Parasite infection rather than tactile stimulation is the proximate cause of cleaning behaviour in reef fish

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Cleaning behaviour is a popular example of non-kin cooperation. However, quantitative support for this is generally sparse and the alternative, that cleaners are parasitic, has also been proposed. Although the behaviour involves some of the most complex and highly developed interspecific communication signals known, the proximate causal factors for why clients seek cleaners are controversial. However, this information is essential to understanding the evolution of cleaning. I tested whether clients seek cleaners in response to parasite infection or whether clients seek cleaners for tactile stimulation regardless of parasite load. Parasite loads on client fish were manipulated and clients exposed to cleaner fish and control fish behind glass. I found that parasitized client fish spent more time than unparasitized fish next to a cleaner fish. In addition, parasitized clients spent more time next to cleaners than next to control fish, whereas unparasitized fish were not attracted to cleaners. This study shows, I believe for the first time, which is somewhat surprising, that parasite infection alone causes clients to seek cleaning by cleaners and provides insight into how this behaviour evolved.

Keywords: cleaning behaviour; mutualism; *Labroides dimidiatus*; Gnathiidae; parasites; proximate causation

1. INTRODUCTION

Cleaning behaviour is often used as a classical example of mutualism (Trivers 1971; Thompson 1994; Hammerstein & Hoekstra 1995; Begon *et al.* 1996), but this has been questioned a number of times (Losey 1979, 1987; Weeks 2000). It has also been used as an example of the limitations of repeated games as defection by clients (eating a cleaner fish) ends the game and, hence, the game is no longer repeated (Hammerstein & Hoekstra 1995). In the marine environment, cleaning behaviour involves hundreds of species (Feder 1966; Kuwamura 1976; Grutter & Poulin 1998a) thereby making it an ideal system for studying multispecies mutualisms.

Grutter (1999a) recently provided conclusive evidence for mutualism in fish cleaning behaviour, i.e. that the ultimate cause of cleaning in clients is ectoparasite removal. However, although cleaning behaviour involves one of the most developed interspecific communication systems, the proximate mechanisms of cleaning behaviour remain controversial. Although the view that clients seek cleaners in response to ectoparasite infection is intuitively predicted and supports the views of cleaning as a mutualism (Limbaugh 1961; Feder 1966), surprisingly this has not been supported. Instead, previous studies have suggested that clients seek cleaners for tactile stimulation with cleaners exploiting the clients' responses to tactile stimulation in order to gain food (Losey & Margules 1974; Losey 1979, 1987). The former view predicts that more heavily parasitized clients should seek cleaners more while the latter predicts that clients will seek cleaners for tactile stimulation regardless of parasite load.

Despite its importance in understanding cleaning interactions, whether parasite infection is a cause of why clients seek cleaners has only been tested once using models of the Hawaiian cleaner fish *Labroides phthiophagus* (Losey 1979). Ectoparasites had little effect on one client species of butterfly fish and only amplified responses in a surgeonfish species. More importantly, clients appeared to learn to respond to models or a bare wire in order to receive a tactile reward. Losey (1979, 1987) proposed that tactile stimuli rather than parasite infection were the proximate cause of cleaning behaviour in client fish. Cleaners thus obtained meals by exploiting the positive responses of the clients to tactile stimuli. However, it is not clear why no counter-adaptations have evolved in clients in order to avoid this exploitation (Poulin & Grutter 1996).

The parasitic juveniles of gnathiid isopods are the main food source of the cleaner fish *Labroides dimidiatus* (Grutter 1996, 1997) on the Great Barrier Reef. Their abundance on the thick-lip wrasse *Hemigymnus melapterus* decreases daily between dawn and sunset in wild and caged fish (Grutter 1999b), probably partly as a result of the 1200 parasites, mostly gnathiids, eaten daily per cleaner (Grutter 1996, 1999a). Gnathiids are therefore ideal for investigating the role of parasites in client cleaning behaviour. In order to examine their effect on clients' tendency to seek cleaners, gnathiids were reared in the laboratory and the behaviour of parasitized clients and unparasitized clients towards cleaners and control fish behind glass was compared.

2. MATERIAL AND METHODS

(a) Fish

All fish were collected from Lizard Island, Great Barrier Reef, following Grutter (1994, 1997), held in running seawater

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Figure 1. Diagram of a plastic 1000-l test tank showing a video camera 1.5 m above the tank and parasite and fish shelter in the centre filled with dead coral. The fish from left to right are the control, cleaner and client, respectively. The shaded areas represent areas in which the client's behaviour was quantified.

and fed prawn pieces *ad libitum* daily. Cleaner fish and control fish were collected on 1 October 1998 (see figure 2*b* for the collection times of client fish).

(b) Parasite infections

Parasitized and unparasitized *H. melapterus* were obtained under controlled conditions using gnathiid isopod juveniles. As gnathiids generally feed for less than 1 h (A. S. Grutter and R. J. G. Lester, unpublished data) and leave hosts when disturbed (Grutter 1995), fish were infected with gnathiids by testing fish in a tank containing a culture of gnathiids. A species of gnathiid from *H. melapterus* (type 1) (Grutter *et al.* 2000) was reared as follows: fish were collected at Lizard Island, Great Barrier Reef, on 12–18 January 1998 and gnathiids were removed (Grutter & Hendrikz 1999). Third stage juveniles were reared to adults (Grutter & Hendrikz 1999). Adult males moulted in eight to nine days. A juvenile with highly visible eggs (after 13–18 days) and a male were placed in a 5-ml vial where juveniles moulted to adult females. New larvae (produced 28–30 days from when gnathiids were first collected) were added to two tanks (figure 1) on 13–19 February 1998. The larvae of two and three pairs, respectively, were added to the two tanks and the larvae of another pair were fixed and counted (27 larvae). The tanks had running seawater and held three *H. melapterus* (10–17 cm standard length) each. A plastic crate (60 cm × 40 cm × 30 cm), which was raised off the bottom with four bricks and filled with dead coral, was placed in the centre of each tank as shelter for gnathiids with the bottom providing shelter for the fish (figure 1).

Whether gnathiid infections had been established in the tanks was examined on 6–9 May 1998. Gnathiids were only found in the tank with larvae from three pairs, with each fish having 3, 9 and 13 gnathiids, respectively. Fish to be used in tests were obtained by adding five new fish to this 'infected' tank and to each of five other similar tanks on 13 May 1998. All fish (8–17 cm standard length) had been 'cleaned' of ectoparasites (Grutter 1999*b*) followed by a 2.5 h bath in the anthelmintic Praziquantel (ICN Biomedicals Inc., Aurora, OH, USA) (1:100 000). So that three of the six tanks had gnathiids, 55 gnathiid larvae of the same species (which were identified by patterns on their dorsal side) obtained from wild fish were added to each of two tanks ('other infected'). In order to deter-

mine whether the numbers of gnathiids were sufficiently high to infect other clients, one fish from the 'infected' tank was sampled on 8 October 1998; it had 18 gnathiids, a number within the range found on wild fish (Grutter 1994). Gnathiids were seen swimming in the two 'other infected' tanks. Prior to trials, all fish, including 'uninfected' controls, were cleaned of ectoparasites following the method of Grutter (1999*b*), a procedure that ensures fish are free of gnathiids. Fish were then held in a similar holding tank for 24 h.

(c) Experimental design

Parasite treatments were obtained by performing trials in a tank with ('infected') and without a gnathiid culture. Fish to be tested were taken from two randomly selected tanks (with and without parasites) on the day prior to testing until all fish in the two tanks (two to four fish per tank) were used. *Hemigymnus melapterus* were given the choice of a cleaner fish or a control fish held in partially submerged separate glass stimulus compartments (aquaria) (40 cm high × 30 cm wide × 60 cm long) at each end of the tank (figure 1). A parasite-free client was added to the tank and allowed to acclimate for 1 h. A cleaner fish and a control fish were then each placed in a stimulus aquarium. Trials were then recorded for 1 h from above using a wide-angle lens (Sony 37mm) and polarizing lens (Arkon V-37) on a Sony TRV 89E video camera. Eight and 11 trials were performed in the tanks with and without parasites, respectively. Trials were conducted between 14 and 18 October 1998 from 08.00 to 17.00, beginning every 2 h. A maximum of five trials were performed per day, alternating between the infected and uninfected tanks, with fish acclimating in one while the other was being tested. The first treatment of the day, in blocks of two days, was determined by tossing a coin. The side of the tank with a cleaner fish or control fish was random but balanced. Each fish was tested once only. After trials, gnathiids were removed from the fish and counted (Grutter & Hendrikz 1999). The mean ($\pm$ s.e.) gnathiid abundance on infected fish was 72 ($\pm$ 21) with a range of 3 to 179 per fish. Gnathiids remain on fish for *ca.* 1 h (A. S. Grutter and R. J. G. Lester, unpublished data). Given a constant emigration rate, the number of parasites on fish after 1 h would have been *ca.* 75% of those present on fish after 2 h (McCallum 2000, p. 291).

(d) Response variables

The variables measured were the total times in minutes per hour that each *H. melapterus* spent in the areas between the outer edges of the shelters and the ends of the tub containing the aquaria with the stimuli fish (shaded areas in figure 1). Posing, which is a measure of a client's willingness to be cleaned (Côté *et al.* 1998), was not measured as *H. melapterus* rarely posed during the study.

(e) Statistical analyses

Non-parametric Mann–Whitney *U*-tests for two independent small samples were used for testing the effects of parasites (presence/absence of gnathiids) and of the side of the tank. Kruskal–Wallis rank sums tests for more than two independent samples were used for testing the effects of time of day and day. (The JMPIN 3.2.6. software of the SAS Institute, Inc. was used for analytical computations). It was assumed that there was no interaction between parasite treatment, side of the tank, time of day and day. In order to determine whether parasitized clients were preferentially attracted to cleaners or to control fish, the ratio of the amount of time in the hour that a client spent near

Figure 2. Box and whisker plots of the behaviour of parasitized versus unparasitized client fish in the experimental tanks. (a) The median time that the client with and without parasitic gnathiids spent near a stimulus aquarium holding a cleaner fish. (b) The median ratio of time a client spent near a stimulus aquarium holding a cleaner fish over time spent near a control (non-cleaner) fish for parasitized clients versus unparasitized fish only. The ratios were compared to the null hypothesis of equal time, i.e. a mean ratio of one. The centre line denotes the median value, the box encloses the inner two quartiles, the error bars indicate the 90th and 10th percentiles and the closed circles indicate outliers. A double asterisk represents a significant difference at a significance level of $p < 0.005$, a single asterisk represents a significant difference from a ratio of one at a significance level of $p < 0.05$ and n.s. represents no significant difference from a ratio of one at a significance level of $p > 0.05$.

the cleaner fish to the time the client spent near the control fish was compared to the null hypothesis of equal time, i.e. a mean ratio of one, using a Wilcoxon's signed-rank test for matched small samples. The same analysis was performed for unparasitized fish. Whether these ratios varied with side of the tank, time of day and day was tested for each of the client groups separately, as for the total response.

3. RESULTS

Client fish parasitized with gnathiids spent more time next to a stimulus aquarium holding a cleaner fish than unparasitized clients (Mann-Whitney U -test, $Z = 2.93$ and $p < 0.005$) (figure 2a). Their responses to stimuli were not significantly affected by side of the tank, time of day or day (Mann-Whitney U -test, $Z = -0.702$ and $p > 0.100$, $\chi^2 = 8.935$, d.f. = 4 and $p = 0.063$ and $\chi^2 = 1.725$, d.f. = 4 and $p = 0.786$, respectively). Fish spent a median of 2.8 times more time near a cleaner fish compared to a control fish within parasitized clients only (Wilcoxon's signed-rank test, $T = 16$ and $p = 0.02$) showing that parasitized clients were specifically attracted to cleaners (figure 2b). In contrast, there was no significant difference in the amounts of time clients spent near a cleaner rather than near a control fish within unparasitized clients (Wilcoxon's signed-rank test, $T = 12$ and $p = 0.32$) indicating that unparasitized clients were not attracted to cleaners (figure 2b). The relative amount of time a client spent near a cleaner fish was not significantly affected by side of the tank, time of day or day (Mann-Whitney U -test, $Z = -0.596$ and $p > 0.100$, $\chi^2 = 1.806$, d.f. = 3 and $p = 0.614$ and $\chi^2 = 3.667$, d.f. = 4 and $p = 0.453$, respectively, for parasitized fish and $Z = 0.822$ and $p > 0.100$, $\chi^2 = 8.258$, d.f. = 4 and $p = 0.083$ and $\chi^2 = 3.712$, d.f. = 4 and $p = 0.446$, respectively, for unparasitized fish). There was a positive although non-significant correlation among parasitized fish ($r = 0.457$, d.f. = 7 and $0.50 > p > 0.20$) between parasite load and the ratio of time a client spent near a cleaner fish over time spent near a control fish.

4. DISCUSSION

This is the first experimental evidence that parasites affect a client's tendency to seek cleaners and it suggests that parasite infection is a proximate cause of cleaning behaviour in the client fish *H. melapterus*. Whether client fish seek cleaners in response to 'itching' by the parasite *per se* or to the wound caused by the parasite is unclear. It is most likely that both factors are involved as fish react to parasitic gnathiids with jolts and shakes immediately after the parasites contact the body of the fish (A. S. Grutter, personal observation) and wounded (from non-parasite sources) fish spend more time at cleaning stations than they do after the healing process is well-advanced (Foster 1985).

The mean number of gnathiids used, which was sufficient for eliciting cleaner-seeking behaviour in clients, was *ca.* 3.5 times higher than that of wild fish that are regularly cleaned (Grutter 1996). High numbers of larger gnathiids can cause mortality in other species of captive fishes (Mugridge & Stallybrass 1983). Whether there is a threshold of gnathiids needed for clients to seek cleaners is unknown. Although there was a trend for fish with more gnathiids to seek cleaners more, not surprisingly, as the sample size was only eight fish, this was not significant.

As the fish were initially parasite free when introduced to the tanks, they were tested using newly settled ectoparasites. Thus, the parasites presented a new stimulus to the fish. Such an event may be a more important perceptual event than having a long-term resident parasite. Parasites

should have been selected in order to minimize perception by hosts, possibly by having stimuli that fish are easily habituated to. However, a new stimulus, which the host may not yet be habituated to, may be a more powerful stimulus for clients to seek cleaners. Parasites such as gnathiid isopods, which only remain on fish for *ca.* 1 h (A. S. Grutter and R. J. G. Lester, unpublished data) may thus form one of the stronger stimuli from a parasite, prior to any habituation in the host.

The experimental design used here ensured that the client fish did not come into contact with cleaners so interactions were not confounded by tactile stimulation (Losey 1979) from cleaners. However, the fact that parasitized clients sought cleaners, even though they could not receive tactile stimulation, demonstrates that the presence of parasites alone is sufficient for motivating clients to seek cleaners.

Interestingly, fish without parasites were not attracted to cleaners. These data do not support the tactile stimuli hypothesis, which predicts that clients will seek cleaners regardless of parasite load (Losey 1979). Losey's (1979) scenario of the evolution of cleaning behaviour, which was aimed at tropical Indo-Pacific cleaning symbiosis involving *Labroides* spp., was that cleaning did not originate as a symbiotic function. Instead, cleaners gained access to a food supply (ectoparasites) by exploiting a pre-existing tendency in their clients to respond to a tactile reward. However, the results of the present study agree more with views depicting a cooperative evolutionary history of cleaning behaviour driven by fitness benefits to both partners (Trivers 1971; Poulin & Vickery 1995; Poulin & Grutter 1996). Clients that are under selective pressure to have parasites removed are hypothesized to have evolved special responses, such as seeking cleaners when parasitized, in order to maximize the adaptive value of the interaction.

The differences between this study and that of Losey (1979) may have been due to phylogenetic differences, as different cleaner and client species were used, the different experimental approaches used and/or spatial variation in the selective pressures on clients, as the client parasite loads at Lizard Island may be higher than in Hawaii (Losey 1972; Grutter 1994). Finally, Losey (1979) stated that 'low sample sizes and high variability between individuals cast doubt on the validity of the conclusion that ectoparasites have no effect' (p. 679).

The latter evolutionary scenario is supported by the integration of ultimate and proximate causations (Drickamer 1998), i.e. 'why' clients seek cleaners (for parasite removal) (Grutter 1999a) and 'how' clients are influenced to seek cleaners (by parasite infection), respectively. It also agrees with the view that parasitism often plays a critical role in the evolution of behaviour (Moore & Gottelli 1990). Numerous examples of behaviours exist where hosts threatened by parasites develop behaviours in order to defend against infestation (Hart 1997). Cleaning behaviour can thus be considered as another, albeit unusual, example of how hosts defend themselves against parasites.

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REFERENCES

- Begon, M., Harper, J. I. & Townsend, C. R. 1996 *Ecology: individuals, populations and communities*. Carlton, Australia: Blackwell Science.
- Côté, I. M., Arnal, C. & Reynolds, J. D. 1998 Variation in posing behaviour among fish species visiting cleaning stations. *J. Fish Biol.* **53**(Suppl. A), 256–266.
- Drickamer, L. C. 1998 Vertebrate behavior: integration of proximate and ultimate causation. *Am. Zool.* **38**, 39–42.
- Feder, H. M. 1966 Cleaning symbiosis in the marine environment. In *Symbiosis*, vol. 1 (S. M. Henry), pp. 327–380. New York: Academic Press.
- Foster, S. A. 1985 Wound healing: a possible role of cleaning stations. *Copeia* **1985**, 875–880.
- Grutter, A. S. 1994 Spatial and temporal variations of the ectoparasites of seven reef fish species from Lizard Island and Heron Island, Australia. *Mar. Ecol. Prog. Ser.* **115**, 21–30.
- Grutter, A. S. 1995 Comparison of methods for sampling ectoparasites from coral reef fishes. *Mar. Freshw. Res.* **46**, 897–903.
- Grutter, A. S. 1996 Parasite removal rates by the cleaner wrasse *Labroides dimidiatus*. *Mar. Ecol. Prog. Ser.* **130**, 61–70.
- Grutter, A. S. 1997 Spatio-temporal variation and feeding selectivity in the diet of the cleaner fish *Labroides dimidiatus*. *Copeia* **1997**, 346–355.
- Grutter, A. S. 1999a Cleaner fish really do clean. *Nature* **398**, 672–673.
- Grutter, A. S. 1999b Infestation dynamics of parasitic gnathiid isopod juveniles on a coral reef fish *Hemigymnus melapterus*. *Mar. Biol.* **135**, 545–552.
- Grutter, A. S. & Hendrikz, J. 1999 Diurnal variation in the abundance of parasitic gnathiid isopod larvae on coral reef fish: its implications in cleaning interactions. *Coral Reefs* **18**, 187–191.
- Grutter, A. S. & Poulin, R. 1998a Cleaning of coral reef fishes by the wrasse *Labroides dimidiatus*: influence of client body size and phylogeny. *Copeia* **1998**, 120–127.
- Grutter, A. S., Morgan, J. A. T. & Adlard, R. D. 2000 Characterising parasitic gnathiid isopod species and matching life stages using ribosomal DNA ITS2 sequences. *Mar. Biol.* **136**, 201–205.
- Hammerstein, P. & Hoekstra, R. F. 1995 Mutualism on the move. *Nature* **376**, 121–122.
- Hart, B. L. 1997 Behavioural defence. In *Host–parasite evolution, general principles and avian models* (ed. D. H. Clayton & J. Moore), pp. 59–77. Oxford University Press.
- Kuwamura, T. 1976 Different responses of inshore fishes to the cleaning wrasse, *Labroides dimidiatus*, as observed in Sirahama. *Publ. Seto Mar. Biol. Lab.* **23**, 119–144.
- Limbaugh, C. 1961 Cleaning symbiosis. *Sci. Am.* **205**, 42–49.
- Losey Jr, G. S. 1972 The ecological importance of cleaning symbiosis. *Copeia* **1972**, 820–833.
- Losey Jr, G. S. 1979 Fish cleaning symbiosis: proximate causes of host behaviour. *Anim. Behav.* **27**, 669–685.
- Losey Jr, G. S. 1987 Cleaning symbiosis. *Symbiosis* **4**, 229–258.

- Losey Jr, G. S. & Margules, L. 1974 Cleaning symbiosis provides a positive reinforcer for fish. *Science* **184**, 179–180.
- McCallum, H. 2000 *Population parameters: estimation for ecological models*. Oxford, UK: Blackwell Science Ltd.
- Moore, J. & Gotelli, N. J. 1990 A phylogenetic perspective on the evolution of altered host behaviours: a critical look at the manipulation hypothesis. In *Parasitism and host behaviour* (ed. C. J. Barnard & J. M. Behnke), pp. 193–233. London: Taylor & Francis.
- Mugridge, R. E. R. & Stallybrass, H. G. 1983 A mortality of eels, *Anguilla anguilla* L., attributed to Gnathiidae. *J. Fish Biol.* **6**, 81–82.
- Poulin, R. & Grutter, A. S. 1996 Cleaning symbioses: proximate and adaptive explanations. *BioScience* **46**, 512–517.
- Poulin, R. & Vickery, W. L. 1995 Cleaning symbiosis as an evolutionary game: to cheat or not to cheat? *J. Theor. Biol.* **175**, 63–70.
- Thompson, J. N. 1994 *The coevolutionary process*. University of Chicago Press.
- Trivers, R. L. 1971 The evolution of reciprocal altruism. *Q. Rev. Biol.* **46**, 35–57.
- Weeks, P. 2000 Red-billed oxpeckers: vampires or tickbirds? *Behav. Ecol.* **11**, 154–160.