The impact of long-term reduced access to cleaner fish on health indicators of resident client fish

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ABSTRACT

In many mutualisms, benefits in the form of food are exchanged for services such as transport or protection. In the marine cleaning mutualism, a variety of ‘client’ reef fishes offer ‘cleaner’ fish Labroides dimidiatus access to food in the form of their ectoparasites, where parasite removal supposedly protects the clients. Yet, the health benefits individual clients obtain in the long term from repeated ectoparasite removal remain relatively unknown. Here, we tested whether long-term reduced access to cleaning services alters indicators of health status such as body condition, immunity and the steroids cortisol and testosterone in four client damselfish species Pomacentrus amboinensis, Amblyglyphidodon curacao, Acanthochromis polyacanthus and Dischistodus perspicillatus. To do so, we took advantage of a long-term experimental project in which several small reefs around Lizard Island (Great Barrier Reef, Australia) have been maintained cleaner-free since the year 2000, while control reefs had their cleaner presence continuously monitored. We found that the four damselfish species from reef sites without cleaners for 13 years had lower body condition than fish from reefs with cleaners. However, immunity measurements and cortisol and testosterone levels did not differ between experimental groups. Our findings suggest that clients use the energetic benefits derived from long-term access to cleaning services to selectively increase body condition, rather than altering hormonal or immune system functions.

KEY WORDS: Cleaning mutualism, Immunocompetence, Condition, Cortisol, Testosterone, Reef fish

INTRODUCTION

Many individuals of different species engage in mutualistic interactions (Bronstein, 2001). Partners in such interactions exchange goods in the form of a reward (food) or service (e.g. transport, pollination or protection) (Bronstein, 2001; Noé et al., 2001). Thus, mutualisms provide excellent study systems to test the evolutionary game theory on the evolution of cooperation without inclusive fitness playing a role (Archetti and Scheuring, 2012; Bshary and Bronstein, 2004; Leimar and Hammerstein, 2010; Noé and Hammerstein, 1995; Sachs et al., 2004). However, the quantification of benefits often involves proxies rather than direct measures of fitness, raising questions about how payoffs translate into ultimate benefits.

An exemplary study system for studying causality in mutualistic interactions is the marine cleaning mutualism, wherein cleaner organisms forage on ectoparasites on the body of their mutualistic partners. Cleaning mutualism plays a central role in coral reef ecosystems, as evidenced in studies on the bluestreak cleaner wrasse (Labroides dimidiatus) and its numerous species of ‘client’ fishes (Bshary, 2003; Grutter et al., 2003). Daily, cleaners engage in iterated mutualistic cleaning interactions with an impressive number of clients, consuming on average 1200 parasites from 2300 clients per day (Grutter, 1996b). Thus, clients visit cleaners to offer them a meal that in return leads to a net reduction of their parasite load (Clague et al., 2011; Grutter, 1999). The most commonly eaten ectoparasite by L. dimidiatus is gnathiid isopods (Grutter, 1996b, 1997a) and consequently, L. dimidiatus’ presence on reefs lowers the abundance and infestation rate of the parasitic stages (Grutter, 1999; Grutter et al., 2018) and the free-living stages of gnathiids (Grutter et al., 2019; Sikkel et al., 2019). Gnathiids feed on blood and negatively affect host physiology (haematocrit, corticosteroids), behaviour (cognition, performance), demographic traits (growth, survival) and community dynamics (blood parasite transmission) (Sikkel and Welicky, 2019). Gnathiids also cause hosts (clients) to seek cleaners (Grutter, 2001). However, conflicts between the mutualistic partners may arise as cleaners prefer to eat clients’ mucus instead of removing ectoparasites (i.e. cheating: Grutter and Bshary, 2003), which may result in increased levels of stress in visiting clients. To compel cleaners to feed against their preference, clients employ partner control mechanisms, i.e. behaviours that reduce the payoff of cheaters, such as partner switching and punishment through aggressive chisling (Bshary and Grutter, 2005). Partly in response to these control mechanisms, cleaners provide another benefit to clients. In essence, they use their pelvic fins to give the clients tactile stimulation (Potts, 1973), an act that reduces cortisol levels (a measure of stress levels) in clients (Soares et al., 2011).

Thus, while cleaners show behaviours that benefit clients, such as removal of parasites and tactile stimulation, they also may harm the client by biting their mucus. There has been a long-standing interest in quantifying the net effects of these different elements of cleaning interactions on client fitness (Clague et al., 2011; Demairé et al., 2020; Limbaugh, 1961; Losey, 1987; Ros et al., 2011; Waldie et al., 2011). Although these studies yielded partly contradicting results, the project leading to the publications by Waldie et al. (2011) and Clague et al. (2011) is the most thorough as it manipulated long-term presence and absence of cleaners using a total of 16 reefs as independent replicates (Grutter et al., 2018). So far, the outcomes of this project have shown positive effects of cleaner presence on client growth and body size (Clague et al., 2011; Waldie et al., 2011). A reasonable hypothesis for these results is that exposure to
ectoparasites warrants increased investment in immunocompetence (Lindström et al., 2004). There is mounting evidence that ectoparasites induce changes in the expression of immune genes (Lindeminen and Begon, 2010; Mills et al., 2010; Sheldon and Verhulst, 1996). Accordingly, Ros et al. (2011) found that resident clients on isolated reefs that are naturally without cleaners had increased immune defense and decreased body condition compared with fish with access to cleaners. Thus, Ros et al. (2011) postulated that by using cleaning interactions as a behavioural parasite avoidance mechanism (i.e. Behringer et al., 2018; Hart, 1990), reef fish gain energetic benefits from access to cleaners as a result of a decreased need to invest in their immune system. A shortcoming in the study of Ros et al. (2011) is that the history of cleaner fish presence on the isolated reefs was unknown and was not experimentally manipulated. The correlative nature of the data collection leaves open the possibility that clients without access to cleaners showed evidence of lower health status because they lived in patches of lower quality. Furthermore, the question remains how the lack of access to cleaner fish affects clients’ health status over long-term periods, i.e. the lifetime of individuals. The experiment of choice to approach these questions is to compare reefs with an experimentally controlled history of presence versus absence of cleaners (e.g. Gutter, 1997b; Losey, 1979). A long-term experiment of that kind has been established at Lizard Island, Great Barrier Reef, Australia. Since 2000, cleaners have been regularly (i.e. around every 3 months) removed from isolated experimental reefs, while their presence was repeatedly confirmed on the control reefs (Gutter et al., 2003; Waldie et al., 2011). This ongoing study substantiated, among others, the negative impact of cleaner absence on the diversity and density of client species (Gutter et al., 2003; Waldie et al., 2011). Furthermore, it showed that small-bodied resident damselfish on reefs with cleaners grow larger than those on reefs without cleaners (Clague et al., 2011; Waldie et al., 2011). Probably, the increase in body growth in fish on control reefs is a consequence of the substantial decrease in ectoparasite load through repeated cleaning interactions (Gutter et al., 2018). Other than condition benefits, access to cleaners can also have cognitive benefits for clients (Binning et al., 2018), though the underlying mechanism remains unknown. More generally, the long-term effects of cleaner removal on client physiology have not been studied. Here, we close this knowledge gap through studying the long-term impact of cleaner presence/absence on resident client fish health indicators.

In two experiments, we tested the effect of cleaner presence/absence on several indicators of health status by using the long-term cleaner removal experimental setup at Lizard Island. First, we tested whether access to cleaner treatment affects body condition, steroid hormone levels (i.e. cortisol and testosterone) and different proxies for the immune system in four damselfish (Pomacentridae) species Pomacentrus amboinensis, Amblyglyphidodon curacao, Acanthochromis polyacanthus and Dischistodus perspicillatus. A range of immunity measures were evaluated to test both the state of the immune system and its response to challenges (reviewed in Norris and Evans, 2000; Watts et al., 2001). First, we tested differences between treatments in chemical defences against bacterial pathogens assessed by measuring lytic activity (Holland and Lambris, 2002) and in cellular defences evaluated through blood leucocyte cell counts and phagocytic activity (Norris and Evans, 2000). Second, in the larger species D. perspicillatus, we tested the effect of treatment on the antibody response to a challenge with the antigen dinitrophenyl keyhole limpet haemocyanin (DNP-KLH) (Herscowitz et al., 1975; Ros et al., 2011). Two alternative predictions were considered to explain previous findings on the relationship between body condition and cleaning. Both are based on the fact that clients on reef patches where cleaners are removed have higher exposure to ectoparasites (Binning et al., 2018; Clague et al., 2011; Gutter, 1999; Gutter et al., 2002, 2018, 2019; Sikkel et al., 2019). First, higher parasite exposure on reefs with long-term cleaner removal would result in higher investment in immune activity and this would trade-off with investment in body condition (Ros et al., 2011). Alternatively, higher ectoparasite exposure on reefs with long-term cleaner removal would in itself be energetically costly as a result of parasites consuming host resources, resulting in low body condition. In the second prediction, the effects of experimental treatment on the immune system, if any, are spuriously related to depletion of energetic resources. Related to such energetic trade-offs, we expected steroid hormones to remain at low basal levels with no difference between long-lasting and thus stable cleaner treatment groups. This is because exposure to chronically high levels of steroid levels is costly (Bonier et al., 2009; MacLeod et al., 2018) and because steroid levels tend to vary less during periods in which the environment is stable (Wingfield and Kitaysky, 2002). Overall, we predicted that client fish with access to cleaners would have a relatively better health status than those with no access.

MATERIALS AND METHODS

Study site and animals

The study was carried out between July and September 2013 at Lizard Island, Great Barrier Reef, Australia (14°40′ 50″S, 145°26′54.5″E). We used the ‘long-term cleaner fish removal project’, which was established by Gutter in the year 2000 (see Gutter et al., 2018). At the start of this project, seven isolated reefs were randomly selected and all L. dimidiatus were removed using hand nets, whereas nine randomly selected control reefs were left undisturbed. The For over 13 years these reefs were inspected for cleaner presence approximately every 3 months during which any L. dimidiatus recruits on ‘removal reefs’ were removed. The mean (±s.e.m.) number of L. dimidiatus was 0.16±0.04 adults and 0.44 ±0.07 juveniles on removal reefs, compared with 2.15±0.10 adults and 0.85±0.10 juveniles on control reefs (see methods in Clague et al., 2011). Experimental reefs (Fig. 1) had a surface area ranging from 60 to 285 m² and were isolated by at least 5 m of sandy substrates from other reefs; there have been no indications that the resident fish population, including the cleaners, move between these reefs (see Gutter, 1996a; Waldie et al., 2011).

The four study species were Pomacentrus amboinensis Bleeker 1868, Amblyglyphidodon curacao (Bloch 1787), Acanthochromis polyacanthus (Bleeker 1855) and Dischistodus perspicillatus (Cuvier 1830). These species are common resident client fish on the reefs around Lizard Island (Triki et al., 2018; Waldie et al., 2011). Fish were caught using a barrier net and hand nets. Because of the relatively large body size of D. perspicillatus (up to 18 cm total body length, TL), we used a larger barrier net (size: 12×2 m,
Experimental design

Experiment 1: effect of long-term removal of cleaners on basal measures

This experiment aimed at testing the effect of cleaner treatment on client health indicators. Here, we caught four P. amboinensis, 10 A. curacao, seven A. polycanthus and nine D. perspicillatus from removal reefs without cleaners, and 12 P. amboinensis, 11 A. curacao, 10 A. polycanthus and 21 D. perspicillatus from control reefs with cleaners. Immediately after fish capture, while still underwater, we transferred the fish individually into quick-sealing plastic bags filled with seawater and a small amount of a sedative (2-phenoxyethanol, Sigma-Aldrich: 0.5 ml l−1 water). We controlled the sedation progress by monitoring gill movements. Once the fish had reached a sedated state (fish not responding but gills still moving), a blood sample was drawn underwater from the caudal vascular vein with a 25 gauge needle (method adapted from Grutter and Pankhurst, 2000). The volume of the sampled blood was approximately 1% of fish body size; for example, up to 50 μl for P. amboinensis and up to 500 μl for D. perspicillatus. All samples were taken within 5 min of capture. After collecting the blood samples, total length and maximum girth (measured behind the gills, at the belly) were measured while fish were still in the plastic bag. The mean (±s.e.m.) TL of the caught fish was: P. amboinensis: 8.2±0.3 cm; A. curacao: 9.7±0.4 cm; A. polycanthus: 12.1±0.3 cm; D. perspicillatus: 18.9±0.5 cm.

Directly after blood sampling, while still underwater, the blood sample was transferred from the syringe to a 2 ml heparinized vacuum container (Vacutainer, BD, Franklin Lakes, NJ, USA). Back on the boat, depending on blood volume (not for the smaller P. amboinensis), half of the blood was transferred a Vacutainer containing CO2-independent medium (see ‘Phagocytic activity’, below). The vials were kept cool on ice-water and quickly brought to the laboratory at the Lizard Island Research Station for further processing. In the laboratory, a small quantity of the blood samples was used for making blood smears and carrying out the phagocytosis protocol. Immediately after this procedure, blood was centrifuged at 2000 g for 10 min and plasma was collected and stored in Eppendorf tubes at −20°C until further processing at the University of Neuchâtel. Based on sample volume, samples were assigned to the different measurements. In the case of the smaller volumes, especially those for P. amboinensis, some measurements (i.e. hormone levels, lytic activity, phagocytosis and/or blood cell counts) were not carried out. Therefore, n-values differ between treatments and these are reported in the respective graphs and Table S1.

Experiment 2: effect of long-term removal of cleaners on the antibody response

The 30 D. perspicillatus captured for blood samples in experiment 1 were tagged underwater with individually recognizable elastomer marks (VIE, Northwest Marine Technology, Anacortes, WA, USA) while fish were still sedated. These fish were used for further analysis as part of immune activity assessment, wherein they received an intraperitoneal injection of DNP-KLH antigen (Merck Calbiochem). DNP-KLH was dissolved in saline (0.9% NaCl) at 500 μg ml−1. The injected amount of DNP-KLH suspension was adjusted to the predicted body mass of the fish based on the measured length of the fish (0.2 ml suspension per 50 g body mass, where body mass=0.028×TL3, based on an average of allometric relationships of damselfishes; Froese and Pauly, 2019). At the end of the procedure, water in the bag was refreshed regularly for at least 10 min to give the fish time to recover from sedation and then the fish was released at its site of capture. The fish was closely followed for another 10–15 min after release to protect it from potential predation attempts. After 14–17 days (some variance due to bad
cell counts. Leucocytes were further classified as lymphocytes and granulocytes (Ellis, 1977). Directly after reaching the laboratory from the field, a drop of fresh blood was smeared over a frosted slide, air dried, fixed with methanol, and stored in a sealed dry environment at ambient temperature. At the University of Neuchâtel, blood smears were examined with a light microscope (BX-50, Olympus, Japan) at 1000× magnification under oil immersion. For each subject, 25 images were captured from the smears from randomly chosen locations at the homogeneous unicellular layer. Total cell numbers in each image were automatically counted using ImageJ software (Abramoff et al., 2004). The fit of human-counted images versus automated counts in ImageJ was found to be Y=0.99X, R²=0.992 with N=193 (Ros et al., 2011). The mean±s.d. number of cells per field was 138±70, with most counts ranging between 50 and 200 cells. Leucocytes were counted directly from the image (without the help of software) and were mostly lymphocytes (Ellis, 1977).

Immune activity

For the estimation of antibody levels to DNP-KLH, an agglutination protocol (Herscowitz et al., 1975; Ros et al., 2011) was applied, using antigen coupled to sheep erythrocytes (sheep red blood cells, SRBC; Harlan, Bicester, UK). In brief, 0.25 ml washed SRBC (100%), 2.5 ml DNP-KLH solution (3 mg ml⁻¹ phosphate-buffered saline, PBS) and 2.5 ml CrCl₃ · 6H₂O solution (1.33 mg ml⁻¹ PBS) were incubated for 35 min at room temperature with occasional mixing. After incubation, the DNP-KLH-coupled SRBC cells were centrifuged (800 g for 5 min) and washed 3 times with PBS. These cells were directly used for the hemagglutination test. To prevent lysis of SRBC by complement, the plasma was heated to 56°C for 30 min (Collazos et al., 1994). After that, the plasma was diluted 1:1 in PBS and then serially diluted in PBS in U-shaped microtitre plates. An equal volume of 0.2% DNP-KLH-coupled SRBC (see Ros et al., 2011) was added to these dilutions, and the plates were incubated at room temperature for 60 min. Antibody titres were scored visually as the highest twofold dilution of plasma showing hemagglutination.

Phagocytic activity

In this assay, the phagocytic capacity of lymphocytes in whole blood was estimated based on the protocol from Millet et al. (2007). After collection, and while still underwater, blood was diluted to 1:20 with CO₂-independent medium (supplemented with 1% penicillin/streptomycin mixture and 4 mmol l⁻¹ L-glutamine, Gibco-Invitro). The assay was conducted within 1.5 h of blood collection. In the laboratory, latex beads were coated with lipopolysaccharide (LPS) from Escherichia coli in PBS plus 2 mmol l⁻¹ sodium azide. Blood samples diluted with CO₂-independent media were incubated with the LPS–latex suspension in 8-well chamber slides (Nalgene Nunc International, Naperville, IL, USA) at 25°C for 90 min. Phagocytic leucocytes engulfed the beads and adhere to the wall of the incubator. Phagocytic activity was stopped by cooling the slide on ice. Afterwards, the 8-well chamber slide was washed 2 times with CO₂-independent media, and the cells were fixed for 2 min with 250 μl methanol on ice. The slides were then dried in air and stored until they were stained with Giemsa stain to count the lymphocyte cells that had enclosed LPS–latex beads.

Lytic activity

A 0.2 g l⁻¹ bacterial suspension was made of lyophilized Micrococcus lysodeikticus (Sigma-Aldrich) in 0.04 mol l⁻¹ KH₂PO₄ (pH 5.75) buffer. A standard chicken lysozyme curve (Sigma-Aldrich) serial dilution was used (12.5–0.75 mg l⁻¹). Blood plasma was centrifuged to get rid of solids and samples were placed on ice. Then, 10 μl of sample and standard were injected into a plate well in duplicate and to each well 250 μl of bacterial suspension was added. The plate was read in a Synergy HT plate reader at 595 nm at 25°C, with 1 s shaking before every reading. In total, 60 readings were carried out at 1 min intervals to measure the speed of reduction in turbidity of the sample. Lytic activity was calculated as the decrease in opacity (OD₅₉₅) over of the bacterial suspension as initial OD₅₉₅ divided by final OD₅₉₅.

Steroid levels

Methods were based on the protocol described by Ros et al. (2015; see also Demaille et al., 2020, for further details). Blood samples were drawn while scuba diving, which allowed us to obtain the samples directly after capture. A similar method used by Grutter and Pankhurst (2000) showed that cortisol plasma levels in the coral reef fish Hemigymnus melapterus increased significantly only after 5–6 min of capture. Therefore, time at the onset of capture was monitored with a stopwatch and only individuals that could be sedated within that time range were sampled, while the others were...
released. Not all steroids were analysed for all species (Table S1). Initially, plasma samples were analysed for cortisol levels. Only larger blood volumes (i.e. collected from the larger species) allowed for analysis of both testosterone and cortisol. Samples were shipped on dry ice to the University of Neuchâtel, Switzerland, where hormones were extracted from the plasma fraction and analysed using UHPLC-MS/MS. This method enables multiple steroids to be quantified simultaneously, and it has a superior dynamic range (Hauser et al., 2008; Koren et al., 2012).

Ethics statement
The research was conducted under permits from the Great Barrier Reef Marine Park Authority (G11/34413.1) with approval from the Queensland Department of Employment, Economic Development and Innovation (DEEDI) Animal Ethics Committee (CA 2012-05-613).

Statistical analysis
Precise sample sizes for each species and measurement are reported in Table S1. We statistically analysed the data and generated the figures with R version 3.5.3 and QGIS version 3.4.12. We ran linear mixed (LMM) and generalized linear mixed (GLMM) effect models to account for the repeated sampling of the same reef sites. Therefore, reef identity was added as a random factor in the models. The treatment of cleaner presence/absence and client species and their interaction were fitted as fixed factors in all the statistical models. To meet the model’s assumptions, such as normality of the residual distribution or homogeneity of the variance, we transformed the data when applicable. Proportion data were arcsine square-root transformed, while continuous variables were log-transformed. We checked for models’ assumptions visually via plots. None of the interactions between cleaner and species effects was statistically significant (for all statistical results, see Table 1), allowing us to focus on the main effect of cleaner alone.

RESULTS
Below, we focus on the effects of cleaner fish removal on separate aspects of health status, i.e. body condition, immune parameters and hormones. Correlation coefficients between all variables measured in each of the four study species are reported in Fig. S1.

Table 1. Mixed effects models summary of the results of cleaner removal, species and their mutual interaction on several physiological traits

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Cleaner removal effect</th>
<th>Species effect</th>
<th>Cleaner removal×species interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>χ²</td>
<td>d.f.</td>
</tr>
<tr>
<td>Condition</td>
<td>78</td>
<td>4.730</td>
<td>1</td>
</tr>
<tr>
<td>Leucocyte proportion</td>
<td>79</td>
<td>0.033</td>
<td>1</td>
</tr>
<tr>
<td>Granulocyte proportion</td>
<td>79</td>
<td>0.101</td>
<td>1</td>
</tr>
<tr>
<td>Lymphocyte proportion</td>
<td>79</td>
<td>0.085</td>
<td>1</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>61</td>
<td>0.101</td>
<td>1</td>
</tr>
<tr>
<td>Lytic activity</td>
<td>82</td>
<td>0.820</td>
<td>1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>67</td>
<td>2.199</td>
<td>1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>30</td>
<td>0.289</td>
<td>1</td>
</tr>
<tr>
<td>Antibody response</td>
<td>30</td>
<td>0.662</td>
<td>1</td>
</tr>
</tbody>
</table>

For details, see Results. Bold indicates significance (P≤0.05).

Notes: Table 1. Mixed effects models summary of the results of cleaner removal, species and their mutual interaction on several physiological traits. The table shows the statistical analysis results for various physiological traits, including condition, leucocyte proportion, granulocyte proportion, lymphocyte proportion, phagocytosis, lytic activity, cortisol, testosterone, antibody response, and their interactions. The table includes the number of observations (N), chi-square (χ²), degrees of freedom (d.f.), and p-values (P) for each factor. The table highlights the significance levels for the main effects and interactions, with bold indicating statistical significance (P≤0.05).
State of the immune system

Across species, there was no effect of cleaner removal on leucocyte proportion (i.e. leucocyte cell count/total cell count, LMM, \( P=0.855 \); Fig. 3A, Table 1). Leucocyte proportions in \( A. \) curacao (mean±s.e.m.: \( A. \) curacao: 0.046±0.010) were higher than in the other three species that were sampled (mean±s.e.m.: \( A. \) polyacanthus: 0.024±0.005; \( D. \) perspicillatus: 0.021±0.002; \( P. \) amboinensis: 0.032±0.009; LMM, \( P=0.035 \)). For the proportions of the two main classes of leucocyte cells, when analysed separately, there was also no effect of cleaner removal (granulocytes: LMM, \( P=0.750 \); lymphocytes: LMM, \( P=0.770 \); Table 1).

Phagocytosis (Fig. 3B) and lytic activity (Fig. 3C) were not affected by cleaner removal (phagocytosis: LMM, \( P=0.751 \); lytic activity: LMM, \( P=0.365 \); Table 1). Lytic activity showed a significant species effect, with \( A. \) polyacanthus and \( D. \) perspicillatus having more than twice the activity of the other two species (Fig. 2C; LMM, \( P<0.0001 \)).

Steroid levels

Neither cortisol nor testosterone was significantly affected by cleaner removal (cortisol: GLMM, \( P=0.138 \); testosterone: LMM, \( P=0.591 \); Fig. 4, Table 1). Means (±s.e.m.) pooled across cleaner treatments for cortisol were: 54.4±9.8 ng ml\(^{-1} \) in \( A. \) curacao, 36.6±5.2 ng ml\(^{-1} \) in \( D. \) perspicillatus, and 20.4±5.2 ng ml\(^{-1} \) in \( P. \) amboinensis (Fig. 4A); and for testosterone 1.23±0.35 ng ml\(^{-1} \) in \( D. \) perspicillatus (Fig. 4B).

**Experiment 2: effect of long-term removal of cleaners on the antibody response**

Antibody responses to DNP-KLH injection were not affected by cleaner removal (Bayesian GLMM, \( P=0.416 \); Fig. 5, Table 1).

**DISCUSSION**

We investigated whether experimentally long-term reduced access to cleaner fish has negative effects on client fish health indicators. Our study provided experimental evidence that prevention of access to cleaning services has a moderate negative impact on an indicator of client fitness, i.e. body condition. However, the reduced access to cleaners did not systematically affect measurements related to the immune system, nor to steroid levels. Below, we discuss in detail each of the studied health indicators in turn.

The current study showed that resident damselfish caught at reefs without cleaners had lower body condition, estimated by girth TL\(^{-1} \), than fish in reefs with cleaners. Maximum girth measurement varies according to muscle mass and fat storage and can thus be used as an excellent correlate for fish body mass (Jones et al., 1999). Our findings are in line with previous research performed on two resident fish species (one being the same species tested here, \( P. \) amboinensis, using the same experimental reefs) where the length of fish was drawn from \( P. \) amboinensis were too small for phagocytosis analysis.

Demairé et al. (2020) did not find a significant effect of cleaner removal on fish condition. Thus, long-term cumulative effects of increased red blood cell loss (Demairé et al., 2020) and/or elevated stress (Bshary et al., 2007; Soares et al., 2011; Sopinka et al., 2016) may need to be experienced in fish to result in a reduced body condition.
No experimental evidence was found for the proposed immune trade-off, in which it was postulated that fish without access to cleaners have to increase investment in immunity because of a higher exposure to parasites and parasite-related pathogen infections (Ros et al., 2011). It has been established that cleaner removal results in an increase in ectoparasites in comparison to control reefs with cleaners (Binning et al., 2018; Clague et al., 2011; Grutter, 1999; Grutter et al., 2002, 2018, 2019; Sikkel et al., 2019). Of immunity measures, antibody response was significantly increased by cleaner absence in the Red Sea (Ros et al., 2011), but not in the current long-term cleaner removal experiment. Moreover, in a recently published short-term cleaner removal experiment (Demairé et al., 2020), leucocyte proportion was found to be lower as a result of cleaner absence, but not in our study and that by Ros et al. (2011). There are many potential explanations for the observed differences between the three studies. Most importantly, it should be noted that the immune system is a complex suite of traits which together defend the individual against pathogenic intrusion (Janeaway et al., 1999). Simplified, these traits embody different barriers that pathogens have to overcome to invade their host, all of which may respond differently to long- or short-term changes in the environment (Demas and Nelson, 2011; Watts et al., 2001). For instance, (a) the chemical barriers against bacterial intrusions measured by lytic activity, and (b) the cellular defences (cell counts) which neutralize pathogens by phagocytosis and can be optimized by (c) antigen-specific antibody responses that are governed by specialized immune cells that produce antibodies (acquired immunity).

Lymphocytes are sensitive to stress (Braude et al., 1999) and short-term changes in access to cleaner fish as in the study by Demaire et al. (2020) might result in higher stress for fish than would be experienced for fish living in long-term (i.e. 13 years) manipulated (and thus stable) reefs, as was the case in the current study. Stress, however, cannot explain the difference between our study and that by Ros et al. (2011). A possible explanation could be season-specific changes in pathogenic challenges (‘winter’ in the current versus ‘summer’ in the study by Ros et al., 2011), and/or species-dependent differences in the immune system (damselfishes in the current study versus a mix of reef fishes in the study by Ros et al., 2011). Beside these considerations, we conclude based on the results of the current experiment that visiting cleaner fish cannot be explained as an alternative strategy for immune protection against parasites (as was proposed in Ros et al., 2011).

The absence of cleaners had no negative effect on basal levels of cortisol in clients. This result is in line with what was expected based on the available literature on basal levels drawn directly from fish in the field (Ros et al., 2011). It is also in line with the general literature on hormonal regulation of emergency responses, in which system-wide changes induced by cortisol may be functional in the short term for survival but are costly when maintained for longer periods (Schreck, 2010; Wingfield et al., 1998). Although basal cortisol levels did not change with cleaner absence, considerable variation was found within and between species. Limited information is available on cortisol levels in the species we studied. Levels reported by Pankhurst (2011) for reef fish sampled within 5 min were 0.9–125 ng ml$^{-1}$. The cortisol levels in damselfishes sampled within 5 min in our previous study (Ros et al., 2011, unpublished values) were 3.1–17.7 ng ml$^{-1}$ ($n=7$) for Dascyllus trimaculatus and 5.2–61.3 ng ml$^{-1}$ ($n=5$) for Amblyglyphidodon leucogaster. Thus, in general, the range of levels found in our study (2.1–141.4 ng ml$^{-1}$) show a large overlap with the ranges documented. Nonetheless, A. curacao had relatively high cortisol levels in comparison to P. amboinensis and D. perspicillatus. However, this
behavioural and metabolic responses, testosterone is metabolized to β-estradiol or 11-ketotestosterone (Borg, 1994). Functions are diverse, ranging from controlling gonadal growth and maturation, to facilitating social behaviour, and learning from social experiences (e.g. Antunes and Oliveira, 2009; Borg, 1994; Oliveira, 2009; Soares et al., 2019). During reproductive periods, when testosterone levels are higher, testosterone might prioritize social interactions and parental behaviour above cleaner interactions, and thus have potential consequences for parasite removal.

In conclusion, we have shown that long-term cleaner absence has a negative impact on the body condition of coral reef fish. Contrary to our expectations, we could not attribute this effect to an increase in investment in immunity or to changes in steroid hormone regulation. Seasonal, client species and ectoparasite differences between this study and that of Ros et al. (2011) may potentially explain the discrepancies between the two studies. However, a major strength of the present study is that it is based on 13 years of reef manipulation, giving unprecedented strength to long-term estimates of cleaner benefits. Protection from repeated blood loss by ectoparasite infestation (see Demaître et al., 2020; Tricker et al., 2016) might be an important factor in such physiological benefits.

Acknowledgements

Special thanks to Eva McClure, and the staff at the Australian Museum Lizard Island Research Station, and especially Lyle and Anne Vail for providing the support and means to carry out the research. We thank S. Gingins, J. R. Paula and J. Messias for assistance in the field.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

This study was supported by the Australian Research Council (DP120102415 to A.S.G. and R.B.), the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (31003AB_135707 and 31003A_153067 to R.B.), and the Swiss Government excellence scholarship (2012.2014 to Z.T.).

Data availability

The detailed script generated in R for the current analysis along with the associated data are available from the figshare digital repository: https://figshare.com/s/e6c75c69e889603d685.

Supplementary information

Supplementary information available online at https://jeb.biologists.org/lookup/doi/10.1242/jeb.231613.supplemental

References

Abramoff, M. D., Magalhães, P. J. and Ram, S. J. (2004). Image processing with ImageJ. Biophotonics Int. 11, 36-43.


Table S1. Overview of N-values for the different measurements taken, tabulated per treatment (Control = “Cleaner present”; Removal = “Cleaner removed”) and per species in the long-term experiment of cleaner presence in Lizard Island.

<table>
<thead>
<tr>
<th>treatment</th>
<th>species</th>
<th>condition</th>
<th>leucocyte proportion</th>
<th>granulocyte proportion</th>
<th>lymphocyte proportion</th>
<th>phagocytosis</th>
<th>lytic activity</th>
<th>cortisol</th>
<th>testosterone</th>
<th>antibody response</th>
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<tr>
<td>control</td>
<td>A. curacao</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>5</td>
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<td>11</td>
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<tr>
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<td>A. polyacanthus</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>11</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>control</td>
<td>D. perspicillatus</td>
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<td>21</td>
<td>21</td>
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<tr>
<td>control</td>
<td>P. ambonensis</td>
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<td>no</td>
<td>11</td>
<td>12</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>removal</td>
<td>A. curacao</td>
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<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>10</td>
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</tr>
<tr>
<td>removal</td>
<td>A. polyacanthus</td>
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<td>D. perspicillatus</td>
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<tr>
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<td>P. ambonensis</td>
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</table>
Figure S1: Correlations between measured parameters for the four study species (a: *Amblyglyphidodon curacao*, b: *Acanthochromis polyacanthus*, c: *Dischistodus perspicillatus*, and d: *Pomacentrus amboinensis*) and per treatment (Control = “Cleaner present”; Removal = “Cleaner removed”) in the long-term experiment of cleaner presence in Lizard Island. The colour code represents the relative strength of the correlation. Significance level set at Spearman’s correlation coefficient ≥ 0.7. Consistent is the correlation between lymphocyte proportions and leucocyte proportion. A more interesting negative relationship seems to exist between phagocytosis and lymphocyte proportions in the removal treatment. This could suggest higher activation of lymphocytes in the removal treatment due to higher recruitment of lymphocytes to peripheral tissue. That would be consistent with higher tissue damage in the removal treatment due to higher ectoparasite loads. However, as the relationship is only reaching the significance criteria for one of the four species such an interpretation would be highly speculative.

Variables reported in figure S1 and how they are named in manuscript:
- `body.condition = condition (girth/TL)`
- `white_cells_prop = leucocyte proportion`
- `lymphocyte_prop = lymphocyte proportion`
- `granulocyte_prop = granulocyte proportion`
- `phagocytosis = phagocytosis`
- `lytic_activity = lytic activity`
- `SRBC = antibody response`
- `cortisol = cortisol`
- `testosterone = testosterone`
A. curacao

Control

S1a)
S1b)

**A. polyacanthus**

**Control**

**Removal**
P. ambonensis

Control

Removal (white blood cells are not added here due to small sample size)