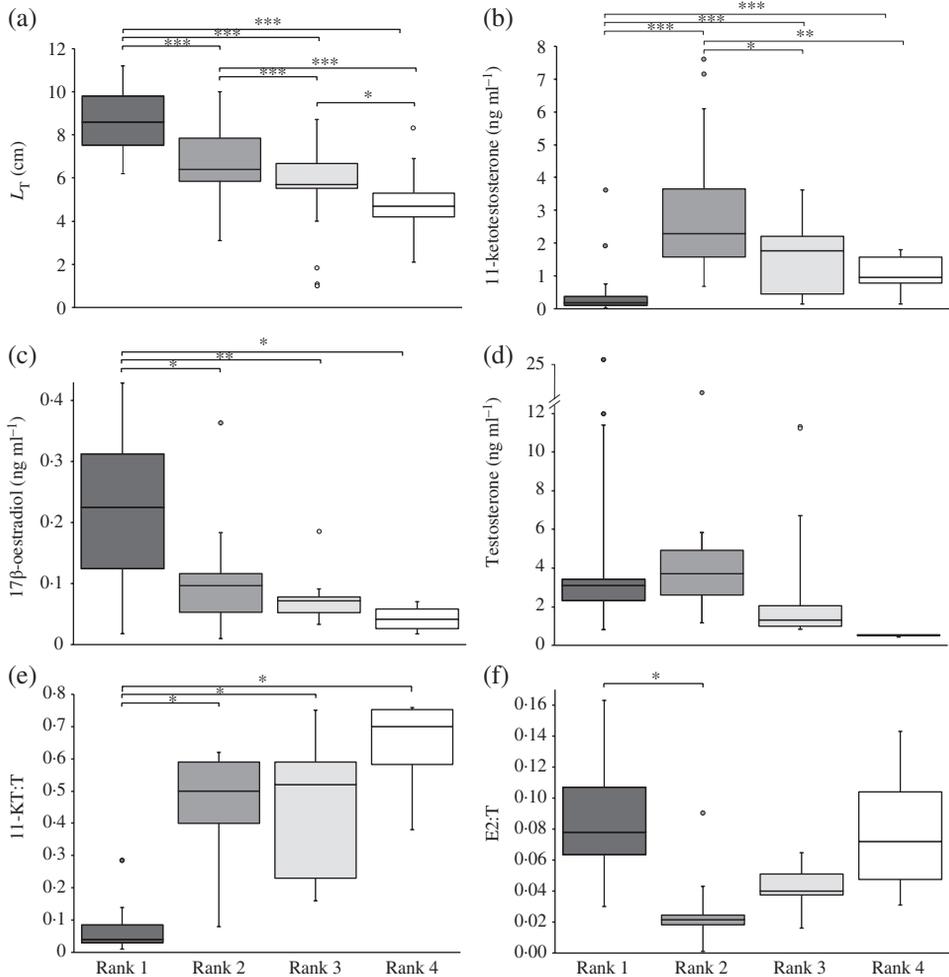


FIG. 2. Boxplots showing mean (—), 25th–75th quartiles, range (whiskers), excluding the outliers (○), of (a) total length (L_T), (b) 11-ketotestosterone, (c) 17β -oestradiol, (d) testosterone, (e) index of conversion of testosterone to 11-ketotestosterone and (f) index of conversion of testosterone to 17β -oestradiol for individual *Amphiprion akallopisos*. ■, 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_2 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_2 :(E_2 + T), which indicate the physiological conversion of T to 11-KT and E_2 respectively, were also compared. Significant differences in the 11-KT:T ratios were found between the ranks [$F_{3,20} = 29.569$,

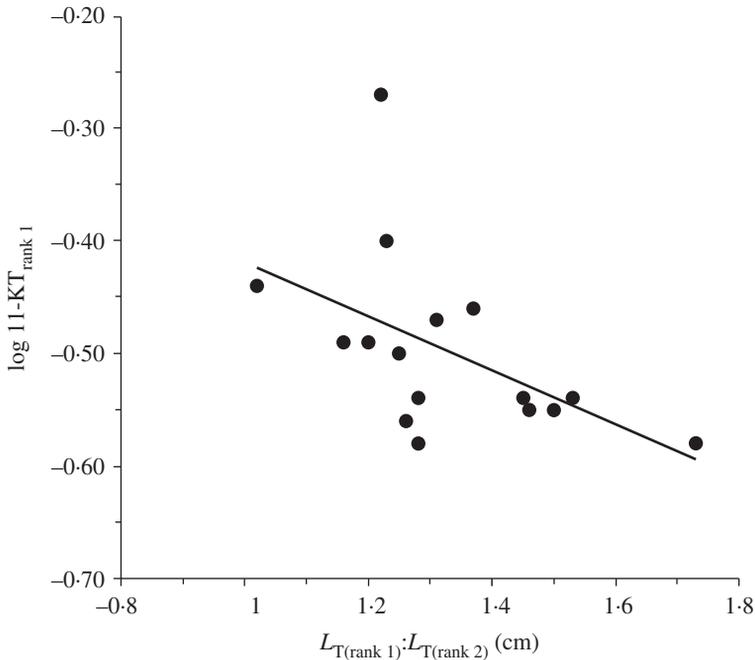


FIG. 3. Relationship between the ratio of total length (L_T) between ranks 1 and 2 and the conversion ratio of plasma 11-ketotestosterone from Testosterone of rank 1 individuals, $F_{1,12} = 4.701$, $P < 0.05$.

$P < 0.001$, $n = 42$; 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 ($y = -0.185 - 0.24x$, $r_s = 0.257$, $F_{1,14} = 4.839$, $P < 0.05$; $n = 15$; 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

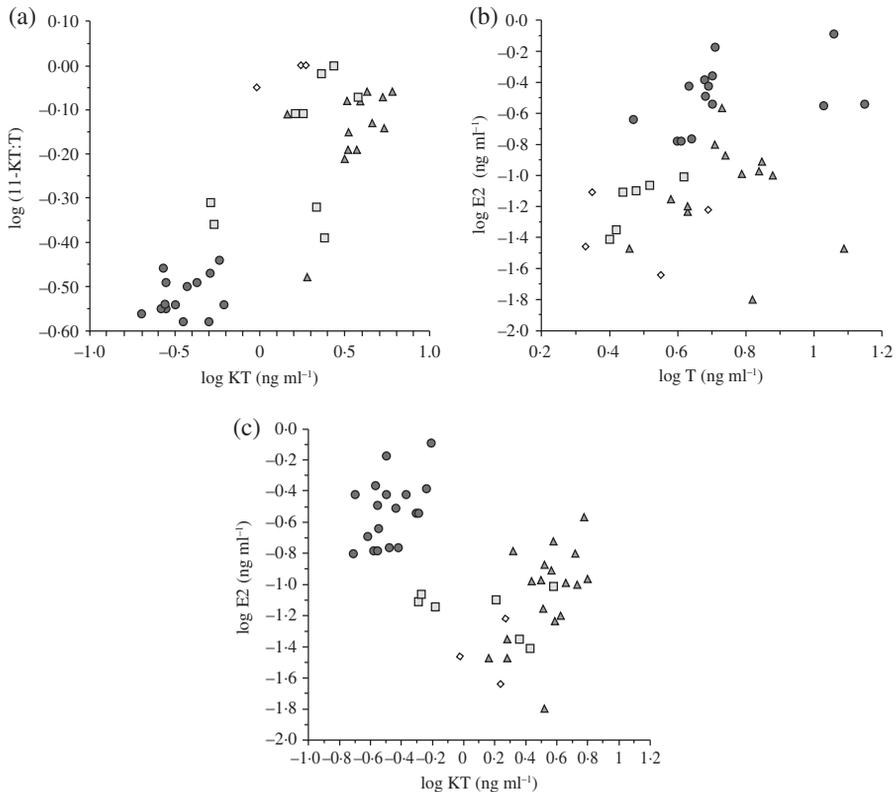


FIG. 4. Correlations between log-transformed hormone levels across the ranks (a) 11-KT:T conversion ratio on 11-KT, (b) E₂ on T and (c) E₂ on 11-KT. ●, rank 1, breeding females; ▲, rank 2, breeding males; □, rank 3, largest non-breeding males; ◇, rank 4, smallest non-breeding males.

relationship was also found between E₂ levels and their conversion ratio from T for all ranks (Spearman $\rho = 0.620$, $P < 0.001$, $n = 36$), which was also driven by individuals of rank 1 (E₂ and E₂:T ratio; Spearman $\rho = 0.625$, $P < 0.05$, $n = 13$).

A significant negative relationship was 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 (E_2), 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 E_2 and 11-KT [Fig. 2(b), (c)]. This is not surprising as E_2 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 E_2 levels were significantly lower in ranks 2 and lower [Fig. 2(c)]. The higher E_2 levels in female compared with male *A. akallopisos* are in agreement with the false clownfish, *Amphiprion ocellaris* Cuvier 1830 (DeAngelis & Rhodes, 2016), the cinnamon clownfish, *Amphiprion 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 E_2 , however, conversion rates of T to E_2 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 E_2 . 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 $E_2:(E_2 + T)$ was elevated for females and positive correlations were found between T and $E_2:(E_2 + T)$, as well as E_2 and $E_2:(E_2 + T)$, these suggest that the biosynthetic pathway for E_2 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 *Amphiprion* 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 protrandrous (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; Møller *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 17 β -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 (Rodríguez *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; Sokołowska *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.

Financial support was provided from INEE INSU-IRD-AAMP-FRB-TAAF-Iles Eparses (Clownfish) to S.C.M. and G.B., Agence National de Recherche (ANR-11-JSV7-012-01/Live and Let Die) to S.C.M., (ANR-14-CE02-0005-01/Stay or Go) to G. Almany, S.C.M. and R.B., Contrat de Projets Etat - Polynésie française to S.C.M., LabEx CORAIL (Where do we go now?) to S.C.M. and R.B., University of California Santa Cruz (UCSC) Department of Ecology and Evolutionary Biology to J.O. and UCSC's Committee on Research (to G.B.) and Sigma Xi to J.O.. We would like to thank the administration of the TAAF (Terres Australes et Antarctiques Françaises) for research permits and the captain and crew of the R.V. *Marion Dufresne 2*. For their assistance with the fieldwork, we wish to thank P. Chabanet, L. Bigot, P. Durville and D. Obura, as well as J.-B. Galves and J. Parodi of the R.V. *Inventive*. We would like to thank B. Marie (www.seaview.photodeck.com) for kindly providing Fig. 1. Our thanks to P. Buston for generating the initial ideas with S.C.M. and reviewing the paper.

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 SI. ANCOVA on homogeneity of slopes for sample dilution *v.* standard dilution curves for testosterone (T), 11-ketotestosterone (11-KT) and 17 β -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 17 β -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: $y = -39.91 x - 4.94$, $R^2 = 0.98$, $n = 9$, $P < 0.001$; 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: $y = -31.31 x - 29.41$, $R^2 = 0.91$, $n = 16$, $P < 0.001$; samples: $y = -31.81 x + 33.92$, $R^2 = 0.95$, $n = 12$, $P < 0.001$); (c) 17β -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; – – →, 50% bound (see Table SI for corresponding dilution factors).

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