The impact of water pH on association preferences in fish

Short title: Impact of water pH on association preferences

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ABSTRACT

Acidification of lakes and rivers, as a consequence of anthropogenic interference, can cause fundamental changes to biological and ecological processes. One of the main consequences of a reduction in water pH for aquatic organisms is the disruption of their chemosensory abilities, as the detection of chemical cues underpins a wide range of decision-making processes; for example, a reduction to low pH has been shown to interfere with predator avoidance and the detection of foraging cues. Moreover, aquatic organisms are known to make widespread use of chemical information to inform their social behaviour, although we have a comparably poor understanding of how this is impacted by water acidification, especially their shoaling behaviour. Using a standard behavioural assay, we therefore investigated the impact of low water pH on the social interactions mediated by diet-derived chemical cues in three-spined sticklebacks (*Gasterosteus aculeatus*), by quantifying social behaviour in water that varied either experimentally or naturally in pH. In both cases we predicted that association patterns would be disrupted by low pH conditions, as reduced pH has shown to interfere with the perception of chemical cues in other non-social contexts. Consistent with this prediction, our results demonstrate that an acute, short-term reduction in water pH caused a breakdown in the diet-mediated social interaction patterns seen in more alkaline water, although, interestingly, the pattern of associations for fish tested in naturally acidic water was both more complex and in a direction that was precisely contrary to our predictions. Overall the findings provide insights into the potential effects of an acute reduction in water pH on fish communication and social interaction patterns, which may have implication for various individual, group, population and community-level processes.

Keywords: anthropogenic interference, chemical cues, *Gasterosteus aculeatus*, social network, sticklebacks, water acidity

Introduction

Anthropogenic acidification of lakes and rivers can have dramatic and far-reaching implications for the structure and function of aquatic ecosystems (Johnson & Webster, 1977; Ikuta, Suzuki, & Kitamura, 2003; Petrin, Englund, & Malmqvist, 2008; Leduc, Munday, Brown, & Ferrari, 2013). Acidification occurs primarily as a result of acidified rain or snow depositions, whereby the emissions of sulfur dioxides and nitrogen oxides into the atmosphere form highly acidic precipitations that can have long lasting effects on freshwater pH (Galloway, Norton, & Church, 1983; Muniz, 1990). Aquatic ecosystems can also be affected by climate-change-related acidification due to the increased uptake of CO_2 from the atmosphere (Caldeira, & Wickett, 2005, Weiss, Pötter, Steiger, Kruppert, Frost, & Tollrian, 2018). Humic acid, a result of degrading organic matter, can be another cause of freshwater acidification (Steinberg, 2013). Although the underlying mechanisms of acidification can differ, the chemical alterations associated with it can lead to fundamental changes at the level of both the individual and the community (reviewed in Leduc et al., 2013).

One particular consequence of the acidification of aquatic ecosystems for the organisms living within them, is the disruption to their chemosensory abilities (Leduc et al., 2013). The detection of chemical cues is important for a variety of decision-making processes, as chemical information is used in a wide range of social contexts including mate selection (Milinski, Griffiths, Wegner, Reusch, Haas-Assenbaum, & Boehm, 2005; Rafferty & Boughman, 2006), kin recognition (Gerlach, Hodgins-Davis, Avolio, & Schunter, 2008; Mehlis, Bakker, & Frommen, 2008) and dominance interactions (Barata, Fine, Hubbard, Almeida, Frade, Sorensen, & Canario 2008), as well as inter- and intra-individual shoaling decisions (Atton, Galef, Hoppitt, Webster, & Laland, 2014; Kleinhappel, Burman, John, Wilkinson, & Pike, 2014b, 2016a, 2016b). Chemical cues also play a major role in homing (Bett, Hinch, Dittman, & Yun, 2016), microhabitat choice (Kim, Grant, & Brown, 2011), prey detection (Atema, Holland, & Ikehara, 1980; Hara, 2006; Derby & Sorensen, 2008) and predator avoidance (Chivers & Smith, 1998; Brown, Paige, & Godin 2000; Wisenden, 2000). As a result, any interference with the chemosensory ability of animals may fundamentally alter their responses to perceived environmental and social information, and therefore impact directly on survival and fitness.

Experimental studies have shown that a reduction in freshwater pH can indeed change an animal's behavioural response towards chemical stimuli. For instance, acute and chronic exposure to a lower than normal water pH can interfere with the detection of foraging cues (Lemly & Smith, 1985; Lemly & Smith, 1987; Royce-Malmgren & Watson, 1987) and weaken or prevent predator avoidance behaviour (Leduc, Kelly, & Brown, 2004; Leduc, Roh, & Brown, 2009; Ou et al., 2015, Weiss et al. 2018). Reduced pH conditions have also been demonstrated to change the reproductive behaviour of aquatic animals (Johnson & Webster, 1977; Ikuta, Munakata, Aida, Amano, & Kitamura, 2001; Ikuta et al., 2003). Overall, experiments indicate that subtle changes in pH can dramatically affect important aspects of behaviour in many aquatic animals. This is supported by recent studies in ocean acidification that have found that elevated CO_2 levels (resulting in acidification) can disrupt shoaling preferences and shoal cohesion in coral reef fish (Nadler, Killen, McCormick, Watson, & Munday 2016; Lopes, Morais, Pimentel, Rosa, Munday, Goncalves, & Faria 2016). However, we still have a relatively poor understanding of the effects of freshwater acidification on shoaling decisions in fish. This is important, as the organisation within social groups can impact on various biological processes, including reproduction, predator avoidance, and resource exploitation (Guevara, Gonzaga, Vasconcellos-Neto, & Avilès, 2011).

The aim of this study was therefore to test whether exposure to relatively acidic freshwater (in the pH range naturally experienced by aquatic organisms) can disrupt social behaviour in an aquatic model species, the three-spined stickleback (*Gasterosteus aculeatus*). Sticklebacks naturally inhabit freshwater with a range of different pH values, from relatively low (e.g. down to around pH 6.0) to relatively high (e.g. up to approximately pH 8.5) (MacColl, El Nagar, & de Roij, 2013). Using an established behavioural assay (Kleinhappel et al., 2014b, 2016a, 2016b), we analysed diet-mediated inter-individual association preferences within groups of fish exposed to pH levels towards the extremes of their natural range by conducting two experiments: fish were tested in (1) experimentally manipulated water pH, and (2) in their natural water, from varying pH habitats. In the first experiment we manipulated water pH to test the impact of a short-term reduction in pH levels which can, for instance, occur as a result of anthropogenic acidification (Leduc et al., 2013). In the second experiment, fish inhabiting lochs of naturally varying acidity on the island of North Uist, Scotland (MacColl et al., 2013) were used to test the effect of water pH on the social interactions of individuals exposed to natural acidity over evolutionary time scales. Previous studies have demonstrated consistent diet-mediated association preferences in this species (Ward, Hart, & Krause, 2004; Atton et al., 2014; Kleinhappel et al., 2014b), with individuals within a shoal associating more frequently with others receiving the same diet, likely mediated by chemical cues. At least some of these studies (Kleinhappel et al., 2014b, 2016a, 2016b) were conducted in water with a relatively alkaline pH, and so we predict that low water pH will disrupt the chemically mediated association preferences induced by different diet treatments, while these will be unaffected at higher pH.

Methods

Experiment 1

Subjects

Adult three-spined sticklebacks (*Gasterosteus aculeatus*) were caught from streams and drainage ditches in Lincolnshire, UK (grid reference: TF 44880 95176) during July 2013 using dip nets, and were housed in the aquatics facility at the University of Lincoln (UK). Fish were kept in mixed sex shoals in four separate 130 l holding tanks at a density of approximately 40 fish per tank (0.3 fishL^{-1}). Holding tanks were filled with filtered and aerated tap water (which maintained a stable pH between 8.2 and 8.4 over time) and contained small plastic plants as enrichment. Fish were kept under a natural photoperiod and water changes were performed daily. Fish in two of the tanks were fed daily with *Daphnia*- and those in the other two with bloodworm (Chironomidae larvae)-based artificial diets (for full details see Kleinhappel et al., 2014b). Fish were kept under these feeding conditions for a minimum of 21 days before the start of the experimental trials. Prior to the start of the testing, fish were non-invasively tagged with a circular barcoded tag (5mm diameter) attached to one of their three dorsal spines and their standard length was measured (for details see Kleinhappel, Al-Zoubi, Al-Diri, Burman, Dickinson, John, Wilkinson, & Pike, 2014a). Males with visible breeding colouration and gravid females were not used for the data collection, however they remained in the holding tanks together with the fish used in the behavioural testing.

Study design

Shoals consisted of three size-matched fish (± 3 mm), selected pseudorandomly (depending on their size) from different holding tanks. We made no differentiation between males and females during the study. During data collection each group consisted of two fish fed with the same diet and one fish fed on a different one, counterbalanced over diet conditions. Shoals were randomly assigned to one of two treatment groups: (1) the low pH condition in which fish were tested in water with a pH ranging between 6.2 to 6.4 (measured using a Benchtop digital pH meter), and (2) the high pH condition in which fish were tested in a pH ranging between 8.3 to 8.5, which was comparable to their normal housing water and the water used in previous experiments (pH range 8.2 to 8.4, unpublished data) (Kleinhappel et al., 2014b, 2016a, 2016b). Experiments were conducted in artificial freshwater, the preparation of which was varied in order to manipulate the water pH.

Artificial freshwater was prepared using the guidelines from the United States Environmental Protection Agency (EPA 2002), which describes standardised methods for producing soft and hard water, which vary in pH but otherwise differ only in the concentration of dissolved minerals. A pH of 6.3 ± 0.1was used for the low pH water treatment, and a pH of 8.4 ± 0.1 for the high pH water treatment.

To prepare the artificial water $\mathrm{MgSO_4}$ (low pH: 7.5 mg/l; high pH: 240 mg/l), $\mathrm{NaHCO_3}$ (low pH: 12 mg/l; high pH: 384 mg/l) and KCl (low pH: 0.5 mg/l; high pH: 16 mg/l) were dissolved in 145 L deionized water and aerated for 12 hours at room temperature. $\mathrm{CaSO_4}$ (low pH: 7.5 mg/l; high pH: 240mg/l) was then dissolved separately in 5 L of deionized water and added. The combined solutions were aerated for an additional 24 hours to stabilize the pH of the medium. The prepared water was then used for housing and testing the fish in the subsequent experiment. The pH of the water was measured daily for two weeks prior to testing to confirm it was stable (low pH: 6.3 ± 0.1; high pH: 8.4 ± 0.1).

Prior to testing, fish were gradually acclimatised over 48 hours to the new water conditions as follows: Depending on the treatment and their final day of testing, fish were transferred from their housing tanks in separate small bare (unenriched) tanks (5 litres) containing a mixture of normal housing water mixed in a ratio of 50:50 with either low pH artificial water, resulting in a pH ranging between 7.5 and 7.7, or high pH artificial water, resulting in a pH ranging between 8.0 and 8.2 (approximately matching the standard pH of their usual housing water). After 24 hours individuals were transferred into further tanks containing only the low or high pH water. They had a further 24 hours to acclimatise before testing. All fish continued to receive their initial (*Daphnia*- or bloodworm-based) diet and were kept in separate tanks in order to control for the impact of possible familiarity between individuals on behaviour in the later observations (Griffiths, 2003). These tanks were opaque and so fish in the different tanks were visually and olfactorily separated from each other.

Behavioural assay

Experimental groups consisted of unique trios of fish, two fed the same diet and the remaining one on a different diet (*i.e.* two bloodworm fed fish and one *Daphnia* fed fish or *vice versa*), with each fish being drawn randomly from different tanks to control for potential familiarity (Griffiths, 2003). At the beginning of each experimental trial, groups were placed into the centre of a black circular testing tank (30cm in diameter) which was filled with 8 cm depth of either high or low pH artificial water, depending on the treatment. Water in the testing tanks was maintained at a constant 11 ºC by placing the experimental tanks within a water bath containing circulating chilled water. A webcam (Microsoft LifeCam) was mounted at the top of each tank and used to collect images every 16 seconds (Kleinhappel et al., 2014b, 2016a, 2016b) for one hour, using custom written Matlab (Mathworks, Natick, MA) code. Image collection started immediately after releasing the groups. A total of 20 replicates for each pH treatment were collected, these were counterbalanced for the diet treatments (i.e. ten replicates with two bloodworm fed fish and one *Daphnia* fed fish or *vice* *versa* for both the high and low pH treatment). A total of 120 fish were therefore used for this study. Individual fish were tested only once.

The individual position of each fish in all images was extracted by finding the spatial coordinates of the centre of their tag, and used to calculate the Euclidean distance between individuals. We assumed that two individuals were associating if they were within two body lengths of each other (i.e. within 64 mm, twice the mean body length of all fish from the study which was 32 ± 1.4 mm). This distance is within the range of inter-individual distances observed in free-ranging shoals (Pitcher & Parrish, 1993) and has previously been used to characterise social interactions in sticklebacks (Kleinhappel et al., 2014b, 2016a). The experimenter was blind to the diet treatments when extracting proximity data from the recorded images as the barcoded tag of individual fish contained no information on the diet treatment they were assigned to.

Two different metrics were subsequently extracted from the proximity data. First, we quantified the overall proportion of time fish spent associating, in order to assess whether our experimental treatments caused measurable differences in sociability (i.e., the tendency to interact with other fish, regardless of which individuals are being interacted with). Specifically, this was defined as the overall proportion of time in which at least one pair of fish was observed to be interacting. However, this tells us nothing about the precise pattern of associations, as equally sociable groups could arise through either random or directed interactions. To quantify the pattern of interactions we therefore constructed weighted social networks using the half weight index (HWI; see Kleinhappel et al., 2014b for details), which quantifies the strength of associations between all pairs of individuals within a group over the time of observation, i.e. a high HWI between any two individuals indicates a high frequency of interactions, while a low HWI which indicates low frequency of interactions. This allowed us to quantify the difference in association patterns depending on the different diet treatments.

Experiment 2

Subjects

Adult three-spined sticklebacks were captured at the end of September and the beginning of October 2013 from ten discrete lochs on the island of North Uist, Scotland, which were characterised as low pH (6.0 to 6.5) and high pH (8.3 to 8.5) habitats (see Table S1). A total of 150 fish, 15 fish per loch, were caught using minnow traps and dip nets. Fish were housed outside in replicated holding tanks (4 tanks per loch, 30 cm in diameter, without enrichment) at a density of 3-4 fish per tank containing aerated water from the source loch, taken from the same location as the fish. We made no differentiation between males and females during the study. Fish were held for 24 hours before testing, during which they were fed to satiation with either frozen bloodworm or *Daphnia*. Although this feeding period was shorter than that used in the laboratory study, it is known to be long enough to elicit diet-mediated shoal choice preferences in this species (Ward, Holbrook, Krause, & Hart, 2005). We also expect no systematic differences between fish from different lochs as Ward et al. (2005) have shown that direct experience, for instance resulting from previous encounters in the source habitat, has little to no impact on their shoal choice preferences when compared to dietary cues.

Behavioural assay

As with the previous experiment, proportion of time fish spent interacting and inter-individual patterns of association were assessed in free swimming fish shoals. Experimental groups consisted of unique trios of fish from the same loch, two fed the same diet and the remaining fish was fed with the alternate diet (e.g. two fish fed with *Daphnia* and one with bloodworm or *vice versa*). Size matched fish (± 3 mm) were drawn pseudorandomly from the different holding tanks and used only once during the study. For data collection, groups were transferred into a grey testing tank (30 cm in diameter) filled with 8 cm depth of their natural habitat water, which was maintained at 10 ± 2 ºC. A camcorder (JVC Everio GZ-MG230) was positioned above each tank for data collection. Tagging fish for individual identification (Kleinhappel et al., 2014a) was not possible, as sticklebacks from the low pH lochs showed a reduction in dorsal and ventral spines (for examples see Figures in Giles, 1983; Magalhaes, D'Agostino, Hohenlohe, & MacColl, 2016). Therefore, each fish was placed into a transparent cylindrical plastic compartment (5 cm in diameter) with colour markings on the top identifying the specific diet treatment. Cylinders were then removed and a video was taken for 60 minutes (1920 × 1080 resolution at 25 frames per second). Data was collected for a total of 40 groups, four replicate groups per loch with 20 replicates for low pH lochs and 20 for the high pH lochs. For each of the lochs, two trios consisted of two bloodworm and one *Daphnia* and the remaining two trios of two *Daphnia* and one bloodworm fed fish.

The position of the fish in each video was manually extracted every 20 seconds by following the movements of each individual fish between sampling points, starting with the known position of each colour-marked cylindrical plastic compartment which was unambiguously identified in the video. Any ambiguities were resolved using the length of each fish. The coordinates of all individuals at each time point were used to compute the Euclidean distance between each fish in the group. As for the first experiment two different metrics were extracted from the proximity data, the overall proportion of time spent interacting and the HWI as a measure of intra-group association patterns.

Data analysis

Differences in the overall proportion of time spent interacting between pH treatments were tested using a two sample t-tests, with logit-transformed data (Warton & Hui, 2011), while differences in diet-mediated association preferences as a function of water pH were tested using permutation tests (Kleinhappel et al., 2014b). We initially tested whether there were any overall differences in association patterns between the low and high pH conditions and, if there were, went on to test for differences within each of the two conditions separately. In these permutation tests, one fish from the two on the same diet treatment was randomly chosen and assigned as the focal fish, and the difference in HWI between this focal fish with the other two individuals (one on the same diet, one on the different diet) was calculated. This was performed for each of the groups in both treatments and the overall mean difference was calculated. This was repeated 10,000 times and the proportion of mean differences less than zero was used to compute the p-value for the test (Kleinhappel, et al. 2014b). When analysing the data from the second experiment, we only permuted within groups from the same loch to control for loch identity (Good, 2013).

Ethical note

All methods used in this study adhered to the ASAB Guidelines for the Use of Animals in Research and gained local institutional ethical approval (UoL 13/45).

Results

Experiment 1

There was no significant difference in the overall proportion of time fish spent associating between the low and the high pH treatment (two-sample t-test: $t = -0.42$, $df = 38$, $p = 0.674$,

Figure 1a). However, the precise pattern of social interactions differed significantly between the two pH treatments (permutation test, $p = 0.026$) with fish tested in the high pH freshwater showing a significant association preference for fish on the same diet treatment (permutation test, $p < 0.001$) while fish tested in the low pH freshwater showed no significant preference for individuals on either diet (permutation test, $p = 0.383$) (Figure 1b).

Experiment 2

As in the first experiment, there was no significant difference in the overall proportion of time fish associated between the low pH and the high pH lochs ($t = -0.45$, $df = 38$, $p = 0.652$; Figure 1c) although the pattern of social interactions differed significantly in direction between the two pH treatments (permutation test, $p = 0.001$). Specifically, fish tested in their naturally low pH water spent significantly more time associating with fish on the same diet treatment as themselves (permutation test, $p = 0.003$), while individuals from the natural high pH water associated significantly more often with fish on the different diet treatment (permutation test, $p = 0.036$) (Figure 1d).

Discussion

The results of this study provide clear evidence that the pH of the water can affect inter-individual shoaling preferences in three-spined sticklebacks. In the first experiment, the artificially reduced water pH disrupted the diet-mediated inter-individual association preferences that have been shown in shoal-choice tasks (Ward et al., 2004; Webster, Goldsmith, Ward, & Hart, 2007), in free shoaling fish (Atton et al., 2014; Kleinhappel et al., 2014b, 2016a, 2016b) and that were also observed in individuals tested in the high pH condition. Interestingly, only the within-group structure of the shoals was affected by the pH manipulation, there was no difference in the overall proportion of time individuals associated with each other.

It has been suggested that behavioural impairment towards chemical cues caused by freshwater acidification can either be mediated by the molecular change of the chemical cues themselves (Brown, Adrian, Lewis, & Tower, 2002; Leduc, Roh, Macnaughton, Benz, Rosenfeld, & Brown, 2010) or caused by a disruption of the fishes’ chemosensory receptor systems (Royce-Malmgren & Watson, 1987; Tierney & Atema, 1988; Moore, 1994; Brown et al., 2002). Free amino acids have been postulated to underpin diet-mediated interactions within shoaling fish (Atton et al., 2014; Kleinhappel et al., 2014b, 2016a) and, as the isoelectric point of different free amino acids lie at different pH values (Lehninger, Nelson, & Cox, 2008), pH could change the charge of the amino acid which could then interfere with the amino acid receptor interaction (Royce-Malmgren & Watson, 1987). An artificial change in pH has indeed been shown to change the behaviour, such as attraction or avoidance, of fish towards free amino acids (Royce-Malmgren & Watson, 1987). This suggests that an acute change in water pH could impact on the way free amino acids are perceived by fish and, as a result, affect their association preferences. In relation to this, a recent study in ocean acidification has shown that exposure to high levels of CO_2 can affect the sensitivity to chemical cues in a marine fish species by compromising both the olfactory system and central brain function (Porteus, Hubbard, Webster, Aerle, Canário, Santos, & Wilson 2018).

Contrary to our findings with the artificial freshwater, in our second experiment, individuals from naturally low pH loch were able to use diet-derived chemicals to mediate their association preference when tested in low pH water (cf. Kleinhappel et al., 2014b, 2016a, 2016b). The acidity in the low pH lochs at North Uist most likely results from humic acid, derived from decaying organic matter, being surrounded by peat-bogs (Giles, 1983). Humic acid, that can consist of several different organic acids derived from degraded organic matter (Steinberg, 2013), is known to affect a variety of biological processes (Sato, Ose, Nagase, & Hayase, 1987; Lovley, Coates, Blunt-Harris, Phillips, & Woodward, 1996; Qiao & Farrell, 2002) and can cause a reduction in the chemosensory responses toward reproductive pheromones (Hubbard, Barata, & Canario, 2002; Mesquita, Canario, & Melo, 2003). However, the findings of our study show that the low pH of the fishes' habitat does not intrinsically cause a disruption in their chemically mediated shoaling decisions, which suggests that the fish adapted to their environment.

Surprisingly, fish from the high pH lochs, showed diet-mediated preferences for the fish from the different diet treatment, this stands in contrast to a variety of studies conducted in this species (Ward et al., 2004; Webster et al., 2007; Atton et al., 2014; Kleinhappel et al., 2014b, 2016a, 2016b). This result was unexpected as the average water pH of 8.4 (± 0.1) was similar to that of previous studies (Kleinhappel et al., 2014b, 2016a, 2016b) and as the main aim of this study was to look at the impact of a reduced pH on individual associations, should have served only as a control compared to the low pH water due to it's similarity with previous studies. One possible explanation could lie in the different habitats in which the fish live, these vary in factors such as food patch availability and food abundance (MacColl et al., 2013), which could make assorting with different, or unfamiliar smelling, fish more adaptive. Juvenile sticklebacks, for instance, prefer unfamiliar over familiar kin if hunger levels are high, which might be an adaptation for avoiding food competition with familiar individuals (Frommen, Luz, & Bakker, 2007). Alternatively, (Spence, Wootton, Barber, Przybylski, & Smith, 2013) found significant differences in personality traits (boldness and shyness) between the different stickleback populations inhabiting the different pH lochs. Personality traits have been shown to influence animal social network structures in variety of species including fish (e.g. Pike, Samanta, Lindstrom, & Royle, 2008; Croft, Krause, Darden, Ramnarine, Faria, & James, 2009) and could be a possible underlying reason for the difference in the association preferences present in this study, although this was not tested in this study. The salience of different mechanisms mediating inter-individual association preferences, e.g. individual personalities, familiarity or chemical cues, on the social structure of shoaling fish needs to be further investigated. Interestingly, (Heuschele & Candolin, 2007) found that an increase of pH enhanced the use of male chemosensory cues, which consisted of various peptides and amino acids, suggesting that a change in pH could facilitate the binding or transportation of olfactory cues. The water conditions in the high pH lochs could therefore have enhanced other chemical cues from the diet treatments that changed the association preferences of individuals.

Although three-spined sticklebacks are able to adapt to a variety of habitats, and the water pH used in the first experiment still lies within the range of stickleback natural habitats (MacColl et al., 2013), reducing the pH of the water can impact on their chemical communication, whilst fish living in low pH water conditions for multiple generations are able to utilise diet-derived chemical cues to mediate their association decisions. This suggests that short-term reductions in water pH can have important implications on social structures in shoaling fish even though they can adapt, given sufficient time, to a variety of habitats and it is therefore likely to impact on their survival and fitness. Our results are comparable with larger scale studies investigating the effects of natural and anthropogenic acidification on species diversity, in which natural acidity had less impact on community structures, which is most likely due to the adaptation of organisms exposed to natural acidity over evolutionary time scales (Petrin et al., 2008). The present findings provide insight into the impact that anthropogenic interference can have on the ecosystem and behaviour of aquatic animals (Johnson & Webster, 1977; Muniz, 1990; Ikuta et al., 2003; Leduc et al., 2013), especially if such changes are relatively quick.

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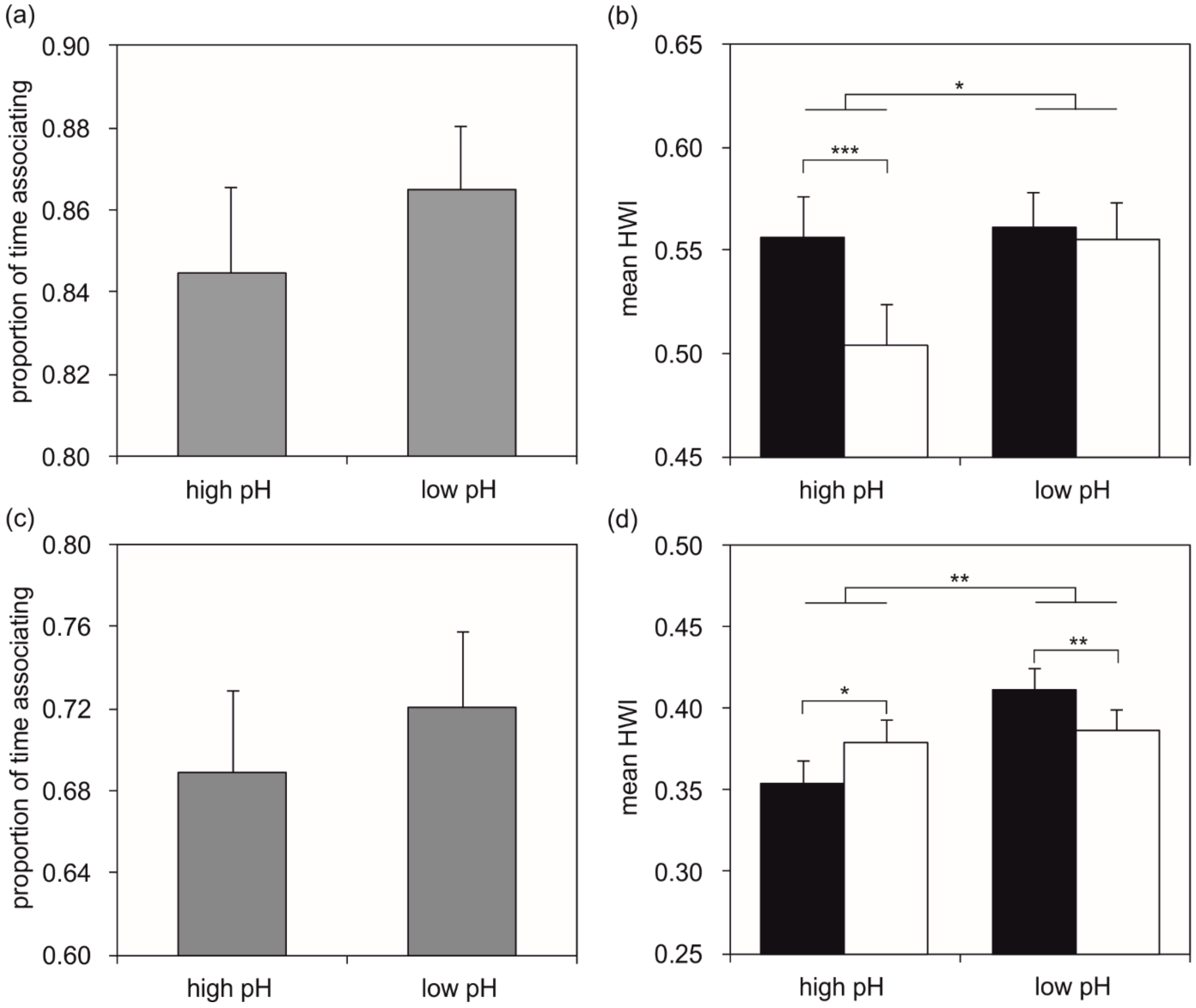
Figure 1. (a, c) Mean ± SE proportion of time two or more fish were in association during the 60 min of observation when tested in water that (a) experimentally or (c) naturally varied in its pH. (b, d) Mean ± bootstrap SE half weight index (HWI) between fish on either the same (black bars) or different (white bars) diet treatments when tested in water that varied (b) experimentally or (d) naturally in pH. Asterisks (*) denote significant differences between groups: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 1.


(a)
0.90
0.88
0.86
0.84
0.82
0.80
proportion of time associating
high pH
low pH
(b)
0.65
0.60
0.55
0.50
0.45
mean HWI
*
***
high pH
low pH
(c)
0.80
0.76
0.72
0.68
0.64
0.60
proportion of time associating
high pH
low pH
(d)
0.50
0.45
0.40
0.35
0.30
0.25
mean HWI
**
*
**
high pH
low pH