Reproductive Morphology of Male Mosquitofish (*Gambusia holbrooki*) Inhabiting Sewage-Contaminated Waters in the South Creek Catchment of the Hawkesbury-Nepean River

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Keywords oestrogenic chemicals, mosquitofish, gonopodium

Abstract

It has recently become apparent that effluent from sewage treatment plants (STPs) may contain substances that are oestrogenic to fish. In this study, we assessed the reproductive morphology of male eastern mosquitofish (*Gambusia holbrooki*) inhabiting sewage-contaminated waters in the South Creek catchment of the Hawkesbury-Nepean River. Male mosquitofish were collected downstream of two STPs, located at St Marys and Quakers Hill. Fish were also collected from upstream of each STP for comparative purposes.

The gonopodium, a modified anal fin that is used to transfer sperm during copulation,'was found to be reduced in length in male mosquitofish collected downstream of St Marys STP compared to fish from upstream of the effluent outfall. The testicular weight of the downstream males was also lower than that of the upstream fish.

Exposure of male mosquitofish to oestrogenic chemicals under laboratory condition has previously been demonstrated to affect gonopodium length and testicular development. There were no differences in the reproductive morphology of male mosquitofish downstream of Quakers Hill STP compared to the upstream fish. Future surveys of mosquitofish populations inhabiting sewage-contaminated waters in the Sydney region should include more direct evidence of oestrogenic exposure, such as vitellogenin induction and gonadal histology.

Introduction

The contamination of aquatic environments with endocrine-disrupting chemicals (EDCs) has raised concerns over the potential effects of these chemicals on the reproductive development and fecundity of aquatic species. Particular attention has focused on chemicals that can mimic the effects of oestrogens. One potential source of oestrogenic chemicals to aquatic systems is sewage treatment plant (STP) effluent. A wide variety of oestrogenic chemicals have been detected in STP effluent, including natural and synthetic estrogens, pesticides, and the degradation products of alkylphenols. In addition, reproductive abnormalities that are consistent with oestrogenic exposure have been reported for wild fish populations inhabiting sewage-contaminated waters, including testicular abnormalities and elevated levels of vitellogenin in male fish (e.g. Jobling *et al.* 2002; Lye *et al.* 1998).

Mosquitofish (*Gambusia holbrooki* and *G. affinis*) are live-bearing, freshwater fish indigenous to central and south America, but introduced around the world as a potential means of mosquito control. They were first introduced into eastern Australia in the 1920s and have since become established in most geographic areas. Mosquitofish exhibit numerous sexually dimorphic characteristics that are under hormonal control, which makes them valuable species for studying the effects of EDCs on fish. The most distinguishable characteristic is the highly modified anal fin of the male, called the gonopodium, which is used to transfer sperm during copulation. The gonopodium is derived from the elongation and modification of the anal fin rays, and forms in response to androgenic stimulation. As such, the gonopodium normally begins to develop in males at the time of sexual maturation, when the testes produce androgens. However, the development of gonopodium-like structures can also be induced in females on exposure to androgenic hormones. In addition, it has recently been demonstrated that exposure of juvenile male mosquitofish to oestrogenic chemicals inhibits the elongation of the gonopodium and disrupts normal testicular development (Doyle and Lim 2002; Dreze *et al.* 2000). Consequently, the reproductive morphology of mosquitofish can be used to assess exposure to androgenic and/or estrogenic chemicals under field conditions.

In this paper, we report on a study of the reproductive morphology of male eastern mosquitofish (*G. hobrooki*) inhabiting sewage-contaminated waters of the South Creek catchment of the Hawkesbury-Nepean River. South Creek is a heavily urbanised catchment situated in the western region of the Sydney metropolitan area. The catchment currently receives effluent from three STPs located at St Marys, Quakers Hill and Riverstone. The combined average dry weather discharge of the three STPs is approximately 66.7 ML of effluent per day, with 97 % of this discharge being contributed by St Marys and Quakers Hill (Lovell *et al.* 2000). We were interested in determining whether male mosquitofish downstream of the St Marys and Quakers Hill STPs were showing evidence of oestrogenic exposure, as indicated by the extent of gonopodial and testicular development, compared with fish collected from upstream of the effluent outfalls.

Methods

The St Marys (StM) STP has a dry weather effluent discharge of 37 ML per day (Lovell *et al.* 2000). The STP receives waste from both residential and industrial areas and services a population of approximately 140,000 persons. The tertiary treated effluent is discharged into a small creek that flows directly into South Creek. The Quakers Hill (QH) STP receives waste from predominantly residential areas and services a population of 110,000 people. Tertiary treated effluent is discharged into Breakfast Creek, which flows into Eastern Creek and subsequently into South Creek. The average dry weather discharge of QH STP is 28 ML per day (Lovell *et al.* 2000).

Sites were selected upstream and downstream of each STP (Figure 1). For StM STP, the upstream site was located approximately 8.5 km above the effluent outfall, while the downstream site was 2.5 km below the outfall. For QH STP, the upstream and downstream sites were 1 and 5 km above and below the outfall, respectively.

Male mosquitofish were collected from each site using a dip net in late August-early September (spring) 2000, which represents the beginning of the mosquitofish breeding season. At this time of year there are very few immature males around, with most males that have survived winter reaching sexual maturity by this stage. Males with a minimum body length of 20 mm were used, as this was the smallest length at which a sexually mature male was observed.

Fish were euthanized by immersion in 400 mg/L benzocaine, weighed and photographed with a digital microscope camera mounted on a stereomicroscope. Morphological measurements were made on the images using the Leica Qwin Image Processing and Analysis System (Leica Microsystems, North Ryde, NSW, Australia). Gonopodial development was assessed using two measures: gonopodium length and gonopodium extension length. Gonopodium length was defined as the length from the anterior base of the anal fin to the gonopodial tip. Gonopodium extension length, which provides a measure of anal fin elongation, was defined as the length of rays 3, 4 and 5 of the anal fin beyond the boundary of the remaining rays. Measurements were also taken of total length. Testes were removed from each fish and weighed.

The gonopodium and gonopodium extension lengths of the collected males were compared between the upstream and downstream sites for each STP using analysis of covariance (ANCOVA) to account for the influence of total length. Comparisons of testis weight were also made using ANCOVA, with body weight as the covariate. Differences in the total length and wet weight of male fish between the upstream and downstream sites for each STP were assessed using *t* tests. All analyses were performed using STATISTICA for Windows (Statsoft, Tulsa, OK, USA).

Results

There were no significant differences ($p > 0.05$) in the total length or wet weight of male fish collected from downstream of either STP compared to fish from the upstream sites (Table 1).

The relationship between gonopodium length and total length for fish collected upstream and downstream of each STP is shown in Figure 2. Regression slopes of total length plotted against gonopodium length did not vary significantly between the upstream and downstream sites for either STP (StM: $p = 0.64$; QH: $p = 0.86$). For StM STP, there were significant differences between the two sites in the adjusted mean gonopodium length ($p < 0.05$) of the collected males. Gonopodium lengths were comparatively shorter for males collected downstream of StM STP compared to males from the upstream site (Table 2). Similar results were observed for the gonopodium extension lengths of the collected males (Table 2). For QH STP, there were no significant differences in the adjusted mean gonopodium length ($p = 0.63$) or gonopodium extension length ($p = 0.56$) between males from the upstream and downstream sites (Table 2).

The relationship between body weight and testis weight is shown in Figure 3. For both STPs, there were no significant differences between the upstream and downstream sites in the slopes of the regressions (StM, $p = 0.27$; QH, $p = 0.99$). For StM STP, the ANCOVA indicated there was a significant difference between the upstream and downstream sites in the adjusted mean testis weight of the collected fish ($p < 0.05$), with the testes of males collected downstream of the effluent outfall weighing significantly less than the testes of fish from upstream of the outfall (Table 3). For QH STP, the difference in adjusted mean testis weight was not significantly different between the upstream and downstream sites ($p = 0.14$; Table 3).

Discussion

There were no differences in the reproductive morphology of male mosquitofish collected upstream and downstream of QH STP. However, the mean gonopodium and gonopodium extension lengths of male mosquitofish inhabiting reaches of South Creek downstream of StM STP were significantly reduced compared to that of fish living upstream of the effluent outfall. In addition, the testicular weight of the downstream fish was also significantly less than that of the upstream fish.

These results are consistent with the reported effects of oestrogenic exposure on male mosquitofish under controlled laboratory conditions. For example, Doyle and Lim (2002) reported an inhibition in the development of the gonopodium in male mosquitofish exposed to the natural oestrogen 17ß-oestradiol, while Dreze *et al.* (2000) observed partially developed gonopodia and atrophied gonads in male mosquitofish exposed to 4-nonylphenol, an oestrogen mimic. Inhibition of testicular development has also been reported for other fish species following exposure to oestrogenic chemicals (e.g. Jobling *et al.* 1996).

Angus *et al.* (2002) recently conducted a survey of male mosquitofish inhabiting sewage-contaminated waters in the USA and found no difference in the mean gonopodium length of sewage-exposed fish compared to non-exposed fish. However, Angus *et al.* used only sexually mature males in their study, with sexual maturity being indicated by the presence of a completely developed gonopodium. Since the reported effects of oestrogenic chemicals on male mosquitofish are an inhibition of gonopodial development and sexual maturation, the use of only sexually mature males would potentially result in the exclusion of oestrogen-affected males from the gonopodium length measurements.

Conversely, including measurements for males with incomplete gonopodia, as we did in this study, may also include immature males that are not necessarily oestrogen-affected but which have simply not yet sexually matured. Such a situation would tend to bias gonopodium length measurements downwards. To reduce the influence of juvenile males on gonopodium length measurements, we sampled just prior to the onset of the breeding season, which is the time of year when there would be the fewest juveniles within a population. Nevertheless, to provide greater evidence that the observed effects were due to oestrogenic exposure and are not an artefact of the experimental design, future surveys of mosquitofish populations should include the use of biomarkers of oestrogenic exposure, such as expression of vitellogenin and histological examination of the testes and livers.

The endocrine-disrupting capacity of STP effluent is dependent on the influent constituents and the treatment processes used, and, therefore, may vary considerably between STPs. Nevertheless, the compounds commonly implicated as being responsible for the oestrogenic activity of STP effluent are the natural and synthetic oestrogens (17β-oestradiol, oestrone, 17α-ethinyloestradiol) and alkylphenolic compounds.

A potential EDC that has been detected in effluent from StM STP is chlorpyrifos, an organophosphate pesticide which has been reported to induce weak oestrogenic responses *in vitro* using the human breast cancer MCF-7 cell proliferation and oestrogen transactivation assays (Anderson et al. 2002). Other potential EDCs have been detected in South Creek that are not necessarily emanating from the STP effluent and which may also be affecting mosquitofish populations. For example, atrazine, a commonly used agricultural herbicide, has recently been detected in water samples from South Creek taken in close proximity to the downstream sample site used in this study (Pablo *et al.* 2002). Atrazine has been demonstrated to induce aromatase activity *in vitro*, which may potentially increase the conversion rate of testosterone to oestrogen (Sanderson *et al.* 2000). Atrazine has also been shown to lower testosterone levels and demasculinize male African clawed frogs (Hayes *et al.* 2002). The source of atrazine to South Creek has not yet been determined. The organochlorine pesticide DDT has also been detected in the sediments of South Creek, although levels are generally highest upstream of the StM STP (Hackney *et al.* 2000). DDT has been demonstrated to possess both oestrogenic and anti-androgenic properties using *in vitro* yeast based assays (Sohoni and Sumpter 1998). Therefore, South Creek is contaminated with a number of EDCs, all of which may potentially be affecting mosquitofish populations downstream of the StM STP.

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