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RESEARCH ARTICLE

The role of ligand-gated chloride channels in behavioural alterations at elevated CO_2 in a cephalopod

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ABSTRACT

Projected future carbon dioxide (CO_2) 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 CO_2-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 CO_2-induced behavioural changes in a cephalopod, the two-toned pygmy squid (*Idiosepius pygmaeus*). We exposed squid to ambient (~450 µatm) or elevated (~1000 µatm) CO_2 for 7 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 CO_2 increased conspecific-directed attraction and aggression, as well as activity levels. For some CO_2-affected behaviours, both gabazine and picrotoxin had a different effect at elevated compared with ambient CO_2, providing robust support for the GABA hypothesis within cephalopods. In another behavioural trait, picrotoxin but not gabazine had a different effect in elevated compared with ambient CO_2, providing the first pharmacological evidence, in fish and marine invertebrates, for altered functioning of ligand-gated chloride channels, other than the $GABA_AR$, underlying CO_2-induced behavioural changes. For some other behaviours, both gabazine and picrotoxin had a similar effect in elevated and ambient CO_2, suggesting altered function of ligand-gated chloride channels was not responsible for these CO_2-induced changes. Multiple mechanisms may be involved, which could explain the variability in the CO_2 and drug treatment effects across behaviours.

KEY WORDS: Ocean acidification, Squid, GABA, Ligand-gated chloride channels, Gabazine, Picrotoxin

INTRODUCTION

Anthropogenic carbon dioxide (CO_2) 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 CO_2 levels are known to alter a range of behaviours in a variety of fishes (Munday et al., 2019). Elevated CO_2-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; Thomas et al., 2020). The behavioural effects of elevated CO_2 are variable. Elevated CO_2 may affect some, but not other, behaviours within the same species. For example, in the blue mussel, elevated CO_2 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 CO_2 on the same behaviour may also be variable among taxa. For example, within molluscs, elevated CO_2 increased locomotion speed in two species of squid (Spady et al., 2014, 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 CO_2 is important to determine why there is such variability in behavioural alterations and to identify which animals will be most vulnerable to rising CO_2 levels. However, the mechanisms underlying behavioural change at elevated CO_2 across the diverse range of marine invertebrates are poorly understood (Thomas et al., 2020).

The prominent mechanistic explanation for elevated CO_2-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 ($GABA_AR$) is a ligand-gated ion channel (LGIC)/ionotropic receptor selectively permeable to chloride (Cl^-) and bicarbonate (HCO_3^-) ions (Bormann et al., 1987; Krnjević, 1974). Under normal conditions, binding of the neurotransmitter GABA opens the $GABA_AR$ channel, which usually allows a net influx of negative charge resulting in hyperpolarisation and inhibition of neuronal firing. At elevated CO_2 conditions, Nilsson et al. (2012) proposed that alterations in Cl^- and HCO_3^- ion gradients across the neuronal membrane, due to acid-base regulation, could alter the function of $GABA_ARs$. 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 $GABA_ARs$, 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 $GABA_AR$ antagonist gabazine (SR-95531) (Heaulme et al., 1986) attenuated CO_2-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 $GABA_AR$ agonist muscimol (Andrews and Johnston, 1979) produced opposite effects in fish exposed to elevated and control CO_2 levels (Hamilton et al., 2013). Recently, the GABA hypothesis has been further refined by the proposal that altered functioning of some $GABA_ARs$ initiates a vicious self-amplifying cycle, explaining how relatively small

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changes in ion gradients can result in large behavioural alterations (Schunter et al., 2019).

Invertebrate GABA also binds to Cl^- and HCO_3^- permeable ionotropic GABA receptors (referred to as $GABA_A$-like receptors or $GABA_A$-like Rs throughout this article 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 CO_2 levels (961 µatm) was restored to control levels by the addition of gabazine (Watson et al., 2014). In a bivalve mollusc, the soft-shell clam (*Mya arenaria*), burrowing behaviours altered at elevated sediment CO_2 levels (9532 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 CO_2 (1380 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 that 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 CO_2-induced behavioural changes. However, the action of gabazine 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 $GABA_A$R antagonists, such as picrotoxin, that are structurally unrelated to gabazine can increase our confidence in the evidence for the GABA hypothesis (Tresguerres and Hamilton, 2017).

Elevated CO_2 may also alter the functioning of other LGICs that are permeable to HCO_3^- and Cl^- ions. Altered glycine receptor functioning at elevated CO_2 levels has been suggested as another potential mechanism in fish because of its similarity to the $GABA_A$R (Tresguerres and Hamilton, 2017). Glycine receptors are not found in invertebrates; however, glutamate (Glu)-gated Cl^- channels are suggested to be the invertebrate equivalent of glycine receptors (Kehoe and Vulfius, 2000; Vassilatis et al., 1997), so may respond similarly to elevated CO_2. Compared with vertebrates, invertebrates also possess a larger range of ligand-gated Cl^- channels (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 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 CO_2 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 CO_2-induced behavioural disturbances in marine invertebrates, indicating a complex assortment of responsible mechanisms.

Elevated CO_2 has been shown to affect activity, defensive and predatory behaviours in cephalopod molluscs (Spady et al., 2014, 2018). Cephalopods are a useful taxon in which to investigate the mechanisms of CO_2-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 (~1000 µatm) CO_2 levels for 7 days followed by sham treatment or treatment with the $GABA_A$R antagonist gabazine or the non-specific $GABA_A$R antagonist picrotoxin. After CO_2 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 GABAR hyperpolarisations (inhibition) and depolarisations (excitation) (Vehovszky et al., 1989). However, there was no evidence for what ion(s) these ionotropic GABARs 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 GABAR hyperpolarisations by antagonising $GABA_A$-like Rs, but the action of gabazine on ionotropic GABAR 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*, although 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 receptors in vertebrates (both ligand-gated Cl^- channels) (Dibas et al., 2002; Lynch, 2004; Masiulis et al., 2019; Wang et al., 2006). In molluscs, the action of picrotoxin has been better studied than that of gabazine (see Table S2). Similar to its role 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,b) as well as Glu-gated Cl^- channels (suggested to be the invertebrate equivalent of glycine receptors: Vassilatis et al., 1997; Piggott et al., 1977). However, compared with vertebrates, molluscs possess a larger

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range of ligand-gated Cl^- channels (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 that picrotoxin acts 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 CO_2 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 CO_2-induced behavioural changes in *I. pygmaeus*. If the behavioural effect of drug treatment at elevated CO_2 is different to the behavioural effect of drug treatment at ambient CO_2 this would suggest that altered function of the drugs target receptor(s) underlies the CO_2-induced behavioural change. The behavioural effect of drug treatment could differ across CO_2 in five ways – opposite, removed, added, diminished or enhanced – with each of these suggesting a slightly different change in receptor function at elevated CO_2 (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 CO_2 conditions this would suggest altered function of the $GABA_A$-like R underlies the CO_2-induced behavioural change, supporting the GABA hypothesis. As gabazine and picrotoxin are structurally unrelated, and the action of picrotoxin within molluscs has been better studied, this would provide more robust support for the GABA hypothesis than previous marine invertebrate pharmacological studies. As picrotoxin, in contrast to gabazine, antagonises Glu-, ACh- and DA-gated Cl^- channels, we hypothesise that differential effects of picrotoxin (but not gabazine) on behaviour across CO_2 conditions would suggest that altered function of these picrotoxin-sensitive ligand-gated Cl^- channels underlies the CO_2-induced behavioural change.

MATERIALS AND METHODS

Animal collection

Male two-toned pygmy squid (*Idiosepius pygmaeus*) were collected in 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 CO_2 (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 (∼1000 µatm) seawater CO_2, 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 CO_2 treatment. Treatment tanks (matte white colour, 40×30×30 cm) held squid individually for 7 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).

CO_2 treatment systems

Experiments were carried out in four interconnected 8000 litre recirculating seawater systems, each with a 3000 litre sump, at James Cook University's research aquarium in Townsville, Australia. Two untreated seawater systems were used for duplicated ambient CO_2 treatments, and a custom-built pH control system dosed CO_2 into the 3000 litre sumps of two seawater systems to create duplicated elevated CO_2 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 CO_2 dosing to maintain the desired pH. The four systems were interconnected by water exchange of approximately 20 litres per hour to maintain similar water quality in each system.

Daily measurements of pH_{NBS} (Ecotrode plus on an 888 Titrando, 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_{CO_2} in the two elevated CO_2 systems. Weekly measurements of salinity by a conductivity sensor (HQ40d, Hach, Loveland, CO, USA), total alkalinity by Gran titration (888 Titrando, 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_{CO_2} values were calculated in CO2SYS v.2.1 (https://cdiac.ess-dive.lbl.gov/ftp/co2sys/CO2SYS_calc_XLS_v2.1/) using the constants K1, K2 from Mehrbach et al. (1973) and refit by Dickson and Millero (1987) and $KHSO_4$ from Dickson et al. (2007). An overview of carbonate chemistry parameters is 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 CO_2 treatment, squid underwent drug treatment for 30 min. In both the gabazine and picrotoxin experiments, squid were individually placed in 100 ml aerated seawater from their CO_2 treatment containing sham or drug treatment, squid were randomly assigned to sham or drug treatment. Gabazine experiment: sham=0.4% distilled water (ambient CO_2, n=22; elevated CO_2, n=22), or gabazine=4 mg l^{-1} (10.86 µmol l^{-1}) gabazine (SR-95531, batch #0000035110, Sigma-Aldrich, St Louis, USA) and 0.4% distilled water (ambient CO_2, n=24; elevated CO_2, n=23). Picrotoxin experiment: sham=0.2% absolute ethanol (ambient CO_2, n=27; elevated CO_2, n=26), picrotoxin=100 µmol l^{-1} picrotoxin (batch #16C/230903, Tocris Bioscience, Bristol, UK) and 0.2% absolute ethanol (ambient CO_2, n=26; elevated CO_2, n=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^{-1} for 30 min reversed the behavioural effect of elevated CO_2. The dose of

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Table 1. Summary of the varying types of different drug effects across CO_2 treatments and how they suggest altered receptor function at elevated CO_2

Drug effect in elevated versus 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 with 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 supressed 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 with 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 with 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 receptors ($GABA_A$-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.

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 µmol l^{-1} 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 (30×30×15 cm) with a mirror taking up the entire area of one wall. The tank was filled with water from their CO_2 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 min behavioural trial and stopped after 16 min 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±s.d.); 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 min 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 CO_2-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 (v.4.2.1, Loligo Systems) was used across the 15 min 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) and number of visits to zone A.

The following information was determined from the videos, at 30 fps, using QuickTime video software (QuickTime v.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 contacting 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: (1) proportion of squid that touched the mirror softly/aggressively; (2) latency to the first soft/aggressive mirror touch (only for squid that touched the mirror); (3) 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 min videos at 15 fps for measures of activity: (1) Time spent active (seconds); (2) total distance moved (cm); (3) average speed (cm s^{-1}).

Squid that inked in the behavioural trial (gabazine experiment sham: ambient CO_2, n=8; elevated CO_2, n=7; gabazine: ambient CO_2, n=10; elevated CO_2, n=7; picrotoxin experiment sham: ambient CO_2, n=6; elevated CO_2, n=5; picrotoxin: ambient CO_2, n=6; elevated CO_2, n=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: ambient CO_2, n=12; elevated CO_2, n=13; gabazine: ambient CO_2, n=12; elevated CO_2, n=16; and picrotoxin experiment sham: ambient CO_2, n=19; elevated CO_2, n=21; picrotoxin: ambient CO_2, n=18; elevated CO_2, n=22.

Statistical analysis

Bayesian modelling was carried out in R (v4.0.2) (https://www.r-project.org/), using RStudio (v1.3.1093) (https://www.rstudio.com/) to test the effects of CO_2 and drug treatment on each behaviour. The Bayesian models were fitted using the package brms (v.2.13.5) (Bürkner, 2017), which uses RStan (v.2.21.2) (https://mc-stan.org/users/interfaces/rstan) 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 fitted using the no U-turn MCMC sampler which ran with 10,000 iterations, a warm-up of 3000 and thinning of 5 for each of 4 chains. Default, weakly informative priors were used. For two response

Table 2. Seawater carbonate chemistry for duplicate ambient and elevated CO_2 treatment systems in the gabazine and picrotoxin experiments, values are mean±s.d.

CO_2 treatment system	Temperature (°C)	Salinity	pH_{total}	Alkalinity (µmol kg^{-1} SW)	P_{CO_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

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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 fitted 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 CO_2 (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 CO_2 and drug; (2) the effect of squid size by including mantle length in cm (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 CO_2 and two levels for elevated CO_2). 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 as less than 14.5% of the Pareto k diagnostic values were 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% probability) in the gabazine experiment and evidence (83.6% probability) 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 from 386 to 670 s, picrotoxin: 1.29-fold increase from 409 to 529 s) (Fig. 1A,B,E,F). There was also strong evidence (93.3% probability) 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% probability); however, there was very strong evidence (97.5% probability) that gabazine decreased the time spent in zone A at elevated CO_2 (0.54-fold decrease from 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% probability) that picrotoxin had a different effect at ambient and elevated CO_2. Specifically, there was strong evidence (91.8% probability) that picrotoxin increased the time spent in zone A at ambient CO_2 (1.41-fold increase from 409 to 577 s), but no evidence of a picrotoxin effect at elevated CO_2 (60.6% probability) (Fig. 1E,F).

There was very strong evidence (99.9% probability) that elevated CO_2 decreased the number of times squid visited zone A in the gabazine experiment (0.24-fold decrease from 15 to 3.6 visits) (Fig. 1C,D). There was also very strong evidence (98.8% probability) that gabazine had a different effect at ambient and elevated CO_2. Specifically, gabazine decreased the number of visits at ambient CO_2 (97.7% probability, 0.37-fold decrease from 15 to 5.6 visits), but increased the number of visits at elevated CO_2 (86.5% probability, 1.63-fold increase from 3.6 to 5.8 visits). In contrast, there was no evidence (56.7% probability) that CO_2 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% probability) that picrotoxin had a different effect at ambient and elevated CO_2 (Fig. 1H).

Soft mirror touch

In the gabazine experiment, there was no evidence (62.2% probability) for an effect of CO_2 on the proportion of sham-treated squid that explored by softly touching the mirror, nor was there any evidence (63.2% probability) that gabazine had a different effect at ambient and elevated CO_2 conditions (Fig. 2A,B). However, in the picrotoxin experiment there was strong evidence (94.4% probability) that elevated CO_2 increased the proportion of sham-treated squid that softly touched the mirror (2.44 odds ratio, increase from 29% to 50%) (Fig. 2G,H). There is no evidence (60.8% probability) that picrotoxin had a different effect at ambient and elevated CO_2, with strong evidence (96.9% and 92.2% probability, respectively) that picrotoxin increased the proportion of squid softly touching the mirror at both ambient CO_2 (2.88 odds ratio, increase from 29% to 54%) and elevated CO_2 (2.31 odds ratio, increase from 50% to 70%) (Fig. 2G,H).

There was some evidence (77.9% probability) in the gabazine experiment and very strong evidence (99% probability) in the picrotoxin experiment that elevated CO_2 decreased the latency to the first soft mirror touch (gabazine: 0.67-fold decrease from 107.7 to 72.5 s; picrotoxin: 0.3-fold decrease from 257.3 to 75.8 s) (Fig. 2C,D,I,J). However, there was no evidence (60.3% probability)

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Table 3. Summary of the CO_2 effect in sham-treated squid, and the type of different drug effect across CO_2 treatments

	CO_2 effect in sham-treated squid				Different drug effect across CO_2 conditions			
	Gabazine		Picrotoxin		Gabazine		Picrotoxin	
	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^{-1})	Evidence	↑	Evidence	↑	None	–	None	–

Evidence: very strong=probability of an effect ≥95%; strong=probability of an effect≥90%; evidence=probability of an effect ≥80%; some evidence=probability of an effect ≥75%. Direction: ↑=increase in the behaviour at elevated compared with ambient CO_2; ↓=decrease in the behaviour at elevated compared with ambient CO_2. Type: Opposite=drug treatment caused an increase in the behavioural trait at ambient CO_2 and a decrease in the behavioural trait at elevated CO_2, or vice versa; Removed=drug treatment increased/decreased the behaviour at ambient CO_2 but had no effect at elevated CO_2; Added=drug treatment had no effect at ambient CO_2 but increased/decreased the behaviour at elevated CO_2; Diminished=drug treatment caused a smaller increase/decrease in behaviour at elevated compared with ambient CO_2.

for a different effect of gabazine across CO_2 conditions. Gabazine increased the latency at both ambient CO_2 (83.2% probability, 1.66-fold increase from 107.7 to 178.6 s) and elevated CO_2 (89.8% probability, 2.03-fold increase from 72.5 to 146.8 s). In contrast, there was very strong evidence (99.1% probability) that picrotoxin had a different effect across CO_2 treatments. Picrotoxin decreased the latency at ambient CO_2 (88.4% probability, 0.55-fold decrease from 257.3 to 140.5 s), whereas it increased the latency at elevated CO_2 (98.8% probability, 2.69-fold increase from 75.8 to 203.2 s) (Fig. 2I,J).

In the gabazine experiment there was strong evidence (93.2% probability), and in the picrotoxin experiment some evidence (78.9% probability), that elevated CO_2 increased the number of soft mirror touches per individual (gabazine: 2.02-fold increase from 13.3 to 27.0 touches; picrotoxin: 1.53-fold increase from 32.1 to 49.1 touches) (Fig. 2E,F,K,L). There was some evidence (78.9% probability) that gabazine had a smaller effect at elevated compared with ambient CO_2. Specifically, gabazine increased the number of soft touches by a median of 2.48-fold at ambient CO_2 (97.1% probability, increase from 13.3 to 33.0 touches) and 1.44-fold at elevated CO_2 (78.7% probability, increase from 27.0 to 38.8 touches) (Fig. 2E,F). By contrast, there was no evidence (62.4% probability) that picrotoxin had a different effect across CO_2 conditions. Picrotoxin decreased the number of soft touches both at ambient CO_2 (86.7% probability, 0.54-fold decrease from 32.1 to 17.5 touches) and at elevated CO_2 (82.5% probability, 0.68-fold decrease from 49.1 to 33.4 touches) (Fig. 2K,L).

Aggressive mirror touch

There was evidence (83.2% probability) in the gabazine experiment and very strong evidence (97.2% probability) in the picrotoxin experiment that elevated CO_2 increased the proportion of sham-treated squid that touched the mirror aggressively (gabazine: 1.82 odds ratio, increase from 31% to 45%; picrotoxin: 3.01 odds ratio, increase from 25% to 50%) (Fig. 3A,B and G,H). In both experiments, there was no evidence (65% and 70.7% probability, respectively) that drug treatment had a different effect at ambient and elevated CO_2 conditions (Fig. 3A,B,G,H).

In the gabazine experiment there was some evidence (76.5% probability), and in the picrotoxin experiment there was evidence (86% probability), that elevated CO_2 increased the latency to the first aggressive mirror touch (gabazine: 1.6-fold increase from 30.8 to 48.9 s; picrotoxin: 1.85-fold increase from 108.1 to 201.8 s) (Fig. 3C,D,I,J). There was also strong evidence (94.2% probability) that the effect of gabazine was smaller at elevated compared with ambient CO_2. Specifically, gabazine increased the latency by a median of 7.6-fold at ambient CO_2 (99.8% probability, increase from 30.8 to 235.3 s) and 2.03-fold at elevated CO_2 (91.3% probability, increase from 48.9 to 97.9 s) (Fig. 3C,D). There was no evidence (55.5% probability) that picrotoxin had a different effect across CO_2 conditions (Fig. 3I,J).

In both experiments, there was strong evidence (97.3% and 93.3% probability, respectively) that elevated CO_2 increased the number of aggressive mirror touches per individual (gabazine: 2.5-fold increase from 11.9 to 29.6 touches; picrotoxin: 1.79-fold increase from 9.7 to 17.4 touches) (Fig. 3E,F and K,L). However, there was no evidence (68.6% and 64.9% probability, respectively) that gabazine or picrotoxin had a different effect across CO_2 conditions (Fig. 3E,F,K,L). There was strong evidence (95.6% and 93.8% probability, respectively) that picrotoxin increased the number of aggressive touches both at ambient CO_2 (2.16-fold increase from 9.7 to 21 touches) and at elevated CO_2 (1.77-fold increase from 17.4 to 30.8 touches) (Fig. 3K,L).

Activity

Elevated CO_2 increased the time squid spent active in both experiments (gabazine: 87.8% probability, 1.41-fold increase from

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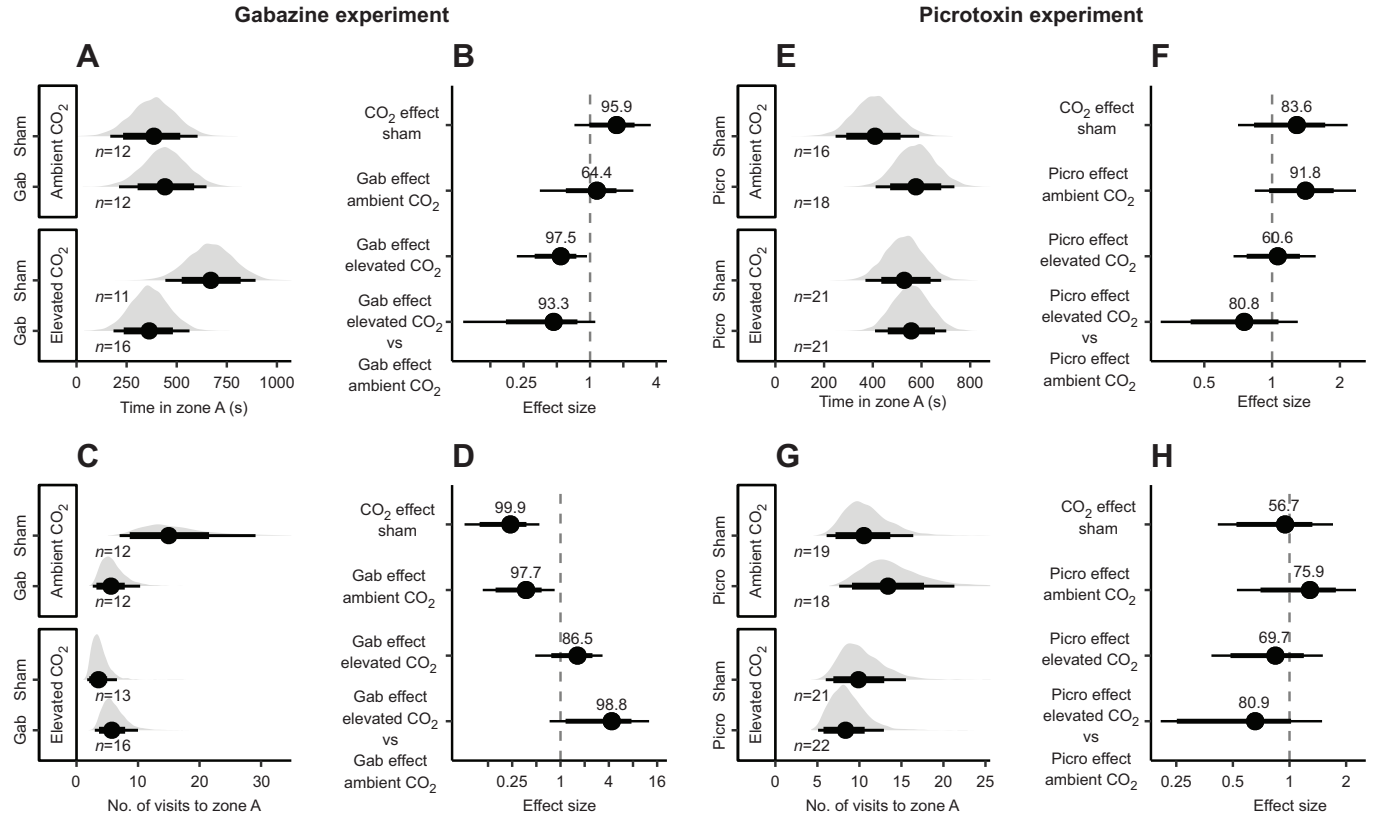

Fig. 1. Effects of CO_2 and drug treatment on space use by the two-toned pygmy squid (*Idiosepius pygmaeus*). (A–D) Gabazine experiment. (E–H) Picrotoxin experiment. (A,E) Partial plots of the time squid spent in zone A. (B,F) Caterpillar plots for the effect of CO_2 and drug treatment on the time squid 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 CO_2 and drug treatment on the number of times squid visited zone A. In partial plots, points represent the Bayesian posterior median value±80% (thick lines) and ±95% (thin lines) highest posterior density interval (HPDI), overlaid with the distribution of Bayesian posterior values. In caterpillar plots, points represent the median effect size (odds ratio for proportion data, fold change for others)±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 percentage probability of this effect (increase or decrease) occurring.

161.5 to 228.2 s; picrotoxin: 86.6% probability, 1.23-fold increase from 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 CO_2 in both experiments. Gabazine had a larger effect at elevated compared to ambient CO_2 (78.9% probability, 1.40-fold); there was no evidence (50.7% probability) at ambient CO_2, but there was evidence (88.7% probability) at elevated CO_2 (1.40-fold increase from 228.2 to 319 s) that gabazine increased active time (Fig. 4A,B). Conversely, picrotoxin had a smaller effect at elevated compared with ambient CO_2 (79.5% probability, 0.8-fold); there was strong evidence (91.6% probability) at ambient CO_2 (1.46-fold increase from 316.1 to 333.9 s), but no evidence (63.5% probability) at elevated CO_2, that picrotoxin increased active time (Fig. 4G,H).

The total distance moved by squid throughout the behavioural trial was also increased by elevated CO_2 in both experiments (gabazine: 90.3% probability, 1.57-fold increase from 360.0 to 566.6 s; picrotoxin: 88.9% probability, 1.36-fold increase 613.5 to 833.9 s) (Fig. 4C,D,I,J). There was some evidence (76.9% probability) that gabazine had a larger effect at elevated compared with ambient CO_2. Specifically, there was no evidence (57.8% probability) at ambient CO_2, but strong evidence (90.7% probability) at elevated CO_2 (1.54-fold increase from 566.6 to 872.2 s) that gabazine increased distance moved (Fig. 4C,D). Conversely, there was some evidence (75.3% probability) that picrotoxin had a smaller effect at elevated compared to ambient CO_2. Specifically, picrotoxin increased distance moved by a median of 1.46-fold at ambient CO_2 (93.4% probability, increase from 613.5 to 892.8 s) and 1.17-fold at elevated CO_2 (78.9% probability, increase from 833.9 s to 976 s) (Fig. 4I,J).

Squid average speed across the behavioural trial was also higher at elevated compared with ambient CO_2 (gabazine: 86.8% probability, 1.12-fold increase from 2.07 to 2.32 cm s^{-1}; picrotoxin: 87.4% probability, 1.10-fold increase from 2.21 to 2.42 cm s^{-1}) (Fig. 4E,F,K,L). There was no evidence (55.9% probability) that gabazine had a different effect across CO_2 conditions, with no evidence (71.2% and 66.6% probability, respectively) of gabazine having an effect at either ambient or elevated CO_2 (Fig. 4E,F). There was also no evidence (54.4% probability) that picrotoxin had a different effect at ambient compared with elevated CO_2; there was strong evidence (95.2% and 98.3% probability, respectively) that picrotoxin increased squid average speed at both ambient CO_2 (1.14-fold increase from 2.21 to 2.53 cm s^{-1}) and elevated CO_2 (1.16-fold increase from 2.42 to 2.81 cm s^{-1}) (Fig. 4K,L).

DISCUSSION

Elevated CO_2 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 CO_2 levels might affect conspecific-directed behaviours or the mechanisms involved in altered marine invertebrate behaviour at elevated CO_2. Here, we found that elevated CO_2 increased male *I. pygmaeus* conspecific attraction, aggression and activity levels in the presence of the squid's mirror image, compared with squid in ambient

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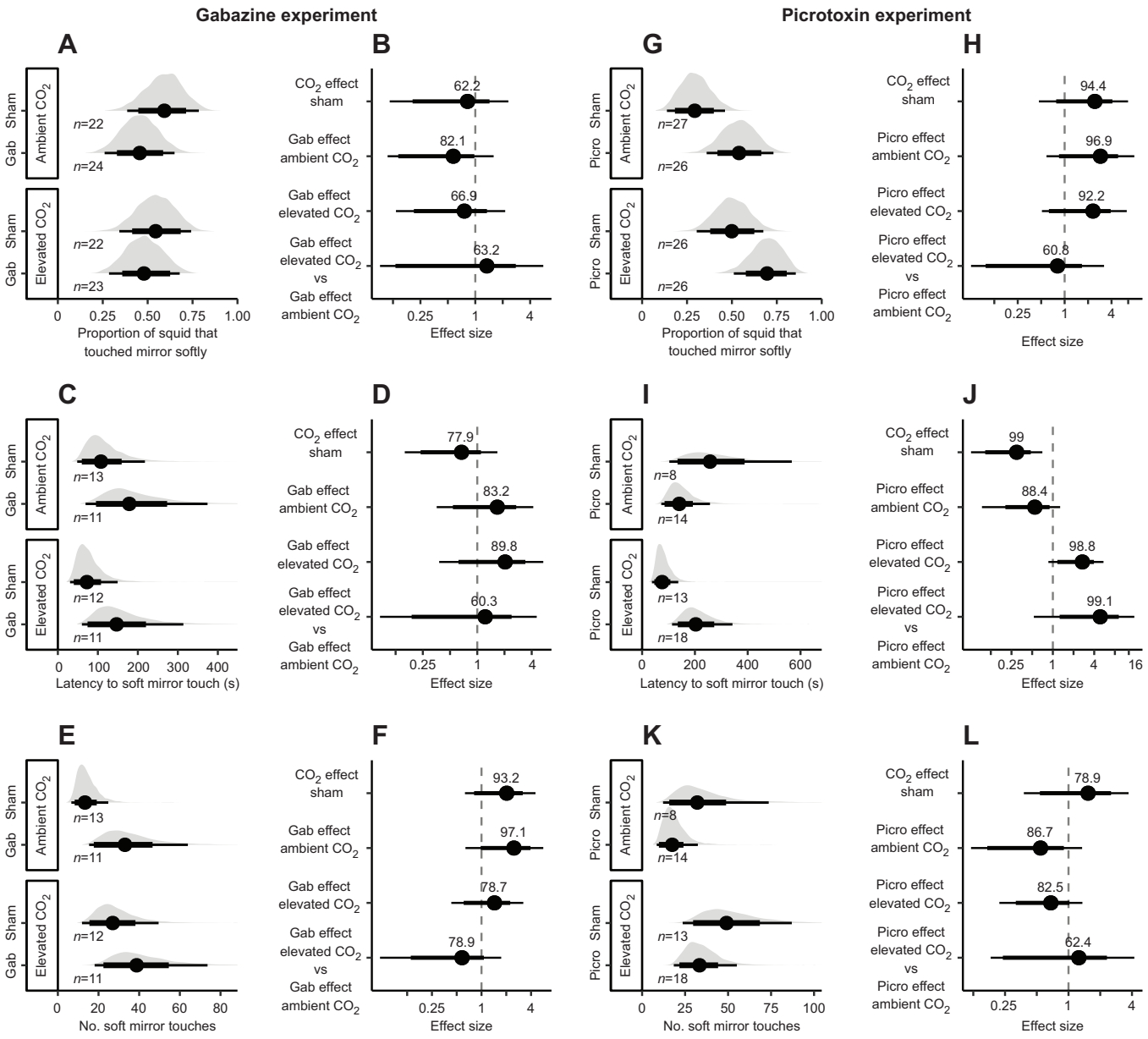

Fig. 2. Effects of CO_2 and drug treatment on measures of soft mirror touching behaviour in squid. (A–F) Gabazine experiment. (G–L) Picrotoxin experiment. (A,G) Partial plots of the proportion of squid that touched the mirror softly. (B,H) Caterpillar plots for the effect of CO_2 and drug treatment on 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 CO_2 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 CO_2 and drug treatment on the total number of soft mirror touches per individual. Partial and caterpillar plot symbols as per Fig. 1.

CO_2 conditions. Treatment with gabazine and picrotoxin had a different effect at elevated compared with ambient CO_2 conditions in some behaviours, providing robust support for the GABA hypothesis within cephalopods, and indicating that altered functioning of Glu-, ACh- and DA-gated Cl^- channels may also underlie CO_2-induced behavioural changes. However, gabazine and picrotoxin had a similar effect in both ambient and elevated CO_2 conditions for other CO_2-affected behavioural traits, suggesting other mechanisms may also be involved.

Behavioural change in response to elevated CO_2

For the majority of the measured behaviours, the effect of elevated CO_2 was consistent across the gabazine and picrotoxin experiments. However, for two behavioural traits there was evidence for an effect of CO_2 in one experiment and not the other (see Table 3). Behaviour is notoriously difficult to measure because of 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 CO_2 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,

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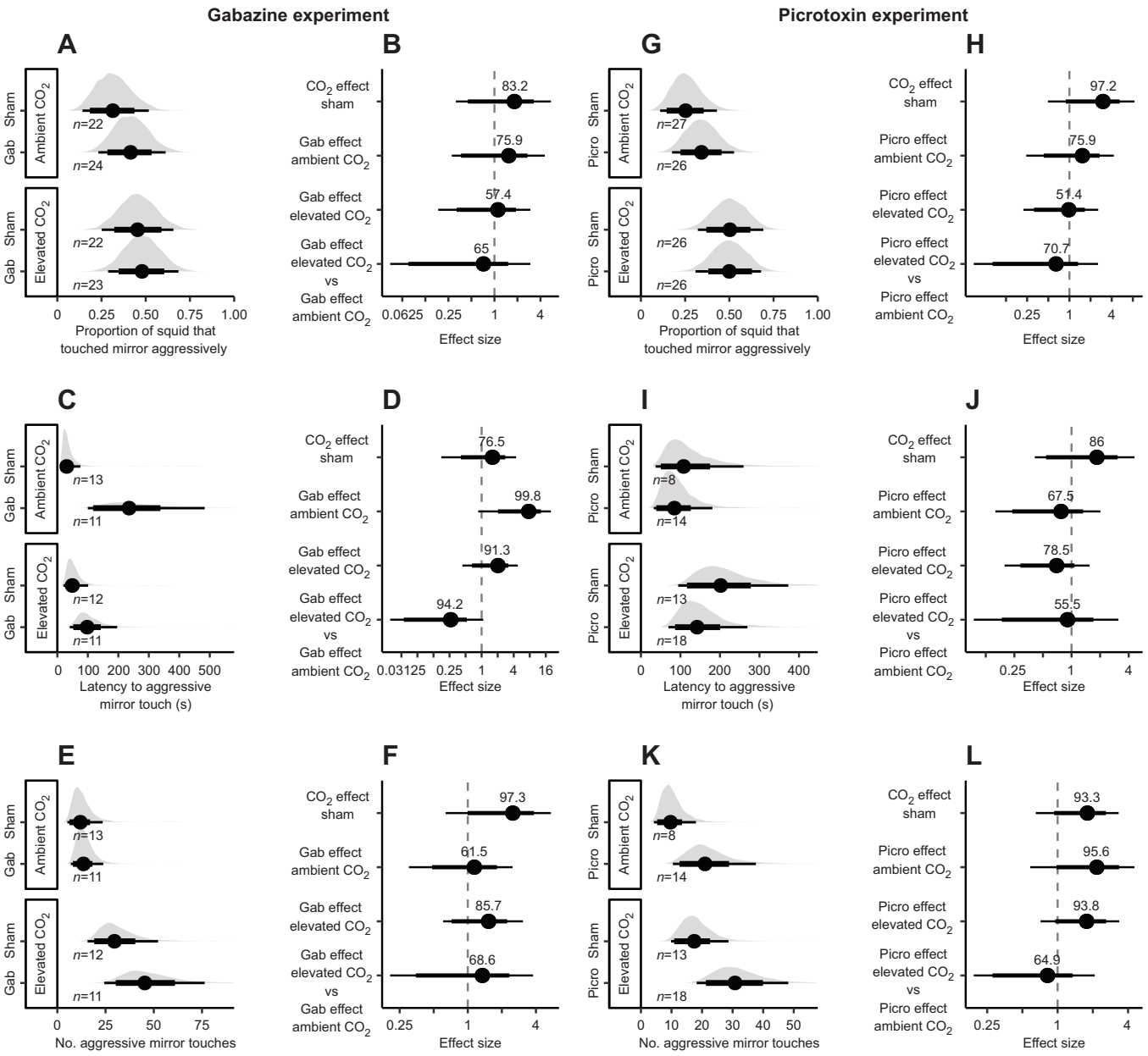

Fig. 3. Effects of CO_2 and drug treatment on measures of aggressive mirror touching behaviour in squid. (A–F) Gabazine experiment. (G–L) Picrotoxin experiment. (A,G) Partial plots of the proportion of squid that touched the mirror aggressively. (B,H) Caterpillar plots for the effect of CO_2 and drug 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 CO_2 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 CO_2 and drug treatment on the total number of aggressive mirror touches per individual. Partial and caterpillar plot symbols as per Fig. 1.

such as temperature or turbidity, and the age of the squid captured. Nevertheless, 9 out of the 11 behaviours measured showed a consistent response to CO_2 across the 2 experiments, suggesting the reliability of these results.

Squid exposed to elevated CO_2 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 with squid at ambient CO_2 conditions. These results suggest that elevated CO_2 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 CO_2-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 CO_2 also increased the proportion of squid that aggressively touched the mirror and the number of aggressive mirror touches, suggesting that elevated CO_2 may increase

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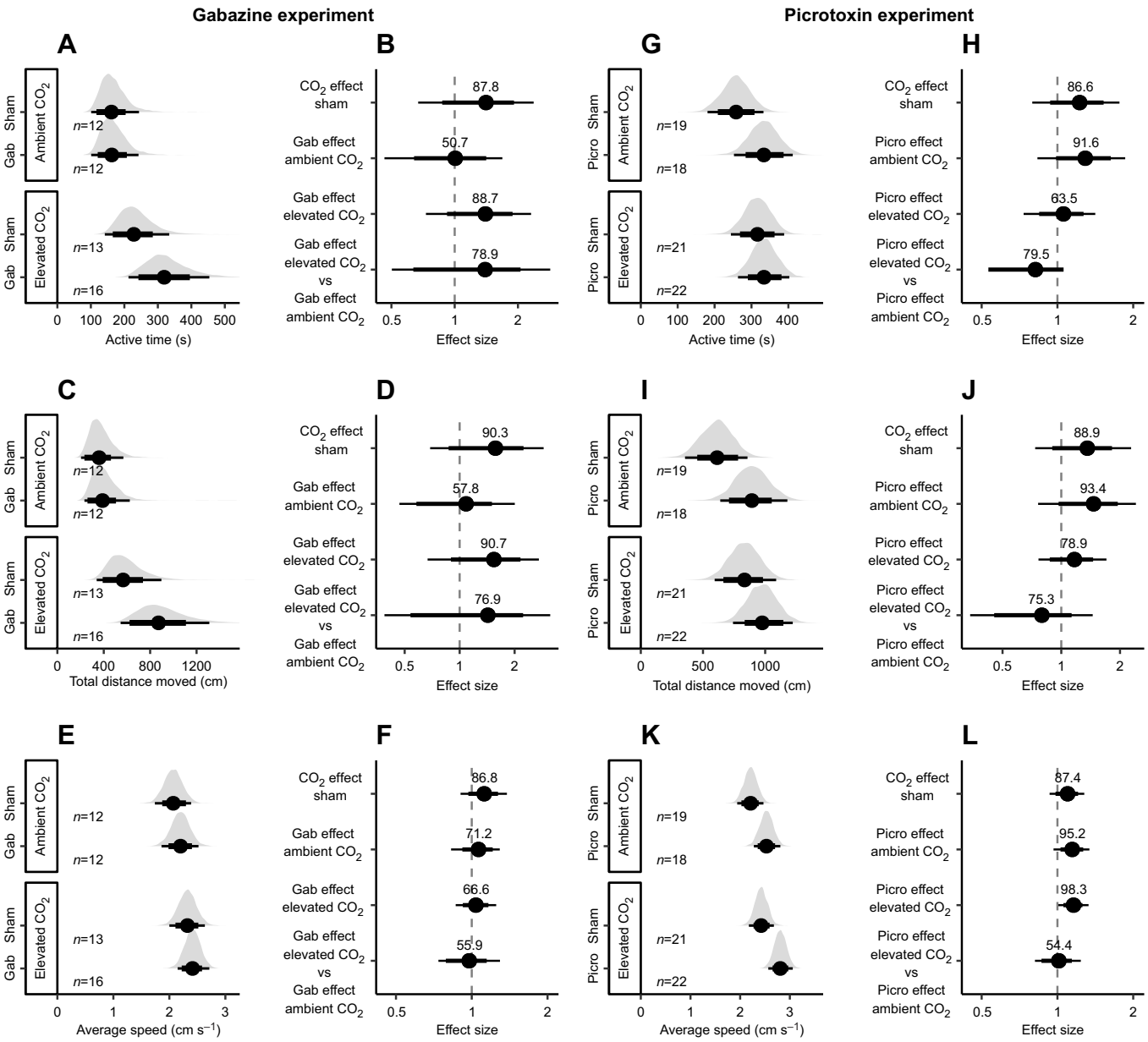

Fig. 4. Effects of CO_2 and drug treatment on activity measures in squid. (A–F) Gabazine experiment. (G–L) Picrotoxin experiment. (A,G) Partial plots of the time squid spent active. (B,H) Caterpillar plots for the effect of CO_2 and drug treatment on the time squid spent active. (C,I) Partial plots of the total distance moved by squid. (D,J) Caterpillar plots for the effect of CO_2 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 CO_2 and drug treatment on squid average speed. Partial and caterpillar plot symbols as per Fig. 1.

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 CO_2. This is consistent with increased activity levels in male *I. pygmaeus* at elevated CO_2 (626 and 956 µatm) 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 CO_2 conditions (935 µatm) (Spady et al., 2018). In contrast, activity decreased at elevated CO_2 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 CO_2 treatment. *I. pygmaeus* and *S. lessoniana* were exposed to elevated CO_2 levels as adults (this study, Spady et al., 2014 and Spady et al., 2018) whereas *D. pealeii* were exposed to elevated CO_2 for the duration of egg development (Zakroff et al., 2018), when there are already high CO_2 levels within the egg (Hu and Tseng, 2017).

It is unknown how CO_2-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 CO_2 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 CO_2-induced behavioural changes, by administration of both gabazine and picrotoxin to male two-toned pygmy squid exposed to ambient or elevated CO_2 conditions.

Behavioural effects of gabazine and picrotoxin

The effects of both gabazine and picrotoxin treatment at ambient CO_2 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 CO_2 on all activity measures, also indicating no convulsant effects. Picrotoxin increased all measures of activity levels at ambient CO_2. 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 to be due to convulsive side effects but rather to the action of these drugs on the underlying neural circuits.

The effect of gabazine and picrotoxin at ambient CO_2 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 are affected by drug administration. Both gabazine and picrotoxin affected multiple measures of space use and soft mirror touching behaviour at ambient CO_2, 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 CO_2 conditions

Gabazine and picrotoxin had different effects across CO_2 treatments on a behavioural measure of space use, and on two activity measures. Gabazine had an 'added' drug effect between CO_2 treatments 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' drug effect across CO_2 on the time in zone A and active time, and a 'diminished' effect across CO_2 on distance moved. The different effect of both gabazine and picrotoxin across CO_2 provides strong evidence for the GABA hypothesis in *I. pygmaeus*. Furthermore, as the different effect of gabazine and picrotoxin across CO_2 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 CO_2. Thus, altered functioning of GABA-, Glu-, ACh- and DA-gated Cl^- channels (and possibly also GABA-gated cation channels) may underlie the CO_2-induced increase in the time spent in zone A and the active time and distance moved by squid.

Gabazine had an opposite effect across CO_2 treatments on the number of visits to zone A, decreasing the number of visits at ambient CO_2 but increasing visits at elevated CO_2. 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 CO_2-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 CO_2 (increased the latency at both ambient and elevated CO_2), whereas picrotoxin had an opposite effect across CO_2 (decreased the latency at ambient CO_2 and increased the latency at elevated CO_2). This suggests that altered functioning of ligand-gated Cl^- channels, other than $GABA_A$-like Rs, underlies the CO_2-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 CO_2, but they do suggest that ligand-gated Cl^- channels other than the $GABA_A$-like R underlie this particular CO_2-induced behavioural change. Furthermore, the opposite effect of picrotoxin across CO_2 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 CO_2 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 CO_2-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.

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In two CO_2-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 CO_2 conditions. This suggests that receptors sensitive to gabazine, but not picrotoxin, may underlie the CO_2-induced change of these behavioural traits, for example GABA-gated cation channels. This suggests the potentially widespread nature of the mechanisms underlying CO_2-induced behavioural changes. However, the lack of evidence for a different effect of picrotoxin across CO_2 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 CO_2-affected behavioural traits showed no evidence of gabazine nor picrotoxin having a different effect across CO_2 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 CO_2-induced change of these specific behavioural traits. Therefore, other mechanisms may be involved in these behavioural alterations.

Overall, our results suggest that elevated CO_2 alters behaviour via multiple mechanisms in *I. pygmaeus*, with different mechanisms underlying the changes of different behavioural traits at elevated CO_2 (Fig. 5). It is possible that elevated CO_2 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 CO_2-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 CO_2 among behaviours.

The complexity of the mechanisms underlying CO_2-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; α1–α6, β1–β3, γ1–γ3, δ, ε, θ, π and ρ1–ρ3 (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 details]. For example, vertebrate $GABA_A$Rs made up of ρ subunits [$GABA_A$-ρ receptors, 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;

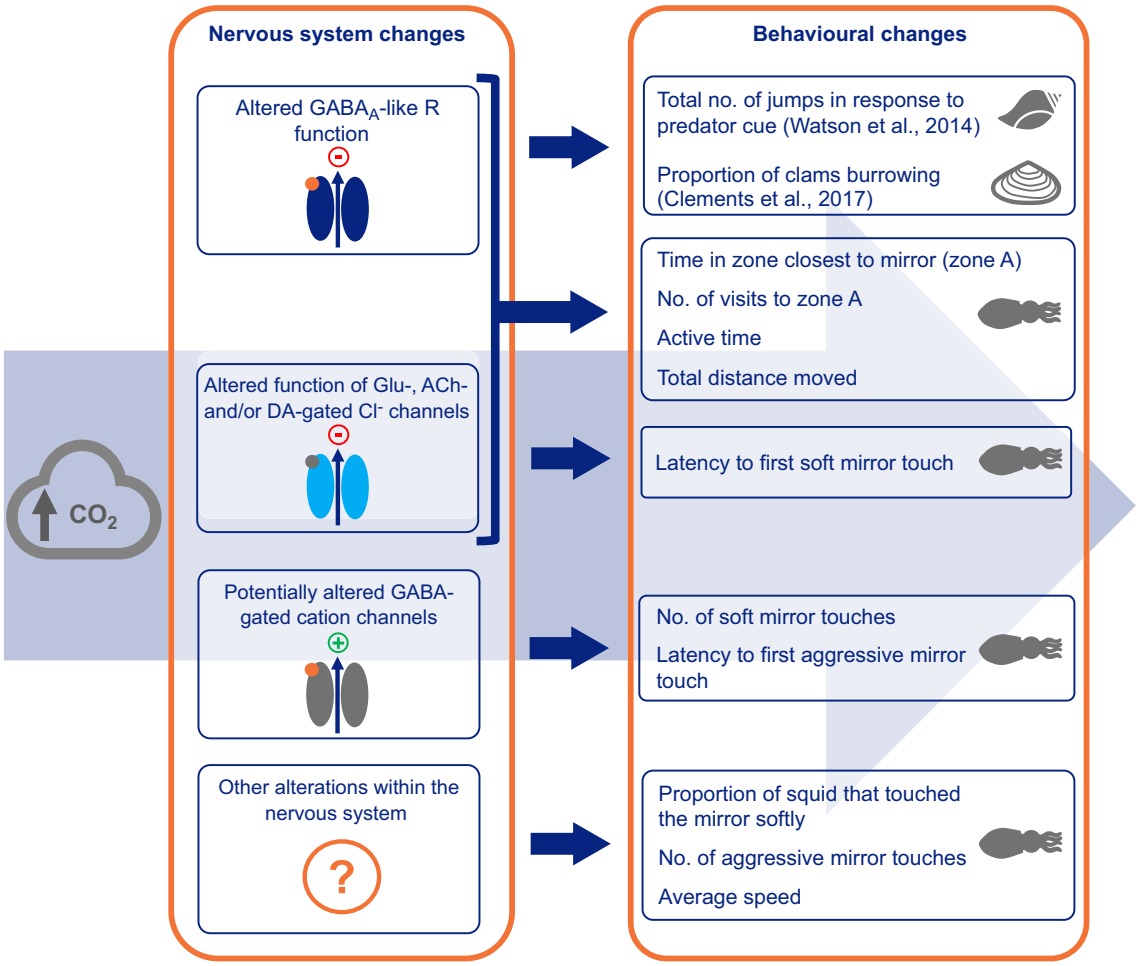

Fig. 5. Evidence within molluscs that elevated CO_2 results in a suite of changes within the nervous system, with each change (or group of changes) altering different behavioural traits. The mechanisms underlying CO_2-induced behavioural change were tested using gabazine in the jumping conch snail and soft-shell clam, and both gabazine and picrotoxin in the two-toned pygmy squid (this study).

Zhang et al., 2008), and $GABA_A$ (α1β2γ2) and $GABA_A$-ρ2 receptors are 10-fold more sensitive to picrotoxin than $GABA_A$-ρ1 receptors (Naffaa et al., 2017). $GABA_A$R subunits have been less studied in molluscs, although molluscan $GABA_A$-like R α and β subunits have been identified (Harvey et al., 1991; Moroz et al., 2006; Stewart et al., 2011) and $GABA_A$-like R 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 GABARs of another neuron within the same mollusc species (Norekian and Satterlie, 1993; Norekian and Malyshev, 2005). Therefore, $GABA_A$-like R 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 CO_2-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 CO_2 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 CO_2-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 CO_2 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 CO_2-induced behavioural changes. We propose that elevated CO_2 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 CO_2, this can potentially explain the variability in the behavioural effects of elevated CO_2, 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 CO_2-induced behavioural change. For example, an antipredator response was recently shown to be altered at elevated CO_2 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 CO_2.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.T.T., P.L.M., S.-A.W.; Methodology: J.T.T., P.L.M., S.-A.W.; Software: J.T.T.; Formal analysis: J.T.T.; Investigation: J.T.T., B.L.S.; Resources: P.L.M., S.-A.W.; Data curation: J.T.T.; Writing - original draft: J.T.T.; Writing - review & editing: J.T.T., B.L.S., P.L.M., S.-A.W.; Visualization: J.T.T.; Supervision: P.L.M., S.-A.W.; Project administration: J.T.T.; Funding acquisition: J.T.T., P.L.M., S.-A.W.

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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 from the ARDC database at: https://researchdata.edu.au/role-ligand-gated-co2-cephalopod/1711446 (doi:10.25903/y6kz-hm11).

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