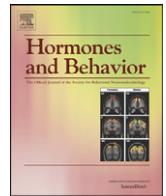




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Does access to the bluestreak cleaner wrasse *Labroides dimidiatus* affect indicators of stress and health in resident reef fishes in the Red Sea?

Albert F.H. Ros^{a,*}, Jeanne Lusa^a, Meghann Meyer^a, Marta Soares^{a,b}, Rui F. Oliveira^{b,c}, Michel Brossard^a, Redouan Bshary^a

^a Department of Biology, University of Neuchâtel, Emile-Argand, 11, 2009 Neuchâtel, Switzerland

^b Unidade de Investigação em Eco-Etologia, Instituto Superior de Psicologia Aplicada, Portugal

^c Champalimaud Neuroscience Programme, Instituto Gulbenkian de Ciência, Portugal

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ABSTRACT

Interactions between the bluestreak cleaner wrasse *Labroides dimidiatus* and its client reef fish are a textbook example of interspecific mutualism. The fact that clients actively visit cleaners and invite inspection, together with evidence that cleaners eat many client ectoparasites per day, indeed strongly suggests a mutualistic relationship. What remains unknown is how parasite removal affects the physiology of clients and thereby their body condition, health, and immune function. Here we addressed these issues in a field study in Ras Mohammed National Park, Egypt. In our study area, small reef patches are inter-spaced with areas of sandy substrate, thereby preventing many species (i.e., residents, including cleaner wrasses) from travelling between the reef patches. This habitat structure leads to a mosaic of resident clients with and without access to bluestreak cleaner wrasses, further referred to as “cleaner access”, on which we focused our study. We found that residents with cleaner access had higher body condition than residents without cleaner access. However, indicators of stress like variation in cortisol levels corrected for handling time and various immune parameters were apparently unaffected by cleaner access. In fact antibody responses were significantly higher in fishes without cleaner access. This suggests that cleaner access decreases the need for active immunity and that this releases resources that might be allocated to other functions such as somatic growth and reproduction.

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Mutualisms involve the exchange of goods or services like food, transport, or protection between partners that belong to different species (Bronstein, 2001; Noë, 2001). A central question is how in different species ecology and decision rules interact to produce mutual benefits. As many mutualistic interactions involve investments – behaviors that reduce the immediate payoff of an actor – individuals often need to control their partner's behavior in order to gain personal benefits. A large body of literature investigates the ecological conditions and the partner control mechanisms that yield stable mutualistic outcomes (Bshary and Bronstein, 2004; Sachs et al., 2004; Foster and Kokko, 2006; Lehmann and Keller, 2006; West et al., 2007; Bshary and Bergmüller, 2008). Although important for testing the conceptual hypotheses yielded by such studies, only a few studies have measured costs and benefits of mutualistic interactions from a physiological perspective and how this would translate to fitness.

Most physiological studies have focused on mutualisms in which at least one partner offers nutrition. Symbiosis often involves exchanges of nutriment, where a key question is how the exchange

is achieved (Kiers and van der Heijden, 2006). Plants and many partner species of ants offer sugar-rich solutions in order to gain transport of pollen or protection by the ants, where several studies investigate how costly the solution is to produce (Pierce et al., 2002; Brandenburg et al., 2009). Detailed knowledge about physiological processes in these systems helps to measure costs and benefits of the interactions in order to make informed guesses about fitness consequences. Such indirect measures of fitness are necessary in most mutualisms as measuring reproductive success is typically virtually impossible (Boucher et al., 1982; Cushman and Beattie, 1991). In addition, understanding physiological processes may help to identify whether and how mutualisms resolve or weaken major trade-offs in the partner species involved.

Here, we present the first detailed data on the physiological effects of the bluestreak cleaner wrasse *Labroides dimidiatus* on other reef fish species that regularly visit and interact with these wrasses (cleaner–client mutualism, Eibl-Eibesfeldt, 1955; Limbaugh, 1961; Bshary and Côté, 2008). The results of a series of studies in a range of reef fishes have validated that ectoparasite removal by cleaner wrasses plays an important role during cleaner–client interactions (Grutter, 1996a, 1999; Bshary and Grutter, 2002b; Grutter and Lester, 2002; Grutter and Bshary, 2003). These results are (i) the presence of parasitic

* Corresponding author. University of Neuchâtel, Department of Behavioural Ecology, Rue Emile-Argand 11, 2009 Neuchâtel, Switzerland. Fax: +41 327183001.

E-mail address: aros@ispa.pt (A.F.H. Ros).

isopods causes clients to visit cleaner wrasses (*Hemigymnus melapterus*: Grutter, 2001), (ii) cleaner wrasses significantly reduce the ectoparasites load of clients (*Pomacentrus vaiuli*: Gorlick et al., 1987; *H. melapterus*: Grutter, 1999; but not in *Pomacentrus moluccensis*: Grutter, 1996b), and (iii) parasites adopt tactics to avoid predation by cleaner wrasses, i.e., gnathiids rapidly flee in case of disturbance and some monogeneans have cryptic coloration (reviewed by Grutter, 2002). In agreement with parasite removal being beneficial, the presence of the bluestreak cleaner wrasse is related with a local increase in reef fish biodiversity (Bshary, 2003; Grutter et al., 2003). Ectoparasites compromise the fitness of their hosts via several mechanisms. They inflict not only direct costs via skin damage and blood consumption but also indirect costs via increasing the chance to secondary infections with opportunistic pathogens (Jones and Grutter, 2005, 2008; Grutter, 2008), and thereby the need to activate inflammatory responses, which are energetically costly (Sheldon and Verhulst, 1996; Watts et al., 2001; Alcorn et al., 2003; Demas, 2004). Furthermore, experimental infection with ectoparasites has been shown to increase the corticosteroid stress response of salmonids (Ruane et al., 1999, 2000; Wagner et al., 2008). Although corticosteroids facilitate adaptive responses to acute stressors, enduring activation of this stress physiology has costly consequences (Sumpter, 1997; Barton, 2002; Yada and Nakanishi, 2002).

In reef fish gnathiid isopods have been shown to cause skin damage, reduced blood volume, reduced growth, and sometimes even death (Jones and Grutter, 2005, 2008; Grutter, 2008, but see Grutter and Pankhurst, 2000). It is thus expected that ectoparasite removal would be beneficial for reef fish. However, several studies have pointed out that clients may incur some costs by interacting with bluestreak cleaner wrasses. First, bluestreak cleaner wrasses often bite their clients in order to eat from their protective protein rich mucus layer (“cheating”), and clients respond to this costly biting by jolting or by responding aggressively (Bshary and Grutter, 2002a, 2005). Second, bluestreak cleaner wrasses give tactile stimulation with their pectoral fins, which has a calming effect on their clients and might be seen as beneficial. However, tactile stimulation occurs often together with cheating and might therefore be a means to manipulate clients (Bshary and Würth, 2001). In order to weigh such costs with the benefits of cleaner visits, more information about the effects of these visits on the overall condition and stress physiology of fish is necessary. In a first study to test the physiological effects of cleaner visits, Bshary et al. (2007) compared reef fishes that lived on isolated small reef patches by comparing clients that did or did not have access to bluestreak cleaner wrasses. The cortisol levels in these fish in response to confinement stress were found to be reduced in client reef fishes with cleaner access in comparison to clients without cleaner access. We used the same method as used in the study of Bshary et al. (2007) to address the question how the observed reduced stress response in clients with cleaner access is linked to condition and other physiological parameters, in particular the functioning of the immune system.

We took advantage of our experience with the reef patch system in the study area, where each patch may repeatedly acquire or lose bluestreak cleaner wrasses in unpredictable ways (Bshary, 2003). This effectively randomizes the factor cleaner access for individuals of species with small home ranges (‘resident’ clients). We took care to select reef patches that were comparable in habitat structure and depth in order to be able to attribute any variation in the physiology of individual residents of these patches to the presence of bluestreak cleaner wrasses. We tested three non-mutually exclusive potential ways how clients may benefit from cleaner access: (1) through an increase in body condition by measuring an index of body weight and body size, (2) through a reduction in stress physiology by measuring baseline plasma levels of cortisol, and (3) through an improved functioning of the immune system by monitoring blood percentages of leukocytes and measuring antibody levels against an experimentally induced immune challenge.

Methods

Field site and study species

The study was carried out from August to October 2008, at Mersa Bareika, Ras Mohammed National Park in Egypt. A high influx of sand in combination with a shallow sub-tidal flat has led to the formation of reef patches that are interspaced with areas of sandy substrate. In this area many of the smaller and intermediate-sized reef fishes settle on a reef patch and seldom move between them (Bshary, 2003). The bluestreak cleaner wrasse, *L. dimidiatus*, is the most abundant cleaning organism at the shallow reef patches of Mersa Bareika (Bshary, 2003; for a description of the behavior, see Potts, 1973). Their presence changes from year to year due to loss or recruitment, resulting in a random distribution over these patches (Bshary, 2003).

Following the design of Bshary et al. (2007), we selected 30 patches in 2- to 6-m depth with or without a cleaner wrasse as a resident. We aimed to sample 1–3 adult individuals per preselected resident species per patch. Study species were preselected based on the local abundance of the species and their mean body size because this limited the volume of blood that we were able to sample. At the maximum, we would draw 1% of the weight of the fish in blood volume (Houston, 1990). Since we aimed on collecting 100 µl of blood (40 µl blood plasma + a blood smear), the minimum weight of fish was 10 g and this excluded the more abundant smaller damselfishes on the reef patches. We therefore collected data from the following resident species, which all belong to the order Perciformes and which all have been observed to interact with cleaner fish at Mersa Bareika (Bshary, 2001): two species of surgeon fish: brown surgeon fish, *Acanthurus nigrofusus* (59 individuals: SL = 10.05 ± 0.02 cm, bodyweight = 44.0 ± 0.2 g), and lined bristletooth, *Ctenochaetus striatus* (16 individuals: SL = 12.4 ± 0.1 cm, bodyweight = 79.1 ± 1.7 g); one species of butterflyfish: the Red Sea banner fish, *Heniochus intermedius* (14 individuals: SL = 13.04 ± 0.06 cm, bodyweight = 111.6 ± 1.3 g); and two species of damselfish: white-belly damselfish, *Amblyglyphidodon leucogaster* (29 individuals: SL = 9.27 ± 0.04 cm, bodyweight = 51.3 ± 0.6 g), and three-spot damselfish, *Dascyllus trimaculatus* (10 individuals: SL = 9.49 ± 0.15 cm, bodyweight = 51.7 ± 2.2 g). All these species are targets of gnathiid isopods with the two species of surgeon fish having the highest gnathiid loads (Soares et al., 2008).

All species in our analysis are monomorphic in appearance. We examined their sex by checking for sperm that can be extruded from the vas deferens by gently pressing the abdomen and by examining the genital papilla (urogenital opening), a structure located ventrally just posterior to the vent. Adapted to egg laying or sperm passage, this structure has been shown to be sexually dimorphic for several species, being shorter and wider in females and longer and thinner in males (Thresher, 1984; Oliveira and Almada, 1995). All fish were examined, however, because the expression of the papilla depends on the reproductive state of the animal and because we had no access to stereomicroscopes at the field site, we were able to classify only a fraction (71%) of the individuals as males or females. Post hoc analyses to detect systematic differences between males and females did not show any significant effect (ANOVA with factors sex and cleaner presence: effect sex: SL, body weight, condition, leukocyte%: $p > 0.17$). We thus pooled our data regarding the sex of the animals in all our analyses.

Behavioral observations

In addition to cleaner wrasses, the reef houses a wide range of species that exhibit cleaning behavior, such as facultative cleaner species like the six line wrasse, *Pseudocheilinus hexataenia*, juveniles of some other species like the lyretail hogfish *Bodianus anthioides*, and several species of shrimp (see Limbaugh, 1961). In order to check for

the possibility that our results comparing reef patches with or without bluestreak cleaner wrasses could be confounded by the potential effect of other cleaning organisms, we recorded all potential cleaning interactions with other cleaning organisms during a total observation time of 45–60 min per patch.

An interaction with a bluestreak cleaner wrasse started when either the cleaner wrasse approached the potential client or the client assumed a slightly oblique and motionless position close to the cleaner wrasse (posing). We recorded the time the two fish were in contact until one of the two swam off. For other species, we used a broad definition in which we included all contact where the client was relatively motionless and in contact with one of the other potentially cleaning species. We checked the fish from as close as possible whenever they went into a cavity to search for cleaner shrimps.

We used the following observation protocol. When no cleaner wrasse was present the observer circled around the patch in order to check for possible cleaner interactions with other species. When one or two bluestreak cleaner wrasses were present, we focused on recording interactions at their territory (e.g., cleaning station). At the latter patches, we regularly checked for cleaning interactions with other cleaner organisms. This was possible since the species we observed were often not in the neighborhood of the cleaner wrasse and this gave the opportunity for us to regularly circle around the patch to scan for potential cleaners.

Sample procedures

At each reef patch a 4 × 1.5 m barrier net was set up (mesh size 2 cm) to intercept fishes by blocking their escape route. The targeted fish was guided to the barrier net where it was captured using a hand net. We captured in the early morning just after sunrise. Given that the fish had to be taken to the shore for measuring and blood sampling, this time was chosen as the best period of day as the weather conditions are cooler than for the rest of the day. Furthermore, this standardization avoids variation in results due to possible daily fluctuations in cortisol levels.

Directly after capture, the fish was placed in a small plastic bag, handed over to a snorkeler, which brought the fish to shore. Once at the shore, the fish was directly anesthetized by placing it in an aerated tank with a solution of MS-222 in seawater (tricaine methanesulfonate, Sigma-Aldrich; dilution 1:10,000). After 1 min, fish reached a deep stage of anesthesia (Summerfelt and Smith, 1990). Blood was drawn from the caudal vasculature with a 1-ml heparinized syringe fitted with a 25-gauge needle (Terumo Europa N.V., Leuven, Belgium) and then placed in 1-ml eppendorf tubes while these were kept in a sealed bag inside cool thermos containers.

Time of capture and time at sampling were recorded and the interval lasted 5–8 min mostly depending on the travel distance to the shore. These values were used to correct for variation in plasma levels of cortisol due to handling time (Grutter and Pankhurst, 2000; Barton, 2002).

Body length was measured to the nearest millimeter using calipers and weighed to the nearest gram by means of a spring balance (Pesola, Switzerland). Thereafter, the fish was injected with a novel antigen (see section on “analysis of antibody responses”) and marked with a visible elastomer tag in order to be able to recapture the individuals (for details, see the next section).

After sampling and handling, the fish were allowed to recover from anesthesia for a period of at least 10 min in an aerated container with fresh sea water and subsequently transported to and released at the exact site of capture.

Condition index

In order to calculate the condition index, we used the ratio between body weight and total length as follows: $\text{weight} \times \text{length}^{-b}$,

where b is the rate of increasing weight with length. This length–weight parameter is close to 3 while it transforms length from a linear scale to a cubic dimension so it can be compared with body weight (Smith, 1984). In order to use a parameter that was species specific but independent of our sample, we searched on fishbase (Froese and Pauly, 2009) for our sampled species and calculated the average b -value. These were 2.967 for the brown surgeon fish, 3.064 for the lined bristletooth, 3.000 for the Red Sea banner fish, 3.000 for the white-belly damselfish, and 2.750 for the three-spot damselfish. The absolute values of the calculated condition ratios differed between species. Since these differences are dependent on the shape of the body, this does not express any information relevant for our research question. Therefore, to facilitate detection of a possible effect of access to cleaners on body condition, within species, we indexed the values to 100% based on the group without cleaner access. We refer to this value as the condition index.

Hormonal analysis

To assess circulating levels of cortisol by radio-immunoassay, the low polar (free) steroid fraction was extracted with diethyl ether from 20 μ l plasma using the method described in Scott and Vermeirssen (1994). Steroid residues were re-suspended in 1 ml assay buffer and stored again at -20°C until being assayed. A commercial polyclonal antibody was used for the radio-immunoassay (Fitzgerald Industries International, Inc.; www.fitzgerald-fii.com catalogue number 20-CR50), which has a cross-reactivity of 5.7% for 11-desoxycortisol, 3.3% for corticosterone, and <0.7% for cortisone. The sensitivity of the assay was 0.4 ng/ml. All samples were processed in a single assay with an intra-assay coefficient of variance (CV) of 1.3%.

Blood cell counts and analysis of antibody responses

Cell counts from blood smears were carried out to assess total and differential white blood cell counts. This method gives a phenotypic examination of the immune system (Blaxhall and Daisley, 1973) and has been used as a sensitive indicator of exposure to stress (Wedemeyer et al., 1990). The conditions in the field (spray of sea water, sand, and the presence of flies) were sub-optimal for making blood smears directly after drawing of the blood sample. Therefore, we decided on first to transfer the samples on ice to the laboratory, which took between 0.5 and 3 h after drawing of the blood samples. A drop of blood was then smeared over a frosted slide, dried on air, and stored in a sealed dry environment at ambient temperature. Immediately after this procedure, blood was centrifuged at 500 × g for 10 min and plasma was collected and stored in screw-capped eppendorf tubes at -20°C until further processing.

At the University of Neuchâtel, blood smears were stained for 20 min with Giemsa's azur eosin methylene blue solution (1:10 dilution in buffer; 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 fields were photographed from randomly chosen places on the part where the smear showed a homogeneous unicellular layer. The leukocyte cell types were classified and counted by eye. ImageJ software (Abramoff et al., 2004) was used to count total cell numbers in each field. The fit of human counted pictures vs. ImageJ counted pictures was $Y = 0.99X$, $R^2 = 0.992$ with $N = 193$. The average number of cells per field was 100 with most counts ranging between 50 and 200 cells.

The following cells were counted (for a review, see Ellis, 1977): (1) granulocytes that are large spherical cells with cytoplasm that contains numerous fine granules. The primary function of these cells is phagocytosis; (2) lymphocytes that are small spherical cells with little cytoplasm. These comprise a variety of cells involved in the production of specific antibodies, like T and B cells; and (3) all other cell types for calculating the total cell count = lymphocytes + granulocytes +

thrombocytes + erythrocytes. We report total leukocyte as percentage of the total cell count (see Ros et al., 2006; Ros and Oliveira, 2009) and lymphocyte and granulocyte counts as differential percentage (percentage of lymphocytes + granulocytes + thrombocytes). We further calculated the H–L ratio as the ratio of granulocytes over lymphocytes. The mean within subject coefficient of variation for the relative counts of leukocytes was 4.1%.

It has been argued that blood smears are difficult to interpret due to the effect of infections on the number of circulating lymphocytes. Without knowledge about changes in lymphocyte numbers, a healthy immunocompetent animal is difficult to distinguish from a diseased animal (Norris and Evans, 2000; but see Ros et al., 2006). Therefore, to have an additional indicator of immunocompetence, we measured the immune response to dinitrophenylated keyhole limpet hemocyanine (DNP-KLH, Merck Calbiochem). DNP-KLH was dissolved in saline at 300 µg/0.6 ml and each subject was immunized with an intraperitoneal injection. The amount of DNP-KLH suspension was adjusted to the body mass of the fish (0.2 ml suspension per 50 g body mass). Each immunized fish was marked individually with elastomer injected under a scale (VIE-tag, Northwest Marine Technology, USA). For assessing the blood titers of specific antibodies to DNP-KLH, 28 marked fish (see Fig. 2) were recaptured 10–14 days later using the barrier net and a second blood sample was drawn using the same sample procedures introduced before.

For the estimation of antibody levels to DNP-KLH, we adopted an agglutination protocol (Herscovitz et al., 1975) using antigen coupled to sheep erythrocytes (SRBC, Harlan, UK). In brief we incubated for 35 min at room temperature with occasional mixing: 0.25 ml washed sheep erythrocytes (100%), 2.5 ml DNP-KLH solution (3 mg/ml PBS), and 2.5 ml CrCl₃ · 6H₂O solution (1.33 mg/ml PBS). 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 lyses of sheep red blood cells by complement, the plasma was heated to 56 °C for 30 min (Collazos et al., 1994). Thereafter, plasma was diluted 1:1 in PBS and then serially diluted in PBS in U-shaped microtiter plates. An equal volume of 0.2% DNP-KLH coupled SRBC was added to these dilutions, and the plates were incubated at 37 °C for 60 min. Antibody titers were scored visually as the highest twofold dilution of plasma showing hemagglutination.

Statistical treatment and data analysis

In this study, we aimed to compare between fish resident in patches with, and fish resident in patches without a bluestreak cleaner wrasse. Therefore, in order to avoid pseudo replication, we set reef patch as our statistical unit except for the data on antibody responses since we were only able to collect a very limited *N*-value per species. As most data were collected on one of our species—the brown surgeon fish, these data allowed for a within species comparison of cleaner access with adequate statistical power.

As expected for behavioral data, the distribution for the number of cleaner interactions comprised many zeros resulting in a strongly skewed distribution. We therefore used non-parametric statistics for testing behavioral data. Body condition, blood cell percentages, and cortisol levels did not significantly deviate from normality (Kolmogorov–Smirnov test, ns) so we used parametric statistics (Pearson's correlation, Student's *t*-test, ANOVA). Analyses were carried out with the SPSS 15.0 package (SPSS Inc., Chicago, IL) and *p*-values represent two-tailed probabilities.

In order to test for the general validity of each result, we followed the within species comparison by a between species analysis in a two-way ANOVA with cleaner access as fixed factor and species as random factor using a hierarchical full factorial model. Levene's test was used to check for violation of the equality of variances assumption. In case Levene's test was significant, we checked whether omission of

extreme variables would result in a change in the interpretation of the test (i.e., a change from statistical significance to non-significance or otherwise).

In the one case for which Levene's test was indicating significant different variations between groups, the interpretation of the test appeared robust and we give the analyses with all values included. In case we did not find significant deviations of the null hypothesis, we carried out a post hoc power analysis using the program G*power (version 3.0.10, Universität Kiel, Germany). We calculated the power based on a substantial biological effect, which we assumed to be a 2 times deviation from the average value of the control group (no cleaner access). Power was calculated assuming the current variance and *N*-values. In case the power at the 2 times change in values was higher than 80%, we estimated the difference from the control group at which we in our test might obtain a 20% chance on a mistake of the second kind (power of 80%).

Variation in *N*-values in our tests resulted from the fact that we were not able to measure all parameters for all individuals and because in some species we could not obtain reliable measurements.

Ethical commitment

Ras Mohammed is a protected area with strict regulations issued not to cause damage to the fragile reefs (see Wood, 2007). We took special care to select procedures that allowed us to collect our samples with a maximum likelihood that all sampled individuals survived. Over all more than 96% of the trapped fish survived the procedures and was successfully reintroduced at the place of capture.

Taking blood samples requires puncturing the caudal vasculature, which is an invasive method. To reduce the level of discomfort experienced by the animals, blood was sampled from fish that had reached a deep stage of anesthesia. The cue for this stage was a total loss of equilibrium and reflexes to stimulation (Summerfelt and Smith, 1990). To check for the stage of anesthesia, we monitored the respiratory opercular movements. Fish were allowed to recover from anesthesia in a small isolation aquarium before they were returned to the site of capture. No side effect of blood sampling on condition was expected (e.g., Ros et al., 2006).

The Egyptian Environmental Affairs Agency (EEAA, Cairo) granted us permission for the study.

Results

Cleaner access effects: intra-specific comparison

Cleaner–client interactions

During the observations of fish on reef patches without bluestreak cleaner wrasses (in total 855 min divided over 13 reef patches), we did not record any interaction of the focal species with other fishes or shrimps in which cleaning took place. Brown surgeon fish had on average 3.14 ± 1.40 s of cleaning interactions per hour on reef patches with cleaner access (Mann–Whitney *U*-test, *N* no access = 13, *N* access = 7, *U* = 7.5, *p* = 0.045).

Body condition

Individuals caught from reef patches with or without cleaner access did not differ in standard length (two-sample *t*-test: $t[23] = 0.44$, *p* = 0.66) or in bodyweight (two-sample *t*-test: $t[23] = 1.14$, *p* = 0.27). However, body condition was found to be 15% higher in individuals on reef patches with cleaner access than on patches without cleaner access (Fig. 1, two-sample *t*-test: $t[23] = 2.76$, *p* = 0.011).

Cortisol levels

The handling time, i.e., the time from capture to blood sampling, ranged between 4 and 10 min (average 6.36 ± 0.04 min). This variation in handling time showed a significantly positive regression

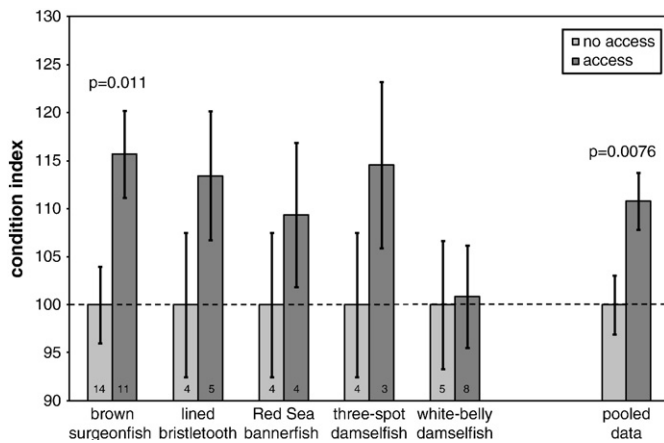


Fig. 1. Effect of access to the bluestreak cleaner wrasse on the body condition for five common resident reef fishes. All data are indexed with the no-access group set at 100. Overall mean and standard errors were calculated over mean values per patch to allow for independent comparisons. The last two bars represent the pooled data over the five species from which data were collected. Numbers refer to the number of patches in the analysis.

with plasma levels of cortisol (average 17.4 ± 0.4 ng/ml; linear regression, $n = 36$ (all data), $R^2 = 0.33$, $p = 0.0025$). A comparison of residual values averaged per reef patch did not show a significant effect of cleaner presence on the clients' cortisol levels (Fig. 2, group difference in cortisol levels = 28%; two-sample t -test, $t[17] = 0.93$, $p = 0.37$). However, the power of this statistical test for rejecting the zero hypothesis at a hypothetical group difference in cortisol levels of 50% was relatively low (35%).

Immunocompetence

None of the variables we measured from the blood smears were significantly different between individuals differing in cleaner access (see Table 1). The power of these tests was high (89% for testing a hypothetical difference of 50% in leukocyte blood cell percentages). There was no indication for leukocytosis in the two groups: leukocyte blood cell percentages ranged between 1.9% and 4.7%, with most cells being lymphocytes (Table 1).

In respect to the antibody response to the KLH-DNP antigen challenge, we did not re-capture enough individuals to allow for testing with sufficient statistical power (see Statistical treatment and

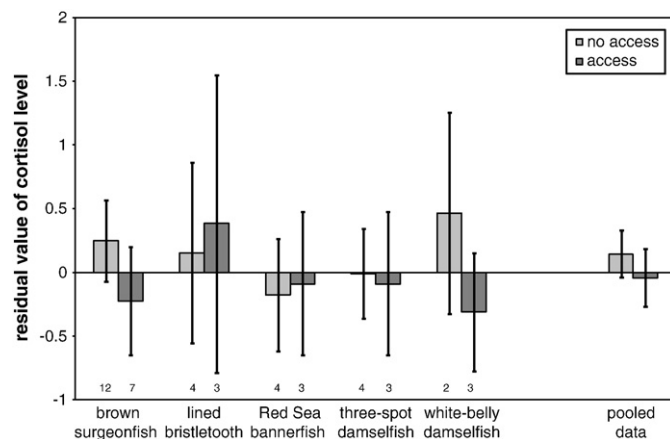


Fig. 2. Effect of access to the bluestreak cleaner wrasse on cortisol levels for five common resident reef fishes. Depicted are standardized residual values from which the effect of handling time was partialled out ($R^2 = 0.33$). Overall mean and standard errors were calculated over mean values per patch to allow for independent comparisons. The last two bars represent the pooled data over the five species from which data were collected. Numbers refer to the number of patches in the analysis.

data analysis sections). Simple comparison of these values revealed that individuals in both groups were able to mount immune responses. Two of eight treated individuals showed no response and these had cleaner access. Because antibody titers refer to a dilution series, average antibody concentrations were more than four times higher in individuals sampled from patches without cleaners than from patches with cleaner wrasses (see Fig. 3).

Cleaner access effects: inter-species comparison

Cleaner–client interactions

The data of the remaining client species (as defined in the methods) on reef patches with cleaner wrasses, i.e., lined bristletooth, Red Sea banner fish, three-spot, and white-belly damselfish, showed a similar trend as the brown surgeon fish: we did not record any cleaner–client interaction on patches without cleaners.

Body condition

The overall ANOVA showed a significant effect of cleaner access (Fig. 1, $F[1,52] = 7.70$, $p = 0.0076$). There were no significant differences between species (ANOVA: interaction effect of cleaner access and species identity: $F[8,52] = 0.63$, $p = 0.26$).

Cortisol levels

Univariate ANOVA using all samples as independent data points showed a highly significant effect of handling time (covariate, $F[1,71] = 33.2$, $p < 0.001$) and species (random factor, $F[4,71] = 4.4$, $p = 0.003$). The latter was due to relatively low cortisol levels in the Red Sea banner fish in comparison to the other species, when this species was removed from the analysis, the effect of species was not significant anymore (random factor, $F[3,60] = 0.81$, $p = 0.49$). A comparison of residual values averaged per reef patch did not show a significant effect of cleaner access on the clients' cortisol levels (Fig. 2, group difference in cortisol levels = 14%; ANOVA, factor cleaner access $F[1,6.8] = 0.32$, $p = 0.59$; interaction cleaner access and species [random] $F[4,35] = 0.39$, $p = 0.81$). The power of this test assuming a 50% group difference was 66%.

Immunocompetence

At 8 patches, we collected data from individuals of the lined bristletooth and Red Sea banner fish, and at 7 patches, we collected data of individuals of the white-belly and three-spot damselfish. An ANOVA including these 4 species and the brown surgeon fish (data collected at 19 patches) showed significant species differences that could be assigned to relatively high leukocyte percentages in the two damselfish in comparison with the other species (see Table 1). The ANOVA did not show a significant effect of cleaner access on total leukocyte blood cell percentages, nor on the relative percentages of lymphocytes and granulocytes (see Table 1; $F[1,39] < 0.67$, $p > 0.4$). Post hoc power analysis yielded a possibility to reject the zero hypothesis with a power of 80% at a difference between group values (corrected for between species variation) of about 1.2%. The differences between groups that we measured were a factor 10 lower.

Repeated sampling for measuring antigen challenges was accomplished for 28 individuals belonging to 4 species: the brown surgeon fish ($N = 8$, 7 patches), the Red Sea banner fish ($N = 10$, 5 patches), the white-belly damselfish ($N = 7$, 6 patches), and the three-spot damselfish ($N = 3$, 3 patches). The results are depicted in Fig. 3. Analyses did not show significant interspecific differences in the antibody response to the challenge ($F[3,14] = 1.05$, $p = 0.40$). All species showed significantly higher antibody responses in patches without cleaner access than those living in patches with cleaner access (Fig. 3, $F[1,14] = 10.4$, $p = 0.0066$).

Table 1
Effect of access to the bluestreak cleaner wrasse on leukocyte blood cell percentage, the differential percentage of lymphocytes and granulocytes, and the H–L ratio (granulocytes/lymphocytes) of five common resident reef fishes. Averages and standard errors were calculated over mean values per patch to allow for independent comparisons. The ANOVA outcome is the result of a two-way analysis with species identity and cleaner access as factors.

	Per patch	Total leukocytes (%)	Lymphocyte (%)	Granulocyte (%)	H–L ratio
Brown surgeon fish (N = 39)	No access (N = 12)	3.53 ± 0.12	86.3 ± 0.5	7.2 ± 0.9	0.086 ± 0.005
	Access (N = 7)	3.15 ± 0.15	82.8 ± 1.7	7.4 ± 0.1	0.098 ± 0.013
	t-test (df = 17)	0.69	0.97	0.11	0.38
	P-value	0.50	0.34	0.92	0.71
Lined bristletooth (N = 14)	No access (N = 5)	3.84 ± 0.37	91.6 ± 0.5	5.4 ± 0.5	0.059 ± 0.005
	Access (N = 3)	3.95 ± 0.41	83.4 ± 1.1	8.0 ± 0.4	0.097 ± 0.004
Red Sea banner fish (N = 14)	No access (N = 4)	5.94 ± 1.05	86.2 ± 2.2	4.7 ± 1.1	0.056 ± 0.013
	Access (N = 4)	5.14 ± 0.97	90.1 ± 2.0	5.6 ± 1.3	0.065 ± 0.016
White-belly damselfish (N = 13)	No access (N = 4)	6.45 ± 0.44	67.7 ± 6.2	7.7 ± 0.8	0.135 ± 0.014
	Access (N = 3)	6.35 ± 1.05	65.9 ± 5.8	14.7 ± 2.1	0.250 ± 0.064
Three-spot damselfish (N = 10)	No access (N = 4)	1.97 ± 0.22	59.1 ± 4.7	21.0 ± 3.7	0.395 ± 0.097
	Access (N = 3)	5.95 ± 1.95	62.0 ± 6.4	9.9 ± 2.4	0.174 ± 0.050
ANOVA outcome	Effect cleaner access				
	F-value	0.67	0.017	0.12	0.19
	P-value	0.42	0.90	0.73	0.66

Discussion

In this study, we asked how cleaner access affects clients' physiology and physical condition. We focused on six perciform client species that are resident at reef patches and clients for the bluestreak cleaner wrasse *L. dimidiatus* in our study area (Bshary, 2001). The distribution of bluestreak cleaner wrasses over these patches is variable from year to year and was not related to habitat structure or dimensions of the patches that were selected (Bshary, 2003; Elena Wagner and Redouan Bshary, unpublished results). Observations confirmed that subjects with cleaner access had cleaning interactions while subjects without access had no cleaning interactions. Individuals on reef patches with a bluestreak cleaner wrasse were found to have higher body condition index than individuals on reef patches without this wrasse. No indication was found for differences in blood percentages of leukocytes, but the antibody response to experimental vaccination with a novel antigen, KLH-DNP, was highest in individuals living in reef patches without a cleaner wrasse. Here we discuss via what mechanisms cleaning interactions might affect the differences in physical condition that we measured and to what extent cortisol might regulate these effects.

Effect of cleaners on indicators of stress of reef fish

As typical for vertebrates, teleost fish respond to unpredictable and noxious stimuli by mounting a stress response (e.g., Barton, 2002). An important element of this response in fish is the activation of the hypothalamus–pituitary–interrenal axis, which results in an increase in circulating levels of cortisol. Bshary et al. (2007) showed for two client species (in the same study site as we used) *Pseudanthias squamipinnis* and *Chromis dimidiatus* that cortisol levels in response to a stressor were higher when these clients were resident on reef patches without cleaner access than with cleaner access. Here we aimed to test whether a similar effect could be measured in the basal levels. As cortisol levels showed a highly significant and positive relationship with capture and handling time, we failed to measure actual basal levels and instead used the residual values corrected for handling time. In these values, we did not find a significant effect of cleaner access on overall levels of cortisol levels but the power of the statistical tests was relatively low. Therefore, we collected additional data in a subsequent field period, where we further optimized our sample protocol to anesthetize and draw blood samples while scuba diving (adapted from Grutter and Pankhurst, 2000). This gave us the opportunity to draw samples of the brown surgeon fish and the white-belly damselfish within 4 min after capture. The basal plasma levels of cortisol measured from these samples were on average 53% and 43% respectively lower in the group with cleaner access than in the group without cleaner access. Again these differences were not statistically significant (brown surgeon fish: $n = 18$, $p = 0.26$, white-belly damselfish: $n = 16$, $p = 0.27$, Albert Ros, Philippe Vuilliod and Redouan Bshary, unpublished results).

Our data together with those of Bshary et al. (2007) suggest that parasite removal by cleaner wrasses might not change average basal levels of cortisol substantially although it might reduce the peak levels of the hormone during stressful events. High peak levels of cortisol indicate a deficiency in the availability of metabolic resources under stressful conditions and this may have lasting and costly consequences for homeostatic processes, immunity, and reproductive behaviors (McEwen and Wingfield, 2003; Schreck, 2010). Another important function of cortisol is its role in increased mobilization of glucose and amino acids to support high locomotor activity (e.g., Vijayan et al., 1997; Leong et al., 2009). This is consistent with the result that the Red Sea banner fish exhibited significantly lower levels of cortisol than all the other species in our study. In comparison with the other client species, Red Sea banner fish are generally slow moving fish, living close to their hiding place, and relatively calm when approached by divers.

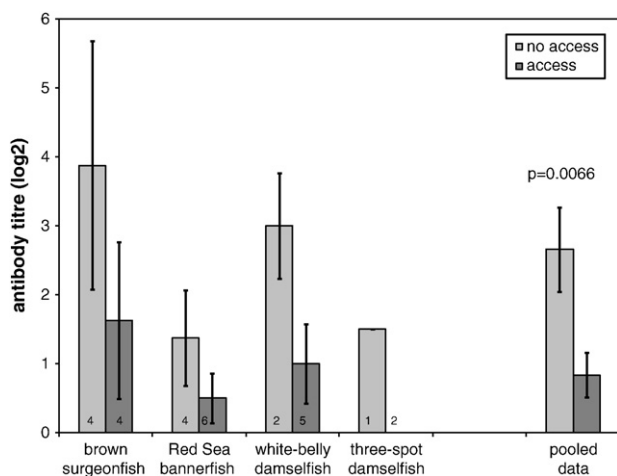


Fig. 3. Effect of access to the bluestreak cleaner wrasse on the antibody response to a single injection of KLH-DNP for four common reef fishes. Mean and standard errors were calculated over individual values. The last two bars represent the pooled data over the four species from which data were collected. Numbers refer to the number of individuals in the analysis.

Our study aimed to map which physiological traits are related with the mutualistic cleaner–client interactions. It has been proposed by Grutter and Pankhurst (2000) in their study of the effects of parasites on the reef fish *H. melapterus* that “it is probably adaptively inappropriate for sustained disease or parasitic challenge to stimulate a classical stress response” because corticosteroids are immunosuppressive (Woo et al., 1987; Pickering and Pottinger, 1989). Such proposed down regulation or tempering of basal stress physiology might mask the calming effect on stress physiology of cleaner access that was shown in the study by Bshary et al., 2007 and thus explain why we did not find significant differences in cortisol levels in this study.

Effect of cleaners on indicators of health of reef fish

The reef fish in our study did not show significant variation in circulating leukocytes related to cleaner access. However, fish that had no cleaner access showed significant higher production of specific antibodies than fish that had cleaner access. Functionally, natural immunity that is coordinated by leukocytes together with the chemical barriers like the complement system and lysozyme comprises the first line of defense against infections (e.g., Braude et al., 1999; Watts et al., 2001). The more pathogen-specific immunity includes humoral immune responses, which primarily protect against extracellular infections (endoparasites or ectoparasites), and cellular immune responses, which protect against intracellular pathogens like viruses. Our results suggest that both groups invested likewise in defense against the primary effects of ectoparasites via the natural immune system (via leukocytes), however, that some difference in the environment between both groups affected the activation of the humoral response to infections. Based on the results, we can explore two possible mechanisms that might explain this difference between groups in humoral immunocompetence.

First, one aim of the present study was to measure levels of the stress steroid hormone cortisol. In general, exposure to elevated levels of cortisol has been shown to suppress many aspects of the immune system including antibody production (Maule and Schreck, 1990; Yada and Nakanishi, 2002). We therefore expected to find a negative relationship between antibody titers and cortisol levels. However, in the present study, we did not find significant differences in cortisol levels between both groups. Moreover and opposite to what would be expected from the higher antibody responses in the group without cleaner access, Bshary et al. (2007) found an increase in stress enhanced cortisol levels in this group compared with the group with cleaner access. Our failure to predict the current relationships demonstrates that we underestimated the complexity of the dynamics of the immune mechanisms involved. For example, acute corticosteroid release may redistribute immune cells, which may enhance rather than suppress immunocompetence (Braude et al., 1999). Furthermore, our knowledge about the effects of parasites on stress physiology of their fish host in natural conditions is still very limited (see Grutter and Pankhurst, 2000).

Second, the increased immune activity in the group without cleaner access might have been a consequence of living in an environment with higher numbers of ectoparasites than in the group with cleaner access. Bluestreak cleaner wrasses remove ectoparasites, and they have been shown to significantly decrease local abundance of gnathiid isopods (Grutter, 1996a; Grutter and Lester, 2002). Thus individuals that are resident on a reef patch with a cleaner wrasse present are most likely to experience a significant reduction in exposure to ectoparasites and thereby in the adverse effects of these parasites. Cleaner mutualism might be viewed as a behavioral strategy to reduce ectoparasite exposure (see Barber et al., 2000). Such a strategy is more likely to evolve if ectoparasite infections are costly and the reduction of these costs may benefit fitness. In addition to the adverse effects of ectoparasites mentioned in the introduction, there is some evidence that immune responses have direct energetic costs (Watts et al., 2001; Alcorn et al., 2003; Demas, 2004) and may suppress androgen steroids,

which play an important role in facilitating reproductive traits (Boonekamp et al., 2008). Thus, because investment in the immune system may trade-off with other traits that are important for fitness, it would be adaptive to adjust activation of humoral immunity to the infection risk that is associated with the environment the individual is living in. In our study, such adaptive regulation of trade-offs might explain the higher body condition we found in the individuals in the group with lower immunocompetence.

Pathogen exposure activates a whole cascade of responses by the immune system among which antibody production. Such exposure may also increase the hosts' investment in the immune system in order to perform more optimal facing further challenges. Additionally and not necessary mutually exclusive, infections may have long-lasting adverse consequences, which decrease performance during future challenges (e.g., Devevey et al., 2010). For example, a high infection rate might result in high allostatic load, which might change stress physiology (McEwen and Wingfield, 2003; Schreck, 2010). An increased allostatic load in animals without cleaner access was proposed by Bshary et al. (2007) to explain the increased cortisol production in response to a stressor they found in these animals. We used a non-pathogenic antigen to challenge the immune system. We assumed that higher antibody titers are indicative of superior immune defense against real-life pathogenic infection but we realize that it would be informative to test other types of immune responses as well in order to have a more complete image about how cleaning interactions might have an impact on immunocompetence and on the fish health in general. Clearly there is a need to further test whether increased antibody responsiveness in the group without cleaner access would be adaptive (resulting in better protection) or a consequence of altered stress physiology. Either way in both scenarios increased immune responses have biologically significant costs associated and thereby ectoparasite exposure would result in selection to maintain cleaner–client mutualistic behavior.

So far the results are in accordance with the hypothesis that reef fish benefit from cleaner access (Grutter, 2001; Grutter and Lester, 2002). Immune activity, body condition (this study), and some aspects of stress physiology (Bshary et al., 2007) have all been shown to be affected by the opportunity to engage in mutualistic interactions. Future work should focus on further manipulations as to ascertain whether these effects are due to parasite removal or to other specific activities of the bluestreak cleaner wrasse such as tactile stimulation (see Losey, 1972; Potts, 1973; Bshary and Würth, 2001). Moreover, our results indicate that the presence of bluestreak cleaner wrasses may decrease the need for reef fish to invest in immunity. Since this investment may trade-off with investment in other traits (for instance, body condition), this might ultimately have positive fitness effects.

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