Behavioural and physical effects of arsenic exposure in fish are aggravated by aquatic algae

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A B S T R A C T

Arsenic contamination has global impacts and freshwaters are major arsenic repositories. Arsenic toxicity depends on numerous interacting factors which makes effects difficult to estimate. The use of aquatic algae is often advocated for bioremediation of arsenic contaminated waters as they absorb arsenate and transform it into arsenite and methylated chemical species. Fish are another key constituent of aquatic ecosystems. Contamination in natural systems is often too low to cause mortality but sufficient to interfere with normal functioning. Alteration of complex, naturally occurring fish behaviours such as foraging and aggression are ecologically relevant indicators of toxicity and ideal for assessing sublethal impacts. We examined the effects of arsenic exposure in the invasive mosquitofish, Gambusia holbrooki, in a laboratory experiment incorporating some of the complexity of natural systems by including the interacting effects of aquatic algae. Our aims were to quantify the effects of arsenic on some complex behaviours and physical parameters in mosquitofish, and to assess whether the detoxifying mechanisms of algae would ameliorate any effects of arsenic exposure. Aggression increased significantly with arsenic whereas operculum movement decreased non-significantly and neither food capture efficiency nor consumption were notably affected. Bioaccumulation increased with arsenic and unexpectedly so did fish biomasses. Possibly increased aggression facilitated food resource defence allowing fish to gain weight. The presence of algae aggravated the effects of arsenic exposure. For increase in fish biomass, algae acted antagonistically with arsenic, resulting in a disadvantageous reduction in weight gained. For bioaccumulation the effects were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. We highlight that multidisciplinary, cross-taxon research, particularly integrating behavioural and other effects, is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

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1. Introduction

Arsenic (As) from both anthropogenic and natural sources has global impacts (Mandal and Suzuki, 2002; Nordstrom, 2002; Rahman and Hasegawa, 2012; Rahman et al., 2012; Smedley and Kinniburgh, 2002) and aquatic systems, including freshwaters, are major repositories for arsenic (Nordstrom, 2002; Smedley and Kinniburgh, 2002). Although some national and international standards are in effect, for example the World Health Organization safe limit for drinking water is 10 μg L⁻¹ (Smith et al., 2002), the toxicity of As is dependent on numerous interacting factors such as its source, concentration and bioavailability; environmental parameters; and organisms’ resistance ability and detoxifying mechanisms (Mandal and Suzuki, 2002; Rahman and Hasegawa, 2012; Smedley and Kinniburgh, 2002). A key factor is its chemical speciation. Inorganic As (iAs) is generally more toxic than organic As, while of the iAs species, arsenite (As³⁺) is more toxic than arsenate (As⁵⁺). However, the organic methylated species (dimethylarsinous acid, DMAA³⁻, and dimethylarsonious acid, MMAA³⁻) are more toxic than their iAs parent compounds (Rahman et al., 2012; Smedley and Kinniburgh, 2002). Quantifying total arsenic in environmental and biological samples is therefore not synonymous with assessment of associated risks. The main chemical species in freshwaters are inorganic arsenics but
methylated and other organic As species are also found (Rahman and Hasegawa, 2012; Rahman et al., 2012). Freshwater ecosystems are extensive and highly dynamic (Moss, 1998) which together with the variable nature of As toxicity makes effects difficult to estimate (Rahman et al., 2012; Smedley and Kinniburgh, 2002; Smith et al., 2002). However, assessment and prediction are essential. In addition to providing water and nutrients for human consumption (Mandal and Suzuki, 2002; Smith et al., 2002; Villéger et al., 2012), freshwater ecosystems may themselves suffer severe impacts from arsenic toxicity (e.g. Rahman and Hasegawa, 2012; Rahman et al., 2012; Scott and Sloman, 2004; Smedley and Kinniburgh, 2002).

Biological activity plays a vital role in As speciation, distribution and cycling in freshwaters (Rahman and Hasegawa, 2012; Rahman et al., 2012). Organismal uptake of arsenic may be direct, through ingestion, inhalation and absorption, or indirect through the food chain (Mandal and Suzuki, 2002; Moss, 1998; Smedley and Kinniburgh, 2002; Smith et al., 2002). Aquatic plants (and bacteria) have important functions in these processes through bio-transformation of As species (Hellweger and Lall, 2004; Rahman and Hasegawa, 2012; Rahman et al., 2012). As\(\text{III}\), the stable and predominant species of arsenic in aquatic environments, has chemical and structural similarities to phosphate (PO\(\text{4}^{3-}\)). Algae mistakes As\(\text{III}\) for PO\(\text{4}^{3-}\) and actively absorb it via the same pathways. Once inside the algal cells, As\(\text{III}\) becomes toxic as this resemblance breaks down, and algae reduce As\(\text{III}\) to As\(\text{II}\), methylate it to MMA\(\text{III}\) and DMA\(\text{AIII}\), and excrete it mostly as As\(\text{V}\) and/or DMA\(\text{AIII}\), which is thought to be a detoxifying mechanism (Hellweger and Lall, 2004; Rahman and Hasegawa, 2012; Rahman et al., 2012). Several factors influence this process. Different algal species have different methylation abilities (Rahman and Hasegawa, 2012) and tolerances to As\(\text{V}\) (e.g. Fatas et al., 2012; Levy et al., 2005; Wang et al., 2013), and not all algae can excrete As\(\text{III}\). For example, both Chlorella sp. and Monoraphidium arcautum take up As\(\text{III}\) and reduce it to As\(\text{II}\) but only M. arcautum excretes it (Levy et al., 2005). Moreover, recent studies indicate that methylation may not be the primary mode of detoxification in freshwater algae. Instead, arsenic is taken up by cells using the phosphate transport system, reduced to As\(\text{II}\) in the cell, and then excreted into the growth medium, probably by an active transport system (Levy et al., 2005; Wang et al., 2013). For example, after exposing Chlamydomonas reinhardtii and Scenedesmus obliquus to different arsenate concentrations, no methylated species could be detected (Wang et al., 2013). Similarly, arsenate and arsenite were the dominant species in the freshwater algae Synechocystis and C. reinhardtii (Yin et al., 2011, 2012). This transformation reaction is suggested to be correlated with algal growth rate and P nutrient status, leading to almost complete methylation under P-limiting conditions and slower methylation and excretion of As\(\text{III}\) into the media if P is in excess (Hellweger and Lall, 2004). Nonetheless, these studies confirm that P has a key role in arsenic toxicity and that biotransformation of As by algae is a central component of aquatic As cycling. Indeed, the use of algae is often advocated for bioremediation of As contaminated water (e.g. Levy et al., 2005; Fatas et al., 2012; Rahman and Hasegawa, 2012; Rahman et al., 2012; Wang et al., 2013).

Fish are a key constituent of aquatic ecosystems and are involved in As mobilization. They are an important component of the aquatic food chain (Agah et al., 2009; Kumar and Banerjee, 2012; Zhang et al., 2013) and even small fish are a source of protein for human consumption (e.g. Moeller et al., 2003). Some fish are also used as bioindicators of various aquatic pollutants (Bhattacharya et al., 2007; Moeller et al., 2003; Moss, 1998; Scott and Sloman, 2004). Bioaccumulation of arsenic in fish occurs directly through absorption across the gills or skin and indirectly via consumption of prey (Rahman et al., 2012); and inorganic, methylated and other organic arsenicals are all found in various fish species (Rahman et al., 2012; Rahman and Hasegawa, 2012). The effects of arsenic toxicity have been examined in numerous species worldwide. For example, bioaccumulation of arsenic has been recorded in fish from California (Moeller et al., 2003), sub-Saharan Africa (Ouedraogo and Amyot, 2013), India (Kumar and Banerjee, 2012), France (Noël et al., 2013), China (Zhang et al., 2013) and the Persian Gulf (Agah et al., 2009). However, most research has focused on parameters such as bioaccumulation, and physiological parameters such as growth (e.g. Kumar and Banerjee, 2012) and metabolic and histopathological effects (e.g. Ahmed et al., 2013; Bhattacharya et al., 2007). One factor that has received much less attention is fish behaviour (e.g. Scott and Sloman, 2004; Weis and Candelmo, 2012; Weis et al., 2001). Contamination in natural systems is often at concentrations well below those that cause mortality, but even low levels of toxicity may be sufficient to interfere with normal functioning. Fish behaviour is ideal for assessing these sublethal impacts (Moss, 1998; Scott and Sloman, 2004; Weis and Candelmo, 2012). Much of the current research focuses on direct behavioural responses to contaminants, for example, avoidance of contaminated sites, respiratory changes and behaviour like body tremors associated with illness. However, alteration of complex, naturally occurring behaviours such as foraging and predation, agonistic interactions, shoaling and reproductive behaviours are more ecologically relevant indicators of toxicity (Scott and Sloman, 2004; Sopinka et al., 2010; Weis et al., 2001). Various environmental toxicants have been shown to affect complex behaviours (reviewed in Atchison et al., 1987; Scott and Sloman, 2004). Arsenic in particular reduces long-term memory in the zebrafish, Danio rerio (de Castro et al., 2005) and is part of a cocktail of chemicals that affects aggressive interactions in the round goby, Neogobius melanostomus (Sopinka et al., 2010). However, the effects of arsenic on fish behaviour have received little attention to date: arsenic is not listed in Scott and Sloman’s (2004) comprehensive review of contaminant effects on fish behaviour. Given the global impacts of arsenic toxicity (e.g. Mandal and Suzuki, 2002; Smedley and Kinniburgh, 2002; Rahman et al., 2012) more work is needed in this field.

In this study, we examined the effects of arsenic on complex behaviours in the invasive mosquitofish, Gambusia holbrooki. This small fish has been introduced worldwide, primarily for mosquito control (Lever, 1996; Pyke, 2008). Although highly tolerant of a variety of stressors (e.g. Evans et al., 2011; Staub et al., 2004; Uliano et al., 2010), G. holbrooki and the closely related Gambusia affinis have been used in toxicity studies (e.g. Tata et al., 1999, 2001) and are known to be affected by arsenic (e.g. Moeller et al., 2003; Newman et al., 1989). As behaviour links physiological functions with ecological processes, an understudied field of research (e.g. Scott and Sloman, 2004; Weis et al., 2001), we also included physiological parameters to assess interrelated effects of arsenic toxicity. Moreover, given the intricacies of the feedback and cycling interactions contributing to arsenic toxicity (e.g. Scott and Sloman, 2004; Weis et al., 2011), field studies may be more general and realistic about environmental effects (Moss, 1998), while laboratory studies allow more controlled quantification of effects, and both provide valuable insight (Weis and Candelmo, 2012). Therefore, we also examined the interacting effects of naturally occurring algae, thus incorporating some of the complexity of natural systems in a laboratory experiment and disentangling some specific processes from whole ecosystem effects.

We addressed two main aims: first to quantify the effects of arsenic on G. holbrooki, and second to assess the interacting effects of algae on arsenic toxicity in this fish species. We examined one direct behavioural response to stress, opercular ventilation rate (Brown et al., 2005; Hawkins et al., 2004), predicting that opercular movement would increase in response to the stress of arsenic exposure; and two complex behaviours, aggression and foraging. As both stress (Folkedal et al., 2012) and physiological effects of contaminants (Weis et al., 2001) can reduce feeding ability.
and motivation, we predicted that food capture and consumption would be decreased with arsenic exposure. For aggression the effects of toxicant exposure are more ambiguous, provoking both increases and decreases in aggression (Scott and Sloman, 2004; Sopinka et al., 2010) so while we expected to see a difference with arsenic exposure we made no directional predictions. Then, for physical parameters, we predicted that fish would gain less weight (e.g. Kumar and Banerjee, 2012) but increase bioaccumulation (Scott and Sloman, 2004) in the presence of arsenic. Finally, given the various and interrelated influences on algal arsenic detoxification capacity we hypothesized that freshwater algal communities will affect $\text{As}^{\text{V}}$ toxicity to fish, but the direction of effects is, a priori, difficult to predict.

2. Methods

2.1. Experiment

Mosquitofish were collected from the Ter (42.0451° N, 3.1960° E), Fluvia (42.1875° N, 3.0851° E) and Muga (42.2527° N, 3.0756° E) rivers and transported to the laboratory where they were placed in 60L stock aquarium (60 cm x 30 cm x 32 cm) each containing a gravel substrate, conditioned water and a filtered air supply. $G.\ holbrooki$ from all three rivers were housed together. Aquaria were maintained at 25 ± 1°C and a constant photoperiod (12:12 light:dark cycle) using 6W bulbs. Fish were fed to satiation once per day with commercial food flakes or frozen bloodworms (Chironomus spp.) and were able to acclimate to laboratory conditions for at least 6 months, with a further month to acclimate to experiment-specific environmental parameters (e.g. temperature: see below).

For the experiment, 12 independent sets of apparatus (experimental units) were set up (Fig. 1). A large (sump) tank (60 cm x 25 cm x 75 cm) was filled with 90L of filtered water. A smaller (fish) tank (31.5 cm x 11 cm x 31.5 cm) containing 4L of filtered water was placed on top, and above this was placed a channel (90 cm x 8.5 cm x 7.5 cm) containing sandblasted glass tiles (1 cm$^2$) to provide substrate for the algal biofilm. 10 $\mu$g L$^{-1}$ each of phosphate and silicate were added once per week to reproduce phosphate limiting conditions for algal growth, i.e. stationary growth phase (Hellweger and Lall, 2004; Moss, 1998; Rahman and Hasegawa, 2012), and to facilitate diatom growth respectively. Water was pumped from the large tank to the head of the algal biofilm channel, passed through this channel into the fish tank, circulated in the fish tank then passed through the overflow back into the sump tank (Fig. 1). The overflow was covered with a fine mesh to prevent algae and fish from leaving via this route. Water levels were monitored throughout the experiment. Water pH was maintained at 7.5 using a pH control system based on CO$_2$ addition (JBL Profiora mi30: JBL, Ludwigshafen, Germany) to provide enough inorganic carbon for algal growth (Favas et al., 2012; Smedley and Kinniburgh, 2002). Illumination (12 h light: 12 h dark) was provided by 120 W LED Grow Lights (Lightech, Girona, Spain) which produce light without heat, and temperature was maintained at 19.5 ± 5°C. This is quite a low temperature for mosquitofish, but well within their tolerance range (Evans et al., 2011), and was necessary for algal growth. The experimental units were left to condition for 1 week prior to the start of the experiment.

Natural algal inocula were obtained from the Llèmena stream, a tributary of the Ter River, by scraping three cobbles from the upstream zone which has minimum human impact (see Serra et al., 2009). On day 1 of the experiment, and at weekly intervals during the following 19 days, the inocula were added to the channels of half of the experimental units so that biofilm was able to colonize the glass tiles. On day 20, 130 $\mu$g L$^{-1}$ of arsenate was added to the sumps of half of the experimental units. $\text{As}^{\text{V}}$ was used as this is the most common arsenic species in freshwater and is the species that is taken up by aquatic algae (Hellweger and Lall, 2004; Rahman and Hasegawa, 2012; Rahman et al., 2011). This gave 3 replicates of 4 experimental conditions: control (C) with neither $\text{As}^{\text{V}}$ nor biofilm, biofilm (B), arsenic (A) and biofilm with arsenic (B+A). On day 24, all fish were weighed to the nearest mg using a balance and total length (TL) was measured to the nearest mm using a ruler. Four fish were added to each experimental unit: 1 male (26.8 ± 2.89 mm TL; mean ± standard deviation) and 3 females (1 small: 28.6 ± 5.51 mm TL; 1 medium: 39.4 ± 1.78 mm TL; 1 large: 45.3 ± 2.96 mm TL). This sex ratio was chosen to reduce sexual harassment of females by males (Evans et al., 2011; Meffe and Snelson, 1989) and as fish numbers were limited. Different sized females were used primarily to allow identification of individuals within a tank so any overlap in sizes between tanks was tolerated. Video recorded observations began on day 25 and continued for 9 days during which arsenic was measured every day and phosphate was measured every 3 days (Table 1). The video camera was placed approximately 50 cm in front of the narrow sides of the fish tanks. Pilot observations showed that fish were not disturbed by the camera. Each day one 10-min video was taken of each tank. Immediately following this, five defrosted frozen bloodworms were added sequentially to each tank such that one prey was consumed before the next was added (also videoed). The order in which tanks were videoed was randomized daily. After observations, all fish were fed to satiation. Any excess food was removed after 1 h and fish were left until the following day. Any fish that died during the experiment (n = 4) were replaced immediately with a same sex, similar sized individual. This occurred only in the first three days of experiments and in all cases except one were males.

After the final observations, all fish were euthanized using an overdose of anaesthetic (clove oil) and weighed and measured as before. Liver and gills were dissected out of each female for analysis of tissue arsenic accumulation. These organs were selected as both are crucial sites of metabolic activity so are likely to accumulate arsenic (e.g. Ahmed et al., 2013; Kumar and Banerjee, 2012). Only females were used for this analysis to avoid biases due to sex differences in bioaccumulation, and as it requires a minimum amount of tissue the single male in each tank was unlikely to be sufficient. To quantify the total amount of As accumulated in fish, the dissected samples were frozen, then freeze dried, then digested with 4 ml of concentrated HNO$_3$ (65% HNO$_3$, Suprapur, Merck, Germany) and 1 ml of H$_2$O$_2$ (31% H$_2$O$_2$, Suprapur, Merck, Germany). Next, a 75-times dilution with milliQ water and acidification (1%) of the samples was performed. Digested samples were analyzed following the procedure used for total As in water. Bioaccumulation was expressed as dissolved As per dry weight ($\mu$g As g DW$^{-1}$). Total dissolved As concentration was measured by ICP-MS (7500 Agilent Technologies, Inc., Wilmington, DE). The detection limit for As was 0.08 $\mu$g L$^{-1}$. Rh was used as the internal standard. The accuracy of the analytical method was checked periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway).

This work followed all national and institutional guidelines for animal experiments and every effort was made to ensure that suffering to the fish was minimized.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Total arsenic and phosphate concentrations ($\mu$g L$^{-1}$; mean ± standard deviation) during the 9 days of observations. For As: n = 9 and P: n = 3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Arsenic</td>
</tr>
<tr>
<td>Control</td>
<td>1.92 ± 0.09</td>
</tr>
<tr>
<td>Biofilm (B)</td>
<td>1.99 ± 0.11</td>
</tr>
<tr>
<td>Arsenic (A)</td>
<td>127.96 ± 5.55</td>
</tr>
<tr>
<td>B + A</td>
<td>124.19 ± 2.64</td>
</tr>
</tbody>
</table>
2.2. Video and statistical analyses

2.2.1. Direct behaviour

The frequencies of opercular movements were recorded for each individual by counting the number of times the operculum opened. As opercula were not always visible, this variable was recorded for a total of approximately 1 min and converted to opercula beats per minute for analyses. In a few cases the fish remained hidden throughout the observation for that day so these observations were excluded from analyses. To assess differences in aggression between treatments, opercula beats min\(^{-1}\) were used in a generalized estimating equation (GEE: an extension of generalized linear models developed for situations where response variables are correlated rather than independent). Experimental unit was the between subjects factor and time (day) was the within subjects factor for the model. The fully factorial analysis included two independent factors, presence and absence of biofilm and arsenic, and time was included as a covariate.

2.2.2. Complex behaviours

We recorded the frequencies of aggressive interactions initiated for each fish. These included lunges (rapid movement towards another fish without physical contact), chases (prolonged movement towards another fish with the recipient individual swimming away from the attacker), and bites (as lunges but with physical contact). As the largest female initiated almost all aggressive interactions in all tanks only these data were used for analyses. We then used the same model as above with number of attacks carried out by the largest female as the dependent variable.

Two foraging parameters were obtained: the time required to locate and capture each food item (capture efficiency), quantified as the interval between the food item touching the surface of the water and the first fish grasping the food; and the interval between capture and when each food item was fully consumed (consumption). The means of each of these variables in each tank for each day were calculated and used in separate GEEs as above.

2.2.3. Physical parameters

We also recorded two physical parameters. First, the change in biomass was obtained by subtracting the weight of each fish at the beginning of the experiment from its weight at the end. Any fish that had replaced a deceased individual were excluded from this analysis. These data were used as the dependent variable in a GEE with experimental unit as the between subjects variable and fish number within each tank as the within subjects variable. The final, factorial model included presence and absence of biofilm and arsenic as independent factors and total length of each fish as a covariate. Second, the tissue concentration of arsenic for the females in each tank was the dependent variable in a factorial generalized linear model (GLM) with the presence and absence of biofilm and arsenic, and the summed changes in biomass for all females in each tank (obtained from the previous analysis) as independent factors.

### Table 2

Results for the generalized estimating equations for variations in operculum movement (beats min\(^{-1}\)) and aggression. Significant results are highlighted in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Operculum movement</th>
<th>Aggression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald χ²</td>
<td>df</td>
</tr>
<tr>
<td>Biofilm (B)</td>
<td>2.977</td>
<td>1</td>
</tr>
<tr>
<td>Arsenic (A)</td>
<td>0.025</td>
<td>1</td>
</tr>
<tr>
<td>Time (T)</td>
<td>110.179</td>
<td>8</td>
</tr>
<tr>
<td>B × A</td>
<td>2.121</td>
<td>1</td>
</tr>
<tr>
<td>B × T</td>
<td>242.592</td>
<td>8</td>
</tr>
<tr>
<td>A × T</td>
<td>40.374</td>
<td>8</td>
</tr>
<tr>
<td>B × A × T</td>
<td>207.470</td>
<td>8</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic diagram of one of the experimental units (see main text for details). The dashed arrows show the direction of water flow.
Table 3
Results for the generalized estimating equations for variations in foraging parameters. Significant results are highlighted in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Capture Wald χ²</th>
<th>df</th>
<th>p</th>
<th>Consumption Wald χ²</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biofilm (B)</td>
<td>5.816</td>
<td>1</td>
<td>0.016</td>
<td>2.759</td>
<td>1</td>
<td>0.097</td>
</tr>
<tr>
<td>Arsenic (A)</td>
<td>0.601</td>
<td>1</td>
<td>0.438</td>
<td>1.075</td>
<td>1</td>
<td>0.300</td>
</tr>
<tr>
<td>Time (T)</td>
<td>25.578</td>
<td>8</td>
<td>-</td>
<td>51.362</td>
<td>8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B × A</td>
<td>0.013</td>
<td>1</td>
<td>0.909</td>
<td>6.611</td>
<td>1</td>
<td>0.010</td>
</tr>
<tr>
<td>B × T</td>
<td>10.303</td>
<td>8</td>
<td>0.001</td>
<td>7.205</td>
<td>8</td>
<td>0.515</td>
</tr>
<tr>
<td>A × T</td>
<td>8.315</td>
<td>8</td>
<td>0.403</td>
<td>6.690</td>
<td>8</td>
<td>0.570</td>
</tr>
<tr>
<td>B × A × T</td>
<td>20.873</td>
<td>8</td>
<td>0.007</td>
<td>13.325</td>
<td>8</td>
<td>0.101</td>
</tr>
</tbody>
</table>

Fig. 2. Mean opercular movements for all four fish in each tank. Trend lines illustrate the relationships between time and the presence and absence of biofilm and arsenic. C = control; B = biofilm; A = arsenic; B × A = biofilm × arsenic.

Analyses were conducted using SPSS v.20. All dependent variables were analyzed with normal distributions and identity link functions.

3. Results

3.1. Direct behaviour

Opercular movement was highest in the control and lowest with just biofilm present. Arsenic produced a lesser decrease in opercular movement whether or not biofilm was present (Fig. 2). Opercular movements increased significantly over time (Table 2, Fig. 2) and there was a significant interaction between time and all other variables while the presence of biofilm and arsenic and their interaction were non-significant (Table 2).

3.2. Complex behaviours

Aggression was lowest in the control. Although biofilm presence initially induced an increase in aggression, this appeared to be returning to the same level as the controls (Fig. 3). Aggression increased almost linearly in the presence of arsenic, and was highest in the presence of both arsenic and biofilm (Fig. 3). The frequency of aggression increased significantly with all three independent factors (Table 2, Fig. 3); however, while the interaction between time and arsenic presence was significant, that between time and biofilm presence was marginally non-significant (Table 2). All other interactions were non-significant (Table 2).

Time had the greatest effect on both foraging variables with capture interval generally significantly decreasing and consumption interval generally significantly increasing over time (Table 3, Fig. 4). However, capture interval increased significantly in the presence of biofilm (Table 3, Fig. 4a), though this may be an artefact resulting from unusually high values in one tank towards the end of the experiment which may have been caused by external disturbance. We retained this outlier in analyses to maintain sample size. The interaction between time, biofilm and arsenic presence was also significant while all other variables and their interactions were non-significant (Table 3). For food consumption interval the only other significant interaction was between the presence of biofilm and the presence of arsenic (Table 3), though again this may reflect the later high values for biofilm presence in one tank (Fig. 4b).

3.3. Physical parameters

All fish gained weight during the experiment (Fig. 5) and there was a significant positive relationship between weight gain and fish length (Table 4, Fig. 5). Biofilm alone showed no effect on weight gain (Table 4) though there was a significant interaction between these two variables (Table 4, Fig. 5a). However, the relationship is unclear. While weight gain increased with fish length, biofilm appears to affect smaller fish more than larger ones and the data is a widely scattered (Fig. 5a). Arsenic had a significant effect on weight gain and showed a significant interaction with both length and biofilm presence and the three-way interaction was likewise significant (Table 4). However, somewhat surprisingly weight gain increased in the presence of arsenic (Fig. 5b) and while the presence of biofilm to some extent appears to ameliorate this effect this is more apparent for smaller than larger fish (Fig. 5c).

For tissue arsenic bioaccumulation, all factors and their interactions were significant with the exception of the three-way interaction which showed just marginal significance (Table 4). Not surprisingly, bioaccumulation was higher when arsenic was added to the water and this increased with fish weight increase (Fig. 6). Biofilm presence alone decreased arsenic bioaccumulation, presumably by removing any naturally occurring arsenic in the water. However, when biofilm and arsenic were present together, tissue
Table 4
Results for the generalized estimating equations for variations in physiological parameters. Significant results are highlighted in bold.

<table>
<thead>
<tr>
<th>Change in biomass</th>
<th>Bioaccumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Wald $\chi^2$</td>
</tr>
<tr>
<td>Biofilm (B)</td>
<td>13.208</td>
</tr>
<tr>
<td>Arsenic (A)</td>
<td>0.876</td>
</tr>
<tr>
<td>Length (L)</td>
<td>639.187</td>
</tr>
<tr>
<td>$B \times A$</td>
<td>15.094</td>
</tr>
<tr>
<td>$B \times L$</td>
<td>18.006</td>
</tr>
<tr>
<td>$A \times L$</td>
<td>3.792</td>
</tr>
<tr>
<td>$B \times A \times L$</td>
<td>13.494</td>
</tr>
</tbody>
</table>

Fig. 4. The mean time taken to (a) capture and (b) consume all five food items in each tank each day. Trendlines have been added to illustrate the relationships between time and the presence and absence of biofilm and arsenic. Note that the y-axis has been curtailed in order to clearly illustrate relationships between treatments which has resulted in the removal of one outlying data point from each graph: (a) treatment B, day 9, value = 12.7; (b) treatment B, day 9, value = 9.8. C = control; B = biofilm; A = arsenic; B + A = biofilm + arsenic.

Fig. 5. The change in weight between the start and end of the experiment for all fish. For clarity each of the treatments are show separately in comparison to the control: (a) biofilm; (b) arsenic; (c) biofilm and arsenic. Trendlines have been added for illustration. C = control; B = biofilm; A = arsenic; B + A = biofilm + arsenic.

4. Discussion

Arsenic produced some effects in mosquitofish, though not exactly as predicted. Aggression increased significantly in the presence of arsenic while for operculum movement and food capture efficiency and consumption rate time, rather than arsenic presence, was the major predictor. Aggression appears to be the major initial behavioural effect of arsenic exposure in this species and continued to increase with exposure duration. Of the behaviours measured, aggression may thus be a suitable biomarker for arsenic toxicity in mosquitofish (Moss, 1998; Scott and Sloman, 2004; Weis et al., 2001). Increased aggression may be induced through stress...
or related physiological changes due to arsenic exposure (e.g. Scott and Sloman, 2004), which may increase the metabolic costs for an individual, thereby leading to increased stress and a potentially damaging feedback cycle. Aggression in some fish species increases with other toxicants. For example, bluegills, Lepomis macrochirus, exposed to copper for 96 h increased the frequency of agonistic acts (Henry and Atchison, 1986), while round gobies from contaminated sites increased their rate of assessment displays compared to fish from a reference site (Sopinka et al., 2010).

In both these cases, dominance status played a role with more dominant bluegills increasing aggression over subordinates (Henry and Atchison, 1986) and reduced dominance establishment in contaminant site gobies (Sopinka et al., 2010). In the present study, almost all agonistic acts were initiated by the largest, presumably dominant, female which may explain the lack of notable effects on foraging parameters. One of the major functions of aggression is resource defence, mainly defence of mates, shelter or food (Huntingford and Turner, 1987; Magellan and Kaiser, 2010). If the largest female was monopolizing most of the food resources, competition for the remaining food by the other individuals may mask any effects of arsenic exposure. However, foraging efficiency was only recorded for the first few food items, after which fish were fed to excess, so later effects may have been overlooked. Time had the greatest effect on foraging, the faster capture efficiency probably being due to fish learning to anticipate food and the slower consumption rate reflecting reduced motivation to feed as they gained weight. However, other factors cannot be ruled out. The concomitant increase in opercular rate over time suggests variation in oxygen demand or efficiency of oxygen uptake which may be induced by the build-up of other chemicals, such as nitrogen, naturally excreted by fish.

These behavioural results can be integrated with the physical results. All fish gained weight during the nine days of observations, probably because the few fish per tank were fed to excess each day so were released from the competition they would have experienced in the stock aquaria, which reflects the foraging results above. Larger fish gained the most weight in all treatments, although unexpectedly arsenic promoted weight gain. The reasons for this result are unknown. The accepted view is that contaminant load should cause a loss of condition (e.g. Kumar and Banerjee, 2012; Scott and Sloman, 2004; Weis et al., 2011). Increased size has been shown in grass shrimps, Palaemonetes pugio, from contaminated sites but this is explained by reduced predation from fish at these locations (Weis et al., 2011). In this study, predation was not a factor although it is interesting that weight gain and aggression varied in parallel, which may imply some effect of resource defence. Increase in fish biomass and bioaccumulation also showed similar patterns, the obvious explanation being that greater weight gain allows more arsenic to be assimilated and fixed in tissues. However, it may also be that fish that gain more weight have characteristics, such as increased aggression and therefore resource holding potential (e.g. Magellan and Kaiser, 2010), that also contribute to As bioaccumulation. Although we provided daily uncontaminated food, mosquitofish also consume algae and diatoms (Garcia-Berthou, 1999). The algae present in the biofilm treatments, some of which dropped into the fish part of the experimental units, were likely to be heavily contaminated with arsenic, which may have promoted bioaccumulation. Finally, small fish such as these mosquitofish, which have a large surface area to volume ratio, are particularly susceptible to absorption of toxins through the skin (Moeller et al., 2003; Rahman et al., 2012), which may be another contributing factor.

Surprisingly, the presence of algae appeared to aggravate, rather than ameliorate, the effects of arsenic exposure in mosquitofish. In terms of increase in fish biomass, although algae acted antagonistically with arsenic, this resulted in a reduction in weight gained which is not likely to be advantageous. This effect is particularly apparent in smaller fish. For bioaccumulation the effects of algae were even more severe, as algae operated additively with arsenic to increase arsenic uptake and/or assimilation. Aggression was also highest in the presence of both algae and arsenic, although in this case the interaction was not significant. One plausible explanation concerns the biotransformation of arsenic by algae as described in Section 1. The exact nature of this transformation depends on algal growth and P nutrient status in the environment (Hellweger and Lall, 2004; Levy et al., 2005; Rahman et al., 2012). Under P-limiting conditions, when algal growth is slow, algae excrete DMAA\(^{III}\). Under P-replete conditions with fast algal growth, PO\(_4^{3-}\) assimilation is up-regulated and As\(^{V}\) uptake increases in parallel. As the transformation of As\(^{V}\) to As\(^{III}\) is faster than that of As\(^{III}\) to DMAA\(^{III}\), As\(^{III}\) builds up within algal cells and is consequently excreted into the environment to keep intracellular As\(^{III}\) at low levels and allow reductase activity (Hellweger and Lall, 2004; Levy et al., 2005; Rahman et al., 2012). The phosphate concentration in our system was selected to simulate P-limiting conditions (Hellweger and Lall, 2004; Moss, 1998; Rahman and Hasegawa, 2012) so should have limited algal growth and consequent arsenic uptake. However, as a recent study showed (Wang et al., 2013), even in P-limiting conditions algal As\(^{V}\) uptake may increase as cells synthesize more P transporters to compensate for the lack of phosphate in the environment. More importantly, however, fish metabolism produces waste, especially ammonia and phosphate. N and P recycling rates vary between species (Vanni et al., 2002; Villéger et al., 2012) and while the exact rate of N and P excretion by fish in this experiment was not quantified, stress is known to strongly stimulate urea (N) excretion in mosquitofish (Uliano et al., 2010). It is therefore likely that the presence of mosquitofish stimulated P-replete conditions and accelerated the biotransformation of As by algae. A further consideration is algal growth. Nutrient supply, in particular phosphorus and nitrogen, is the most important determinant of algal production (Moss, 1998; Rahman and Hasegawa, 2012; Villéger et al., 2012). Algal growth, nutrient concentration, and As are thus intricately linked. Research has shown a positive correlation between As\(^{III}\) concentration and primary productivity (Rahman and Hasegawa, 2012) and the presence of fish is likely to contribute to this effect. Other elements such as oxygen (Smedley and Kinniburgh, 2002; Wang et al., 2013) and iron (Senn and Hemond, 2002) also influence arsenic speciation. Whatever the exact mechanisms here, it is evident that these various processes interacted to promote biotransformation of arsenic by algae. The end products of this transformation, in particular As\(^{III}\), are less toxic to algae, but more toxic to fish (Rahman et al., 2012; Smedley and Kinniburgh, 2002), so even if the overall aquatic As
concentration is reduced by algae, this may be counterproductive at an ecosystem scale.

For mosquitofish, the effects of arsenic exposure are overall detrimental. Despite the increased biomass seen here with arsenic, bioaccumulation of arsenic is harmful (de Castro et al., 2009; Moeller et al., 2003; Sopinka et al., 2010) and increased aggression may increase the chance of physical damage (e.g. Huntingford and Turner, 1987) and exacerbate physiological effects of arsenic exposure (e.g. Scott and Sloman, 2004). Moreover, in addition to, or as a consequence of, the effects documented here other functions and interactions are likely to be disrupted. For example, both mate recognition (e.g. Fisher et al., 2006) and predator recognition (e.g. Mandrillon and Saglio, 2007) are compromised by alteration of the chemical environment. The mechanisms underlying the behavioural changes demonstrated in this study may involve sensory, hormonal, neurological and metabolic systems (Scott and Sloman, 2004) all of which may also affect other behaviours including locomotory behaviours like predator avoidance or swimming performance. The increase in aggression and lack of effects on feeding behaviour in this study suggest locomotory functions were not affected. However, the exposure treatments here were neither particularly acute nor chronic and increased exposure concentrations or durations are likely to lead to more serious impacts. Finally, here we used an invasive, highly tolerant fish as a model. The effects of arsenic exposure on potentially endangered native species would be both more difficult and more critical to evaluate.

In conclusion, we have shown here that changes in complex behaviours are practical, ecologically relevant measures of toxicological effects (e.g. Scott and Sloman, 2004; Weis et al., 2001). Aggression in particular should be considered in assessment of arsenic impacts as it is a highly dynamic and responsive process that may show immediate impacts and can influence several other aspects of behaviour. In common with other authors, we also highlight interacting effects of contaminant exposure, both through integration of behavioural and physical mechanisms (e.g. Scott and Sloman, 2004; Weis et al., 2001) and consideration of different taxa together (e.g. Scott and Sloman, 2004; Weis et al., 2011). In particular, toxicant responses in multi-trophic, natural ecosystems are often found to be different from single-species laboratory studies. Multi-trophic studies are therefore crucial to elucidate the real effects of toxicants. An important finding in this respect from the current study is the aggravating influence of algae on the impacts of arsenic exposure in fish. Bioremediation of arsenic contaminated waters using aquatic algae should therefore be carried out with consideration of entire ecosystem effects. Such multidisciplinary, cross-taxa research is crucial for understanding the impacts of arsenic toxicity and thus restoration of aquatic ecosystems.

**Conflict of interest**

The authors declare no conflict of interest.

**Contributors**

Concept: HG, KM, EGB; experimental design: HG, KM, EGB; field collection: EGB, KM, HG; carried out experiments: KM, LBF, MR, GU, HG; video analyses: KM, PS; biochemical analyses: LBF, MR, PS, GU, HG; statistical analyses: KM; wrote the paper: KM; edited, revised and wrote small sections of the manuscript: HG, EGB, MR, LBF, GU.

All authors have approved the final article.

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