

The role of ligand-gated chloride channels in behavioural alterations at elevated CO₂ in a cephalopod

Jodi T Thomas^{1*}, Blake L Spady^{2,3}, Philip L Munday¹, Sue-Ann Watson^{1,4}

¹ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia

²Coral Reef Watch, National Oceanic and Atmospheric Administration, College Park, Maryland, United States

³ReefSense Pty Ltd., Cranbrook, Queensland, Australia

⁴Biodiversity and Geosciences Program, Museum of Tropical Queensland, Queensland Museum Network, Townsville, Queensland, Australia

* Correspondence: Jodi T Thomas jodi.thomas@my.jcu.edu.au

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Summary Statement

We pharmacologically demonstrate that ionotropic GABA receptors and other ligand-gated chloride channels may underlie the alterations of some conspecific-directed behaviours at elevated seawater CO₂ in a cephalopod.

Abstract

Projected future carbon dioxide (CO₂) levels in the ocean can alter marine animal behaviours. Disrupted functioning of γ -aminobutyric acid type A (GABA_A) receptors (ligand-gated chloride channels) is suggested to underlie CO₂-induced behavioural changes in fish. However, the mechanisms underlying behavioural changes in marine invertebrates are poorly understood. We

pharmacologically tested the role of GABA-, glutamate-, acetylcholine- and dopamine-gated chloride channels in CO₂-induced behavioural changes in a cephalopod, the two-toned pygmy squid (*Idiosepius pygmaeus*). We exposed squid to ambient (~450 μatm) or elevated (~1,000 μatm) CO₂ for seven days. Squid were treated with sham, the GABA_A receptor antagonist gabazine, or the non-specific GABA_A receptor antagonist picrotoxin, before measurement of conspecific-directed behaviours and activity levels upon mirror exposure. Elevated CO₂ increased conspecific-directed attraction and aggression, as well as activity levels. For some CO₂-affected behaviours, both gabazine and picrotoxin had a different effect at elevated compared to ambient CO₂, providing robust support for the GABA hypothesis within cephalopods. In another behavioural trait, picrotoxin but not gabazine had a different effect in elevated compared to ambient CO₂, providing the first pharmacological evidence, in fish and marine invertebrates, for altered functioning of ligand-gated chloride channels, other than the GABA_A R, underlying CO₂-induced behavioural changes. For some other behaviours, both gabazine and picrotoxin had a similar effect in elevated and ambient CO₂, suggesting altered function of ligand-gated chloride channels was not responsible for these CO₂-induced changes. Multiple mechanisms may be involved, which could explain the variability in the CO₂ and drug treatment effects across behaviours.

Introduction

Anthropogenic carbon dioxide (CO₂) emissions are being absorbed by the oceans at an increasing rate, resulting in reduced seawater pH referred to as ocean acidification (Bindoff et al., 2019). Elevated CO₂ levels are known to alter a range of behaviours in a variety of fishes (Munday et al., 2019). Elevated CO₂-induced behavioural alterations also occur in some marine invertebrates, including in cnidarians, polychaetes, echinoderms, arthropods and molluscs, across a variety of behavioural traits (reviewed in Clements and Hunt (2015), Nagelkerken and Munday (2015) and Thomas et al. (2020)). The behavioural effects of elevated CO₂ are variable. Elevated CO₂ may affect some, but not other, behaviours within the same species. For example, in the blue mussel elevated CO₂ decreased predator cue-induced defensive behaviours (Kong et al., 2019) and feeding rates (Gu et al., 2019; Meseck et al., 2020), but did not alter a startle response behaviour (Clements et al., 2021). The effects of elevated CO₂ on the same behaviour may also be variable among taxa. For example, within molluscs elevated CO₂ increased locomotion speed in two species of squid (Spady et

al., 2014; Spady et al., 2018) but reduced speed in a third squid species (Zakroff et al., 2018), a sea hare (Horwitz et al., 2020), and two whelk species (Fonseca et al., 2020; Queirós et al., 2015). A mechanistic understanding of behavioural change at elevated CO₂ is important to determine why there is such variability in behavioural alterations and to identify which animals will be most vulnerable to rising CO₂ levels. However, the mechanisms underlying behavioural change at elevated CO₂ across the diverse range of marine invertebrates are poorly understood (Thomas et al., 2020).

The prominent mechanistic explanation for elevated CO₂-induced behavioural alterations is the GABA hypothesis, first demonstrated in two species of tropical coral reef fish (Nilsson et al., 2012). In vertebrates, the γ -aminobutyric acid type A receptor (GABA_A R) is a ligand-gated ion channel (LGIC)/ionotropic receptor selectively permeable to chloride (Cl⁻) and bicarbonate (HCO₃⁻) ions (Bormann et al., 1987; Krnjević, 1974). Under normal conditions, binding of the neurotransmitter GABA opens the GABA_A R channel, which usually allows a net influx of negative charge resulting in hyperpolarisation and inhibition of neuronal firing. At elevated CO₂ conditions, Nilsson et al. (2012) proposed that alterations in Cl⁻ and HCO₃⁻ ion gradients across the neuronal membrane, due to acid-base regulation, could alter the function of GABA_A Rs. The change in ion gradients was suggested to reverse the net flow of negative ions, resulting in a net efflux of negative charge from some GABA_A Rs, switching their function from inhibitory to excitatory, thus influencing behavioural responses (see Heuer et al. (2019) for a detailed explanation). Pharmacological studies have supported the GABA hypothesis in fish; administration of the GABA_A R antagonist gabazine (SR-95531) (Heaulme et al., 1986) attenuated CO₂-induced behavioural alterations (Chivers et al., 2014; Chung et al., 2014; Lai et al., 2015; Lopes et al., 2016; Nilsson et al., 2012; Regan et al., 2016) and the GABA_A R agonist muscimol (Andrews and Johnston, 1979) produced opposite effects in elevated and control CO₂ exposed fish (Hamilton et al., 2013). Recently, the GABA hypothesis has been further refined by the proposal that altered functioning of some GABA_A Rs initiates a vicious self-amplifying cycle, explaining how relatively small changes in ion gradients can result in large behavioural alterations (Schunter et al., 2019).

Invertebrate GABA also binds to Cl⁻ and HCO₃⁻ permeable ionotropic GABA receptors (referred to as GABA_A-like Rs throughout this manuscript to differentiate invertebrate from vertebrate GABA_A Rs) (Kaila and Voipio, 1987; Lunt, 1991); therefore, the GABA hypothesis should theoretically apply to invertebrates as well. Administration of the GABA_A R antagonist gabazine has been used to test the GABA hypothesis in several marine invertebrates. In a gastropod mollusc, the jumping conch snail

(*Gibberulus gibberulus gibbosus*), impaired escape behaviour at elevated CO₂ levels (961 µatm) was restored to control levels by gabazine (Watson et al., 2014). In a bivalve mollusc, the soft-shell clam (*Mya arenaria*), burrowing behaviours altered at elevated sediment CO₂ levels (9,532 µatm) were also restored to control levels by gabazine (Clements et al., 2017). In contrast, gabazine did not restore lost chemical-cue induced photosensitive behaviour at elevated CO₂ (1,380 µatm) in Asian shore crab larvae (*Hemigrapsus sanguineus*) (Charpentier and Cohen, 2016). Electrophysiological studies have shown crustacean GABA_A-like Rs to be insensitive to gabazine (El Manira and Clarac, 1991; Jackel et al., 1994; Pearstein et al., 1996) and thus gabazine may not have tested the GABA hypothesis in *H. sanguineus*. However, as pharmacological sensitivity of receptors can vary between differing cell types (Lee and Maguire, 2014) and only specific neurons were tested in these electrophysiological studies, it is possible that other crustacean cell types are sensitive to gabazine. Thus, systemic gabazine administration by Charpentier and Cohen (2016) could have tested the GABA hypothesis. If this is the case, it suggests the GABA hypothesis may apply to some, but not other, invertebrate taxa.

To date, pharmacological studies assessing the GABA hypothesis in marine invertebrates have exclusively used gabazine. This has provided a useful starting point to understand elevated CO₂-induced behavioural changes. However, gabazine's action in the invertebrate taxa studied to date is not well characterised. The pharmacological profile of invertebrate GABA_A-like Rs differs from that of vertebrate GABA_A Rs (Lunt, 1991; Walker et al., 1996). Furthermore, the invertebrates span an enormous amount of phylogenetic variation and invertebrate LGICs can differ across taxa (Tsang et al., 2007; Wolstenholme, 2012). Thus, the action of GABA_A R antagonists, such as gabazine, may differ between invertebrates and vertebrates and also among invertebrate taxa. The use of GABA_A R drugs shown to work in the taxa of interest, and interpreting the results appropriately based on the abundance or scarcity of research into the drug's action in the studied taxa, will be important to further assess the GABA hypothesis. Furthermore, using other GABA_A receptor antagonists that are structurally unrelated to gabazine, such as picrotoxin, can increase our confidence in the evidence for the GABA hypothesis (Tresguerres and Hamilton, 2017).

Elevated CO₂ may also alter the functioning of other LGICs that are permeable to HCO₃⁻ and Cl⁻ ions. Altered glycine R functioning at elevated CO₂ levels has been suggested as another potential mechanism in fish due to its similarity to the GABA_A R (Tresguerres and Hamilton, 2017). Glycine Rs are not found in invertebrates, however glutamate (Glu)-gated Cl⁻ channels are suggested to be the

invertebrate equivalent of glycine Rs (Kehoe and Vulfius, 2000; Vassilatis et al., 1997), so may respond to elevated CO₂ similarly. Invertebrates also possess a larger range of ligand-gated Cl⁻ channels than vertebrates (Wolstenholme, 2012), including acetylcholine (ACh)- (Kehoe, 1972; Putrenko et al., 2005; Schmidt and Calabrese, 1992; van Nierop et al., 2005; Yarowsky and Carpenter, 1978b), dopamine (DA)- (Carpenter et al., 1977), serotonin- (Gerschenfeld and Tritsch, 1974; Ranganathan et al., 2000) and histamine- (Gisselmann et al., 2002; Zheng et al., 2002) gated Cl⁻ channels. Genes encoding for the LGICs (and associated proteins) of GABAergic, glycinergic-like, glutamatergic and cholinergic synapses were found to be differentially expressed in pteropod molluscs (*Heliconoides inflatus*) exposed to elevated CO₂ levels (617 – 720 μatm) compared with ambient controls (380 - 410 μatm) (Moya et al., 2016). Therefore, a range of ligand-gated Cl⁻ channels could play a role in CO₂-induced behavioural disturbances in marine invertebrates, indicating a complex assortment of responsible mechanisms.

Elevated CO₂ has been shown to affect activity, defensive and predatory behaviours in cephalopod molluscs (Spady et al., 2014; Spady et al., 2018). Cephalopods are a valuable taxa in which to investigate the mechanisms of CO₂-induced behavioural effects because of their well-developed nervous system and complex behaviours rivalling those of fishes (Hanlon and Messenger, 2018). In this study, male two-toned pygmy squid *Idiosepius pygmaeus* (Steenstrup 1881) were exposed to ambient (~450 μatm) or elevated (~1,000 μatm) CO₂ levels for seven days followed by sham or treatment with the GABA_A R antagonist gabazine or the non-specific GABA_A R antagonist picrotoxin. After CO₂ and drug treatment, conspecific attraction, exploratory and aggressive behaviours, and activity levels were measured while squid were exposed to a mirror.

The pharmacological profile of gabazine in molluscs is not well characterised (see Table S1). The only study using electrophysiological methods to investigate gabazine's action in a mollusc demonstrated that gabazine inhibits both ionotropic GABA R hyperpolarisations (inhibition) and depolarisations (excitation) (Vehovszky et al., 1989). However, there was no evidence for what ion(s) these ionotropic GABA Rs were permeable to. Molluscs possess both hyperpolarising and depolarising GABA-gated Cl⁻ channels (GABA_A-like Rs) (Rubakhin et al., 1996). It is also suggested that excitatory GABA-gated cation channels may be present in molluscs (Miller, 2019; Norekian, 1999; Yarowsky and Carpenter, 1978a), as seen in other invertebrates (Beg and Jorgensen, 2003; Gisselmann et al., 2004). Therefore, gabazine likely inhibited ionotropic GABA R hyperpolarisations by antagonising GABA_A-like Rs, but gabazine's action on ionotropic GABA R depolarisations may have been due to

gabazine's action on GABA-gated cation channels. Furthermore, as the action of gabazine has not been tested on other molluscan receptors, the specificity of gabazine within molluscs is unknown. To the best of our knowledge no studies have assessed the action of gabazine on cephalopod receptors, but GABA_A-like Rs are present in squid (Conti et al., 2013). In the current study, gabazine administration was used to test the functioning of GABA_A-like Rs in *I. pygmaeus*, though GABA-gated cation channels and other closely related LGICs may have also been antagonised by gabazine.

Picrotoxin is a relatively non-specific GABA_A R antagonist, inhibiting GABA_A as well as glycine Rs in vertebrates (both ligand-gated Cl⁻ channels) (Dibas et al., 2002; Lynch, 2004; Masiulis et al., 2019; Wang et al., 2006). In molluscs, picrotoxin's action has been better studied than that of gabazine (see Table S2). Similar to in vertebrates, picrotoxin antagonises molluscan GABA_A-like Rs (Jing et al., 2003; Rubakhin et al., 1996; Wu et al., 2003; Yarowsky and Carpenter, 1978a; Yarowsky and Carpenter, 1978b) as well as Glu-gated Cl⁻ channels (suggested to be the invertebrate equivalent of glycine Rs (Vassilatis et al., 1997)) (Piggott et al., 1977). However, molluscs possess a larger range of ligand-gated Cl⁻ channels than vertebrates (Wolstenholme, 2012) and picrotoxin also inhibits molluscan ACh- (Yarowsky and Carpenter, 1978b) and DA- (Magoski and Bulloch, 1999) gated Cl⁻ channels. As far as we are aware, no studies have assessed the action of picrotoxin on cephalopod ligand-gated Cl⁻ channels. However, local injection of GABA and picrotoxin into the optic lobe of a cuttlefish had opposite effects on locomotion (Chichery and Chichery, 1985) suggesting picrotoxin acted on an unidentified type of GABA receptor. In the current study, picrotoxin administration was used to investigate the functioning of GABA_A-like Rs as well as Glu-, ACh- and DA- gated Cl⁻ channels in *I. pygmaeus*.

Our study aimed to: 1) determine if elevated CO₂ alters a range of conspecific-directed behaviours and activity levels in *I. pygmaeus*, 2) provide robust evidence for or against the GABA hypothesis within a cephalopod mollusc, and 3) determine whether ligand-gated Cl⁻ channels other than the GABA_A-like R (Glu-, ACh- and DA- gated Cl⁻ channels) could also underlie CO₂-induced behavioural changes in *I. pygmaeus*. If the behavioural effect of drug treatment at elevated CO₂ is different to the behavioural effect of drug treatment at ambient CO₂ this would suggest that altered function of the drugs target receptor(s) underlies the CO₂-induced behavioural change. The behavioural effect of drug treatment could differ across CO₂ in five ways; opposite, removed, added, diminished or enhanced, with each of these suggesting a slightly different change in receptor function at elevated CO₂ (see Table 1 for definitions and explanations). As the common target of gabazine and picrotoxin

is the GABA_A-like R, if both gabazine and picrotoxin alter behaviour in a similar manner across CO₂ conditions this would suggest altered function of the GABA_A-like R underlies the CO₂-induced behavioural change, supporting the GABA hypothesis. As gabazine and picrotoxin are structurally unrelated, and picrotoxin's action within molluscs has been better studied, this would provide more robust support for the GABA hypothesis than previous marine invertebrate pharmacological studies. As picrotoxin antagonises Glu-, ACh- and DA- gated Cl⁻ channels, but gabazine does not, if picrotoxin, but not gabazine, has a different effect on behaviour across CO₂ conditions this would suggest altered function of these picrotoxin-sensitive ligand-gated Cl⁻ channels underlies the CO₂-induced behavioural change.

Materials and methods

Animal Collection

Male two-toned pygmy squid (*Idiosepius pygmaeus*) were collected between August – October 2019 (picrotoxin experiment) and November – December 2019 (gabazine experiment) by dip net from the inshore waters around the Townsville breakwater complex (19°15'S, 146°50'E) (Queensland Government General Fisheries Permit number 199144). Males were identified by visual inspection of the testis at the tip of the mantle and transported immediately to the experimental facilities. Only males were used due to the potential for sex-specific responses to elevated CO₂ (Ellis et al., 2017; Spady et al., 2014) and GABA_A R antagonists (Manev et al., 1987; Peričić et al., 1986). Squid were acclimated in groups at ambient seawater conditions for 1 - 6 days before transferral to treatment tanks set at either current-day ambient (~450 μatm) or elevated (~1,000 μatm) seawater CO₂, consistent with end of century projections under Representative Concentration Pathway (RCP) 8.5 (Collins et al., 2013). Squid were randomly assigned to tanks within their CO₂ treatment. Treatment tanks (matte white colour, 40 x 30 x 30 cm) held squid individually for seven days, which is approximately 10% of the total lifespan and 25% of the adult lifespan of male *I. pygmaeus* (Jackson, 1988). Squid were provided with PVC pipes for shelter and fed glass shrimp (*Acetes sibogae australis*) daily *ad libitum*. Glass shrimp were collected from the same location as squid and housed at ambient conditions. This study followed the animal ethics guidelines at James Cook University (JCU animal ethics number A2644).

CO₂ Treatment Systems

Experiments were carried out in four interconnected 8,000 L recirculating seawater systems, each with a 3,000 L sump, at James Cook University's research aquarium in Townsville, Australia. Two untreated seawater systems were used for duplicated ambient CO₂ treatments, and a custom-built pH control system dosed CO₂ into the 3,000 L sumps of two seawater systems to create duplicated elevated CO₂ treatments. An inline pH sensor (Tophit CPS471D, Endress+Hauser, Reinach, Switzerland) measured pH continuously and communicated via a transmitter (Liquiline CM442, Endress+Hauser, Reinach, Switzerland) with the computerised controller (OMNI C40 BEMS, Innotech, Brisbane, Australia) to regulate CO₂ dosing to maintain the desired pH. The four systems were interconnected by water exchange of approximately 20 L per hour to maintain similar water quality in each system.

Daily measurements of pH_{NBS} (Ecotrode plus on an 888 Titrande, Metrohm AG, Switzerland), and temperature (Comark C26, Norfolk, UK) were taken from each of the four systems. Dosing set points were adjusted as required to maintain the target *p*CO₂ in the two elevated CO₂ systems. Weekly measurements of salinity by a conductivity sensor (HQ40d, Hach, Loveland, CO, USA), total alkalinity by Gran titration (888 Titrande, Metrohm AG, Switzerland), and pH on the total scale (pH_T) by spectrophotometry (Spectronic 200E, Thermo-Scientific, Madison, USA) using m-cresol purple as an indicator dye (Dickson and Millero, 1987; Dickson et al., 2007) were taken from each of the four systems. Titration calibrations remained within 1% of certified reference material from Prof. A.G. Dickson (Scripps Institution of Oceanography, batch #136) throughout the experiments. Daily pH_T values were estimated by comparing pH_{NBS} and pH_T values. *p*CO₂ values were calculated in CO2SYS version 2.1 (Pierrot et al., 2006) using the constants K1, K2 from Mehrbach et al. (1973) and refit by Dickson and Millero (1987), and KHSO₄ from Dickson et al. (2007). An overview of carbonate chemistry parameters are in Table 2. Temperature, salinity, alkalinity and ambient pH were chosen to be similar to the natural conditions where the squid were collected.

Drug Treatment and Behavioural Trials

After CO₂ treatment, squid underwent drug treatment for 30 minutes. In both the gabazine and picrotoxin experiments, squid were individually placed in 100 mL aerated seawater from their CO₂ treatment containing sham or drug treatment, squid were randomly assigned to sham or drug treatment. Gabazine experiment: sham = 0.4% distilled water ($n_{\text{ambient CO}_2} = 22$, $n_{\text{elevated CO}_2} = 22$), or gabazine = 4 mg/L (10.86 μM) gabazine (SR-95531, batch #0000035110, Sigma-Aldrich, St Louis, USA) and 0.4% distilled water ($n_{\text{ambient CO}_2} = 24$, $n_{\text{elevated CO}_2} = 23$). Picrotoxin experiment: sham = 0.2% absolute ethanol ($n_{\text{ambient CO}_2} = 27$, $n_{\text{elevated CO}_2} = 26$), picrotoxin = 100 μM picrotoxin (batch #16C/230903, Tocris Bioscience, Bristol, UK) and 0.2% absolute ethanol ($n_{\text{ambient CO}_2} = 26$, $n_{\text{elevated CO}_2} = 26$). The dose of gabazine was chosen based on *in vivo* studies in fish (Chivers et al., 2014; Hamilton et al., 2013; Nilsson et al., 2012) and another mollusc (Watson et al., 2014) that showed bath application of 4 mg/L for 30 minutes reversed the behavioural effect of elevated CO₂. The dose of picrotoxin was chosen based on *in vivo* studies in a mollusc (Biscocho et al., 2018) and barnacle (Rittschof et al., 1986), which showed altered behaviour after 100 μM picrotoxin treatment. Distilled water and ethanol were used to dissolve gabazine and picrotoxin, respectively.

Immediately after drug treatment, squid were placed in the middle of a matte white acrylic tank (30 x 30 x 15 cm) with a mirror taking up the entire area of one wall. The tank was filled with water from their CO₂ treatment to a depth of 3 cm (to limit vertical movement for tracking). The tank was illuminated by an LED strip hung approximately 5 cm to the side of the arena walls, around the entire tank. The behavioural trial was filmed with a digital camera (Canon PowerShot G15 or G16) placed on white Corflute® directly above the tank, 70 cm from the water surface. Filming was at 30 frames per second (fps) and started before squid were placed in the behavioural arena to minimize disturbance. Filming continued for the 15 minute behavioural trial and stopped after 16 minutes to eliminate possible disturbances when approaching the camera. Squid mantle length (ML) was measured at the conclusion of each behavioural trial; gabazine experiment ML = 9.99 ± 0.99 mm (mean \pm standard deviation), picrotoxin experiment ML = 9.54 ± 0.94 mm.

Behavioural Analysis

For each experiment, the squid's interaction with the mirror was analysed across the 15 minutes following introduction into the behavioural arena. Other cephalopod species appear to recognise their mirror image as a conspecific (Ikeda and Matsumoto, 2007; Palmer et al., 2006; Shashar et al., 1996). Therefore, a mirror was used to analyse visual conspecific-directed behaviours while controlling for possible confounding factors of using a live conspecific, such as size, movement and chemical cues. Furthermore, using a mirror image limited the senses that influenced behaviour to vision only. This reduced the potential of altered sensation as a mechanism underlying any elevated CO₂-induced behavioural changes (Thomas et al., 2020), which allowed us to focus on central mechanisms, such as altered LGIC function.

Videos were analysed with the observer blinded to treatment. LoliTrack tracking software (version 4.2.1, Loligo Systems) was used across the 15 minute videos with the framerate subsampled to 15 fps. A 3 cm wide zone along the full length of the mirror (Zone A) was created in LoliTrack to determine space use:

- Time spent in Zone A (seconds) (only for squid that did enter Zone A)
- Number of visits to Zone A

The following information was determined from the videos, at 30 fps, using QuickTime video software (QuickTime version 7.7.5, Apple Inc.):

Mirror touching was categorized into two groups. Soft mirror touches were exploratory and defined as only the arm tips touching the mirror. Aggressive mirror touches occurred when the arms splayed upon coming in contact with the mirror, usually at high speed and accompanied by flashing body colour. Other parts of the squid's body coming in contact with the mirror (e.g. mantle) were not counted as mirror touches. Mirror touching measures determined were:

- Proportion of squid that touched the mirror softly/aggressively
- Latency to the first soft/aggressive mirror touch (only for squid that touched the mirror)
- Number of soft/aggressive mirror touches – squid's arms had to detach from the mirror completely between mirror touches before a successive touch was counted (only for squid that touched the mirror)

LoliTrack was also used across the 15 minute videos at 15 fps for measures of activity:

- Time spent active (seconds)
- Total distance moved (centimeters)
- Average speed (cm/s)

Squid that inked in the behavioural trial (gabazine experiment sham: $n_{\text{ambient CO}_2} = 8$, $n_{\text{elevated CO}_2} = 7$ and gabazine: $n_{\text{ambient CO}_2} = 10$, $n_{\text{elevated CO}_2} = 7$; and picrotoxin experiment sham: $n_{\text{ambient CO}_2} = 6$, $n_{\text{elevated CO}_2} = 5$ and picrotoxin: $n_{\text{ambient CO}_2} = 6$, $n_{\text{elevated CO}_2} = 2$) were excluded from the tracking analysis because LoliTrack could not distinguish between the squid and the ink. Furthermore, squid that were in very close proximity to the mirror for an extended period of time, e.g. attached to the mirror, were also excluded from the tracking analysis because LoliTrack could not distinguish between the squid and the squid's mirror image. A total of 38 squid (gabazine experiment) and 25 squid (picrotoxin experiment) were excluded from the tracking analysis in LoliTrack. Therefore, sample sizes for tracked data are as follows: gabazine experiment sham $n_{\text{ambient CO}_2} = 12$, $n_{\text{elevated CO}_2} = 13$, gabazine $n_{\text{ambient CO}_2} = 12$, $n_{\text{elevated CO}_2} = 16$; and picrotoxin experiment sham $n_{\text{ambient CO}_2} = 19$, $n_{\text{elevated CO}_2} = 21$, picrotoxin $n_{\text{ambient CO}_2} = 18$, $n_{\text{elevated CO}_2} = 22$.

Statistical Analysis

Bayesian modelling was carried out in R (v4.0.2) (R Core Team, 2020), using RStudio (v1.3.1093) (RStudio Team, 2020) to test the effects of CO₂ and drug treatment on each behaviour. The Bayesian models were fit using the package brms (v2.13.5) (Bürkner, 2017), which uses RStan (v2.21.2) (Stan Development Team, 2020) to interface with the statistical modelling platform Stan.

All count data were modelled against a negative binomial distribution with a log link, binomial data were modelled against a Bernoulli distribution with a logit link, and continuous data were modelled with a linear model (Gaussian distribution with an identity link) or against a gamma distribution with a log link. The models were fit using the no u-turn MCMC sampler which ran with 10,000 iterations, a warm-up of 3,000 and thinning of 5 for each of 4 chains. Default, weakly informative priors were used. For two response variables in the picrotoxin experiment, number of visits to Zone A and the latency to the first aggressive mirror touch, acceptable MCMC diagnostics were maintained by

adjusting the MCMC sampler to run with 120,000 iterations, a warm-up of 40,000 and thinning of 80 for each of 4 chains, Stan was forced to take smaller steps by increasing `adapt_delta` from the default of 0.8 to 0.99, and the priors were specified. See Tables S3 and S4 for the distribution and link function, and the priors used for each response variable (behaviour).

Variable selection was used to choose a model with the best subset of explanatory variables for each response variable (behaviour). A set of six biologically plausible candidate models were fit for each response variable, with each model hypothesized *a priori* to represent a particular aspect of biology that could affect the response variable. All models included the interaction of CO₂ (fixed factor with two levels; ambient and elevated) and drug (fixed factor with two levels; sham and gabazine/picrotoxin). The six models tested: 1) only the interaction of CO₂ and drug, 2) the effect of squid size by including mantle length in centimeters (continuous), 3) the effect of the methods used for behavioural testing by including the behavioural tank used (fixed factor with two levels - behavioural trials were carried out in two different tanks of the same dimensions) and the time of day the behavioural test was carried out (continuous), 4) the effect of drug lot by including the drug test number (fixed factor with three levels in the gabazine experiment or seven levels in the picrotoxin experiment – gabazine solution was made up directly before the trial and the same solution was used for up to three separate trials, while picrotoxin solution was always only used for one trial but all picrotoxin solutions were made up at the start of the day and up to seven picrotoxin solutions were used across one day), 5) the effect of housing conditions by including the number of days squid were acclimated at ambient seawater conditions before transferal to treatment tanks (fixed factor with 6 levels - squid were acclimated for 1 - 6 days) and the day squid underwent the behavioural trial (continuous), 6) the effect of the duplicate seawater systems by including system (fixed factor - two levels for ambient CO₂ and two levels for elevated CO₂). Leave-one-out cross-validation information criterion (LOOIC) values, which have the same purpose as the frequentist Akaike Information Criterion (AIC) values, were calculated for each model. All LOOIC values were considered reliable due to less than 14.5% of the Pareto k diagnostic values being larger than 0.7. The chosen model for each response variables had a LOOIC within 1 of the best model. See Tables S3 and S4 for the explanatory variables included in the model for each response variable.

All chosen models followed the nominated distribution, tested with Q-Q plots and the Kolmogorov-Smirnov test, showed no over- or under-dispersion, no outliers and the residuals showed no patterns. MCMC diagnostics for each of the chosen models suggested that the chains were well mixed and converged on a stable posterior. MCMC diagnostics included trace plots for visual inspection of chain mixing, $\hat{R} < 1.05$, autocorrelation factor < 0.2 and effective sample size $> 50\%$. Posterior probability checks suggest that the priors did not influence the data.

If the drug effect at elevated CO_2 was found to be different to the drug effect at ambient CO_2 this was considered support for altered function of the drugs target receptor(s) underlying the CO_2 -induced behavioural change. The drug effects could differ across CO_2 in five ways; an opposite, removed, added, diminished or enhanced drug effect, with each effect suggesting a slightly different change in receptor function at elevated CO_2 (see Table 1 for definitions and explanations of each of these effects).

Results

A summary of the effects of elevated CO_2 on squid behaviour (in sham-treated individuals) and the drug effect across CO_2 treatments is shown in Table 3.

Space Use

There was very strong evidence (95.9%) in the gabazine experiment and evidence (83.6%) in the picrotoxin experiment that elevated CO_2 increased the time that sham-treated squid spent in Zone A (for those squid that entered Zone A at least once) (gabazine: 1.74-fold increase 386 to 670 s, picrotoxin: 1.29-fold increase 409 to 529 s) (Fig. 1A,B,E,F). There was also strong evidence (93.3%) that gabazine had a different effect at ambient and elevated CO_2 conditions. Specifically, there was no evidence of a gabazine effect at ambient CO_2 (64.4%), however there was very strong evidence (97.5%) that gabazine decreased the time spent in Zone A at elevated CO_2 (0.54-fold decrease 670 s to 363 s) (Fig. 1A,B). The time spent in Zone A by gabazine-treated squid at elevated CO_2 was very similar to sham-treated squid at ambient CO_2 (386 and 363 s, respectively) (Fig 1A). There was also evidence (80.8%) that picrotoxin had a different effect at ambient and elevated CO_2 . Specifically, there was strong evidence (91.8%) that picrotoxin increased the time spent in Zone A at ambient CO_2

(1.41-fold increase 409 to 577 s), but no evidence of a picrotoxin effect at elevated CO₂ (60.6%) (Fig. 1E,F).

There was very strong evidence (99.9%) that elevated CO₂ decreased the number of times squid visited Zone A in the gabazine experiment (0.24-fold decrease 15 to 3.6 visits) (Fig. 1C,D). There was also very strong evidence (98.8%) that gabazine had a different effect at ambient and elevated CO₂. Specifically, gabazine decreased the number of visits at ambient CO₂ (97.7% confidence, 0.37-fold decrease 15 to 5.6 visits), but increased the number of visits at elevated CO₂ (86.5% confidence, 1.63-fold increase 3.6 to 5.8 visits). In contrast, there was no evidence (56.7%) that CO₂ affected the number of visits to Zone A by sham-treated squid in the picrotoxin experiment (Fig. 1G,H). However, there was evidence (80.9%) that picrotoxin had a different effect at ambient and elevated CO₂ (Fig. 1H).

Soft mirror touch

In the gabazine experiment, there was no evidence (62.2%) for an effect of CO₂ on the proportion of sham-treated squid that explored by softly touching the mirror, nor was there any evidence (63.2%) that gabazine had a different effect at ambient and elevated CO₂ conditions (Fig. 2A,B). However, in the picrotoxin experiment there was strong evidence (94.4%) that elevated CO₂ increased the proportion of sham-treated squid that softly touched the mirror (2.44 odds ratio, increase 29% to 50%) (Fig. 2G,H). There is no evidence (60.8%) that picrotoxin had a different effect at ambient and elevated CO₂, with strong evidence (96.9% and 92.2%, respectively) that picrotoxin increased the proportion of squid softly touching the mirror at both ambient CO₂ (2.88 odds ratio, increase 29% to 54%) and elevated CO₂ (2.31 odds ratio, increase 50% to 70%) (Fig. 2G,H).

There was some evidence (77.9%) in the gabazine experiment and very strong evidence (99%) in the picrotoxin experiment that elevated CO₂ decreased the latency to the first soft mirror touch (gabazine: 0.67-fold decrease 107.7 to 72.5 s, picrotoxin: 0.3-fold decrease 257.3 to 75.8 s) (Fig. 2C,D,I,J). However, there was no evidence (60.3%) for a different effect of gabazine across CO₂ conditions. Gabazine increased the latency at both ambient CO₂ (83.2% confident, 1.66-fold increase 107.7 to 178.6 s) and elevated CO₂ (89.8% confident, 2.03-fold increase 72.5 to 146.8 s). In contrast, there was very strong evidence (99.1%) that picrotoxin had a different effect across CO₂ treatments. Picrotoxin decreased the latency at ambient CO₂ (88.4% confident, 0.55-fold decrease 257.3 to 140.5

s), whereas it increased the latency at elevated CO₂ (98.8% confident, 2.69-fold increase 75.8 to 203.2 s) (Fig. 2I,J).

In the gabazine experiment there was strong evidence (93.2%), and in the picrotoxin experiment some evidence (78.9%), that elevated CO₂ increased the number of soft mirror touches per individual (gabazine: 2.02-fold increase 13.3 to 27.0 touches, picrotoxin: 1.53-fold increase 32.1 to 49.1 touches) (Fig. 2E,F,K,L). There was some evidence (78.9%) that gabazine had a smaller effect at elevated compared to ambient CO₂. Specifically, gabazine increased the number of soft touches by a median of 2.48-fold at ambient CO₂ (97.1% confidence, increase 13.3 to 33.0 touches) and 1.44-fold at elevated CO₂ (78.7% confidence, increase 27.0 to 38.8 touches) (Fig. 2E,F). By contrast, there was no evidence (62.4%) that picrotoxin had a different effect across CO₂ conditions. Picrotoxin decreased the number of soft touches both at ambient CO₂ (86.7% confident, 0.54-fold decrease 32.1 to 17.5 touches) and at elevated CO₂ (82.5% confident, 0.68-fold decrease 49.1 to 33.4 touches) (Fig. 2K,L).

Aggressive mirror touch

There was evidence (83.2%) in the gabazine experiment and very strong evidence (97.2%) in the picrotoxin experiment that elevated CO₂ increased the proportion of sham-treated squid that touched the mirror aggressively (gabazine: 1.82 odds ratio, increase 31% to 45%, picrotoxin: 3.01 odds ratio, increase 25% to 50%) (Fig. 3A, B and G, H). In both experiments, there was no evidence (65% and 70.7%, respectively) that drug treatment had a different effect at ambient and elevated CO₂ conditions (Fig. 3A,B,G,H).

In the gabazine experiment there was some evidence (76.5%), and in the picrotoxin experiment there was evidence (86%), that elevated CO₂ increased the latency to the first aggressive mirror touch (gabazine: 1.6-fold increase 30.8 to 48.9 s, picrotoxin: 1.85-fold increase 108.1 to 201.8 s) (Fig. 3C,D,I,J). There was also strong evidence (94.2%) that the effect of gabazine was smaller at elevated compared to ambient CO₂. Specifically, gabazine increased the latency by a median of 7.6-fold at ambient CO₂ (99.8% confident, increase 30.8 to 235.3 s) and 2.03-fold at elevated CO₂ (91.3% confident, increase 48.9 to 97.9 s) (Fig. 3 C,D). There was no evidence (55.5%) that picrotoxin had a different effect across CO₂ conditions (Fig. 3I,J).

In both experiments, there was strong evidence (97.3% and 93.3%, respectively) that elevated CO₂ increased the number of aggressive mirror touches per individual (gabazine: 2.5-fold increase 11.9 to 29.6 touches, picrotoxin: 1.79-fold increase 9.7 to 17.4 touches) (Fig. 3E, F and K, L). However, there was no evidence (68.6% and 64.9%, respectively) that gabazine or picrotoxin had a different effect across CO₂ conditions (Fig. 3E,F,K,L). There was strong evidence (95.6% and 93.8%, respectively) that picrotoxin increased the number of aggressive touches both at ambient CO₂ (2.16-fold increase 9.7 to 21 touches) and at elevated CO₂ (1.77-fold increase 17.4 to 30.8 touches) (Fig. 3K,L).

Activity

Elevated CO₂ increased the time squid spent active in both experiments (gabazine: 87.8% confident, 1.41-fold increase 161.5 to 228.2 s, picrotoxin: 86.6% confident, 1.23-fold increase 258.4 to 316.1 s) (Fig. 4A,B,G,H). There was some evidence that drug treatment had a different effect at ambient and elevated CO₂ in both experiments. Gabazine had a larger effect at elevated compared to ambient CO₂ (78.9% confident, 1.40-fold); there was no evidence (50.7%) at ambient CO₂, but there was evidence (88.7%) at elevated CO₂ (1.40-fold increase 228.2 to 319 s) that gabazine increased active time (Fig. 4A,B). Conversely, picrotoxin had a smaller effect at elevated compared to ambient CO₂ (79.5% confident, 0.8-fold); there was strong evidence (91.6%) at ambient CO₂ (1.46-fold increase 316.1 to 333.9 s), but no evidence (63.5%) at elevated CO₂, that picrotoxin increased active time (Fig. 4G,H).

The total distance moved by squid throughout the behavioural trial was also increased by elevated CO₂ in both experiments (gabazine: 90.3% confident, 1.57-fold increase 360.0 to 566.6 s, picrotoxin: 88.9% confident, 1.36-fold increase 613.5 to 833.9 s) (Fig. 4C,D,I,J). There was some evidence (76.9%) that gabazine had a larger effect at elevated compared to ambient CO₂. Specifically, there was no evidence (57.8%) at ambient CO₂, but strong evidence (90.7%) at elevated CO₂ (1.54-fold increase 566.6 to 872.2 s) that gabazine increased active time (Fig. 4C,D). Conversely, there was some evidence (75.3%) that picrotoxin had a smaller effect at elevated compared to ambient CO₂. Specifically, picrotoxin increased distance moved by a median of 1.46-fold at ambient CO₂ (93.4% confident, increase 613.5 to 892.8 s) and 1.17-fold at elevated CO₂ (78.9% confident, increase 833.9 s to 976 s) (Fig. 4I,J).

Squid average speed across the behavioural trial was also higher at elevated compared to ambient CO₂ (gabazine: 86.8% confident, 1.12-fold increase 2.07 to 2.32 cm/s, picrotoxin: 87.4% confident, 1.10-fold increase 2.21 to 2.42 cm/s) (Fig. 4E,F,K,L). There was no evidence (55.9%) that gabazine had a different effect across CO₂ conditions, with no evidence (71.2% and 66.6%, respectively) of gabazine having an effect at either ambient or elevated CO₂ (Fig. 4E,F). There was also no evidence (54.4%) that picrotoxin had a different effect at ambient compared to elevated CO₂; there was strong evidence (95.2% and 98.3%, respectively) that picrotoxin increased squid average speed at both ambient CO₂ (1.14-fold increase 2.21 to 2.53 cm/s) and elevated CO₂ (1.16-fold increase 2.42 to 2.81 cm/s) (Fig. 4K,L).

Discussion

Elevated CO₂ levels can alter marine invertebrate behaviour (reviewed in Clements and Hunt (2015), Nagelkerken and Munday (2015) and Thomas et al. (2020)); however, little is known about how elevated CO₂ levels might affect conspecific-directed behaviours or the mechanisms involved in altered marine invertebrate behaviour at elevated CO₂. Here, we found that elevated CO₂ increased male *I. pygmaeus* conspecific attraction, aggression and activity levels in the presence of the squid's mirror image, compared to squid in ambient CO₂ conditions. Treatment with gabazine and picrotoxin had a different effect at elevated compared to ambient CO₂ conditions in some behaviours, providing robust support for the GABA hypothesis within cephalopods, and indicating altered functioning of Glu-, ACh- and DA- gated Cl⁻ channels may also underlie CO₂-induced behavioural changes. However, gabazine and picrotoxin had a similar effect in both ambient and elevated CO₂ conditions for other CO₂-affected behavioural traits, suggesting other mechanisms may also be involved.

Behavioural change in response to elevated CO₂

For the majority of the measured behaviours, the effect of elevated CO₂ was consistent across the gabazine and picrotoxin experiments. However, for two behavioural traits there was evidence for an effect of CO₂ in one experiment and not the other (see Table 3). Behaviour is notoriously difficult to measure due to its complexity (Niepoth and Bendesky, 2020), and behavioural effects are known to be influenced by subtle environmental changes, even when in controlled laboratory conditions (Crabbe et al., 1999). There are many potential explanations for why the CO₂ effect was not the same between experiments for the number of times squid visited the zone closest to the mirror (Zone A) and the proportion of squid that touched the mirror softly. For example, the gabazine experiment was carried out at a later date than the picrotoxin experiment. This may have altered factors including the natural environmental conditions squid were exposed to before capture, such as temperature or turbidity, and the age of the squid captured. Nevertheless, nine out of the 11 behaviours measured showed a consistent response to CO₂ across the two experiments, suggesting the reliability of these results.

Squid exposed to elevated CO₂ spent more time in the zone closest to the mirror, tended to remain in this zone rather than move in and out of it, exhibited a decreased latency until the first soft mirror touch, and an increased number of soft mirror touches compared to squid at ambient CO₂ conditions. These results suggest that elevated CO₂ conditions may increase the exploratory behaviours of squid directed towards their mirror image and an increased attraction of squid to conspecifics. This contrasts with the elevated CO₂-induced loss of conspecific chemical cue attraction in larval banded coral shrimp (Lecchini et al., 2017). These opposing results may be due to the differing baseline behaviours of the studied species. Larval shrimp are attracted to conspecific chemical cues as a signal for settlement (Lecchini et al., 2017), whereas adult *I. pygmaeus* are solitary and individuals are thought to avoid each other (Moynihan, 1983). Differences may also be due to the differing senses and taxa tested. Furthermore, coral shrimp were offered a binary choice (conspecific versus heterospecific chemical cues). However in our experiments, squid did not have a choice of different cues, only whether to interact with the mirror image or not.

Elevated CO₂ also increased the proportion of squid that aggressively touched the mirror and the number of aggressive mirror touches, suggesting elevated CO₂ may increase conspecific-directed aggression in *I. pygmaeus*. All measures of activity (time spent active, distance moved and average speed) were also increased with exposure to elevated CO₂. This is consistent with increased activity levels in male *I. pygmaeus* at elevated CO₂ (626 and 956 μ atm) when measured by mean number of line crosses (Spady et al., 2014). In the bigfin reef squid, active time, total distance moved and average speed were also higher at elevated CO₂ conditions (935 μ atm) (Spady et al., 2018). In contrast, activity decreased at elevated CO₂ levels in para-larvae of the longfin inshore squid *Doryteuthis pealeii* (Zakroff et al., 2018). Differences between studies may be due to the different species used, or differences in the timing of CO₂ treatment. *I. pygmaeus* and *S. lessoniana* were exposed to elevated CO₂ levels as adults (this study, Spady et al. (2014) and Spady et al. (2018)) whereas *D. pealeii* were exposed to elevated CO₂ for the duration of egg development (Zakroff et al., 2018), in which there is already high CO₂ levels within the egg (Hu and Tseng, 2017).

It is unknown how CO₂-induced increases in conspecific attraction, aggression, and activity levels in *I. pygmaeus* may translate to changes in the wild. More conspecific interactions, particularly aggressive ones, could increase the prevalence of injuries in elevated CO₂ conditions. Increased activity levels may adversely affect the finely tuned energy budgets of squid (Rodhouse, 1998) and increase detection by predators (Draper and Weissburg, 2019).

Behavioural change in response to drug treatment

Within marine invertebrates, the GABA hypothesis has been assessed in gastropod molluscs (Moya et al., 2016; Watson et al., 2014; Zlatkin and Heuer, 2019), a bivalve mollusc (Clements et al., 2017) and decapod crustaceans (Charpentier and Cohen, 2016; de la Haye et al., 2012; Ren et al., 2018). Here, we test the GABA hypothesis for the first time in a cephalopod, and assess whether Glu-, ACh- and DA- gated Cl⁻ channels may also be involved in the CO₂-induced behavioural changes, by administration of both gabazine and picrotoxin to male two-toned pygmy squid exposed to ambient or elevated CO₂ conditions.

Behavioural effects of gabazine and picrotoxin

The effects of both gabazine and picrotoxin treatment at ambient CO₂ on specific behavioural traits in the current study suggests that receptors in *I. pygmaeus* are sensitive to gabazine and picrotoxin. Furthermore, the concentrations used did not cause any obvious convulsions, which can be produced by the excitotoxic effects of GABA_A R antagonists when administered systemically (Hinton and Johnston, 2018). The concentrations used are based on previous *in vivo* studies showing behavioural change with no convulsant effects reported (Biscocho et al., 2018; Chivers et al., 2014; Hamilton et al., 2013; Nilsson et al., 2012; Rittschof et al., 1986; Watson et al., 2014). There is no evidence for an effect of gabazine at ambient CO₂ on all activity measures, also indicating no convulsant effects. Picrotoxin increased all measures of activity levels at ambient CO₂. This is likely due to the action of picrotoxin on the neural circuits underlying locomotion, rather than causing excitotoxicity. At a synapse within the central pattern generator for pteropod mollusc swimming, pre-synaptic release of ACh causes post-synaptic inhibition via an increase in Cl⁻ permeability (Panchin et al., 1995; Panchin and Sadreyev, 1997), and picrotoxin antagonizes inhibition at this synapse (Arshavsky et al., 1985). Furthermore, picrotoxin has been shown to affect cephalopod mollusc locomotion (Chichery and Chichery, 1985). Receptors that are sensitive to picrotoxin but not gabazine, such as ACh-gated Cl⁻ channels, may be involved in the generation of mollusc swimming behaviour explaining the effect of picrotoxin, but not gabazine, on *I. pygmaeus* activity. Thus, the behavioural effects of gabazine and picrotoxin in *I. pygmaeus* are likely not convulsive side effects but rather due to the action of these drugs on the underlying neural circuits.

The effect of gabazine and picrotoxin at ambient CO₂ on some, but not other, behavioural traits is likely due to different neural circuits underlying different behaviours. Only those behaviours in which the drug's target receptor(s) play an important role in are affected by drug administration. Both gabazine and picrotoxin affected multiple measures of space use and soft mirror touching behaviour at ambient CO₂, suggesting that both gabazine-sensitive receptors (GABA_A-like Rs and possibly also GABA-gated cation channels) and picrotoxin-sensitive ligand-gated Cl⁻ channels may be important in different aspects of squid attraction and exploratory behaviour towards their mirror image/conspecific. Interestingly, gabazine and picrotoxin mostly had opposite effects on these behavioural traits which may be due to the different target receptors of gabazine and picrotoxin. Gabazine and picrotoxin also affected various measures of aggressive mirror touching behaviour, suggesting that GABA_A-like Rs (and possibly also GABA-gated cation channels) and/or picrotoxin-

sensitive ligand-gated Cl⁻ channels may also be important for producing conspecific-directed aggressive behaviours. As far as we are aware, no research has assessed what receptor(s) are involved in conspecific-directed behaviours in marine invertebrates. However, vertebrate GABA_A Rs have also been found to be involved in mammalian conspecific-directed behaviours; GABA_A R agonist administration into the rat brain increased social approach and conspecific-directed aggressive behaviours, while antagonising GABA_A Rs decreased conspecific-directed aggressive behaviours (Depaulis and Vergnes, 1985).

Drug treatment effects across CO₂ conditions

Both gabazine and picrotoxin had a different effect across CO₂ treatments on a behavioural measure of space use, and on two activity measures. Gabazine had an added effect across CO₂ on the time spent in the zone closest to the mirror (Zone A), active time and distance moved by the squid. Picrotoxin had a removed effect across CO₂ on the time in Zone A and active time, and a diminished effect across CO₂ on distance moved. The different effect of both gabazine and picrotoxin across CO₂ provides strong evidence for the GABA hypothesis in *I. pygmaeus*. Furthermore, as the different effect of gabazine and picrotoxin across CO₂ were in different directions (added effect versus removed or diminished effect, respectively) this suggests that it is not just the common target receptor, the GABA_A-like Rs, but the target receptors of both gabazine and picrotoxin that are affected by elevated CO₂. Thus, altered functioning of GABA-, Glu-, ACh- and DA-gated Cl⁻ channels (and possibly also GABA-gated cation channels) may underlie the CO₂-induced increase in the time spent in Zone A and the active time and distance moved by squid.

Gabazine had an opposite effect across CO₂ treatments on the number of visits to Zone A, decreasing the number of visits at ambient CO₂ but increasing visits at elevated CO₂. This suggests that a reversal in the flow of ions through, and a switch in function of, the GABA_A-like R may underlie the CO₂-induced decrease in the number of visits to Zone A.

For the latency to the first soft mirror touch, gabazine had a similar effect across CO₂ (increased the latency at both ambient and elevated CO₂), whereas picrotoxin had an opposite effect across CO₂ (decreased the latency at ambient CO₂ and increased the latency at elevated CO₂). This suggests that altered functioning of ligand-gated Cl⁻ channels, other than GABA_A-like Rs, underlies the CO₂-induced decrease in the latency to the first soft mirror touch. In molluscs, Glu-, ACh- and DA-gated Cl⁻ channels are all antagonised by picrotoxin (Magoski and Bulloch, 1999; Piggott et al., 1977; Yarowsky and Carpenter, 1978b). Serotonin-gated Cl⁻ channels exist in nematodes (Ranganathan et al., 2000) and possibly also molluscs (Gerschenfeld and Tritsch, 1974), but it is unknown whether serotonin-gated Cl⁻ channels are picrotoxin-sensitive. Our results cannot distinguish which specific ligand-gated Cl⁻ channels are involved in the behavioural effects of elevated CO₂, but they do suggest that ligand-gated Cl⁻ channels other than the GABA_A-like R underlie this particular CO₂-induced behavioural change. Furthermore, the opposite effect of picrotoxin across CO₂ suggests there is a reversal in ion flow through, and a switch in function of, these ligand-gated Cl⁻ channels. This finding agrees with previous suggestions that other ligand-gated Cl⁻ channels, such as vertebrate glycine receptors or invertebrate Glu-gated Cl⁻ channels, are likely involved due to their similarity to the GABA_A R (Thomas et al., 2020; Tresguerres and Hamilton, 2017). Our results also agree with molecular work showing altered expression of genes encoding for the LGICs (and associated proteins) of glycinergic-like, glutamatergic and cholinergic synapses at elevated CO₂ in a pteropod mollusc (Moya et al., 2016). However, this result does not necessarily preclude the possibility of GABA_A-like Rs also being involved in the CO₂-induced decrease in the latency to the first soft mirror touch. If gabazine acts on GABA-gated cation channels, the influence of these GABA-gated cation channels could potentially mask the effect of GABA_A-like Rs.

In two CO₂-affected behavioural traits, the number of soft mirror touches and the latency to the first aggressive mirror touch, gabazine had a diminished effect whereas picrotoxin had a similar effect across CO₂ conditions. This suggests that receptors sensitive to gabazine, but not picrotoxin, may underlie the CO₂-induced change of these behavioural traits, for example GABA-gated cation channels. This suggests the potentially widespread nature of the mechanisms underlying CO₂-induced behavioural changes. However, the lack of evidence for a different effect of picrotoxin across CO₂ conditions does not necessarily rule out the involvement of GABA_A-like Rs. For example, if the GABA_A-like Rs are the only ligand-gated Cl⁻ channels involved in these particular behavioural changes, the influence of other ligand-gated Cl⁻ channels could mask the effect of the GABA_A-like Rs.

A range of other CO₂-affected behavioural traits showed no evidence of gabazine nor picrotoxin having a different effect across CO₂ conditions. This suggests that GABA_A-like Rs as well as other gabazine-sensitive (possibly GABA-gated cation channels) and picrotoxin-sensitive (Glu-, ACh- and DA- gated Cl⁻ channels) receptors are not involved in the mechanisms underlying the CO₂-induced change of these specific behavioural traits. Therefore, other mechanisms may be involved in these behavioural alterations.

Overall, our results suggest that elevated CO₂ alters behaviour via multiple mechanisms in *I. pygmaeus*, with different mechanisms underlying the changes of different behavioural traits at elevated CO₂ (Fig. 5). It is possible that elevated CO₂ results in a suite of changes within the nervous system, including, but not limited to, altered functioning of GABA-, Glu-, ACh- and DA-gated Cl⁻ channels as well as possibly GABA-gated cation channels. As different neural circuits produce different behaviours, only some of the CO₂-induced changes within the nervous system may result in the alteration of a particular behavioural trait. This can potentially explain the variability in the effects of elevated CO₂ among behaviours.

The complexity of the mechanisms underlying CO₂-induced behavioural changes is further increased by the fact that receptors can vary in subunit composition, and therefore pharmacological sensitivity, between differing subcellular, cellular and tissue locations (Lee and Maguire, 2014). For example, GABA_A Rs are composed of 5 subunits and there are 19 different subunit genes identified in humans; α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π and ρ 1-3 (Simon et al., 2004). These subunits can combine in various ways to form GABA_A R subtypes that differ in location, the functions (including behaviours) they are involved in, and their pharmacological profile (see Olsen and Sieghart (2009) for more detail). For example, vertebrate GABA_A Rs made up of ρ subunits (GABA_A- ρ Rs, which are part of the GABA_A R family (Olsen and Sieghart, 2008) but sometimes called GABA_C Rs) are less sensitive to gabazine than GABA_A Rs not composed of ρ subunits (Feigenspan and Bormann, 1994; Woodward et al., 1993; Zhang et al., 2008), and GABA_A (α 1 β 2 γ 2) and GABA_A- ρ 2 Rs are 10-fold more sensitive to picrotoxin than GABA_A- ρ 1 Rs (Naffaa et al., 2017). GABA_A R subunits have been less studied in molluscs, though molluscan GABA_A R-like α and β subunits have been identified (Harvey et al., 1991; Moroz et al., 2006; Stewart et al., 2011) and GABA_A R-like α , β , γ and ρ subunit sequences have been predicted in molluscs (for example GenBank BioProject PRJNA551489 and PRJNA625562). Picrotoxin antagonised GABA_A-like R hyperpolarisations in some neurons, but had no effect on ionotropic GABA Rs of another neuron within the same mollusc species (Norekian and Satterlie, 1993; Norekian and

Malyshev, 2005). Therefore, GABA_A R-like subtypes that have differing pharmacological sensitivities are likely present in molluscs. Systemic drug administration allowed us to determine what receptors may be involved in the CO₂-induced behavioural changes, but it cannot address the heterogeneity of receptor subtypes between different subcellular locations, cell types and tissues.

Conclusions and future work

We found that elevated CO₂ increased conspecific-directed attraction and aggression as well as activity levels in male two-toned pygmy squid. Previous studies exclusively using gabazine have provided evidence for altered GABA_A-like R functioning as a mechanism for CO₂-induced behavioural changes in a gastropod and bivalve mollusc. Our study now also supports the GABA hypothesis in a cephalopod mollusc. Furthermore, we have provided more robust support for the GABA hypothesis in molluscs by using both gabazine and picrotoxin, which is structurally unrelated to, and has a better studied molluscan pharmacological profile than, gabazine. Therefore, altered GABA_A-like R functioning may be a common mechanism underlying behavioural change at elevated CO₂ across marine molluscs. The use of both gabazine and picrotoxin also showed, for the first time in any marine invertebrate taxa, that altered functioning of ligand-gated Cl⁻ channels other than the GABA_A-like R may be involved in CO₂-induced behavioural changes. We propose that elevated CO₂ leads to a suite of changes within the nervous system. As different neural circuits underlie different behaviours, and these different neural circuits may have various sensitivities to elevated CO₂, this can potentially explain the variability in the behavioural effects of elevated CO₂, both among behaviours and among species. The use of model animals will be important for future research to assess the complexities of the mechanisms underlying elevated CO₂-induced behavioural change. For example, an antipredator response was recently shown to be altered at elevated CO₂ levels in *Aplysia californica* (Zlatkin and Heuer, 2019), a well-studied model organism for neurobiological work. Drug administration in a species whose pharmacological profile is well known, as well as other techniques such as gene knockdown and transcriptomics, will be important to understand the complexities of the mechanisms underlying behavioural change at elevated CO₂.

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Competing interests

No competing interests declared.

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Data availability

Raw carbonate chemistry data, raw behavioural data, behavioural videos, R code and the Bayesian modelling results in table format (estimates and contrasts) are available at DOI: [10.25903/y6kz-hm11](https://doi.org/10.25903/y6kz-hm11) (Thomas et al., 2021).

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Figures

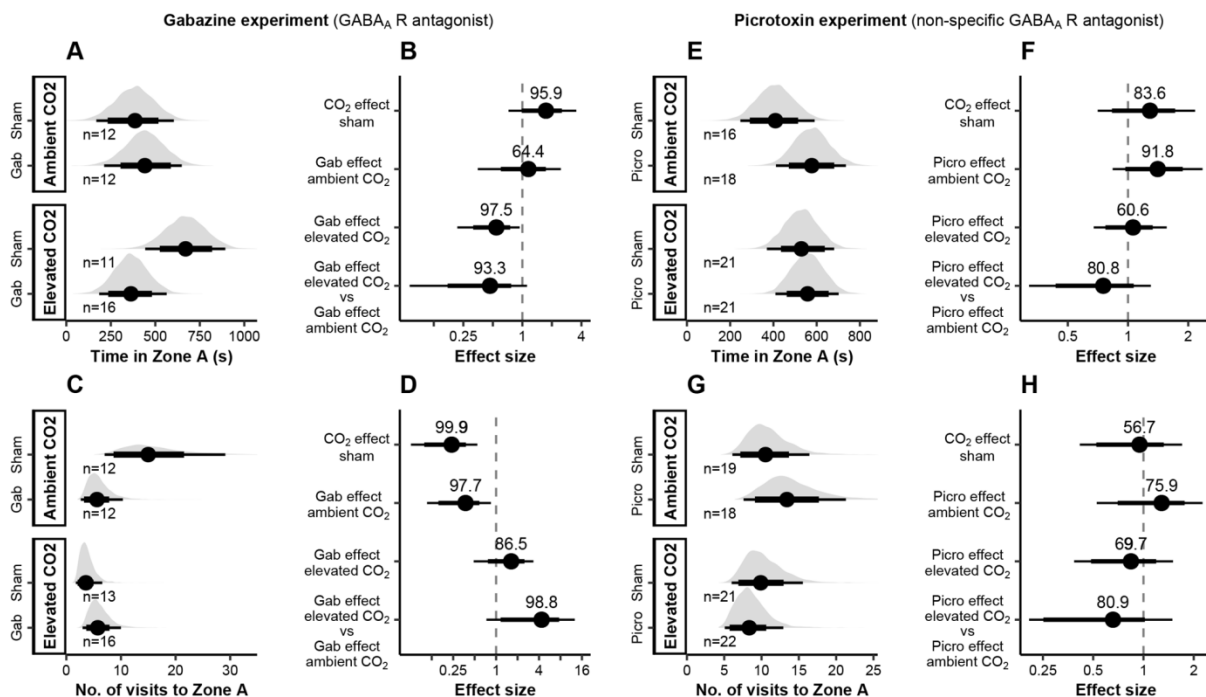


Figure 1. Effects of CO₂ and drug treatment on space use. A - D gabazine experiment, **E - H** picrotoxin experiment. **A & E** partial plots of the time squid spent in Zone A, **B & F** caterpillar plots for the effect of CO₂ and drug treatment on the time squid spent in Zone A, **C & G** partial plots of the number of times squid visited Zone A, **D & H** caterpillar plots for the effect of CO₂ and drug treatment on the number of times squid visited Zone A. Partial plots: point represents the Bayesian posterior median value \pm 80% (thick lines) and 95% (thin lines) highest posterior density interval (HPDI), overlaid with the distribution of Bayesian posterior values. Caterpillar plots: point represents the median effect size (odds ratio for proportion data, fold change for others) \pm 80% (thick lines) and 95% (thin lines) HPDI. The dashed vertical line indicates an effect size of 1 (no effect) and the numbers above each point are the percent probability of this effect (increase or decrease) occurring.

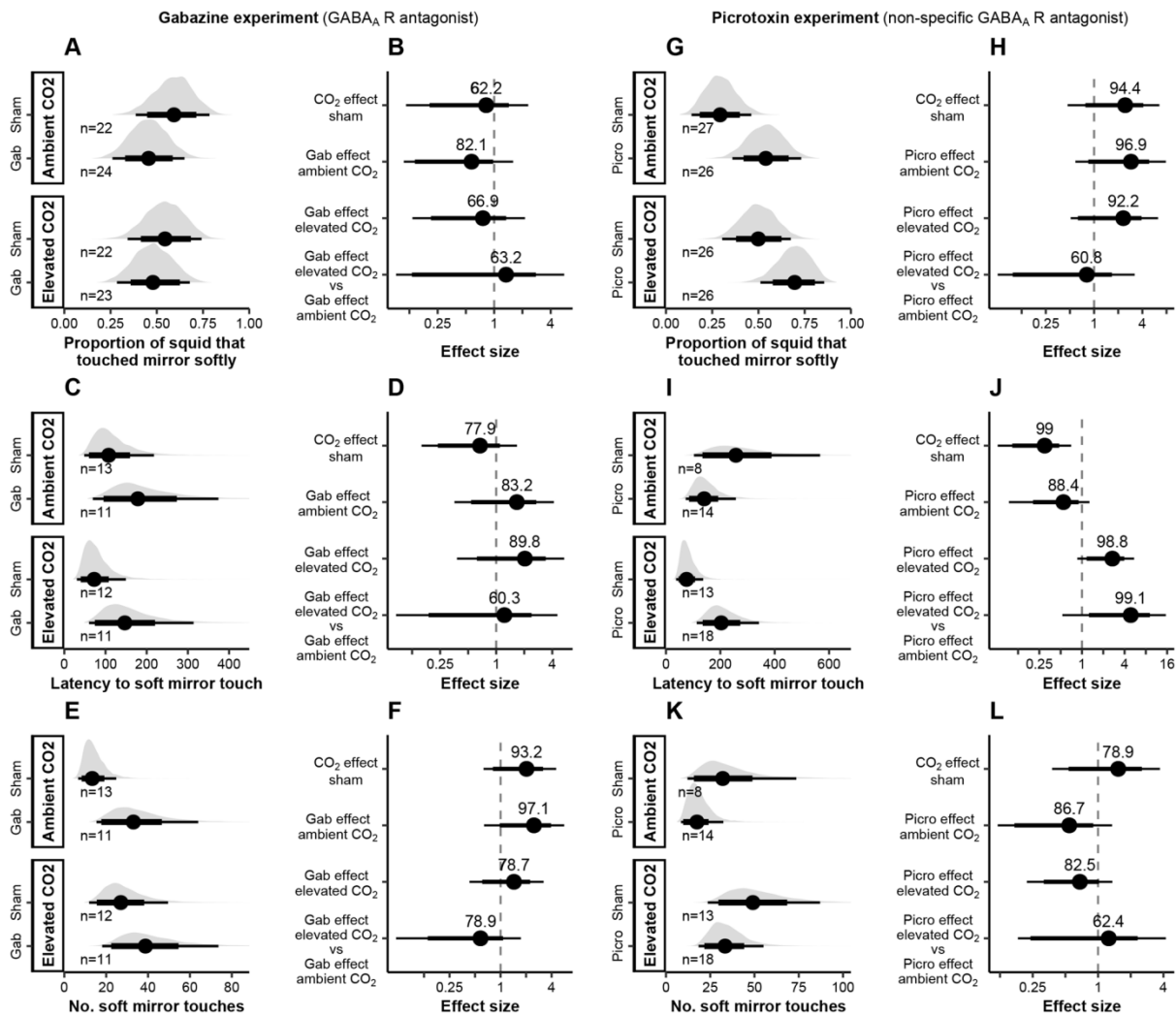


Figure 2. Effects of CO₂ and drug treatment on measures of soft mirror touching behaviour. A - F gabazine experiment, **G - L** picrotoxin experiment. **A & G** partial plots of the proportion of squid that touched the mirror softly, **B & H** caterpillar plots for the effect of CO₂ and drug treatment on the proportion of squid that touched the mirror softly, **C & I** partial plots of the latency from introduction to the first soft mirror touch (seconds), **D & J** caterpillar plots for the effect of CO₂ and drug treatment on the latency from introduction to the first soft mirror touch (seconds), **E & K** partial plots of the total number of soft mirror touches per individual, **F & L** caterpillar plots for the effect of CO₂ and drug treatment on the total number of soft mirror touches per individual. Partial and caterpillar plot symbols as per Figure 1.

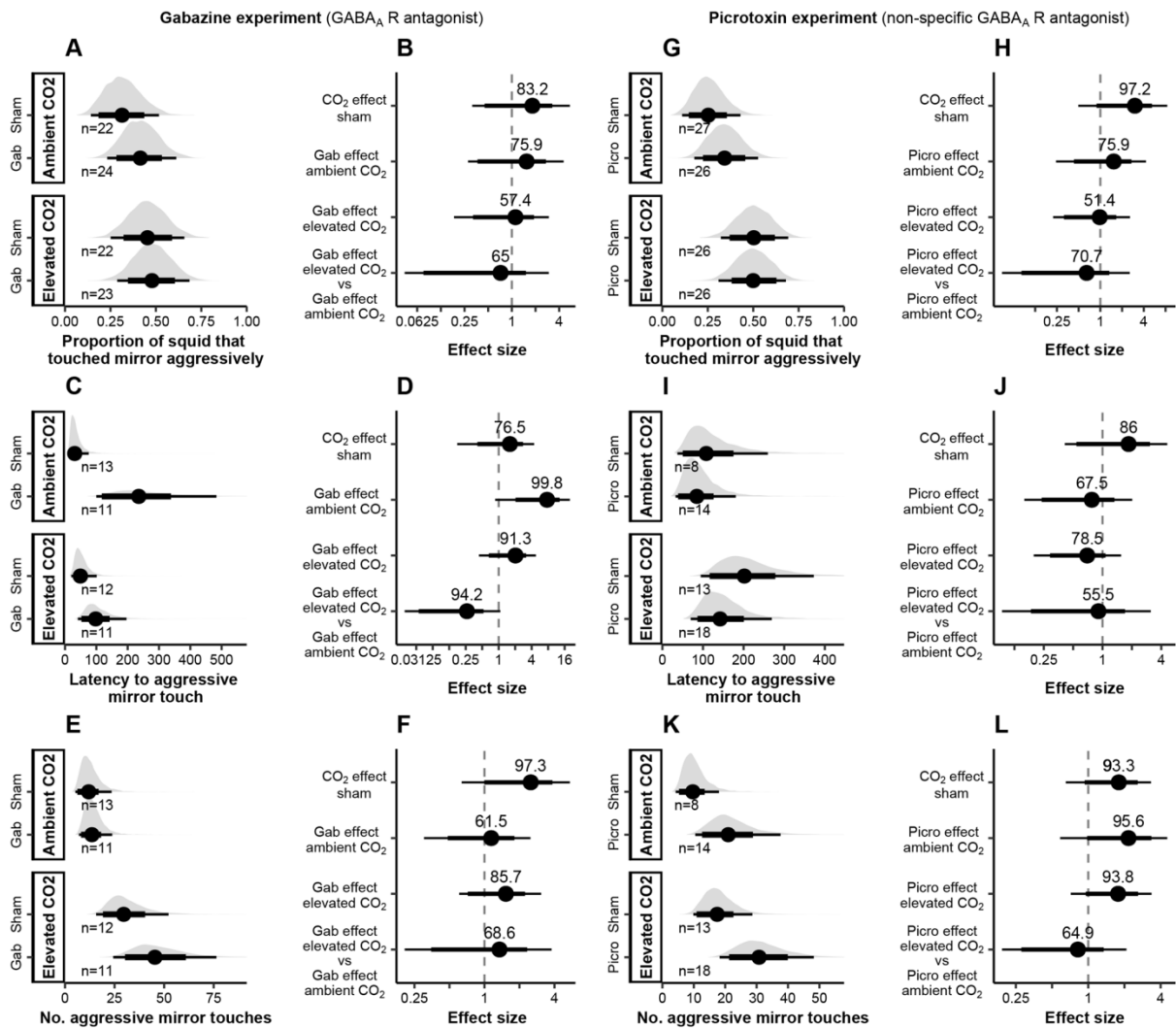


Figure 3. Effects of CO₂ and drug treatment on measures of aggressive mirror touching behaviour. A - F gabazine experiment, G - L picrotoxin experiment. A & G partial plots of the proportion of squid that touched the mirror aggressively, B & H caterpillar plots for the effect of CO₂ and drug treatment on the proportion of squid that touched the mirror aggressively, C & I partial plots of the latency from introduction to the first aggressive mirror touch (seconds), D & J caterpillar plots for the effect of CO₂ and drug treatment on the latency from introduction to the first aggressive mirror touch (seconds), E & K partial plots of the total number of aggressive mirror touches per individual, F & L caterpillar plots for the effect of CO₂ and drug treatment on the total number of aggressive mirror touches per individual. Partial and caterpillar plot symbols as per Figure 1.

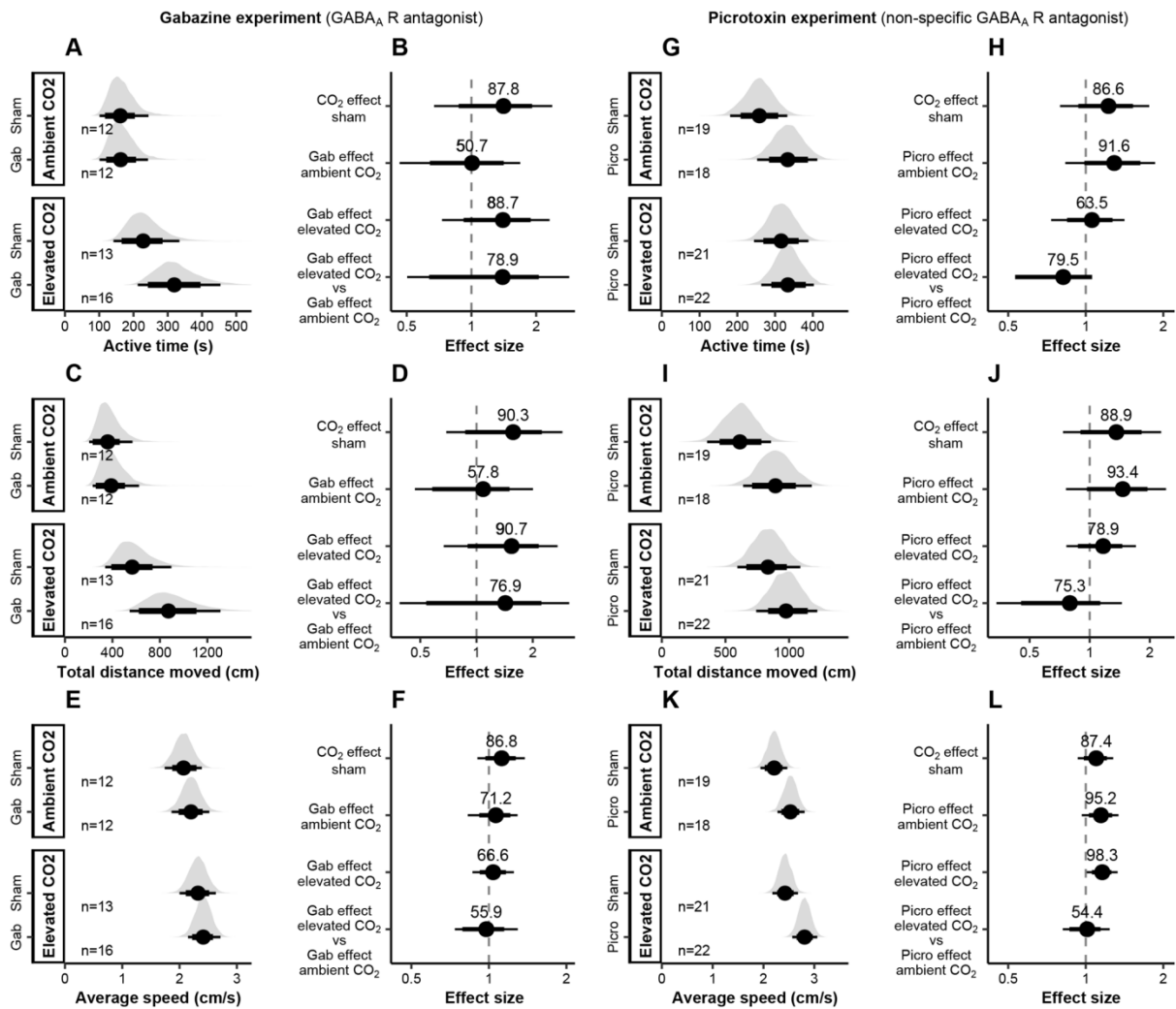


Figure 4. Effects of CO₂ and drug treatment on activity measures. A - F gabazine experiment, **G - L** picrotoxin experiment. **A & G** partial plots of the time squid spent active, **B & H** caterpillar plots for the effect of CO₂ and drug treatment on the time squid spent active, **C & I** partial plots of the total distance moved by squid, **D & J** caterpillar plots for the effect of CO₂ and drug treatment on the total distance moved by squid, **E & K** partial plots of squid average speed, **F & L** caterpillar plots for the effect of CO₂ and drug treatment on squid average speed. Partial and caterpillar plot symbols as per Figure 1.

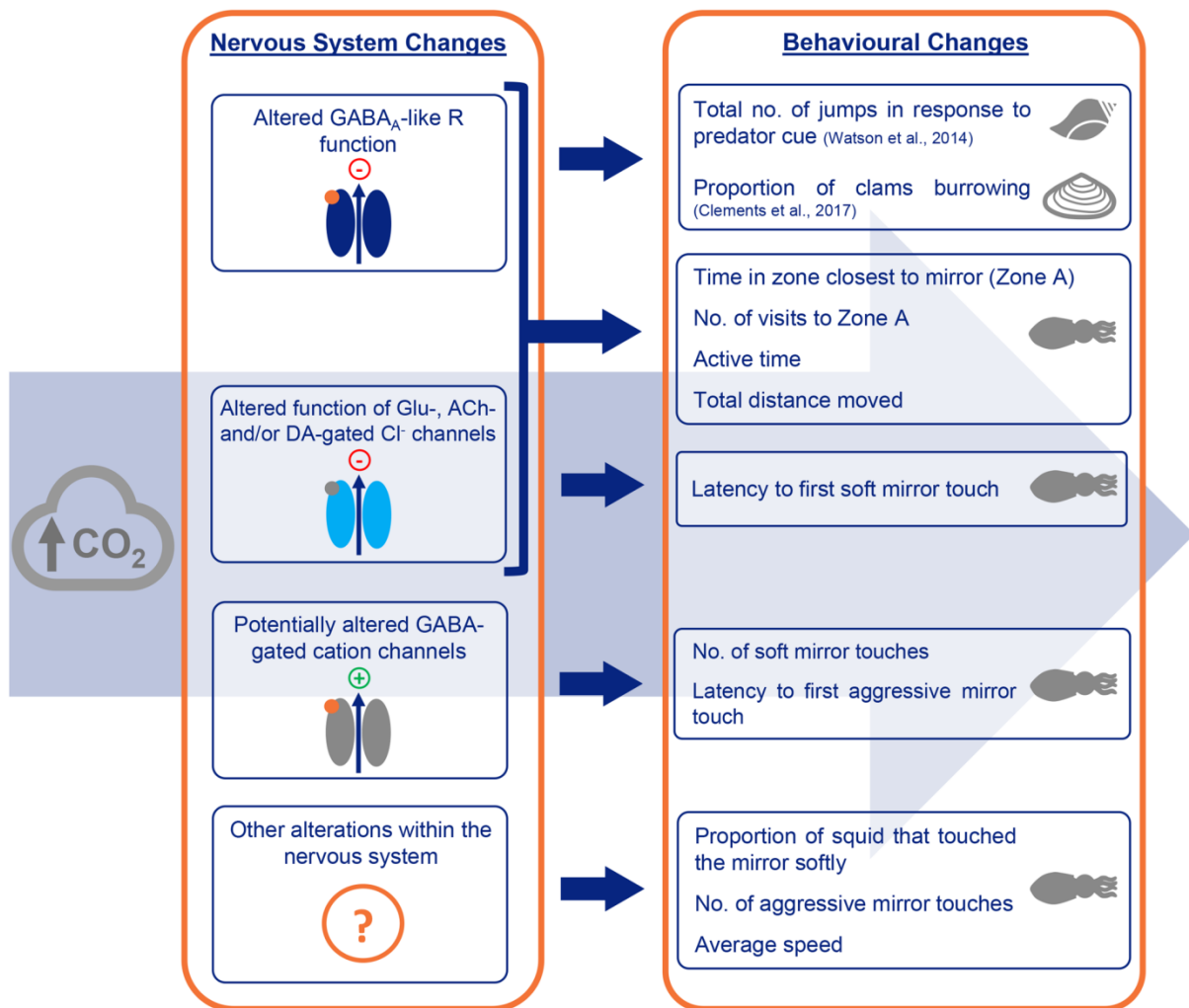


Figure 5. Evidence within molluscs that elevated CO₂ results in a suite of changes within the nervous system, with each change (or group of changes) altering different behavioural traits. The mechanisms underlying CO₂-induced behavioural change were tested using gabazine in the jumping conch snail and soft-shell clam, and both gabazine and picrotoxin in the two-toned pygmy squid (this study).

Table 1. Summary of the varying types of different drug effects across CO₂ treatments and how they suggest altered receptor function at elevated CO₂.

Drug effect in elevated vs ambient CO ₂ treatments	Definition	Suggested change in ion flow and receptor function at elevated CO ₂	Example for how the different drug effect at elevated compared to ambient CO ₂ suggests a change in receptor function
Opposite	Drug treatment increases the measured behaviour at ambient CO ₂ but decreases the behaviour at elevated CO ₂ (or vice versa).	Reversal in ion flow through the receptor channel resulting in a switch in function of the target receptor(s).	A behaviour is suppressed by GABA _A -like R-induced inhibition and generated by GABA _A -like R-induced excitation. At ambient CO ₂ , there is a net influx of negative charge through the GABA _A -like R having an inhibitory effect on the behaviour. Drug treatment at ambient CO ₂ antagonises the GABA _A -like R-induced inhibition and suppression of the behaviour is removed, resulting in an increase in the measured behaviour. At elevated CO ₂ , ion flow reverses and there is a net efflux of negative charge through the GABA _A -like R resulting in excitation. Thus, the measured behaviour is higher at elevated compared to ambient CO ₂ . Drug treatment at elevated CO ₂ antagonises the GABA _A -like R-induced excitation and generation of the behaviour is decreased, resulting in a decrease in the measured behaviour.
Removed	Drug treatment increases or decreases the behaviour at ambient CO ₂ but has no effect at elevated CO ₂ .	Reduction in ion flow through the receptor channel resulting in a loss of function of the target receptor(s).	The ion gradient across the neuronal membrane is decreased at elevated compared to ambient CO ₂ . At ambient CO ₂ , ligand binding produces a flow of ions through the GABA _A -like R having an inhibitory effect which alters behaviour. Therefore, drug treatment at ambient CO ₂ antagonises the GABA _A -like R-induced inhibition and has a behavioural effect. However, at elevated CO ₂ ligand binding results in a very small, or no, flow of ions through the GABA _A -like R and produces very weak or no inhibition. This very weak (or absence of) GABA _A -like R-induced inhibition has no effect on behaviour and thus drug treatment has no behavioural effect at elevated CO ₂ .
Added	Drug treatment has no effect at ambient CO ₂ but increases or decreases the behaviour at elevated CO ₂ .	Increased ion flow through the receptor channel resulting in a gain of function of the target receptor(s).	The ion gradient across the neuronal membrane is increased at elevated compared to ambient CO ₂ . At ambient CO ₂ , ligand binding results in a very small, or no, flow of ions through the GABA _A -like R producing very weak or no inhibition. This very weak (or absence of) GABA _A -like R-induced inhibition has no effect on behaviour and thus drug treatment has no behavioural effect at ambient CO ₂ . However, at elevated CO ₂ ligand binding produces a flow of ions through the GABA _A -like R resulting in inhibition which affects behaviour. Therefore, drug treatment at elevated CO ₂ antagonises the GABA _A -like R-induced inhibition and

			has a behavioural effect.
Diminished	Drug treatment causes a smaller increase or decrease in behaviour at elevated CO ₂ compared to ambient CO ₂ .	Reduction in ion flow through the receptor channel resulting in a decreased function of the target receptor(s) (direction of ion flow remains the same).	The ion gradient across the GABA _A -like R is smaller at elevated compared to ambient CO ₂ . Therefore, ligand binding produces a smaller flow of ions through the GABA _A -like R and a weaker inhibitory effect at elevated compared to ambient CO ₂ , although the direction of ion flow remains the same at both CO ₂ conditions. Drug treatment antagonises the strong inhibition at ambient CO ₂ and the weaker inhibition at elevated CO ₂ . Consequently, drug treatment has a large behavioural effect at ambient CO ₂ and a smaller behavioural effect at elevated CO ₂ .
Enhanced	Drug treatment causes a larger increase or decrease in behaviour at elevated CO ₂ compared to ambient CO ₂ .	Increased ion flow through the receptor channel resulting in an enhanced function of the target receptor(s) (direction of ion flow remains the same).	The ion gradient across the GABA _A -like R is larger at elevated compared to ambient CO ₂ . Therefore, ligand binding produces a larger flow of ions through the GABA _A -like R and a stronger inhibitory effect at elevated compared to ambient CO ₂ , although the direction of ion flow remains the same at both CO ₂ conditions. Drug treatment antagonises the weak inhibition at ambient CO ₂ and the stronger inhibition at elevated CO ₂ . Consequently, drug treatment has a small behavioural effect at ambient CO ₂ and a larger behavioural effect at elevated CO ₂ .

The examples in this table all refer to inhibitory GABA_A-like Rs. However, these explanations similarly refer to all receptors investigated in this study; gabazine tested the functioning of inhibitory and excitatory GABA_A-like Rs (and possibly GABA-gated cation channels) and picrotoxin tested the functioning of inhibitory and excitatory GABA_A-like Rs, and Glu-, ACh- and DA- gated Cl⁻ channels.

Table 2. Seawater carbonate chemistry for duplicate ambient and elevated CO₂ treatment systems in the gabazine and picrotoxin experiments, values are mean ± standard deviation.

CO ₂ Treatment System	Temperature (°C)	Salinity	pH _{total}	Alkalinity (μmol/kg SW)	pCO ₂ (μatm)
Gabazine Experiment					
Ambient 1	26.1 ± 0.2	34.9 ± 0.2	8.02 ± 0.03	2390 ± 114	450 ± 37
Ambient 2	26.2 ± 0.2	34.7 ± 0.3	7.99 ± 0.03	2385 ± 110	483 ± 43
Elevated 1	26.0 ± 0.2	35.0 ± 0.1	7.72 ± 0.02	2404 ± 113	1014 ± 83
Elevated 2	26.1 ± 0.3	35.1 ± 0.1	7.72 ± 0.02	2405 ± 120	1006 ± 54
Picrotoxin Experiment					
Ambient 1	26.0 ± 0.1	34.6 ± 0.2	8.09 ± 0.13	2619 ± 48	413 ± 55
Ambient 2	26.1 ± 0.1	34.6 ± 0.2	8.10 ± 0.05	2623 ± 44	401 ± 59
Elevated 1	26.0 ± 0.2	35.2 ± 0.4	7.73 ± 0.03	2648 ± 46	1075 ± 88
Elevated 2	26.1 ± 0.1	35.5 ± 0.5	7.73 ± 0.02	2655 ± 50	1066 ± 48

Table 3. Summary of the CO₂ effect in sham-treated squid, and the type of different drug effect across CO₂ treatments.

	CO ₂ effect in sham-treated squid				Different drug effect across CO ₂ conditions			
	Gabazine Experiment		Picrotoxin Experiment		Gabazine Experiment		Picrotoxin Experiment	
	Evidence	Direction	Evidence	Direction	Evidence	Type	Evidence	Type
Space Use								
Time in Zone A (s)	Very strong	↑	Strong	↑	Strong	Added	Evidence	Removed
No. of visits to Zone A	Very strong	↓	None	-	Very strong	Opposite	Evidence	Removed
Soft mirror touch								
Proportion of squid that touch mirror softly	None	-	Strong	↑	None	-	None	-
Latency to first soft mirror touch (s)	Some evidence	↓	Very strong	↓	None	-	Very strong	Opposite
No. of soft mirror touches	Strong	↑	Some evidence	↑	Some evidence	Diminished	None	-
Aggressive mirror touch								
Proportion of squid that touch mirror aggressively	Evidence	↑	Very strong	↑	None	-	None	-
Latency to first aggressive mirror touch (s)	Some evidence	↑	Evidence	↑	Strong	Diminished	None	-
No. of aggressive mirror touches	Very strong	↑	Strong	↑	None	-	None	-

Activity measures								
Active time (s)	Evidence	↑	Evidence	↑	Some evidence	Added	Some evidence	Removed
Total distance moved (cm)	Strong	↑	Evidence	↑	Some evidence	Added	Some evidence	Diminished
Average speed (cm/s)	Evidence	↑	Evidence	↑	Evidence	-	None	-

Evidence: Very strong = probability of an effect $\geq 95\%$, strong = probability of an effect $\geq 90\%$, evidence = probability of an effect $\geq 80\%$, some evidence = probability of an effect $\geq 75\%$. CO₂ effect in sham treated squid, Direction: ↑ = increase in the behaviour at elevated compared to ambient CO₂, ↓ = decrease in the behaviour at elevated compared to ambient CO₂. Different drug effect across CO₂ conditions, Type: Opposite = drug treatment caused an increase in the behavioural trait at ambient CO₂ and a decrease in the behavioural trait at elevated CO₂, or vice versa, Removed = drug treatment increased/decreased the behaviour at ambient CO₂ but had no effect at elevated CO₂, Added = drug treatment had no effect at ambient CO₂ but increased/decreased the behaviour at elevated CO₂, Diminished = drug treatment caused a smaller increase/decrease in behaviour at elevated compared to ambient CO₂

Table S1. Studies that have investigated the mechanism of action of gabazine in molluscs. All studies in molluscs have been done in gastropod molluscs. Target receptor: ion? = unknown which ion(s) the receptor is permeable to, hyper/depolarising? = unknown whether activation of the receptor results in hyperpolarisation (inhibitory) or depolarisation (excitatory). Administration method: bath application = animal/tissue/neuron sitting in the solution. Gabazine effect: the effect of gabazine on the behaviour measured or electrophysiological recording (neurotransmitter-induced hyper or depolarisation): - = no effect, X = completely blocked, ↓ = decrease, ↑ = increase.

Target receptor	Species Common name	Life stage	Gabazine conc. (uM)	Administration method	Method of measurement	Gabazine effect	Reference
Ionotropic GABA R (Cl ⁻ , hyper/depolarising?)	<i>Tritia obsoleta</i> Marine mud snail	Larvae	0.1, 1, 10, 100	Bath application	<i>In vivo</i> Percentage of larvae metamorphosing	-	Biscocho et al. (2018)
Ionotropic GABA R (ion?, hyperpolarising)	<i>Helix aspersa</i> Terrestrial snail	Not stated	10	Bath application then by micropipette	<i>In vitro</i> Electrophysiology	X	Vehovszky et al. (1989)
Ionotropic GABA R (ion?, depolarising)	<i>Helix aspersa</i> Terrestrial snail	Not stated	10	Bath application then by micropipette	<i>In vitro</i> Electrophysiology	↓	Vehovszky et al. (1989)
GABA R (type unknown)	<i>Nassarius obsoletus</i> Eastern mud snail (marine)	Larvae	1, 10 100, 1,000	Bath application	<i>In vivo</i> Percentage of larvae metamorphosing	- ↑	Welch (2015)

Table S2. Studies that have investigated the mechanism of action of picrotoxin in molluscs. All studies are in gastropod molluscs, apart from one study in a cephalopod mollusc (Chichery and Chichery, 1985). Target receptor: ion? = unknown which ion(s) the receptor is permeable to, hyper/depolarising? = unknown whether activation of the receptor results in hyperpolarisation (inhibitory) or depolarisation (excitatory). Administration method: superfusion = continuous flow over the outside of the tissue/neuron, perfusion = continuous flow through the tissue/neuron, bath application = animal/tissue/neuron sitting in the solution. Picrotoxin effect: The effect of picrotoxin on the behaviour measured or the electrophysiological recording (neurotransmitter-induced hyper or depolarisation): - = no effect, X = completely blocked, ↓ = decrease, ↑ = increase.

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Table S3. Explanatory variables, distribution family and link function as well as the priors used for the chosen model of each response variable in the gabazine experiment. Explanatory variables: * = interactive effect, + = additive effect. Priors: - = priors not applicable for this parameter, improper uniform = improper uniform priors were used (= uniform priors from infinity to -infinity).

Response variable	Explanatory variables	Family (link)	Intercept prior	Slope prior	sigma prior	shape prior
Time in Zone A (s)	CO2 * Drug	Gaussian (identity)	student_t(3, 316, 458.1)	improper uniform	student_t(3, 0, 458.1)	-
No. of visits to Zone A	CO2 * Drug + Mantle length	Negative binomial (log)	student_t(3, 1.4, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Proportion of squid that touched mirror softly	CO2 * Drug	Binomial (logit)	student_t(3, 0, 2.5)	improper uniform	-	-
Latency to first soft mirror touch (s)	CO2 * Drug	Gamma (log)	student_t(3, 3.7, 2.5)	improper uniform	-	gamma(0.01, 0.01)
No. of soft mirror touches	CO2 * Drug + Behavioural tank + Time of test	Negative binomial (log)	student_t(3, 2.9, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Proportion of squid that touched mirror aggressively	CO2 * Drug	Binomial (logit)	student_t(3, 0, 2.5)	improper uniform	-	-
Latency to first aggressive mirror touch (s)	CO2 * Drug + Behavioural tank + Time of test	Gamma (log)	student_t(3, 3.7, 2.5)	improper uniform	-	gamma(0.01, 0.01)
No. of aggressive mirror touches	CO2 * Drug	Negative binomial (log)	student_t(3, 2.9, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Active time (s)	CO2 * Drug	Gamma (log)	student_t(3, 5.3, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Total distance moved (cm)	CO2 * Drug	Gamma (log)	student_t(3, 6, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Average speed (cm/s)	CO2 * Drug	Gaussian (identity)	student_t(3, 2.1, 2.5)	improper uniform	student_t(3, 0, 2.5)	-

Table S4. Explanatory variables, distribution family and link function as well as the priors used for the chosen model of each response variable in the picrotoxin experiment. Explanatory variables: * = interactive effect, + = additive effect. Priors: - = priors not applicable for this parameter, improper uniform = improper uniform priors were used (= uniform priors from infinity to -infinity).

Response variable	Explanatory variables	Family(link)	Intercept	Slope	sigma	shape
Time in Zone A (s)	CO ₂ * Drug + Behavioural tank + Time of test	Gaussian (identity)	student_t(3, 677, 315.8)	improper uniform	student_t(3, 0, 315.8)	-
No. of visits to Zone A	CO ₂ * Drug + System	Negative binomial (log)	normal(0, 8)	normal(0, 2.5)	-	gamma(0.01, 0.01)
Proportion of squid that touched mirror softly	CO ₂ * Drug	Binomial (logit)	student_t(3, 0, 2.5)	improper uniform	-	-
Latency to first soft mirror touch (s)	CO ₂ * Drug	Gamma (log)	student_t(3, 4.4, 2.5)	improper uniform	-	gamma(0.01, 0.01)
No. of soft mirror touches	CO ₂ * Drug + Mantle length	Negative binomial (log)	student_t(3, 2.8, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Proportion of squid that touched mirror aggressively	CO ₂ * Drug	Binomial (logit)	student_t(3, 0, 2.5)	improper uniform	-	-
Latency to first aggressive mirror touch (s)	CO ₂ * Drug + System	Gamma (log)	normal(0, 10)	normal(0, 2.5)	-	gamma(0.01, 0.01)
No. of aggressive mirror touches	CO ₂ * Drug + Number of acclimation days + Date introduced to treatment tank	Negative binomial (log)	student_t(3, 3.1, 2.5)	improper uniform	-	gamma(0.01, 0.01)
Active time (s)	CO ₂ * Drug	Gaussian (identity)	student_t(3, 297.8, 206.5)	improper uniform	student_t(3, 0, 206.5)	-
Total distance moved (cm)	CO ₂ * Drug	Gaussian (identity)	student_t(3, 737.1, 641.1)	improper uniform	student_t(3, 0, 641.1)	-
Average speed (cm/s)	CO ₂ * Drug	Gaussian (identity)	student_t(3, 2.4, 2.5)	improper uniform	student_t(3, 0, 2.5)	-

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