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Ocean warming and acidification degrade shoaling performance and lateralisation of novel tropical–temperate fish shoals

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Abstract

Gregarious behaviours are common in animals and provide various benefits such as food acquisition and protection against predators. Many gregarious tropical species are shifting poleward under current ocean warming, creating novel species and social interactions with local temperate taxa. However, how the dynamics of these novel shoals might be altered by future ocean warming and acidification remains untested. Here we evaluate how novel species interactions, ocean acidification, and warming affect shoaling dynamics, motor lateralisation, and boldness of range-extending tropical and co-shoaling temperate fishes under controlled laboratory conditions. Fishes were exposed to one of twelve treatments (combinations of three temperature levels, two $_pCO_2$ levels, and two shoal type levels: mixed-species or temperate-only) for 38 days. Lateralisation (a measure of asymmetric expression of cognitive function in group coordination and predator escape) of tropical and temperate species was right-side biased under present-day conditions, but side bias significantly diminished in tropical and temperate

fish under ocean acidification. Ocean acidification also decreased shoal cohesion irrespective of shoaling type, with mixed-species shoals showing significantly lower cohesion than temperate-only shoals irrespective of climate stressors. Tropical fish became bolder under ocean acidification (after four weeks), and temperate fish became bolder with increasing temperature, while ocean acidification dampened temperate fish boldness. Our findings highlight the direct effect of climate stressors on fish behaviour and the interplay with the indirect effects of novel species interactions. Because strong shoal cohesion and lateralisation are key determinants of species fitness, their degradation under ocean warming and acidification could adversely affect species performance in novel assemblages in a future ocean, and might slow down tropical species range extensions.

Introduction

Climate change has facilitated marine and terrestrial taxa to undergo poleward range extensions, shifts and contractions (Chen *et al.,* 2011; Poloczanska *et al.,* 2016). Range shifts are often seen as an attempt to escape the effects of warming or an opportunity to take advantage of colder conditions at higher latitudes/deeper waters now suitable for invasion (Walther *et al.,* 2002; Hoegh-Guldberg & Bruno, 2010), or could simply be an artefact of strengthening boundary currents moving dispersive larvae further poleward. Ectotherms such as fish are particularly susceptible to ocean warming and are frequently observed at the forefront of marine range extensions (Walther *et al.,* 2002; Booth *et al.,* 2011). Marine fishes

entering new environments often modify their behaviour through novel interactions with resident species (Nagelkerken & Munday, 2016; Coni *et al.,* 2021b). These novel interactions can determine the rate of range extension and, ultimately, a species range extension success. Previous studies assessing these interactions between marine range-extending and native species have focused on ocean warming effects but have not evaluated how the concurrent effects of ocean acidification may modify the effects of range extensions into high latitudes (Figueira *et al.,* 2009; Figueira *et al.,* 2019).

Marine organisms shift biogeographically faster than terrestrial species (marine species: 28 km/decade; terrestrial: 6.1 km/decade; Burrows *et al.,* 2011). Poleward boundary currents are the primary driver of marine biographic range shifts (Sorte *et al.,* 2010; Wu *et al.,* 2012). Ocean warming has made range shifts possible by increasing the accessibility of previously thermally unsuitable environments for dispersive tropical marine organisms (Walther *et al.,* 2002). The concurrent effects of ocean warming and intensification of boundary currents have increased the dispersal volume of tropical organisms into higher latitudes (Parmesan & Yohe, 2003; Wu *et al.,* 2012), inducing a widening of the tropical belt known as tropicalisation (Seidel *et al.,* 2007).

Regions exposed to poleward boundary currents (e.g. Kuroshio in Japan, East Australian Current in Australia, and Gulf Stream in North-east America) have warmed 3-4 times faster (0.037 ºC/yr) than global averages (Wu *et al.,* 2012). Globally, poleward boundary currents have facilitated tropicalisation. In eastern Africa, an inflow of fishes into sub-tropical latitudes has been observed directly due to the strengthening of the Agulhas Current (Lloyd *et al.,* 2011). In eastern Japan, the Kuroshio Current has propagated poleward extensions of tropical fishes, especially corallivores (Nakamura *et al.,* 2013). The arrival of tropical reef fishes in eastern Japan has aligned with the poleward movement of coral species, which form suitable habitats for many of the arriving tropical fishes observed (Yamano *et al.,* 2011). In

76   Australia, the East Australian Current has acted as a direct dispersal mechanism for over 150

77   tropical fish species and multiple species of tropical corals (Feary *et al.,* 2014; Booth and Sear,

78   2018). Range-shifting tropical ecosystem engineers such as herbivorous fish have also

79   mediated community phase shifts through habitat modification in regions including the

80   Mediterranean, Japan, and Australia (Verges *et al.,* 2014; Verges *et al.,* 2016). Such evidence

81   demonstrates how invading tropical fishes travel to temperate latitudes, yet few studies have

82   assessed how interactions between range-shifting and native species may mediate

83   tropicalisation (but see Coni et al., 2021a; 2021c).

84   Shallow marine ecosystems susceptible to tropical invasions are often exposed to

85   above-average rates of ocean warming (Lough, 2008; Wu *et al.,* 2012; Verges *et al.,* 2014) and

86   acidification (Scanes *et al.,* 2020). These climatic stressors can, directly and indirectly, alter

87   species interactions through shifts in behaviour (Malavasi *et al.,* 2013; Nagelkerken & Munday,

88   2016), decision making (Domenici *et al.,* 2014), learning (Briffa *et al.,* 2012), neurological

89   function (Nilsson *et al.,* 2012), development (Warren *et al.,* 2016), metabolism (Ishimatsu *et

90   al.,* 2005), and sensory function (Munday *et al.,* 2009; Rossi *et al.* 2016;) of the interacting

91   species. Oceans are expected to experience a pH reduction of ~3.3 units and warm by up to 4.5

92   ⁰C by 2100 (Caldeira & Wickett, 2003; Feely *et al.,* 2009; IPCC, 2021). Hence, it is necessary

93   to assess how ocean warming and acidification affect novel species interactions to predict

94   range-extension outcomes and their effects on marine ecosystem function.

95   Shoaling with natives may be an advantageous strategy for invading tropical species

96   for a few reasons: (1) social learning from heterospecifics (Smith *et al.,* 2018; Santiago-

97   Arrellano *et al.,* 2021); (2) reduced predation pressure as temperate predators more often target

98   native species (potentially overriding oddity effects; Mathis & Chivers, 2003); and (3)

99   increased foraging efficiency (Camacho-Cervantes *et al.,* 2014; Paijmans *et al.,* 2020).

100   However, because ocean warming and acidification can modify a wide range of behaviours,

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101 these benefits during the initial stages of range extension could be lost under future warming

102 and acidification, driving functional shifts in shoaling dynamics of vagrant and native fishes.

103 This could lead to decreased species performance, consequently altering range-extension

104 outcomes in marine ecosystems.

105 Gregarious fish species often possess some degree of brain asymmetry, whereby they

106 turn to one side more often than the other under predation stress (Miletto Petrazzini *et al.,*

107 2020). For shoaling species, such lateralisation can be an effective strategy to minimise

108 predation risk through dilution and confusion effects (e.g., when an entire shoal turns right, it

109 reduces the focal fish's probability of predation (Turner and Pitcher, 1986). Furthermore,

110 lateralised individuals have increased cognition (Bisazza & Brown, 2011; Bibost & Brown,

111 2013) and form more cohesive and coordinated shoals than non-lateralised fish (Bisazza &

112 Dadda, 2005). Hence, cohesive shoals inherently possess a competitive advantage over less

113 cohesive and less lateralised shoals through superior performance of behavioural tasks such as

114 predator vigilance, predator escape reactivity and prey acquisition (Bisazza & Dadda, 2005;

115 Dadda & Bisazza, 2006; Dadda *et al.,* 2010). Ocean warming and acidification have been

116 shown to alter lateralisation (Domenici *et al.,* 2012; 2014; Vila Pouca *et al.,* 2018), shoaling

117 dynamics (Colchen *et al.,* 2016; Lopes *et al.,* 2016; Nadler *et al.,* 2016) and, anti-predator

118 performance (Dadda *et al.,* 2010; Domenici *et al.,* 2019) in fishes. Thus, the performance of

119 novel species shoals composed of vagrant and native fishes could strongly modulate the

120 persistence of species undergoing range shifts.

121 Here we used a controlled laboratory experiment to test the interactive effects of ocean

122 warming, ocean acidification, and novel species interaction on the lateralisation, shoaling

123 dynamics, and anti-predator performance of a common tropical vagrant and temperate co-

124 shoaling fish species across a one-month exposure period. Video recordings were used to

125 analyse shoaling behaviour, while motor lateralisation was repeatedly tested over four weeks

126 using a standard detour test. We aimed to test how climate change stressors and novel

127 interactions might alter the shoaling dynamics, boldness, and lateralisation of tropical vagrant

128 fish and native co-shoaling temperate fish. We then discuss how changes to these behavioural

129 proxies might facilitate or restrict tropicalisation in high latitude ecosystems.

130

131

132 **Materials and Methods**

133

134 **Fish Collection and Acclimation**

135

136　　In Australia, strengthening of the East Australian Current and faster than average ocean

137 warming at high latitudes (34 ºS) along the east coast (Ridgway, 2007; Ridgway *et al.,* 2009;

138 Lough, 2008) has acted as a direct dispersal mechanism for dispersive larval-stage tropical

139 fishes from the Great Barrier Reef into temperate southeastern Australian waters (Booth *et al.,*

140 2007; Booth *et al.,* 2011). Southeastern Australia's cooler conditions have so far restricted

141 tropical fish from establishing breeding populations, as temperatures drop below their thermal

142 minima in winter (~17 ºC; Figueira *et al.,* 2009; Figueira & Booth, 2010). However, ocean

143 warming in southeastern Australia (Ridgway, 2007) is expected to facilitate the successful

144 establishment of breeding tropical populations (Feary *et al.,* 2014). These range-extending fish

145 species are commonly termed 'vagrants'. One of the most common species of vagrants, Indo-

146 Pacific Sergeants (*Abudefduf vaigiensis*), is often observed shoaling with temperate species,

147 including Eastern Hulafish (*Trachinops taeniatus*) and, more frequently, Australian Mado

148 (*Atypichthys strigatus*) (Smith *et al.,* 2018).

149   *A. vaigiensis* is omnivorous, exhibits a dispersive larval stage, has high site fidelity in

150   the adult stage, and reaches up to 20 cm in length (Beck *et al.,* 2016). Co-shoaling temperate

151   species, *A. strigatus*, grows up to 25 cm in length and is also omnivorous (Kuiter, 2000).

152   Flow-through 20-litre transparent tanks (IKEA product number: 898.914.70;

153   dimensions: 39×28×28 cm, height×length×width; n = 120) were installed across two adjacent

154   flowthrough temperature/pH control rooms at the Sydney Institute of Marine Science. Two 0.5

155   cm (diameter) holes were inserted 4 cm from the top of each tank (lengthways) to allow water

156   to flow bilaterally out of each tank. Each pairing tank was allocated a tank identifier and ocean

157   acidification (OA) treatment (Table S1). Tanks were randomly assigned one of the two $CO_2 \times$

158   3 temperature treatments (N = 6 treatments; Table S1). The ocean warming and ocean

159   acidification treatments reflected those forecasts during summer and winter for the New South

160   Wales coastline by 2100 under an RCP 8.5 scenario (20 ⁰C: project future winter temperature,

161   23 ⁰C: current summer temperature, and 26 ⁰C: projected future summer temperature; Ridgway

162   2007). We added 10 cm lengths of PVC piping (diameter: 10 cm) to each tank for shelter

163   (Figure S1). Tanks were maintained at experimental target temperatures and pH units

164   throughout the experiment (Table S1). Solenoid feedback systems were used to maintain the

165   designated temperature within a range of ± 0.25 ⁰C of the tanks target temperature. Ocean

166   acidification treatments were reached by bubbling pure CO_2 into header tanks (one header tank

167   for every four fish tanks) to reflect future values. Header tank water flowed into fish tanks at a

168   rate of 150 ml.min^{-1}. pH was decreased at a rate of 0.06 pH/day by gradually increasing the

169   pCO$_2$ concentration in header tanks and monitored daily until target pH levels were reached.

170   Sixty *A. vaigiensis* and 180 *A. strigatus* were collected during 7-24 March 2020, using

171   barrier and hand nets at Little Manly Cove (-33.806771 ⁰S, 151.285644 ⁰E) and Freshwater

172   Beach (-33.781688 ⁰S, 151.294088 ⁰E) at depths of 0.1–2 metres. Following collection, fish

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173 were transported to ambient holding tanks (size: 100 litres) at the Sydney Institute of Marine

174 Science.

175   Tropical vagrants benefit from shoaling with temperate residents, growing faster and

176 surviving longer into winter than conspecific tropical shoals in temperate ecosystems (Smith

177 et al., 2018). Hence, the rationale for selecting temperate + temperate and tropical + temperate

178 pairs lies in understanding how novel co-shoaling species in temperate ecosystems (i.e. range-

179 extending tropical) might alter temperate fish performance in mixed-species shoals (i.e. future

180 conditions) compared to temperate-only pairs (i.e. present-day conditions), and how this is

181 mediated by the direct effect of climate change stressors. This comparison of shoaling types

182 can inform us about potential declines of temperate fishes in tropicalisation hotspots and the

183 rate of tropicalisation in a future ocean. As such, and also because of space limitations, we did

184 not include pairs of tropical species.

185   Fish were randomly assigned to pairing tanks (tropical vagrants, N: 60; mixed-species

186 temperates, N: 60; temperate-only paired fish, N: 120) across climate-manipulated rooms.

187 Temperate-only selected fishes were paired with individuals of similar body sizes to mitigate

188 competitive advantages that larger fish could develop in obtaining food and shelter. However,

189 mixed-species shoaling fish displayed different body sizes to reflect the current field situation

190 of tropical vagrants being smaller than their co-shoaling temperate species (Smith *et al.,* 2018;

191 Table S26). Before commencement of the experiments, all individual fish had their initial wet

192 weight measured on digital scales (to the nearest 0.01 g) and initial standard length measured

193 (to the nearest 0.01 mm) with a sliding calliper from the tip of the upper lip to the beginning of

194 the caudal fin. Initial wet weight (mean $\pm$ SE) was: tropical fish 0.31 ± 0.04 g; mixed-species

195 temperate fish 1.24 ± 0.15; temperate-only fish 4.04 ± 0.20. For initial standard length, this

196 was: tropical fish 18.61 ± 0.68 mm; mixed-species temperate fish 29.34 ± 1.52 mm; temperate-

197 only fish 53.28 ± 0.80 mm).

Fish were acclimated from ambient temperature conditions by raising or decreasing temperatures by 0.5 ⁰C per day to minimise stress generated from rapid temperature changes (Alfonso *et al.,* 2020). Fish introduction and acclimation to experimental tanks were staggered to prevent fish in control treatments (23 ⁰C, pH 8.1) from gaining an acclimation advantage over fish in treatments that required more acclimation steps (e.g. 20 ⁰C and pH 7.7 or 26 ⁰C and pH 7.7). Fish were exposed to climate treatments for six days before the commencement of motor lateralisation testing. API^{TM} Stress Coat solution was added to each tank daily during acclimation and experimental periods. No fish died during the acclimation period. All fish were fed under a restricted feeding regime of 2.86 g of Ocean Nutrition^{TM} frozen *Artemia* mixed into 60 ml fresh seawater twice daily. Fish were exposed to treatment conditions for a maximum length of 40 days.

Water Chemistry

Total alkalinity values were estimated by Gran titration (Metrohm 888 TitrandoTM) from 60 ml water samples from all tanks at days 24 and 25. Mean pCO$_2$ values were calculated using CO2SYS (Pierrot *et al.,* 2006) for Excel with constants from Mehrbach *et al.* (1973) refit by Dickson and Millero (1987) (Table S1).

Lateralisation Test

Motor lateralisation was evaluated using a standard detour test (T-test) (Bisazza *et al.,* 1998). The detour test is commonly used to evaluate behavioural asymmetries in fish (Miletto Petrazzini *et al.,* 2020). The arena used in this study was based on a design used previously by Bisazza *et al.* (1998; Fig. S2; note: our arena design differed slightly, as we removed the visual stimulus cues at each end of the arena used in Bisazza *et al.,* 1998), and it consists of a two-way T-shape runway which allows scoring the direction of the turn (e.g. left or right) of each

223　individual over consecutive trials. The experimental arena consisted of a transparent plastic

224　tank (IKEA product number: 198.856.46; 39×28×14 cm, length×width×height), with a runway

225　in the middle (20×8 cm, length×width). At both ends of the runway, plywood barriers

226　(12×12×1 cm) were positioned perpendicular to the runway. The runway was created by

227　placing custom wooden plywood inserts onto each side of the arena to form the runway

228　(20×8×15 cm). Water in the arena was 15 cm deep and ran directly from a flowthrough control

229　system into both sides of the arena, at a rate of 150 ml.min^{-1}, to maintain target temperature

230　and pH in the arena. Two parallel 0.5 cm (diameter) holes were added at each end of the

231　runaway to minimise bias in directional water flow.

232　　　　At the start of each trial, a single fish was introduced from a pairing tank (tropical +

233　temperate or temperate + temperate) into the experimental arena and left to acclimate to the

234　novel arena environment for 2 minutes. We conducted 12 consecutive turning trials for each

235　individual. During each trial, we chased the focal fish with a handheld net along the runway to

236　force a left or right turning choice at the end of the runway (see Vinogradov *et al.,* 2021). The

237　experimenter was always positioned at the same end of the arena to minimise side bias. Upon

238　completion of the 12 turning decisions, fish were returned to their pairing tank. The

239　experimental arena was emptied and refilled with treatment seawater following each trial until

240　all treatments had been tested.

241　　　　Mean relative lateralisation index (L_R) was calculated from the 12 turning decisions

242　(per fish, per trial week) and used to identify the strength of laterality and turn preference (e.g.

243　bias in left or right turns). For the L_R index, individuals were classified between the extreme

244　values of '+100' (fish that turned right on all 12 turning decisions) and '−100' (fish that turned

245　left on all 12 turning decisions). An individual L_R close to zero indicates that a fish is neither

246　left- nor right-biased in its turning tendency (Bisazza *et al.,* 1998). Because individuals could

247　be left-biased (all negative scores) or right-biased (all positive scores), the average L_R across

248 replicate fish within a treatment could hypothetically be zero (i.e. even number of fully left- vs

249 right-biased fish). This would not reveal the presence of a strong lateralisation response that is

250 independent of turning direction. Therefore, the absolute lateralisation index (L_A) was also

251 calculated (Domenici *et al.,* 2014). This, in contrast to L_R, evaluates individual lateralisation

252 strength irrespective of the direction of their turns. The L_A index corresponds to the absolute

253 value of L_R, thus ranging from 0 (an individual that turned in equal proportion to the right and

254 the left) to 100 (an individual that turned right or left on all 12 trials).

255   Each fish performed 12 turning decisions at alternating ends of the runway, on trial

256 days 6, 17 and 25, from which the mean score of the turning direction (L_R) and the mean

257 absolute lateralisation (L_A) for each fish were obtained for each trial day. Lateralisation tests

258 were not performed on fish showing signs of stress (i.e. irregular swimming pattern).

259   Tropical and temperate fish were tested between 7 am - 10 pm on days 6, 17, and 25 of

260 the experiment. One researcher set up the treatment water in the experimental arena on each

261 trial day. The sequence of treatments being tested was selected randomly. The second person

262 who performed the scoring of lateralisation was blinded to the treatment type to minimise

263 observer bias.

264

Video Observations of Shoaling Dynamics and Startle Escape Response

266   On days 19 and 27 (recording days selected to minimise repeated experimental stress

267 following T-tests on days 17 and 25) of treatment exposure, we filmed all fishes' shoaling

268 dynamics (inter-individual distance, amplitude variability, and oscillation frequency) and

269 startle escape distance in 9-min video recordings. Paired fish (mixed species or temperate-only

270 pairs) were kept in their pairing tanks during video observations (Figure S1). Tanks were

271 illuminated by LED strip lighting (750 lumens) placed above the water surface outside the tank.

272 White sheeting surrounded the back of the tank to minimise any external influences on tested

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273 fish during video recordings. We placed GoPro Hero 7 Silver cameras 30 cm away from pairing

274 tanks and commenced recording. Video recordings lasted for 9 min., excluding 3 min of

275 acclimation time to the cameras, and were split into three 3-min periods (period 1: shoaling

276 dynamics, period 2: food administration, period 3: startle escape distance). Researchers were

277 blinded to treatments of each video recording during video analysis until data collection was

278 completed.

279

280 **Period 1: Shoaling Dynamics**

281   We measured three different proxies for shoal dynamics of the fishes (mixed-species

282 and temperate-only pairs) during the first 3-min period of the video analysis:

283 (1) Inter-individual distance (IID). The distance between individuals within each tank was

284 measured every 3 sec for 3 min (N = 60 measurements per tank) of video recording. The

285 average distance between fish was calculated to obtain the average distance for shoals (mixed-

286 species or temperate-only; Figure S2). When both fish in a tank were observed residing within

287 the shelter, the inter-individual distance was scored as a distance of 0 cm. If both fish could not

288 be observed simultaneously, a 'not applicable' distance value was given for that time point.

289 (2) Amplitude variability. From the sixty repeated measurements of shoaling distance within

290 each tank, the standard deviation of inter-individual distance was calculated for each tank, and

291 the mean, standard deviation for each treatment type was calculated to determine amplitude

292 variability. (3) Oscillation frequency. The inter-individual distance (IID) of shoaling fishes is

293 not fixed (Miller & Gerlai, 2008) but oscillates between a maximum and minimum distance.

294 We measured the time (seconds) elapsed between sequential maximum peak distances across

295 the 60 IID samples taken across the 180 sec shoaling video period to calculate the mean time

296 between maximum distance points (the oscillation frequency).

297   Paired fish often were aligned with each other at the front of the tank (the side nearest

298   to the camera), reducing the overall effect that tank depth had on calculating the distance

299   between the individuals.

300

Period 2: Food Administration

302   Approx. 1.43 g of Ocean Nutrition[TM] frozen *Artemia* mixed into 60 ml fresh seawater

303   was administered via a syringe into each tank to draw fish out of the shelter in preparation for

304   startle escape distance testing. Tanks were left undisturbed for 3 minutes following the addition

305   of food to tanks.

306

Period 3: Startle Escape Distance

308   We used a startle escape distance test as a proxy to measure the boldness and anti-

309   predator behaviour of paired fish. Startle escape distance was defined as 'the distance at which

310   a fish initiated a rapid movement away from the simulated threat'. We used a white ceramic

311   weight (size: 3×3×3 cm; weight 500 g) as the simulated threat.

312   After 6 min of video recording, a ceramic weight was handheld above a tank (to mimic

313   a looming visual stimulus). The ceramic weight was then released from 3 cm above the water

314   surface between the two paired fish to induce a startle escape response. In case a fish did not

315   elicit a startle escape response or was within the shelter provided, a 'not applicable' score was

316   given.

317   During video analysis, videos were paused to measure the distance between the focal

318   fish and the ceramic weight at which startle escape response was initiated. Distance calibration

319   during video analysis was accomplished by using the shelter size (10 cm) as a reference.

320

Statistical Analysis

322   Using Primer version 6, a permutational multivariate analysis of variance (MANOVA)

323   was used to examine the effect of temperature, pCO_2, and pairing type treatments on the various

324   non-independent behaviours, i.e. those measured from the same individuals (relative

325   lateralisation, absolute lateralisation, inter-individual distance, mean distance amplitude,

326   oscillation frequency, and startle escape distance) for the tropical and temperate fishes. A four-

327   way MANOVA was performed for the tropical fish (factors: Ocean acidification (OA),

328   Temperature (Te), Time (Ti) and Fish ID (Fi)), and a five-way MANOVA (addition of fixed

329   factor: pairing type (Pa)) for the temperate fish. Fixed factors were temperature, ocean

330   acidification and pair type. Fish ID was nested within all factors. Time was set as a repeated

331   measure random factor. Fish who scored 'not applicable' scores in startle escape distance tests

332   were excluded from MANOVAs. The data was first standardised (to adjust the values of the

333   different behavioural types to a similar scale). The MANOVA datasets were square-foot

334   transformed and converted with a Bray-Curtis resemblance matrix. Permutations were set to

335   9999. Permutational MANOVAs were performed, followed by pairwise post hoc tests

336   (significance accepted at p < 0.05; Anderson 2001).

337   Lateralisation (L_R and L_A) and startle response distance were also tested separately for

338   the two species among the twelve treatments using four-way ANOVAs for tropical fish (Ocean

339   Acidification (OA), Temperature (Te), Time (Ti) and Fish ID (Fi)) and five-way ANOVAs

340   (addition of fixed factor: pairing type (Pa)) for temperate fish. Fish ID was set as a random

341   repeated measure factor and nested within the main factors (OA, Te, and Pa). Values for

342   lateralisation and startle response distance were square-root transformed. Proxies of shoaling

343   dynamics (inter-individual distance, mean distance amplitude, oscillation frequency) were

344   tested using five-way ANOVAs. Pairing type, temperature, and ocean acidification were set as

345   fixed factors. Tank ID was nested within the main effect factors. Time was set as a repeated

346   random measure factor. Values for shoaling amplitude and inter-peak oscillation frequency

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347 were square-root transformed. The permutation method used was a permutation of residuals

348 under a reduced model. A Euclidean Distance dissimilarity resemblance matrix was used for

349 univariate statistical analyses. The number of replicates for the above ANOVAs was larger

350 than that used in the MANOVAs, as the latter could only include individuals for which all 6

351 behaviours were measured, excluding those with missing values for a particular behaviour),

352 whilst the former included all fish.

353   We performed ANCOVAs to test how both inter-individual distance and startle escape

354 distance were related to relative laterality (i.e. did an increased inter-individual distance

355 correlate with decreased laterality). For ANCOVAs, we first tested for homogeneity of slopes

356 by including interactions between covariates and main factors. In cases where interactions were

357 not significant, these were removed from the final test statistics. Other assumptions for

358 ANCOVAs (linearity, normality) were tested via residuals and boxplots. We found no

359 association between laterality and shoaling or startle escape distance and hence did not discuss

360 this result in detail (see the statistical output in Table S28-31).

361 On R version 3.6.2, we used the function ' rpt' located in R package 'rptR' (Stoffel *et al.,* 2017)

362 to test the relative repeatability (L_R) across the three trial series for individuals of each species

363 across treatments. This differed from testing the 'time' effect in the repeated measures

364 ANOVAs because R package 'rptR' provides a measure of the consistency of relative

365 lateralisation scores of individual fish among treatments across time, while repeated measures

366 ANOVAs testing time effects on relative lateralisation analyse differences in a samples mean

367 relative lateralisation across time. Mean repeatability values for species across treatments

368 ranged from 0 (non-repeatable) to 1 (fully repeatable) and provided a standardised measure of

369 the consistency of phenotypes across time (Nakagawa & Schielzeth, 2010). We specified

370 Gaussian error distributions and 1,000 bootstrapping and permutation iterations to calculate

371 95% confidence intervals (CIs). Repeatability was calculated separately for species (tropical or

15

372   temperate), pCO$_2$ levels (control or OA), temperature (20, 23, or 26 ºC) and pair types (mixed-

373   species or temperate-only). Fish ID was added as a random effect, as per Roche *et al.* (2020).

374   The raw data used in the statistical analysis is publicly available (Mitchell *et al.,* 2021).

375

376

377   **Results**

378

379   **Multivariate Analysis of Behaviours**

380   Behaviours of the tropical fish were significantly altered under ocean acidification at

381   20 ºC (20 ºC: control vs OA, p = 0.049; Table S2, S4) and differed between 20 ºC and 26 ºC

382   under ocean acidification (p = 0.023; Table S2, S3). Behaviours of temperate-only paired fish

383   significantly shifted from 20 ºC to 26 ºC (p = 0.013; Table S5, S6). Temperature had no effect

384   on the behaviours of mixed-species paired temperate fish (p > 0.05, Table S6). Behaviours also

385   differed between temperate-only and mixed-species temperate fish at 20 ºC and 26 ºC

386   (temperate-only vs mixed-species, 20 ºC: p = 0.022, 26 ºC: p = 0.015; Table S7), but not at 23

387   ºC (p > 0.05; Table S7).

388

389   **Individual Behaviours**

390   Both tropical and temperate fishes showed a right-side turning bias at population level

391   (L$_R$), which was significantly reduced under ocean acidification (Figs. 1A, C, E, S3A, C, E;

392   tropical: p = 0.003, Table S10; mixed- and temperate-only paired temperate: p = 0.004, Table

393   S8). Mixed-species paired temperate fish showed significantly greater lateralisation at the

394   population level than temperate-only paired fish (Figs., 1C, E, S3C, E; p = 0.037, Table S8).

395   Absolute lateralisation (L$_A$) was not affected by climate treatments in temperate or tropical fish

396 (Figs. 1B, D, F, S3B, D, F; Tables S9, S11). Time had no effect on individual or population-

397 level lateralisation (p > 0.05; Table S8-S11).

398   Mixed-species shoals showed significantly greater inter-individual distance than

399 temperate-only pairs (Figs. 2A vs 2B; p < 0.001, Table S12). Ocean acidification significantly

400 increased inter-individual distance in both pairing types (p = 0.036, Table S12). The mean

401 amplitude of shoaling distance of mixed-species and temperate-only pairs was not affected by

402 climate treatments but was greater in mixed-species pairs than temperate-only pairs (Figs. 2C

403 vs 2D; p < 0.001, Table S13). In both shoal types, mean amplitude only decreased from day 19

404 to day 27 (p = 0.016; Figs. 2C, D, Table S13). The oscillation frequency of inter-individual

405 distance of mixed-species and temperate-only pairs was not affected by ocean acidification or

406 temperature (Figs. 2 E, F) but was longer in mixed-species pairs than temperate-only pairs (p

407 < 0.001, Table S14).

408   Startle escape distance of tropical fish exposed to ocean acidification significantly

409 decreased from day 19 to day 27 in tropical fish (Fig. 3A; p = 0.030, Tables S21, S25). In

410 temperate fish (both pairing types), startle escape distance was significantly shorter under

411 warming (20 ºC vs 23 ºC and 26 ºC, p < 0.001, Tables S15, S16); however, temperate fish

412 exposed to ocean acidification showed a greater startle escape distance than fish not exposed

413 to ocean acidification, irrespective of warming, shoal type and time (Figs. 3B, C; p = 0.047,

414 Table S15). In mixed-species temperate fish, startle escape distance significantly increased

415 from day 19 to day 27 across all climate treatments (Fig. 3B; p = 0.044, Tables S15, S20).

416

417 **Repeatability of Lateralisation**

418   Relative lateralisation of individuals was not significantly repeatable over time in

419 tropical or temperate fish across treatment types (p > 0.05, Table S27) expect in tropical fish at

420 26 ºC under control pCO$_2$ levels (R = 0.38 ± 0.193, CI = 0-0.701, p = 0.038, Table S27). In

17

contrast, repeated-measures ANOVAs showed no effect of time on the reduced lateralisation

observed under ocean acidification (OA×Ti interaction: p = 0.563 for tropical and p = 0.390

for temperates, Table S8, S10).
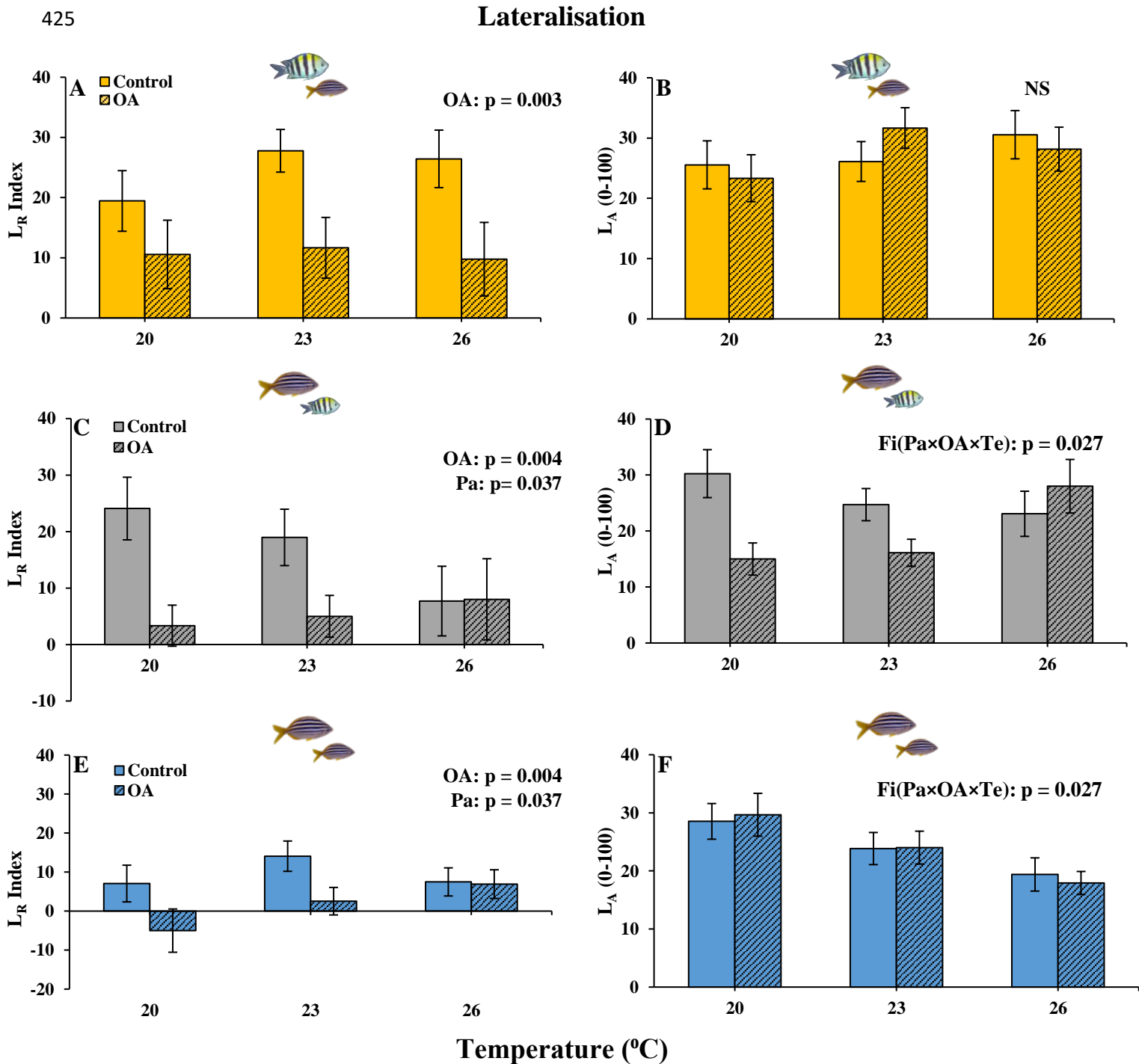
Figure 1: Mean difference (± SE) in relative lateralisation (L_R) and absolute lateralisation (L_A)

across three repeated trials for mixed-species shoaling *A. vaigiensis* (A and B), mixed-species

shoaling *A. strigatus* (C and D), and temperate-only shoaling *A. strigatus* (E and F) among temperature (Te) treatments (20 ºC: project future winter temperature, 23 ºC: current summer temperature, and 26 ºC: projected future summer temperature) and ocean acidification (OA) treatments (control pH: 8.1, projected future pH: 7.7; Tables S3 - S6). NS = not significant, Fi = Fish individual, Pa = Pair Type. Error bars represent standard errors.

Shoal cohesion dynamics
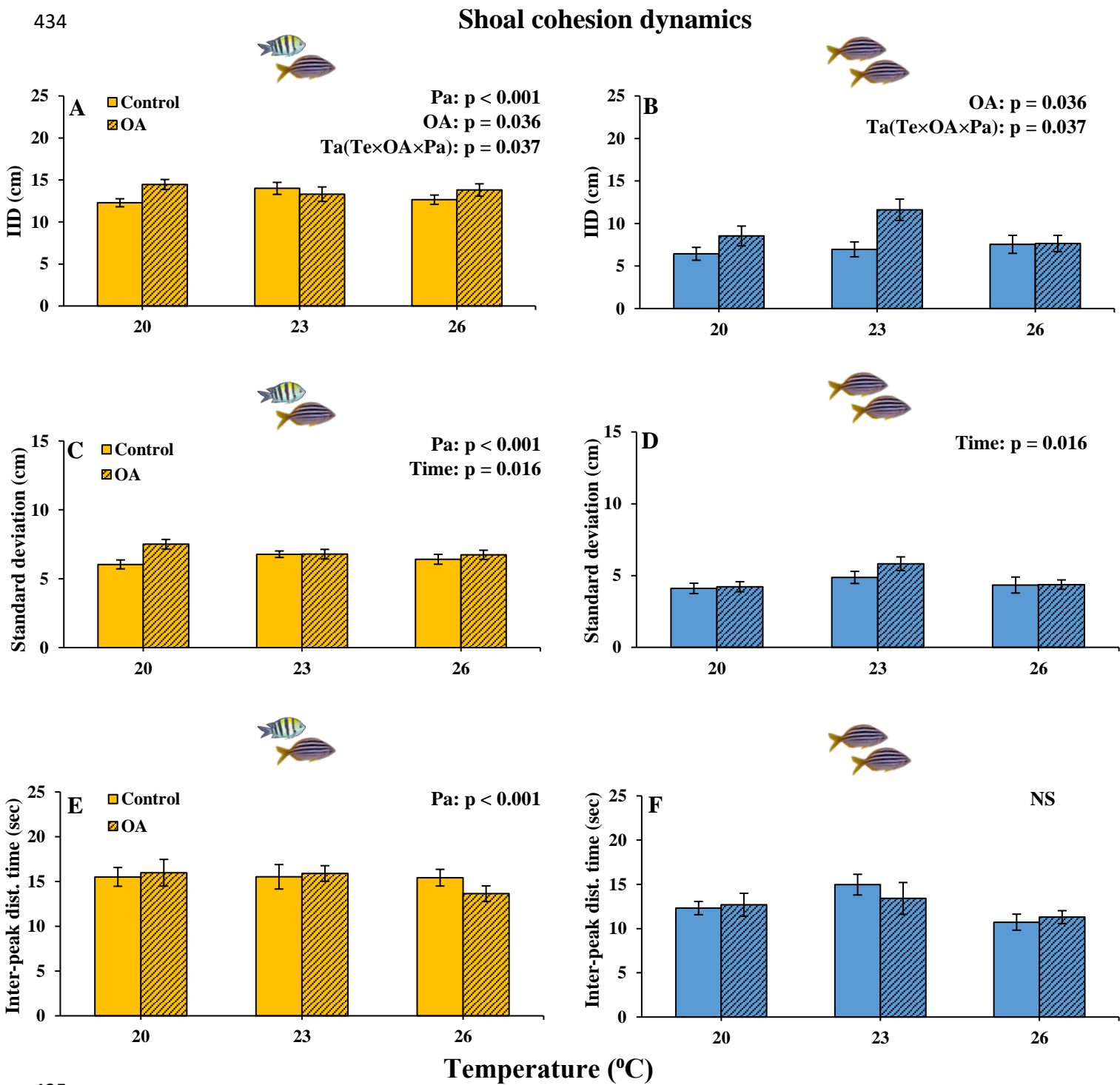
436 **Figure 2:** Mean inter-individual distance (IID, cm), mean distance amplitude of inter-
437 individual distance (standard deviation, cm), and the inter-peak distance oscillation frequency
438 of inter-individual distance (sec) across mixed-species pairs (panels: A, C and E) and
439 temperate-only pairs (panels: B, D and F) shoals (Tables S7 - S9). Data for video trials at days
440 19 and 27 were pooled for visualisation purposes. NS = not significant. Pa = paring type
441 (tropical + temperate vs temperate only), Ta = Tank ID. Error bars represent standard errors.

442

443 **Startle escape distance**
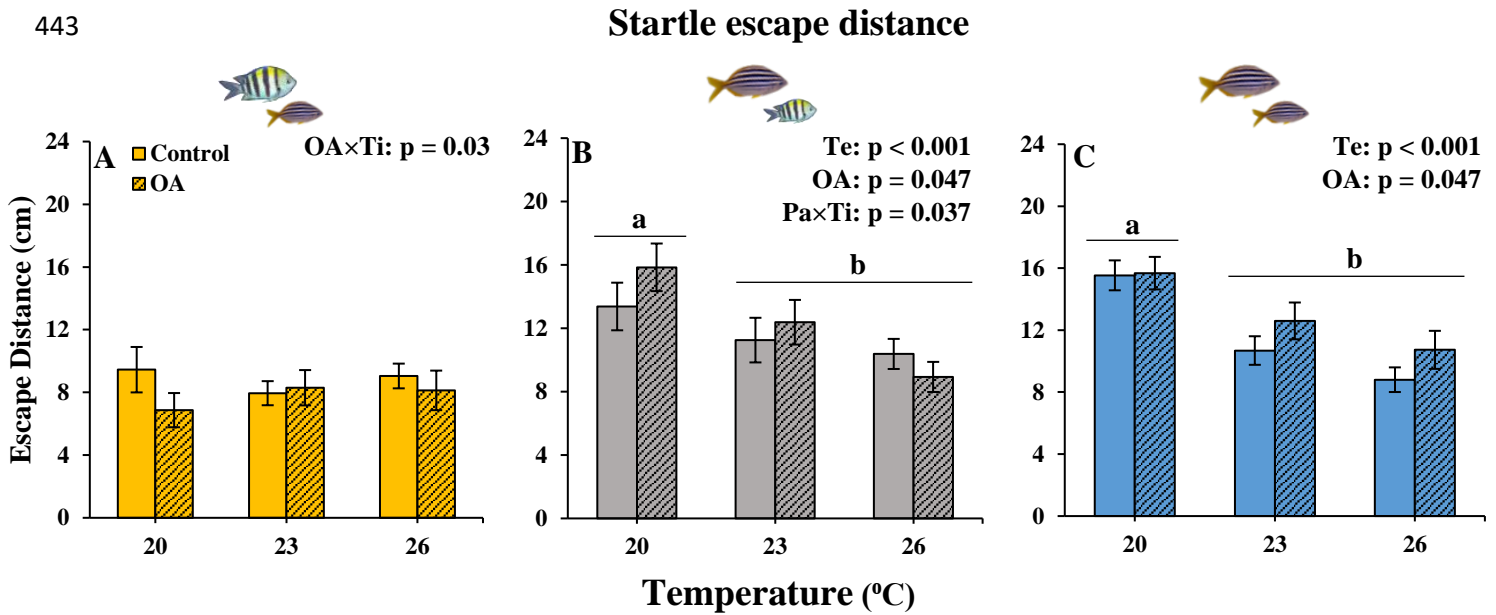
444 **Figure 3:** Startle response distance (cm) responses for tropical (A) and temperate (B, C) fishes
445 across temperature (Te), ocean acidification (OA) and shoaling type (Pa) treatments (the larger
446 fish in A and B indicate the focal fish species, whilst the smaller one indicates the co-shoaling
447 fish species). Ti = time (days). Data for days 20 and 27 were pooled for visualisation purposes.
448 The different letter above bars indicates significant differences among treatments (Tables S10
449 - S20). Error bars represent standard errors.

450

451

452 **Discussion**

453

454   Here we show that ocean warming and acidification can disrupt the lateralisation and
455 shoaling behaviour of temperate fish pairs as well as novel mixed-species pairs composed of
456 tropical and temperate fishes in climate range-shifting hotspots. Changes to critical behavioural

20

457   traits, such as reduced lateralisation, shoal cohesion, and startle escape distance (only in

458   temperate fish under warming) under ocean warming or acidification, could decrease temperate

459   and tropical fish performance (e.g. feeding and predator avoidance). Reduced lateralisation of

460   tropical vagrants and co-shoaling temperates under ocean acidification could decrease

461   individual cognitive performance (Bibost & Brown, 2014) and performance of novel shoals,

462   increasing predation risk of both tropical and temperate fish. Ocean acidification may buffer

463   tropicalisation directly through reduced lateralisation in tropical vagrants and indirectly

464   through degrading the shoaling dynamics of novel tropical-temperate pairs (Bisazza & Dadda,

465   2005; Dadda *et al.,* 2010; Lopes *et al.,* 2016). The latter could reduce behavioural plasticity

466   and learning (e.g., novel prey and predators; Bibost & Brown, 2014) of vagrants entering high-

467   latitude ecosystems. In addition, ocean acidification could switch currently dominant habitat

468   types at high latitudes from barrens to turf-dominated habitats, reducing the recruitment of

469   tropical fishes and hence retard the rate of tropicalisation at high latitudes (Coni *et al.,* 2021).

470   We conclude that ocean acidification may decrease the performance of common temperate and

471   tropical co-shoaling species through disruption of their lateralisation and shoaling dynamics,

472   which might slow the rate of tropicalisation in SE Australia and other temperate ecosystems.

473   Ocean warming increased the boldness (i.e. reduced startle escape distance) of

474   temperate fish irrespective of shoaling type, while the combination with ocean acidification

475   appears to dampen the boldness of the temperate fish. This, together with a decrease in shoal

476   cohesion and lateralisation under ocean acidification, could compromise the survival of

477   temperate species at their current equatorward distributions (i.e. trailing edges) in a future

478   ocean. Reduced shoaling cohesion is likely to decrease anti-predator shoal performance by

479   extending the time taken before a minimum relative number of shoal mates initiate a critical

480   behaviour (e.g. left turn to evade a predator; Lemasson *et al.,* 2018; Ward *et al.,* 2008).

481   Lateralized, more cohesive, and polarised shoals benefit from faster information transfer

482   between shoal mates (Bisazza & Dadda, 2005; Ward *et al.,* 2018). Hence, a destabilisation of

483   shoal cohesion and relative lateralisation can slow down information transfer time between

484   shoal mates, thus decreasing decision-making and reaction time in critical fast-event situations

485   (e.g. predator attacks). Altered shoaling dynamics could further modify the performance of

486   temperate species via shifts in behavioural trade-offs (e.g. foraging activity versus predator

487   vigilance) typically observed in shoaling fish (Hintz & Lonzarich, 2018). Under ocean

488   warming, bolder temperate fish may benefit from increased prey access (Weetman *et al.,* 1999).

489   However, boldness benefits may be dampened by decreased lateralisation and altered shoaling

490   dynamics (this study), concurrently increasing predation mortality risk and decreasing foraging

491   efficiency (Miletto Petrazzini *et al.,* 2020). Such decreases in the startle escape distance and

492   altered shoaling dynamics of temperate fish as a consequence of novel species interaction and

493   climate change are likely to reduce the performance of shoaling temperate fishes, possibly

494   speeding up range contraction rates at the trailing range edges of temperate residents and

495   lowering their overall abundance in temperate tropicalisation hotspots.

496   The dampening of right-side bias of co-shoaling tropical and temperate fish under ocean

497   acidification may have negative ecological implications on species performance. Our

498   lateralisation results align with previous work showing a decrease from right-side bias relative

499   lateralisation in fish exposed to ocean acidification (Domenici *et al.,* 2014). Strong relative

500   lateralisation is more common in gregarious species, as lateralisation can provide benefits

501   relating to cognition (Bibost & Brown, 2014), shoal cohesion (Bisazza & Dadda, 2005; Bibost

502   & Brown, 2013), and escape performance (Dadda *et al.,* 2010). We assume that the relative

503   lateralisation pattern detected in control groups is representative of wild populations of tropical

504   vagrants and temperate co-shoaling fish. Thus, ocean acidification will likely diminish any

505   advantage provided by relative lateralisation to gregarious tropical and temperate fish.

506   Relative lateralisation was not repeatable at the individual level yet on average (i.e.

507   across individuals) did not change over time (4 weeks). Our findings suggest fish populations

508   can maintain side bias across extended periods (days to weeks), irrespective of individuals

509   possessing some degree of plasticity in their laterality responses (Stamps *et al.,* 2012). In an

510   ecological context, the predictability of lateralised behaviours can be ecologically detrimental

511   to the fitness of shoaling fishes (Vallortigara & Rogers, 2005). Reduced predictability of

512   predator-escape behaviours through variability in individual lateralisation responses within a

513   population provides ecological benefits in predator-prey encounters. Plasticity of lateralisation

514   might be an artefact of the evolutionary consequences of gregarious species responding to

515   predation due to the costs associated with predictable and strongly lateralised behaviours

516   (Chivers *et al.,* 2016). The plasticity of lateralised behaviour may provide ecological benefits

517   to gregarious species through intra-individual variability (Stamps *et al.,* 2012) and may account

518   for the non-significant individual repeatability we detected. For example, individual fish in a

519   shoal might reduce the predictability of their lateralised behaviours through intra-individual

520   variation whilst the collective shoal maintains relative side bias over time, supported by our

521   non-significant time effects detected in our relative lateralisation results. Plasticity in laterality

522   can enhance species fitness and concurrently maintain ecological advantages attributed to

523   relative laterality (Bibost & Brown, 2014; Bisazza & Dadda, 2005; Dadda *et al.,* 2010). Our

524   findings suggest that relative lateralisation and how this is altered by ocean acidification is

525   repeatable over time despite intra-individual variability within lateralised populations.

526   Novel shoaling interactions between tropical vagrants and temperate natives can

527   diminish shoal dynamics irrespective of climatic stressors. Here we show that in mixed

528   tropical-temperate species pairs, shoal cohesion and amplitude/oscillation frequency of inter-

529   individual distances increased compared to temperate-only pairs. Slower oscillation

530   frequencies may indicate destabilisation of the trade-offs between foraging and predator

23

vigilance observed in single-species shoals (Miller & Gerlai, 2007; 2008). Periodic changes in the inclination of vagrants to match a temperate shoal mate's orientation and vice versa (Partridge & Pitcher, 1980; Partridge, 1981) may also contribute to slower oscillations. Hence, due to the historical unfamiliarity of two species with each other, orientation alignments take longer, leading to a slower information transfer time between mixed-species pair mates, inhibiting the reactivity and responsiveness of the collective mixed-species shoal during critical events. The larger shoaling amplitude observed in mixed-species shoals may be explained by the species (historical) unfamiliarity and account for unfamiliar shoal behaviour compared to the homogenous behaviour characteristic of highly cohesive and polarised single-species shoals (Miller & Gerlai, 2008). Slower oscillation frequency, lower shoal cohesion, greater amplitude, and shorter startle response distance in mixed-species shoals compared to temperate shoals implicates that anti-predator and shoaling performance of temperate species can degrade in novel shoal compositions due to the indirect effect of species range extensions.

A limitation of our study is that we tested the shoaling dynamics of fish pairs. Group size, social and physical factors can alter group dynamics and structure (Krause, 1993; Partridge, 1980). While fish pairs can be considered a shoal (Partridge, 1980), group size and tropical-temperate shoal composition may alter shoal-level responses to climate change (Coni *et al.,* 2021a). To support and generalise our findings, we suggest future climate experiments focus on scaling up from pairs to larger shoals.

Under current climate conditions, novel species interactions could concurrently boost the growth of tropical vagrants (Smith *et al.,* 2018), restrict vagrant foraging performance (Coni *et al.,* 2021a), and reduce the shoaling performance of temperate fish in mixed-species shoals (this study). Under future climate conditions, however, lower temperate and tropical fish fitness may arise through the degradation of relative lateralisation, shoaling and anti-predator performance (Figure 4). Ocean warming will provide tropical organisms with thermally

556 suitable ecosystems to invade at high latitudes, while temperate species face physiologically

557 undesirable conditions, likely driving abundance declines at their trailing range edges (Coni et

558 al. 2021a; 2021c). The rate at which temperate population levels decrease or are impacted,

559 however, may be slowed under ocean acidification by destabilising the shoaling behaviour

560 performance of tropical vagrants and depressing settlement success (reduced competition). We

561 suggest that in future oceans, reduced lateralisation, altered shoaling dynamics and decreased

562 anti-predator performance resulting from co-occurring climate effects may have negative

563 ecological implications on co-shoaling tropical and temperate fish.

564 Previous tropicalisation studies did not account for ocean acidification effects on

565 vagrant range-extension success. Most studies still focus primarily on how ocean warming

566 facilitates tropicalisation at higher latitudes, increasing tropical fish abundances and reducing

567 that of temperate fishes. Current climatic conditions and novel species interactions allow for

568 the coexistence of tropical vagrants and co-shoaling temperate fish (Coni *et al.,* 2021a) at

569 tropical vagrant cold-edge ranges due to the wide trophic niche breadth of tropical vagrants

570 (Kingsbury *et al.,* 2019), their relative dietary generalism (Monaco *et al.,* 2020), physiological

571 benefits accrued from novel tropical-temperate shoaling (Smith *et al.* 2018), and the mosaic of

572 suitable habitats found in temperate tropicalisation hotspots (Coni *et al.,* 2021c). However, the

573 coexistence of interacting gregarious temperate and tropical fish may be compromised under

574 future climatic conditions. We suggest that a temperature increase of 3–4 ⁰C coinciding with

575 ocean acidification and ensuing habitat shifts (caused by ocean acidification; Coni *et al.,* 2021c)

576 at high latitude tropicalisation hotspots could simultaneously slow tropical fish range-extension

577 and decrease temperate fish abundance, through degradation of shoaling performance across

578 novel mixed species and temperate shoals, in southeastern Australia and possibly in other

579 tropicalisation zones as well.

580

Conclusions

Ocean warming facilitates the tropicalisation of temperate ecosystems, but ocean acidification might partly negate this effect by reducing the lateralisation, shoaling dynamics and anti-predator performance of vagrant tropical fish in novel mixed-species shoals. Resident temperate species face negative implications from novel species interactions and ocean warming on their shoaling and anti-predator performance, while ocean acidification might partly reduce boldness and degrade lateralisation of shoaling temperate fishes. We conclude that the degradation of lateralisation and shoaling dynamics in novel tropical-temperate shoals by ocean acidification could slow down the rate of tropicalisation in some climate change hotspots.

Ethics Statement

This experiment was conducted according to The University of Adelaide Animal Ethics and University of Technology guidelines and permits: S-2020-13 and 2017-1117, and under New South Wales DPI Scientific Collection Permit: F94/696(A)-9.0.

Conflict of Interests

The authors acknowledge there was no conflict of interest during any part of the creation of

this research.

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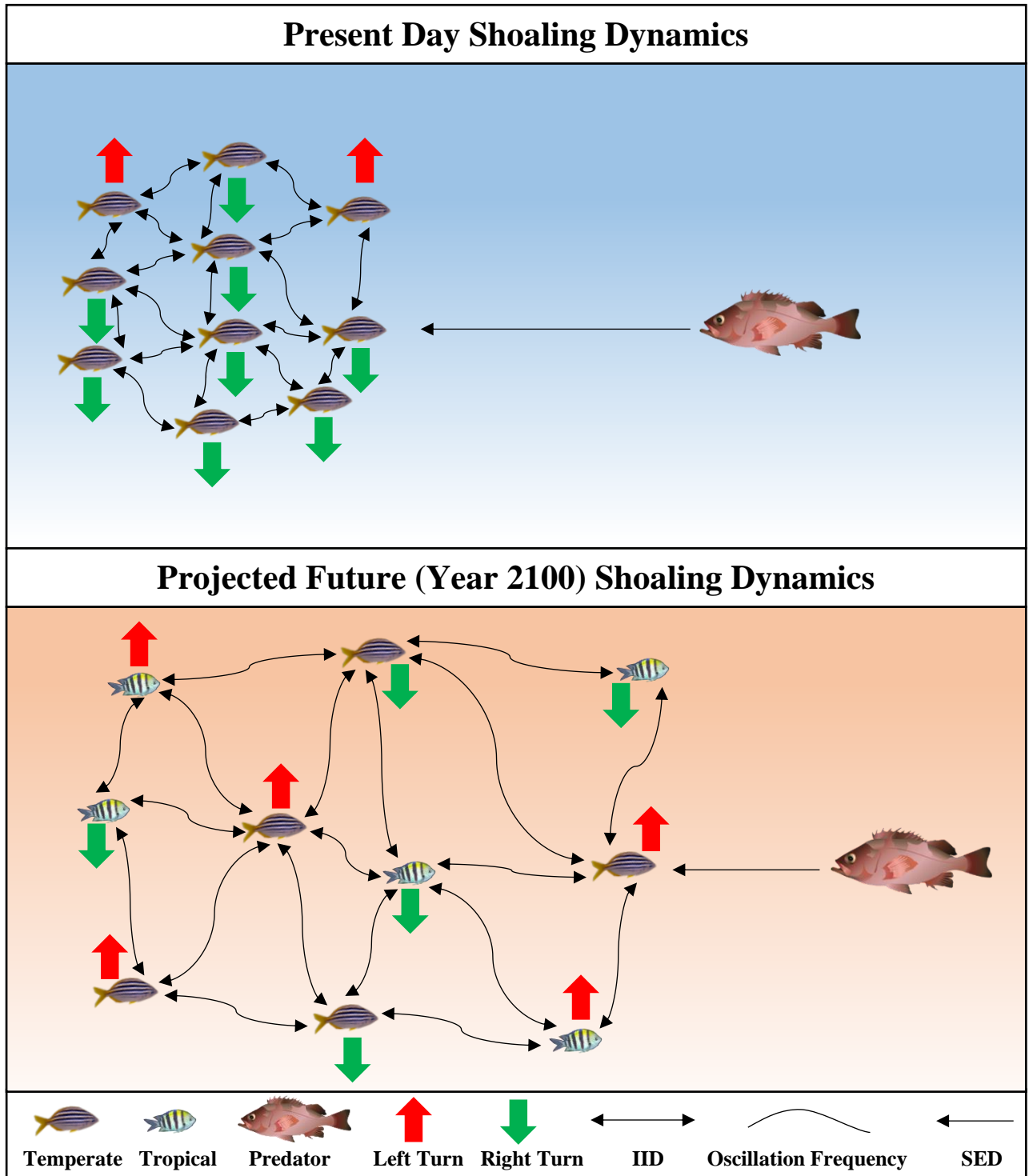
Figure 4: Conceptual diagram showing how the future combined effect of temperature and CO_2 increases can reduce the inter-individual distance (IID), relative lateralisation (L_R), and

startle escape distance (SED) of novel shoal types of local temperate and vagrant tropical fishes. Integration of tropical vagrants into residing temperate shoals slows down shoal oscillation frequency (~), increases shoal cohesion amplitude and decreases shoal cohesion compared to temperate-only shoals who exhibit higher shoal cohesion and lower amplitude faster oscillation frequency.

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