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Presence of conspecifics reduces between-individual variation and increases avoidance of multiple stressors in bluegill

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Individual animals differ in their responses to external stressors, and sociability has been shown to impact whether or not an individual will avoid a stressor. However, the effect of collective group behaviour on individual avoidance in response to a stressor has not been elucidated. In this study, we sought to determine whether stressor avoidance behaviour in individuals is affected by the behaviour of a familiar shoal, and if social personality is a driver of avoidance behaviour. Bluegill, *Lepomis macrochirus*, were exposed to either carbon dioxide or rising temperatures in a shuttle box choice tank. All bluegill were exposed to a stressor in isolation, then their social personalities were quantified using a social network assay. Bluegill were then exposed to the same stressor in the presence of a familiar shoal, with the entire shoal being able to respond to the stressor. We found that being in a shoal significantly decreased individual avoidance thresholds to both carbon dioxide and temperature, but neither avoidance behaviour in isolation nor individual social personality type was predictive of this response. The presence of the shoal was the primary driver of the difference in avoidance behaviour when bluegill were in isolation versus when they were in groups. Potential mechanisms, both behavioural and physiological, for the relationship between group behaviour and stressor avoidance are discussed. Our results provide evidence that group movements impact individual avoidance of stressors, which may have implications for the behaviour of animals in response to decreasing habitat quality.

Living in groups provides animals with many benefits, including enhanced foraging opportunities (Herbert-Read et al., 2016), improved predator avoidance (Herbert-Read et al., 2017; Krause, 1994; Roberts, 1996; Ward, Herbert-Read, Sumpter, & Krause, 2011), a reduction in energy expenditure (Herskin & Steffensen, 1998; Killen, Marras, Steffensen, & McKenzie, 2012; Marras et al., 2015) and more reproductive opportunities (Bekkevold, Hansen, & Loeschcke, 2002; Pilastro, Benetton, & Bisazza, 2003). For animals living in groups, these benefits must outweigh potential costs, including disease transfer (Han, Park, Jolles, & Altizer, 2015), visibility to predators (Krause, 1994; Krause & Ruxton, 2002) and intraspecies competition (Webster & Hart, 2006). Furthermore, individuals within a group are forced to compromise resources and fitness for the good of the group (Borowiec, O'Connor, Goodick, Scott, & Balshine, 2017; Cooper, Adriaenssens, & Killen, 2018; Ward et al., 2016). To promote the benefits of group living while managing for the inherent differences between individuals, a social structure for information sharing and cohesive decision making should be in place (Conradt & Roper, 2009; Couzin et al., 2011; Couzin, Krause, Franks, & Levin, 2005; Miller, Garnier, Hartnett, & Couzin, 2013).

Within any group of animals, there will be phenotypic variation between individuals that leads to differing personalities (Réale et al., 2010; Réale, Reader, Sol, McDougall, & Dingemanse, 2007; Sih, Bell, & Johnson, 2004). This includes variation in social personalities, or how individuals interact with each other. These interindividual differences in social personalities have been proposed to be responsible for social cohesion in animal groups, as some individuals are more likely to take the lead while others are more likely to follow (Harcourt, Ang, Sweetman, Johnstone, & Manica, 2009; Jolles et al., 2015; Kurvers et al., 2009; Marras & Domenici, 2013; Nakayama, Harcourt, Johnstone, & Manica, 2012). Leadership, defined as the initiation of movement by an individual that is then followed by a group (Krause, Hoare, Krause, Hemelrijk, & Rubenstein, 2000), is generally assumed by individuals that are bold, or more willing to take risks (Jolles et al., 2015; Jolles, Ostojić, & Clayton, 2013; Kurvers et al., 2010a;

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Leblond & Reebs, 2006). In contrast, shy individuals are more likely to group with others and respond to information provided by group members as 'followers' rather than as 'leaders' (Couzin et al., 2011; Harcourt, Biau, Johnstone, & Manica, 2010; Kurvers, van Oers, et al., 2010b; Pike, Samanta, Lindström, & Royle, 2008; Trompf & Brown, 2014). A link between sociability and leadership has also been suggested. Specifically, leaders are more likely to be asocial and disperse from a group while social individuals form strong associations with other group members and are unlikely to leave the group (Cote & Clobert, 2007; Cote, Fogarty, & Sih, 2012; Réale et al., 2007). Frequency-dependent selection most likely facilitates the balance between leaders and followers within a group, as both behavioural types are needed to maintain a well-functioning group that is capable of both efficient decision making and social cohesion (Dall, Houston, & McNamara, 2004; Miller et al., 2013). Social interactions between individuals can be quantified through a social network, which describes the relative sociability, cliquishness and aggressiveness of each individual based on observed interactions (Croft, James, & Krause, 2008). Social network analysis can be used to identify individuals in a group with leadership personalities (bold, asocial), which then allows researchers to observe how leadership and social dynamics can change in different contexts.

Social dynamics can influence how a group responds to external stressors. In a study examining the trade-off between association with a conspecific and exposure to hypoxia, Borowiec et al. (2017) found that a fish is more likely to stay in a hypoxic zone to associate with a conspecific. Cooper et al. (2018) also found that fish will ignore individual temperature preferences to remain with their shoal. However, the studies mentioned above presented an all-or-nothing situation, in which a single, focal individual had to choose between social interaction (with the group not exposed to the negative stimulus) or preferential conditions. Furthermore, the conditions presented in these studies are not representative of natural conditions, in which environmental parameters can change-over time and animals in groups are exposed to challenges simultaneously, rather than only one individual being exposed to the challenge. The potential impact of individual social personality type and social interactions on the avoidance of an environmental stressor has not been elucidated, specifically when the entire group of individuals is able to respond to the stressor.

In the present study, we used bluegill sunfish, *Lepomis macrochirus* Rafinesque, 1819, as a model species to quantify the effects of conspecifics and social personality types on avoidance behaviour in response to two different environmental stressors. Bluegill are an ideal study species because they are highly social and display variation in many behavioural types (Wilson & Godin, 2009). We compared avoidance thresholds, defined as the amount of a stressor needed to induce a fish to choose to leave an environment, of individual bluegill exposed to either elevated partial pressure of free carbon dioxide gas in water (pCO_2) or elevated temperature, to avoidance by that same individual when in a shoal. Results will allow us to become better informed about how being part of a shoal may increase an individual's avoidance response to stressors, which could be another benefit of group living versus solitary living.

METHODS

Study Site and Animals

Bluegill sunfish ($N = 75$; mean ± SD total length (TL) = 136.71 ± 8.5 mm) were gathered from water bodies across Illinois by the Illinois Department of Natural Resources prior to being held at the Jake Wolf Memorial Fish Hatchery in Topeka, Illinois, U.S.A. for 2 weeks. The age and sex of the bluegill were unknown. Bluegill were transported from the hatchery to the Illinois Natural History Survey Aquatic Research Facility in Urbana, Illinois. All experiments took place indoors between 11 June 2018 and 4 July 2018 (total holding period = 24 days). Bluegill were held in four 568-litre tanks ($N = 25$ fish per tank) for 1 week prior to behavioural testing to recover from hauling and transport stress and to acclimate to laboratory conditions. During this holding period water was supplied to the indoor tanks from an outdoor earthen-bottom pond and treated with Quick Start (0.3 ml/litre, API, Chalfont, PA, U.S.A.), Stress Coat (0.1 ml/litre, API) and Aquarium Salt (1 g/litre, API), as per manufacturer's instructions to prevent ammonia toxicity and fungal infections and to promote mucous production. Each tank was also outfitted with an ultraviolet sterilizer (Vecton-6, American Aquarium Products, Grants Pass, OR, U.S.A.), an aquarium heater/chiller (TK-500, Teco US) and canister filter (FX6, Fluval Aquatics, Mansfield, MA, U.S.A.). Temperature, dissolved oxygen (DO) (YSI, 550A Yellow Springs Instruments, Irvine, CA, U.S.A.), chlorine (Insta-Test Free and Total Chorine, LaMotte Company, Chestertown, MD, U.S.A.), ammonia (Ammonia Test Kit, API) and nitrites (Nitrite Test Kit, API) were measured daily (Appendix, Table A1). Bluegill were fed frozen bloodworms (Brine Shrimp Direct, Ogden, UT, U.S.A.) daily ad libitum. For identification, individual bluegill were tagged with a passive integrated transponder (PIT) tag (HPT12, Biomark, Boise, ID, U.S.A.) containing a unique identification number. Fish were tagged after the 1-week acclimation period and were then given an additional 4 days to recover from tagging before experiments began.

Individual Shuttle Box Assessments

To quantify stressor avoidance, we used a 'shuttle box' apparatus (Loligo Systems, Denmark). This is a commonly used choice tank for studies examining voluntary movement of fish in response to environmental conditions (Cupp, Tix, et al., 2017b; Dennis, Wright, & Suski, 2016; Kates, Dennis, Noatch, & Suski, 2012; Tucker, Suski, Philipp, Jeffrey, & Hasler, 2019), because it allows for simultaneous and continuous manipulation of water quality in two different arenas, resulting in a binary response to an environmental challenge (stay put or shuttle) that makes avoidance thresholds easy to quantify (Fig. 1). Briefly, a shuttle box consists of two circular arenas (1.5 m diameter, 0.5 m depth) connected by a narrow tunnel (20 cm width, 0.5 m depth). Water is pumped out of each arena into an external water tower reservoir, where it can either be treated (i.e. increased CO_2 or increased temperature) or untreated before gravity draws the water back into the associated arena. Because the arenas are circular and the pumps generate a slight current around the tank, mixing through the narrow tunnel is minimal such that water conditions in the two arenas remain distinct, and water quality on the treated side of the arena can be manipulated without affecting the untreated side (Kates et al., 2012). The entire shuttle box was surrounded by black fabric to prevent fish from being disturbed by external stimuli. A camera (iDS uEye 1480-C camera, iDS, Obersulm, Germany) was mounted over the shuttle box and connected to an adjacent computer to track the fish movements and position.

For each trial, a single bluegill ($N = 75$) was identified using its PIT tag and placed into one side of the shuttle box (determined at random with a coin flip) and given a 10 min acclimation period. Preliminary trials ($N = 5$) confirmed that an acclimation time of 10 min was sufficient for the fish to cease exploratory behaviours and become stationary in the shuttle box. In addition, during this acclimation period, the two sides of the shuttle box were separated by thin mesh connected to clear fishing line to keep bluegill confined to one side before the start of the experiment. After the 10 min acclimation period, the mesh barrier was slowly lifted to allow the fish to access the other side of the shuttle box

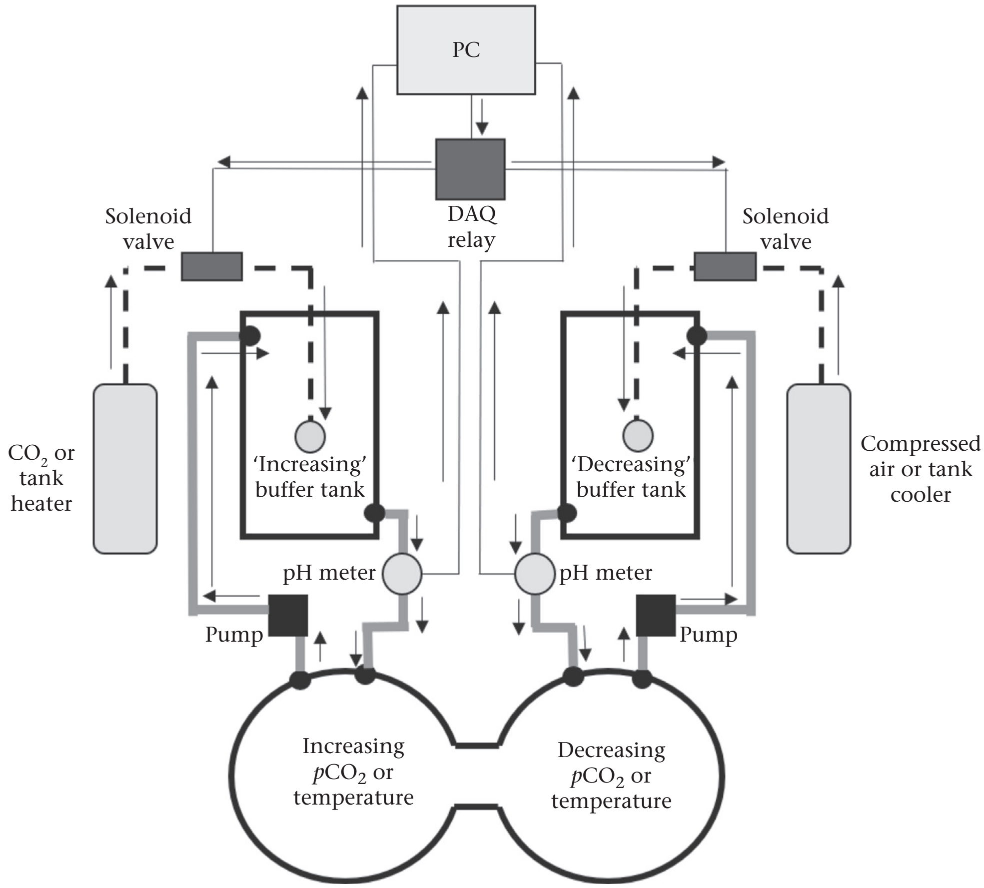

Figure 1. Shuttle box choice tank. One side of the tank receives an input of carbon dioxide (or increasing temperature) via the buffer tank while the other side receives compressed air. The entire system is connected to a computer and ShuttleSoft v.2 software (Loligo Systems, Copenhagen, Denmark) is used to control the system. A camera mounted above the shuttle box allows the researcher to observe the movements of the fish while tracking water quality through the probe vessels and buffer tanks. Thick grey lines = water tubing; dashed black lines = gas tubing; thin black lines = electrical wires; arrows show the direction of water, gas or electrical flow.

(preliminary trials indicated that removing this barrier did not startle or change the behaviour of the fish) and either (1) CO_2 was added to the external water tower reservoir on the side of the shuttle box containing the fish while compressed air was added to the other water reservoir ($N = 40$), or (2) aquarium heaters were added to the water reservoir on the side of the shuttle box containing the fish while the opposite water reservoir was maintained at a constant temperature using an aquarium chiller ($N = 35$). This manipulation resulted in the slow increase of either the quantity of CO_2 or temperature (0.2 °C/min) over time within the side of the shuttle box that contained the single bluegill. Compressed air was also added along with the aquarium heaters to maintain elevated dissolved oxygen. The position of bluegill in the shuttle box was continuously monitored via the overhead camera, and the time taken to move from the treated arena to the untreated arena was recorded. Once bluegill 'shuttled' to the arena that was not being treated, the experiment ended and the fish was returned to its holding tank. All bluegill were only tested once to avoid habituation to the stressor (Clingerman, Bebak, Mazik, & Summerfelt, 2007). In addition, we recorded the pH or temperature at the start of the experiment and at the time of shuttling. For CO_2 trials, water samples were taken from both the water tower reservoir that received CO_2 injections as well as the second water tower that did not receive a CO_2 injection, and the dissolved CO_2 content was determined by titration (Model CA-23, Hach Company, Loveland, CO, U.S.A.). Dissolved CO_2, pH, air pressure (recorded from http://w1.weather.gov/data/obhistory/KCMI.html) and alkalinity (measured by titration; Model AL-AP, Hach Company) were plugged into CO_2Calc software for calculation of the partial pressure of free carbon dioxide in the water, pCO_2 (Robbins, Hansen, Kleypas, & Meylan, 2010). For temperature trials, temperature was continuously recorded in the reservoirs using a combination temperature and dissolved oxygen probe (YSI, 550A Yellow Springs Instruments, Irvine, CA). If a bluegill did not shuttle within 1 h, the trial was terminated, and those fish were excluded from the study. The shuttle box was emptied and refilled between each trial. Additionally, a heater/chiller (Tank TK-1000, Teco, Ravenna, Italy) was used to keep the temperature of the water in the shuttle box within 2 °C of the temperature used in the holding tanks, as CO_2 avoidance has been shown to be affected by temperature (Tix et al., 2018).

Social Network Assay

Immediately following the shuttle box assay, the same bluegill that were used in the shuttle box assay above were used in a social network assay to identify social personalities (tendency to be near other individuals and tendency towards aggression). Bluegill were randomly assigned to groups of five individuals. Mean fish size was not different across groups (mean ± SD total length (TL) = 136.71 ± 3.9 mm; one-way ANOVA: $F_{13,55} = 1.03$, $P = 0.43$), and the difference between the largest and smallest fish in each group ranged from 12 to 19%. To differentiate between individual bluegill in social groups via video, we attached a small plastic tag to the dorsal fin of each fish (Webster & Laland, 2009) so that each fish in a social group had a uniquely coloured tag. None of the bluegill in this study displayed any signs of altered swimming patterns or agitation as a result of the tag. Each group was housed in its own 117 l aquarium (81 × 40 cm and 36 cm high), which was surrounded by an opaque white covering to prevent fish from being disturbed by observers or adjacent aquaria. Groups were given 48 h to acclimate to the aquarium and establish social hierarchies after the individual shuttle box trials (Louison, 2018). After 48 h, each group was filmed for 1 h using GoPro® cameras (HERO3, HERO Session, HERO5; GoPro, Inc., San Mateo, CA) mounted above each aquarium.

Videos of each social group were assessed for social and aggressiveness parameters, following commonly used protocols (Croft et al., 2008), to quantify social personalities of the bluegill. Still images at 5 min intervals over the 1 h monitoring period were used to quantify the proximity of each individual to other fish in the group. Proximity was defined as being within one body length of another fish. Fish that were within one body length of a nearby fish were also considered to be in proximity to any other fish that happened to be within one body length of the other fish (Pike et al., 2008). The resulting data were organized as a symmetrical matrix that represented the diversity and number of interactions that each fish had with every other fish in its social group (i.e. the ‘strength’; Barthélemy, Barrat, Pastor-Satorras, & Vespignani, 2005). We analysed this matrix for social degree (frequency of interactions with other fish), cliques (diversity of interactions with other fish) and clustering coefficient (impact of individual fish on the structure of the network) using the software UCInet (Borgatti, Everett, & Freeman, 2002) to determine the relative sociability of each fish (Barthélemy et al., 2005; Pike et al., 2008). Aggressiveness parameters were obtained by continuous observation of the full 60 min recording period. Aggressive interactions included chasing (one fish quickly swims toward another fish, and the other fish quickly swims away at least one body length), displacement (one fish slowly swims towards another fish, and the other fish slowly swims away at least one body length) and attacks (one fish swims quickly towards another fish and makes direct physical contact with them, and the other fish swims quickly away at least one body length) (Cañon Jones et al., 2010). Each aggressive interaction was entered into an unsymmetrical matrix relating aggressors to their targets (e.g. if the yellow-tagged fish chased the green-tagged fish, this interaction would be logged in the yellow row (aggressor) and the green column (target)). We analysed the resulting matrix for outdegree (aggressive actions by a fish towards another fish) and indegree (aggressive actions received by a fish) using UCInet v.6 (Borgatti et al., 2002; Analytic Technologies, Lexington, KY, U.S.A.; http://www.analytictech.com/).

Group Shuttle Box Assessments

Immediately following the social network assay, each social group was placed together into one side of the shuttle box to quantify the response of the group to thermal and CO_2 stressors ($N = 6$ groups of 5 bluegill tested for CO_2, $N = 6$ groups of 5 bluegill tested for temperature). The procedure for the shuttle box experiment with groups was identical to the procedure listed above for trials with individual fish. However, in addition to recording the pH and temperature at the start of the experiment, we also recorded the time at shuttling and the pH or temperature at the time of shuttling for each individual bluegill within a group using the coloured tags on their dorsal fins. Water samples were collected from the external water tower reservoir at each shuttling point for each fish in the group and the dissolved CO_2 content was determined by titration (Model CA-23, Hach Company). The partial pressure of free carbon dioxide gas in the water (pCO_2) was again calculated using CO_2 Calc as described above. During thermal avoidance trials, temperature was continuously recorded in the external water tower reservoir using the same oxygen/temperature probe described above. Control groups ($N = 2$ groups of 5 bluegill tested for CO_2, $N = 1$ group of 5 bluegill tested for temperature) consisted of individual bluegill that had also participated in the social network assay ($N = 10$ individuals for CO_2, $N = 5$ individuals for temperature). These fish were tested again in the shuttle box individually rather than with their social group to determine whether participation in the social network assay altered shuttling behaviour in the absence of their social group.

Animal Welfare Note

The procedures in our study were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (Protocol no. 17238). In total, 75 bluegill were used.

During transport of the bluegill from the hatchery to the research facility in Urbana, Illinois, aeration was supplied to ensure that the fish received enough oxygenation during the trip. The fish were immediately moved into their holding tanks upon arrival at the facility. The tanks were equipped with temperature control, aeration and both UV and carbon-based filtration. Stress Coat was added to the tanks to aid in formation of the slime coat of the fish after handling of fish (i.e. when moved from the truck to the holding tanks, after tagging, when moved from the holding tanks to the social arenas, etc.). Fish were given multiple days between handling events to recover from the stress of handling.

No complications were incurred as a result of PIT tagging and none of the bluegill in this study displayed any signs of altered swimming patterns or agitation as a result of the coloured fin tags used to differentiate between individual bluegill in social groups via video. Each coloured fin tag (1.27 cm diameter) was attached to the dorsal fin of each fish using thin jewelry wire (Webster & Laland, 2009). At the conclusion of the study, we removed the fin tags from each individual by gently snipping the jewelry wire to release the tag, and no fin damage was observed.

As part of our study, fish were exposed to either carbon dioxide or rising temperatures during their shuttlebox trials. For both stressors, the environmental conditions began at ambient levels, and either carbon dioxide or temperature was gradually increased in the experimental arena until the fish voluntarily left the arena, signalling the end of the trial. None of the fish lost equilibrium or showed signs of surface gulping during the procedure, and they were all able to escape from the experimental arena into the control arena, which was maintained at ambient conditions.

At the conclusion of the study, all bluegill were released into an outdoor retention pond at our facility.

Statistical Analyses

All statistical tests were run in R v.3.4.8 (R Core Team, 2017) using the base package and other packages listed below. Social network scores generated by UCInet were analysed using principal component analysis (PCA), which allowed for the simplification of multiple, correlated behavioural variables. Suitability for PCA was confirmed using the Kaiser–Meyer–Olkin (KMO) test and Bartlett's test for sphericity (Budaev, 2010; Fouladi & Steiger, 1993) through the ‘psych’ package (Revelle, 2017). Components with eigenvalues greater than 1 were rotated using varimax rotation (Grossman, Nickerson, & Freeman, 1991). Factors with loadings greater than or equal to an absolute value of 0.5 were considered to be primary drivers for its associated principal component (PC) (Budaev, 2010). Rotated loadings were then used to determine the PC scores of each fish.

We compared the pCO_2 or temperature at the time of shuttling between the first trial (before social network assay) and second trial (after social network assay) using a linear mixed effects model through the 'lme4' package (Bates, 2010) with trial, total length and PC scores generated by PCA as fixed effects, and fish identity (ID) as a random effect (McLean, Persson, Norin, & Killen, 2018). P values for post hoc tests were generated using the 'lmerTest' package (Kuznetsova, Brockhoff, & Christensen, 2017), and the marginal and conditional R^2 values were estimated using the package 'MuMin' (Barton, 2018). pCO_2 was rank-transformed (Conover & Iman, 1981), which improved model performance as indicated by analysis of residuals. The same test was used to compare pCO_2 or temperature at the time of shuttling for fish between the first trial (isolated, before social network assay) and the second trial (in a group, after social network assay) for fish in the experimental group. We estimated the intraclass correlation (ICC) and its 95% confidence intervals using the ICC package (Wolak, Fairbairn, & Paulsen, 2012) to determine whether shuttling behaviour was repeatable for control individuals (Bartko, 1966).

We used a one-way ANOVA with repeated measures (Briff & Elwood, 2010) to quantify whether the colour of the dorsal fin tags had an effect on the PC scores generated from the social network assay, using the 'car' package (Fox & Weisberg, 2019) with tag colour as a random effect. PC scores were compared to the order of shuttling in group trials (e.g. first fish to shuttle, second fish to shuttle, etc.) using ANOVA with repeated measures to determine whether there was an effect of social personality on the position of a fish in the shuttling order, using shuttling order as a random effect. Results were considered significant at $P < 0.05$, and residual plots for all statistical models were generated and examined to confirm suitability of the model for the data (Zurr, Ieno, Walker, Saveliev, & Smith, 2009). Figures were generated using 'ggplot2' (Wickham, 2009).

RESULTS

Social Personality Assessment

Five variables (sociability degree, indegree, outdegree, clustering coefficient and cliquishness) were used for principal component analysis (KMO = 0.69; $\chi^2_{10} = 138.85$, $P < 0.0001$). The resulting PCA produced two components with eigenvalues >1 that explained 75.45% of the total variance (Table 1). PC1 explained 53.22% of the total variance and had positive loadings for sociability degree, clustering coefficient and cliquishness. PC2 explained 22.22% of the total variance and was associated with negative loadings for outdegree and positive loadings for indegree. High PC1 scores were associated with high sociability, high clustering coefficients and high cliquishness, meaning that fish with high PC1 scores were more likely to have many interactions but with only certain other individuals. High PC2 scores were associated with low outdegree scores and high indegree scores, meaning that fish with high PC2 scores were more likely to be targets of aggression. A bluegill's PC scores were not influenced by the colour of the dorsal fin tag (one-way repeated measures ANOVA: PC1: $F_{4,8} = 0.70$, $P = 0.61$; PC2: $F_{4,8} = 0.39$, $P = 0.81$). Furthermore, personality type did not differ across the shuttling order of the fish (one-way repeated measures ANOVA: PC1: $F_{4,8} = 0.53$, $P = 0.72$; PC2: $F_{4,8} = 0.76$, $P = 0.58$).

Table 1
Principal component analysis (PCA) results from social network assay following varimax rotation

Variable	PC1	PC2	Communalities
Social degree score	**0.567**		0.567
Clustering coefficient	**0.543**	−0.152	0.083
Cliquishness	**0.573**		0.573
Outdegree centrality	0.159	**−0.786**	0.125
Indegree centrality	0.175	**0.586**	0.103
Variance explained	53.22	22.22	
Eigenvalue	2.66	1.11	

Groups of five bluegill were filmed for 1 h and the videos were scored for interaction strength metrics and aggression metrics using UCInet. The output from UCInet was used for PCA, and only principal components (PC) with eigenvalues >1 were used for further analysis. PC1 was related primarily to the social degree score, clustering coefficient and cliquishness, indicating that the drivers of this component were the frequency of interactions and the variety of interactions that one fish had with other fish in its network. PC2 was driven by the two aggressiveness metrics, namely outdegree centrality (actor of aggression) and indegree centrality (recipient of aggression). Significant PC loadings are shown in bold.

Avoidance of CO_2 in Isolation and in Groups

Bluegill in groups shuttled at significantly lower pCO_2 when they were in a group compared to when those same individuals were exposed to CO_2 individually (Table 2, Fig. 2a). The strongest driver of shuttling between isolated bluegill and groups of bluegill was the type of trial (isolated versus group), while total length and PC scores did not significantly influence pCO_2 at shuttling (Table 2). The mean(±SD) pCO_2 at shuttling for bluegill in isolation was 7705 ± 11 212 μatm, while the mean pCO_2 at shuttling for those same bluegill when they were in a group dropped to 530 ± 698 μatm. Additionally, isolated bluegill had shuttling times ranging from 0.23 min to 9.48 min, while bluegill in groups shuttled at 0.38 min to 3.05 min. When bluegill were in groups, all five fish in a group shuttled within 0.82 min of the first fish.

There was no significant difference between the first and second shuttling trial for control fish exposed to pCO_2 in isolation. Total length and PC scores were also not significant drivers of shuttling (Table 2, Fig. 2b). Repeatability of shuttling was poor, as the ICC was 0.29 and the 95% confidence interval for this score did not include zero (0.42, 0.76).

Avoidance of Temperature in Isolation and in Groups

Bluegill tested in groups shuttled at lower temperature changes relative to bluegill tested in isolation (Table 2, Fig. 3a). Only the trial type (isolation versus group) was a significant driver of the model, while total length, PC1 and PC2 did not significantly influence shuttle temperature (Table 2). In isolation, bluegill shuttled at temperature increases of 1.28 ± 1.62 °C above ambient, and at times ranging from 0.22 min to 58.5 min. In groups, bluegill shuttled at temperature increases of 0.19 − 0.16 °C above ambient, and at times ranging from 0.22 min to 4.97 min. When bluegill were tested in groups, all five fish in the group shuttled within 1.18 min of the first fish.

As in the fish exposed to pCO_2, there was no significant difference between the first and second shuttling trial for control fish exposed to elevated temperatures in isolation. Total length and PC scores also did not significantly influence shuttle temperature for bluegill in the control treatment (Table 2, Fig. 3b). Shuttling in response to temperature was not repeatable within individuals, as the ICC was 0.42 (95% confidence interval: -0.49, 0.92).

DISCUSSION

Our study showed that bluegill in a group shuttled at lower pCO_2 and temperature than bluegill tested individually. Individual fish are known to voluntarily leave areas with high pCO_2 (between 40 000 and 80 000 μatm) (Hasler et al., 2019; Kates et al., 2012; Tierney, 2016), and they are also known to behaviourally select

Table 2
Results of linear mixed effects models examining factors affecting the pCO_2 or temperature change at shuttling

	Estimate	SEM	*df*	*t*	*P*	$r^2_{marginal}$	$r^2_{conditional}$
Response: pCO_2 at shuttling							
Intercept	42.51	32.74	54.00	1.30	0.20	0.60	0.60
Isolated vs group trial (experimental fish)	**26.37**	**2.85**	**54.00**	**9.25**	**<0.0001**		
Total length	−0.18	0.23	54.00	−0.76	0.45		
PC1	−1.24	1.43	54.00	−0.87	0.39		
PC2	2.76	1.89	54.00	1.46	0.15		
Response: pCO_2 at shuttling							
Intercept	−0.44	28.90	6.56	−0.02	0.99	0.22	0.63
Trial 1 vs trial 2 (control fish)	−0.18	1.82	8.40	−0.10	0.92		
Total length	0.06	0.21	6.50	0.30	0.77		
PC1	−0.45	2.08	5.98	−0.22	0.84		
PC2	2.75	1.52	6.32	1.81	0.12		
Response: Temperature change at shuttling							
Intercept	31.62	28.61	55.00	1.11	0.27	0.29	0.29
Isolated vs group trial (experimental fish)	**18.3**	**3.85**	**55.00**	**4.75**	**<0.0001**		
Total length	−0.07	0.21	55.00	−0.33	0.74		
PC1	−2.65	2.30	55.00	−1.16	0.25		
PC2	−1.51	2.42	55.00	−0.62	0.54		
Response: Temperature change at shuttling							
Intercept	83.82	56.54	1.00	1.48	0.38	0.41	0.48
Trial 1 vs trial 2 (control fish)	−0.58	1.48	4.00	−0.39	0.72		
Total length	−0.61	0.40	1.00	−1.53	0.37		
PC1	−4.15	2.91	1.00	−1.42	0.39		
PC2	−3.57	4.70	1.00	−0.76	0.59		

All fish were first tested in a shuttle box in isolation. Following a social network assay, fish were retested in the shuttle box as part of a group (experimental fish). A subgroup was retested in the shuttle box in isolation rather than as part of a group (control fish). Fish identity (ID) was used as a random effect. Results from four separate models are shown, separated by the response variable. PC1 = principal component 1 (sociability), PC2 = principal component 2 (aggressiveness), generated using PCA analysis. Significant outcomes are shown in bold.

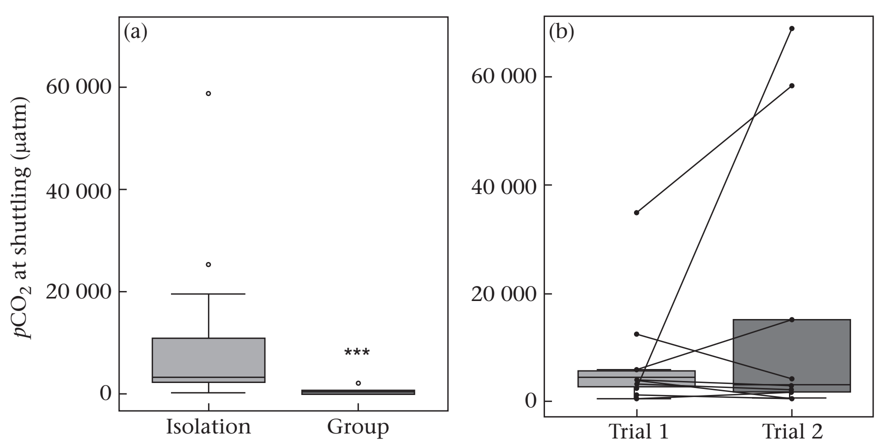

Figure 2. pCO_2 at shuttling in bluegill. (a) Bluegill were exposed to CO_2 first in isolation. Following a social network assay, bluegill were exposed to CO_2 again in the same groups that they tested with in the social network assay ($N = 8$ groups, 5 fish per group). $^{***}P < 0.0001$. (b) Bluegill that served as controls were exposed to CO_2 first in isolation (trial 1). Following a social network assay, bluegill were again exposed to CO_2 in isolation (trial 2) to test the potential effect of the social network assay ($N = 2$ groups, 5 fish per group). Each line represents an individual fish.

optimum temperatures (Cooper et al., 2018; Crawshaw & Podrabsky, 2011; Killen, 2014). While previous studies have shown that social fish will choose to remain near conspecifics even when water quality is degrading, the conspecifics in those studies were either (1) not exposed to the same conditions as the focal fish (Borowiec et al., 2017), or (2) unable to leave the area of degrading water quality (Cooper et al., 2018). Our study provides evidence that the avoidance behaviour of conspecifics that are exposed to the same conditions, and can choose to avoid those conditions, will influence an individual fish's response to degrading water quality. Groups of bluegill either shuttled in one group behind the first fish, or shuttled in two distinct groups within 1 min of the first fish shuttling. A similar effect was found by Marras and Domenici (2013) in a study that showed the behaviour of the first responder to a predator within a school of fish was the strongest influence on group escape strategy. This suggests that a tendency to ‘follow the leader’ is the primary driver of group shuttling.

There are three potential hypotheses to explain the patterns of avoidance seen by bluegill in this study. First, the first fish in a group to shuttle in response to an environmental stressor could be the fish in the group with the lowest tolerance to that stressor, such that the first fish to shuttle is looking to avoid adverse conditions. This would be indicated by lower pCO_2 at shuttling during the individual shuttling trials relative to other individuals tested in isolation. However, in our study, individual shuttling trial results could not predict which fish in a group would be the ‘leading’ fish during group shuttling trials, indicating that this hypothesis probably is not applicable in the current study. Second, being part of a group can alter an individual's physiology, rendering it more or less responsive to an environmental stressor (Killen, Marras, Metcalfe,

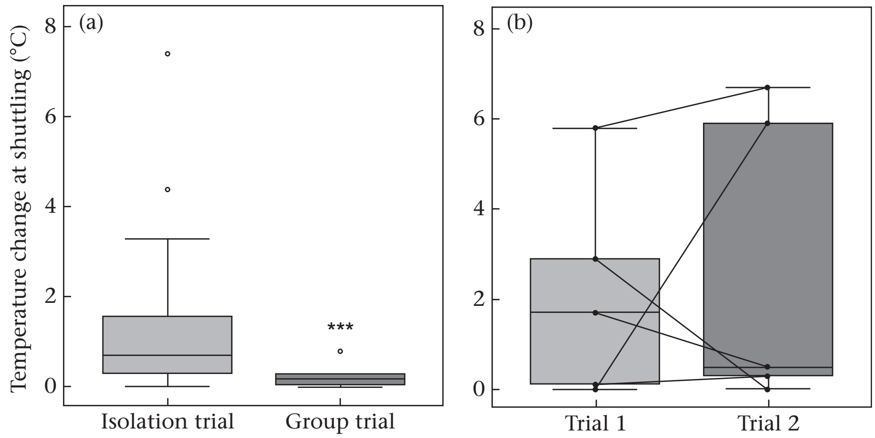

Figure 3. Temperature change at shuttling in bluegill. (a) Bluegill were exposed to increasing temperature first in isolation. Following a social network assay, bluegill were exposed to increasing temperature again in the same groups that they tested with in the social network assay ($N = 8$ groups, 5 fish per group). $^{***}P < .0001$. (b) Bluegill that served as controls were exposed to increasing temperature first in isolation (trial 1). Following a social network assay, bluegill were again exposed to increasing temperature in isolation (trial 2) to test the potential effect of the social network assay ($N = 1$ group, 5 fish per group). Each line represents an individual fish.

McKenzie, & Domenici, 2013). Plasma cortisol may play a role in avoidance of CO_2 in largemouth bass, as fish with lower levels of plasma cortisol showed lower thresholds of CO_2 avoidance (Tucker et al., 2019). While the mechanism behind this phenomenon is not clear, it has been hypothesized that cortisol may either (1) have a buffering effect on the blood, reducing the impact of increased pCO_2 on blood pH, or (2) cause a shift in the coping mechanism of an individual, such that a fish with higher cortisol 'freezes' in response to a stressor rather than fleeing (Koolhaas et al., 1999). Being part of a group has been shown to reduce ventilation rates and metabolic rates in fish (Lefrançois, Ferrari, Moreira da Silva, & Domenici, 2009; Nadler, Killen, McClure, Munday, & McCormick, 2016), suggesting a 'calming effect' of shoaling. While we did not measure plasma cortisol in our study, shoaling could potentially lower overall plasma cortisol levels in fish, thereby lowering their response thresholds for CO_2 or temperature. The third hypothesis relates to animal personality. In the study of animal behaviour, personality is sometimes called a 'behavioural syndrome', which describes a set of correlated behaviours that are expressed consistently across multiple contexts (Sih et al., 2004). A 'leadership' behavioural syndrome would indicate that an animal is risk taking and asocial. More specifically, leadership in groups of animals, such as a fish shoal, can be positively associated with boldness and negatively associated with social attraction (Jolles et al., 2015; Leblond & Reebs, 2006), and is the basis behind the 'social indifference' hypothesis, which suggests that a leader in a group of animals will direct the movements of the group based on its own needs (e.g. food, shelter) over its own desire to be social (Conradt, Krause, Couzin, & Roper, 2009). Under this hypothesis, bluegill in our study with low PC1 scores (low sociability) would be more likely to shuttle first. However, PC scores were not significant predictors of shuttling order for either temperature or CO_2 exposure. It is likely that, while sociability is often correlated with boldness and leadership, sociability on its own does not predict leadership in a group. Boldness is defined as a tendency to take risks, and is often measured by placing a fish in a novel environment and observing how long it takes for a fish to emerge from a shelter or to interact with a novel object (Wilson & Godin, 2009). Boldness differs from leadership in that leadership specifically refers to whether or not the movements of one animal will be followed by others (Leblond & Reebs, 2006). While boldness was not quantified in the present study, Tucker et al. (2019) found that there was no significant effect of boldness on avoidance of CO_2 in individual bluegill. The impact of boldness on avoidance behaviour may be augmented by the presence of conspecifics. The mechanism behind what causes the 'leading' fish to shuttle first remains to be elucidated and should be a subject of future studies. Regardless of the driving factor behind what prompts the 'leading' fish to initiate shuttling, our study provides evidence that lower pCO_2 or temperature is required to induce avoidance in groups of fish when compared with individuals.

Habitation to a stressor could possibly explain the reduction in pCO_2/temperature needed to induce avoidance behaviour in bluegill. In animals that are exposed to a repeated stimulus, negative-feedback on the hypothalamic–pituitary–interrenal (HPI) axis has been shown to cause a dampening of the stress response in the animal (Thompson, 2009), which can lead to habituation. Additionally, habituation is often accompanied by associative learning, in which an animal learns to predict the onset of a stressor and adjusts its behaviour to avoid it (Grissom & Bhatnagar, 2009). All bluegill in our study were exposed to either CO_2 or elevated temperatures in the shuttle box twice, thereby providing the opportunity for both habituation and learning. If habituation or learning influences shuttling behaviour, bluegill in the control group should have consistently reduced pCO_2/temperature at shuttling. While ICC estimates lower than 0.5 indicate that shuttling behaviour of individuals is not repeatable within individuals (Bartko, 1966), the pCO_2 and temperature that induced shuttling did not differ significantly between the first and second trial for the control group, thus suggesting that habituation and learning did not occur in bluegill, despite being tested twice during the study.

Different outcomes could be expected depending on whether an individual or a group is exposed to an environmental stressor. The results of this study suggest that fish in a group will leave a degraded environment (high CO_2 or high temperature) sooner than if those same fish were alone, which implies that shoaling fishes might be less likely to be found in degraded habitat if better habitat is accessible. Previous studies found that, when suitable habitat was not available, individual fish had a tendency to endure the degraded environment longer in order to associate with conspecifics (Borowiec et al., 2017; Cooper et al., 2018). However, some stressors, including CO_2, are known to disrupt shoal familiarity in some species (McCormick, Nadler, Munday, Watson, & Killen, 2016). Disruption of social dynamics in this case could further inhibit the ability of individuals to avoid the stressor, based on the tendency of individuals to follow their group as shown in this study.

When predicting the impact of a stressor on fish in a natural setting, it is therefore important to consider (1) whether or not the fish species is inherently social and (2) whether the fish are able to leave the degraded area. For example, thermal pollution from a power plant (e.g. Madenjian, Jude, & Tesar, 1986) can be avoided by fishes in some cases, but increased temperatures and pCO_2 as a result of climate change (Feely, Doney, & Cooley, 2009; Hasler, Butman, Jeffrey, & Suski, 2016) is relatively unavoidable.

The results of this study can be applied to the use of CO_2 as a deterrent to prevent the movement of invasive fishes (Cupp et al., 2017a; Donaldson et al., 2016; Noatch & Suski, 2012). A nonphysical barrier of dissolved CO_2 has been proposed in the management of bigheaded carps, *Hypophthalmichthys nobilis* (Cupp et al., 2017a; Donaldson et al., 2016; Kates et al., 2012), round goby, *Neogobius melanostomus* (Cupp, Tix, et al., 2017b), and sea lamprey, *Petromyzon marinus* (Dennis et al., 2016) as these invasive fishes have been shown to actively swim away from zones of high CO_2, deterring their movement and spread. One of the major drawbacks in the implementation of a CO_2 barrier is the interindividual variation in the pCO_2 required to elicit avoidance behaviours of fish within the same species, making target CO_2 levels difficult to define. For example, data from the current study found that the pCO_2 required to induce avoidance for individual bluegill ranged from 100 μatm to 20 000 μatm, while Kates et al. (2012) showed that variation for individual bluegill was approximately 100-fold. Data from the current study shows that these shoaling fishes respond to lower levels of CO_2 relative to that of individuals. While several studies have confirmed that the responses of fishes to elevated CO_2 is conserved across species (Kates et al., 2012), work should be conducted with invasive fishes, especially those that shoal, such as bigheaded carps, to confirm that this reduction in shuttle pCO_2 for groups occurs.

One potential limitation of this study is related to nonindependence of grouped data. Nonindependence must be controlled in situations where investigators are interested in individual-level data in a grouped situation, or when individuals are measured more than once (Grawitch & Munz, 2004). By measuring pCO_2 or temperature avoidance for fish individually first and within a group second, rather than randomizing the order of these avoidance treatments, the data are subject to nonindependence. However, this experimental design was chosen for two reasons. First, running group shuttling trials prior to the social network assay would have increased the risk that the fish would be interacting with each other in the shuttle box rather than with the CO_2 or temperature, thus potentially compromising the shuttling data. Second, conducting the social network assay after group shuttling would lead to fish in the social network assay not being ‘strangers’ to each other, potentially compromising our social network data. More importantly, the intent for group-level analyses was to quantify the behaviour of the entire group. As there was no interest in individual-level analyses for animals within the groups, nonindependence of the group data does not need to be controlled for (Grawitch & Munz, 2004). Furthermore, one purpose of this study was to determine the level of nonindependence, and whether the behaviour of fish in groups differed from that of individual fish. To ensure statistical rigour, we employed a mixed model approach, which can account for the repeated sampling of fish (Bauer & Curtin, 2018). Thus, while there is potential for nonindependence of the group-level data in this study, the current statistical approach accounts for the effects of individual- and group-level sampling, and also demonstrates that fish most likely experienced some degree of mutual influence.

In conclusion, the results of this study provide evidence that fish in groups show increased avoidance of elevated CO_2 and temperature relative to fish tested individually. This effect appears to be driven by the fact that fish tested in groups tended to move together, rather than factors such as habituation, personality, learning or interindividual variation in tolerance to adverse conditions. Individual shuttling time and personality type did not influence group shuttling behaviour in response to elevated CO_2 or temperature. Together, these results indicate that lower thresholds of some environmental stressors can be used to elicit avoidance behaviours in shoaling fishes.

Competing Interests

We have no competing interests to declare.

Acknowledgments

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Appendix

Table A1
Water quality parameters for bluegill holding tanks

Parameter	Mean	SD
Temperature (°C)	21.92	1.58
Dissolved oxygen (mg/litre)	7.79	0.92
Ammonia (mg/litre)	0.71	1.35
Nitrite (mg/litre)	0.58	0.74
Chlorine (mg/litre)	0.00	0.00
Alkalinity (mg/litre $CaCO_3$)	13.50	3.47

During the course of experiments utilizing bluegill, water quality was measured daily. Ammonia and nitrites were slightly elevated at the beginning of the holding period after addition of fish to the indoor tanks, but a nitrogen cycle was fully established by the end of the first week of holding and the levels of both ammonia and nitrites dropped to 0.