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Ocean Acidification Does Not Affect Fish Ectoparasite Survival

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Abstract: The juveniles of gnathiid isopods are one of the most common fish ectoparasites in marine habitats and cause deleterious effects on fish by feeding on host blood and lymph. Reef fishes tend to engage in cooperative interactions with cleaning organisms to reduce their ectoparasite load. Ocean acidification (OA) pose multiple threats to marine life. Recently, OA was found to disrupt cleaner fish behaviour in mutualistic cleaning interactions. However, the potential effects of ocean acidification on this common ectoparasite remains unknown. Here, we test if exposure to an acidification scenario predicted by IPCC to the end of the century (RCP 8.5 – 980 µatm pCO_2) affects gnathiid survival. Our results show that ocean acidification did not have any effects on gnathiid survival rate during all three juvenile life stages. Thus, we advocate that the need for cleaning interactions will persist in potentially acidified coral reefs. Nevertheless, to better understand gnathiid resilience to ocean acidification, future studies are needed to evaluate ocean acidification impacts on gnathiid reproduction and physiology as well as host-parasite interactions.

Keywords: CO_2; environmental change; cleaning symbiosis; gnathiids; isopods; coral reefs

1. Introduction

Human-induced environmental changes currently represent the single greatest threat to global diversity. Earth's atmospheric concentration of carbon dioxide (CO_2) has been increasing at an unparalleled rate. Currently, 30% of the anthropologically emitted CO_2 is being dissolved into the ocean, which has decreased seawater pH by 0.1 units during the last decade [1]. When CO_2 is dissolved in seawater, CO_2 concentration increases and combines with water to produce carbonic acid (H_2CO_3); this dissociates into bicarbonate (HCO_3^-) and hydrogen ions decreasing seawater pH. The increased concentration of hydrogen ions can also interact with carbonate ions (CO_3^{2-}) to form more bicarbonate, reducing the saturation of seawater aragonite and calcite, crucial for shells and skeletons of marine organisms. This phenomenon, known as ocean acidification (OA), is projected to decrease seawater pH between 0.14–0.42 units by the end of this century [1,2].

Different biological responses to OA have been observed across multiple taxa, with sensitivity varying according to the measured trait, life stage, species and exposure duration [3]. OA is known to affect growth, survival, reproduction and behaviour of multiple species. In coral reefs, calcification is one of the most critical functions to be affected by OA. Lower calcification rates in corals under

OA can result in slower coral growth and more fragile structures, making corals more susceptible to disturbances [4]. OA can also lead to the reduced abundance of crustose coralline algae, crucial to larval recruitment of invertebrates [5] and reduce zooplankton community biomass [6].

Although coral reef fish can regulate their acid-balance [7], coral reef fishes have been suggested to be susceptible to physiological and behavioural alterations under OA [7–9], yet, recent studies have also documented low or no effect of OA on fish behaviour [10–12], suggesting at least variability in fish behavioural responses to OA. During cleaning interactions, cleaner fishes inspect the body of their clients for ectoparasites, dead tissue and mucus [13]. Recently, Paula et al. (2019) [14] described a loss in motivation in cleaner wrasse *Labroides dimidiatus* (the most abundant cleaner fish species in the Indo-Pacific [15]) to interact with a client reef species.

Gnathiid isopods (family: Gnathiidae) are the most common ectoparasites found on coral reef fishes [16], and they can lower blood volume of their host, cause tissue damage, transmit blood-borne protozoan parasites and, in large numbers, can even cause death to adult fish [17,18]. Fish larvae and juveniles are especially vulnerable to the effects of ectoparasite infection, as they are small relative to the parasite, and can experience reduced performance and even mortality when infected [18,19]. Cleaning interactions can significantly lower the gnathiid loads on fish [20] and can indirectly affect gnathiid populations [21]. When not feeding on hosts, these ectoparasites are part of the demersal zooplankton community [22].

Nevertheless, despite the effects of OA on cleaner fish motivation and the ecological relevance of gnathiid ectoparasites, until now, the effect of OA on gnathiids has not been tested. To understand the effects of OA on gnathiids, we tested whether the survival rate of a cultured gnathiid species, *Gnathia aureamaculosa,* is altered when exposed to projected OA conditions (~980 µatm pCO_2, RCP8.0 2100, in IPCC 2013 [1]).

2. Materials and Methods

2.1. Parasite Culture and Gnathiid Collection

Gnathiids *G. aureamaculosa* were obtained during February 6–10, 2019 from a parasite culture maintained at Lizard Island Research Station (Lizard Island, Australia 14°40′S, 145°28′E) since 2001 [23,24]. Gnathiids were collected in the morning (ranging from n = 32-142 per day, total n = 510) by moving a black tray around the culture tank to simulate the movement of a host. Gnathiids are briefly attracted to the tray, allowing them to more easily be captured using a pipette. Following collection, gnathiids were placed together into 75 mL containers filled with seawater. From these, they were individually transferred into 5 mL labelled vials that were half-filled with pre-conditioned seawater according to treatment (see below). Vials were held underwater in plastic baskets (17 × 17 × 10 cm), one for each treatment and replicate. The vials were randomly allocated to the seawater treatment and system replicate (n = 510 vials; 6 baskets: 2 CO_2 treatments × 3 replicates). Vial lids were only labelled with the combination of one letter (I, K, L, M, O or X) and one number (from 1 to 100). Thus, the experimenter was blind to the treatment, eliminating any potential observer bias. Baskets had four mesh (1 mm^2) windows (12 × 5 cm) on the sides and one in the lid (12 × 12 cm) to allow water flow. A dive weight was used to submerge the baskets in experimental tanks.

2.2. Seawater CO_2 Manipulation and Aquatic Systems

Gnathiids were maintained in a closed vial, half full of seawater with one of two treatments, control (~405 µatm pCO_2) or OA/high CO_2 (~980 µatm pCO_2). For each treatment, seawater was collected from established flow-through aquatic systems (n = 6; 2 treatments × 3 replicates) to maintain correct levels of total alkalinity, dissolved inorganic carbon and pH. In these systems, natural seawater was pumped from the sea into three 10 m^3 seawater storage tanks. From the storage tanks, seawater was supplied to a mixing tank and experimental tank. pCO_2 control was performed indirectly by adjusting pH to a nominal pH value defined by CO2SYS software using measured salinity, total

alkalinity, temperature and desired pCO_2 as input variables. Levels of pH were monitored and automatically adjusted by a control unit (Profilux 3.1N, GLH, Rheinland-Pfalz, Germany), that was downregulated by direct injection of CO_2 gas (BOC, North Ryde, Australia) and upregulated through aeration with atmospheric air in mixing tanks. Seawater temperature was maintained at a level similar to current reef temperature, due to the flow of recently captured seawater. Before adding water to the vial, we used handheld equipment to complement the automatic systems and measured seawater temperature (Hanna CheckTemp 1C, Woonsocket, RI, USA), salinity (V2 refractometer, TMC, Lisbon, Portugal) and pH (VWR pHenomenal pH 1100H, connected to a glass electrode calibrated with TRIS-HCl and 2-aminopyridine-HCl buffers). Seawater carbonate parameters were calculated from total alkalinity (titration) and pH measurements. Bicarbonate and pCO_2 values were calculated using CO2SYS software. Seawater parameters are summarised in Table S1.

2.3. Gnathiid Survival and Stage Determination

Gnathiid survival was checked daily by direct observation under a microscope (Zeiss Olympus, Munich, Germany) through the unopened clear vial, until they were identified as dead. Each basket was removed from the experimental tank, placed in an insulated container with treatment seawater to maintain temperature and transported to the microscope room. Gnathiid death was confirmed by prolonged complete cessation of any movement (from swimming to movements of body parts) after a gentle shake, or signs of body decomposition. When death was confirmed, 4% of formalin was added to the vial for further determination of the larval stage. This was determined by measuring the headwidth (HW) of fixed gnathiids, where stage one had HW < 0.2 mm, stage two 0.2 mm < HW < 0.248 mm and stage three HW > 0.248 mm [25]. From the total of 510 gnathiids collected we discarded 35 that had an engorged gut to avoid confounding effects of feeding in our experiments. From the total of 475 gnathiids used, 260 were stage one, 177 stage two and 34 stage three.

2.4. Statistical Analysis

Since we have time-to-event data, we performed a survival analysis to compare the number of days alive according to CO_2 treatment and gnathiid stage. We used a proportional hazards Cox mixed-effects model fit by maximum likelihood with CO_2 treatment and gnathiid stage as categorical fixed effects, tank as a random factor and gnathiid headwidth as a covariate. Both headwidth and stage were maintained in the model as headwidth varies within stage [25], thus survival could also vary within stage. Both models with and without headwidth were fitted, yet only the full model complied with the assumption of proportional hazards (see Figures S1 and S2). We used the function "coxme" in the package "Coxme" [26] and function "Anova" in the package "Car" [27]. We verified compliance with the assumption of proportional hazards using the global test statistic in the function "coxph" from the R package "survival" [26] and graphically using a smoothed spline plot of the Schoenfeld residuals relative to time. Survival curves were plotted as Kaplan-Meier plots using the function "ggsurvplot" from the R package "survminer" [28]. Both statistical analysis and graphs were performed in R, version 3.4.3 [29].

2.5. Ethics

All applicable national laws and institutional guidelines for animal testing, animal care and use of animals were followed by the authors.

3. Results

The total number of gnathiids used per stage was distributed relatively evenly within CO_2 treatments (control and high CO_2, stage one: 124, 136; stage two: 95, 82; stage three: 12, 22). Survival rate was significantly different according to an interaction between headwidth and stage ($\chi^2 = 11.63$; d.f. = 2; $p = 0.003$, Table 1; Table S2). Larger gnathiids within each stage and older gnathiids (stage

3 > stage 2 > stage 1) had higher survival rate. Survival rate was not significantly affected by CO_2 treatment ($\chi^2 = 3.29$; d.f. = 1; $p = 0.07$ Table 1, Figure 1).

Table 1. Analysis of deviance table (Type II tests) of gnathiid survival among CO_2 treatments, gnathiid stages and size for Cox mixed effect model.

	D.f.	χ^2	*p*
CO_2 treatment	1	3.29	0.070
Larval stage	2	6.35	**0.042**
Headwidth	1	27.35	**<0.001**
CO_2 treatment × Headwidth	1	2.10	0.147
CO_2 treatment × Larval stage	2	1.18	0.555
Stage × Headwidth	2	11.63	**0.003**
CO_2 treatment × Stage × Headwidth	2	0.80	0.680

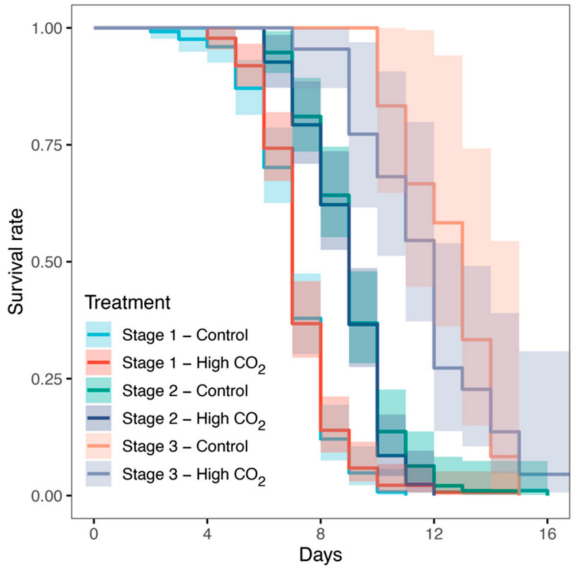

Figure 1. Effects of CO_2 treatment and larval stage on gnathiid survival rate according to treatment exposure day. Survival was verified every 24 h. Kaplan–Meier survival trajectories illustrate the different survival trajectories according gnathiid stage and CO_2 treatment. Lines represent rate of live gnathiids at each exposure day. Shaded areas are 95% confidence intervals.

4. Discussion

OA has the potential to reduce the abundance of demersal zooplankton that reside in tropical coral reefs [6]. However, in our study, we did not observe an effect of OA on the short-term survival of the gnathiid *G. aureamaculosa*, an organism that forms part of the tropical reef demersal zooplankton community. Since all gnathiids considered here were not fed (i.e., no potential host was provided), all gnathiids reached death during this study most likely due to starvation. Our results indicate that, although survival was dependent on larval stage and on headwidth within a larval stage, gnathiid survival was not significantly affected by OA. Overall, there was a non-significant tendency for third stages to survive longer, with 50% of individuals surviving after 12 to 13 days, compared with 7 and 9 days for stage one and two, respectively (Figure 1). The gnathiid survival increase with age might be related to different resource allocation, as, for example, third stage gnathiids have to allocate resources to prepare reproductive organs [25]. Moreover, gnathiid survival increases with size and varies with age (AS Grutter personal communication), however this response could have varied if gnathiids were fed.

Determining which species are sensitive to OA is crucial to determine the impacts of OA on ecosystem function [30,31]. Previous studies have shown that extreme OA (2380 µatm pCO_2) has

little to no impact on the survival of non-calcifying zooplankton species, such as copepods [32]. However, in a naturally acidified reef, Smith et al. (2016) [6] observed a loss of reef-associated demersal zooplankton abundance, including zooplankton from the Order Isopoda, without any shift in diversity. The authors suggested that although this loss could be driven by: (i) an indirect effect of physiological or behavioural impacts of OA, and (ii) reduced habitat complexity (i.e., higher abundance of branching corals at control sites, compared to a domination of massive bouldering corals in high CO_2 sites). Although, in the case of gnathiids, loss of habitat complexity can be beneficial since a previous study demonstrated that gnathiids (*Gnathia marleyi*) prefer less complex habitats [33].

During our study, gnathiids were isolated in small vials and left to starve. We cannot ignore that potential OA effects on gnathiid physiology, digestion or behaviour (e.g., host detection and attachment success) could have indirect effects on gnathiid survival. OA can induce alterations of stomach pH in marine invertebrates leading to decreased digestive efficiencies [34]. Other studies also showed that host-parasite dynamics can vary with OA. Namely, increased infection rates of trematodes (*Maritrema novaezealandensis*) in amphipods have been described under severe acidification (pH 7.4 ~1980 pCO_2, 2300 scenario) [35]. Contrarily, no effects were observed in infection rates of *Perkinsus marinus* in *Crassostrea virginica* [36]. Moreover, exposure to ocean acidification decreased cercarial survival of four parasite species (*M. novazelandensis*, *Philophthalmus* sp., *Parorchis* sp., and *Galactosum* sp.) [37]. Thus, further studies are necessary to understand hosts' susceptibility to gnathiids and the attachment success of gnathiids onto hosts, as well as the biological interactions in other parasite systems under OA.

Environmental perturbations, such as bleaching and cyclones, can lower cleaner fish abundance considerably [38] and OA has the potential to disrupt cleaning interactions [14]. Such perturbations could lead to disruptions in cleaners' control of gnathiid abundances [39]. Our results, indicating an apparent tolerance of these fish ectoparasites to OA, suggest that a potential cascading impact of OA on the cleaning symbiosis may include the continued need for cleaners' parasite removal services in clients under projected OA conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2673-1924/1/1/3/s1, Table S1: Seawater physicochemical parameters in all experimental setups. Table S2: Summary output for Cox mixed effects model of gnathiid survival among CO_2 treatments, gnathiid stage and headwidth; Figure S1: Smoothed spline plots of Shoenfeld residuals for the Cox mixed effects model relative to time; Figure S2: Smoothed spline plots of Schoenfeld residuals of the Final Cox mixed effects model relative to time (with headwidth).

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Conflicts of Interest: The authors declare no conflict of interest.

Data Availability: The datasets generated and analysed during this study are available in the Figshare repository, doi: 10.6084/m9.figshare.11354063.

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