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Wilson, Alexander

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1 **Individual and collective behavior of fish subject to differing risk-level treatments with**
2 **a sympatric predator**

3

4 Alexander D M Wilson^{1,2}, Timothy M Schaerf³, Ashley J W Ward²

5

6 1 School of Biological & Marine Sciences, University of Plymouth, Devon PL4 8AA United
7 Kingdom

8 2 School of Life and Environmental Sciences, University of Sydney, Sydney, NSW, Australia

9 3 School of Science and Technology, University of New England, Armidale, NSW, Australia

10

11 Corresponding author: Alexander Wilson: alexander.wilson@plymouth.ac.uk

12

13 **ORCID iD**

14 **Wilson:** 0000-0002-7696-1671

15 **Schaerf:** 0000-0001-6642-8374

16 **Ward:** 0000-0003-0842-533X

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18 **Declarations**

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25 approved by the University of Sydney's animal ethics committee, 2016/1077.

26 **Consent for Publication:** All authors consent to publication.

27 **Significant statement:** The ability to adapt behavior according to ambient risk is vital to the
28 success of many organisms. However, risk is unpredictable across time/space, representing a
29 research challenge. We use five treatments, varying from low to high threat, to present a
30 detailed analysis of how fishes, individually and collectively, adapt their behavior to perceived
31 predation threat. We show that fish produce both quantitative and qualitative graded changes
32 in their behavior as a function of proximate threat levels.

33 **ABSTRACT**

34 The ability to adapt behavior according to context is vital to the success of many organisms,
35 particularly when it comes to mitigating risk, across time and space, in unpredictable
36 environments. While many studies have examined the role of predation threat in shaping
37 behavior, very few have quantitatively investigated prey responses across different elements of
38 perceived risk or ecological contexts. Here, we present a detailed analysis of how mosquitofish
39 (*Gambusia holbrooki*) adapt their individual and collective behavior in five experimental
40 treatments designed to replicate different core elements of predator threat. As risk increased,
41 within and across treatments, fish decreased movements across key regions of their
42 environment, swimming speed and nearest neighbour distances, reflecting a threat-sensitive
43 approach. Furthermore, movement predictability also decreased when fish occupied higher risk
44 areas. Within and between treatments, increased risk shaped the local interactions among fish.
45 Local density, group polarisation, and alignment of near neighbours increased, while patterns
46 of local density differed according to whether the fish were in a preferred (region of low
47 perceived risk) or non-preferred (region of high perceived risk) area. Groups in non-preferred
48 areas became elongated, suggesting more rapid locomotion. The rate of turning response and
49 changes in speed relative to the positions of near neighbours increased as a function of risk in
50 safer areas but became less coordinated in areas and treatments associated with higher risk.
51 Importantly, the interactions between fish under the greatest risk also showed a novel
52 qualitative shift in patterns, with fish prioritising group cohesion over group order. Taken
53 together, these results demonstrate the considerable flexibility of fish to adapt according to risk,
54 producing both quantitative and qualitative changes in behavior as a function of proximate
55 threat levels.

56

57 **KEYWORDS:** Predator-Prey, Threat, Behavioral flexibility, Shoaling, Collective Movement

58 Introduction

59

60 The ability to adapt behavior according to context is critical for survival. A key element of this,
61 central to the behavioral repertoire of prey animals, involves gauging and responding
62 appropriately to the level of ambient threat. However, while predation threat is ubiquitous for
63 many animals, the degree or severity of threat may vary considerably in both time and space
64 (Lima and Dill 1990; Kohl et al. 2018; Gaynor et al. 2019). In order to determine the proximate
65 threat level, prey animals are known to assess diverse sources of ecological information,
66 including predator behavior and the chemical cues that they produce (Ward and Mehner 2010;
67 Kent et al. 2019a; Crane et al. 2020). Such assessments enable prey to respond adaptively to
68 the level of risk, tailoring their behavior to context and improving overall fitness by reducing
69 the costs of anti-predator behaviors (Helfman 1989; Adamo et al. 2017).

70

71 A broad range of behavioral responses to predation risk have been documented, including
72 increased vigilance, changes (often reductions) in activity, and increases in refuge use (Krause
73 et al. 1998; Beauchamp 2007; Creel et al. 2014; Schaerf et al. 2017). Group-living animals
74 show additional, characteristic responses to risk, particularly increases in group cohesion that
75 result from group members decreasing their nearest neighbour distances (Hoare et al. 2004).
76 Indeed, group-living is thought to represent an adaptive means of mitigating risk (Krause and
77 Ruxton 2002; Ward and Webster 2016). Collective behavior, which is typically associated with
78 synchronous and coherent group movement, is often most pronounced in the presence of risk
79 (Procaccini et al. 2011). In this latter case, group-level behavioral patterns emerge as a result
80 of local interactions between neighbouring animals (Sumpter 2010). In experimental studies,
81 descriptions of these local interactions have followed the predictions of theoretical models
82 based on principles of self-organization, where group members adjust their position, speed, and
83 heading direction relative to those of near neighbours (Aoki 1982; Reynolds 1987; Romey
84 1996; Couzin and Krause 2003; Lukeman et al. 2010; Herbert-Read et al. 2011). These ‘rules
85 of interaction’ adapt to context. For instance, tetras (*Pristella maxillaris*) in an annular arena
86 decreased their responsiveness to other group-members when they detected chemical cues
87 indicating the presence of food, and increased their responsiveness when they detected alarm
88 cues, in each case resulting in different patterns of global group structure (Schaerf et al. 2017).
89 Nonetheless, a criticism of experimental studies in this field is that they tend to examine threat
90 in a binary manner, usually based on the presence or absence of risk. Further, targeted efforts
91 toward a more holistic approach that includes both individual and collective group responses

92 to graded levels of threat across ecological contexts are almost completely lacking in the
93 current literature. For while it is known that animals adapt their individual and collective
94 behavior according to context, how animals adapt the underlying mechanisms that drive these
95 patterns is poorly understood and represents a significant research need.

96

97 Here, we present a broad-ranging and comprehensive analysis of how prey animals
98 (mosquitofish, *Gambusia holbrooki*) adapt their behavior across five different ecological
99 treatments, each of which represent a different graded level of potential predation threat.
100 Mosquitofish are primarily surface-dwelling, are facultatively social (being found singly but
101 more typically in groups) (Pyke 2005; Pyke 2008 and references therein), showing strong
102 individual and collective responses to predation threat (Kent et al. 2019a). We assessed their
103 individual behavior in relation to their patterns of movement as well as their interactions with
104 group mates. We predicted that the level of perceived risk would be higher across increasing
105 treatment number (i.e. treatment 1 to treatment 5) and that fish both individually and
106 collectively would alter their behavior in a threat-sensitive manner (e.g. reduce activity level
107 and form closer more coherent shoals) to reduce individual risk of predation. We further
108 predicted that these changes would be more apparent in (a) sections of the arena within
109 treatments (refugia vs open water) as well as (b), between treatments as perceived risk level
110 increased. To date, no studies that we are aware of have examined how individual and
111 collective motion interactions vary across ecological contexts or along a predation risk gradient
112 across treatments in a structured manner.

113

114 **Materials and Methods**

115 *Experimental subjects and protocol*

116 Mosquitofish (*G. holbrooki*, total body length: range 23 ± 2 mm) were wild caught using hand
117 nets from Manly Dam Reservoir (33° 46'38" S; 151° 15'3" E), New South Wales, Australia in
118 July 2016. Following collection, all fish were held communally in large 150 L aquaria for ~ 2
119 weeks prior to experimentation in the animal holding rooms at the University of Sydney.
120 During holding, fish were kept under a 12 h:12 h (light:dark) photoperiod and fed commercial
121 flake food (Nutrafin Max) to satiation once daily. All fish were fed at 16:00 hrs the day before
122 trials were to begin, and experimental trials began at 09:00 hrs the following morning.

123 The experimental arena was rectangular in shape (internal dimensions: 120 x 30 x 30
124 cm, L x W x D) consisting of two shallow platform zones separated by a central deeper open-
125 water zone (Fig. 1). The platforms (22 x 30 x 15 cm (L x W x D) were positioned so that the

126 central zone also measured 22 x 30 cm. The arena was filled to a depth of 17 cm with aged,
127 conditioned tap water at the same temperature as the main holding tanks (25 °C); water was
128 replaced between trials. The water depth over the platforms (2cm) reflect where these fish can
129 be found in nature in the presence of sympatric predators, including their location of capture,
130 and thus is indicative of a shallow water refugia from piscivorous predators. In all trials
131 mosquitofish had access to the predator-free areas represented by the platforms with entry to
132 the central, potentially risky, zone being volitional (Fig. 1). The entire experimental arena,
133 including platforms, was built using 1 cm thick, white, opaque acrylic plastic. Both platforms
134 were fitted with a 30cm high piece of white acrylic plastic on the back to prevent fish from
135 moving behind the platforms during trials.

136

137 At the onset of an experimental trial, groups of 8 novel mosquitofish were selected haphazardly
138 from holding aquaria and transferred via a dipnet and a small bucket to the right platform of
139 the experimental arena. Each treatment consisted of 10-13 replicates of 8 fish (i.e. T1 = 13, T2
140 =10, T3 = 12, T4 =11, T5=13 trials). Mosquitofish within each group replicate were collected
141 from the same holding aquaria and were not re-used in the study to eliminate risk of bias based
142 on previous experience with the arena, predator, or experimental procedure. Only females were
143 used in the experiment since the presence of males can confound group dynamics due to sexual
144 harassment (e.g. Dadda et al. 2005; Agrillo et al. 2006). Fish were given fifteen minutes to
145 adjust to the experimental environment prior to the start of a given trial (Pazmino et al. 2020),
146 during which time they could move around freely. We recorded each trial for a total of 20 min
147 using a Canon Legria HF G30 camera positioned 1.5 m above the experimental arena and
148 filming at 25 frames per second at a resolution of 1080p. Our experimental protocol consisted
149 of 5 different treatments representing graded levels of increasing predation threat including (a)
150 control - empty arena (T1), (b) predator visually absent – chemical/ olfactory cues of predator
151 satiated on dead mosquitofish present (T2), (c) predator present – satiated on commercial pellet
152 fish food (T3), (d) predator present – satiated on dead mosquitofish (T4), (e) predator present
153 – hungry (T5). For those treatments where a predator was required, we used a locally sympatric
154 predator, the jade perch (*Scortum barcoo*, total length 128 mm) obtained from a commercial
155 supplier. As an aspect of experimental design and for consistency, the same perch was used for
156 all predator trials to reduce unintended biases associated with using predators of different size,
157 appearance (mouth, eye dimensions), chemical profile or behavior. As we were only interested
158 in the response of naïve fish to the presence of a predator, and not the predator itself, it was
159 important to minimize variability in this regard. During trials, the perch was consistently active

160 and would pursue mosquitofish, however, no mosquitofish were captured during trials. To
161 account for any predator effects in trials we quantified the number of lunges that the predator
162 made in each trial for treatments T3-T5. A lunge was defined as occurring when the predator
163 was oriented at a mosquitofish and moved a quarter or greater of its body length toward that
164 prey fish in $<1/10$ of a second (2 frames when filming at 25 fps). In general T3 and T4 had
165 fewer lunges than T5, but there were no significant differences between T3 and T4 (SI S2.2,
166 Fig. S2, Tables S2-S6).

167 In treatments T4 and T5, the predator was added to the central open water zone 90 min
168 prior to mosquitofish being added with the exception of the predator cues treatment (T2) where
169 it was placed in one of the outer chambers for the same duration (Fig. 1c). In this case, the
170 predator, while not visible to the focal fish, was detectable via olfactory cues that were present
171 throughout the arena via passive diffusion over time. Both the afternoon prior, and
172 immediately before the start of a given predator trial, the predator was fed either dead
173 mosquitofish (T2, T4) or commercial pellets (T3) (New Life Spectrum). For the hungry
174 predator treatment (T5) the jade perch was not fed at either time interval but was fed
175 immediately following the day's experimental trials. Each treatment was conducted on a
176 separate day to prevent confounding variables, but treatment order was randomized.

177

178

179

180 *Data Collection*

181

182 While the fish were free to move throughout the arena within each treatment, fish tended to
183 prefer either the central zone, or the platforms, according to whether there was a predator in
184 the central zone (respective analyses presented below). Consequently, in treatments T3, T4 and
185 T5, mosquitofish showed a preference for the platforms, while in T1 and T2, they showed a
186 preference for the central zone. This corresponds with the intended function of the shallow
187 water platforms acting as refugia. Since our initial observations suggested that fish showed
188 different behavior according to whether they were in their preferred zone, or in their non-
189 preferred zone, we incorporated 'zone preference' ('Preferred' or 'Non-Preferred') as a factor
190 in subsequent analytical models as we consider this to be more biologically-relevant than 'zone
191 type' ('Platform' or 'Central'). However, for the sake of completeness, we carried out parallel
192 analyses using zone type as an alternative, and the results of these are included in the
193 Supplementary Information.

194

195 Post experimental trials, time series of each individual's coordinates, $(x(t), y(t))$ in millimetres
196 (body centroid), within each trial were obtained via automatic visual tracking as applied by
197 CTrax (Branson et al. 2009), with any ambiguities relating to the trajectories resolved using
198 the associated *fixerrors* GUI in MATLAB. At 25 frames per second for a duration of 20
199 minutes each time series thus consisted of 30000 discrete time steps of coordinate data. The
200 complete data set for this study consists of 8 such trajectories (one for each individual) for
201 each of 13 trials for the T1 (control) treatment, 10 trials for the T2 (chemical cue) treatment,
202 12 trials for the T3 (pellet-fed predator) treatment, 11 trials for the T4 (mosquitofish-fed
203 predator) treatment, and 13 trials for the T5 (hungry predator) treatment. In addition, we
204 recorded the coordinates of the corners of each platform and the central zone for each
205 experimental trial. We used the coordinates of the corners of each region of the tank
206 (platforms or central zone), the time series of each fish's coordinates and MATLAB's built-in
207 *inpolygon* function to identify the zone occupied by each fish for each tracked frame. For the
208 reporting of data and model predictions in figures and tables the units of distance and time
209 were converted to millimetres (where 1 mm was equal to 1.3 pixels) and seconds (where 1 s
210 was equal to 25 frames), respectively. To minimize observer bias, blinded methods were use
211 when all behavioral data were recorded and/or analyzed.

212

213 *Statistical Analyses*

214

215 All data were analysed using R version 3.4.3 (R Development Core Team 2011) and
216 MATLAB. Assumptions of normality were assessed using Q-Q plots and Shapiro-Wilk tests.
217 Data for time spent on platforms violated the assumptions, so we used Wilcox's Robust
218 ANOVA in the WSR2 package and the *lincon* function in R to provide post-hoc comparisons
219 (within the *lincon* function, confidence intervals are automatically adjusted to control
220 familywise error). Other individual-based analyses (number of zone changes; mean speed;
221 mean nearest neighbour distance; mean mutual information and mean entropy rate) were
222 analysed using linear mixed effects models (*lmer*), with treatment and zone preference (and
223 their interaction) as the fixed factors and individual ID nested within group as our random
224 effect. Post-hoc comparisons were generated using the *glht* function in the *multcomp* package
225 for R; these post-hoc comparisons also accounted for familywise error (Hothorn et al. 2008).

226

227 We determined the time spent by each individual in each form of zone (platform or central
228 zone) by counting the number of video frames that each individual occupied each zone during
229 each experimental trial, with the aid of the individual and zone-defining coordinate data
230 described above. Inferential analysis was applied to the proportion of time that individuals
231 spent on the platforms. We broadly quantified the activity level of individuals based on the
232 number of movements between each of the distinct zones in the tank throughout each trial.

233

234 The trajectory data was used to estimate the speed and nearest neighbour distances for each
235 individual at each time step, as detailed in section S1.3 of the Supplementary Information (SI,
236 equations (2) and (4)). In addition, we calculated the group polarisation to examine the
237 instantaneous degree of alignment in directions of motion of group members (see for example
238 (e.g. Vicsek et al. 1995; Couzin et al. 2002; Tunstrøm et al. 2013); further details are included
239 in section S1.3 of the SI.

240

241 We refined the methods described in Schaerf et al (2017) to use the information theory
242 measures of mutual information and entropy rate associated with individual changes in
243 position and velocity to examine the predictability of movements across different treatments
244 and zones within the experimental arena. In the context of mutual information, here
245 predictability refers to how much a change in position or velocity of an individual over a brief
246 observation period (19 to 21 frames/0.76 to 0.84 seconds) informs an observer about the
247 change in position or velocity over the next (or previous) observation period. Relatively low
248 mutual information values suggest that an individual gives away little about its next move
249 based on its current movement, whereas higher values suggest that an individual telegraphs
250 its next move more clearly. The entropy rate is associated with the variety of movement
251 choices (in terms of changes in position or velocity) exhibited by an individual, with a higher
252 entropy rate associated with a greater range of movement choices being employed. Both
253 measures thus can be used to examine the moment-to-moment predictability of an
254 individual's movement, with the movements of an individual that is less predictable
255 characterised by relatively lower mutual information and relatively higher entropy rate. See
256 section S1.4 of the SI for full details of the calculation of both measures.

257

258 We applied the force-mapping approach as detailed in Schaerf et al. (2017), and similar to that
259 applied in other studies (see Herbert-Read et al. 2011, 2017; Katz et al. 2011; Zienkiewicz et
260 al. 2018; Escobedo et al. 2020) to examine how individuals adjusted their velocity on average

261 as a function of the relative coordinates of their group mates, in component form via changes
262 in speed and direction of motion. Such calculations are targeted at inferring the underlying
263 rules of interaction that drive collective motion, in a form equivalent to that hypothesised and
264 applied in theoretical models (Couzin et al. 2002; D’Orsogna et al. 2006). The calculations
265 applied here are reliable in terms of their ability to reveal the presence of repulsion (collision
266 avoidance) and attraction (group joining/cohesion) interaction rules, but may not accurately
267 reflect the sizes of the regions over which such rules are applied by individuals, nor the
268 associated magnitudes of associated changes in speed or direction (see Escobedo et al. 2020
269 for further discussion on other potential issues with the approach; Mudaliar and Schaerf 2020).
270 The graphs that result from the force-mapping, referred to as force-maps, are constructed in a
271 consistent frame of reference that places each individual at the origin of the coordinate system,
272 with this “focal” individual’s direction of motion aligned here with the positive x -axis. In
273 interrelated calculations, we examined local group structure in the same relative coordinate
274 system via graphs that illustrate the relative frequency that neighbours occupy given relative
275 (x, y) coordinates (as in Lukeman et al. 2010; Katz et al. 2011; Schaerf et al. 2017), and
276 statistics of the relative alignment of neighbours with the focal individual (Davis et al. 2017;
277 Schaerf et al. 2017, 2021; Ward et al. 2017). We applied the mean absolute difference
278 randomisation test described in Schaerf et al. (2021) modified for across-group comparisons
279 (see Encel et al. 2021), to identify statistical differences between equivalent force-maps and
280 local structure plots across treatments. Significance levels for the randomisation tests were
281 corrected according to the Holm-Bonferroni method to take into account the multiple pairwise
282 comparisons made across treatments for each measure (Holm 1979). We note that there are
283 other viable methods for inferring collective motion interactions from trajectory data that are
284 potentially superior to the force-map approach applied here, especially in terms of decoupling
285 interaction rules (Calovi et al. 2018; Escobedo et al. 2020) and handling more independent
286 variables and interactions with multiple neighbours (Heras et al. 2019), but these have yet to
287 be coupled with a statistical significance test. For completeness, full details of the calculation
288 of the force-maps, local structure, and the associated randomisation tests are provided in section
289 S1.5 of the SI.

290

291 **RESULTS**

292

293 *Individual Behavior*

294 Measures of space use, zone changes, speed, neighbour distances and entropy showed
295 consistent patterns according to levels of perceived risk across treatments (see below and
296 Supplementary Information).

297

298 *Distribution and zone changes within the arena*

299 Time spent in platform zones varied according to treatment (Wilcox Robust ANOVA: $F_{4,14.33}$
300 = 188.68, $p < 0.001$, explanatory measure of effect size: 0.78). Fish in treatments where a
301 predator was visibly present (T3, T4, T5) spent significantly more time in the platform zones
302 than treatments where a predator was not visibly present (T1, T2) (Fig. S1, Table S1). All
303 treatments were significantly different from each other, with the exception of satiated predator
304 treatments (T3 and T4), which indicates that mosquitofish distribute themselves according to
305 their perceptions of risk in the arena. Furthermore, it suggests that the level of risk perceived
306 by mosquitofish does not differ when a satiated predator is visible, irrespective of the
307 chemical/olfactory cues expressed by the predator. The proportion of time spent over the
308 platform regions was approximately the same as the proportion of the arena occupied by the
309 platforms (two thirds of the arena) for T3 and T4 (Fig. S1). Such distribution of the fish in T3
310 and T4 may occur if individuals occupied random coordinates in the arena at every instant, but
311 here was very likely driven by the presence of the predator, given the clear preference for the
312 central zone in the control case (T1).

313

314 The numbers of movements between zones also varied across treatments ($X^2_{[4]} = 77.41$, $P <$
315 0.001). Fish paired with either predator olfactory cues (T2) or a hungry predator (T5) showed
316 similar, reduced levels of movement between zones that were significantly lower than that in
317 control trials (T1) or in treatments with satiated predators, regardless of diet (T3, T4) (Fig. S3,
318 Table S7). The reduced movements between the zones in the T2 and T5 treatments were
319 correlated with much greater proportions of time spent in a single form of zone (the central
320 region in the case of T2, and the platforms for T5; see Fig. S1).

321

322 *Individual Speed, Nearest Neighbour Distances and Polarisation*

323 There was a significant interaction between treatment and zone in relation to mean speed, mean
324 nearest neighbour distance (NND), and polarisation (Fig. 2, Table 1; see also Fig S4 and Table
325 S8). Generally, in non-preferred zones fish showed greater speed as well as increased
326 polarisation and greater NNDs as compared to the preferred zones. This pattern is likely a

327 response to the perceptions of risk in the non-preferred zones, in particular travelling at a
328 greater speed, which may result in increased spacing between individuals in the short term.

329

330 *Mutual Information and Entropy Rate*

331 There was a significant interaction between treatment and zone in entropy rates and mutual
332 information associated with changes in displacement/position and changes in velocity (see Fig.
333 3, S5, Table 2). Fish in non-preferred zones showed greater entropy rates and decreased mutual
334 information than when they were in preferred zones, indicating that their movement is less
335 predictable when risk is elevated (see section S2.5 in the SI for further details).

336

337 *Interactions Between Individuals*

338 Overall, animals changed the way in which they interacted with near neighbours according to
339 the level of perceived predation risk, and this was correlated with adaptive context-dependent
340 changes in group-level patterns. Specifically, in treatments with the highest levels of perceived
341 risk, T2 and T5, fish showed the most cohesive grouping behavior, with the highest
342 concentration of groupmates in close proximity as compared to fish in other treatments of lower
343 perceived risk (T1, T3, T4) (Fig. 4). Overall, the local density distributions of fish differed
344 significantly between almost all possible pairwise treatment comparisons, with the exception
345 of T3 and T4 (see Table S9). In addition, we found similar dramatic changes in the basic
346 patterns of grouping structure when comparing preferred versus non-preferred zones across
347 treatments. In preferred zones, groups were more cohesive than those in non-preferred zones,
348 correlated with an observed tendency of fish to move aimlessly in their preferred areas of
349 habitat. Furthermore, group morphology appears to differ between the zones, with those in non-
350 preferred zones being more elongated, which likely reflects the greater speed of the animals in
351 non-preferred zones.

352

353 In addition, we found differences in patterns of social responsiveness in the magnitude of
354 changes in speed as well as turning responses of focal fish to near neighbours, as a function of
355 the relative coordinates of these neighbours, across treatments (Fig. 5, 6). Pairwise comparisons
356 revealed significant differences between treatments in the mean changes in speed of the fish as
357 a function of the relative coordinates of their group mates (see Table S10). Similarly, there
358 were significant differences between treatments in the mean changes in direction of the
359 mosquitofish as a function of the relative coordinates of group mates (See Table S11). There
360 were significant differences in both these parameters between the control treatment, T1, and

361 the higher risk treatment, T5, but no significant differences between the T1 and T2 treatments,
362 or between T3 and T4. Fish in higher risk treatments (particularly T5) exhibited higher
363 magnitude turning responses toward near neighbours than fish in lower risk treatments and
364 greater differences in their responses to near neighbours between the preferred and non-
365 preferred zones.

366

367 Focal fish tended to align themselves most closely with group mates either in front or behind
368 them across treatments. Specifically, focal fish were most aligned on average with neighbours
369 three to four body lengths in front and behind. Pairwise comparisons between treatments of
370 local alignment patterns of mosquitofish as a function of their position relative to a focal
371 individual revealed significant differences between all treatments, with the exceptions of T1
372 and T2, and T3 and T4 (see Table S12). Alignment was strongest in non-preferred versus
373 preferred zones (Fig. 7). Thus, these results are consistent with those from earlier in the study
374 (Table 1, Fig.2). Individuals in non-preferred zones, and particularly those in higher risk
375 treatments, showed a change in collective motion from low polarity, non-coherent movements
376 in the preferred zones, to rapid collective movements as they crossed the non-preferred zone,
377 characterised by faster movement and stronger alignment in less tightly packed groups. In
378 addition, the groups in the non-preferred zone tended to be elongated in the statistical
379 distribution of near neighbours and featured a less intense turning response to group-mates at
380 either side of themselves.

381

382 **DISCUSSION**

383 Our results provide a novel, broad-ranging and detailed analysis of how fish respond to risk in
384 a graded manner both individually and collectively. In control and low risk treatments, the
385 observed patterns were similar to previous studies in other species of shoaling fishes in benign
386 conditions, including mosquitofish (Herbert-Read et al. 2011). Changes in the local interactions
387 between group members coincided with changes in global properties, and patterns of space use
388 were mechanistically tied to the presence and status of ambient predator olfactory cues within
389 and across treatments. However, importantly, in situations of unseen threat (T2) or immediate
390 threat (T5), we found that fish not only quantitatively but also qualitatively shift the way that
391 they interact with near neighbours relative to other treatments. Such previous unobserved
392 context-dependent qualitative changes in behavior in response to imminent threat may be
393 ecologically-important and widespread across taxa, warranting further investigation.

394

395 Predation threat, particularly the imminent threat provided by the proximity of a hungry
396 predator, produced wide-ranging effects on the behavior of groups of mosquitofish.
397 Distribution patterns were associated with differences in ambient perceived predation threat.
398 Fish in treatments with visible predator presence showed a preference for spending time on
399 platform areas, while fish without a visible predator presence preferred the open water zone.
400 From an ecological standpoint, this result matches the life history and environmental
401 preferences of mosquitofish in the wild as this species is subject to heavy predation in the wild
402 from an array of piscivorous fish and birds (Pyke 2008). In the presence of aquatic predators,
403 shallow waters provide refuge, while in contrast, without an identifiable source of threat, such
404 as with the control or olfactory cues treatments, deeper water potentially reduces the chance of
405 predation by increasing the available avenues of potential escape from both aerial and aquatic
406 sources. In the current experiment, predation risk also influenced behavior between treatments
407 such that when a predator was visibly present, mosquitofish appeared to be more threat
408 sensitive, showing reduced activity measured via movements between zones of the arena,
409 slower movement and grouping more cohesively. When comparing within treatments, we see
410 a largely similar pattern. Generally, fish in non-preferred zones, likely those they perceived to
411 be more risky, showed increased mean swimming speeds and lower nearest neighbour
412 distances than in preferred zones (Anholt et al. 2000; Hoare et al. 2004).

413

414 The predictability of fish movements, measured both in terms of mutual information and
415 entropy rate, was strongly affected both by treatment and by the zone of the arena the fish were
416 in. Interestingly, fish in the treatments where risk was apparently perceived to be greatest (T2
417 and T5), showed the greatest predictability while in the preferred zones, but shifted strongly in
418 non-preferred zones to be the least predictable among all treatments. Such reductions in the
419 predictability of movement are thought to reduce the likelihood of capture by an attacking
420 predator (Hu et al. 2015; Herbert-Read et al. 2017; Schaerf et al. 2017). Why might fish in the
421 riskier treatments be the most predictable in their preferred zones? A possible explanation for
422 this is that while increased entropy in response to predation threat may mitigate some aspects
423 of predation risk, it can also interfere with group cohesion and the ability of fish to synchronize
424 their behavior with near neighbours. As such, increased mutual information may reflect the
425 adaptive benefits for such coordinated actions prior to engaging in moves across the non-
426 preferred zone, or to dealing with an imminent threat more generally (Ward and Webster 2016).
427 Understanding this functional trade-off in terms of collective phenomena and behavioral
428 predictability is a key area of current and future research need.

429

430 The remarkable flexibility of collective responses to threat are exemplified by clear differences
431 in responses to near neighbours across all treatments and between zones in the arena (Bode et
432 al. 2010; Herbert-Read et al. 2017; Schaerf et al. 2017). Groups of fish showed greatest
433 cohesion in treatments most associated with danger but exhibited different patterns according
434 to the zone in which the fish were located. Fish switched from broadly circular groups with a
435 peak occurrence of neighbours alongside a focal while in their preferred zones to elongated
436 travelling groups with peak occurrence in front and behind while making collective movements
437 across riskier portions of their environment (Ward et al. 2017; Kent et al. 2019b). This is
438 supported by patterns of alignment, which was strongest in the presence of a predator and in
439 non-preferred zones, particularly T5. In addition, the area immediately surrounding the focal
440 fish, often interpreted as the repulsion zone, was markedly smaller in the riskiest treatments,
441 characteristic of individuals condensing into tight groups under threat (Hamilton 1971).

442

443 Fish adjusted their speed relative to near neighbours in a manner similar to that observed in
444 previous studies (Herbert-Read et al. 2011, 2017; Jolles et al. 2017; Kent et al. 2019b), avoiding
445 collisions at close proximity and either speeding up to catch those further in front, or slowing
446 to allow those behind to join them. However, a significant difference between this and any
447 previous study is seen in T5, where focal individuals in their preferred zone increased their
448 speed rapidly when their partners were very close, irrespective of if their partners were in front
449 or behind, a result that may be allied with the reduction in the repulsion zone. At greater
450 distances in this same zone and treatment, focal individuals slowed down irrespective of
451 whether far neighbours were in front or behind. In contrast, when they were in the central zone,
452 speed regulation relative to the position of near neighbours showed no clear pattern, aside from
453 large magnitude changes of speed.

454

455 In their preferred zones, fish showed strong turning responses toward near neighbours, and the
456 strength of this response tended to increase according to the degree of risk associated with the
457 treatment. While some of these characteristics align with other work, (Herbert-Read et al. 2011;
458 Schaerf et al. 2021), these highly organized patterns disappeared when focal individuals were
459 in their non-preferred zone in T2 and T5, marking a significant departure from any previous
460 studies. This, in conjunction with our findings of both changes in speed relative to near
461 neighbours and the decrease in predictability of individuals' trajectories in the non-preferred

462 zones, especially in T2 and T5, suggests that coherent patterns of collective motion break down
463 under conditions of perceived high risk.

464

465 In addition, focal individuals typically turned away from nearby conspecifics that were ahead
466 of them (within one body length), likely as a means of collision avoidance in the majority of
467 treatments, especially T3 and T4, but also T1 in their non-preferred-zone. In contrast, however,
468 in the presence of a hungry predator (T5), these patterns were reversed with focal individuals
469 turning towards partners directly in front of them, and away from partners directly behind. We
470 can only speculate why this is, but the unexpected shift in the rules of interaction may reflect a
471 change in strategy by focal individuals, minimizing risk by limiting their domain of danger and
472 maintaining the closest possible distance between themselves and near neighbours (Morrell et
473 al. 2011). However, such an unprecedented pattern needs to be explored further, particularly in
474 terms of its adaptive value and this represents a priority for future work.

475

476

477 **Figure Legends**

478

479 Fig. 1 Schematic representation of experimental arena used across risk gradient treatments.
480 The arena consisted of three primary zones as represented by a central open water zone (A)
481 flanked by two shallow platform zones (B) on either side. When a predator (a jade perch;
482 *Scortum barcoo*) was present visually (T3-T5) it was located in the central zone. Across
483 treatments, mosquitofish (*Gambusia holbrooki*) could move across the three platforms freely
484 but were prevented from reaching the outermost areas (C) beyond the platforms by an opaque
485 partition. In the predator cues treatment (T2), the predator was located in one of these two
486 outermost areas on either side of the platform zones and water (containing olfactory cues)
487 was mixed with the experimental observation area

488

489 Fig. 2 Speed, nearest neighbour distance (NND) and polarisation in preferred and non-
490 preferred zones across treatments. (a) Median speed, (b) nearest neighbour distance and (c)
491 polarisation in preferred (left panels) and non-preferred (right panels) zones in five treatments
492 across a risk gradient. Following standard conventions, the boxes within the plots extend
493 from the first (Q_1) to third (Q_3) quartiles of the data for the given measure and treatment, with
494 the median (Q_2) represented by a thicker horizontal line. The boxes encompass the
495 interquartile range (IQR) for each plot, such that $IQR = Q_3 - Q_1$. Whiskers on the plots
496 extend from Q_1 to the lower threshold (LT), given by $LT = Q_1 - 1.5IQR$, and from Q_3 to the
497 upper threshold (UT), given by $UT = Q_3 + 1.5IQR$. Outliers, points that lie below the lower
498 threshold or above the upper threshold, are represented by solid black circles

499

500 Fig. 3 Mutual information and entropy rate of fish trajectories associated with changes in
501 displacement (position) in preferred and non-preferred zones. Mutual information (left), and
502 entropy rate (right) associated with changes in displacement for 5 treatments across a risk
503 gradient (T1, black; T2, green; T3, blue; T4, magenta; T5, red). Differences across measures
504 are shown in the context of preferred (P) versus non-preferred (NP) zones. The calculations
505 used to generate these plots are detailed in the Supplementary Materials

506

507 Fig. 4 Detail of the relative frequencies, p , that group members occupied (x, y) coordinates
508 relative to the location and direction of motion of a focal individual that was located in the
509 preferred or non-preferred region of the tank across treatments T1 to T5 for $-100 \leq x \leq 100$

510 (mm), $-100 \leq y \leq 100$ (mm). In these plots, the focal individual is located at the origin, $(0, 0)$
511 , moving parallel to the positive x -axis

512

513

514 Fig. 5 Detail of the mean change in speed over time of a focal individual located in their
515 preferred on non-preferred region of the tank as a function of the relative (x, y) coordinates of
516 group mates for T1 to T5 with $-100 \leq x \leq 100$ (mm), $-100 \leq y \leq 100$ (mm). In these plots,
517 the focal individual is located at the origin, $(0, 0)$, moving parallel to the positive x -axis.

518 Redder regions on these plots indicate that the focal individual increased their speed when
519 their groupmates occupied points in these regions; bluer regions indicate that the focal
520 individual decreased their speed when groupmates occupied points in these regions

521

522 Fig. 6 Detail of the mean change in angle of motion over time of a focal individual located in
523 their preferred on non-preferred region of the tank as a function of the relative (x, y)

524 coordinates of group mates for T1 to T5 with $-100 \leq x \leq 100$ (mm), $-100 \leq y \leq 100$ (mm).

525 In these plots, the focal individual is located at the origin, $(0, 0)$, moving parallel to the
526 positive x -axis. Redder regions on these plots indicate that the focal individual turned anti-
527 clockwise when their groupmates occupied points in these regions; bluer regions indicate that
528 the focal individual turned clockwise when groupmates occupied points in these regions

529

530 Fig. 7 Detail of the average directions of motion of group members at (x, y) coordinates
531 relative to the location and direction of motion of a focal individual that was located in the
532 preferred or non-preferred region of the tank across treatments T1 to T5 for $-100 \leq x \leq 100$
533 (mm), $-100 \leq y \leq 100$ (mm). In these plots, the focal individual is located at the origin, $(0, 0)$

534 , moving parallel to the positive x -axis. Arrows point in the average direction of motion of
535 other group members located at specific (x, y) coordinates relative to the focal individual.

536 Colours in the plots represent R , a measure of the focus of the angles contained in each bin
537 used to construct the plot about the mean angle within the same bin, with possible values

538 such that $0 \leq R \leq 1$. $R = 1$ indicates perfect alignment between all angles of motion

539 contained within a bin, with lower values of R indicating greater scatter or variance for the
540 binned angles of motion

541

542

543

544 **Table 1 Output of glmms examining differences in mean speed, mean nearest neighbour**
545 **distance and mean polarisation in preferred and non-preferred zones across treatments. *P*-**
546 **values less than 0.05 are flagged with a single asterisk (*), *P*-values less than 0.01 are flagged with**
547 **two asterisks (**) and *P*-values less than 0.001 are flagged with three asterisks (***).**

548

Speed	Chisq	Df	Pr(>Chisq)
Treatment	15.972	4	0.0031 **
Zone	426.109	1	< 0.0001 ***
Treatment*Zone	325.103	4	< 0.0001 ***
Nearest Neighbour Distance			
Treatment	12.685	4	0.0129 *
Zone	329.339	1	< 0.0001 ***
Treatment*Zone	261.98	4	< 0.0001 ***
Polarisation			
Treatment	53.494	4	< 0.0001 ***
Zone	348.542	1	< 0.0001 ***
Treatment*Zone	102.314	4	< 0.0001 ***

559

560 **Table 2 Output of glmms examining difference in mutual information and entropy rate with**
 561 **respect to displacement and velocity in preferred and non-preferred experimental zones across**
 562 **treatments**

	Chisq	Df	Pr(>Chisq)
Mutual Information			
(a) Displacement			
Zone	182.672	1	<0.0001
Treatment	85.039	4	<0.0001
Zone:Treatment	121.447	4	<0.0001
(b) Velocity			
Zone	183.727	1	<0.0001
Treatment	88.399	4	<0.0001
Zone:Treatment	123.537	4	<0.0001
Entropy Rate			
(c) Displacement			
Zone	211.273	1	<0.0001
Treatment	91.759	4	<0.0001
Zone:Treatment	136.454	4	<0.0001
(d) Velocity			
Zone	214.606	1	<0.0001
Treatment	93.853	4	<0.0001
Zone:Treatment	137.690	4	<0.0001

563

564

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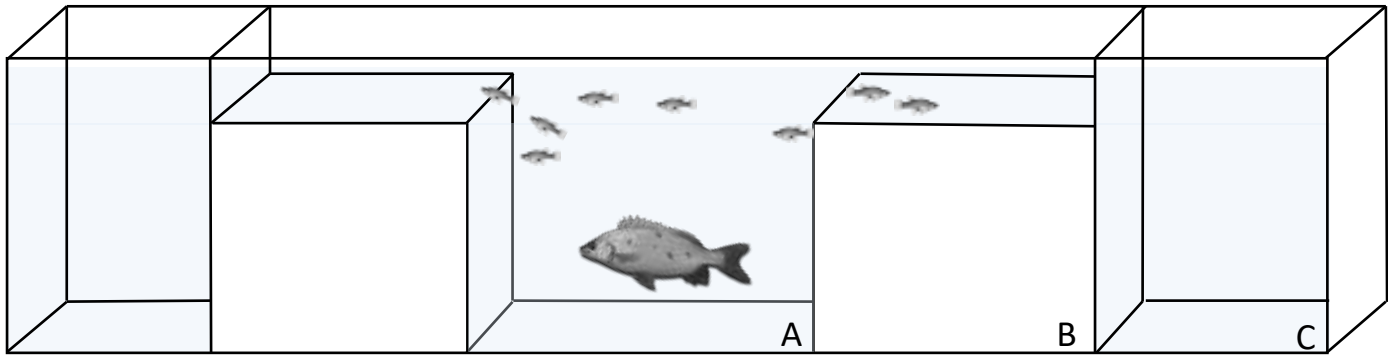
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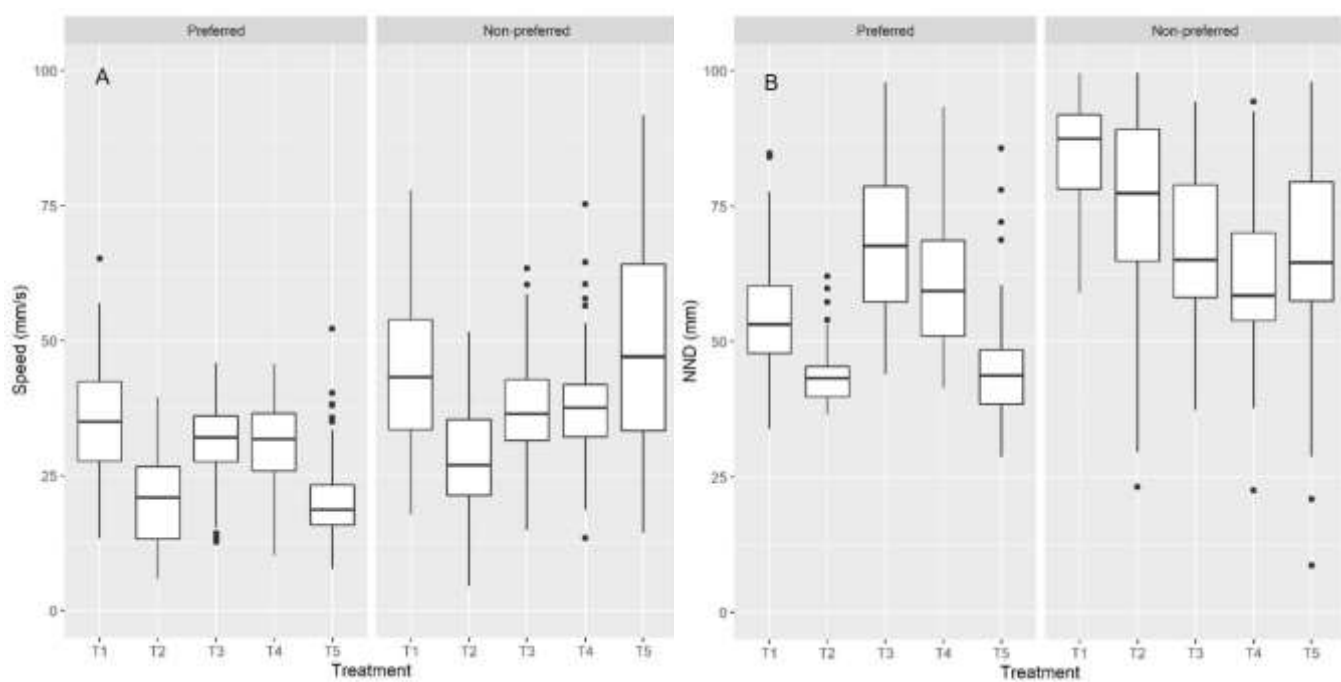
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676 Fig. 1

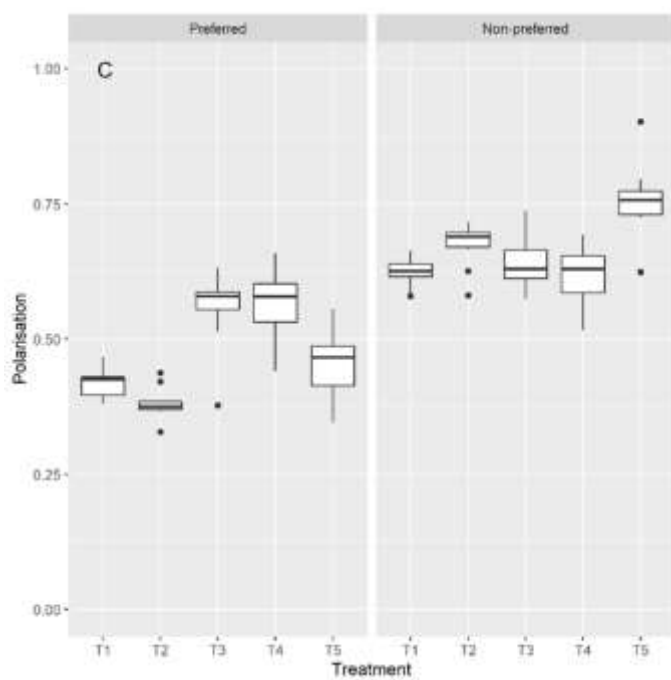
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678 Fig. 2



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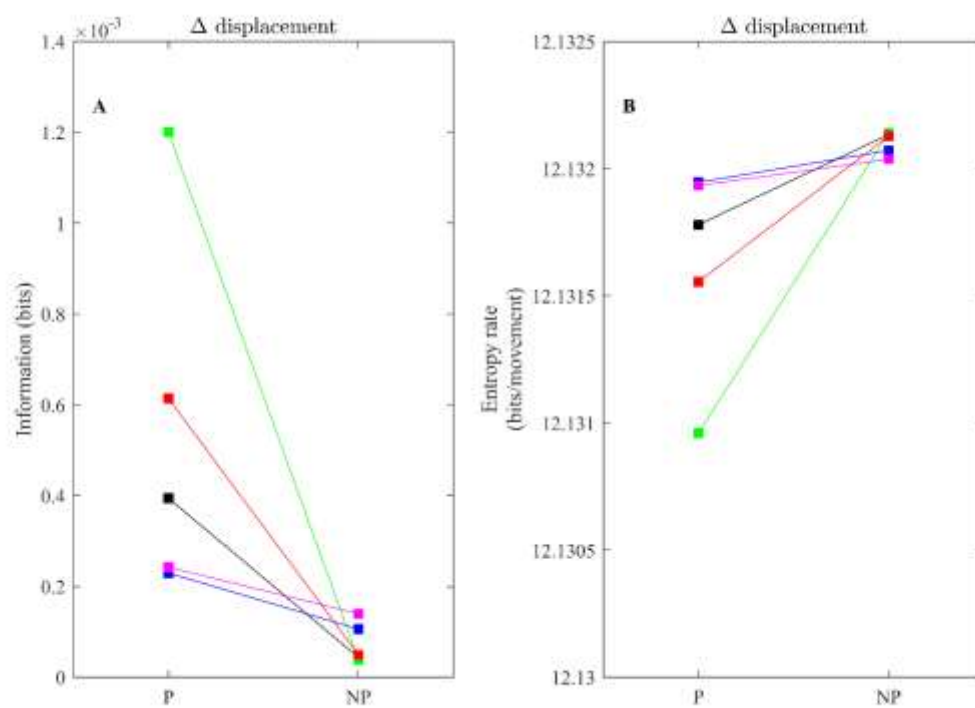


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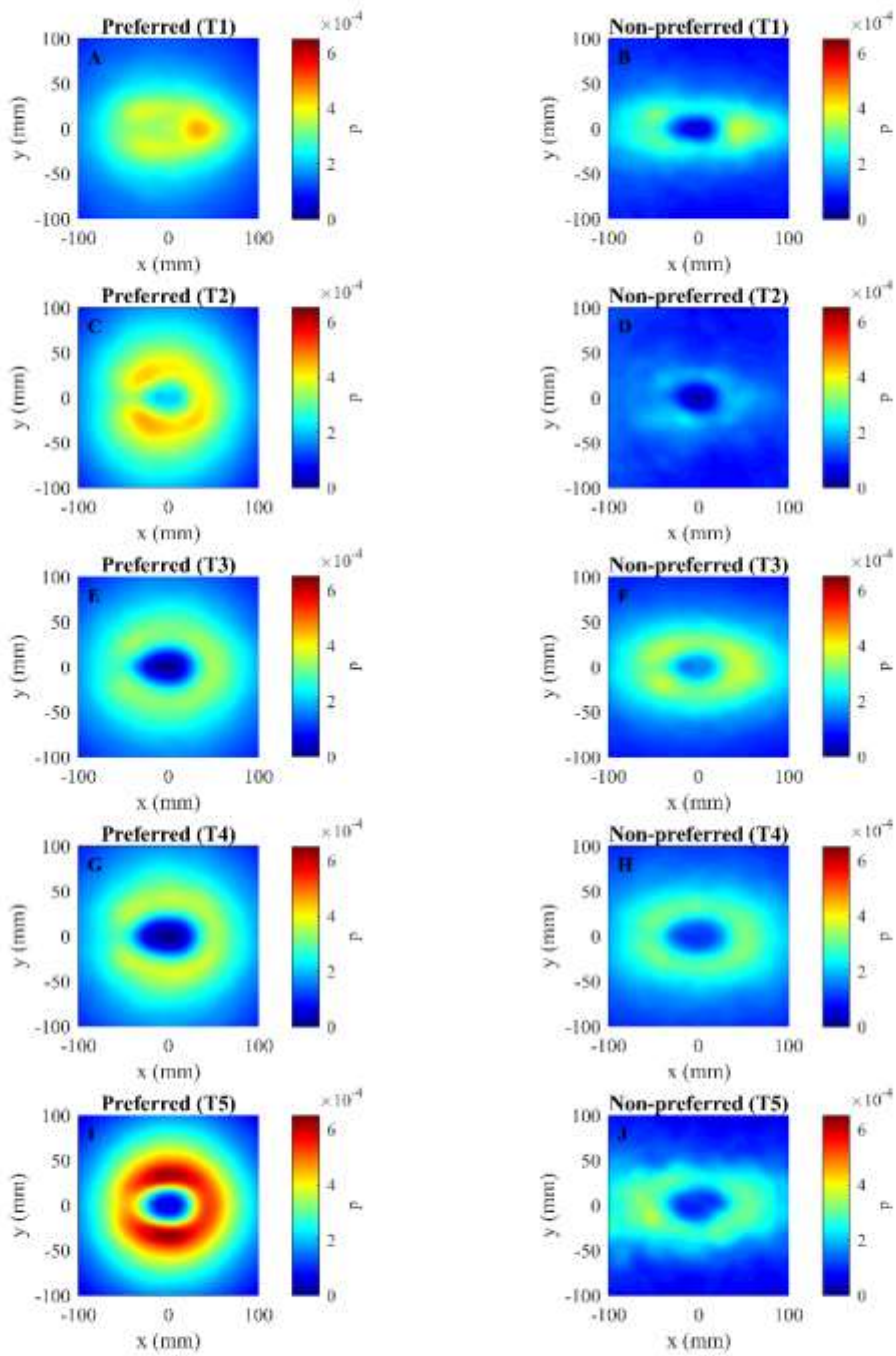
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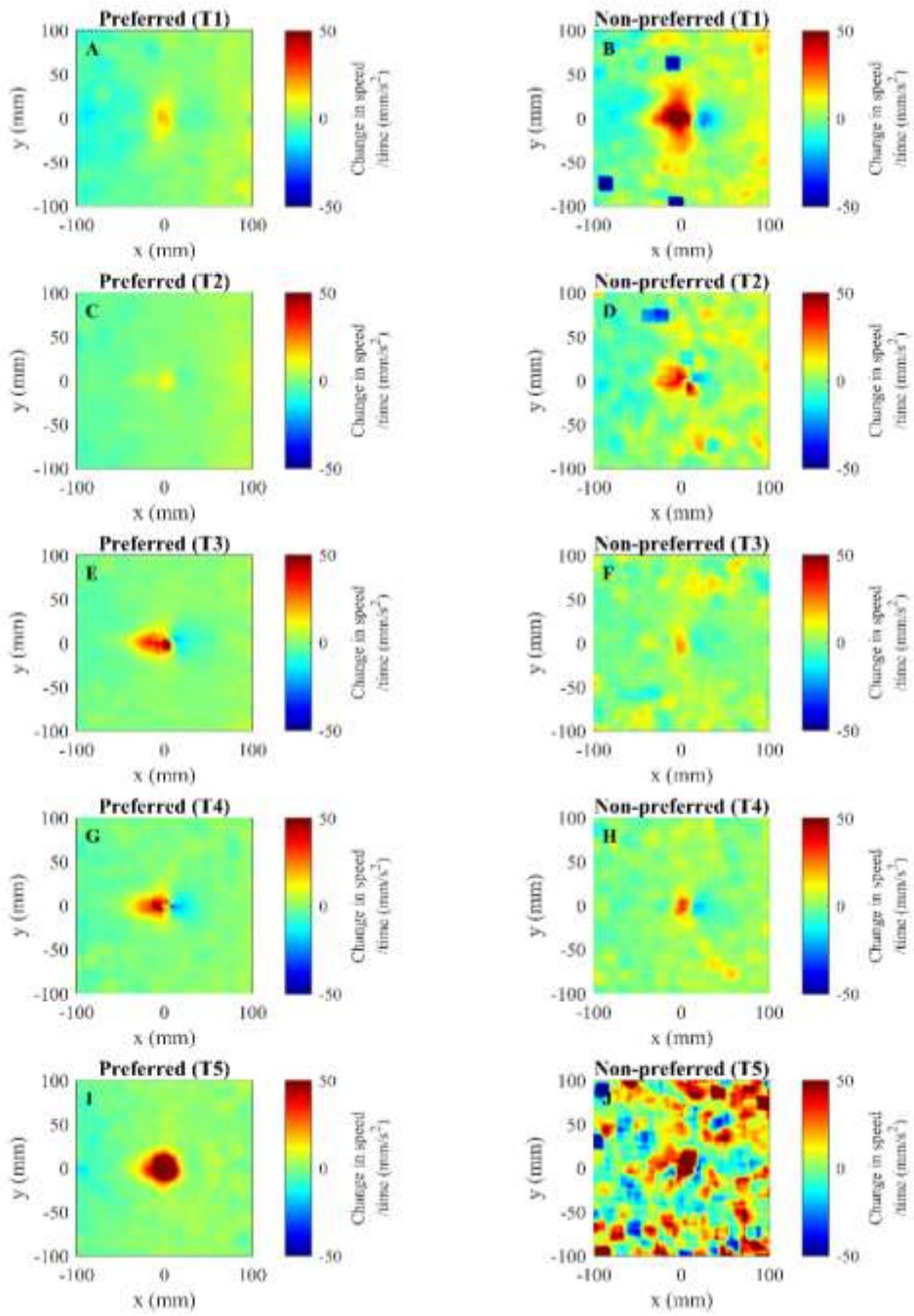
683 Fig. 3



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