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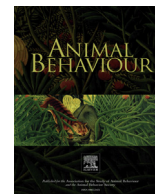
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GABAergic role in the disruption of wild cleaner fish behaviour under high CO₂

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Ocean acidification is considered to affect fish behaviour through the disruption of GABAergic neurotransmission in controlled laboratory conditions, but less is known of the GABAergic role on fish behavioural performance in the wild. Most coral reef fishes engage in complex cleaning interactions, where they benefit from ectoparasite removal and stress relief. Here, we tested whether potential ocean acidification impairment of wild cleaning interactions, between the cleaner fish *Labroides dimidiatus* and its clients, can be explained by the GABA_AR model. We used, the GABA_A receptor agonist (muscimol) and antagonist (gabazine) for the first time in the wild and tested their effects on cleaning behaviour in Moorea Island (French Polynesia) to address natural interactions and recovery capacity. After exposure to expected ocean acidification conditions, the proportion of time spent advertising cleaning services, a measure of motivation to interact, dropped significantly relative to controls. Furthermore, the GABAergic antagonist gabazine recovered most CO₂-induced behavioural alterations to control levels, consistent with the GABA_AR model of altered Cl⁻ flux in ocean acidification-exposed fish. However, muscimol treatment only produced the same behavioural alterations found with CO₂ exposure in time spent advertising cleaning. Our results support the evidence that ocean acidification alters some components of cleaning behaviour through GABA_A receptor modulation with potential cascading effects on coral reef health and structure.

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Climate change and its associated stressors are known to impact the marine biosphere (Pörtner et al., 2014), but the impacts on the behaviour of interacting species as well as the proximate mechanisms governing them are less well known. The oceans absorb approximately 30% of the CO₂ emitted into the atmosphere, changing sea water chemistry and reducing sea water pH, a process known as ocean acidification (Dupont & Pörtner, 2013). These changes in ocean CO₂ partial pressure (pCO₂) are known to impact multiple taxa, including invertebrates and fish (Cattano et al., 2018; Melzner et al., 2020; Rosa et al., 2017). Moreover, previous studies

identified that ocean acidification could directly impact fish behaviour (Goldenberg et al., 2018; Munday et al., 2019; Paula, Repolho, et al., 2019), cognition (Ferrari et al., 2014; Paula, Baptista, et al., 2019) and disrupt several sensory mechanisms such as hearing, vision and olfaction (Chung et al., 2014; Porteus et al., 2018; Rossi et al., 2016). These behavioural effects were observed after a period of high CO₂ exposure, and some were observed even when fish were subsequently tested in control water (Munday et al., 2016). However, other studies documented little or no effect of ocean acidification on fish behaviour (Clark et al., 2020; Raby et al., 2018; Sundin et al., 2017), suggesting variability in fish behavioural responses to ocean acidification. However, most of these past studies were performed under laboratory conditions with captive-reared animals and data collected in the wild are still

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mostly lacking. Thus, to understand variability in behaviour, it is important to study the mechanistic drivers of the different responses using wild animals.

One of the suggested mechanisms for behavioural disruption under ocean acidification is the interference of CO₂ (and subsequently low pH) on GABAergic neurotransmission (Hamilton et al., 2014; Nilsson et al., 2012). Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain (20–50% of central nervous system synapses are GABAergic) and mediates inhibition of neuronal pathways via GABA_A receptors (Paredes & Ågmo, 1992). Exposure to ocean acidification in the water results in a proportional increase in CO₂ in the plasma (known as hypercapnia). Fishes restore their acid–base balance by increasing H⁺ excretion and accumulating HCO₃⁻. The increased [HCO₃⁻] in plasma is associated with a decreased chloride concentration [Cl⁻] and these changes in [Cl⁻] exchange in GABA_A receptors are believed to be the primary cause of behavioural disruption (Hamilton et al., 2014; Nilsson et al., 2012; Tresguerres & Hamilton, 2017). Under normal conditions, Cl⁻ has a higher concentration in the neuron extracellular medium relative to the intracellular. When GABA (or agonistic drugs such as muscimol) binds to a GABA_A receptor, the gate receptors open, Cl⁻ moves from the extracellular medium into the neuron and hyperpolarizes the membrane, having an inhibitory function on the neuronal pathway. This reduces neuronal activity and subsequently inhibits behaviours, such as movement and activity (Paredes & Ågmo, 1992). However, under ocean acidification, since the equilibrium potential for Cl⁻ is affected by a decrease in extracellular medium [Cl⁻] (due to the increased H⁺ excretion to counteract acidosis), the binding of GABA (or muscimol) now leads to a net Cl⁻ movement out of the neuron into the extracellular medium, causing membrane depolarization which results in an excitatory function and causes behavioural disruption (Hamilton et al., 2014; Heuer & Grosell, 2014; Tresguerres & Hamilton, 2017). Molecular evidence also supports the GABAergic hypothesis. Quantitative PCR studies revealed a CO₂-induced upregulation of GABA_A receptor alpha-subunits (Lai et al., 2017) and whole-brain transcriptomes showed a generalized upregulation of genes involved in GABA_A function under high CO₂ (Schunter et al., 2018). Both studies suggest that fishes overexpress GABAergic machinery to increase inhibitory signalling, offsetting their neurobiological system's overexcitation. However, since high CO₂ leads to the reversal of function of GABA_A receptors in the direction of neural excitation, overexpression of GABAergic systems will further increase overexcitation, thus suggesting the presence of a CO₂-induced positive feedback loop (Schunter et al., 2019). However, this hypothesis has only been tested in laboratory studies and needs to be examined in the wild.

Cleaning symbioses are crucial for the functioning of coral reef ecosystems (Côté, 2000), as the presence of cleaner fishes (e.g. the bluestreak cleaner wrasse, *Labroides dimidiatus*) not only impacts client ectoparasite loads but can also drive fish diversity, recruitment, growth and abundance (Cheney & Côté, 2001; Clague et al., 2011; Grutter, 1999; Grutter et al., 2018; Waldie et al., 2011). During a cleaning interaction, cleaners remove ectoparasites and dead skin from their clients and contribute to their stress relief and cognitive performance (Binning et al., 2018; Grutter, 1996, 1999; Ros et al., 2011; Soares et al., 2011). However, as some cleaners prefer to eat mucus from their clients rather than cleaning them (a behaviour known as cheating or dishonesty), a conflict arises between cleaners and clients, the latter usually responding with jolts and chases (Bshary, 2011; Bshary & Bronstein, 2004; Bshary & Grutter, 2002; Côté & Mills, 2020; Grutter & Bshary, 2003; Mills & Côté, 2010). Simultaneously, cleaners use reconciliation strategies to increase the odds of future interactions with clients, for example by providing tactile stimulation while cleaning (Bshary &

Grutter, 2005; Oates et al., 2010). Environmental disturbances are known to impact the cleaner–client symbiosis, by reducing cleaner cooperation and client retaliation in response to motorboat noise (Nedelec et al., 2017), decreasing cleaners' strategic sophistication (the ability to adjust one's behaviour flexibly given the specifics of a situation) after cyclones and bleaching events (Triki et al., 2018), or disrupting their cleaning motivation and interaction quality under laboratory acclimation to ocean warming and acidification (Paula, Repolho, et al., 2019).

To our knowledge, the potential effects of ocean acidification on cleaning interactions have only been investigated under laboratory conditions and with the bluestreak cleaner wrasse responding to only one client, the surgeon fish, *Naso elegans* (Paula, Repolho, et al., 2019). Here, we address this research gap by evaluating the effects of ocean acidification on cleaning interactions in the wild and determine the role of GABAergic neurotransmission on behavioural disruption, using an agonist (muscimol) and an antagonist (gabazine) of GABA_A receptors, in natural cleaning interactions after exposure to ocean acidification (ca. 1000 µatm; high CO₂). More specifically, at the behavioural level, we analysed both cleaner fish motivation to interact (e.g. the proportion of clients inspected and the proportion of interactions started by cleaners) and the quality of these interactions (e.g. mean interaction duration, client jolts and tactile stimulation) as in Paula, Repolho, et al. (2019). We predicted that (1) ocean acidification will disrupt cleaner fish behaviours (as in Paula, Repolho, et al., 2019), and (2) if the GABA_AR model is the mechanism by which ocean acidification impacts behaviour, gabazine, as an antagonist of GABA_A receptors, will likely reverse the disruption of ocean acidification on fish behaviour to levels similar to those found under control CO₂ levels. No study has addressed the effect of GABAergic manipulation on cleaning behaviour. Nevertheless, due to its anxiogenic nature, (3) we predicted that, under control conditions, gabazine might decrease motivation to interact with other fishes. Furthermore, as a second test to show that GABAergic neurotransmission drives behavioural disruption under ocean acidification, (4) we predicted that muscimol, as an agonist of GABA_A receptors, under control CO₂ levels should inhibit cleaner fish motivation to interact to similar levels to that found under ocean acidification conditions, whereas under ocean acidification, muscimol should either increase its behavioural effects or have no consequences (if the behavioural response is already at maximum levels).

METHODS

Ethical Note

Animal experimentation met the ASAB/ABS guidelines for ethical treatment of animals. The experiments were conducted under the approval of Faculdade de Ciências da Universidade de Lisboa animal welfare body (ORBEA-FCUL) and Portuguese Direção-Geral de Alimentação e Veterinária (DGAV – Permit 2018-05-23-010275) following the requirements imposed by the Directive 2010/63/EU.

Collection and Acclimation

We used bluestreak cleaner wrasses (females only, so that we had fish of similar size, as males are larger than females in this species; $N = 62$, mean \pm SE or SD standard length = 5.5 ± 0.5 cm) captured using barrier and hand nets while SCUBA diving in the reefs around Moorea, French Polynesia ($17^{\circ}32' 19''S$, $149^{\circ}49'46''W$), between October and November 2017. Cleaner wrasses were transported by boat to CRIOBE, Opunohu Bay, Moorea. The boat transport never took longer than 30 min and the cleaner wrasses

were kept in thermally controlled containers filled with sea water with an air stone connected to an air pump to provide sufficient aeration. No mortality was observed during transport and all fish were healthy on arrival. Cleaner fish were then placed in individual aquaria (25 × 25 cm and 20 cm high; approximately 12.5 litres of water, with bubbling air stones to ensure maintenance of oxygen levels close to air saturation) in flow-through systems. These systems were maintained outdoors and protected by an opaque roof to maintain natural daylight cycles. Fish were fed *ad libitum* with mashed prawn (acquired frozen and whole from the local supermarket). A 10 cm PVC pipe was provided as shelter for each fish. Fish were laboratory acclimated for 5 days in natural sea water conditions (pumped from Opunohu Bay; temperature = 28.7 ± 0.3 °C, pH = 8.05 ± 0.01, salinity = 35 ppt).

CO₂ Treatment

Each fish was then exposed to one of two CO₂ treatments for 15 days: control scenario (pH = 8.05 ± 0.01; pCO₂ ca. 400 µatm) or high CO₂ scenario (pH = 7.70 ± 0.02; pCO₂ ca. 1000 µatm, according to the 2100 projection of the IPCC RCP 8.5 scenario (Stocker et al., 2014)). The exposure time was in line with previous studies and did not cause any mortality (Hamilton et al., 2014; Munday et al., 2016; Nilsson et al., 2012). Each aquarium was part of a flow-through system where sand-filtered water directly pumped from Opunohu Bay fed six upper mixing tanks (approximately 50 × 40 cm and 40 cm high; approximately 80 litres of water each), and where CO₂ or air and water were mixed according to the treatment. The conditioned water was drained (gravity fed) to each aquarium (each mixing tank fed 8–12 aquaria). The pH levels were automatically adjusted every 2 s by direct injection of CO₂ gas (Profilux 3.1N, GLH, Rheinland-Pfalz, Germany). We used handheld equipment to complement the automatic systems with manual daily monitoring of sea water temperature (Hanna TempCheck, Hanna Instruments, Póvoa de Varzim, Portugal), salinity (V2 refractometer, TMC Iberia, S. João do Tojal, Portugal) and pH (YSI Professional Plus, Handheld Multiparameter Instrument, Yellow Springs, OH, U.S.A.). We quantified pH (in Profilux 3.1N, GLH, Rheinland-Pfalz, Germany) using pH probes connected to glass electrodes (VWR pH DJ 113, Allentown, PA, U.S.A.), calibrated with TRIS-HCl (TRIS) and 2-aminopyridine-HCl (AMP) sea water buffers. The sea water carbonate system was calculated twice a week from total alkalinity (Titration – Hach Digital Titrator) and pH measurements (Sarazin et al., 1999). The pCO₂ and bicarbonate values were calculated using CO2SYS (<https://www.ncei.noaa.gov/access/ocean-carbon-acidification-data-system/oceans/CO2SYS/co2rprt.html>). Seawater parameters of experimental set-ups are summarized in Appendix Table A1.

GABAergic Manipulation

GABA_A receptor antagonist (gabazine, 4 mg/litre, 10.9 mM, Sigma-Aldrich SR-95531 S106, Sigma-Aldrich, St Louis, MI, U.S.A.) and agonist (muscimol, 1 mg/litre, 8.8 mM, Sigma-Aldrich M1523) were dissolved in 500 ml of sea water (with pCO₂ according to the treatment). These concentrations followed previous studies in marine fish and never resulted in mortality (Hamilton et al., 2014; Nilsson et al., 2012). Gentle stirring and a vortex were used to dissolve the compounds. As a control, 500 ml of sea water alone was used (sham treatment).

Behavioural Observations

Since previous studies have shown that ocean acidification effects can be tested in control water (Munday et al., 2016), we tested

cleaner fish behavioural responses outside their CO₂ treatment water, i.e. in their natural habitat. During each test, two fish (one per diver) were taken in their aquaria, covered with a lid, by boat (transport was <30 min), to their capture site. Upon arrival, fish were placed in 500 ml individual vials sealed with a tight lid, containing treatment solutions: sham (sea water), gabazine or muscimol for 30 min (Hamilton et al., 2014). Fish were taken in their vial to their release site (same as collection site) by a diver just before the end of the exposure and released after 30 min of treatment exposure. Divers retreated immediately after release and cleaner fish behaviour was recorded for 30 min from a distance of 2–3 m with an underwater-housed digital camera (Nikon Coolpix or Canon G7 Mark II). Fish were not recaptured after release. Cleaning behaviour was grouped into two categories: cleaner fish motivation to engage in cleaning interactions and interaction quality, according to Paula et al. (2015). To characterize 'cleaner fish motivation to interact', we measured the proportion of (1) clients inspected (number of clients cleaned/total number of visits), (2) interactions initiated by cleaners and (3) time advertising a cleaning service (time performing advertising dance/time, not in interaction; cleaners perform advertising dance to attract potential clients; Grutter, 2004). Interaction quality was determined considering (1) the proportion of interactions with client jolts (conspicuous signals that indicate cheating; Soares et al., 2008), (2) the proportion of interactions with tactile stimulation (tactile stimulation of clients with cleaners' pectoral fins which reduces stress levels and can prolong interaction duration; Grutter, 2004; Soares et al., 2011) and (3) mean interaction duration. We excluded the first 5 min from the video analysis to account for cleaner fishes' behavioural acclimation after release (Nanninga et al., 2017). All behavioural videos were analysed by a blind observer (L.C.), who was absent for the entirety of the experiment at Moorea, using the event-logging software 'Boris' (Friard & Gamba, 2016). For six videos, an interobserver analysis test was performed by a second observer (R.O.).

Statistical Analysis

Data exploration was performed according to the protocol given in Zuur et al. (2010). From the initial 62 individuals, we excluded five from the analysis (final $N = 57$): one was eaten by a predator lizard fish during the behavioural observations, three were away from the camera more than 50% of the observational time (average 3%) and one spent 44% (average 1.7%) of the time fighting with another cleaner (so no cleaning interactions could be observed). This resulted in the following sample size: CO₂ treatment: 29 control and 28 high CO₂; GABAergic treatment: 21 sham, 18 gabazine and 18 muscimol. This resulted in 10 individuals per combined CO₂ and GABAergic treatment, except for high CO₂ with gabazine and control with muscimol where the sample size was eight individuals.

Interobserver reliability was analysed using a linear model with the observer as categorical fixed factor. No differences between observers were detected (number of interactions: $\chi^2_1 = 0.002$, $P = 0.957$). We analysed behavioural data using generalized linear mixed-effect models (GLMM). These models used CO₂ treatment (factor with two levels: control and high CO₂) and GABAergic treatment (factor with three levels: sham, gabazine and muscimol) as categorical fixed factors and acclimation system as a random factor according to Zuur and Ieno (2016). In these models, the Gaussian distribution was used for continuous data (mean interaction duration) and the negative binomial distribution for proportions (the proportion of clients inspected, the proportion of interactions started by cleaners, the proportion of time advertising cleaning services, the proportion of interactions with tactile

stimulation and the proportion of interactions with client jolts). Regarding the proportion of time spent advertising cleaning service, initial data exploration using Cleveland dot-plots, according to [Zuur et al. \(2010\)](#), showed the presence of an outlier (value more than 17 times higher than the upper quartile, see Cleveland dot-plot in [Fig. A1](#)). Thus, this value was not included in the model (although it is still included in the raw data provided). The full models, with all possible interactions, were tested using the function 'glmmTMB' from the package 'glmmTMB' ([Brooks et al., 2017](#)) and the function 'Anova' from the package 'car' ([Fox & Weisberg, 2011](#)) in R, version 3.4.3 ([R Core Team, 2017](#)). Post hoc multiple comparisons were performed using the package 'emmeans' ([Lenth, 2020](#)) with Tukey corrections. Model assumptions and performance were validated using the package 'performance' ([Lüdecke et al., 2021](#)). Data exploration used the HighstatLibV10 R library from Highland Statistics ([Zuur et al., 2009](#)).

RESULTS

Cleaner Fish Motivation to Engage in Cleaning Interactions

The proportion of clients inspected was not significantly affected by the interaction between GABAergic treatment and CO₂ treatment or CO₂ alone, but it was significantly altered by the GABAergic treatment ([Table 1, Fig. 1a](#)). Post hoc pairwise comparisons revealed that the proportion of clients inspected increased significantly with gabazine compared to sham under high CO₂, but not with muscimol or in control conditions ([Table 2](#)).

The proportion of interactions started by cleaners was significantly affected by the interaction between GABAergic treatment and CO₂ treatment ([Table 1, Fig. 1b](#)). Post hoc pairwise comparisons revealed that the proportion of interactions started by cleaners was only significantly lower with gabazine in high CO₂ compared to sham in control CO₂ ([Table 2](#)).

Regarding the proportion of time spent advertising their cleaning service, there was a significant interaction between CO₂ and GABAergic treatment ([Table 1, Fig. 1c](#)). Post hoc pairwise comparisons revealed CO₂ significantly decreased cleaning advertisement in all GABAergic treatments except muscimol ([Table 2](#)). Moreover, while no effect was observed between gabazine and sham under control conditions, muscimol significantly decreased advertisement compared to sham ([Table 2](#)).

Interaction Quality

Within cleaning interaction quality measures, the proportion of interactions with client jolts was not altered by the interaction between GABAergic treatment and CO₂ treatment, CO₂ or GABAergic treatment ([Table 1, Fig. 1d](#)).

There was a significant interaction between CO₂ and GABAergic treatment on the proportion of interactions with tactile stimulation ([Table 1, Fig. 1e](#)). Here, post hoc pairwise comparisons revealed that after gabazine administration CO₂ significantly increased tactile

stimulation ([Table 2](#)). Gabazine administration under high CO₂ also led to significantly more tactile stimulation compared to sham under control conditions and to muscimol under high CO₂ ([Table 2](#)).

Lastly, the mean interaction duration was not altered by the interaction between GABAergic treatment, CO₂ or GABAergic treatment ([Table 1, Fig. 1f](#)).

DISCUSSION

The present study shows, for the first time, that high CO₂ also alters cleaning interactions of populations in the wild. In agreement with our first hypothesis, cleaner wrasses subjected to higher concentrations of CO₂ ($p\text{CO}_2$ ca. 1000 μatm) showed less motivation to interact with clients (reduced time spent advertising cleaning services, [Fig. 1c](#)) and slightly lowered the use of reconciliation strategies after cheating, although not significantly (tactile stimulation, [Fig. 1e](#)). In agreement with the GABA_AR model being a mechanism by which ocean acidification impacts fish behaviour, the GABAergic antagonist gabazine partly recovered CO₂-induced behavioural alterations closer to control levels including motivation to interact with clients (i.e. proportion of time spent advertising cleaning services, [Fig. 1c](#)) and the use of reconciliation strategies after cheating (i.e. proportion of interactions with tactile stimulation, [Fig. 1e](#)). In further agreement with this proposed mechanism, muscimol treatment produced the same behavioural alterations in motivation to interact with clients (time spent advertising cleaning services) in control conditions as found with high CO₂ exposure ([Fig. 1c](#)).

Cleaner wrasses subjected to higher concentrations of CO₂ appeared to be less motivated to interact with clients (i.e. a lower proportion of time advertising cleaning service and a nonsignificant trend of a lower proportion of clients inspected) which is consistent with aquaria-based studies ([Paula, Repolho, et al., 2019](#)). However, in this study, and contrary to [Paula, Repolho, et al. \(2019\)](#), the proportion of interactions started by cleaners did not decrease significantly with CO₂. We argue that this difference between studies is probably due to key differences in cleaner–client interaction networks. While in [Paula, Repolho, et al. \(2019\)](#) both cleaners and clients were deprived of cleaning interactions for 45 days and the cleaners only had access to one individual client (*N. elegans*) during the behavioural trial, here (1) cleaners had access to multiple clients, (2) clients had access to different cleaning stations and (3) only cleaners were deprived of interactions. Thus, in the present study, a better measure of cleaner wrasse motivation is time spent advertising its cleaning services, which, as in [Paula, Repolho, et al. \(2019\)](#), decreased under high CO₂.

Consistent with the GABA_AR model ([Nilsson et al., 2012](#)), our study suggests that, for two of the six behaviours measured, GABA_A receptor modulation is one mechanism by which ocean acidification impairs cleaner wrasse motivation to interact. First, gabazine (GABA antagonist) reversed the motivational drop under ocean acidification as (1) no effect of CO₂ was observed on the proportion of time spent advertising after gabazine administration ([Fig. 1c](#))

Table 1
Analysis of deviance table (type II tests) for cleaner fish behaviour

Predictors	Proportion of clients inspected			Proportion of interactions started by cleaners			Proportion of time advertising			Proportion of interactions with jolts			Proportion of interactions with tactile stimulation			Mean interaction duration		
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
CO ₂ treatment	1	1.672	0.196	1	2.675	0.102	1	152.674	<0.001	1	0.431	0.512	1	0.019	0.890	1	1.432	0.231
GABAergic treatment	2	7.305	0.025	2	2.966	0.227	2	62.761	<0.001	2	0.948	0.622	2	9.750	0.007	2	3.972	0.137
CO ₂ * GABAergic treatment	2	3.699	0.157	2	7.485	0.024	2	84.132	<0.001	2	0.609	0.738	2	13.281	<0.001	2	2.030	0.362

Values of $P < 0.05$ are shown in bold.

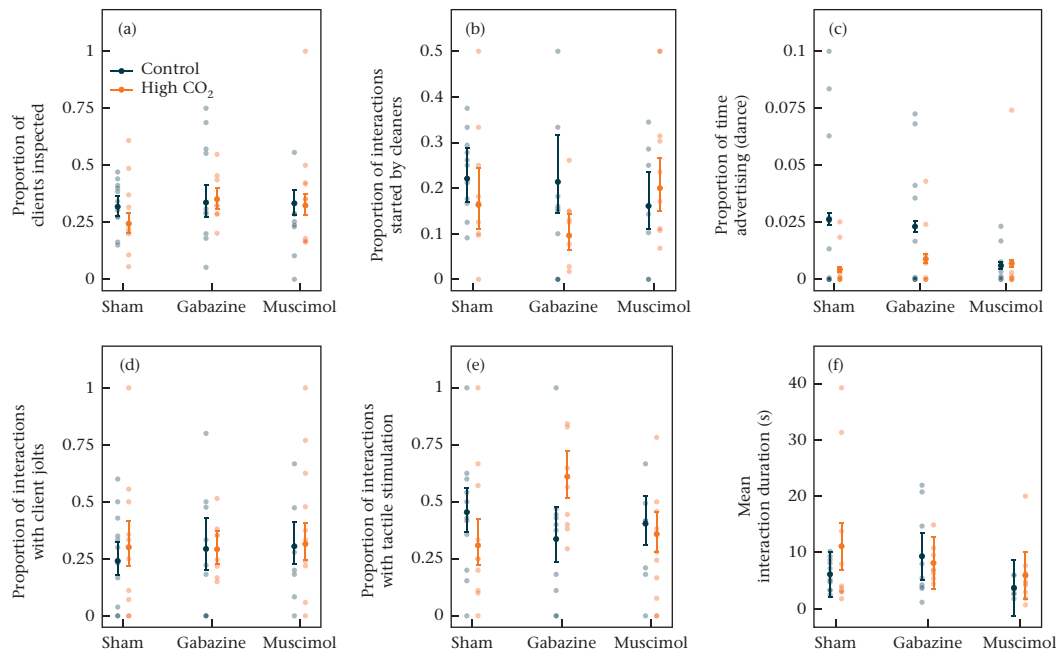


Figure 1. Natural cleaning behaviour on Moorea coral reefs following cleaner wrasse exposure to high CO₂ and GABAergic manipulation. Cleaner fish motivation to engage in cleaning interactions was measured as (a) proportion of clients inspected (number of clients cleaned/total number of visits); (b) proportion of interactions started by cleaners (number of interactions started by cleaners/number of interactions); (c) proportion of time advertising cleaning services (time spent performing advertising dance/time not in interaction). Cleaning interaction quality was measured as (d) proportion of interactions with jolts (number of interactions with tactile stimulation/number of interactions); (e) proportion of interactions with tactile stimulation (number of interactions with tactile stimulation/number of interactions); (f) mean interaction duration (s). GABAergic treatments are represented by sham, gabazine and muscimol. CO₂ treatments: dark blue = control (pCO₂ ca. 400 μatm); orange = high CO₂ (ocean acidification scenario, pCO₂ ca. 1000 μatm). (a–f) Back-transformed predicted means ± 95% confidence interval from the model and raw data values are presented. (g) Natural cleaning interaction between *L. dimidiatus* and a client in Moorea, French Polynesia. Photo: Frederic Zuberer.

suggesting recovery of behaviour, (2) under high CO₂ there was an effect of gabazine administration compared to sham on the proportion of clients inspected (Fig. 1a) and the proportion of interactions with tactile stimulation (Fig. 1e) again suggesting recovery of behaviour. However, contrary to the GABA_AR model predictions, gabazine and CO₂ exposure reduced the proportion of interactions started by cleaners, which was not significantly reduced under CO₂ exposure alone (Fig. 1b). Hamilton et al. (2014) also found that gabazine did not reverse the effect of CO₂ and that gabazine and CO₂ increased rockfish, *Sebastes diploproa*, anxiety to maximal levels. If CO₂ and gabazine also increased cleaner fish anxiety in our study, this could have prevented cleaners from proactively initiating cleaning interactions, producing the same result as observed with rockfish (Hamilton et al., 2014). Also, this may suggest that other mechanisms could be involved in the modulation of these behaviours under high CO₂, as seen in Paula, Repolho, et al. (2019) and discussed further below.

As a second test of the GABA_AR model, our study found that muscimol (GABA agonist) produced the same behavioural disruption as exposure to ocean acidification (decreased the proportion of time spent advertising cleaning service, Fig. 1c). Moreover, muscimol did not produce the same behavioural response as high CO₂, nor did it intensify the response under CO₂ on any other behavioural variable. Besides Hamilton et al. (2014) and our experiment, no other study has looked at the effect of muscimol on fish behaviour under high CO₂. Unlike Hamilton et al. (2014), we analysed behaviours that depend on interactions between at least two individuals, and only one of the interacting partners was exposed to CO₂ and muscimol. Thus, we cannot exclude that the absence of muscimol effects could be due to interdependency of these behaviours. Supporting this hypothesis is the decrease in time advertising cleaning services with muscimol and high CO₂, as this

behaviour is performed independently of the interaction partner. Therefore, a clear double support of the GABA_AR model for disruption of cleaner fish motivation was only found for the proportion of time spent advertising cleaning services.

The quality of cleaning interactions can determine whether clients return to the same cleaning station where they were previously inspected, and bystanders choose cleaning stations where high-quality interactions occur (Bshary & Schaffner, 2002). In a previous study, under laboratory conditions, exposure to high CO₂ led to an increase in a cleaner's investment in interaction quality (increase in tactile stimulation) without an increase in its dishonesty, i.e. client jolts (Paula, Repolho, et al., 2019). Here, although we also did not observe an effect on client jolts, the proportion of interactions with tactile stimulation was not significantly altered under high CO₂, but increased significantly when cleaners were treated with gabazine under high CO₂ (Fig. 1e). This suggests that under high CO₂ the use of reconciliation strategies is context dependent, that is, one client available in an aquarium versus a multitude of clients in a natural reef. The different results between studies are likely to be related to the clients inspected, since here these interactions were recorded in the wild and occurred with clients that were not manipulated, and in the previous study, the interactions occurred with only one client that was also acclimated to high CO₂. As gabazine increased the use of tactile stimulation during interactions, these results suggest that GABA_A receptors might be involved in the modulation of this behaviour; however, further studies are needed to understand this result. It is worth noting that other neurotransmitter systems, such as dopamine and serotonin, are known to disrupt cleaner fish motivation and interaction quality under ocean acidification (Paula, Repolho, et al., 2019). Moreover, as GABAergic activation is known to reduce the serotonergic response and increase the inhibitory influence in

Table 2
Contrasts of the post hoc multiple comparisons

Post hoc comparison	Proportion of clients inspected		Proportion of interactions started by cleaners		Proportion of time advertising		Proportion of interactions with jolts		Proportion of interactions with tactile stimulation		Mean interaction duration	
	t	P	t	P	t	P	t	P	t	P	t	P
Sham Control <-> Sham High CO ₂	-	-	1.228	0.821	13.098	<0.001	1.979	0.369	-	-	-	-
Sham Control <-> Gabazine Control	-0.442	0.898	0.136	1.000	1.712	0.531	1.427	0.711	-	-	-	-
Sham Control <-> Gabazine High CO ₂	-	-	3.386	0.017	9.267	<0.001	-2.141	0.285	-	-	-	-
Sham Control <-> Muscimol Control	-0.406	0.913	1.344	0.759	11.240	<0.001	0.685	0.983	-	-	-	-
Sham Control <-> Muscimol High CO ₂	-	-	0.492	0.996	11.125	<0.001	1.444	0.701	-	-	-	-
Sham High CO ₂ <-> Gabazine Control	-	-	0.934	0.934	12.013	<0.001	0.359	0.999	-	-	-	-
Sham High CO ₂ <-> Gabazine High CO ₂	-3.238	0.006	1.840	0.451	-4.354	0.001	-3.719	0.007	-	-	-	-
Sham High CO ₂ <-> Muscimol High CO ₂	-2.415	0.050	-0.806	0.965	-2.875	0.063	-0.733	0.977	-	-	-	-
Sham High CO ₂ <-> Muscimol Control	-	-	-0.056	1.000	1.913	0.408	1.284	0.792	-	-	-	-
Gabazine Control <-> Gabazine High CO ₂	-	-	2.800	0.074	8.027	<0.001	-2.991	0.047	-	-	-	-
Gabazine Control <-> Muscimol Control	0.082	0.996	1.026	0.907	10.115	<0.001	-0.816	0.963	-	-	-	-
Gabazine Control <-> Muscimol High CO ₂	-	-	0.266	1.000	9.877	<0.001	-0.287	1.000	-	-	-	-
Gabazine High CO ₂ <-> Muscimol Control	-	-	1.837	0.453	-2.411	0.173	-2.619	0.112	-	-	-	-
Gabazine High CO ₂ <-> Muscimol High CO ₂	0.822	0.692	-2.915	0.057	1.599	0.603	3.555	0.011	-	-	-	-
Muscimol Control <-> Muscimol High CO ₂	-	-	-0.902	0.944	-0.896	0.946	0.661	0.985	-	-	-	-

P values were adjusted using Tukey corrections from the R package emmeans. Values of P < 0.05 are shown in bold.

dopaminergic neurons (Summers et al., 2003; Tritsch et al., 2012), we cannot discard a possible interaction between GABA, dopamine and serotonin in the modulation of the observed cleaner fish behavioural impairment.

As in Paula, Repolho, et al. (2019), the present findings support the hypothesis that ocean acidification can alter cleaning behaviour between cleaner wrasses and their clients. Here we acknowledge that (1) we only exposed cleaner wrasses to high CO₂ and not the clients, (2) during the behavioural trials cleaners were in control CO₂ water, and (3) this study also only considered acute exposure to high CO₂ within one generation. Future studies trying to understand the dynamics of natural cleaning interactions could use CO₂ seeps to address these caveats (Munday et al., 2014). Many ocean acidification studies do not consider the potential of species adaptation to an increase in CO₂ or consider variation in tolerance among or within populations. A recent study showed that, although high CO₂ decreased cognitive performance in cleaner fishes, some individuals showed CO₂ tolerance revealing the potential for adaptation (Paula, Baptista, et al., 2019). Here, we never observed a complete cessation of cleaning interactions, and variation in responses was observed for all behavioural variables. Thus, if selection on motivation to interact and interaction quality is strong, as these traits are heritable, then we could expect adaptation potential. However, the interaction effects of other climate change-related stressors (such as temperature or hypoxia) could hamper organismal response and the potential for adaptation through directional selection.

In conclusion, we show that cooperative cleaning interactions are disrupted by ocean acidification, even when tested in natural conditions. Moreover, we confirmed that GABA_A receptors are involved in ocean acidification disruption of certain cleaning behaviours, probably due to Cl⁻ efflux. Unravelling behavioural effects with neurobiological links is a priority for ocean acidification research, as it provides the opportunity to improve our understanding of the physiological basis of CO₂-induced behavioural impairments. At the ecosystem level, changes in cleaning mutualism integrity (e.g. motivation for cooperative interactions) can lead to an eventual breakdown of the mutualism with cascading effects to coral reef ecosystems (Kiers et al., 2010; Sachs & Simms, 2006). Nevertheless, we acknowledge that short-term studies like ours fail to address the potential for adaptation to ocean acidification (Sunday et al., 2014) and studies addressing adaptation capacity of these mutualisms are urgently needed to improve our understanding of the future impacts on these charismatic interactions.

Author Contributions

J.R.P., S.C.M. and R.R. designed the study. J.R.P., C.F.S., R.B., S.C.M. and R.R. performed the experiment. L.C. was the sole blind annotator of all behavioural videos using Boris software. R.O. did the interobserver analysis. J.R.P., E.O. and R.R. analysed the data provided by L.C. behavioural annotation. J.R.P., S.C.M. and R.R. wrote the manuscript. All authors discussed the results and their implications and commented on the manuscript at all stages.

Data Availability

The data sets generated and analysed for this study can be found in the repository Figshare <https://doi.org/10.6084/m9.figshare.13473156>.

Declaration of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix

Table A1

Sea water physicochemical parameters in all experimental set-ups

	Control	High CO ₂
Measured		
Temperature (°C)	28.7 ± 0.3	28.7 ± 0.3
pH	8.05 ± 0.01	7.70 ± 0.02
AT (μmol/kg sea water)	2428.2 ± 11.8	2428.2 ± 11.8
Salinity (ppt)	35	35
Calculated		
TC (μmol/kg sea water)	2072.9 ± 12.5	2263.9 ± 12.5
pCO ₂ (μatm)	400.8 ± 15	1029.2 ± 44.0
Ω Arg	4.1 ± 0.1	2.2 ± 0.1

Salinity, pH and temperature were measured daily and averaged over the whole experimental period. The combination of total alkalinity (AT) and pH_T (pH total scale) was used to calculate carbonate system parameters [pCO₂ (carbon dioxide partial pressure), TC (total inorganic carbon) and Ω Arg (aragonite saturation state)]. Values are represented as mean ± SD.

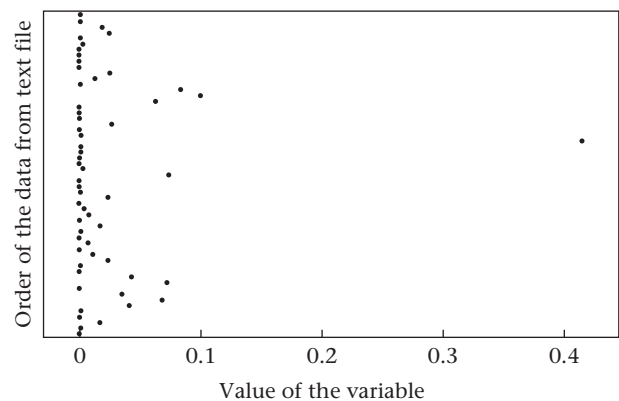


Figure A1. Cleveland plot for proportion of time advertising (dance).