

## Regular article

## Cortisol mediates cleaner wrasse switch from cooperation to cheating and tactical deception

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## ARTICLE INFO

## Article history:

Received 16 January 2014

Revised 7 June 2014

Accepted 10 June 2014

Available online 18 June 2014

## Keywords:

Glucocorticoids

Cortisol

Cleaner fish

Cooperative levels

Tactical deception

*Labroides dimidiatus*

## ABSTRACT

Recent empirical research, mostly done on humans, recognizes that individuals' physiological state affects levels of cooperation. An individual's internal state may affect the payoffs of behavioural alternatives, which in turn could influence the decision to either cooperate or to defect. However, little is known about the physiology underlying condition dependent cooperation. Here, we demonstrate that shifts in cortisol levels affect levels of cooperation in wild cleaner wrasse *Labroides dimidiatus*. These cleaners cooperate by removing ectoparasites from visiting 'client' reef fishes but prefer to eat client mucus, which constitutes cheating. We exogenously administered one of three different compounds to adults, that is, (a) cortisol, (b) glucocorticoid receptor antagonist mifepristone RU486 or (c) sham (saline), and observed their cleaning behaviour during the following 45 min. The effects of cortisol match an earlier observational study that first described the existence of "cheating" cleaners: such cleaners provide small clients with more tactile stimulation with their pectoral and pelvic fins, a behaviour that attracts larger clients that are then bitten to obtain mucus. Blocking glucocorticoid receptors led to more tactile stimulation to large clients. As energy demands and associated cortisol concentration level shifts affect cleaner wrasse behavioural patterns, cortisol potentially offers a general mechanism for condition dependent cooperation in vertebrates.

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## Introduction

What conditions make an individual help another unrelated individual, i.e. increase the recipient's direct fitness? Today, a large variety of functional concepts describe conditions and strategies that explain how the helper gains direct fitness benefits as well (Bshary and Bronstein, 2011; Lehmann and Keller, 2006; Leimar and Hammerstein, 2010; Sachs et al., 2004; West et al., 2007). In contrast, explicit studies on the link between physiology and helping behaviour among unrelated individuals are currently rare and are largely restricted to humans. For example, Kosfeld et al. (2005) showed that the application of oxytocin increases trust in humans and hence their tendency to cooperate in situations where cheating by the partner is an obvious risk. Also in humans, lower levels of the neurotransmitter serotonin reduce cooperative play during an Iterated Prisoners Dilemma Game (Wood et al., 2006) while its enhancement seems to contribute to the increased cooperative communication and play during Mixed-Motive Games (Tse and Bond, 2002a, 2002b). Recently, the neuropeptide arginine vasotocin was

implicated in the regulation of cooperative behaviour in a fish cleaning mutualism (Soares et al., 2012), which was a strong indication of the potential role of cortisol as another candidate modulator of cooperative levels and defection. For example, in meerkats, the level of investment of helpers when raising offspring depended on cortisol levels, with higher levels associated with a greater investment (Carlson et al., 2006).

Glucocorticoids (GCs) are a key component of the stress response, which modulates a variety of biological processes that prepare animals for novel, and sometimes extremely challenging, social and environmental shifts (Dallman, 2005; Lupien et al., 2009). GCs coordinate multiple modes of actions, some of which are fast enough (seconds to minutes) to contribute to rapid behavioural adaptation (Tasker et al., 2005, 2006). In humans, rapid central effects of GCs are related to fear detection and consolidation of memories that are linked to strong emotional contexts, which can be negative or positive (Lupien et al., 2007). In non-human models, fast, non-genomic GC actions are known to mediate an increase in locomotion, food intake, ingestion of carbohydrates, vocalization, and aggressive behaviour, while contributing to a decrease in sexual clasping, memory, and adrenocorticotrophic hormone (ACTH) secretion (Dallman, 2005). Furthermore, changes in baseline glucocorticoids (i.e. cortisol) levels are also known to affect

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attention levels and alertness (Chapotot et al., 1998). However, much remains to be discovered when it comes to the contribution of GCs to social decision-making processing in non-human animals.

An ideal model animal to study the effects of manipulating circulating levels of cortisol on cooperative behaviour is the cleaner wrasse *Labroides dimidiatus*. These cleaners provide a service to so-called client fish by removing ectoparasites, but also mucus and scales (Bshary and Côté, 2008; Côté, 2000; Randall, 1958). Male cleaner wrasses are harem holders and most frequently live and clean in pairs, usually with the largest female of his harem, although the other females are regularly visited (Robertson, 1972). A conflict of interest exists between cleaners and clients because the cleaners prefer mucus over ectoparasites, where eating mucus constitutes cheating (Grutter and Bshary, 2003). Clients use various partner control mechanisms to keep cleaner service quality high, including the threat of reciprocity by predatory clients, partner switching, punishment, and even image scoring when acting as bystanders in an interaction (Bshary, 2011; Pinto et al., 2011). As a consequence of clients exerting partner control, cleaners have to decide in each interaction how frequently they dare to eat their client fish's mucus, despite the risk of negative client responses. Interestingly, cleaners may vary in how they respond to this trade-off. Bshary (2002a) provided a first description of two very different cleaner behavioural strategies, which are not fixed (Bshary and D'Souza, 2005): the majority of 'normal' cleaners show low interest in small clients and rarely cheat larger clients, while a small minority of 'biting' cleaners cheat large non-predatory clients with approximately six times higher frequency (Bshary, 2002a). Interestingly, such biting cleaners seek small clients and mainly provide them with tactile stimulation (with their pectoral and pelvic fins) (Bshary, 2002a). Tactile stimulation lowers basal and acute cortisol levels in clients (Soares et al., 2011), and cleaners normally use it to build relationships with new clients, to reconcile after having cheated and also as a pre-conflict management strategy with predators (Bshary, 2002b; Bshary and Würth, 2001; Grutter, 2004). Because clients arriving at a cleaning station are most likely to invite inspection if they witness an ongoing interaction without conflict (Bshary, 2002a; Bshary and Grutter, 2006; Pinto et al., 2011), providing regularly tactile stimulation to small clients will attract any potential observer (Bshary, 2002a). Thus, large clients that happen to observe such an interaction are deceived by biting cleaners due to a signal out of its typical context (Bshary, 2002a): clients rely on false information to invite for inspection and are then cheated. 'Biting' individuals were invariably females, and biting was typically documented during the spawning season (Bshary and D'Souza, 2005).

A change in reproductive status is typically accompanied by a rise in GC concentration, which implies an increase in the costs of maintaining homeostasis, e.g. an increase in allostatic load (Goymann and Wingfield, 2004). Shifts in social status are also known to have a profound influence on animals' allostatic load (Abbott et al., 2003; Creel, 2001; Goymann and Wingfield, 2004). Cleaner wrasses are protogynous hermaphrodites, i.e. individuals first reproduce as females and eventually change sex into males that control a harem of females (Robertson, 1972). It is thus conceivable that female cleaner wrasse, first, should experience a rise in energetic demands during reproduction, and second, this may be enhanced by selection pressure on fast growth in order to become a male and achieve a relatively higher reproductive output (Robertson, 1972; Sakai et al., 2001). This rise in allostatic load should be related to an elevation of female GCs levels, which might play a role in the decision to switch (even if only temporarily) to become a 'biting' cleaner. This would occur under the assumption that the biting strategy increases current energy uptake via the ingestion of higher amounts of client energy-rich mucus (which cleaners prefer when compared with ectoparasites; Grutter and Bshary, 2003). In addition, it would occur at the expense of future benefits because visiting clients are known to respond to a poor service by switching to different stations for their next inspection (Bshary and Schäffer, 2002; Soares et al., 2013).

The role of stress-related mechanisms on the modulation of cleaner fish levels of cooperation remains little understood. Cleaner wrasses' ability to switch between behavioural tactics revealed the existence of a conditional strategy (Bshary, 2002a); however, the underlying physiological mechanisms are unclear. Here, we aimed to discover the potential role of changes in cortisol levels and the ability of cortisol signalling pathways to operate on the social decision-making process of the cleaner wrasse. We conducted our study in natural conditions to determine whether exogenous administration of GCs (cortisol and the GC receptor antagonist mifepristone RU486) would produce variations in their degree of cooperation (tactile stimulation and cheating, the latter measured as client "jolts" in response to a feeding bite; Bshary and Grutter, 2002; Soares et al., 2008) when dealing with interspecific partners. Because the data from Bshary (2001) suggest that changes in service quality may vary according to a client's value as a food patch, we recorded client size as a correlate of this value (Bshary, 2001; Grutter, 1994).

## Methods

### Field methods

Field experiments were carried out on 10 different reefs around Lizard Island (Lizard Island Research Station, Australia, 14° 40'S, 145° 28'E) between August and September 2011, in which 24 female cleaner fish were tested. Larval settlement of *L. dimidiatus* at these reefs mostly occurs in November and December (larvae settle about 3 weeks after hatching, Brothers et al., 1983), which indicates that cleaner spawning occurs between October and December (Grutter, 2012; Waldie et al., 2011) and that our field experiments therefore occurred in a "non-spawning" season. We thus assumed that all sampled females were "normal" cleaners. All manipulations and observations were made by two SCUBA divers, between 10:00 and 16:00 h. Cleaner fish were selected haphazardly across the reefs and cleaning stations varied in depth between 1.5 and 12 m. Individuals were captured using a barrier net and measured to the nearest mm (TL—total length). TL of the fish ranged from 6.0 to 8.7 cm. Body weight was then estimated from a length–weight regression (Soares et al., 2012). We then gave the focal female an intra-muscular injection of one of three compounds: (a) hydrocortisone ("cortisol"), dosage 1 µg per gram of body mass (gbm, Sigma, H4001), (b) GC receptor antagonist Mifepristone RU486, dosage 3 µg per gbm (Sigma, M8046) and (c) saline (0.9 NaCl). The steroids were first dissolved in 50 µl of ethanol and only then were the solutions made with saline (and left overnight to complete ethanol evaporation). The control solution used (saline) was also prepared with an equivalent amount of ethanol as the treatment groups. Injection volumes ranged from 20 to 50 µl. Fish handling never exceeded 3 min. Once an individual was released, it was then videotaped for the next 45 min, using video cameras in waterproof cases (Sony HDR-XR155). The order of the treatments was randomized for each dive and all treatments used different cleaner fish. Because this study was done exclusively in field conditions with limitations of time and number of fish used (collecting permit allowance), and because the removal of blood would equate to animal death, dosages chosen were based on previous studies (DiBattista et al., 2005; Remage-Healey and Bass, 2004) and not through dosage effect tests.

### Behavioural data collection

Video recordings were made from a distance of 2–3 m. During each video analysis, we recorded the following measures: (a) family identification and TL of each client (estimated visually to the nearest cm, using the focal cleaner fish's size estimation as proxy) visiting the cleaning station; (b) the number of tactile stimulations provided

(where a cleaner touches, with ventral body and fins, the body of the client and no feeding is involved) and (c) the number of jolts by clients and the client's reaction following each jolt.

### Ethical Note

No fish suffered any detectable injury or mortality as a result of the injections or behavioural testing. The methods for animal handling and experimental protocols were first assessed and approved by the by the Portuguese Veterinary Office (Direcção Geral de Veterinária, license no. 0420/000/000/2009) and then by The University of Queensland Animal Ethics Committee (permit SBS/130/11/FCT).

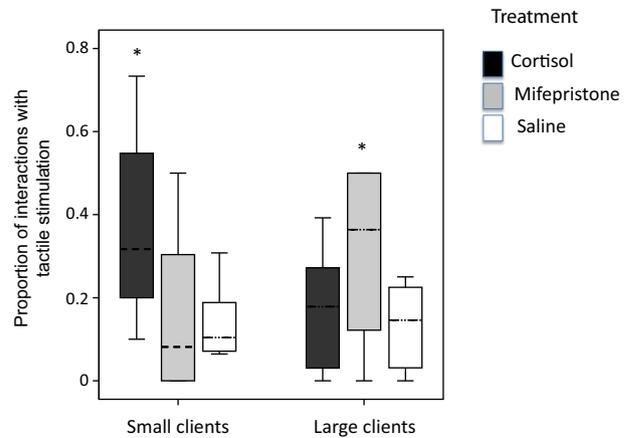
### Statistical analysis

All cleaner fish were haphazardly selected and were independent measures. Regarding the clients, we could not apply the distinction by Bshary (2002a) between 'resident species' with access to only one cleaning station and 'visitor species' with access to several cleaning stations. This distinction is easily observable in the patch reef habitat used by Bshary (2002a) but not on fringing reefs (Oates et al., 2010), like the ones we used, where a species' size may allow some rough correlation with the number of cleaning stations accessible. Because Bshary (2002a) found that client size affected cheating rates by biting cleaners quite independently of client category (resident or visitor), we distinguished two size categories for our video analyses according to their TL estimate: small clients (<14 cm) and large clients ( $\geq 14$  to 45 cm). In the smaller clients' category, the majority of individuals belonged to the following families: Pomacentridae (damselfishes), Chaetodontidae (butterflyfishes) and Labridae (wrasses and parrotfishes); while the larger clients mostly belonged to: Acanthuridae (surgeonfishes), Mullidae (goatfishes) and Caesionidae (fusiliers). Client size is usually considered to be a good correlate of cleaner fish food value as it has been demonstrated to be a reliable indicator of parasite numbers and of nutritious mucus quantity (Arnal and Morand, 2001; Grutter, 1995). Interspecific cleaner fish behaviour towards clientele was measured with two different behavioural categories: (a) tactile stimulation provided, measured by the proportion of interactions in which tactile stimulation was used by cleaners, and (b) frequency of jolts per 100 s of inspection—jolts are whole-body shudders, in response to cleaner fish mouth contact that are a good correlate of cheating by cleaners, i.e. eating mucus (Bshary and Grutter, 2002; Soares et al., 2008). Data were analysed using non-parametric tests because the assumptions for parametric testing were not met. We therefore compared each hormonal treatment with the control (saline) group by using Mann–Whitney  $U$  tests, as is recommended for a small set of planned comparisons (Ruxton and Beauchamp, 2008). Bonferroni correction was applied to account for multiple testing, thus reducing the significance level to  $\alpha = 0.025$ .

## Results

### Effect of glucocorticoids on tactile stimulation of clients

Compared with the saline control, cleaners treated with cortisol provided a higher proportion of tactile stimulation to small clients (cortisol vs saline:  $U = 9$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.02$ ; Fig. 1), but there was no significant effect when cleaners interacted with large clients (cortisol vs saline:  $U = 23.5$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.37$ ; Fig. 1). The effects of the GC receptor antagonist mifepristone were different to those found with cortisol: no significant effect was found when cleaners interacted with small clients (mifepristone vs saline:  $U = 22.5$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.32$ ; Fig. 1), but a higher proportion of tactile stimulation was provided to large clients (mifepristone vs saline:  $U = 10.5$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.02$ ; Fig. 1).



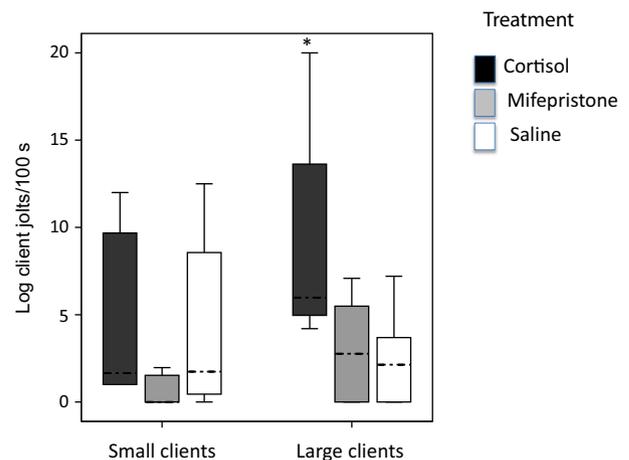
**Fig. 1.** The effect of glucocorticoids on the proportion of tactile stimulation provided by cleaner fish *Labroides dimidiatus* to client fish, compared with a control (saline) for clients of two different size categories. Medians (dashed lines) and interquartile range are presented in boxes. \* above bars represent significant  $P$  values which refer to Mann–Whitney  $U$  tests for each glucocorticoid treatment against the reference (saline) group (\*,  $<0.025$ ). Sample sizes (number of individual cleaner fish) are  $n = 8$  for all groups of treatments.

### Effect of glucocorticoids on jolt frequencies of clients

Compared with saline, large clients jolted more often when attended by cleaners that were treated with cortisol (cortisol vs saline:  $U = 5$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.01$ ; Fig. 2), while no difference was found with those treated with mifepristone ( $U = 30.5$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.87$ ; Fig. 2). For small clients no significant effects of treatment were found (cortisol vs saline:  $U = 28$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.67$ ; mifepristone vs saline:  $U = 13$ ,  $n_1 = 8$ ,  $n_2 = 8$ ,  $p = 0.04$ ; Fig. 2).

## Discussion

Our findings show that changes in cortisol levels and in cortisol signalling (via GC receptor antagonism) are associated with significant behavioural changes in the cleaning service provided by cleaners to their clients. When compared with a saline control, cleaners treated with cortisol provided a greater proportion of tactile stimulation to small clients, indicating they were more cooperative towards them. This same



**Fig. 2.** The effect of glucocorticoids on the dishonesty level (jolts) of cleaner fish *Labroides dimidiatus*, measured in the field and compared with a control (saline), using the frequency of jolts per 100 s of inspection for clients of two different size categories. See Fig. 1 for boxplot definitions. Symbols above bars and sample sizes per treatment were the same as in Fig. 1.

treatment also resulted in cleaners causing more jolts to large clients, indicating that these cleaners were more dishonest. In contrast, when GC receptor signalling was blocked, cleaners provided a greater proportion of tactile stimulation to large clients, indicating they were more cooperative towards them. These results coincide with an earlier field observational study that revealed that cleaner wrasses can be categorised into 'biting' (cleaner wrasse's that cheat more often as expressed by client jolts) and 'normal' cleaner wrasse (Bshary, 2002a). 'Biting' ones behave more cooperatively with small resident clients, which apparently allow them to gain access to large visiting clients that ultimately were cheated more frequently. Since visiting clients visually assess the cleaner wrasse's behaviour in ongoing interactions and base their decision to invite inspection on this (i.e. image scoring; Nowak and Sigmund, 1998), that explained why cooperative interactions with small clients may help to attract large clients (Bshary, 2002a). Our results provide a first physiological mechanism to explain this behavioural dichotomy and hence cleaner wrasse's cooperative flexibility: the increased cortisol levels facilitate expression of "biting" in cleaners.

Animals respond to stressors with a series of endocrine and neural changes, which mobilize energy by inhibiting other physiological processes that are not necessary for immediate survival (Creel, 2001). One of the primary responses to stress, via activation of the hypothalamic–pituitary–adrenal (HPA) axis for mammals or hypothalamic–pituitary–interrenal (HPI) axis for teleost fish, is one that is responsible for an increase of available circulating GCs (Creel et al., 2013). Changes in social status for example may predict GCs levels expressed. For instance, in many cooperative breeder species, dominant animals experience elevated GCs, which have associated costs, but this is usually accompanied by higher access to mates or resources (Creel, 2001). Moreover, the rise in metabolic demands (and as a consequence, increased GC concentration) is associated with several other social conditions such as parenting and lactation, or partnership instability (Goymann et al., 2001). Our findings suggest that higher levels of circulating cortisol are associated with an alternative behavioural strategy ("biting" cleaners, Bshary, 2002a), which should occur in response to an increase in energetic requirements. The existence of this behavioural strategy by cleaners in the wild might thus reflect a change in reproductive effort, growth effort, or possibly a rise of intra-couple (male–female) competition that contextualize females' change in social rank. For example, Sakai et al. (2001) observed that females often move to other harems in which they succeed to increase in rank status and thus shorten the time it would take to become a dominant male. GC level variations might help prepare females for these life-history changes, which in turn may affect behavioural strategies, including levels of cooperation.

Cleaner wrasses are known to adjust their foraging strategic options in accordance with how clients react to their cheating behaviour (Bshary, 2011). For example, predatory clients typically receive the highest service quality, whereas non-predatory clients punish or switch cleaners to control cleaners' cheating. Furthermore, because clients arriving at a cleaning station observe any ongoing interaction and avoid cheating cleaners, the cleaners behave more cooperatively with current clients in the presence of bystanders (Bshary and Grutter, 2006; Pinto et al., 2011). However, while client control mechanisms typically lead to high levels of cooperation by cleaners, a small minority of cleaner females switch temporarily to biting, apparently during spawning episodes (Bshary, 2002a; Bshary and D'Souza, 2005). These biting cleaners primarily cheat large clients but increase the amount of tactile stimulation given to small clients (Bshary, 2002a). Apparently, the tactile stimulation to small clients serves to attract potential bystander clients that will then be exploited, as in a global analysis of strings of interactions, cheating systematically occurred in interactions directly after those consisting of tactile stimulation (Bshary, 2002a). In the current study, we could replicate the behavioural changes—tactile stimulation to small clients and biting of large clients—by injecting cortisol. We can therefore suggest that the

increase in metabolic demands, either during changes in reproductive or social status, which triggers cortisol release, may influence female cleaner wrasse's need for a different behavioural strategy.

If changes in reproductive and social status have a key effect on allostatic load (and GC concentration variations), other social conditions such as social support and social stability within cooperative groups or pairs may, on the other hand, reduce the scope for conflict and competition (which will be reflected in reduced GC concentrations). For instance, in baboons, social support is thought to function as a buffer for variation in GCs levels (Virgin and Sapolsky, 1997), while lower stress levels exhibited by low ranking males ultimately contribute to the maintenance of higher affiliative relationships (such as non-sexual bouts of reciprocal grooming, (Smuts, 1999; Virgin and Sapolsky, 1997). In our system, cleaners treated with the GC antagonist provided more tactile stimulation (in almost half of those interactions) to high value (large) clients. Cleaners' behavioural shift caused by an inhibition of GC receptor signalling at a cellular level, therefore, becomes a positive improvement that may lead to the reinforcement of current and future relationships aimed specifically to high value clients. Better cleaning services should translate into a higher likelihood that these clients would revisit the same cleaner (Bshary and Schäffer, 2002). However, the shift in behaviour elicited by antagonism of the GC receptors may further contribute to the relationship stability of cleaner wrasse pairs. Female cleaner wrasses are more often punished when they are closer in size to males (this is correlated with an increase in competition levels via a change in social rank) and when cheating high value clients (Raihani et al., 2012). By providing more tactile stimulation to large clients, these females may be directly contributing to a decrease of intra-couple conflict levels.

Future research may also focus on the potential organizational effects of GCs on the early development of cleaner wrasses. This may lead to consistent individual differences with respect to their behavioural flexibility, such as in further activation effects of GCs in 'subordinate' female cooperative levels. Because energy demands and cortisol levels may associate with changes in cleaner wrasse behavioural patterns, cortisol could potentially offer a general mechanism for condition dependent cooperation in vertebrates.

### Funding statement

This study was funded by Fundação para a Ciência e Tecnologia (grant PTDC/MAR/105276/2008 to MCS) and by the Swiss National Foundation (grant given to RB). RFO was funded by FCT strategic project PEst-OE/MAR/UI0331/2011. MCS is supported by the Project "Genomics and Evolutionary Biology", co-financed by North Portugal Regional Operational Programme 2007/2013 (ON.2–O Novo Norte), under the National Strategic Reference Framework (NSRF), through the European Regional Development Fund (ERDF).

### Author contributions

MCS designed the study. MCS and SCC collected the data. MSC, SCC, ASG, RFO and RB analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

### Acknowledgments

We thank the Lizard Island Research Station (Directors and staff) for their support and friendship and Dr. Albert Ros for necessary guidance during the planning of experiments and ordering of chemicals.

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