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ORIGINAL PAPER

Reduced access to cleaner fish negatively impacts the physiological state of two resident reef fishes

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Abstract

Many "client" coral reef fishes have their ectoparasites removed by the "cleaner" wrasse *Labroides dimidiatus* in mutualistic interactions. Clients regularly receiving cleaning services reportedly benefit from increased growth and cognitive performance, but the underlying physiological changes that covary with such benefits are unknown. Here, we tested whether reduced access to cleaning services affects physiological state in two species of damselfish, *Amblyglyphidodon curacao* and *Acanthochromis polyacanthus.* We performed an in situ removal experiment on the Great Barrier Reef, Australia, whereby 47% of cleaners on a natural reef were removed. Since cleaners occupy defined territories (called "cleaning stations"), this removal created areas where small, resident clients, including *A. polyacanthus* and *A. amblyglyphidodon*, had no access to cleaning services. One month following cleaner removal, we measured body condition and collected blood samples from both damselfish species from territories with and without access to cleaners. Blood was used for estimating haematocrit levels, hormonal analyses, and immune cell counts. We tested for correlations among all these parameters to explore potential trade-offs in terms of growth, aerobic capacity, immune activation, and/or reproduction as a result of the loss of cleaning benefits. In both species, we found that fish without access to cleaners had lower haematocrit, testosterone levels, and lymphocyte counts than fish with access. There were no significant changes in fish body condition, leukocytes, granulocytes, or plasma cortisol levels between fish with access to cleaners or not. However, testosterone levels correlated negatively with the proportion of granulocytes in the blood of fish with access to cleaners. Our results suggest that even a relatively short-term reduction in access to cleaning services can have negative physiological outcomes for clients. Thus, the presence of cleaners on coral reefs appears to have important benefits for coral reef fish community health.

Introduction

Parasites exploit their host species (Anderson and May 1981), often causing major negative effects at both the individual and population levels (Ebert et al. 2000). However, hosts can behaviourally defend themselves against infection either through prophylactic or therapeutic movement behaviours (Binning et al. 2017). Prophylactic behaviours can include selective foraging at non-infected areas (Hutchings et al. 2001), defecating at sites away from their own nest or den (Ezenwa 2004), or avoiding grouping (Mooring and Hart 1992). Once individuals are infected, therapeutic behaviours, including ectoparasite removal by cleaning organisms, can help to reduce parasite burdens. Such strategies include some of the best documented examples of intraspecific cooperative interactions (social grooming/preening) as well as interspecific mutualistic cleaning interactions (MacFarland and Reeder 1974; Hutchins and Barash 1976; Dean and MacDonald 1981; Kilham 1982; Hart 1990; Cheney and Côté 2005). Among the best studied cleaning mutualisms are the interactions between the cleaner wrasse *Labroides dimidiatus* (hereafter "cleaner") and its client reef fishes (Losey 1979). Cleaners interact with both "visitor"

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clients, which have access to multiple cleaners, and with "resident" clients, which have access to a single cleaner. The difference in access between residents and visitors is entirely based on the species territory/home-range size in relation to cleaner wrasse density on a given reef (Bshary 2001). Interactions between cleaners and clients have individual benefits for both players. Cleaners gain food by removing clients' ectoparasites (Grutter 1999), and clients experience reduced parasite loads (Grutter 1995; Grutter et al. 2017), which is linked to increased growth, greater body condition, and higher cognitive performance when individuals have access to cleaning stations (Ros et al. 2011; Clague et al. 2011; Binning et al. 2018). Indeed, limited access to cleaners increases clients' cortisol levels (Bshary et al. 2007), likely due to increased ectoparasite infection (Triki et al. 2016).

Cleaner presence also has flow-on community effects: reef patches lacking cleaners have lower client species richness, abundance, and juvenile settlement than nearby areas with cleaners (Bshary 2003; Sun et al. 2015; Wagner et al. 2015). Although this growing body of literature highlights the ecological importance of cleaning organisms in nature, we have a much more limited understanding of the physiological mechanisms underlying the health benefits experienced by clients. Studies documenting client health benefits as a result of access to cleaning services either focus on experimental infection and cleaning behaviour under laboratory settings (Jones and Grutter 2005; Triki et al. 2016), or evaluate the effects of long-term cleaner removal or absence in natural habitats (Bshary et al. 2007; Ros et al. 2011; Waldie et al. 2011; Clague et al. 2011). However, it is unknown whether reduced cleaning services for a relatively short-term period also impact client physiology state. This question is ecologically relevant as severe weather events such as the 2016 mass bleaching on the Great Barrier Reef were linked to a decrease in cleaner densities (Triki et al. 2018) and fluctuations in the densities of a common ectoparasite gnathiids (Sikkel et al. 2019) in the months following the perturbation. Therefore, studying the impact of such short-term perturbations might provide a further understanding of the benefits of the cleaning mutualism for client host organisms.

In nature, clients are repeatedly infected by mobile ectoparasites such as gnathiids (Grutter 2003), and, therefore, seek cleaning services up to 140 times per day (Grutter 1995). Over time, these interactions appear to contribute to improved client body condition and growth (Clague et al. 2011). However, fish body condition alone is a vague indicator of the physiological effects caused by infection with marine ectoparasites. Various blood components, such as circulating hormones, immune cells and antigens, or blood cell composition, can provide more detailed insights about an individuals' physiological state (Sopinka et al. 2016). For example, haematocrit, the red blood cell component of blood volume, can be used as a proxy to estimate oxygen transport capacity (Jones et al. 1990). As most fish ectoparasites feed on host blood and body fluids (Grutter 1994, 2003), they can cause a reduction in host haematocrit after 24 h of exposure (Triki et al., 2016). These findings make haematocrit a critical measure of physiological state. Parasite infection can also trigger an immune response in hosts, which can be energetically costly to mount (Sheldon and Verhulst 1996). Ros et al. (2011) found that fish living on reef patches naturally lacking cleaners had higher antibody activity than fish with access to cleaners, suggesting that a lack of cleaning service increases the need for active immunity. Immune cells (leukocytes), such as lymphocytes and granulocytes play a significant role in the innate and adaptive immune response in vertebrates (Bayne and Gerwick 2001). As such, evaluating the relative proportion of immune cells present in the blood of fish can also be a reliable indicator of immune activation and individual physiology state (Davis et al. 2008).

Hormone levels can also be informative for understanding individual physiological state and trade-offs between various life-history strategies, such as individual investment in growth versus reproduction, which may be linked to differences in behaviour, physiology, and environmental conditions. For instance, glucocorticoids, including cortisol, are the primary indicators of a stress response in fish (Sopinka et al. 2016) and can act to suppress the immune response (Saha et al. 2003) and reproductive function (see review by Schreck 2010). Reproductive suppression as a result of cortisol exposure can arise indirectly via a loss of body mass due to long-term energy mobilisation to increase survival (Van Weerd and Komen 1998). It can also occur directly via a negative feedback on the release of androgens such as testosterone and its oxidised form, 11-ketotestosterone (11-keto) (Hu et al. 2008), which are crucial for reproduction in fish (Brantley et al. 1993). Conversely, high levels of androgens can also interfere with individual's physiological state indirectly via their immunosuppressive activity (Slater and Schreck 1997). Assessing the various physiological indicators together may yield important insights regarding how individuals manage important trade-offs in their overall energy budget. Thus, exploring potential correlations among such measurements can provide a more accurate assessment of an individual's physiology and life-history state than interpreting each physiological indicator alone.

To test whether an acute reduction in cleaner access affects client fish physiological indicators, we performed an in situ cleaner removal experiment on a continuous natural coral reef on the Great Barrier Reef in Australia. Cleaner densities were experimentally reduced by half, creating areas within the reef where resident fish had limited or no access to a cleaning station within their home range. One month following cleaner removal, we collected individuals of two species of damselfish, the staghorn damselfish (*Amblyglyphidodon curacao*) and the spiny chromis

(*Acanthochromis polyacanthus*). Both *A. curacao* and *A. polyacanthus* are mid-sized, common, monomorphic (i.e., not possible to differentiate between females and males from body shape or colouration) resident reef fishes which are regularly cleaned by *L. dimidiatus* (Bshary 2001). We chose these species, because most of the Pomacentrids are territorial fish (Randall et al. 1997), and it is likely that these two species have small home-range sizes (Cowlishaw 2014) encompassing no more than one cleaning station (Bshary 2001). Individuals were collected from territories with cleaner wrasse and from territories where cleaners had been experimentally removed. Damselfish were transported by boat to the laboratory for body measurements and blood collection, and then returned to their respective site of capture. The following physiological indicators were measured: body condition, haematocrit level, immune cells, circulating plasma cortisol, testosterone, and 11-keto levels. If regular cleaning interactions yield immediate benefits to clients, we predicted that individuals without regular access to cleaners would have a relatively lower physiology state (i.e., lower body condition, low haematocrit, increased immune cell count, higher cortisol, and lower testosterone) than those with access to cleaners despite potential differences between the two study species regarding their baseline physiological characteristics.

Materials and methods

Fieldwork was conducted at the Lizard Island Research Station (LIRS), Great Barrier Reef, Australia (14°40′08.0S 145°27′34.0E), between July and September 2015. The study site is located on the southwestern edge of the island, at a depth ranging from 2 to 8 m and is approximately 7000 m^2 in surface area (14°41′17.8"S 145°26′33.2"E).

Experimental design

Two SCUBA divers estimated the number of cleaners at the study site by swimming 2 m-wide circular transects from the outside of the reef towards its centre. Cleaners typically occupy small territories called cleaning stations, where they are routinely visited by client reef fish to get their ectoparasites removed (Losey 1979). Sixty-six cleaners were counted at the study site on 23rd July 2015. The following day, 31 cleaners were removed approximately evenly across the study site: the aim was to reduce cleaner density by approximately 50% on the reef. Cleaners were collected by divers on SCUBA with a barrier net (2 × 1 m; 0.5 cm^2 mesh size) and hand nets. Removed cleaners were transported back to the aquarium facilities at LIRS, and held in individual, flow-through aquaria for the duration of the study. Following the study, cleaners were released back at the study site.

One month following the cleaners' removal (August 25th, 2015), divers returned to the study site for observation of client fishes. Individual adult *A. curacao* and *A. polyacanthus* were observed underwater for 5 min each to assess their access to the remaining cleaners. Adults were identified as having a standard length (SL) greater than 5 cm for *A. curacao* (Curtis-Quick et al. 2012) and greater than 6 cm for *A. polyacanthus* (Kingsford and Hughes 2005). Tags made of small floats and fishing line were used to mark the territories of 60 individuals (30 *A. curacao*; 30 *A. polyacanthus*) that showed no apparent signs of injury or illness. For each species, 15 fish were scored as "with access" and 15 fish as "without access" to cleaners based on the initial 5 min behavioural assessment. Divers returned to the site twice more on consecutive days and re-observed the individuals on marked territories for 5 min each time. Each fish was observed for a total of 15 min, this was done to confirm that individuals had been correctly categorised as either having or lacking access to cleaners during the initial assessment.

Following these observations, the 60 focal damselfishes were collected, in two trips, from tagged territories by divers on SCUBA. On the first trip, 30 captured fishes (i.e., 15 individuals of each species) were immediately placed in labelled 20 L buckets: fishes with or without cleaner access were kept separate. Within 1 h of capture, fishes were transported to LIRS and housed in one of two large aquaria (110 × 60 × 50 cm, one for each treatment). On the second trip, we followed similar steps as in the first trip. During the holding period, fish were provided with aerated seawater pumped directly from the nearby reef and pieces of cut PVC pipe (13 × 24 cm) serving as shelters.

Blood sampling and fish body condition

Blood sampling was performed on fish 4–10 h after capture. Thus, our cortisol assay did not measure basal levels (which would have required sampling within 4 min after capture; Pankhurst, 2011). Rather, our sampling aimed to measure a stress response to cumulative stressors such as catching and transporting (Barton et al. 1986). Before blood sampling, fish were placed in a 20 L bucket with aerated seawater containing a sedative bath of 30 mg L^{-1} Aqui-S (Triki et al. 2016). Once the fish were sedated (~ 1 min) which was determined by a loss of equilibrium, a blood sample (~ 1% of the fish's body weight) was drawn from the caudal vein with a 1 mL heparinised syringe (Ros et al. 2011; Binning et al. 2015; Triki et al. 2016). Fish from the two species and treatments were sampled in a counterbalanced (a.k.a. alternate) order.

Following the blood sampling, we measured fish total body length (TL) with callipers and body mass on a digital balance. The mean ± SD TL was 9.3 ± 0.7 cm, $n = 30$ for *A. curacao*, and 9.4 ± 1.0 cm, $n = 30$ for *A. polyacanthus*. Mean ± SD body mass was 24.2 ± 5.7 g, $n = 30$ for *A. curacao*, and 27.6 ± 9.4 cm, $n = 30$ for *A. polyacanthus*. The body condition index was calculated as the ratio of body mass to TL following the formula ($\text{Mass} \times \text{Length}^{-b}$), where b is a coefficient calculated for each species from a power regression curve of length and weight (Ros et al. 2011). We used this formula to facilitate further comparisons of the results to those in the study Ros et al. (2011). Fish were allowed to recover at LIRS for 2 days after sampling and fed with tropical fish flakes ad libitum, twice daily. Fish were then released back at the study site.

Blood analyses

Blood samples were centrifuged for 5 min at 3500 rpm. The collected plasma was frozen at − 20 °C for subsequent steroid analyses at the University of Neuchâtel, Switzerland (see methods in the supplementary material). Haematocrit measurements were recorded immediately from the freshly sampled blood. Micro-haematocrit capillary tubes (Hirshman®, Germany) were filled with blood and centrifuged at 12,000 rpm for 2 min. Haematocrit was calculated as the length of the red section (i.e., the volume of red blood cells) divided by the total length of the centrifuged sample in the capillary tube. Blood smears were performed by smearing one drop of fresh blood on a frosted microscope slide. All blood smears were prepared in double, dried and stored in an air-conditioned room (temperature set at 25 °C) to avoid any damage caused by humidity.

Blood smears were prepared for examination at the University of Neuchâtel, Switzerland (see methods in the supplementary material). Two cell subtypes were identified based on the shape of different leukocytes (Fig. S1). Large spherical cells (i.e., granulocytes) were distinguishable by the presence of numerous cytoplasmic granules, and small spherical cells (i.e., lymphocytes) were distinguishable by a large nucleus and low cytoplasmic volume (Ros et al. 2011).

Steroid assays

Fish blood plasma was assessed for circulating levels of cortisol, testosterone, and 11-keto. Frozen blood plasma samples kept at − 20 °C were extracted and analysed using liquid chromatography–mass spectrometry HPLC–MS. Detailed protocols of steroid extraction, analyses, and data readings are reported in the supplementary material. 11-keto was undetectable in most samples, suggesting that most individuals were females (Sangalang et al. 1978).

Statistical analysis

Statistical analyses were carried out in *R* version 3.3.2 (*R* Core Team 2017). Assumptions of linear models were assessed with standard diagnostic plots (QQ plots, plots of residuals vs fitted values) and statistical tests (Bartlett's test). We ran linear models for the response variables: haematocrit, granulocytes, lymphocytes, cortisol, and testosterone, and non-parametric tests for the response variables: body condition, and leucocytes. We tested the effect of client species and access to cleaners (fixed factors) on the physiological parameters of interest. Fish mass was included as a covariate in the models for haematocrit and steroid levels to control for the potential effect of fish body size on these variables. Specifically, we fitted linear models (LM) to the steroid and granulocyte data using the function *lm()* and generalised least-squares models (GLS) to the haematocrit and lymphocyte data using the function *gls()*. GLS models were used to account for unequal variances among groups. We specified Type II sum of squares using the *Anova* function in the package *car*. Haematocrit was log-transformed, and lymphocyte and granulocyte proportions were logit transformed to fulfil model assumptions. Body condition index and the proportion of leukocytes were analysed with Wilcoxon tests due to violations of the assumptions of normality and homogeneity of variance. We did not perform statistical analyses on the 11-keto data due to the very low sample size (*A. curacao*: with cleaner access, $n = 4$; without cleaner access, $n = 4$; *A. polyacanthus:* $n = 0$). We also tested for correlations between physiological indicators to explore potential trade-offs that might indicate shifts in individual life-history strategies. To do so, we created four matrices of the different physiological measurements for each species and category: *A. curacao* or *A. polyacanthus* with either access to cleaners or without access. We used Spearman correlations (function *corr.test* in the package *psych*).

Results

Body condition and haematocrit

The body condition index did not differ between fishes with and without access to cleaners in either *A. curacao* (Wilcoxon signed-ranks test $N = 30$, $W = 121$, $P = 0.743$), or *A. polyacanthus* (Wilcoxon signed-ranks test: $N = 30$, $W = 157$, $P = 0.067$) (Fig. 1a, Table 1). Haematocrit differed between fishes with and without access to cleaners (GLS $N = 56$, $\chi^2_{(1)} = 5.894$, $P = 0.015$); there was no effect of species (GLS $N = 56$, $\chi^2_{(1)} = 2.003$, $P = 0.156$) nor an interaction between species and cleaner access (GLS $N = 56$, $\chi^2_{(1)} = 0.058$, $P = 0.809$) (Fig. 1b).

White blood cells

White blood cells (leukocytes) proportions were affected by the restricted access to cleaners in *A. curacao*

(Wilcoxon signed-rank $N = 30$, $W = 169$, $P = 0.018$), but not in *A. polyacanthus* (Wilcoxon signed-ranks $N = 28$, $W = 126$, $P = 0.210$) (Fig. 2a). With regards to specific white blood cells, the proportion of granulocytes did not differ between fish with and without access to cleaners (LM $N = 58$, $F_{1,54} = 0.476$, $P = 0.492$); but there was a client species effect (LM $N = 58$, $F_{1,54} = 4.436$, $P = 0.039$), while no significant effect was recorded for the interaction between species and cleaner access (LM $N = 58$, $F_{1,54} = 2.105$, $P = 0.152$) (Fig. 2b). Conversely, fishes with cleaner access had a higher proportion of lymphocytes than fishes without cleaner access (GLS $N = 58$, $\chi^2_{(1)} = 9.141$, $P < 0.01$); the main effect of species (GLS $N = 58$, $\chi^2_{(1)} = 3.357$, $P = 0.067$) and the interaction between species and cleaner access (GLS: $N = 58$, $\chi^2_{(1)} = 2.818$, $P = 0.093$) were non-significant (Fig. 2c).

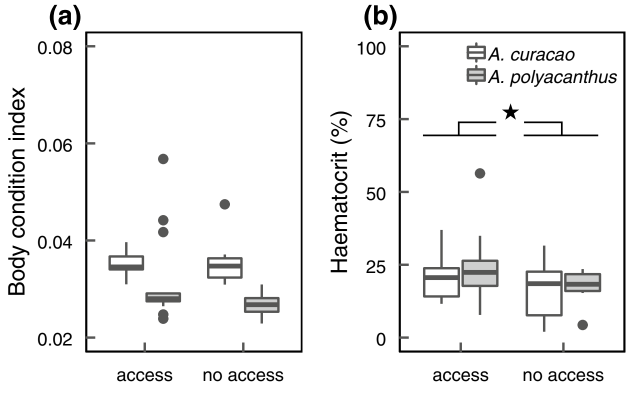

Fig. 1 Effect of access to cleaner wrasse on physiology indiactors in two resident client fishes. Box plots of median, interquartile range, upper and lower quartiles, and outliers of (**a**) body condition index of: *A. curacao* (with access: $n = 15$; without access: $n = 15$), and *A. polyacanthus* (with access: $n = 15$; without access: $n = 15$), and (**b**) haematocrit percentage of: *A. curacao* (access, $n = 13$; no access, $n = 15$) and *A. polyacanthus* (access, $n = 14$; no access, $n = 14$). $^*p < 0.05$

Table 1 Summary table of the size effect of reduced cleaner wrasse access on physiological markers in two resident client species (*A. curacao* and *A. polyacanthus*)

Physiological marker	*Amblyglyphidodon curacao*	*Acanthochromis polyacanthus*
Body condition index	−0.51% (n.s.)	**−15.17%** (·)
Haematocrit	**−21.77% (*)**	**−24.57% (*)**
Leukocyte proportion	**−46.96%** (*)	−13.97% (n.s.)
Granulocyte proportion	−33.66% (n.s.)	4.44% (n.s.)
Lymphocyte proportion	**−62.83% (*)**	**−36.03% (*)**
Cortisol levels (ng mL^{-1})	−16.26% (n.s.)	−25.56% (n.s.)
Testosterone levels (ng mL^{-1})	**−52.54% (*)**	**−33.83% (*)**

Percentage values indicate the mean change in individuals without access to cleaners compared to fish with access. Values in bold font refer to statistically significant differences ($p \leq 0.05$). (*) refers to statistically significant differences (alpha set at $p < 0.05$); (·) refers to a tendency, while (n.s.) refers to statistically non-significant differences

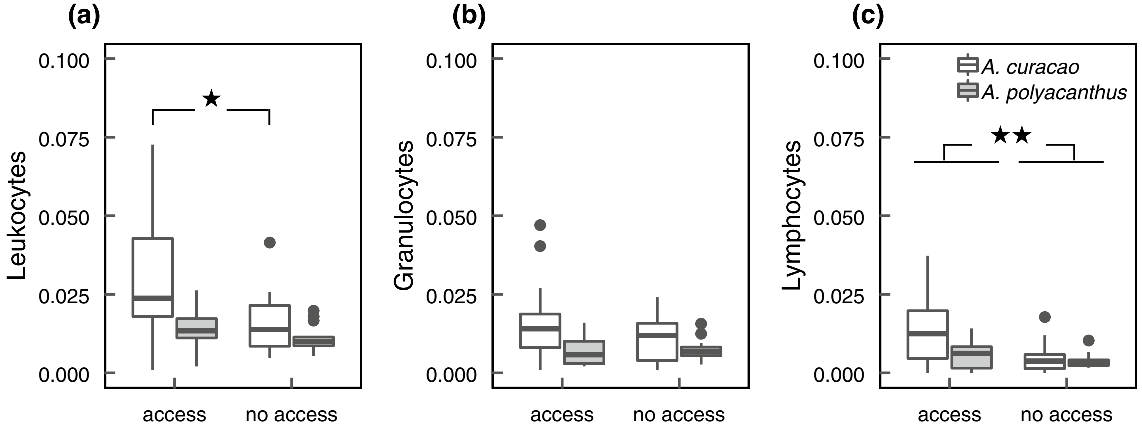

Fig. 2 Effect of access to cleaners on white blood cell proportions in two resident client fishes. Box plots of median, interquartile range, upper and lower quartiles, and outliers of (**a**) the proportion of leukocytes, (**b**) proportion of granulocytes, and (**c**) proportion of lymphocytes blood cells from the total blood cell count of *A. curacao* (access, $n = 15$; no access, $n = 15$), and *A. polyacanthus* (access, $n = 14$; no access, $n = 14$). $^*p \leq 0.05$; $^{**}p \leq 0.001$

Steroid levels

Cortisol levels were unaffected by client access to cleaners (LM $N = 56$, $F_{1,51} = 0.185$, $P = 0.669$) or species (LM $N = 56$, $F_{1,51} = 0.006$, $P = 0.939$); the interaction between species and cleaner access was also non-significant (LM $N = 56$, $F_{1,51} = 0.103$, $P = 0.749$) (Fig. 3a). However, fish with access to cleaners exhibited higher levels of testosterone than fish without access (LM $N = 44$, $F_{1,39} = 10.888$, $P < 0.01$). There was also a significant difference between species with *A. polyacanthus* displaying overall higher testosterone levels than *A. curacao* (LM $N = 44$, $F_{1,39} = 15.261$, $P < 0.001$). There was no interaction between species and cleaner access (LM $N = 44$, $F_{1,39} = 3.143$, $P = 0.084$) (Fig. 3b).

Correlations

Testosterone levels were negatively correlated with the proportion of granulocytes in *A. curacao* when they had access to cleaners (Spearman rank correlation: *A. curacao*, $rs = -0.81$, $N = 8$, $p = 0.01$). There was a similar trend in *A. polyacanthus*, but the correlation was not statistically significant (Spearman rank correlation, $rs = -0.36$, $N = 12$, $p = 0.26$, Supplementary Fig. S2). In contrast, there was no relationship between testosterone and the proportion of blood granulocytes in fish without access to cleaners (Spearman rank correlation: *A. curacao*, $rs = -0.12$, $N = 12$, $p = 0.73$; *A. polyacanthus*, $rs = -0.13$, $N = 12$, $p = 0.68$, Supplementary Fig. S3). The other physiological parameters were either weakly correlated or species-specific rather than being generally affected by the absence of cleaners (Supplementary Fig. S2 and Fig. S3).

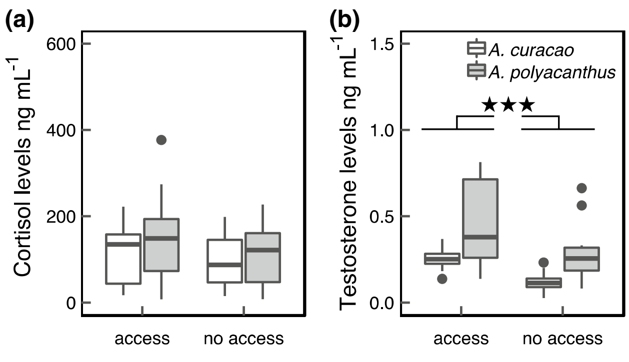

Fig. 3 Effect of access to cleaners on plasma steroid levels in two resident client fishes. Box plots of median, interquartile range, upper and lower quartiles, and outliers of (**a**) cortisol levels in ng mL^{-1} in *A. curacao* (access, $n = 13$; no access, $n = 15$) and *A. polyacanthus* (access, $n = 14$; no access, $n = 14$), and (**b**) testosterone levels in ng mL^{-1} in *A. curacao* (access, $n = 8$; no access, $n = 11$) and *A. polyacanthus* (access, $n = 13$; no access, $n = 12$). $^{***}p \leq 0.001$

Discussion

By experimentally removing cleaners, we set out to determine the effects of a relatively short period of reduced access to cleaning services on client physiology under natural conditions. In both species of resident clients tested, we found that fish with reduced access to a cleaner had lower haematocrit, testosterone, and lymphocyte levels, whereas no differences were found in body condition, leukocytes, granulocytes, and cortisol levels.

Body condition and haematocrit

In both species tested, there was no difference in body condition between individuals with or without access to cleaners. These results are contrary to the findings of Ros et al. (2011), who reported a clear overall lower body condition in several resident fish species. This discrepancy is likely due to short-term vs long-term impact. The fish in Ros et al. were caught on patches naturally lacking cleaners. Thus, the resident fishes had likely experienced reduced access to cleaning services over an extended period of time. Sopinka et al. (2016) suggest that body condition is a reliable indicator of long-term, but not short-term stress. It is, hence, possible that slow-changing physiological indicators such as body condition are robust to changes in cleaning services in the short-term.

Blood haematocrit levels were negatively affected by the absence of cleaning services. Fishes without access to cleaners had lower haematocrit than those with access. Cleaners can remove up to 4.8 ectoparasites per minute during interactions with some clients (Grutter 1996). Thus, fish without access to cleaners can harbour more parasites than the ones with access (Grutter 1999; Clague et al. 2011). In our study, it is likely that fish caught from territories without cleaners experienced much higher levels of ectoparasite infection than those with access to cleaning services. Some of the most common parasites on coral reefs, including gnathiid isopods, are hematophagous, and might diminish an individual's circulating blood volume. Hosts tend to compensate for this loss of blood volume through homoeostatic mechanisms which include increasing plasma volume (Small et al. 1983). This mechanism results in diluted blood, consequently yielding low haematocrit readings. Although, we failed to document the parasite load from the studied fish due to logistic limitations, our findings are in line with the previous research where marine hematophagous parasites have been shown to decrease haematocrit (Jones and Grutter 2005; Triki et al. 2016). The decline in haematocrit levels can hence have serious repercussion on fish fitness, through a reduced aerobic capacity (Jones et al. 1990; Clark et al. 2013).

White blood cells

Cleaner manipulation affected the leukocyte proportion only in *A. curacao*, but not in *A. polyacanthus*, wherein *A. curacao* without access to cleaners had lower proportions in comparison to *A. curacao* with access. Nevertheless, analyses carried out on leukocyte cell subtypes showed that the proportion of lymphocytes was lower in all fishes without access to cleaners. This outcome was unexpected as we hypothesised lower physiological status in fish without regular access to cleaners, and thus higher immune activity in the form of higher lymphocyte counts, in fish without access to cleaners as demonstrated by Ros et al. (2011). Intriguingly, lymphocytes are the immune-competent cells that form the adaptive immune system, and they are highly sensitive to stress hormones (Ellis 1977). Stress hormones can decrease lymphocyte loads dramatically, whereas granulocytes do not seem to be affected by the stress response (Weyts et al. 1998). Fish lymphocyte cells, as in mammals, possess glucocorticoid receptors, with a high affinity for cortisol. The binding of cortisol to these receptors in vitro induces lymphocyte apoptosis (Saha et al. 2003). Although we did not find a significant effect of cleaner removal on client fish cortisol levels, our methods of cortisol analysis may have been insufficient to detect baseline stress levels in our individuals (see discussion about cortisol below). Furthermore, our analyses focused exclusively on evaluating white blood cell counts rather than their activity. White blood cells counts have been repeatedly used as a reliable proxy for physiology state (Chung et al. 2015). However, further research exploring immune activity in addition to white blood cells counts would be useful to establish the mechanisms by which environmental challenges might interfere with immunity.

Steroids

Removal of cleaners was predicted to induce an environmental stress on resident clients that would impair their response to parasitic infection (Grutter 1996). Unexpectedly, we failed to demonstrate a difference in cortisol levels based on whether fishes had access to cleaners or not. All fishes in our experiment experienced stress during catching, transporting, and handling in the 4–10 h after capture. Previous work on Chinook salmon (*Oncorhynchus tshawytscha)* showed that repeated stressors may generate a “cumulative stress response”, wherein the amount of cortisol detected in the blood covaries positively with the number of the repeated handling stress (Barton et al. 1986; Barton 2002). Despite the long-time gap between the repeated stressors and the cortisol sampling, Barton et al (1986) still found significant differences in cortisol readings up to 12 h post-exposure (Barton et al. 1986). We expected to observe a cumulative stress response in our fish, such that the stress induced by the removal of cleaners would simply add to that induced by the catching and handling stressors. Nevertheless, it is possible that our individuals reached maximum levels of circulating blood cortisol, which remained high during the sampling period (Grutter and Pankhurst 2000; Pankhurst 2011). For instance, our cortisol readings in *A. polyacanthus* were 18-fold higher than readings from the same species, but without stressors such as handling (Pankhurst et al. 1999). It appears that the sampling methods to assess circulating cortisol have a major influence on the results: Bshary et al. (2007) did find higher cortisol levels in clients without access to cleaners, but Ros et al. (2011) failed to find similar differences in the circulating levels of cortisol in their study. Intriguingly, Bshary et al. (2007) measured cortisol levels in the holding water where fish were housed for one hour after capture, while Ros et al. (2011) measured the circulating cortisol from blood plasma sampled within a very short time lapse (i.e., 8 min maximum) after capture. Together, it suggests that holding water cortisol can be an alternative method for assessing baseline or acute stress levels in fishes (Bshary et al. 2007; Félix et al. 2013). This method is a non-invasive procedure to evaluate cortisol levels that depends strongly on the clearance time of the hormone from the blood plasma through the urine, or by passive diffusion through the gills (Félix et al. 2013).

Our steroid analyses showed that fish without access to cleaners experienced a decrease in their circulating testosterone levels. In the absence of cleaning service, clients experience increased parasite infection rates (Grutter et al. 2017). Therefore, fishes should switch life-history strategies and invest energy in immune defences rather than in reproduction following the trade-off hypothesis (Slater and Schreck 1997; Kurtz et al. 2007). Our findings are in line with the trade-off hypothesis: we found that fishes with access to cleaners had relatively higher testosterone levels as well as a negative correlation between testosterone and granulocytes. Since granulocytes possess androgen receptors, this observation is not the result of an immunosuppressive effect on these cells due to high testosterone levels (Slater and Schreck 1993; Slater et al. 1995; Águila et al. 2013). Rather, fishes with access to cleaners are likely investing less in immune defence, since they have their parasites removed regularly. Therefore, these fishes should preferentially invest energy in reproduction instead of immunity. Although testosterone levels were lower in fish without access to cleaners, there were no apparent trade-offs between testosterone and other physiological indicators. One month is, perhaps, inadequate to detect such trade-offs, and this hypothesis should be further explored in repeated sampling of sites following cleaner removal over the course of several months.

Conclusion

Our findings suggest that cleaner absence creates a challenging environment for the resident clients sampled. We found evidence suggesting that a client's energy allocation in terms of reproduction versus immunity can adjust even within one month period. Our findings add to the growing body of literature on the direct benefits of repetitive cooperative interactions in nature. They suggest that acquired benefits, in the presence of such relationship, can improve physiological state, and shape an individuals' energy allocation patterns towards reproduction rather than immune defence.

Data availability

The data and the statistical analyses script that support the findings of the present study are available in the repository Figshare: (https://doi.org/10.6084/m9.figshare.5032262).

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Author contributions RB, ZT and CD conceived the study. ZT, CD, DGR and SAB finalized data collection design. CD and ZT collected the data with assistance from DGR and SAB. GG performed the HPLC-MS analyses. ZT ran statistical analyses and generated the figures. ZT and CD wrote the first draft of the manuscript, and ZT incorporated comments from all co-authors.

Conflict of interest All authors declare that they have no conflict of interest.

Ethical standards The Animal Ethics Committee of the Queensland government (DAFF) approved the project (CA 2015/06/869).

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