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Altered reproductive behaviours in male mosquitofish living downstream from a sewage treatment plant

Minna Saaristo[a,e,*], Jackie Myers[b], Rowan Jacques-Hamilton[a], Mayumi Allinson[c], Atsushi Yamamoto[d], Graeme Allinson[c], Vincent Pettigrove[b], Bob B.M. Wong[a]

[a] *School of Biological Sciences, Monash University, Victoria, Australia*
[b] *Centre for Aquatic Pollution Identification and Management (CAPIM), Department of Zoology, The University of Melbourne, Victoria, Australia*
[c] *Centre for Aquatic Pollution Identification and Management (CAPIM), School of Chemistry, The University of Melbourne, Victoria, Australia*
[d] *Urban Environment Group, Osaka City Institute for Public Health and Environmental Sciences, Osaka, Japan*
[e] *Åbo Akademi University, Department of Biosciences, Turku, Finland*

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ABSTRACT

Freshwater environments are common repositories for the discharge of large volumes of domestic and industrial waste, particularly through wastewater effluent. One common group of chemical pollutants present in wastewater are endocrine disrupting chemicals (EDCs), which can induce morphological and behavioural changes in aquatic organisms. The aim of this study was to compare the reproductive behaviour and morphology of a freshwater fish, the mosquitofish (*Gambusia holbrooki*), collected from two sites (wastewater treatment plant (WWTP) and a putative pristine site). The mosquitofish is a sexually dimorphic livebearer with a coercive mating system. Males inseminate females using their modified anal fin as an intromittent organ. Despite this, females are able to exert some control over the success of male mating attempts by selectively associating with, or avoiding, certain males over others. Using standard laboratory assays of reproductive behaviour, we found that mosquitofish males living in close proximity to WWTP showed increased mating activity compared to those inhabiting a pristine site. More specifically, during behavioural trials in which males were allowed to interact with females separated by a transparent divider, we found that WWTP-males spent more time associating with females. Concordant with this, when males and females were subsequently allowed to interact freely, WWTP-males also spent more time chasing and orienting towards the females. As a result, females from both sites showed more interest towards the WWTP-site males. Male anal fin morphology, however, did not differ between sites. Our study illustrates that lifetime exposure to WWTP-effluents can greatly affect male behaviour. The results underscore the importance of behaviour as a potential tool for investigating unknown contaminants in the environment.

Introduction

Urban and peri-urban waters can contain a large number of organic chemicals from a diverse range of sources, such as roadside run-off, waste water treatment plant (WWTP) inputs, stormwater and agricultural enterprises. Some of these pollutants may prove directly toxic to aquatic organisms (*e.g.* Guillette et al., 1995), while others may elicit more subtle effects (Vandenberg et al., 2012). For example, pollutants such as endocrine disrupting chemicals (EDCs) present in WWTP effluents and surface waters (Johnson and Sumpter, 2001; Viganò et al., 2008; Thorpe et al., 2009; Allinson et al., 2010), can interfere with the endocrinology of aquatic and terrestrial organisms (Scott and Sloman, 2004; Waring and Harris, 2005; Kidd et al., 2007; Ankley et al., 2009). Intense research on the topic during the last two decades has shown that EDCs can cause a wide range of physiological and morphological changes (Oppen-Berntsen et al., 1992; Jobling et al., 1998; Christensen et al., 1999; Ankley et al., 2003). Nevertheless, there has been a growing number of studies in recent years looking at the impacts of EDCs

* Corresponding author at: School of Biological Sciences, Monash University, Clayton Campus, 3800 Victoria, Australia. Tel.: +61 3 99020756.
E-mail addresses: minna.saaristo@monash.edu (M. Saaristo), jhmyers@unimelb.edu.au (J. Myers), rowan.jacques.hamilton@gmail.com (R. Jacques-Hamilton), mayumia@unimelb.edu.au (M. Allinson), yamamoto@iphes.city.osaka.jp (A. Yamamoto), gallinson@unimelb.edu.au (G. Allinson), vpet@unimelb.edu.au (V. Pettigrove), bob.wong@monash.edu (B.B.M. Wong).

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on behaviour (see reviews: Zala and Penn, 2004; Clotfelter et al., 2004; Scott and Sloman, 2004; Söffker and Tyler, 2012). Behaviour has proven to be a sensitive early-warning indicator of contamination (Bell, 2001; Martinovic et al., 2007; Saaristo et al., 2009b; Hallgren et al., 2011; Shenoy, 2012) and, most importantly, altered sexual behaviours are predicted to have important ecological and evolutionary consequences (Candolin and Wong, 2012)

So far, only a few studies have explored the impact of industrial and WWTP effluents on the reproductive behaviour of fish – and the results have been equivocal. While some studies have detected impacts on behaviour, others have not. For instance, fathead minnow (*Pimephales promelas*) males failed in competing for nest sites and mates after being exposed to estrogenic WWTP effluent for three weeks (Martinovic et al., 2007), while three-spined stickleback (*Gasterosteus aculeatus*) males showed altered reproductive behaviour after short-term exposure to anti-androgenic sewage effluents (Sebire et al., 2011). By contrast, in two separate studies on eastern mosquitofish (*Gambusia holbrooki*), no differences were found in male behaviours between contaminated and reference sites, even though morphological indicators (anal fin length, testis size, liver weight) showed clear impact of EDCs (Toft et al., 2003, 2004). Clearly, the causal link between behavioural responses and WWTP effluents warrants further investigation.

Our study species, the eastern mosquitofish (*G. holdbrooki*) is an excellent model system for studying contaminants in WWTP effluents. The species has a widespread, cosmopolitan distribution in shallow freshwater habitats in both urban and agricultural areas (Pyke, 2005). The mosquitofish is a small (size 20–40 mm) sexually dimorphic livebearer, with males inseminating females using their gonopodium (a modified anal fin), as an intromittent organ (Constantz, 1984). Male mosquitofish do not court females but, instead, attempt forced copulations by thrusting their gonopodia towards the female's genital pore (McPeek, 1992; Bisazza and Marin, 1995). Despite the coercive mating system, female mosquitofish have been shown to be choosy (Bisazza et al., 2001; Kahn et al., 2010) and are able to exert some control over the success of male mating attempts by selectively approaching certain males over others (Bisazza et al., 2001). Due to their internal mode of fertilisation, male mosquitofish need to be in close proximity to females before any mating attempts can be made (Martin, 1975). Females prefer males with larger gonopodia, and so male genitalia appears to be a sexual ornament that is under the influence of sexual selection (Kahn et al., 2010). Morphologically, mosquitofish present a valuable local indicator species for exposure to EDCs because the development of the male gonopodium is androgen dependent. Indeed, previous research has found that embryonic exposure to androgenic hormones can increase the length of gonopodium of males in relation to body size (Angus et al., 2001; Leusch et al., 2006), and induce gonopodial development in females (Turner, 1942; Bortone and Davis, 1994; Angus et al., 2001).

The aim of our study was to examine the effects of aquatic contaminants on male and female reproductive behaviour and morphology of wild collected fish. Specifically, we compared male and female reproductive behaviours and anal fin morphology in mosquitofish that had been living in close proximity to a WWTP and a comparatively more pristine (*i.e.* reference) site. Laboratory-based studies of EDC exposure are often criticised for lacking ecological relevance (see review by Zala and Penn, 2004), as they tend to be conducted using single compounds or laboratory reared model species rather than individuals from wild populations. On the other hand, field observations alone, without follow up experimental studies, are often insufficient to establish cause and effect. By bringing wild fish into the laboratory, our approach combines field exposure with laboratory-based behavioural assays.

Material and methods

Sampling sites and collection of fish

The study was carried out in Victoria, Australia, during March–April 2011, which corresponds to the late breeding season of mosquitofish (Haynes and Cashney, 1995). The collection sites Brodies Lake (reference site) and Jackson's Creek (WWTP-site) were chosen according to an EDC-monitoring program being carried out by The Centre of Aquatic Pollution, Identification and Monitoring (CAPIM) in 2010–2011 (Chinathamby et al., 2013). Brodies Lake is a relatively pristine site (total estrogen equivalent concentration: 0.5 ng/EEQ/L: Chinathamby et al., 2013) located adjacent to a reservoir that supplies drinking water to parts of suburban Melbourne. Jackson's Creek, by contrast, was 100 m downstream from the outlet of a WWTP (total estrogen equivalent concentration: 12.5 ng/L/EEQ: Chinathamby et al., 2013). We collected 300 fish from each site and took water samples to determine the concentration of estrone (E1) estradiol (E2) and ethinyl estradiol (EE2); and the androgen androstenedione. Fish were caught with dip nets and brought back to the laboratory where they were separated by sex and acclimated to laboratory conditions (12:12 h light regime) for two weeks in 54 L tanks (20 fish per tank), before the commencement of the behavioural trials (Fig. 1). Fish were kept in filtered freshwater tanks during the acclimation period. Water temperature in the tanks was monitored daily and ranged from 19 to 23 °C. Fish were fed *ad libitum* with commercial fish flakes (Otohime Hirame, Aquasonic) once a day.

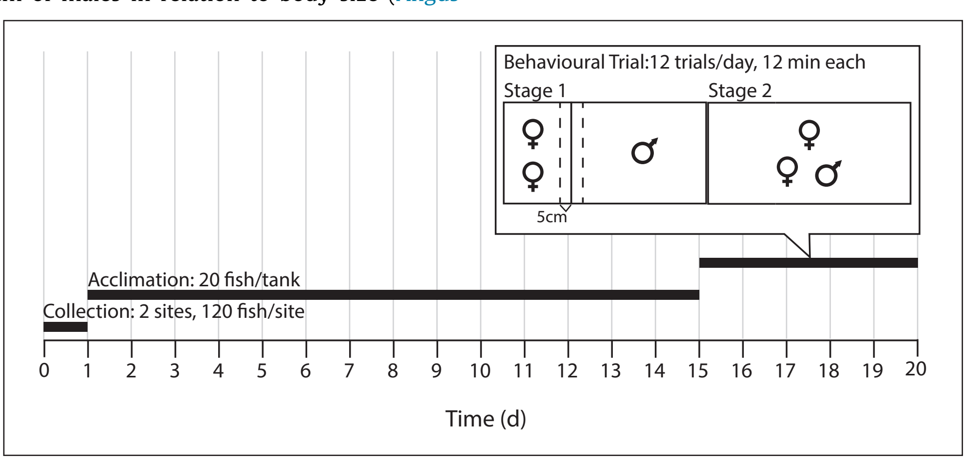

Fig. 1. Experimental timeline showing the time of fish collection, acclimation period and behavioural assay. A detailed description of the behavioural assay is also presented.