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Individual and collective behaviour of fish subject to differing risk-level treatments with a sympatric predator

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

56

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

58 Introduction

59

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

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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 show additional, characteristic responses to risk, particularly increases in group cohesion that 74 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 synchronous and coherent group movement, is often most pronounced in the presence of risk 78 79 (Procaccini et al. 2011). In this latter case, group-level behavioral patterns emerge as a result of local interactions between neighbouring animals (Sumpter 2010). In experimental studies, 80 descriptions of these local interactions have followed the predictions of theoretical models 81 based on principles of self-organization, where group members adjust their position, speed, and 82 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 decreased their responsiveness to other group-members when they detected chemical cues 86 87 indicating the presence of food, and increased their responsiveness when they detected alarm cues, in each case resulting in different patterns of global group structure (Schaerf et al. 2017). 88 Nonetheless, a criticism of experimental studies in this field is that they tend to examine threat 89 in a binary manner, usually based on the presence or absence of risk. Further, targeted efforts 90 91 toward a more holistic approach that includes both individual and collective group responses

92 to graded levels of threat across ecological contents 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.

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

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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.

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

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

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

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

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

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180 Data Collection

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

194

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

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213 Statistical Analyses

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

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

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

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We refined the methods described in Schaerf et al (2017) to use the information theory 241 242 measures of mutual information and entropy rate associated with individual changes in position and velocity to examine the predictability of movements across different treatments 243 244 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 observation period (19 to 21 frames/0.76 to 0.84 seconds) informs an observer about the 246 change in position or velocity over the next (or previous) observation period. Relatively low 247 mutual information values suggest that an individual gives away little about its next move 248 based on its current movement, whereas higher values suggest that an individual telegraphs 249 its next move more clearly. The entropy rate is associated with the variety of movement 250 choices (in terms of changes in position or velocity) exhibited by an individual, with a higher 251 252 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 characterised by relatively lower mutual information and relatively higher entropy rate. See 255 256 section S1.4 of the SI for full details of the calculation of both measures. 257

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

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

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298 Distribution and zone changes within the arena

Time spent in platform zones varied according to treatment (Wilcox Robust ANOVA: F_{4.14.33} 299 300 = 188.68, p < 0.001, explanatory measure of effect size: 0.78). Fish in treatments where a predator was visibly present (T3, T4, T5) spent significantly more time in the platform zones 301 302 than treatments where a predator was not visibly present (T1, T2) (Fig. S1, Table S1). All treatments were significantly different from each other, with the exception of satiated predator 303 treatments (T3 and T4), which indicates that mosquitofish distribute themselves according to 304 their perceptions of risk in the arena. Furthermore, it suggests that the level of risk perceived 305 by mosquitofish does not differ when a satiated predator is visible, irrespective of the 306 307 chemical/olfactory cues expressed by the predator. The proportion of time spent over the platform regions was approximately the same as the proportion of the arena occupied by the 308 309 platforms (two thirds of the arena) for T3 and T4 (Fig. S1). Such distribution of the fish in T3 and T4 may occur if individuals occupied random coordinates in the arena at every instant, but 310 here was very likely driven by the presence of the predator, given the clear preference for the 311 312 central zone in the control case (T1).

313

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

321

322 Individual Speed, Nearest Neighbour Distances and Polarisation

There was a significant interaction between treatment and zone in relation to mean speed, mean nearest neighbour distance (NND), and polarisation (Fig. 2, Table 1; see also Fig S4 and Table S8). Generally, in non-preferred zones fished showed greater speed as well as increased polarisation and greater NNDs as compared to the preferred zones. This pattern is likely a response to the perceptions of risk in the non-preferred zones, in particular travelling at agreater speed, which may result in increased spacing between individuals in the short term.

329

330 Mutual Information and Entropy Rate

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

336

337 Interactions Between Individuals

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

352

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

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

366

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

381

382 **DISCUSSION**

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

Predation threat, particularly the imminent threat provided by the proximity of a hungry 395 predator, produced wide-ranging effects on the behavior of groups of mosquitofish. 396 Distribution patterns were associated with differences in ambient perceived predation threat. 397 Fish in treatments with visible predator presence showed a preference for spending time on 398 platform areas, while fish without a visible predator presence preferred the open water zone. 399 From an ecological standpoint, this result matches the life history and environmental 400 preferences of mosquitofish in the wild as this species is subject to heavy predation in the wild 401 402 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 as with the control or olfactory cues treatments, deeper water potentially reduces the chance of 404 predation by increasing the available avenues of potential escape from both aerial and aquatic 405 sources. In the current experiment, predation risk also influenced behavior between treatments 406 407 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, slower movement and grouping more cohesively. When comparing within treatments, we see 409 410 a largely similar pattern. Generally, fish in non-preferred zones, likely those they perceived to be more risky, showed increased mean swimming speeds and lower nearest neighbour 411 412 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 entropy rate, was strongly affected both by treatment and by the zone of the arena the fish were 415 416 in. Interestingly, fish in the treatments where risk was apparently perceived to be greatest (T2) and T5), showed the greatest predictability while in the preferred zones, but shifted strongly in 417 non-preferred zones to be the least predictable among all treatments. Such reductions in the 418 predictability of movement are thought to reduce the likelihood of capture by an attacking 419 420 predator (Hu et al. 2015; Herbert-Read et al. 2017; Schaerf et al. 2017). Why might fish in the riskier treatments be the most predictable in their preferred zones? A possible explanation for 421 422 this is that while increased entropy in response to predation threat may mitigate some aspects of predation risk, it can also interfere with group cohesion and the ability of fish to synchronize 423 424 their behavior with near neighbours. As such, increased mutual information may reflect the adaptive benefits for such coordinated actions prior to engaging in moves across the non-425 preferred zone, or to dealing with an imminent threat more generally (Ward and Webster 2016). 426 Understanding this functional trade-off in terms of collective phenomena and behavioral 427 428 predictability is a key area of current and future research need.

429

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

442

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

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

464

In addition, focal individuals typically turned away from nearby conspecifics that were ahead 465 of them (within one body length), likely as a means of collision avoidance in the majority of 466 treatments, especially T3 and T4, but also T1 in their non-preferred-zone. In contrast, however, 467 in the presence of a hungry predator (T5), these patterns were reversed with focal individuals 468 turning towards partners directly in front of them, and away from partners directly behind. We 469 can only speculate why this is, but the unexpected shift in the rules of interaction may reflect a 470 change in strategy by focal individuals, minimizing risk by limiting their domain of danger and 471 maintaining the closest possible distance between themselves and near neighbours (Morrell et 472 al. 2011). However, such an unprecedented pattern needs to be explored further, particularly in 473 terms of its adaptive value and this represents a priority for future work. 474 475

477 Figure Legends

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Fig. 1 Schematic representation of experimental arena used across risk gradient treatments. 479 The arena consisted of three primary zones as represented by a central open water zone (A) 480 flanked by two shallow platform zones (B) on either side. When a predator (a jade perch; 481 Scortum barcoo) was present visually (T3-T5) it was located in the central zone. Across 482 treatments, mosquitofish (Gambusia holbrooki) could move across the three platforms freely 483 but were prevented from reaching the outermost areas (C) beyond the platforms by an opaque 484 485 partition. In the predator cues treatment (T2), the predator was located in one of these two outermost areas on either side of the platform zones and water (containing olfactory cues) 486 was mixed with the experimental observation area 487 488

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

499

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

507 Fig. 4 Detail of the relative frequencies, p, that group members occupied (x, y) coordinates

relative to the location and direction of motion of a focal individual that was located in the

preferred or non-preferred region of the tank across treatments T1 to T5 for $-100 \le x \le 100$

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

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Fig. 5 Detail of the mean change in speed over time of a focal individual located in their 514 preferred on non-preferred region of the tank as a function of the relative (x, y) coordinates of 515 group mates for T1 to T5 with $-100 \le x \le 100$ (mm), $-100 \le y \le 100$ (mm). In these plots, 516 the focal individual is located at the origin, (0, 0), moving parallel to the positive x -axis. 517 Redder regions on these plots indicate that the focal individual increased their speed when 518 their groupmates occupied points in these regions; bluer regions indicate that the focal 519 520 individual decreased their speed when groupmates occupied points in these regions 521 Fig. 6 Detail of the mean change in angle of motion over time of a focal individual located in 522 their preferred on non-preferred region of the tank as a function of the relative (x, y)523 coordinates of group mates for T1 to T5 with $-100 \le x \le 100$ (mm), $-100 \le y \le 100$ (mm). 524 525 In these plots, the focal individual is located at the origin, (0, 0), moving parallel to the positive x-axis. Redder regions on these plots indicate that the focal individual turned anti-526 clockwise when their groupmates occupied points in these regions; bluer regions indicate that 527 528 the focal individual turned clockwise when groupmates occupied points in these regions

529

Fig. 7 Detail of the average directions of motion of group members at (x, y) coordinates 530 relative to the location and direction of motion of a focal individual that was located in the 531 preferred or non-preferred region of the tank across treatments T1 to T5 for $-100 \le x \le 100$ 532 (mm), $-100 \le y \le 100$ (mm). In these plots, the focal individual is located at the origin, (0, 0)533 , moving parallel to the positive x-axis. Arrows point in the average direction of motion of 534 other group members located at specific (x, y) coordinates relative to the focal individual. 535 Colours in the plots represent R, a measure of the focus of the angles contained in each bin 536 used to construct the plot about the mean angle within the same bin, with possible values 537 538 such that $0 \le R \le 1$. 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 binned angles of motion 540

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*-

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					
549	Speed	Chisq	Df	Pr(>Chisq)	
550	Treatment	15.972	4	0.0031 **	
551	Zone	426.109	1	< 0.0001 ***	
552	Treatment*Zone	325.103	4	< 0.0001 ***	
553 Nearest Neighbour Distance					
554	- (• • • • • • • • • • • • • • • • • •	-			
	Treatment	12.685	4	0.0129 *	
555	Zone	329.339	1	< 0.0001 ***	
556	Treatment*Zone	261 98	4	< 0.0001 ***	
557		201.90	•		
FEQ	Polarisation				
556	Treatment	53.494	4	< 0.0001 ***	
559	Zone	348.542	1	< 0.0001 ***	
	Treatment*Zone	102.314	4	< 0.0001 ***	

- 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	

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Fig. 2











686 Fig. 4











692 Fig. 6





695 Fig. 7



