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RESEARCH ARTICLE

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; Noë 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; Noë 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 chasing (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

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ectoparasites warrants increased investment in immunocompetence (Lindström et al., 2004). There is mounting evidence that ectoparasites induce changes in the expression of immune genes (Lindenstrøm et al., 2004) and infiltration of the skin by lymphocytes (Haond et al., 2003). By causing skin and tissue damage (Adlard and Lester, 1995), ectoparasites may also facilitate secondary infections and parasites may enter the body. In the absence of cleaners, active immunity might have to be upregulated to prevent secondary infections. While endoparasites may also affect clients in similar ways, such as lowering cognitive performance (Binning et al., 2018), there is no evidence that interactions with cleaners lower endoparasite load (Binning et al., 2018).

Mounting an immune response is a costly trait, as implied by trade-offs with investment in other fitness-related traits, including body condition and growth (Alcorn et al., 2003; Beldomenico 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 defence 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. Grutter, 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 (Grutter 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 (Grutter 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 (Grutter 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; Grutter, 1999; Grutter 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 Grutter in the year 2000 (see Grutter 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 m^2 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 Grutter, 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,

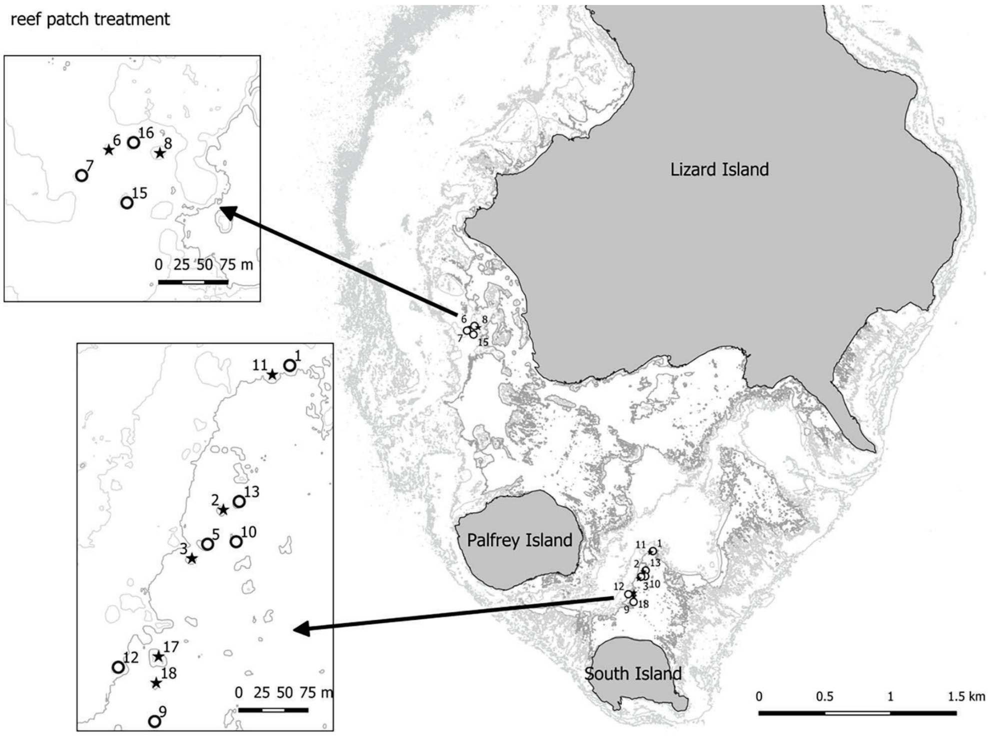

Fig. 1. Overview of the location and treatment of isolated reefs in the long-term experiment on cleaner presence in Lizard Island, Australia. Black stars represent reefs at which cleaners were experimentally removed. White circles represent reefs at which cleaners were continuously present. The map was redrawn using qGIS from GeoEye imagery taken on 24 September 2005.

mesh size: 1 cm) for it, whereas for the other three small species (body size up to 10 cm TL) a smaller barrier net (size: 1×2 m, mesh size: 5 mm) was used. For the large-bodied species, scuba-divers guided the individuals towards the barrier net. For the small-bodied species, however, we used bait such as bread and oat flakes to attract them towards the barrier net. Handling after capture depended on the experiment we were running (see 'Experimental design', below).

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. polyacanthus* and nine *D. perspicillatus* from removal reefs without cleaners, and 12 *P. amboinensis*, 11 *A. curacao*, 10 *A. polyacanthus* and 21 *D. perspicillatus* from control reefs with cleaners. Immediately after fish capture, and 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. polyacanthus*: 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 CO_2-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=$0.028 \times TL^{3.1}$, 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 weather), 30 experimentally treated *D. perspicillatus* were recaptured to collect another blood sample. Capture and return procedures were similar to those of the first capture. The second blood sample was used for estimating the antibody response potentially triggered by the DNP-KLH antigen injection.

Measurement of health indicators

Body condition

To estimate body condition, the length and maximum girth of the fish were used to calculate a condition index using the variation in girth relative to length (girth TL^{-1}). This measures the relative thickness of the fish and is thus related to the storage of fat (Jones et al., 1999). Girth relative to length varies with food supply, gonad development and season (e.g. Santos et al., 2006). This girth TL^{-1} index is hereafter referred to as 'condition based on girth'.

Immunity function measurements

As the immune system is a complex suite of traits which together defend the individual against pathogenic intrusion (Demas and Nelson, 2011; Janeway et al., 1999), several indicators for natural and pathogen-specific immunity were measured in this study (Norris and Evans, 2000; Watts et al., 2001). These measurements involved: (1) leucocyte cell counts (differentiated into lymphocytes and granulocytes) as a phenotypic measure of cellular defences (Blaxhall and Daisley, 1973); (2) the antibody response to a DNP-KLH injection as a measurement of the ability of the immune system to mount a specific immune response (Herscowitz et al., 1975; Ros et al., 2011); (3) phagocytosis as a measurement of cellular responses to pathogens; and (4) lytic activity as a functional measurement of chemical constitutive defences against parasites (Harris et al., 1998; Holland and Lambris, 2002; Jones, 2001). Variation from control levels in the case of leucocyte counts is indicative of increased immune challenges, but may also indicate increased exposure to stress (Wedemeyer et al., 1990). Lower values of the three other measurements indicate lower immunocompetence. Measurements were taken from several species, except for the antibody response, for which measurements were taken from the larger species, *D. perspicillatus*, only.

Leucocyte cell counts

Cell counts from blood smears were carried out to assess leucocyte 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 stained for 20 min with Giemsa's azur–eosin–Methylene Blue solution (1:10 dilution in PBS; Merck KGaA, Darmstadt, Germany). 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 (Abràmoff et al., 2004). The fit of human-counted images versus automated counts in ImageJ was found to be Y=0.99X, R^2=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 $CrCl_3 \cdot 6H_2O$ 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 CO_2-independent medium (supplemented with 1% penicillin/streptomycin mixture and 4 mmol l^{-1} L-glutamine, Gibco-Invitrogen). 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 CO_2-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 engulf 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 CO_2-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} KH_2PO_4 (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 (OD_{595}) over of the bacterial suspension as initial OD_{595} divided by final OD_{595}.

Steroid levels

Methods were based on the protocol described by Ros et al. (2015; see also Demairé 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

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

		Cleaner removal effect			Species effect			Cleaner removal×species interaction		
Response variable	*N*	χ^2	d.f.	*P*	χ^2	d.f.	*P*	χ^2	d.f.	*P*
Condition	78	4.730	1	**0.030**	218.573	3	**<0.0001**	0.943	3	0.815
Leucocyte proportion	79	0.033	1	0.855	8.624	3	**0.035**	2.338	3	0.505
Granulocyte proportion	79	0.101	1	0.750	9.014	3	**0.029**	2.028	3	0.567
Lymphocyte proportion	79	0.085	1	0.770	14.249	3	**0.003**	1.819	3	0.611
Phagocytosis	61	0.101	1	0.751	2.183	2	0.336	1.816	2	0.403
Lytic activity	82	0.820	1	0.365	103.901	3	**<0.0001**	2.023	3	0.568
Cortisol	67	2.199	1	0.138	13.298	2	**0.001**	0.157	2	0.925
Testosterone	30	0.289	1	0.591		n.a.			n.a.	
Antibody response	30	0.662	1	0.416		n.a.			n.a.	

For details, see Results. Bold indicates significance ($P\leq0.05$).

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.

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

Body condition

Our findings show that cleaner removal resulted in an overall poorer body condition of clients (LMM: P=0.030, Fig. 2, Table 1). This effect was found across all four species as the interaction term between cleaner removal treatment and client species was not statistically significant (LMM: P=0.82). While body condition differed among species (LMM: P<0.0001), this simply reflects expected species differences in the girth TL^{-1} ratio.

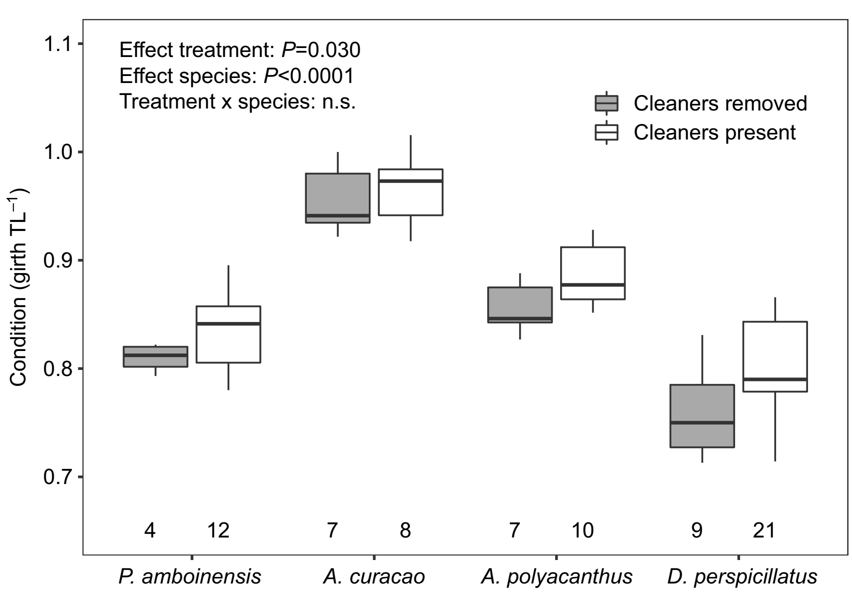

Fig. 2. Effects of long-term treatment (cleaner presence/absence) on condition. Condition was expressed as girth TL^{-1} (TL=total length) for fish in reefs from which cleaners were experimentally removed (grey) or were continuously present (white). *N*-values are given above the *x*-axis. Boxplots show the median, 25th and 75th quantiles, and range. Reported *P*-values are two-tailed mixed effects model statistics.

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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 affect 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} ratio, 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 measured and found to be on average smaller for those living in reefs from which cleaners had been removed (Waldie et al., 2011). Furthermore, the results are in accordance with the findings of Ros et al. (2011), who measured a condition index based on body mass and length relationships in other client fish species in the Red Sea.

The presence of cleaners leads to faster growth in clients (Clague et al., 2011). This and the current results suggest that continuous deprivation from cleaning services results in a decline in energy resources that are available for the fish. Indeed, a recent study showed that client fish had lower haematocrit levels (an indicator of red blood cell volume) as a result of short-term (i.e. 1 month) reduced access to cleaner fish (Demairé et al., 2020), probably because of a higher hematophagous ectoparasite load (Triki et al., 2016). However,

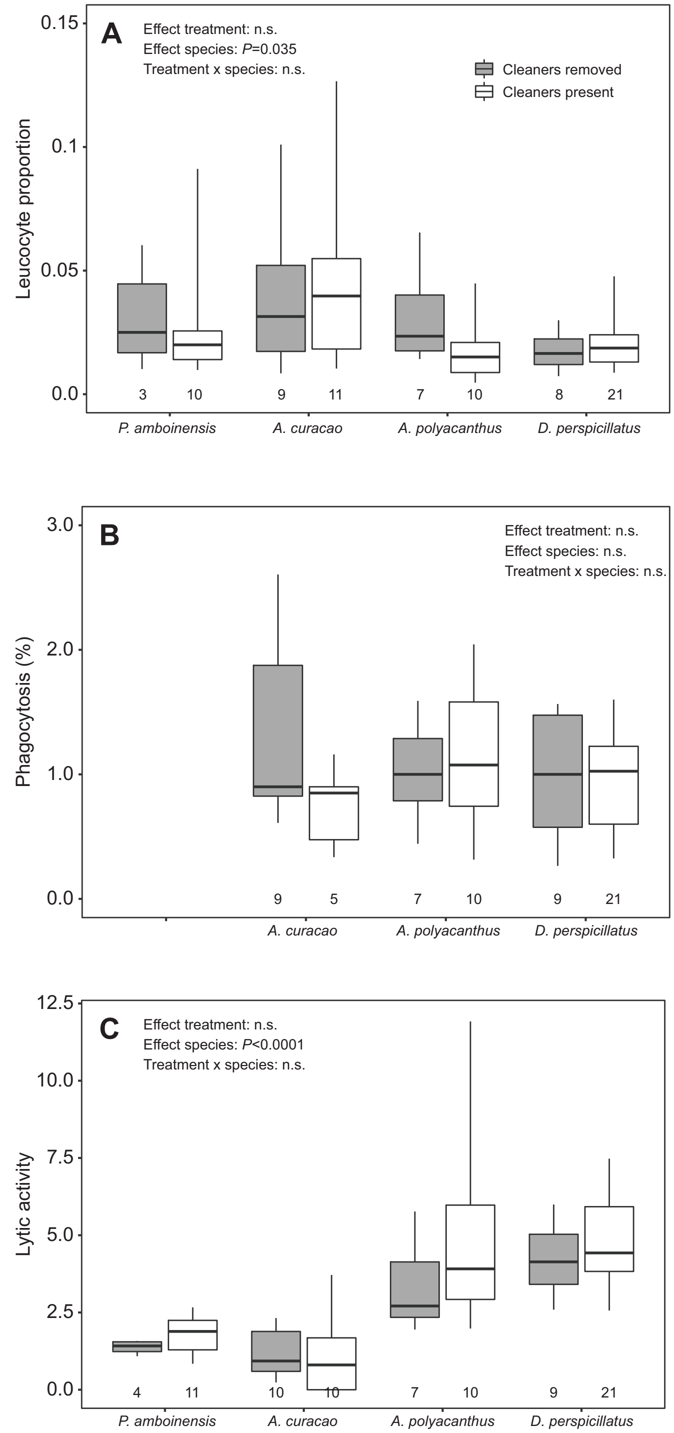

Fig. 3. Effects of long-term treatment (cleaner presence/absence) on proxies for immune function. Leucocyte proportion (A), phagocytosis (B) and lytic activity (C) levels of fish in reefs from which cleaners were experimentally removed (grey) or were continuously present (white). *N*-values are given above the *x*-axis. Boxplots show the median and 5th, 25th, 75th and 95th quantiles. Reported *P*-values are two-tailed mixed effects model statistics. Blood volumes 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.

Journal of Experimental Biology (2020) 223, jeb231613. doi:10.1242/jeb.231613

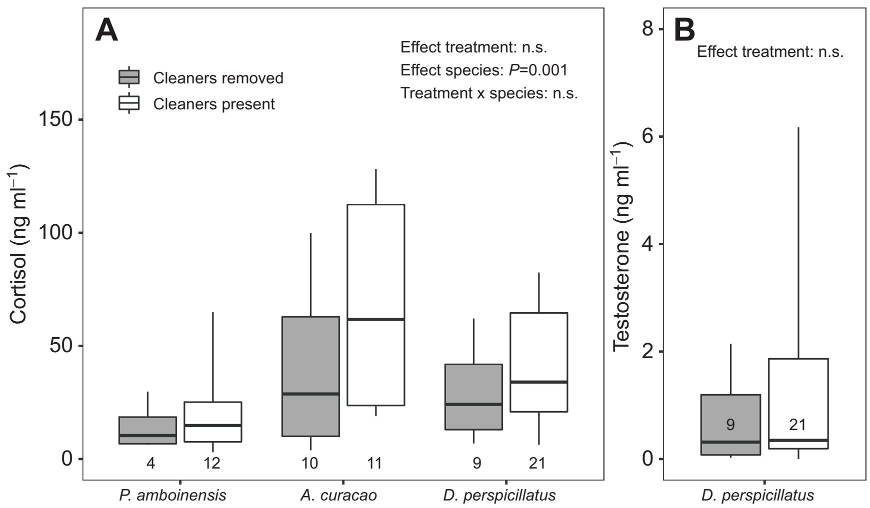

Fig. 4. Effects of long-term treatment (cleaner presence/absence) on blood plasma levels of cortisol and testosterone. Cortisol (A) and testosterone (B) levels of fish in reefs from which cleaners were experimentally removed (grey) or were continuously present (white). *N*-values are given above the *x*-axis. Boxplots show the median and 5th, 25th, 75th and 95th quantiles. Reported *P*-values are two-tailed mixed effects model statistics.

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 (Janeway 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).

Fig. 5. Effects of long-term treatment (cleaner presence/absence) on the antibody response to dinitrophenyl keyhole limpet haemocyanin antigen (DNP-KLH). DNP-KLH response of *P. perspicillatus* in reefs from which cleaners were experimentally removed (grey) or were continuously present (white). *N*-values are given above the *x*-axis. The difference is not significant (two-tailed mixed effects model). Boxplots show the median and 5th, 25th, 75th and 95th quantiles.

Lymphocytes are sensitive to stress (Braude et al., 1999) and short-term changes in access to cleaner fish as in the study by Demairé 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

Journal of Experimental Biology

does not necessarily imply higher stress physiology in this species than in the other two species, as species-specific differences in cortisol sensitivity might exist through differences in receptor type and density (Mommsen et al., 1999).

Results from basal levels may not represent possible differences in the scope of the cortisol response during a stressful event. For example, Bshary et al. (2007) measured cortisol using a confinement stress paradigm from holding water after 1 h containment in a small aquarium after capture. Interestingly, the cortisol levels measured in this condition were significantly higher in fish captured from reefs where cleaners were absent. This indicates that cleaners do have a calming effect on stress physiology that might not be measured from basal levels of the hormone. Such differences in the stress response may fade again during chronic stress (Bshary et al., 2007). Indeed, Demairé et al. (2020) found no significant differences between cleaner treatment groups in samples taken 4–10 h after capture (including handling and transport from the field to the laboratory).

Cleaner absence also did not alter testosterone level, which was only measured in *D. perspicillatus*. This species defends a year-round territory that contains algae important for their survival. Although comparison with literature data is limited, testosterone levels were in the lower range of those found in other tropical damselfish species (Damjanovic et al., 2015; Pankhurst, 1995; Ros et al., 2014). The fish might thus not have been reproductively active. In both sexes in fishes, testosterone levels are highest during the reproductive period, but levels may also be increased during territorial competition (reviewed in Goymann et al., 2007). To bind to receptors that regulate behavioural and metabolic responses, testosterone is metabolized to 17β-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 presence on client body condition. Therefore, the results of the current study favour the hypothesis that reef fish mainly incur energetic benefits from living with cleaners. Protection from repeated blood loss by ectoparasite infestation (see Demairé et al., 2020; Triki et al., 2016) might be an important factor in such physiological benefits.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.F.H.R., A.S.G., R.B.; Methodology: A.F.H.R., A.S.G., R.B.; Validation: A.F.H.R.; Formal analysis: A.F.H.R., D.N., Z.T., R.B.; Investigation: A.F.H.R., D.N., R.B.; Resources: R.B.; Data curation: A.F.H.R., D.N.; Writing - original draft: A.F.H.R.; Writing - review & editing: A.F.H.R., D.N., Z.T., A.S.G., R.B.; Visualization: A.F.H.R., D.N., Z.T.; Supervision: R.B.; Project administration: A.S.G., R.B.; Funding acquisition: A.S.G., R.B.

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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/e9c75c69e88980a46d85.

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

References
Abràmoff, M. D., Magalhães, P. J. and Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics Int.* **11**, 36-43.
Adlard, R. D. and Lester, R. J. G. (1995). The life-cycle and biology of *Anilocra pomacentri* (Isopoda, Cymothoidae), an ectoparasitic isopod of the coral-reef fish, *Chromis nitida* (Perciformes: *Pomacentridae*). *Aust. J. Zool.* **43**, 271-281. doi:10.1071/ZO9950271
Alcorn, S. W., Pascho, R. J., Murray, A. L. and Shearer, K. D. (2003). Effects of ration level on immune functions in Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* **217**, 529-545. doi:10.1016/S0044-8486(02)00369-1
Antunes, R. A. and Oliveira, R. F. (2009). Hormonal anticipation of territorial challenges in cichlid fish. *Proc. Natl. Acad. Sci. USA* **106**, 15985-15989. doi:10.1073/pnas.0900817106
Archetti, M. and Scheuring, I. (2012). Game theory of public goods in one-shot social dilemmas without assortment. *J. Theor. Biol* **299**, 9-20. doi:10.1016/j.jtbi.2011.06.018
Behringer, D. C., Karvonen, A. and Bojko, J. (2018). Parasite avoidance behaviours in aquatic environments. *Phil. Trans. R. Soc. B.* **373**, 20170202. doi:10.1098/rstb.2017.0202
Beldomenico, P. M. and Begon, M. (2010). Disease spread, susceptibility and infection intensity: vicious circles? *Trends Ecol. Evol.* **25**, 21-27. doi:10.1016/j.tree.2009.06.015
Binning, S. A., Roche, D. G., Grutter, A. S., Colosio, S., Sun, D., Miest, J. and Bshary, R. (2018). Cleaner wrasse indirectly affect the cognitive performance of a damselfish through ectoparasite removal. *Proc. R. Soc. B* **285**, 20172447. doi:10.1098/rspb.2017.2447
Blaxhall, P. C. and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.* **5**, 771-781. doi:10.1111/j.1095-8649.1973.tb04510.x
Bonier, F., Martin, P. R., Moore, I. T. and Wingfield, J. C. (2009). Do baseline glucocorticoids predict fitness? *Trends Ecol. Evol.* **24**, 634-642. doi:10.1016/j.tree.2009.04.013
Borg, B. (1994). Androgens in teleost fishes. *Comp. Biochem. Phys. C* **109**, 219-245. doi:10.1016/0742-8413(94)00063-G
Braude, S., Tang-Martinez, Z. and Taylor, G. T. (1999). Stress, testosterone, and the immunoredistribution hypothesis. *Behav. Ecol.* **10**, 345-350. doi:10.1093/beheco/10.3.345
Bronstein, J. L. (2001). The costs of mutualism. *Integr. Comp. Biol.* **41**, 825. doi:10.1093/icb/41.4.825
Bshary, R. (2003). The cleaner wrasse, *Labroides dimidiatus*, is a key organism for reef fish diversity at Ras Mohammed National Park, Egypt. *J. Anim. Ecol.* **72**, 169-176. doi:10.1046/j.1365-2656.2003.00683.x
Bshary, R. and Bronstein, J. L. (2004). Game structures in mutualistic interactions: what can the evidence tell us about the kind of models we need? *Adv. Study Behav.* **34**, 59-101. doi:10.1016/S0065-3454(04)34002-7
Bshary, R. and Grutter, A. S. (2005). Punishment and partner switching cause cooperative behaviour in a cleaning mutualism. *Biol. Lett.* **1**, 396. doi:10.1098/rsbl.2005.0344
Bshary, R., Oliveira, R. F., Oliveira, T. S. F. and Canário, A. V. M. (2007). Do cleaning organisms reduce the stress response of client reef fish? *Front. Zool.* **4**, 21. doi:10.1186/1742-9994-4-21
Clague, G. E., Cheney, K. L., Goldizen, A. W., McCormick, M. I., Waldie, P. A. and Grutter, A. S. (2011). Long-term cleaner fish presence affects growth of a coral reef fish. *Biol. Letters* **7**, 863-865. doi:10.1098/rsbl.2011.0458
Collazos, M. E., Barriga, C. and Ortega, E. (1994). Optimum conditions for the activation of the alternative complement pathway of a cyprinid fish (*Tinca tinca* L.). Seasonal variation in the titres. *Fish Shellfish Immun.* **4**, 499-506. doi:10.1006/fsim.1994.1044

Damjanovic, K., Glauser, G., Bshary, R. and Ros, A. F. H. (2015). Intra-and interspecific social challenges modulate the levels of an androgen precursor in a seasonally territorial tropical damselfish. *Horm. Behav.* **71**, 75-82. doi:10.1016/j.yhbeh.2015.04.011

Demairé, C., Triki, Z., Binning, S. A., Glauser, G., Roche, D. G. and Bshary, R. (2020). Reduced access to cleaner fish negatively impacts the physiological state of two resident reef fishes. *Mar. Biol.* **167**, 48. doi:10.1007/s00227-020-3658-2

Demas, G. and Nelson, R. J. (2011). *Ecoimmunology*. Oxford: Oxford University Press.

Ellis, A. E. (1977). The leucocytes of fish: a review. *J. Fish Biol.* **11**, 453-491. doi:10.1111/j.1095-8649.1977.tb04140.x

Froese, R. and Pauly, D. (2019). *FishBase*. World Wide Web electronic publication. www.fishbase.org, (02/2019).

Goymann, W., Landys, M. M. and Wingfield, J. C. (2007). Distinguishing seasonal androgen responses from male–male androgen responsiveness—revisiting the challenge hypothesis. *Horm. Behav.* **51**, 463-476. doi:10.1016/j.yhbeh.2007.01.007

Grutter, A. S. (1996a). Experimental demonstration of no effect by the cleaner wrasse *Labroides dimidiatus* (Cuvier and Valenciennes) on the host fish *Pomacentrus moluccensis* (Bleeker). *J. Exp. Mar. Biol. Ecol.* **196**, 285-298. doi:10.1016/0022-0981(95)00135-2

Grutter, A. S. (1996b). Parasite removal rates by the cleaner wrasse *Labroides dimidiatus*. *Mar. Ecol. Prog. Ser.* **130**, 61-70. doi:10.3354/meps130061

Grutter, A. S. (1997a). Spatio-temporal variation and feeding selectivity in the diet of the cleaner fish *Labroides dimidiatus*. *Copeia* **1997**, 346-355. doi:10.2307/1447754

Grutter, A. S. (1997b). Effect of the removal of cleaner fish on the abundance and species composition of reef fish. *Oecologia* **111**, 137-143. doi:10.1007/s004420050217

Grutter, A. S. (1999). Cleaner fish really do clean. *Nature* **398**, 672-673. doi:10.1038/19443

Grutter, A. S. (2001). Parasite infection rather than tactile stimulation is the proximate cause of cleaning behaviour in reef fish. *Proc. R. Soc. Lond. B* **268**, 136-365. doi:10.1098/rspb.2001.1658

Grutter, A. S. and Bshary, R. (2003). Cleaner wrasse prefer client mucus: support for partner control mechanisms in cleaning interactions. *Proc. R. Soc. Lond. B* **270**, S242-S244. doi:10.1098/rsbl.2003.0077

Grutter, A. S. and Pankhurst, N. W. (2000). The effects of capture, handling, confinement and ectoparasite load on plasma levels of cortisol, glucose and lactate in the coral reef fish *Hemigymnus melapterus*. *J. Fish Biol.* **57**, 391-401. doi:10.1111/j.1095-8649.2000.tb02179.x

Grutter, A. S., Murphy, J. M. and Choat, J. H. (2003). Cleaner fish drives local fish diversity on coral reefs. *Curr. Biol.* **13**, 64-67. doi:10.1016/S0960-9822(02)01393-3

Grutter, A. S., Deveney, M. R., Whittington, I. D. and Lester, R. J. G. (2002). The effect of the cleaner fish *Labroides dimidiatus* on the capsalid monogenean *Benedenia lolo* parasite of the labrid fish *Hemigymnus melapterus*. *J. Fish Biol.* **61**, 1098-1108. doi:10.1111/j.1095-8649.2002.tb02458.x

Grutter, A. S., De Brauwer, M., Bshary, R., Cheney, K. L., Cribb, T. H., Madin, E. M. P., McClure, E. C., Meekan, M. G., Sun, D., Warner, R. R. et al. (2018). Parasite infestation increases on coral reefs without cleaner fish. *Coral Reefs* **37**, 15-24. doi:10.1007/s00338-017-1628-z

Grutter, A. S., Blomberg, S. P., Box, S., Bshary, R., Ho, O., Madin, E. M. P., McClure, E. C., Meekan, M. G., Richardson, M. A., Sikkel, P. C. et al. (2019). Changes in local free-living parasite populations in response to cleaner manipulation over 12 years. *Oecologia* **190**, 783-797. doi:10.1007/s00442-019-04451-8

Haond, C., Nolan, D. T., Ruane, N. M., Rotllant, J. Wendelaar Bonga, S. E. (2003). Cortisol influences the host-parasite interaction between the rainbow trout (*Oncorhynchus mykiss*) and the crustacean ectoparasite *Argulus japonicus*. *Parasitology* **127**, 551-560. doi:10.1017/S0031182003004116

Harris, P. D., Soleng, A. and Bakke, T. A. (1998). Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) mediated by host complement. *Parasitology* **117**, 137-143. doi:10.1017/S003118209800287X

Hart, B. L. (1990). Behavioral adaptations to pathogens and parasites: five strategies. *Neurosci. Biobehav. R* **14**, 273-294. doi:10.1016/S0149-7634(05)80038-7

Hauser, B., Deschner, T. and Boesch, C. (2008). Development of a liquid chromatography tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine. *J. Chromatogr. B* **862**, 100-112. doi:10.1016/j.jchromb.2007.11.009

Herscowitz, H. B., Diblasio, R. C. and Rosenberg, J. B. (1975). A hemolytic plaque assay for the detection of direct and indirect antibody-forming cells to keyhole limpet hemocyanin. *J. Immunol. Methods* **6**, 331-345. doi:10.1016/0022-1759(75)90004-6

Holland, M. C. H. and Lambris, J. D. (2002). The complement system in teleosts. *Fish Shellfish Immunol.* **12**, 399-420. doi:10.1006/fsim.2001.0408

Janeway, C. A., Travers, P., Walport, M. and Capra, J. D. (1999). *Immunobiology: The Immune System in Health and Disease*. London; San Francisco; New York: Current Biology Limited; Garland Pub. Inc.

Jones, S. R. M. (2001). The occurrence and mechanisms of innate immunity against parasites in fish. *Dev. Comp. Immunol.* **25**, 841-852. doi:10.1016/S0145-305X(01)00039-8

Jones, R., Petrell, R. and Pauly, D. (1999). Using modified length-weight relationships to assess the condition of fish. *Aquacult. Eng.* **20**, 261-276. doi:10.1016/S0144-8609(99)00020-5

Koren, L., Ng, E. S. M., Soma, K. and Wynne-Edwards, K. E. (2012). Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation. *PLoS ONE* **7**, 1-7. doi:10.1371/annotation/7493e5d2-4c1a-43eb-a83f-16814861ff13

Leimar, O. and Hammerstein, P. (2010). Cooperation for direct fitness benefits. *Phil. Trans. R. Soc. B* **365**, 2619-2626. doi:10.1098/rstb.2010.0116

Limbaugh, C. (1961). Cleaning symbiosis. *Sci. Am.* **205**, 42-49. doi:10.1038/scientificamerican0861-42

Lindenstrøm, T. C., Secombes, J. and Buchmann, K. (2004). Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. *Vet. Immunol. Immunopathol.* **97**, 137-148. doi:10.1016/j.vetimm.2003.08.016

Lindström, K. M., Foufopoulos, J., Pärn, H. and Wikelski, M. (2004). Immunological investments reflect parasite abundance in island populations of Darwin's finches. *Proc R. Soc. Lond. B* **271**, 1513-1519. doi:10.1098/rspb.2004.2752

Losey, G. S., Jr (1979). Fish cleaning symbiosis: proximate causes of host behaviour. *Anim. Behav.* **27**, 669-685. doi:10.1016/0003-3472(79)90004-6

Losey, G. S., Jr (1987). Cleaning symbiosis. *Symbiosis* **4**, 229-256.

MacLeod, K. J., Sheriff, M. J., Ensminger, D. C., Owen, D. A. S. and Langkilde, T. (2018). Survival and reproductive costs of repeated acute glucocorticoid elevations in a captive, wild animal. *Gen. Comp. Endocr.* **268**, 1-6. doi:10.1016/j.ygcen.2018.07.006

Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* **31**, 188-201. doi:10.1016/j.dci.2006.05.013

Mills, S. C., Grapputo, A., Jokinen, I., Koskela, E., Mappes, T. and Poikonen, T. (2010). Fitness trade-offs mediated by immunosuppression costs in a small mammal. *Evolution* **64**, 166-179. doi:10.1111/j.1558-5646.2009.00820.x

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fisher* **9**, 211-268. doi:10.1023/A:1008924418720

Noë, R. and Hammerstein, P. (1995). Biological markets. *Trends Ecol. Evol.* **10**, 336-339. doi:10.1016/S0169-5347(00)89123-5

Noë, R., Hooff, J. A. R. A. M. and Hammerstein, P. (2001). *Economics in Nature: Social Dilemmas, Mate Choice and Biological Markets*. Cambridge University Press.

Norris, K. and Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* **11**, 19-26. doi:10.1093/beheco/11.1.19

Oliveira, R. F. (2009). Social behavior in context: hormonal modulation of behavioral plasticity and social competence. *Integr. Comp. Biol.* **49**, 423-440. doi:10.1093/icb/icp055

Pankhurst, N. W. (1995). Hormones and reproductive behavior in male damselfish. *B. Mar. Sci.* **57**, 569-581.

Pankhurst, N. W. (2011). The endocrinology of stress in fish: an environmental perspective. *Gen. Comp. Endocrinol.* **170**, 265-275. doi:10.1016/j.ygcen.2010.07.017

Potts, G. W. (1973). The ethology of *Labroides dimidiatus* (Cuv. & Val.)(Labridae, Pisces) on Aldabra'. *Anim. Behav.* **21**, 250-291. doi:10.1016/S0003-3472(73)80068-5

Ros, A. F. H., Lusa, J., Meyer, M., Soares, M., Oliveira, R. F., Brossard, M. and Bshary, R. (2011). Does access to the bluestreak cleaner wrasse *Labroides dimidiatus* affect indicators of stress and health in resident reef fishes in the Red Sea? *Horm. Behav.* **59**, 151-158. doi:10.1016/j.yhbeh.2010.11.006

Ros, A. F. H., Vullioud, P., Bruintjes, R., Vallat, A. and Bshary, R. (2014). Intra-and interspecific challenges modulate cortisol but not androgen levels in a year-round territorial damselfish. *J. Exp. Biol.* **217**, 1768-1774. doi:10.1242/jeb.093666

Ros, A. F. H., Damjanovic, K., Glauser, G. and Bshary, R. (2015). No scope for social modulation of steroid levels in a year-round territorial damselfish. *J. Exp. Zool. Part A* **323**, 80-88. doi:10.1002/jez.1900

Sachs, J. L., Mueller, U. G., Wilcox, T. P. and Bull, J. J. (2004). The evolution of cooperation. *Q. Rev. Biol.* **79**, 135-160. doi:10.1086/383541

Santos, M. N., Canas, A., Lino, P. G. and Monteiro, C. C. (2006). Length–girth relationships for 30 marine fish species. *Fish. Res.* **78**, 368-373. doi:10.1016/j.fishres.2006.01.008

Schreck, C. B. (2010). Stress and fish reproduction: the roles of allostasis and hormesis. *Gen. Comp. Endocr.* **165**, 549-556. doi:10.1016/j.ygcen.2009.07.004

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321. doi:10.1016/0169-5347(96)10039-2

Sikkel, N. M. and Welicky, R. L. (2019). The ecological significance of parasitic crustaceans. In *Parasitic Crustacea: State of Knowledge and Future Trends* (ed. N. J. Smit, N. L. Bruce and K. A. Hadfield), pp. 421-477. Cham, Switzerland: Springer.

Sikkel, P. C., Richardson, M., Sun, D., Narvaez, P. and Grutter, A. S. (2019). Changes in abundance of fish-parasitic gnathiid isopods associated with warm-water bleaching events on the northern Great Barrier Reef. *Coral Reefs* **38**, 721-730. doi:10.1007/s00338-019-01835-3

Soares, M. C., Oliveira, R. F., Ros, A. F. H., Grutter, A. S. and Bshary, R. (2011). Tactile stimulation lowers stress in fish. *Nat. Commun.* **2**, 534. doi:10.1038/ncomms1547

Soares, M. C., Mazzei, R., Cardoso, S. C., Ramos, C. and Bshary, R. (2019). Testosterone causes pleiotropic effects on cleanerfish behaviour. *Sci. Rep.* **9**, 15829. doi:10.1038/s41598-019-51960-w

Sopinka, N. M., Donaldson, M. R., O'Connor, C. M., Suski, C. D. and Cooke, S. J. (2016). Stress indicators in fish. *Fish Physiol.* **35**, 405-462. doi:10.1016/B978-0-12-802728-8.00011-4

Triki, Z., Grutter, A. S., Bshary, R. and Ros, A. F. H. (2016). Effects of short-term exposure to ectoparasites on fish cortisol and hematocrit levels. *Mar. Biol.* **163**, 187. doi:10.1007/s00227-016-2959-y

Triki, Z., Wismer, S., Levorato, E. and Bshary, R. (2018). A decrease in the abundance and strategic sophistication of cleaner fish after environmental perturbations. *Glob. Change Biol.* **24**, 481-489. doi:10.1111/gcb.13943

Waldie, P. A., Blomberg, S. P., Cheney, K. L., Goldizen, A. W. and Grutter, A. S. (2011). Long-term effects of the cleaner fish *Labroides dimidiatus* on coral reef fish communities. *PLoS ONE* **6**, e21201. doi:10.1371/journal.pone.0021201

Watts, M., Munday, B. L. and Burke, C. M. (2001). Immune responses of teleost fish. *Aust. Vet. J.* **79**, 570-574. doi:10.1111/j.1751-0813.2001.tb10753.x

Wedemeyer, G. A., Barton, B. A. and McLeay, D. J. (1990). Stress and acclimation. In *Methods for Fish Biology* (ed. C. B. Schreck and P. B. Moyle), pp. 451-489. Bethesda, MD: American Fisheries Society.

Wingfield, J. C. and Kitaysky, A. S. (2002). Endocrine responses to unpredictable environmental events: stress or anti-stress hormones? *Integr. Comp. Biol.* **42**, 600-609. doi:10.1093/icb/42.3.600

Wingfield, J. C., Maney, D. L., Breuner, C. W., Jacobs, J. D., Lynn, S., Ramenofsky, M. and Richardson, R. D. (1998). Ecological bases of hormone—behavior interactions: the "emergency life history stage". *Am. Zool.* **38**, 191-206. doi:10.1093/icb/38.1.191

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.

treatment	species	condition	leucocyte proportion	granulocyte proportion	lymphocyte proportion	phagocytosis	lytic activity	cortisol	testosterone	antibody response
control	*A. curacao*	8	11	11	11	5	10	11	no	no
control	*A. polyacanthus*	10	10	10	10	10	10	2	no	no
control	*D. perspicillatus*	21	21	21	21	21	21	21	21	21
control	*P. ambonensis*	12	10	10	10	no	11	12	no	no
removal	*A. curacao*	7	9	9	9	9	10	10	no	no
removal	*A. polyacanthus*	7	7	7	7	7	7	no	no	no
removal	*D. perspicillatus*	9	8	8	8	9	9	9	9	9
removal	*P. ambonensis*	4	3	3	3	no	4	4	no	no

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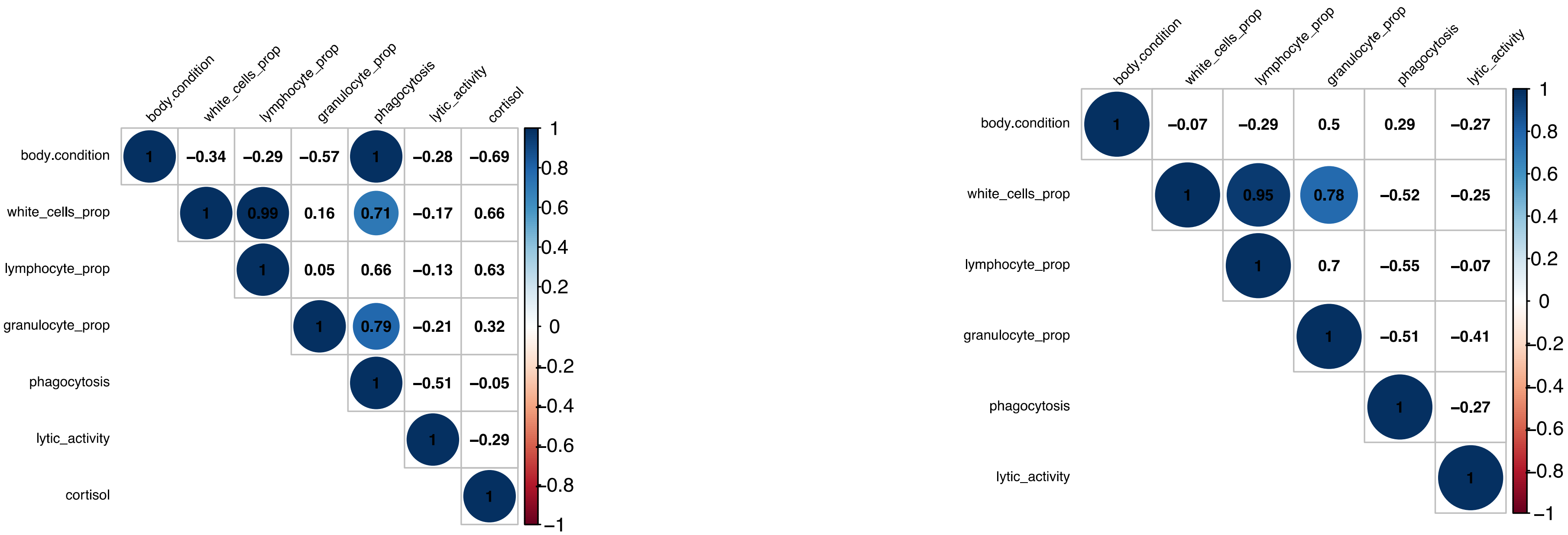
Figure S1: Correlations between measured parameters for the four study species (a: *Amblyglyphidodon curacao*, b: *Acanthochromis polyacanthus*, c: *Dischistodus perspicillatus*, and d: *Pomacentrus amboinensis*) and per treatment (Control = “Cleaner present”; Removal = “Cleaner removed”) in the long-term 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= granulocypte proportion
phagocytosis = phagocytosis
lytic_activity = lytic activity
SRBC = antibody response
cortisol = cortisol
testosterone = testosterone

S1a)

A. curacao

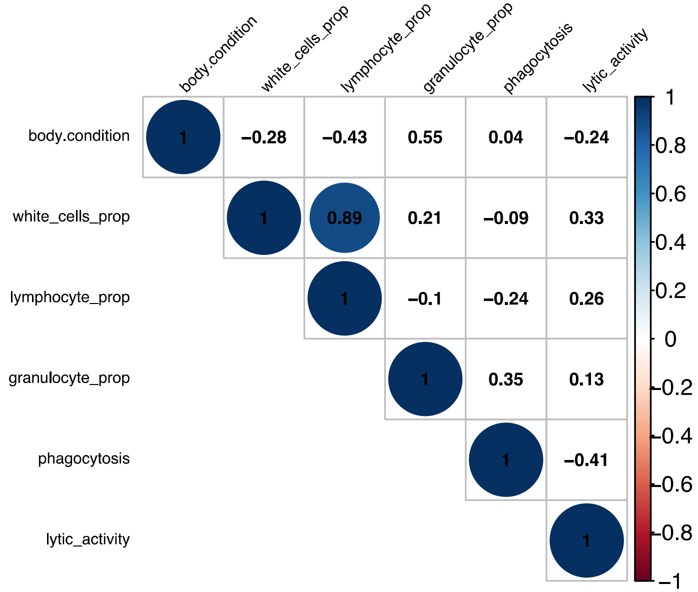


Control

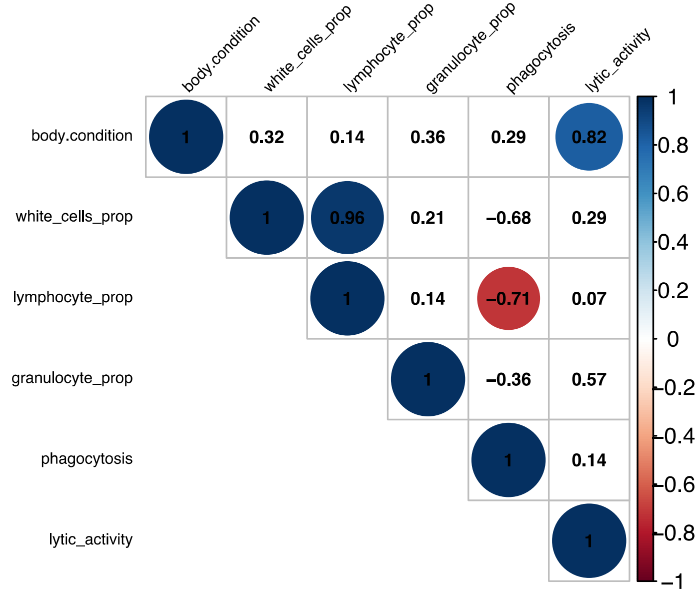
Removal

S1b)

A. polyacanthus



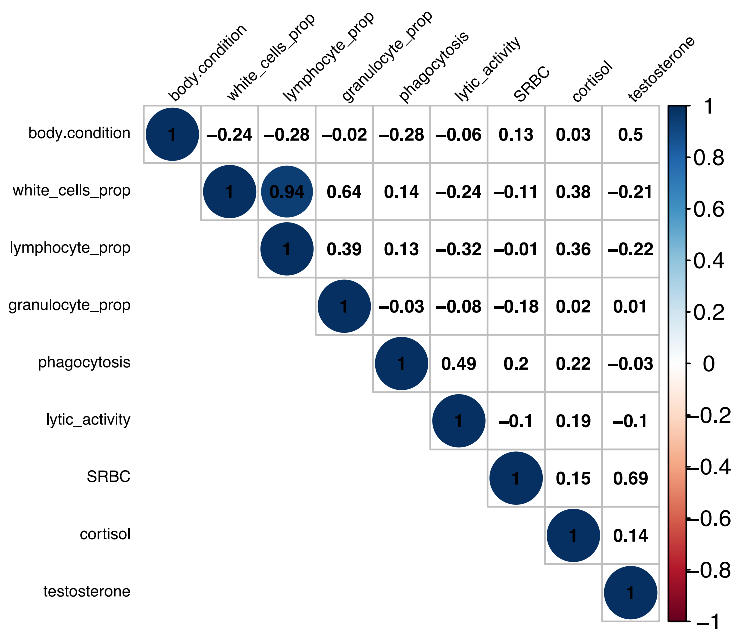
Control



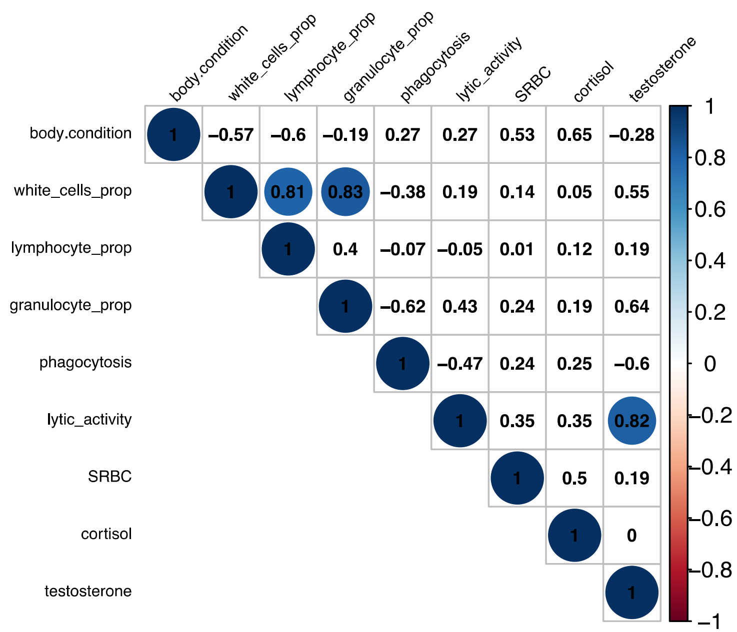
Removal

S1c)

D. perspicillat



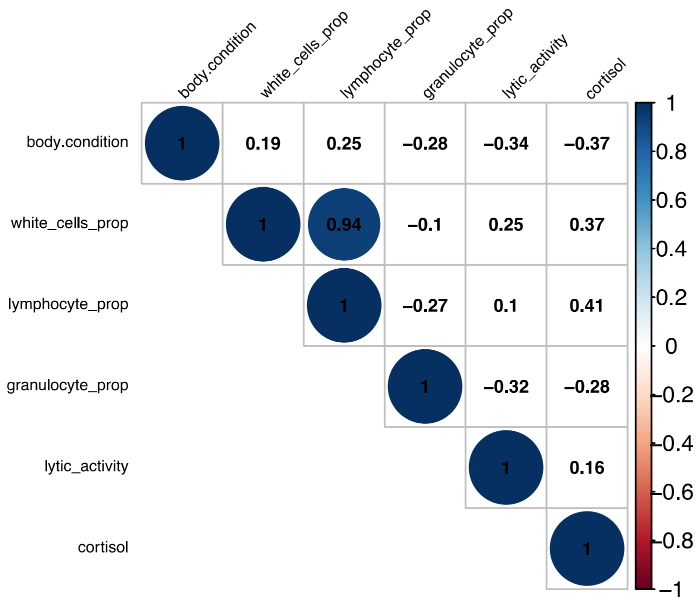
Control

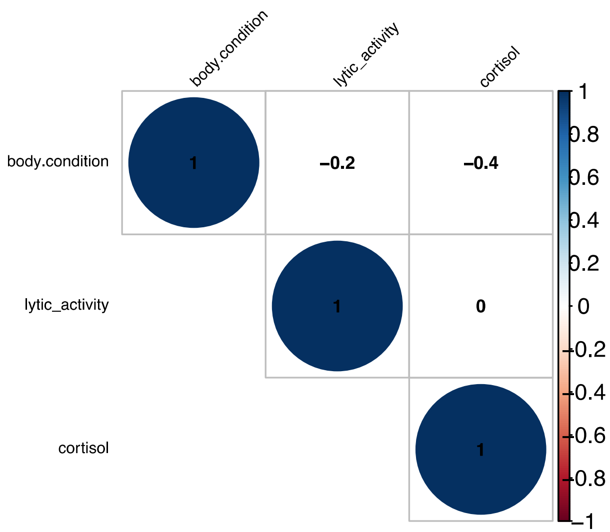


Removal

S1d)

P. ambonensis





Control

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