



Research article

Osmotic response of *Dotilla fenestrata* (sand bubbler crab) exposed to combined water acidity and varying metal (Cd and Pb)Babatunde Adeleke^{*}, Deborah Robertson-Andersson, Gan Moodley

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ABSTRACT

This study assessed the interactive effects of near-future coastal acidification in combination with varying sub lethal metal concentrations on the haemolymph osmolality of *Dotilla fenestrata*. Crabs were exposed to acute combination of near-future pH scenarios of estuarine systems (7.2, 7.4 and 7.6) by bubbling CO₂ into holding tanks and metal concentrations (Cd = 0.50, 0.75, and 1.00 mg/l), (Pb = 6.50, 8.50 and 10.50 mg/l) and (Cd & Pb = 4.50, 5.75 and 7.00 mg/l) at 32 psu salinity and 18 °C for 96 h and compared with the control group that were acclimated in water medium (salinity 32 psu, temperature 18 °C and pH 8.1). Mean haemolymph osmolality of crabs exposed to a combination of varying pH and metal concentrations were not significantly different (ANOVA HSD: df 9; p > 0.05) from the crabs acclimated close to background water parameters. The study showed that near-future coastal pH has no significant effect on the haemolymph osmolality of the crab *Dotilla* exposed to sublethal concentrations of Cd and Pb at salinity level of 32 ppt.

1. Introduction

Trace metals can be taken up and accumulated from the environment and food sources by aquatic organisms (Ali et al., 2019). Although many of these metals perform essential metabolic roles in marine animals, however, they could all have potential ecotoxicological effects at elevated levels in organisms (Rainbow, 1997). Cadmium (Cd) and lead (Pb), are nonessential and highly toxic heavy metals of environmental concern because of their adverse consequences in aquatic organisms (Zyadah and Abdel-Baky, 2000; Rainbow, 2002). These metals occur in the marine environment due to natural processes and rising anthropogenic input, which is predominant in coastal and estuarine areas (Grosell et al., 2006).

Trace metals uptake from water by crustaceans usually follows either one of two transport routes which are either passive or active transport (Rainbow, 1997). Trace metals bind with a metal-binding protein epithelial surface membrane firstly before going through a thermodynamic gradient of metal-binding ligands that have increasing affinities for metal, in a passive diffusion medium (Rainbow 1997). Cadmium, calcium and some heavy metals could follow paths of key metal ion uptake, which is finally propelled by the epithelial cell membrane pump (Rainbow 1997). Metal uptake rates in euryhaline crustaceans are determined by the physicochemistry of metal speciation, i.e. bioavailable

form of such metal in the aquatic environment with the interaction of the organism's physiological functions (Rainbow 1997).

The Intergovernmental Panel on Climate Change (IPCC) predicts that global climate change (GCC) will significantly influence environmental conditions such as ocean acidification (OA) with potential impacts on the sensitivity of organisms to environmental toxicants (Solomon et al., 2007; Kimberly and Salice, 2014). Ocean acidification, therefore, has the potential to change the physiological responses of marine organisms when exposed to environmental pollutants (Kimberly and Salice, 2014). The impacts of temporal variability in exposures to contaminants and climatic stressors – a situation where organism exposures to climatic stressors such as OA increase susceptibility to subsequent toxicant exposure is known as climate-induced toxicant sensitivity (CITS) (Hooper et al. 2013). Climate-induced toxicant sensitivity scenarios result in alteration of chemical toxicity in organisms due to changes in climatic conditions (Hooper et al., 2013).

Alterations in seawater chemistry as a result of ocean acidification can affect solubility, speciation and distribution of heavy metals in sediments and water, potentially affecting the toxicity of metals to marine organisms (Ivanina and Sokolova, 2015). Low pH increases the solubility of heavy metals and can cause metal desorption from the sediments and organic ligands, resulting in a higher influx of the dissolved metals into the water column (Ivanina and Sokolova, 2015). In line with this trend,

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elevated CO₂ levels within the range of predicted near-future ocean acidification scenarios of ~700–1,500 µatm pCO₂, increases heavy metal solubility and metal (Ni, Zn, and Fe) influx from the sediments into the water column (Breitbart et al., 2010; Roberts et al., 2013). Also, mobilization of Cd, Cu, Pb and Zn into the water column increases at lowered pH of 7.5 and 6.5 depending on the binding strength between the metal and the sediment particles with the degree of mobilization dependent on the strength of the association (Riba et al., 2003).

From recent studies on the effects of seawater pCO₂/pH on uptake and accumulation of metals, an intricate pattern which cannot be easily predicted from the chemical models of metal speciation and ligand binding is depicted (Ivanina and Sokolova, 2015). The effects of pCO₂/pH on uptake, accumulation and toxicity of metals are dependent on the species, organism's life stage and the OA levels rather than the predicted concentrations of the free metals in seawater (Ivanina and Sokolova, 2015). Uptake and accumulation of Cd, one of the most studied metals about ocean acidification-metal interactions could serve as a useful reference illustration for species and environment-dependent variability of responses to pCO₂. Cadmium speciation, unlike Cu and Fe, is independent of pH and pCO₂ (Ivanina and Sokolova, 2015).

The physiological effects of OA in many marine organisms have been extensively studied (Das and Mangwani, 2015), but the potential for OA to interact with other environmental stressors remains poorly understood (Crain et al., 2008). Till date, such studies have focused on combining OA with either temperature, salinity or hypoxia (Lewis et al., 2016). Of particular interest for environmental assessment, however, is the understanding of how near-future OA will change the behaviour and bioavailability of persistent marine contaminants, notably heavy metals. Studies by Lewis et al. (2016), revealed that near-future OA scenarios significantly increases the sub-lethal toxicity responses of two key coastal marine invertebrates, namely mussels (*Mytilus edulis*) and urchins (*Paracentrotus lividus*) to relevant concentrations of copper in the marine environment. Copper-induced damage to DNA of both marine invertebrates was significantly greater when the animals were exposed to nominal 0.1 µM copper under OA (high pCO₂/low pH) conditions compared with animals exposed under extant pCO₂ levels (Lewis et al., 2016).

Osmoregulation is an essential physiological function in most marine crustaceans because it permits them to adjust to ionic concentration changes within their bodies and environments (Romano and Zeng, 2012), and has been broadly studied in many crustaceans (Mantel and Farmer, 1983; Pequeux, 1995; Silvestre et al., 2005; Romano and Zeng, 2006; Freire et al., 2008; Charmantier et al., 2009). The capacity of crustaceans to osmoregulate is frequently assessed by the haemolymph osmolality measurement, which is the sum of osmolytes Na⁺ and Cl⁻ which is expressed in milliosmoles/kg as compared to the inhabited environment (Romano and Zeng 2012). Crustaceans inhabiting marine environments regulate their haemolymph osmolytes continuously, except for the strict osmoconformers as an adaptive strategy in adjusting to rapid changes in concentration of ions in their habitat (Romano and Zeng 2012). Although the extent of osmoregulatory response is mainly dependent on salinity and is highly species-specific, ocean/coastal acidification and heavy metal exposures could also impact the physiological functions of crustaceans by decreasing the measure of energy to be used for growth and reproduction thus impacting survival and performance (Romano and Zeng 2012; Ivanina and Sokolova 2015). Crustaceans usually have to compensate for the influx of ion through hypo-osmoregulation whenever the osmolality of the environment is hyper-osmotic to the haemolymph osmolality (Rainbow and Black, 2001). When the osmolality of the haemolymph is however, hyper-osmotic to the environmental osmolality, it compensates for the loss of ions from the haemolymph by hyper-osmoregulation (Rainbow and Black, 2001). When the external and internal osmolality equilibrates, i.e. iso-osmotic, marine crustaceans usually still have to osmoregulate to an extent due to significant difference between the ionic composition of the haemolymph and the environment (Rainbow and Black, 2001).

Crabs are capable of taking up and accumulating trace metals in their tissues and are, therefore a suitable bioindicator for environmental contamination assessment (Kumar et al., 2000; Bastami et al., 2012). *Dotilla fenestrata* (Hilgendorf, 1869), the sand-bubbler crab, is a small species and is about 1cm across the carapace (Dray and Paula, 1998; Gherardi et al., 2002; Flores et al., 2005). They belong to the Ocypodidae family of brachyuran crabs and are widely distributed along the East African coast from Kenya to South Africa and also found in Madagascar and The Comoro Islands (Hartnoll, 1973; Bulcao and Hodgson, 2012). They are burrowing decapod crustaceans and occur abundantly on soft sediment shores in tropical and sub-tropical climates (Maitland, 1986; Bulcao and Hodgson 2012). Sand bubbler crabs are distributed mainly in the north of Durban, South Africa (29° 52' S; 31° 04'E), although small numbers are found in warm temperate regions as far south of the Breede River estuary (Day, 1974, 1981; Rius et al., 2010). *Dotilla fenestrata* inhabits a range of coastal systems, including intertidal sandflats or mudflats and mangroves (Hartnoll 1973; Dray and Paula 1998; Bulcao and Hodgson 2012), but prefers sheltered sandflats habitat where it can reach the greatest densities (Hartnoll 1973; Gherardi and Russo, 2001; Gherardi et al., 2002; Flores et al. 2005). *Dotilla fenestrata* plays important ecological roles like other burrowing crustaceans as a deposit feeder and bioturbator within its habitat (Flores et al., 2005). Its bioturbation function, i.e. the process that is responsible for a rapid rate of sediment turnover that results in a change in the physical, chemical and biological characteristics of the sediment (Branch and Pringle, 1987; Dray and Paula 1998; Flores et al., 2005) has been shown to affect the productivity of sandy shores and changing of meiofaunal communities (Flores et al. 2005). This study aims to determine the effects of near-future coastal acidification (a climatic stressor) and the concentrations of metals (Cd and Pb) (a chemical stressor) on the haemolymph osmolality of *Dotilla spp.*

2. Materials and methods

2.1. Crab sampling and experimental design

Dotilla fenestrata (N = 540; 7 ± 1 mm carapace width) collected from Durban Bay Harbour, KwaZulu-Natal in line with the recommended ethical and governmental requirements, were cleaned with filtered seawater to remove debris and washed with 30 % artificial seawater to eliminate unwanted contaminants. They were subsequently acclimated in a constant temperature room at 18 °C and 32 psu (ambient salinity), controlled photoperiodic duration of 12L:12D with pH of 8.1 (field level pH at Durban Harbour where the crabs were collected) for 72 h. Crabs in the control group were acclimated to near-field level water physico-chemical parameters only.

The acute exposures of crabs were carried out according to the standard methodology such as those of the FAO (Ward and Parrish, 1982; Reich and Oshida, 1987) and the American Public Health Association (Apha, 1992). Prior to exposures and preparations of stock solution, all glassware was soaked in 10 % nitric acid and rinsed thoroughly with double distilled water and deionized water. A three by three experimental grid design was adopted, and a range-finder test was run to select applicable metal concentrations for the crabs to be exposed to in 96 h. Crabs were exposed to three varying metal concentrations and pH in triplicates per combined concentrations of metal and pH by bubbling CO₂ using an automated CO₂ system into glass tanks containing 5 L of (Fatoki and Mathabatha, 2001) homogenized stock solutions of sub-lethal concentrations of Cd (0.5, 0.75 and 1.0 mg/l), Pb (6.5, 8.5 and 10.5 mg/l) and Cd & Pb (4.5 (0.50 Cd & 4.00 Pb), 5.75 (0.75 Cd & 5.00 Pb) and 7.0 (1.00 Cd & 6.00 Pb) mg/l) freshly prepared by dissolving the appropriate analytical grade of metal salts (CdCl₂·5H₂O for Cd, and Pb (NO₃)₂) for Pb in deionized water with standard glass flasks. These sub-lethal concentrations fall between the range of Cd and Pb concentrations obtained by Fatoki and Mathabatha (2001) in water and sediment from East London, and Port Elizabeth Harbours in South Africa and represent about 1000 % order of magnitude above the South African marine water quality

guideline target values (Cd 4.0 µg/g and Pb 12.0 µg/g). Stock solutions were acidified by the controlled bubbling of CO₂ into stock solution (Chapman, 1978) to obtain varying pH groups of 7.2, 7.4 and 7.6 to simulate predicted near-future coastal pH. A coastal pH of 7.80 was recorded at one of the sampling sites of the crab *Dotilla*, therefore, to simulate near-future coastal acidification, pH of 7.2, 7.4 and 7.6 were considered for near-future and extreme coastal acidification scenarios. Tanks were covered with plastic lids to minimize evaporation and small holes bored through the centre to allow for bubbling of air and CO₂ throughout the experiment. Test solutions were set up and running 24 h before the introduction of crabs. The experiment was conducted in a constant temperature room at 18 °C and 32 psu and a controlled photoperiodic duration of 12L: 12D. Before each experiment, 20 active crabs were gently introduced and submerged into each tank. Water quality parameters were monitored periodically throughout the 96-hour duration of the experiment to ensure that all variables were within experimental limits.

2.2. Haemolymph osmolality analysis

After the exposure period, 0.5 ml of haemolymph was pooled from crabs in each group of the replicates through the arthroal membrane at the base of the pereopod using a 1 ml hypodermic syringe and 27-gauge needle. To prevent coagulation of the haemolymph samples, 0.25M stock solution of EDTA was added to the haemolymph stored in micro-centrifuge tubes using 1:1 ratio (0.5 ml EDTA: 0.5 ml haemolymph) and subsequently centrifuged for 10 min at 3000 rpm according to standard procedure. After centrifuging, 50 µl of supernatant was extracted using a micropipette into 0.5 ml microcentrifuge tubes and the osmolality was determined using a cryoscopic osmomat 030-D osmometer (1 mOsm/kg resolution, with a 3 point automated calibration). To correct for the addition of EDTA (anticoagulant), 25µl of the 0.25M EDTA stock solution was mixed with 25µl of distilled water using 10 replicates. The mean osmolality of the replicates was used as a correction factor for all the haemolymph osmolality readings.

2.3. Statistical analysis

After testing for normality and equality of variance, main effects analysis of variance (ANOVA) and pairwise comparisons analysis using Tukey's HSD test were used to test for significant difference in mean haemolymph osmolality of crabs due to the interactive effects of metal concentrations and varying pH using Statistica 13.0.

3. Results

3.1. Mean haemolymph osmolality

The mean haemolymph osmolality of the crabs exposed to combinations of Cd concentrations of 0.50, 0.75 and 1.00 mg/l, with varying pH of 7.2, 7.4 and 7.6 at the salinity of 32 psu were not significantly different (ANOVA HSD: df 9; $p > 0.05$) from the haemolymph osmolality of the control group acclimated at a pH of 8.1 at the salinity of 32 psu which was similar to background conditions of the environment at the time of sampling (See Figure 1 and Table 1). The mean haemolymph osmolality of the crabs exposed to combinations of Pb concentrations of 6.50, 8.50 and 10.50 mg/l, with varying pH of 7.2, 7.4 and 7.6 at the salinity of 32 psu were not significantly different (ANOVA HSD: df 9; $p > 0.05$) from the haemolymph osmolality of the control group acclimated at a pH of 8.1 at the salinity of 32 psu which was similar to background conditions of the environment at the time of sampling (See Figure 1 and Table 1).

The mean haemolymph osmolality of the crabs exposed to combined metals of Pb and Cd concentrations of 4.50, 5.75 and 7.00 mg/l, with varying pH of 7.2, 7.4 and 7.6 at the salinity of 32 psu were not significantly different (ANOVA HSD: df 9; $p > 0.05$) from the haemolymph osmolality of the control group acclimated at a pH of 8.1 at the salinity of

32 psu which was similar to background conditions of the environment at the time of sampling (See Figure 2 and Table 1).

4. Discussion

There is a dearth of literature on the combined effects of heavy metal concentrations and ocean acidification on osmoregulation capacity of decapod crustaceans. Therefore, this study provides an initial investigation into the interactive effects of metal concentrations and pH on haemolymph osmolality of *Dotilla*.

The mean haemolymph osmolality of the crabs exposed to combinations of Cd concentrations of 0.50, 0.75 and 1.00 mg/l, varying pH of 7.2, 7.4 and 7.6 and salinity of 32 psu were not significantly different from the haemolymph osmolality of the control group acclimated at a pH of 8.1 and salinity of 32 psu which was similar to background conditions of the environment at the time of sampling (See Table 1). Similar scenarios were experienced with the crabs exposed to varying Pb (6.50; 8.50 and 10.50 mg/l) and Pb & Cd (4.50; 5.75 and 7.00 mg/l) concentrations, varying pH and salinity of 32 psu (See Table 1). The lack of any significant effect of pH and varying trace metal concentrations on haemolymph osmolality could relate to the biochemical behaviour of trace metals and physiological interaction of crustaceans with salinity (Ivanina and Sokolova, 2015). Alteration in seawater chemistry due to lowering pH could impact solubility, speciation and mobility of heavy metals in water, thus potentially affecting uptake and toxicity to organisms (Ivanina and Sokolova, 2015). Although reduced pH increases metal desorption from sediments and solubility in water column (De Orte, Lombardi, et al., 2014; De Orte, Sarmiento, et al., 2014), uptake of metals by crustaceans from water is physiologically controlled through osmoregulatory mechanism, therefore are more resistant to metal at high salinity (Putranto et al., 2014). Crustaceans inhabiting contaminated sites do make physiological regulation to avoid uptake of contaminant from their environment (Capparelli et al., 2016). The amphipod *Gammarus marinus* from a clean river were reported by Wright (1986) with higher uptake rates of Cu, Zn and Pb than three other metal-polluted rivers in south-west England.

Exposure of euryhaline crustaceans to low salinity media causes their body fluid to be hyper-osmotic to the external water column. Therefore, water enters osmotically and thus increases urine efflux from the antennary glands which are iso-osmotic to haemolymph leading to losses of dissolved salts (Mantel and Farmer, 1983; Putranto et al., 2014). The lost salt is replaced via energy-dependent ion uptake, which includes metal ions across the gills (Mantel and Farmer (1983); Towle (1993). Likewise, euryhaline species showed more sensitivity to metal ions in very low salinity medium while hyper-osmoregulating (Hall and Anderson, 1995; Grosell et al., 2006). Moreover, the overriding effects of salinity over the combined effect of pH and metal concentrations on the haemolymph osmolality of *Dotilla* in this study also relate to the findings of Withers (1992) and Bervoets et al.; where prawns were found to possess significantly higher permeability at low salinities for key cations thus leading to increased uptake of metals which usually share similar pathways. In addition, some authors also reported osmoregulation disruption in crustaceans exposed to metals with reductions in major haemolymph ions (Na⁺, Ca²⁺, K⁺, Mg²⁺ and Cl⁻) concentrations and exhibited gill inhibition of Na⁺ - K⁺ - ATPase activity, which is majorly responsible for osmotic and ionic regulations (Bjerregaard and Vislie, 1986; Weeks et al., 1993; Spicer et al., 1998; Issartel et al., 2010). Salinity media of 32 psu used in this study irrespective of the combined metal concentrations and pH, could have significantly lowered the permeability of the crabs to metal ions in the water column which resulted in low uptake of metals. It will be expedient to carry out a further study using combinations of varying salinities, metal concentrations and pH to ascertain their combined effects on crab osmoregulation. It can, therefore, be inferred that changes in osmolality can strongly impact metal uptake and toxicity to *D. fenestrata*, i.e. uptake of heavy metals reduces when haemolymph osmolality of the crab is at equilibrium with the external medium osmolality (Roast et al., 2001).

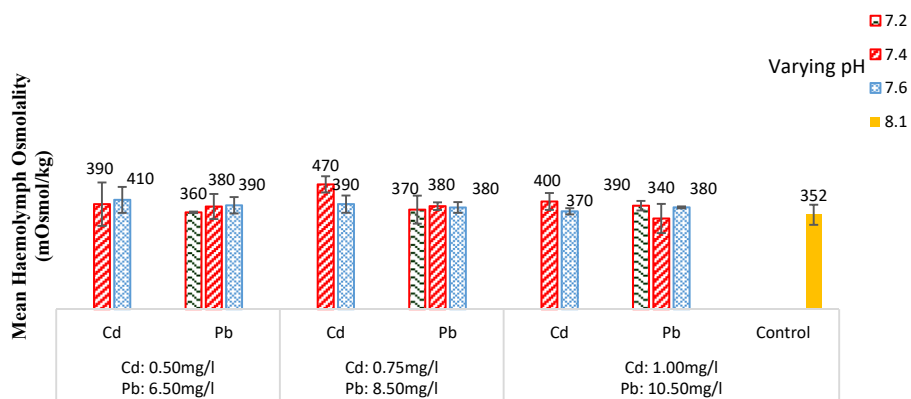


Figure 1. Mean haemolymph osmolality (mOsmol/kg ± SD) of *Dotilla* spp. exposed to varying concentrations of Cd only and Pb only, with pH.

Table 1. Pairwise test (pH and metal effects) on haemolymph osmolality of crabs exposed to different pH and metal (Cd, Pb and Cd/Pb) combinations.

Metals Exposures	pH	Cd df = 6	Pb df = 9	Cd/Pb df = 9	Control Group df = 6
(Cd 0.50 mg/l)	7.2	-	360 ± 0	390 ± 20	352 ± 40
(Pb 6.50 mg/l)	7.4	309 ± 80	380 ± 50	380 ± 30	
(Cd/Pb 4.50 mg/l)	7.6	410 ± 50	390 ± 30	410 ± 30	
(Cd 0.75 mg/l)	7.2	-	370 ± 50	420 ± 30	
(Pb 8.50 mg/l)	7.4	470 ± 30	380 ± 10	370 ± 50	
(Cd/Pb 5.75 mg/l)	7.6	390 ± 30	380 ± 20	370 ± 10	
(Cd 1.00 mg/l)	7.2	-	390 ± 20	390 ± 30	
(Pb 10.50 mg/l)	7.4	400 ± 30	340 ± 50	410 ± 90	
(Cd/Pb 7.00 mg/l)	7.6	370 ± 10	380 ± 0	350 ± 30	

(Mean value (mOsmo/kg) ± SD). n = 84 (*) denotes significant difference (p < 0.05), (**) denotes high significant difference (p < 0.01).

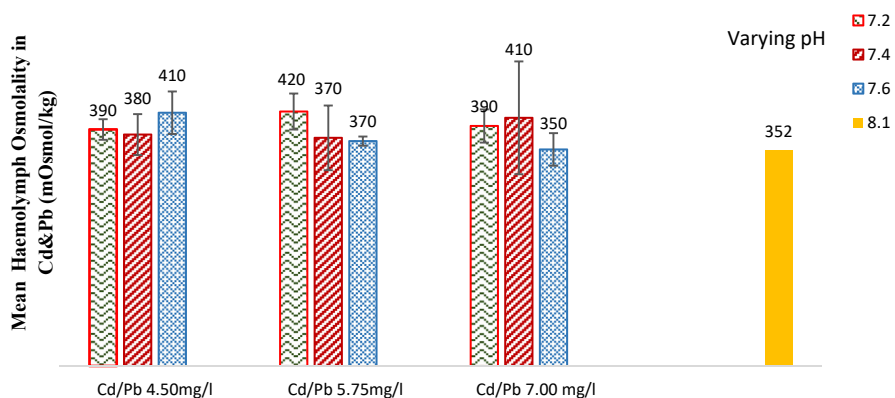


Figure 2. Mean haemolymph osmolality (mOsmol/kg ± SD) of *Dotilla* spp. exposed to varying concentrations of combined Cd & Pb with pH.

5. Conclusion

Findings of this study showed that varying concentrations of metal Cd and Pb in combination with near-future pH scenarios (7.2; 7.4 and 7.6) did not have a significant impact on the crab's haemolymph osmolality at the background salinity of 32 psu. Therefore, future studies are recommended on the combined effects of varying salinities, metal concentrations and pH on the osmotic response of this crab in order to better understand the interactive effects of lowering pH due to acidification, metal concentrations and varying salinity on the crab *Dotilla*. The osmolality of the crabs exposed to varying concentrations of Cd and pH of 7.2 could not be determined by the cryoscopic osmometer as the instrument could not freeze these

samples to the required freezing point even after using several replicates; thus, no results were reported for these exposures. It is therefore, recommended that future studies use vapour pressure osmometer due to the difficulties experienced obtaining readings with some samples as discussed.

Declarations

Author contribution statement

Babatunde Ayoade Adeleke: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Robertson-Andersson Deborah: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Moodley Gan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

The authors do not have permission to share data.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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