Behaviour alterations of coral reef fish due to elevated ocean CO_2 levels and the need for long-term research.

Abstract:

Due to the rapid increase in anthropogenic atmospheric CO_2, seawater CO_2 concentrations are also increasing at an alarming rate. Levels of dissolved seawater CO_2 are predicted to reach 800 to 1,000 ppmv at the end of the century. This increase in CO_2 is part of a phenomena called ocean acidification and reportedly has detrimental effects on the behaviour of coral reef fish species. The complete reversal of predator cue recognition is often reported to be one of the most alarming behavioural effects of elevated CO_2 concentrations. However, recent replication studies testing with larger sample sizes or trials with longer intervals do not find this result. Since ocean acidification is a phenomenon with vast implications, there exists a need for long-term research in order to quantify the effect of elevated CO_2 concentrations and reach a undivided scientific consensus on these effects.

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1. Introduction

In the last 300 hundred years the level of atmospheric CO_2 on earth has increased from 280 ppmv (parts per million by volume) to over 418 ppmv at the end of 2022 (Doney *et al.,* 2009; IPCC, 2007; Lan *et al.,* 2023). For the past 400,000 years these CO_2 concentrations have oscillated between 200 and 280 ppmv (Figure 1) (Feely *et al.,* 2004; IPCC, 2007). The current concentration however, exceeds the highest concentration of at least the last 800,000 years (Hönisch *et al.,* 2012; Lüthi *et al.,* 2008) and the rate of increase is the highest observed for millions of years (Doney and Schimel, 2007). Because around 30% of the atmospheric CO_2 is sequestered by the Earth's oceans (Feely *et al.,* 2004), this increase in atmospheric CO_2 will also lead to an increase in ocean CO_2 concentrations. Dissolved CO_2 concentrations in seawater are predicted to possibly reach levels of 800 – 1,000 ppmv at the end of the century (IPCC, 2007). This is the highest concentration for marine life in the past 30 million years (Hönisch *et al.,* 2012). Furthermore, this elevated CO_2 concentration alters ocean chemistry and possibly lowers the pH by as much as 0.3 – 0.4 units (See box 1 in the appendix on ocean acidification chemistry) (Caldeira and Wickett, 2003; IPCC, 2022). These alterations are commonly referred to as Ocean Acidification (OA).

The subject of OA has seen a significant increase in research over the last two decades (Browman, 2016). First literature published on the topic primarily focussed on changes in seawater chemistry, due to elevated CO_2 concentrations, and the implications it has for calcification and physiological processes in marine life. Research on the alterations of behaviour appeared later on. Commonly, articles published in these early days of OA research reported detrimental effects and large effect sizes of short-term exposure to elevated CO_2 concentrations in a number of different aspects of marine life. Specifically for the behavioural research, a whole range of different behavioural traits have been reported to be significantly affected by OA (Clements and Hunt, 2015).
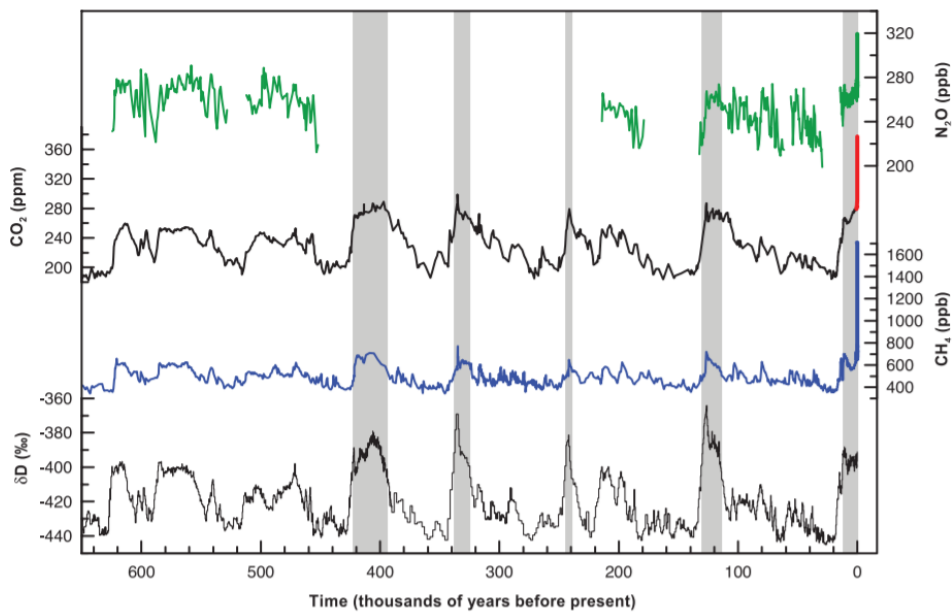
GLACIAL-INTERGLACIAL ICE CORE DATA

Figure 1 | Variations in deuterium (δD), carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) from ice cores and recent atmospheric measurements over a period of 650,000 years. Shaded bands indicate interglacial warm periods. (IPCC, 2007)

Behaviour is a vital component in the functioning and the formation of populations, communities and ecosystems. Complex interactions of organisms, both inter- and intraspecific, are fundamental for ecological processes in ecosystems (e.g. predation, competition, symbiosis). Environmental conditions can affect the behaviour of organisms, which can lead to changes in species interactions and can affect population dynamics and ultimately biodiversity (Nagelkerken and Munday, 2016). The first response of animals to a changing environment is mostly through modification of their behaviour (Tuomainen and Candolin, 2011), which can be seen as an early warning system for altering or deteriorating ecosystems (Hellou, 2010). Nowicki *et al.* (2012) already reported that climate change induced alterations of marine environments, such as elevated sea surface temperatures, influence the behaviour of marine organisms. Therefore, there exist an interaction between the physical environment and the outcome of ecological processes, in which the behaviour of organisms plays an important role (Hay, 2009).

Lots of behavioural OA research primarily focusses on the modification of a single variable, such as single species, life phase or a single type of behaviour, despite the important interaction between environmental change, behaviour, and ecological processes (Nagelkerken and Munday, 2016). For instance, a lot of studies concentrate on tropical fish species, particularly ones associated with coral reefs (Briffa *et al.*, 2012; Clements and Hunt, 2015). Species of *Pomacentridae* (damselfishes) are often used in behavioural OA research and are regarded as model species because they belong to a diverse family of coral reef fish species and are easily reared in captivity (Munday *et al.*, 2009c). Early studies which used larval fish stages of these species reported detrimental effects of elevated CO_2 concentrations to behaviour and a heightened sensitivity of this group to OA (Munday *et al.*, 2009a, 2010). Measurements of elevated CO_2 (anthropogenic CO_2) levels in ocean waters, however, indicate that CO_2 levels are rising over a wide range of latitudes on Earth (Figure 2) (Feely *et al.*, 2004). This suggests that the rapidly increasing effect of OA could potentially impact a broad spectrum of different ocean ecosystems and species, all over Earth. Previous research already indicated the fragility of coral reef ecosystems in relation to the impact of climate change (Hoegh-Guldberg, 1999), however, the
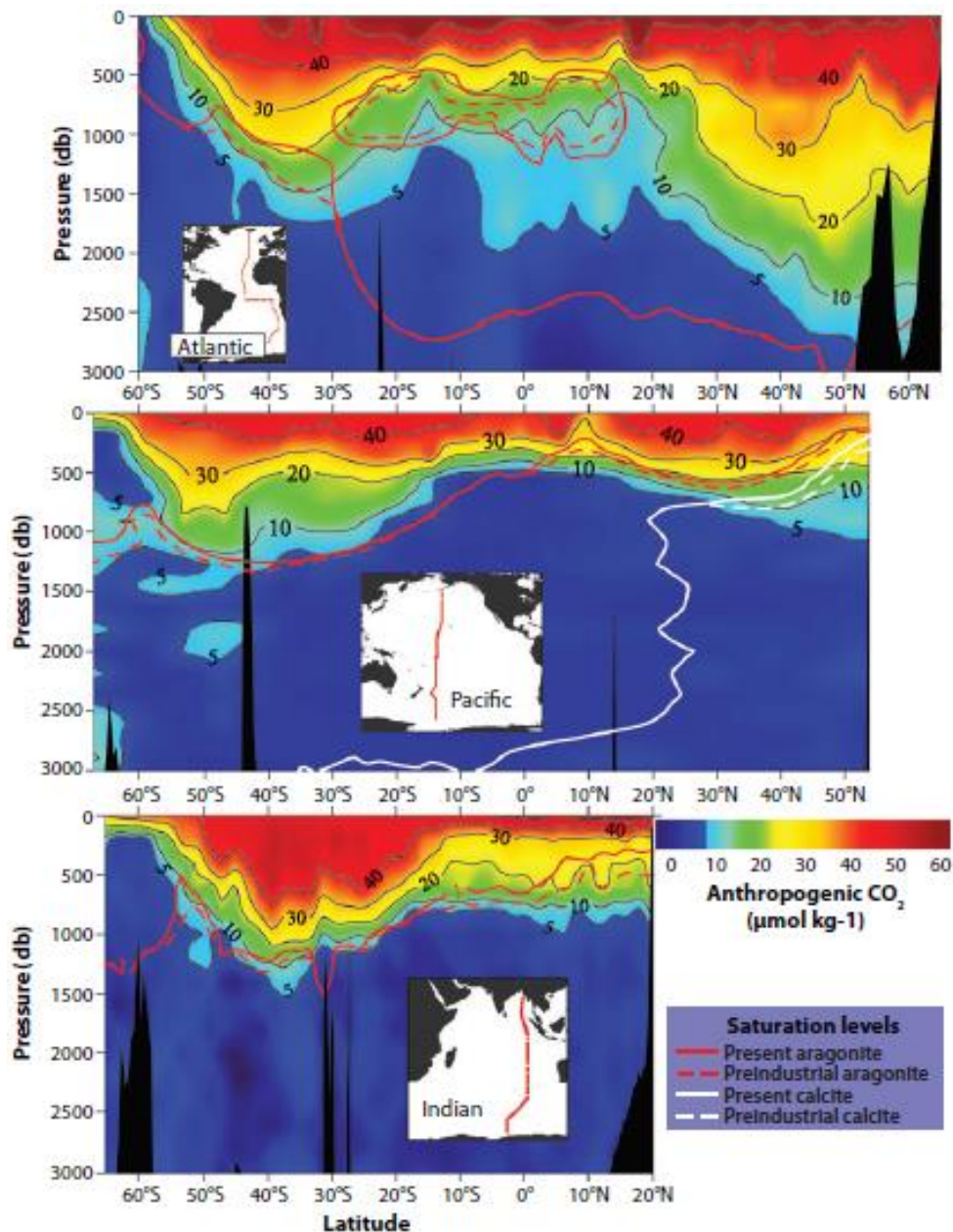
Figure 2 | Vertical distribution of anthropogenic CO₂ and saturation levels for aragonite and calcite along a north-south transect in the Atlantic (top), Pacific (middle) and Indian (bottom) Oceans (Feely et al., 2004).

broad effect of OA will remain poorly understood if behavioural research continues to focus on a singular group of organisms in a single type of ecosystem.

Pioneering behavioural research within OA often concluded strong effects (e.g. becoming attracted to predator cues instead of avoiding them (Munday *et al.*, 2010)) and low variability among tested individuals, even though individuals were tested after only short exposure to elevated CO_2 concentrations (4 days). Already in 2008, Ishimatsu and co-authors concluded that long-term exposure experiments covering entire life stages were needed to realistically predict effects of OA, due to long-term energy expenditure and increased mortality in fish populations. In contrast, more recently published studies often do not find such strong effects (Clements *et al.*, 2022; Sundin, 2023). When replicating the research with increased exposure time or increased sample size, multiple studies have

found no significant alteration of behaviour as a result of elevated CO_2 levels (e.g. Clark *et al.*, 2020; Sundin *et al.*, 2017a, 2017b, 2019).

Due to the lack in understanding of the consequences of OA to the behaviour of marine organisms, even though this behaviour is an important factor for the functioning of ecosystems, combined with the pressuring timeframe to quantify this research, this paper reviews some of the behavioural studies within OA regarding predator-prey interactions and activity levels, highlighting important findings. Furthermore, it will examine some points of discussion regarding the behavioural research over the years. Because the amount of research within this topic has increased rapidly over recent years, reviewing the entirety of the topic reaches far beyond the scope of this paper. It therefore discusses some of the highly cited research regarding predator-prey interactions and levels of activity of coral reef fish species. These are seen as important because they directly influence the energy expenditure and survivability of organisms, driving population dynamics and ultimately biodiversity within an ecosystem.

2. Alteration of behaviour

Over time, behavioural research within OA has covered different types of behaviour within a range of behavioural contexts. Feeding behaviour, predator-prey interaction, cue recognition, learning ability, swimming activity, boldness, exploratory behaviour, lateralization, auditory response and choice are all behavioural processes that have been studied in relation to OA and are reported to be affected in some way (Briffa *et al.*, 2012; Clements and Hunt, 2015; Nagelkerken and Munday, 2016). Below are mentioned some studies regarding predator-prey interactions and swimming activity. Any physical effect of OA on marine organisms may result in the alteration of the energy budget of that animal, for example by affecting the aerobic performance (Munday *et al.*, 2009b). This could result in the offset of timing and performance in predator strikes or escape attempts by predatory and prey fish (Allan *et al.*, 2013). Changes in these behavioural processes may affect species survival, population dynamics and ecosystem functioning.

Predator-prey interactions

Chemical cues and signals play a varied but critical role in the behaviour of organisms in marine ecosystems. They, for instance, determine feeding behaviour and predator avoidance, whether to interact with other organisms or not (e.g. mating choice) and are of influence in locating save habitats (Brown, 2003; Hay, 2009). Correctly recognising and using chemical cues can greatly increase an individual's fitness (Hay, 2009). Change in this type of behaviour (such as the complete reversal of predator cue recognition) can be detrimental for an individual's survival and is therefore often remarked as the most alarming effect of OA (Clark *et al.*, 2020). Behavioural alteration in cue recognition can impact species mortality rates and population dynamics.

Predator cue recognition

In 2010, Munday *et al.* tested the ability of clownfish larvae (*Amphiprion percula*) to recognise predator cues in CO_2 enriched environments. Because of the high mortality rate when transitioning from the planktonic larval stage to the benthic adult stage, correct assessment of predator cues is important for survival of clownfish in these early life stages (Munday *et al.*, 2010). In order to test this ability, directly after hatching larval clownfish were reared in one of four CO_2 environments. In accordance with end of the century predictions, these consisted of 550, 700 and 850 ppmv CO_2 for the elevated conditions and~390 ppmv CO_2 for the control conditions (approximate ambient seawater CO_2 concentration at the time of the experiment).

For up to 10 days after hatching the behavioural response of the larvae to a cue of a common reef predator (*Cephalopholis cyanostigma*) was tested in a two-channel choice flume (Figure 3). In this
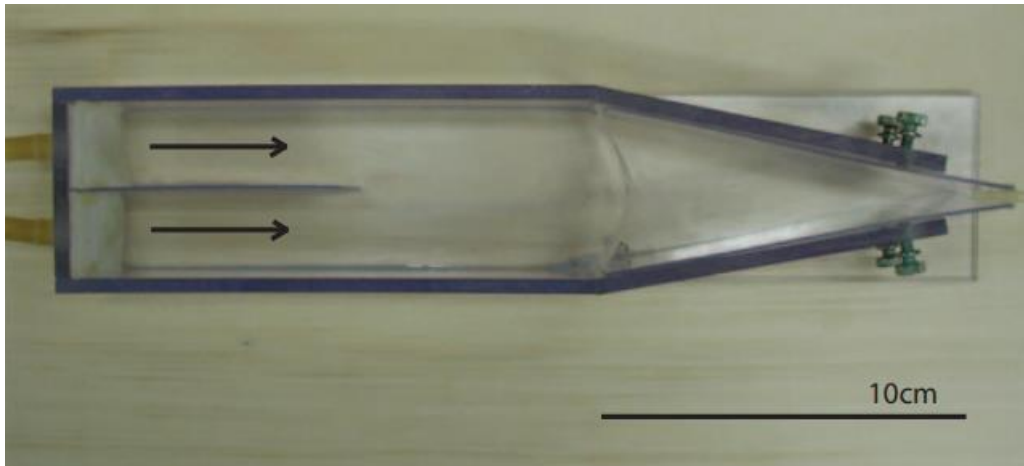
Figure 3 | A two-channel choice flume as used in multiple behavioural studies. Water from two different sources enters the flume on the left and flows in the direction of the arrows. Fish would be positioned in the central part of the flume for testing. Photo from Munday et al., 2009a.

setup ambient seawater containing the predator cue was fed through one of two channels, the other channel was fed seawater containing no additional cue. For the experiment, larvae were placed downstream in the flume where they were free to move to either side. Preference or avoidance for a side of the setup was then recorded. As a control for side habituation, water sources were switched halfway through the experiment. Approximately 20 individuals were tested for each CO_2 treatment each day and individuals were never tested twice (n=18-25).

Both the clownfish in the control group and clownfish reared at 550 ppmv CO_2 showed strong avoidance of the predator cue from day 1 and completely avoided the cue by day 8 (Figure 4). Larvae reared at 700 ppmv CO_2 initially avoided the predator cue, but started to become more attracted to the cue from day 4 (~30-45% of time spent on cue side) (Figure 4). Notably, a duality in response was present among these larvae. About half of the tested individuals continued to avoid the predator cue, with stronger avoidance of the cue over time. The other half of individuals became strongly attracted to the cue, spending 74-88% of their time in the stream containing the predator cue. Clownfish reared at 850 ppmv CO_2 avoided the cue on day 1, but subsequently became more attracted to the cue, spending over 94% of their time on the cue side of the flume at day 8 (Figure 4). No duality in response was observed within this group.
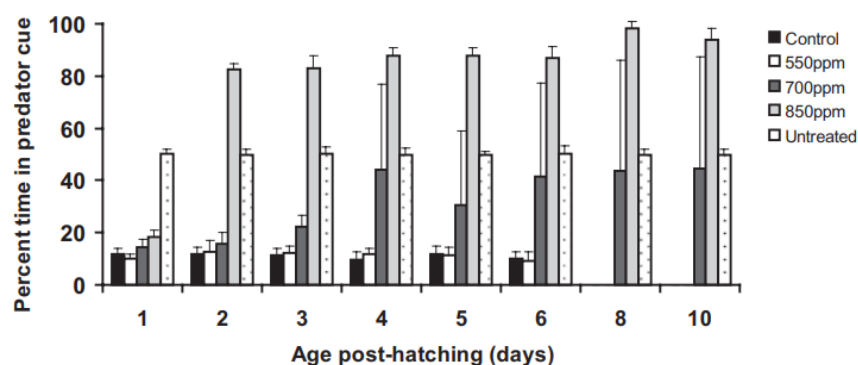

Figure 4 | Effects of elevated CO_2 concentrations on olfactory ability of clownfish larvae (Munday et al., 2010). Bars indicate mean time (±SD) spent in predator cue by clownfish larvae. Untreated category shows time spent on either side of the flume when no cue was added to the setup.

Habitat cue recognition

In a comparable study on olfactory impairment, Munday *et al.* (2009a) analysed the change in response of clownfish larvae (*A. percula*) to olfactory cues from different habitats in elevated CO_2 conditions. Similar to predator cues, correct assessment of habitat cues is important for the sustainability of populations of clownfish on coral reefs (Munday *et al.*, 2009a). Habitat cues from adult reefs benefit the recruitment of juvenile clownfish, ensuring settlement in suitable environments. Any change in the ability of clownfish larvae to recognise these cues potentially affects population dynamics.

For experimentation, clownfish were reared in one of three different pH levels from the egg-phase up to the settlement stage of the larvae (11-12 days posthatching). Rearing conditions (obtained through increasing CO_2 concentrations of water in the rearing tanks, but measured here in pH) consisted of pH 8.15, 7.8 and 7.6. Ambient seawater with a pH of 8.15 was used as a control, pH 7.8 and 7.6 corresponded to ~1,000 and ~1,700 ppm CO_2 respectively. Testing for behavioural response occurred at 11 days posthatching. In a two-channel choice flume, similar as described above, preference or avoidance of clownfish larvae to four different habitat cues and ambient seawater (seawater without additional cue, for control) was recorded. Prior research already indicated that clownfish larvae use water-borne cues from vegetated islands near coral reefs when locating suitable reef habitats (Dixson *et al.*, 2008). Therefore, attracting cues from a tropical rainforest tree (*Xanthostemon chrysanthus*), repelling cues from a swamp tree (*Melaleuca nervosa*), a neutral cue from a species of grass (*Megathyrsus maximus*) and cues from a host anemone were used in the experiment.

As expected, larvae reared in pH 8.15 seawater showed strong preference for cues of *X. chrysanthus* and the host anemone, avoided the *M. nervosa* cue and had no preference for the *M. maximus* cue (Figure 5). In comparison, larvae reared at pH 7.8 showed a reduced response to the *X. chrysanthus* and host anemone, while significantly increasing their preference for the *M. nervosa* and *M. maximus* cues. In case for the swamp tree cues, fish reared at pH 7.8 now spend over 80% of their time in the stream containing this cue, whereas control larvae completely avoided this cues (Figure 5). Larvae reared at pH 7.6 (not shown in figure) were not responsive to olfactory cues in testing at all. Clownfish of this group remained passive inside the experimental setup, even when testing was conducted using control seawater. Larvae reared in control seawater and tested in water with pH 7.6 did not show significant different behaviour from fish reared and tested in control seawater. Thus indicating that olfactory impairment is a result of prolonged exposure to low pH seawater rather than an immediate alteration of the chemosensory capability of clownfish larvae.
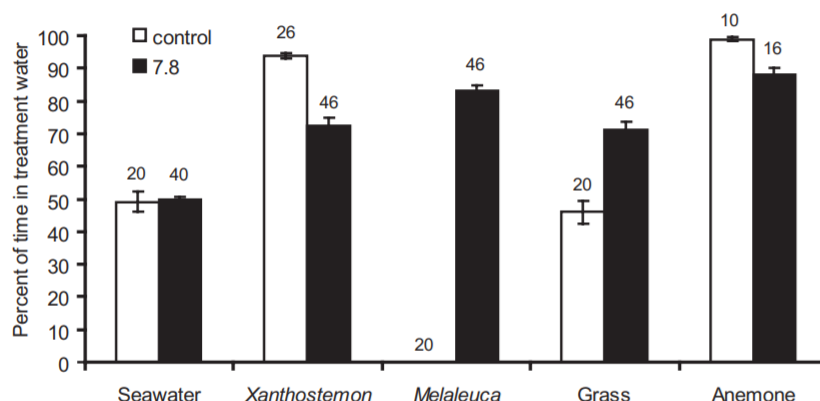

Figure 5 | Effect of pH on olfactory ability of clownfish larvae to recognise habitat cue (Munday et al., 2009a). Bars indicate mean time (±SD) spent in cue by clownfish larvae. 'Seawater' category shows time spent on either side of the flume when no cue was added to the setup. Numbers above bars are the number of replicates for each test.

Visual risk assessment

In addition to the chemosensory impairment of cue recognition, Ferrari *et al.* (2012) indicated that also the visual assessment of risks by coral reef prey fish is affected by elevated CO_2 levels. Much like how chemical cues are of importance for the survival of marine organisms, correct assessment of visual cues from predator species can greatly decrease the changes of being captured by a predator. Alteration of visual responses, especially during the transition of marine species from pelagic to a benthic life stage, could therefore be of great influence to biodiversity of coral reef ecosystems (Ferrari *et al.*, 2011).

For the 2012 study, wild-caught juvenile damselfish *Pomacentrus amboinensis* were reared in one of four different CO_2 concentrations for four days (440 (present-day control), 550, 700 and 850 µatm CO_2). After four days of CO_2 treatment the visual risk assessment of individuals, before and after introduction of an adult spiny chromis *Acanthochromis polyacanthus*, was investigated by examining a number of behavioural traits. These traits consisted of foraging, activity level, area use, minimum approach distance to predator and the occurrence of bobbing behaviour (quick, oscillating, vertical movement). A combination of these behavioural traits are common antipredator behaviour for *P. amboinensis*. When detecting a threat, damselfish will decrease foraging, level of activity and area usage, while increasing the distance to the predator and bobbing behaviour (Ferrari et al. 2010).
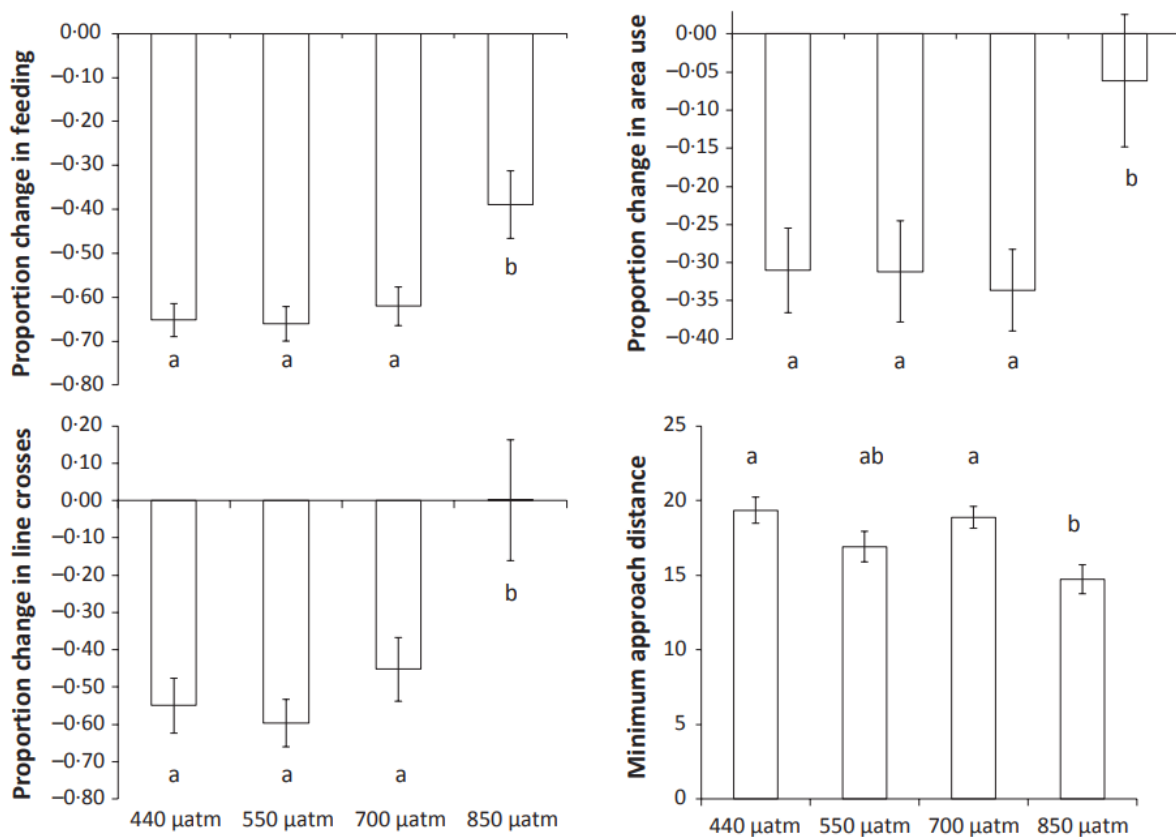

Figure 6 | Mean proportion of change (±SE) in feeding strikes (top left panel), area use (top right panel), line crosses (bottom left panel) and mean (±SE) minimum approach distance (bottom right panel) for juvenile damselfish of different CO_2 treatments and exposed to the sight of a spiny chromis (Ferrari et al., 2012). Different letters refer to statistical differences at a 0.05 α level. N = 22-25 per treatment.

Results from these experiments showed no significant difference in response of the damselfish to the spiny chromis between CO_2 treatments of 440, 550 and 700 µatm CO_2 (Figure 6). There was, however, a significant reduction in antipredator response of damselfish exposed to 850 µatm CO_2. Fish of this CO_2 group showed less reduction of feeding, activity and area use, as well as lower minimum approach

distance and less bobbing behaviour (bobbing behaviour not shown in figure) compared to the other CO_2 treatment groups (Figure 6). This indicated that damselfish became less threatened by presence of a predatory fish in high pCO_2 seawater.

Results from both the chemosensory studies and the visual assessment study indicate impairment of antipredator behaviour of coral reef prey fish. In some cases the complete reversal of cue recognition could be observed, fish would become attracted to predator or non-safe cues that would normally be avoided. Ocean CO_2 concentrations predicted for the end of the century, or in cases even earlier, seem to be detrimental for the antipredator behaviour of these fish species, potentially influencing biodiversity and ecosystem functioning. However, despite these results, more recent research does not support the same conclusion.

Replication studies

In a multi-year study, Clark *et al.* (2020) tested individuals of six different species of the *Pomacentridae* family (*Acanthochromis polyacanthus*, *Chromis atripectoralis*, *Dascyllus aruanus*, *Dischistodus perspicillatus*, *Pomacentrus amboinensis* and *Pomacentrus moluccensis*) for the alteration of predator avoidance behaviour and activity levels after exposure to seawater with elevated CO_2 concentrations. Up until this study, the behaviour of four of these reef species had already been described to be significantly affected in high CO_2 environments (Ferrari *et al.*, 2012; Munday *et al.*, 2014; Welch *et al.*, 2014). Because of the large effect sizes and low variation reported among these studies, similar results were expected for the replications in the multi-year research programme.

In total, predator cue recognition was quantified for 560 individuals of six species in experiments across a period of three years. Prior to experimentation, fish were reared in either seawater with ambient CO_2 concentrations as a current-day control (410 µatm for 2014 and 2015, 520 µatm for experiments in 2016) or in seawater with 1,000 µatm CO_2 for at least 4 days and up to 16. Both adult and juvenile fish were used in testing. Predator avoidance behaviour was tested in a two-channel choice flume, similar to choice flumes described in previous studies. Established protocols and methodology described in these studies were followed as closely as possible. To minimise observer bias, experiments were recorded and analysed using automated tracking software.

Despite the strong effect of elevated CO_2 levels on the antipredator behaviour observed by previous researchers, results of the replication study showed no such effect. Both the test and control group of fish tested in 2014 showed no alteration of antipredator behaviour and significantly avoided the
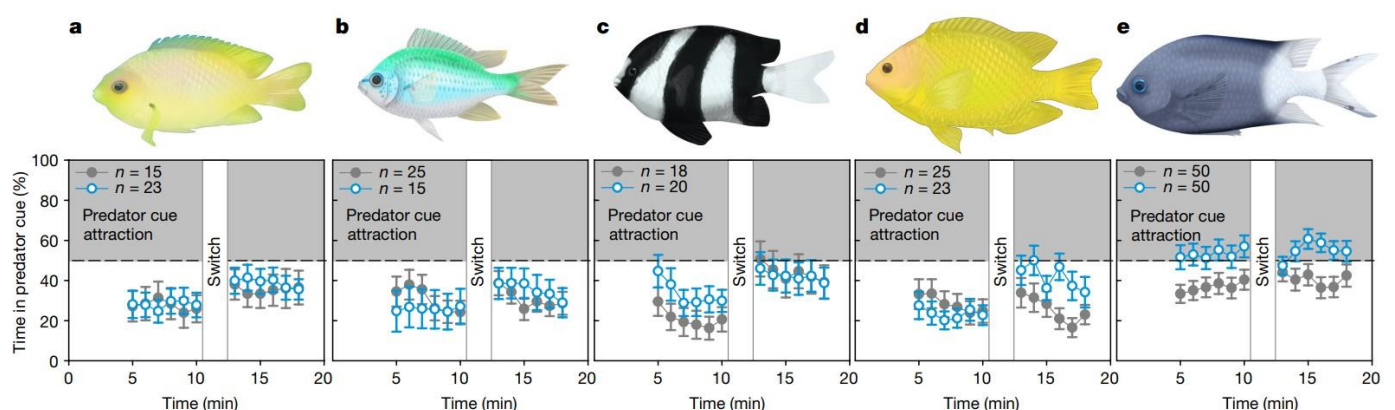

Figure 7 | Predator avoidance in coral reef damselfish exposed to present-day (grey circles) and end-of-century (blue circles) levels of CO_2 (Clark et al., 2020). Species: (a) P. amboinensis, (b) C. atripectoralis, (c) D. aruanus, (d) P. moluccensis, (e) A. polyacanthus. Shown is percentage of time (mean ± s.e.m.) that fish spent in water containing chemical cues of a predator. Results of species D. perspicillatus not shown in figure.

predator cue (Figure 7a-d). In 2015 however, slight preference for the predator cue by the test group was observed (Figure 7e). Captive bred juvenile *A. polyacanthus* spent ~54% of their time on the side of the flume with the predator cue, whereas individuals of the control group spent ~39% of their time on this side (n=100, P <0,001). When tested again in 2016, neither juveniles nor adult *A. polyacanthus* showed this alteration (Figure 8a-b). Furthermore, two other species of fish also tested that year did not show significant alteration of antipredator behaviour as a result of CO_2 treatment (Figure 8c-e). After the introduction of the predator cue, fish tended to spend most of their time on the side of the flume without the cue.
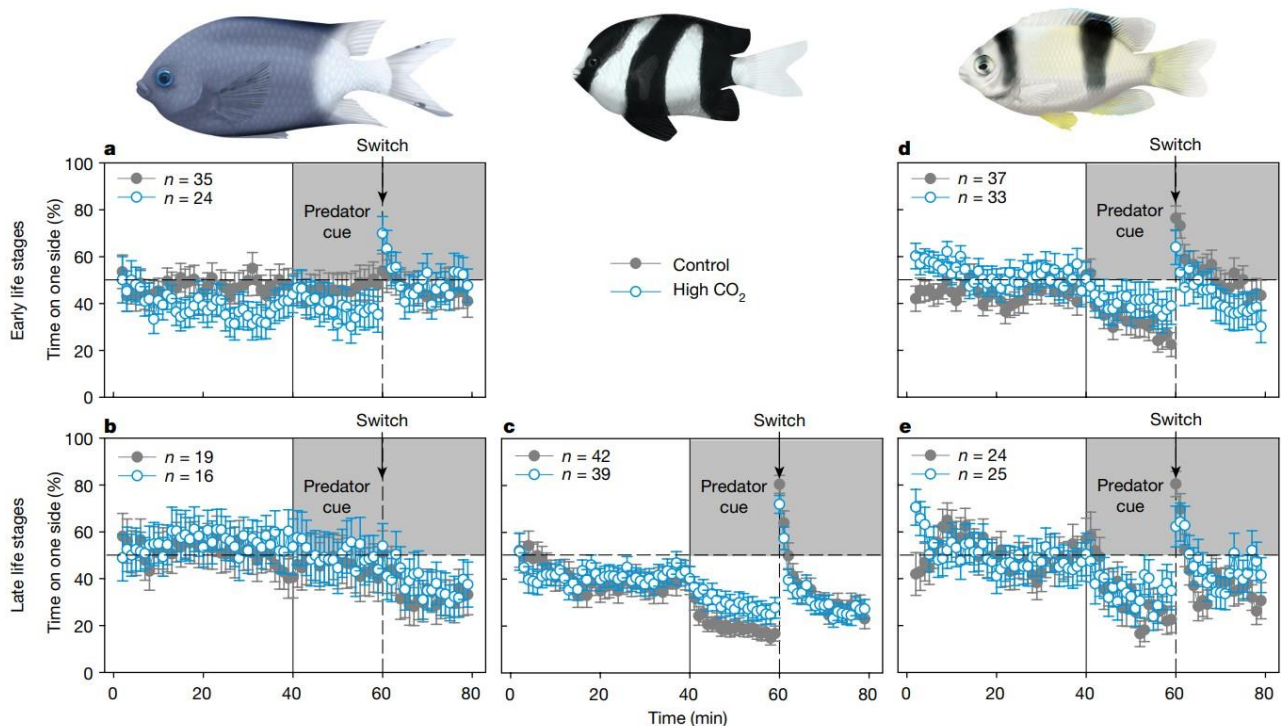

Figure 8 | Predator cue avoidance by damselfish when exposed to present-day (grey circles) or end-of-century (blue circles) CO_2 levels (Clark et al., 2020). Species: (a, b) A. polyacanthus, (c) D. aruanus, (d, e) D. perspicillatus. Shown is percentage of time (mean ± s.e.m.) that fish spent in water containing chemical cues of a predator.

All in all, over a period of three years Clark *et al.* (2020) only found slight alteration of antipredator behaviour as a result of CO_2 treatment in one species tested in one year. Most of the individuals from the six species tested showed no significant alteration of antipredator behaviour after being exposed to seawater with elevated CO_2 concentrations (1,000 µatm). No reversal of antipredator behaviour or attraction to the predator cue, such as found in earlier studies, was observed in the multi-year replication study.

Swimming activity

As mentioned before, any alterations in the energy budget of fish or other marine organism can alter the survivability of these organisms, which in turn could change ecosystem functioning. To that end, OA behavioural research has studied the effect of elevated CO_2 concentrations on the swimming activity of marine species.

Increased swimming activity

For a study published in 2013, Munday *et al.* tested activity levels of 40 juvenile common coral trout *Plectropomus leopardus* after exposure to elevated CO_2 levels for 4 weeks. CO_2 concentrations used consisted of 490 µatm CO_2 for the control concentration and 570, 700 and 960 µatm CO_2 for the experimental concentrations. Behavioural traits tested for in order to quantify activity consisted of
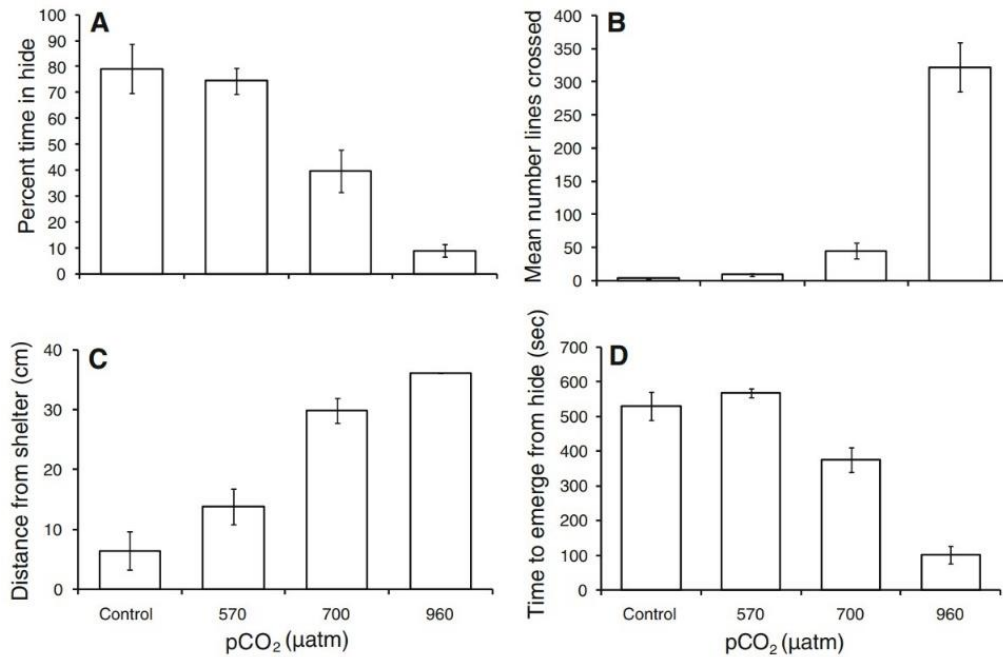
Figure 9 | Behaviour and activity levels of juvenile coral trout (Munday et al., 2013). Shown are the percent time in shelter (A), activity level (B), maximum distance ventured from shelter (C) and time to reemerge from shelter after being chased into the shelter (D). Activity level was assessed as the number of lines crossed on a transparent grid placed in front of the tank. N = 10 for each CO2 treatment.

time spent in shelter, activity levels (number of virtual lines crossed in an aquarium), distance moved from shelter and time taken to reemerge from shelter after being chased into a shelter.

Results from this experiment show a significant increase in activity and boldness of fish reared in higher CO_2 environments (Figure 9). Over the 10 minute trial both the fish from the control and 570 µatm CO_2 treatment spent over 75% of their time inside the shelter, whereas individuals from the 700 and 960 µatm CO_2 treatment spent approximately 40% and less than 10% of their time in hiding respectively (Figure 9A). Furthermore, fish that spent more time outside of the shelter also tended to be more active inside the tanks (Figure 9B). Individuals from the control and 570 µatm CO_2 treatments displayed the lowest amount of activity with only a few lines crossed during the trial, whereas fish of the 960 µatm CO_2 treatment were vastly more active, crossing almost 90 times more lines than the control group (321 to 3.6 lines crossed). Juveniles from the 700 µatm CO_2 treatment showed relative low amounts of activity, crossing around 10 times more lines than controls (45 to 3.6 lines crossed).

Maximum distance travelled from the shelter also rose with higher CO_2 treatments (Figure 9C). The distance travelled increased incrementally from 5-6 cm by fish from the control group to 35 cm (maximum distance inside the tanks) by individuals from the most active 960 µatm CO_2 treatment group. Lastly, the time it took fish to reemerge from a shelter also differed among CO_2 treatments (Figure 9D). Juveniles from both the control and 570 µatm CO_2 treatment took on average 9 minutes to emerge, while individuals of the 700 and 960 µatm CO_2 treatment took 6 and less than 2 minutes respectively.

Results of this study indicate the significant increase in activity of fish reared in seawater with elevated CO_2 concentrations. Predicted seawater CO_2 concentrations for the end of the century could have a strong effect on the energy budget of fish, thereby significantly altering species survival.

No change in swimming activity

Over recent years multiple studies have replicated these swimming activity experiments. In the multi-year research programme by Clark *et al.* (2020) researchers not only tested for predator cue recognition, but also tested for alterations of swimming activity after exposure to elevated CO_2 concentrations. Using 582 individuals from the same six species of damselfish as mentioned above (*A. polyacanthus*, *C. atripectoralis*, *D. aruanus*, *D. perspicillatus*, *P. amboinensis* and *P. moluccensis*) Clark *et al.* quantified activity levels with the use of automated tracking software. Prior to experimentation individuals (juveniles and adults) were reared either at ambient seawater CO_2 concentrations (410 µatm for 2014 and 2015, 520 µatm for experiments in 2016) or at elevated CO_2 concentrations of 1,000 µatm for 4 to 16 days. Activity was then measured for a period of 27 minutes while the fish was inside a flow-through tank or choice flume.

In 2014, no significant difference was observed between the activity levels of the two CO_2 treatments for three out of five species tested (*C. atripectoralis*, *P. amboinensis* and *P. moluccensis;* not every species was tested each year; Figure 10c-e). However, individuals of the high CO_2 treatment group from both *D. aruanus* and *A. polyacanthus* did show significant higher activity compared to the controls (approx. 50-92% increase in activity) (Figure 10a-b). Specifically, an interaction existed between CO_2 treatment and standard length of individuals, indicating that smaller fish exhibited higher levels of activity (P = 0.009 for Figure 10a; P = 0.03 for Figure 10b). It must be noted that *A. polyacanthus* was tested with a relative small sample size (Figure 10a). For testing in 2015 sample size of *A. polyacanthus* was increased from 16 to 66 individuals. This year, no interaction of CO_2 treatment and standard length was observed. Furthermore, replications in 2016 with *A. polyacanthus*, *D aruanus* and *D. perspicillatus* yielded no significant effect of elevated CO_2 concentrations on activity levels (Figure 10a, b and f).

All in all, over a period of three years and across fish of six coral reef species, Clark *et al.* (2020) found little evidence for an interaction between elevated CO_2 concentrations and swimming activity. They further noted the large variability in activity levels among individuals and the need for large sample sizes in order to reduce the risk of type-I errors (Figure 10).
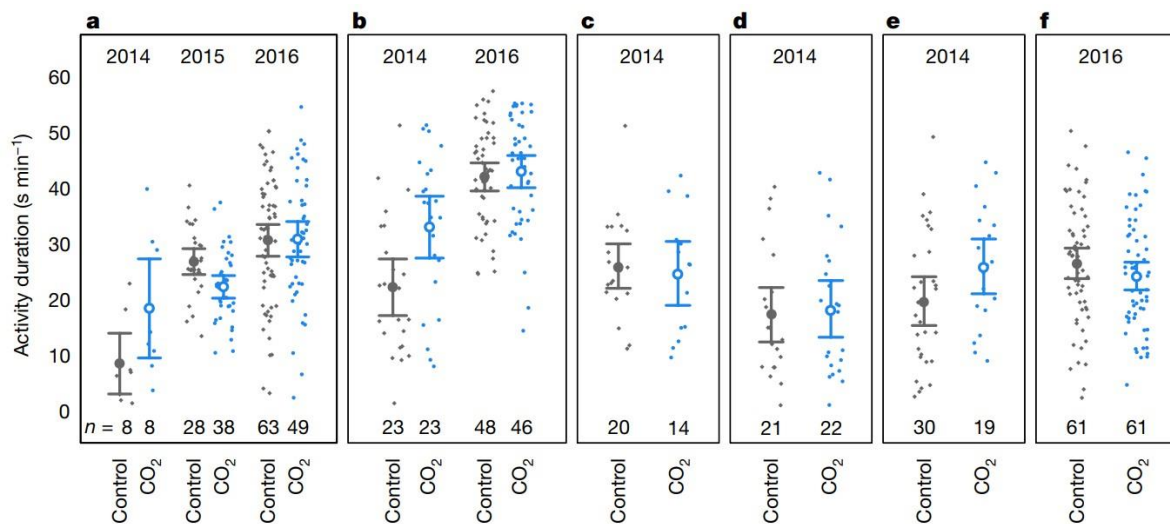

Figure 10 | Activity levels of six species of coral reef damselfish tested across a period of six years after acclimation to control (grey bars) or elevated CO_2 concentrations (blue bars) (Clark et al., 2020). Mean values with ± 95% confidence intervals are shown for each group. Data for (a) A. polyacanthus, (b) D. aruanus, (c) C. atripectoralis, (d) P. amboinensis, (e) P. moluccensis and (f) D. perspicillatus. N numbers shown on the bottom of the panels.

Furthermore, in two studies by Sundin *et al.* (2017a, 2019) researchers found no significant alteration of swimming activity after prolonged exposure of damselfish *A. polyacanthus* to elevated concentrations of CO_2. For these experiments fish were reared in either ambient CO_2 levels of ~420 μatm CO_2 or in a high CO_2 environment of ~1,000 μatm CO_2 for 78 to 82 days. Swimming activity was monitored using automated tracking software. In both experiments fish of the high CO_2 environments did not show any difference in swimming activity from fish of the control group, indicated no effect of CO_2 concentration even after prolonged exposure to a high CO_2 environment.

The replication studies mentioned above are a couple among a growing number of publications indicating the lack of evidence found for the detrimental effect of elevated CO_2 concentrations on the behaviour of coral reef fish. Even though OA poses a threat to ocean ecosystems, the specific finding by early OA behaviour studies that end-of-century predicted concentrations of CO_2 in seawater will drastically alter fish behaviour must be reassessed. Replications of these studies using transparent and improved methods (e.g. automated tracking software, larger sample sizes) must be used to reach a consensus on the effect of elevated CO_2 concentrations on fish behaviour.

3. Discussion

Coral reef ecosystems naturally have large diurnal and seasonal swings in seawater CO_2 concentrations due to processes like respiration and calcification (Ohde and van Woesik, 1999). Concentrations of 100 to 1,300 μatm CO_2 are regularly observed on coral reef ecosystems (Shaw *et al.*, 2012). These concentrations even exceed predicted CO_2 levels for the end of the century. Fish have well-developed acid-base regulatory systems to maintain tissue pH in these high CO_2 environments (Ishimatsu *et al.*, 2005). Therefor, it has long been considered that fish would be able to cope with predicted CO_2 concentrations. Furthermore, this ability to self-regulate tissue pH must be considered as a mechanisms of fish to possibly adapt to increasing CO_2 concentrations over time. This is further strengthened by the large variation in behaviour of fish tested with late-century (around 700 μatm) CO_2 concentrations, as observed in studies by Munday *et al.* (2010, 2013). This variation could benefit selection for more tolerant individuals. Long-term intergenerational studies in the context of OA are scarce, but can be of great importance in understanding the effect of OA to fish over longer timeframes.

4. Conclusion

In a meta-analysis published in 2022, Clements *et al.* indicated that more than a decade of OA research on fish behaviour can be defined by the 'decline effect' (Figure 11). This effect describes the tendency of initial scientific findings (often strong effects with large effect sizes) in a field to diminish over time. Already in 2008, Ishimatsu *et al.* argued for the use of realistic CO_2 concentrations (in line with predictions) in behavioural research, as well as the need for long-term research. This type of research has become more prevalent over recent years, but is still scarce. However, a large part of the publications reporting strong effects are still cited to this day. Furthermore, OA research is a vastly expanding topic increasing with a large number of publications each year (Browman, 2016; Sundin, 2023). Therefore, there exists a great need for quantified research, using transparent methods. This is especially true when studies reporting strong effects can have great implications for management measures and policy making on a widespread phenomenon as ocean acidification.
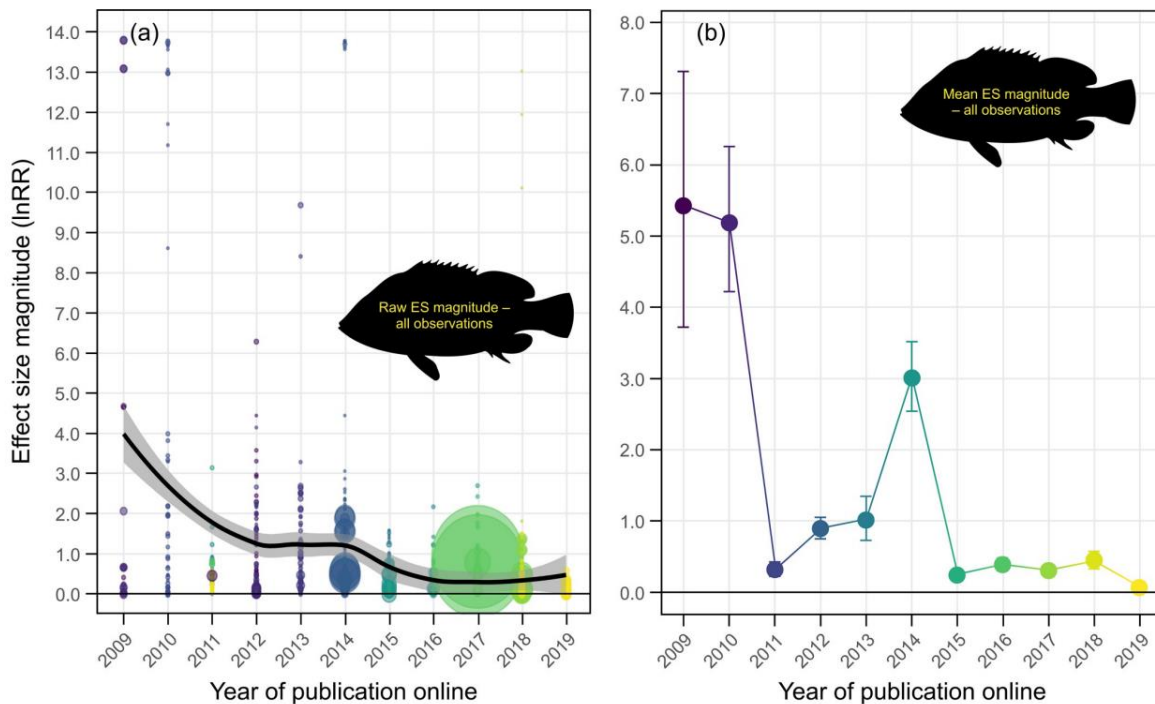
Figure 11 | The decline effect in ocean acidification research on fish behaviour (Clements et al., 2022), (a) raw effect size magnitude over time and (b) mean effect size magnitude over time with upper and lower confidence bounds.

5. References

Allan, B.J.M., Domenici, P., McCormick, M.I., Watson, S.-A., Munday, P.L. (2013). Elevated CO_2 affects predator-prey interactions through altered performance. *PLoS ONE*, **8**(3), Article e58520, doi:10.1371/journal.pone.0058520.

Briffa, M., de la Haye, K., Munday, P.L. (2012). High CO_2 and marine animal behaviour: Potential mechanisms and ecological consequences. *Marine Pollution Bulletin*, **64**(8): 1519–1528, doi:10.1016/j.marpolbul.2012.05.032.

Brown, G.E. (2003). Learning about danger: chemical alarm cues and local risk assessment in prey fishes. *Fish and Fisheries*, **4**(3): 227–234, doi:10.1046/j.1467-2979.2003.00132.x.

Browman, H.I. (2016). Applying organized scepticism to ocean acidification research. *ICES Journal of Marine Science*, **73**(3): 529–536, doi:10.1093/icesjms/fsw010.

Caldeira, K. and Wickett, M.E. (2003). Anthropogenic carbon and ocean pH. *Nature*, **425**(6956): 365–365, doi:10.1038/425365a.

Clark, T.D., Raby, G.D., Roche, D.G., Binning, S.A., Speers-Roesch, B., Jutfelt, F., Sundin, J. (2020). Ocean acidification does not impair the behaviour of coral reef fishes. *Nature*, **577**: 370–375, doi: https://doi.org/10.1038/s41586-019-1903-y.

Clements, J.C. and Hunt, H.L. (2015). Marine animal behaviour in a high CO_2 ocean. *Marine Ecology Progress Series*, **536**: 259–279, doi:10.3354/meps11426.

Clements, J.C., Sundin, J., Clark, T.D., Jutfelt, F. (2022). Meta-analysis reveals an extreme "decline effect" in the impacts of ocean acidification on fish behavior. *PLoS Biology*, **20**(2), Article e3001511, doi:10.1371/journal.pbio.3001511.

Dixson, D.L., Jones, G.P., Munday, P.L., Planes, S., Pratchett, M.S., Srinivasan, M., Syms, C., Thorrold, S.R. (2008). Coral reef fish smell leaves to find island homes. *Proceedings of the Royal Society B–Biological Sciences*, **275**(1653): 2831–2839, doi:10.1098/rspb.2008.0876.

Doney, S.C. and Schimel, D.S. (2007). Carbon and climate system coupling on timescales from the Precambrian to the Anthropocene. *Annual Review of Environment and Resources*, **32**: 31–66, doi:10.1146/annurev.energy.32.041706.124700.

Doney, S.C., Fabry, V.J., Feely, R.A., Kleypas, J.A. (2009). Ocean acidification: The other CO_2 problem. *Annual Review of Marine Science*, **1**: 169–192, doi:10.1146/annurev.marine.010908.163834.

Feely, R.A., Sabine, C.L., Lee, K., Berelson, W., Kleypas, J., Fabry, V.J., Millero, F.J. (2004). Impact of anthropogenic CO_2 on the $CaCO_3$ system in the oceans. *Science*, **305**(5682): 362–366, doi:10.1126/science.1097329.

Ferrari, M.C.O., Wisenden, B.D., Chivers, D.P. (2010). Chemical ecology of predator-prey interactions in aquatic ecosystems: a review and prospectus. *Canadian Journal of Zoology*, **88**(7): 698–724, doi:10.1139/Z10-029.

Ferrari, M.C.O., McCormick, M.I., Munday, P.L., Meekan, M.G., Dixson, D.L., Lonnstedt, O., Chivers, D.P. (2011). Putting prey and predator into the CO_2 equation - qualitative and quantitative effects of ocean acidification on predator-prey interactions. *Ecology Letters*, **14**(11): 1143–1148, doi:10.1111/j.1461-0248.2011.01683.x.

Ferrari, M.C.O., McCormick, M.I., Munday, P.L., Meekan, M.G., Dixson, D.L., Lonnstedt, O., Chivers, D.P. (2012). Effects of ocean acidification on visual risk assessment in coral reef fishes. *Functional Ecology*, **26**(3): 553–558, doi:10.1111/j.1365-2435.2011.01951.x.

Hay, M.E. (2009). Marine chemical ecology: Chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*, **1**: 193–212, doi:10.1146/annurev.marine.010908.163708.

Hellou, J. (2010). Behavioural ecotoxicology, an "early warning" signal to assess environmental quality. *Environmental Science and Pollution Research*, **18**(1): 1–11, doi:10.1007/s11356-010-0367-2.

Hönisch, B., Ridgwell, A., Schmidt, D.N., Thomas, E., Gibbs, S.J., Sluijs, A., Zeebe, R., Kump, L., Martindale, R.C., Greene, S.E., Klessling, W., Ries, J., Zachos, J.C., Royer, D.L., Barker, S., Marchitto Jr., T.M., Moyer, R., Pelejero, C., Ziveri, P., Foster, G.L., Williams, B. (2012). The geological record of ocean acidification. *Science*, **335**(6072): 1058–1063, doi:10.1126/science.1208277.

Hough-Guldberg, O (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Marine and Freshwater Research*, **50**(8): 839–866, doi:10.1071/MF99078.

Intergovernmental Panel on Climate Change (IPCC) (2007). *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996 pp.

Intergovernmental Panel on Climate Change (IPCC) (2022). Changing Ocean, Marine Ecosystems, and Dependent Communities. In *The Ocean and Cryosphere in a Changing Climate: Special Report of the Intergovernmental Panel on Climate Change* (pp. 447-588). Cambridge: Cambridge University Press. doi:10.1017/9781009157964.007.

Ishimatsu, A., Hayashi, M., Lee, K.-S., Kikkawa, T., Kita, J. (2005). Physiological effects on fishes in a high-CO_2 world. *Journal of Geophysical Research-Oceans*, **110**: Article C09S09, doi:10.1029/2004JC002564.

Ishimatsu, A., Hayashi, M., Kikkawa, T. (2008). Fishes in high-CO_2, acidified oceans. *Marine Ecology Progress Series*, **373**: 295–302, doi:10.3354/meps07823.

Lan, X., Tans, P. and K.W. Thoning (2023, May 2). *Trends in globally-averaged CO2 determined from NOAA Global Monitoring Laboratory measurements.* https://gml.noaa.gov/ccgg/trends/global.html Version 2023-04 https://doi.org/10.15138/9N0H-ZH07.

Lüthi, D., Le Floch, M., Bereiter, B., Blunier, T., Barnola, J., Siegenthaler, U., Raynaud, D., Jouzel, J., Fischer, H., Kawamura, K., Stocker, T.F. (2008). High-resolution carbon dioxide concentration record 650,000–800,000 years before present. *Nature*, **453**(7193): 379–382, doi:10.1038/nature06949.

Millero, F.J., Pierrot, D., Lee, K., Wanninkhof, R., Feely, R., Sabine, C.L., Key, R.M., Takahashi, T. (2002). Dissociation constants for carbonic acid determined from field measurements. *Deep-Sea Research Part I-Oceanographic Research Papers*, **49**(10): 1705–1723, doi:10.1016/S09670637(02)00093-6.

Munday, P.L., Dixson, D.L., Donelson, J.M., Jones, G.P., Pratchett, M.S., Devitsina, G.V., Døving, K.B. (2009a). Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences*, **106**(6): 1848–1852, doi:10.1073/pnas.0809996106.

Munday, P.L., Crawley, N.E., Nilsson, G.E. (2009b). Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Marine Ecology Progress Series*, **388**: 235–242, doi:10.3354/meps08137.

Munday, P.L., Donelson, J.M., Dixson, D.L., Endo, G.G.K. (2009c). Effects of ocean acidification on the early life history of a tropical marine fish. *Proceedings of the Royal Society B–Biological Sciences*, **276**(1671): 3275–3283, doi:10.1098/rspb.2009.0784.

Munday, P.L., Dixson, D.L., McCormick, M.I., Meekan, M., Ferrari, M.C.O., Chivers, D.P. (2010). Replenishment of fish populations is threatened by ocean acidification. *Proceedings of the National Academy of Sciences*, **107**(29): 12930–12934, doi:10.1073/pnas.1004519107.

Munday, P.L., Pratchett, M.S., Dixson, D.L., Donelson, J.M., Endo, G.G.K., Reynolds, A.D., Knuckey, R. (2013). Elevated CO_2 affects the behaviour of an ecologically and economically important coral reef fish. *Marine Biology*, **160**(8): 2137–2144, doi:10.1007/s00227-012-2111-6.

Munday, P.L., Cheal, A.J., Dixson, D.L., Rummer, J.L., Fabricius, K.E. (2014). Behavioural impairment in reef fishes caused by ocean acidification at CO_2 seeps. *Nature Climate Change*, 4(6): 487–492, doi:10.1038/NCLIMATE2195.

Nagelkerken, I. and Munday, P.L. (2016). Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. *Global Change Biology*, **22**(3): 974–989, doi:10.1111/gcb.13167.

Nowicki, J.P., Miller, G.M., Munday, P.L. (2012). Interactive effects of elevated temperature and CO_2 on foraging behavior of juvenile coral reef fish. *Journal of Experimental Marine Biology and Ecology*, **412**: 46–51, doi:10.1016/j.jembe.2011.10.020.

Ohde, S. and van Woesik, R. (1999). Carbon dioxide flux and metabolic processes of a coral reef, Okinawa. *Bulletin of Marine Science*, **65**(2): 559–576.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M., Yamanaka, Y., Yool, A. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**(7059): 681–686, doi:10.1038/nature04095.

Shaw, E.C., McNeil, B.I. and Tilbrook, B. (2012). Impacts of ocean acidification in naturally variable coral reef flat ecosystems. *Journal of Geophysical Research-Oceans*, **117**: Article C03038, doi:10.1029/2011JC007655.

Sundin, J., Amcoff, M., Mateos-González, F., Raby, G.D., Jutfelt, F., Clark, T.D. (2017a). Long-term exposure to elevated carbon dioxide does not alter activity levels of a coral reef fish in response to predator chemical cues. *Behavioral Ecology and Sociobiology*, **71**(8): Article 108, doi:10.1007/s00265-017-2337-x.

Sundin, J., Vossen, L.E., Nilsson-Sköld, H., Jutfelt, F. (2017b). No effect of elevated carbon dioxide on reproductive behaviors in the three-spined stickleback. *Behavioral Ecology*, **28**(6): 1482–1491, doi:10.1093/beheco/arx112.

Sundin, J., Amcoff, M., Mateos-González, F., Raby, G.D., Clark, T.D. (2019). Long-term acclimation to near-future ocean acidification has negligible effects on energetic attributes in a juvenile coral reef fish. *Oecologia*, **190**(3): 689–702, doi:10.1007/s00442-019-04430-z.

Sundin, J. (2023). The effects of ocean acidification on fishes – history and future outlook. *Journal of Fish Biology*, Advance online publication, doi:10.1111/jfb.15323.

Tuomainen, U. and Candolin, U. (2011). Behavioural response to human-induced environmental change. *Biological Reviews*, **86**(3): 640–657, doi:10.1111/j.1469-185X.2010.00164.x.
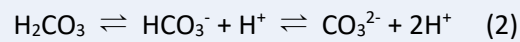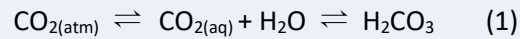
Welch, M.J., Watson, S.-A., Welsh, J.Q., McCormick, M.I., Munday, P.L. (2014). Effects of elevated CO_2 on fish behaviour undiminished by transgenerational acclimation. *Nature Climate Change*, **4**(12): 1086–1089, doi:10.1038/NCLIMATE2400.

Appendix:

Box 1: Ocean acidification chemistry

Ocean acidification refers to the process in which the pH and alkalinity levels of the oceans decrease (Doney *et al.* 2009). This process is fuelled by the increase in atmospheric carbon dioxide that dissolves in seawater and reacts to form carbonic acid (H_2CO_3) (Reaction 1) (Doney *et al.* 2009). In turn, this carbonic acid forms bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions by losing hydrogen ions (Reaction 2) (Doney *et al.* 2009). These reactions are in equilibrium and reversible for a current oceanwater acidity of 8.1 pH (Millero *et al.* 2002).

$$CO_{2(atm)} \rightleftharpoons CO_{2(aq)} + H_2O \rightleftharpoons H_2CO_3 \qquad (1)$$

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \rightleftharpoons CO_3^{2-} + 2H^+ \qquad (2)$$

Increased concentrations of dissolved CO_2 result in elevated concentrations of bicarbonate and hydrogen ions (Doney *et al.* 2009). Due to the increased concentration of hydrogen ions, which bond to carbonate to form bicarbonate, the level of carbonate ions will decrease (Doney *et al.* 2009). Ocean acidification is predicted to decrease the pH by 0.3 to 0.4 units by the end of the 21st century (Caldeira and Wickett 2003, Orr *et al.* 2005; IPCC, 2022).