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Escape responses of the Japanese anchovy *Engraulis japonicus* under elevated temperature and CO$_2$ conditions

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Abstract An increasing number of papers have been published on the effects of ocean acidification and warming on fishes over the last several years. However, there is little information on how these environmental changes affect the swimming behavior of fish. This study examined the escape response under elevated CO$_2$ concentration and temperature of the Japanese anchovy *Engraulis japonicus*. Following acclimation to four conditions (CO$_2$ 400/1000 ppm, temperature 15/19 °C) for 1 month, the fish were tested for escape response through kinematic analysis of startle reactions to a mechanical stimulus. The response was recorded with a high speed video camera of 500 frames per second. The result showed turning rate was significantly higher at 19 °C than at 15 °C. Neither CO$_2$ nor temperature affected the kinematic parameters analyzed (the escape trajectory, swimming velocity, acceleration, escape direction, or frequency of single and double bends), with the exception of the turning rate that was significantly higher at 19 °C than at 15 °C. However, we must clarify how future oceanic environmental changes affect escape responses of schooling fish and prey-predator interactions under more rigorous experimental conditions, to elaborate our prediction

capacity for the trajectory of anchovy populations and thereby assess possible implications for anchovy fisheries.

Keywords C-start · Escape response · Japanese anchovy · Ocean acidification · Ocean warming

Introduction

The atmospheric CO$_2$ concentration has increased from 280 ppm in the late 1700s to 390 ppm in 2011, mainly due to the burning of fossil fuels and land use change [1]. The CO$_2$ concentration is projected to reach 940 ppm in 2100 in accordance with the representative concentration pathway (RCP) 8.5, the most carbon-intensive future projection scenario among the RCPs [1, 2]. The ocean has absorbed about 30 % of the anthropogenic CO$_2$ emitted to the air, which has resulted in a decrease of average surface ocean pH by 0.1 units since the beginning of the industrial revolution [1]. The reduction in seawater pH, together with associated changes in seawater carbonate chemistry, is termed ocean acidification. Under the RCP 8.5 projection, average surface ocean pH will further decrease by 0.30–0.32 units by the end of the twenty-first century [1]. The rapidly changing oceanic environmental conditions will likely alter the structure and function of marine ecosystems [3, 4], which will undermine productivity of fisheries and other ecosystem services of the ocean [1].

Reflecting mounting concern about the fate of marine ecosystems in the coming decades, there has been a rapid growth of scientific literature attempting to predict how marine species and ecosystems will respond to these environmental changes [4–7]. Although earlier studies of ocean acidification focused mainly on invertebrates, more attention has been paid to fishes during the last few years.

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Recent papers on the ocean acidification effect on fish have demonstrated that ocean acidification disrupts olfactory discrimination of chemical cues [8], visual perception of a predator fish [9], which may be related to impaired retinal function [10], and auditory ability to discriminate sounds appropriate for settling [11]. In addition to these findings on sensory functions, several studies have also shown that CO_2 could have detrimental impacts on the brain function of fish, which may have direct implications for fish survival. For example, Ferrari et al. [12] demonstrated clear evidence that acclimation to CO_2 (850 ppm) would suppress the learning ability of juvenile fish. Elevated CO_2 also disrupts the behavioral lateralization of fish, which may influence efficacy of an escape response [13]. More directly, coral reef fish larvae pretreated with CO_2 (700 and 850 ppm) and released to a natural reef showed significantly higher mortality, though the cause-and-effect relationship of this finding is somewhat obscure [14]. In contrast, the data by Allan et al. [15] demonstrated that when predators acclimated to control CO_2 conditions are interacted with prey fish acclimated to 880 ppm CO_2, capture success rate decreased with no change in predation rate (capture success/number of attack). There is some evidence for the involvement of a neurotransmitter, gamma-aminobutyric acid (GABA), in alterations of sensory and behavioral functions under elevated CO_2 [16–18], but the mechanism(s) for the cognitive and behavioral disturbance by CO_2 needs more scrutiny. In comparison, much less is known about swimming responses to elevated ambient CO_2 [19–22].

One crucial behavior that determines survivorship of fish is the escape response [23]. In response to a predation risk, fish generally show a behavior called C-start, which determines whether or not fish can avoid a predator. The escape response (C-start) is the highest swimming speed attainable by a fish and occurs during the "flight or fight" response to predators. C-start begins with unilateral contraction of trunk musculature, bending the fish's body into a C shape (stage 1) and may be followed by contraction of the contralateral trunk musculature (stage 2) [24]. An escape response consists of non-locomotion variables, which relate to sensory perception of a stimulus and processing of the sensory input to trigger a response (responsiveness, i.e., whether a fish responds to a stimulus or not, escape latency, and directionality) and locomotion variables, which directly govern kinematics of the escape response (turning rate, distance, swimming speed, and acceleration) [25]. Because escape responses are usually triggered by a specific type of cells in the central nervous system (the Mauthner cells) [26], one might expect that CO_2 would somehow modify or disrupt escape responses of fish.

Since ocean acidification will proceed concurrently with temperature rise [1, 2], it is crucial to understand how these two environmental changes will affect marine species and ecosystems in concert. Under the RCP 8.5 pathway, surface ocean temperature is projected to rise 2 °C by the year 2100 [2]. Numerous papers have already been published on the effect of temperature on various aspects of fish biology [see [27], [28] for review] because temperature is one of the most powerful environmental factors to modulate biological activities [29]. Similar to all other animal groups, temperature almost invariably exerts strong influences on development [30–32], behavior [33–35], metabolism [36, 37], growth [38–40], and swimming [41–43] of fishes. It has also been reported that temperature affects tail-beat frequency, muscle twitch contraction, and aversive behavior of fish [44–46] and that increasing CO_2 levels can reduce the swimming ability of both predators and prey [15, 47]. Domenici et al. [48] showed that elevated temperature attenuated the magnitude of lateralization in a marine damselfish without affecting the directionality of turning behavior, whereas elevated CO_2 significantly reversed the turning bias. To our knowledge, no study has been conducted on the interactions of temperature and CO_2 on the escape response of fish.

The aim of this study was to examine both separate and combined effects of temperature and CO_2 on the escape responses of fish. The Japanese anchovy *Engraulis japonicus* was selected in our study for the following two reasons. First, it is commercially very important, being ranked ninth in the list of major marine fish species in production, amounting to the annual global catch of 1.3 million tonnes in 2012 [49]. In fact, both the top and 21st species in the list are from the genus *Engraulis* (Peruvian anchovy *E. ringens* and European anchovy *E. encrasicolus*, respectively), underpinning the importance of the genus in fisheries production. Second, the Japanese anchovy plays an important role in the marine ecosystem as prey of higher tropic predators such as skipjack tuna [50] and, as such, experiences a high mortality rate due to predation. The Japanese anchovy is found in the western north and central Pacific including the Yellow Sea, East China Sea, and Sea of Japan, showing changes in distribution pattern with resource abundance [51, 52]. These ocean regions are the main distribution areas of the Japanese anchovy and are among the most rapidly warming large marine ecosystems around the world [53]. Thus, studying the escape response of Japanese anchovy to elevated CO_2 and temperature should provide useful information to foresee the trajectory of this important fishery species into the future ocean.

Materials and methods

Ethics statement

Animal care and experimental procedures for the experiment were approved by the Animal Care and Use Committee of the Institute for East China Sea Research, Nagasaki University (Permit no. ECSER13-01) in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

Experimental animals

In January 2013, ~200 adult Japanese anchovy [total length = 99.3 ± 9.9 mm (mean ± SD, $N = 25$)] were purchased from a fisherman in Saikai-shi, Nagasaki, Japan (33°05′ N, 129°41′ E), who maintained the anchovies in fish cages as bait for skipjack tuna fishing. The fish were transferred to the Institute for East China Sea Research of Nagasaki University, Japan and stocked in two 500 L tanks supplied with a continuous flow of fresh seawater at a flow rate of 4 L/min. Fish were fed artificial pellet (diameter 2.3 mm, Otohime EP2, Marubeni Nisshin Co. Ltd., Tokyo) by using a custom-made automatic feeding machines every 30 min from 9 am to 5 pm with a daily ration of 5 % body weight. The Japanese anchovy turned out to be highly sensitive to handling stress, and a high mortality occurred within 3 days of transportation. The fish were maintained for 2 weeks before being acclimated to the experimental conditions described below.

Acclimation

After 2-weeks transport, 80 fish were randomly chosen, divided into four groups of 20 each and acclimated to four conditions: control condition (seawater equilibrated with ambient air containing 400 ppm CO_2 and temperature at 15 °C), high CO_2 condition (seawater equilibrated with air containing CO_2 at a concentration of 1000 ppm and temperature at 15 °C), high temperature condition (CO_2 same as control and temperature at 19 °C), and combined condition (CO_2 1000 ppm and temperature at 19 °C. There were two acclimation tanks for each treatment (a total of eight tanks), and 10 fish were stocked in each tank (20 fish per treatment). The acclimation tanks were 100 L in capacity and supplied with filtered (1 μm) seawater at a flow rate 0.4 L/min. The overflow from the tanks was drained without recirculating. Seawater in the acclimation tanks was bubbled with atmospheric air for control or CO_2-enriched air for high CO_2 conditions at a flow rate of 20 L/min. Water temperatures of 15 and 19 °C were controlled by a submersible heater and a thermostat. The fish were fed artificial pellets at a daily ration of 5 % of body weight in three portions. Excess food was removed daily by siphoning. Water quality in each tank was monitored at 10:00 am daily for dissolved oxygen (DO) concentration, pH (NBS scale), and salinity. DO concentration was measured with a digital DO meter (YSI proODO dissolved oxygen meter, US) and never fell below 85 % saturation. Salinity was measured with a digital salinometer (Mettler-Toledo GmbH SG3, Switzerland). Water pH was measured with a digital pH meter (Mettler-Toledo GmbH SG8, Switzerland), calibrated with standard buffer solutions of pH 4.01, 6.86, and 9.18 (Nacalai Tesque Inc. Kyoto Japan). Alkalinity was measured with a total alkalinity titrator (Kimoto, ATT-05, Japan) at weekly intervals. Partial pressure of CO_2 (pCO_2) was calculated from measured seawater pH, temperature and total alkalinity by using the program CO2SYS [54]. The concentration of ammonia in seawater was measured twice weekly with an ammonia electrode (Orion 9512, Thermo Scientific, USA), calibrated with a standard solution of 1000 ppm NH_3. The fish were acclimated to the four experimental conditions for 1 month before testing, between 4 February and 7 March 2013. The data of seawater temperature, oxygen saturation, and carbonate chemistry during acclimation are shown in Table 1.

We used every precaution to avoid any stressful treatment to the anchovies during acclimation, but daily

Table 1 Seawater temperature, pH_{NBS}, salinity, oxygen saturation (SO_2), ammonia (NH_3) concentration, total alkalinity (A_T), and calculated CO_2 partial pressure (pCO_2) values (mean ± SD) during acclimation

Treatments	Measured						Calculated
	Temperature (range, °C)	pH_{NBS}	Salinity PSU	SO_2 (%)	NH_3 ppm	A_T μmol/l	PCO_2^a μatm
Control	14.8–16.3	8.12 ± 0.03	34.61 ± 0.49	89 ± 2	0.0017 ± 0.0004	2246 ± 60	424 ± 26
High CO_2	14.7–16.2	7.83 ± 0.02	34.61 ± 0.49	90 ± 2	0.0036 ± 0.0009	2233 ± 29	939 ± 31
High temp.	18.3–20.1	8.11 ± 0.03	34.61 ± 0.49	90 ± 3	0.0017 ± 0.0005	2227 ± 35	443 ± 15
Combined	18.2–20.2	7.84 ± 0.02	34.61 ± 0.49	87 ± 3	0.0044 ± 0.0007	2237 ± 28	946 ± 23

[a] Calculated from measured temperature, pH_{NBS}, salinity, and A_T. In a gas phase, 1 μatm = 1 ppm at the barometric pressure of 1 atm; $n = 56$

cleaning of the tanks was apparently stressful to them. Many fish jumped out from the tanks or into the net covering the tanks. Thus, the number of fish decreased from 80 to 48 (Control 15, high CO_2 12, high temperature 10, and combined 10) during the 1-month acclimation period.

Procedure

Seven dead fish (total length = 98.4 ± 4.5 mm and wet body mass = 8.80 ± 0.71 g; mean ± SD) were used to estimate the position of the center of mass (CM). The CM was estimated by hanging dorsally each dead fish with a needle. The balancing point of the body indicated the position of the CM at 43.64 ± 1.70 mm, i.e., 44.3 ± 0.2 % of the total length, from the snout. A black polyethylene tank (capacity 500 L, diameter 150 cm, height 80 cm) with a light yellow sheeting on the bottom was used for recording escape responses. The tank was filled with seawater to a depth of 20 cm. Escape responses were studied under the same CO_2 and temperature conditions as during acclimation. The stimulus (a rubber parcel) was released through a 75 cm long PVC tube with a diameter of 6 cm by triggering an electromagnetic device. To avoid visual stimulation, the PVC tube was positioned at the center of the experimental tank, with the lower end 5 mm above the water surface. A high speed video camera (HAS-L1, Ditect Co., Japan) was installed 2 m above the experimental tank. A 180 W spotlight was mounted on the upper edge of the experimental tank and two 32 W fluorescent lamps were installed above the tank. The light intensity at the water surface of the testing tank was about 1800 lx. Each fish was tested individually and only once. Before triggering the electromagnetic stimulus, the anchovies were allowed to habituate themselves in the test tank for about 10 min until they started to swim in a normal manner. When the fish swam to the center of the tank, the stimulus was activated. A high speed video recorded the escape response at 500 frames/s. The video recording was triggered two seconds before the stimulus was dropped and lasted for 4 s.

Measured variables

The fish movement was sequentially tracked frame by frame by using ImageJ 1.46r (National Institutes of Health, USA). The x–y coordinates of the CM and the tip of the head were digitized in each frame. The following variables were calculated according to Lefrançois et al. [55]: (1) response latency, i.e., the time interval between the stimulus hitting the surface and the first detectable movement of the escape behavior; (2) response type, termed single bend (SB), when the tail did not recoil completely after the formation of the C or double bend (DB), when a full return
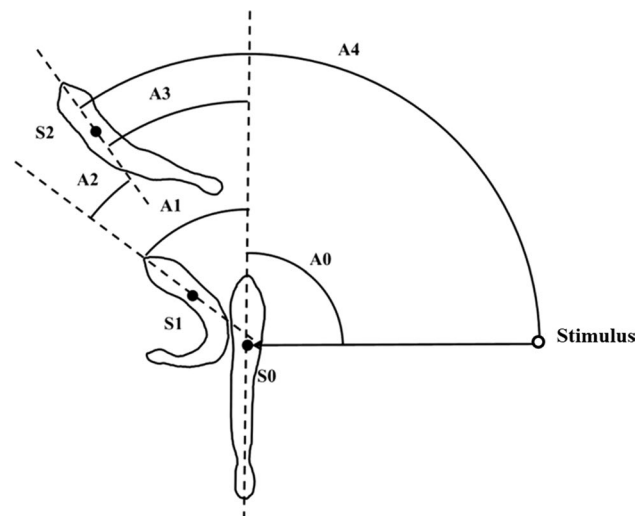

Fig. 1 Schematic drawing of angular variable. The *solid circles* indicate the positions of the center of mass. The *open circle* indicate the position of stimulus hitting the water surface. S0, position of fish at the onset of stage 1; S1, position at the end of stage 1; S2, position at the end of stage 2; A0, initial orientation; A1, stage 1 angle; A2, stage 2 angle; A3, escape angle; A4, escape trajectory angle

flip of the tail occurred after initial contraction; (3) directionality, the "away" and "toward" responses, which were defined on the basis of the first detectable movement of fish being either oriented away or toward the stimulus; (4) stage 1 duration, the time between the first detectable fish movement and the onset of the return tail flip; (5) stage 2 duration, the time between the end of stage 1 and the end of the return tail flip; (6) total duration, the sum of the stage 1 and stage 2 durations; (7) initial orientation (A0), the angle between the line passing through the stimulus and the CM, and the line passing through the CM and the tip of the head at the onset of stage 1 (as shown in Fig. 1); (8) stage 1 angle (A1), the angle between the lines passing through the CM and the tip of the head at the onset and the end of stage 1; (9) stage 2 angle (A2), the angle between the lines passing through the CM and the tip of the head at the end of stage 1 and at the end of stage 2; (10) escape angle (A3), the sum of A1 and A2; (11) escape trajectory angle (A4), the sum of A0 and A3; (12) turning rate, calculated by dividing A1 by stage 1 duration; and (13) the cumulative distance (D), maximum speed (V_{max}), and maximum acceleration (A_{max}) were determined within a fixed 48 ms duration which was the time needed to complete stages 1 and 2 (see Results). Speed and acceleration were calculated by differentiation and double differentiation, respectively, by the cumulative distance for the time-series. A five point smoothing with polynomial regression was applied to calculate the speed and acceleration using QuickSAND software [56].

Table 2 Statistical analysis of the effects of temperature, CO_2 and their interaction on the escape response of the Japanese anchovy *Engraulis japonicas*

Treatments	Initial orientation (A0)		Escape trajectory angle (A4)	
	U	P	W	P
Control-high CO_2	33.0	0.005*	3.1898	0.3183
Control-high temp.	73.0	0.921	1.4708	0.4936
Control-combined	51.0	0.183	1.7159	0.4388
High CO_2-high temp.	18.0	0.006*	4.0339	0.200
High CO_2-combined	51.0	0.553	4.3998	0.1225
High temp.-combined	28.0	0.096	0.4584	0.8044

* Significantly different. Mann–Whitney *U* test and Mardia-Watson-Wheeler test were used for the pairwise comparisons of initial orientation and escape trajectory angle between treatments, respectively; $n = 47$

Table 3 Response latency and durations of stage 1 (s1) and stage 2 (s2) of the Japanese anchovy *Engraulis japonicas* in control, high CO_2, high temperature and combined treatments (mean ± SD)

Treatments	Response latency ms	s1 duration ms	s2 duration ms
Control	6.3 ± 4.0	22.1 ± 5.4	21.7 ± 3.4
High CO_2	7.3 ± 2.6	24.8 ± 10.6	21.4 ± 4.1
High temp.	4.8 ± 1.7	21.8 ± 9.4	22.3 ± 3.5
Combined	4.8 ± 3.0	27.1 ± 10.9	21.3 ± 3.0

Statistical analysis

Because of the unexpected mortality during the acclimation period, a total of 48 fish were used for testing C-start response. One fish in the high temperature group failed to respond to the stimulus. Therefore, data from 47 individuals were used for analysis (15 in the control treatment, 12 in the high CO_2 treatment, 10 each in high temperature and combined treatments). The SPSS 16 was used to analyze all the measurement parameters. Two-way ANOVA with temperature and CO_2 as fixed factors was used to compare response latency. A binary logistic regression was applied to analyze response type and directionality (away and toward the stimulus), in which the response type or directionality was regarded as the objective variable, and temperature, CO_2 and their interaction were regarded as explanatory variables. The significance of these explanatory variables were assessed by using the Wald *t* test. The initial orientation was compared by a Kruskal–Wallis test, followed by Mann–Whitney *U* test for pairwise comparison. Mardia-Watson-Wheeler test was applied to detect differences in angular variance of escape direction between treatments. Because of multiple pairwise testing, Bonferroni correction was applied to adjust the alpha for each comparison. There were six hypotheses being tested at *P* value 0.05, and therefore, the new critical *P* value is either 0.05/6 or 0.008. Two-way ANCOVA was applied to assess the effect of temperature and CO_2 on the s1 and s2 durations, turning rate, cumulative distance, maximum velocity, and maximum acceleration. Webb [57] observed that fast-start increased with size while Domenici and Blake [58] interpreted that the initial orientation of the fish had affected the directionality of escape. Therefore, the effect of both fish size and initial orientation were also included as explanatory variables to estimate all the parameters.

Results

Initial orientation and timing variable

The median of initial orientation angle was found to be 150.8, 98.8, 146.7, and 113.8 degrees in each of the control, high CO_2, high temperature and combined treatments, respectively. The initial orientation angle was significantly smaller in high CO_2 treatment, compared to the control and high temperature treatments (Table 2). The data on response latency, s1 and s2 durations are shown in Table 3. Two-way ANOVA detected no significant effect of temperature, CO_2 or their interaction on the response latency (Table 4). Similarly, two-way ANCOVA failed to detect significant effects of temperature, CO_2, or their interaction on the s1 duration or s2 duration (Table 4).

Directionality and escape trajectory

The proportions of fish whose first detectable movement was away from the stimulus in control, high CO_2, high temperature and combined treatments were 40, 50, 60, and 60 %, respectively. Binary logistic regression indicated that temperature or CO_2 did not have statistically significant effect on directionality (Table 4). Multiple Mardia-Watson-Wheeler test with adjusted *P* value showed no significant difference in escape trajectory angle between treatments (Table 2; Fig. 2).

Turning rate and response type

The turning rate was calculated for both single bend (SB) and double bend (DB) responses. The turning rate at 19 °C was significantly higher than those at 15 °C (Table 4; Fig. 3a). Binary logistic regression showed neither temperature nor CO_2 affected response type (Table 4; Fig. 3b).

Table 4 Statistical analysis of the effects of temperature, CO_2 and their interaction on the escape response of the Japanese anchovy *Engraulis japonicas*

Factors	L_t		T_r		s1 duration		s2 duration		D		V_{max}		A_{max}		D_t		R_t	
	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	$F_{(1,46)}$	P	Wald t	P	Wald t	P
Temp.	3.417	0.071	16.18	0.001*	0.185	0.670	0.055	0.816	0.690	0.415	0.076	0.785	1.043	0.313	2.275	0.131	0.057	0.812
CO_2	0.329	0.057	0.180	0.893	0.780	0.781	1.127	0.266	0.044	0.835	0.519	0.476	0.002	0.961	0.872	0.351	0.893	0.345
Temp. × CO_2	0.329	0.057	2.291	0.137	0.300	0.864	1.193	0.281	3.090	0.860	1.259	0.268	2.873	0.096				

* Significantly different. Two-way ANOVA was used for the analysis of response latency (L_t), two-way ANCOVA for turning rate (T_r), stage 1 (s1) and stage 2 (s2) durations, cumulative distance (D), maximum velocity (V_{max}) and maximum acceleration (A_{max}), and binary logistic regression for directionality (D_t) and response type (R_t)

Kinematics

The mean (±SD) values of cumulative distance (D), maximum velocity (V_{max}), and maximum acceleration (A_{max}) within the average s2 duration (48 ms) of control, high CO_2, high temperature and combined treatments are shown in Fig. 4. ANCOVA showed that there were no significant effects of temperature, CO_2 or their interaction on D, V_{max}, or A_{max} (Table 4).

Discussion

This is the first study that has examined separate and combined effects of elevated temperature and CO_2 on the escape response of fish. The results demonstrated that neither elevated temperature nor CO_2 concentration or their combination affected any of the measured variables of solitary anchovies except turning rate, which was significantly higher at 19 °C than at 15 °C irrespectively of CO_2 levels.

The maximum velocity (1.4–1.6 m/s) found in this study is in good agreement with the relationship between body length and V_{max} obtained from the data from five species of fish (1.3 m/s for body length 0.1 m) [59]. The observed ranges of A_{max} (63–117 m/s^2) and turning rate (1.7–3.2 degrees/ms) are both well within the range reported for other fishes. Both the sum of stage 1 and stage 2 durations (44–48 ms) and response latency (5–7 ms) are relatively short, but are still within the range reported for other fishes from other studies [24, 25]. The relatively low proportion of away response (40–60 %) may have resulted from the initial orientation angles being larger than 90°. It has been shown that the proportion of away response can be as high as 90 % when a fish is stimulated sideways, but it decreases when the initial orientation angles deviate from 90° [25]. Our video recording showed that 50–75 % of fish was stimulated sideways.

Response latency is a behavioral measure directly correlated to the activity in the brainstem escape network that includes the two Mauthner cells [26], and thus the measurement of response latency yields important information with respect to the temperature dependence of the system. It has been reported that response latency decreases with temperature [60, 61]. The response latency in our study tended to be shorter at higher temperature (Table 3), but the difference was not statistically significant. This may have been due to the relatively small temperature difference used (4 °C) as compared with the much wider habitat temperature range of the Japanese anchovy (15–29 °C) [62].

The escape responses observed for the Japanese anchovy are somewhat different from those reported by Allan et al. [47], who studied the effects of ocean acidification on escape response in juvenile coral reef fish, *Amphiprion*

Fig. 2 Circular frequency distribution of escape trajectory angle of the Japanese anchovy defined as swimming direction at the end of stage 2 with respect to the stimulus orientation at 0°. The *number* beside a sector represents the number of fish that had an escape trajectory angle within each central angle division of 36°. **a** control, **b** high CO_2, **c** high temperature, and **d** combined
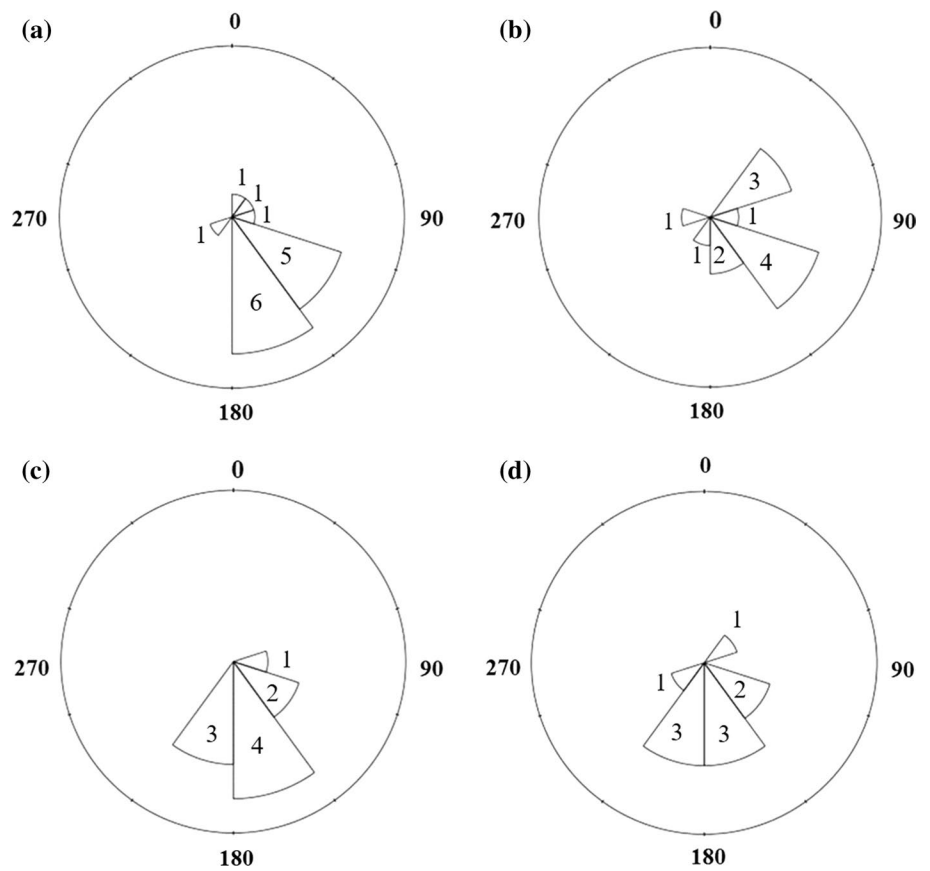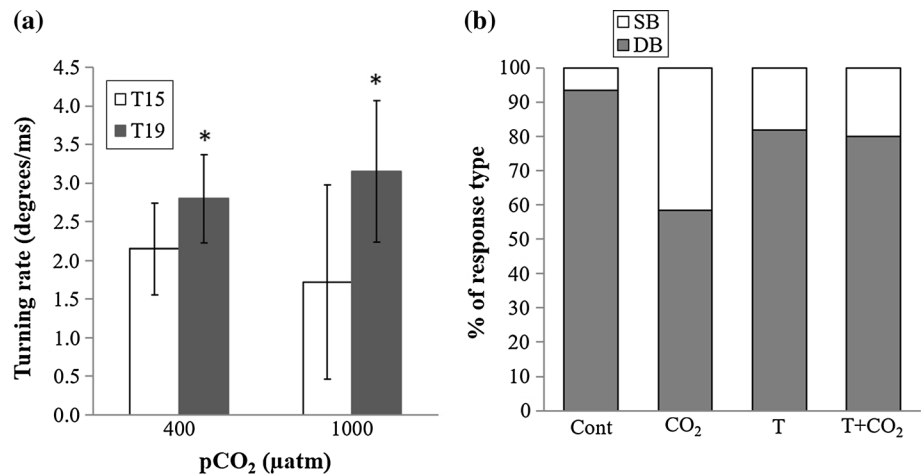

Fig. 3 **a** Comparison of turning rate between the four treatments (mean ± SD). **b** Percent frequency of single (SB) and double bend (DB) response in each treatment. *Significant difference between 15 and 19 °C data ($P < 0.05$, Two-way ANOVA). *Cont* control, CO_2 high CO_2, *T* high temperature, $T + CO_2$ combined

melanopus. These authors tested escape responses in control (400 µatm) and high (1087 µatm) CO_2 conditions, using juveniles produced by the parents that had been kept in the respective conditions for 11 days. The comparison between control and CO_2-treated juveniles from control parents detected significant reductions in: (1) response distance (the total distance covered by the fish during the entire escape response until the fish stopped movement); (2) mean swimming speed; (3) V_{max}; (4) the proportion of fish that showed away response in directionality; and (5) response duration (the duration of entire escape response). In addition, a significant increase was detected for the proportion of non-reactive fish, while no difference was found in response latency. CO_2-treated juveniles from the parents acclimatized to high CO_2 conditions largely showed intermediate responses. Data from this study showed no change in V_{max} or the proportion of non-reacting fish (with only one fish in high temperature group failing to respond). It
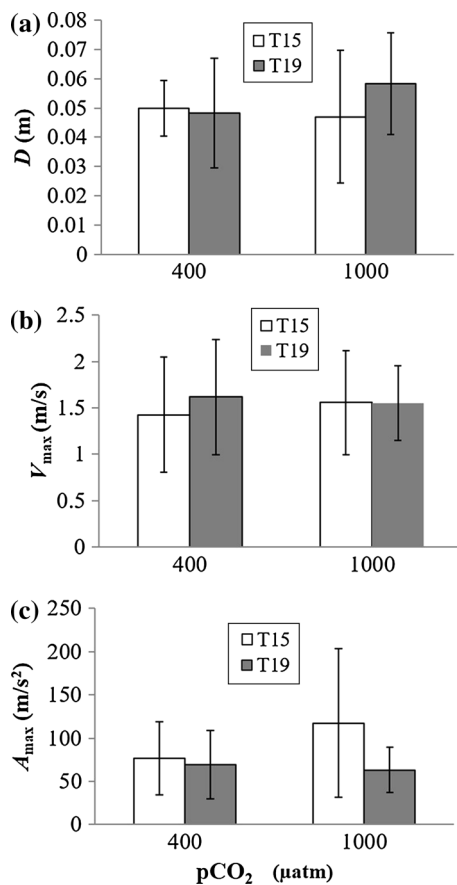
Fig. 4 Comparison of swimming kinematics of the Japanese anchovy in four treatments (mean ± SD). **a** cumulative distance, **b** maximum velocity, and **c** maximum acceleration

is not clear whether these differences in escape response between the species are due to the differences in life stages of test fish (i.e., juveniles vs. adults), test temperature (15 and 19 °C vs. 28.5 °C) or acclimation period (11 days vs. 1 month) or the neuromechanical nature of the kinematic behavior inherent to each species.

The turning rate was the only parameter that showed a significant difference between treatment groups in this study (Fig. 3a). Turning rate determines how fast a fish turns its body into C shape following a stimulus, and therefore, a higher turning rate would potentially lead to a higher rate of escape from predators. Walker et al. [63] demonstrated that the following abilities of the prey affected evasion outcome; the ability of the prey to generate rapid tangential acceleration (V_{max} and A_{max}), and the ability of the prey to rapidly rotate during the initial stage of the fast start (i.e., a higher turning rate), together with the evasion path of the prey relative to the strike path. An elevation of 4 °C increased turning rate from 0.65 degrees/ms (400 ppm CO_2) to 1.44 degrees/ms (1000 ppm CO_2). Temperature is also known to increase reaction distance, latency, responsiveness, distance, speed, and acceleration in fish [25]. These effects may be substantial when a test fish is subjected to acute temperature change, but will usually subside after temperature acclimation [44]. Considering the range of temperature rise projected (4–5 °C), which will gradually occur over the next decades, temperature will probably exert a minor, if any, direct influence on fish escape behavior. It is likely that increasing daily temperature fluctuations during extreme weather events would have much stronger effects on the behavior.

Although our data demonstrated the relative robustness of escape response by the Japanese anchovy under simulated future conditions, this does not imply that the fish will be subjected to a lower risk when attacked by a predator in the high CO_2, warmer oceans. For instance, there is now ample evidence that the predicted CO_2 level in 2100 will affect the sensory perception of external stimuli in fish, which is a crucial step in initiating an escape response (see Introduction). Thus, there is a possibility that prey fish would incur a higher risk of predation through impaired perception of approaching predators. On the other hand, how future oceanic environment may affect attack behaviour of predators needs to be examined for the mechanistic understanding of prey-predator interaction in changing marine conditions. Another important consideration is each species' tendency to form schools or aggregations. The Japanese anchovy, like many other small pelagic fishes, forms a large school in nature [51]. Although schooling individuals can become solitary when they are sequentially attacked by predators [64], Domenici and Batty [65] reported that escape responses shown by fish in school may differ quantitatively in kinematics from those of solitary individuals, and they speculated that a solitary herring (a schooling species) may employ more toward responses, as a result of an alternative strategy aiming at increasing the unpredictability of the response. Namely, schooling fish can take advantage of the confusion effect while solitary fish cannot. However, these authors also pointed out that individual herrings in a school can be in effect solitary for a brief period. Therefore, to understand how these small pelagic fish will be affected by the future oceanic environmental changes and how the effect will propagate to higher trophic levels, it is crucial to analyze escape responses of the fish in both school and solitary conditions, and to investigate how prey-predator interaction of solitary and schooling fish will be influenced under simulated future oceanic conditions.

Recently, Cornwall, and Hurd (2015) reported that 94 % of papers published since 1993 in the field of ocean acidification research had employed experimental designs that fall short of the rigorous statistical requirements; treatments within manipulation experiments must all contain adequate numbers of randomly interspersed and independent treatment replicates [66]. To satisfy the level of independence of each experimental unit these authors recommend, each

anchovy must have been acclimated to an experimental condition in a separate tank, or multiple anchovies must have been acclimated to multiple tanks with three or more header tanks per treatment. Though ideal, these designs would have been logistically highly demanding and practically quite difficult to achieve because the Japanese anchovy is a species that does not easily acclimate to laboratory conditions and needs extreme care to handle, which was evident from the unfortunate high mortality we experienced in this study. Repeated investigations from independent research institutions on different species of commercially important, small plegic fishes would improve our prediction capacity for the trajectory of these fishes and fishery resources in the future oceanic environment, as long as they meet statistical requirements to the maximum feasible extent to prevent pseudoreplications in the experimental design. With these limitations in mind, we still hope that the present results form a basis for further research to predict how commercially important, small pelagic fish will be affected by warmer and more acidified future oceans.

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