Answering questions on the impact of recycled water on wildlife using *Gambusia holbrooki* as a surrogate

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ABSTRACT

The disruption of endocrine systems due to environmental contaminants potentially impacts on developmental, behavioural, regulatory, and reproductive systems of wildlife. A major source of exposure of wildlife (terrestrial and aquatic) to endocrine disrupting compounds is through contact with contaminated surface waters. Current testing routines in aquatic systems have not been designed specifically to assess endocrine disruption properties or, alternatively, methods tend not to be fully developed. An alternative approach is to use a response sensitive to these chemicals such as gonopodium length in the pest species *Gambusia holbrooki* as an indicator. This species was used as a surrogate for native aquatic species to assess endocrine disruption in wetlands that are used for the storage of stormwater or treated sewage effluent. These were compared with adjacent wetlands used for watering stock (farm dams) that were not contaminated with these pollutants. Deformities in the mosquito fish were found that were consistent with endocrine disruption caused by sex steroids and/or their mimics in the first in the sequence of each pollution type but not in farm dams. We concluded that the first wetland in the sequence of recycling stormwater or treated sewage effluent could cause detrimental effects to wildlife but not in subsequent wetlands in a sequence of water storage systems. Since all waters are moved through at least three wetlands before being used for irrigation, or released into the river system, the impact on wildlife is likely to be minimal beyond the first wetlands.

Key words: Endocrine disruption, Recycled water, Biomarker, Stormwater effluent, Treated sewage effluent, Mosquito fish.

Introduction

The disruption of endocrine systems due to environmental contaminants potentially impacts on the developmental, behavioural, regulatory and reproductive systems of wildlife (Brevini *et al.* 2005; Colborn *et al.* 1993; Jobling *et al.* 1998; Lintelmann *et al.* 2003; Tyler *et al.* 1998). In wildlife, these contaminants may act as (a) an agonist and antagonist of steroid hormone receptors, (b) a modulator of enzymes involved in synthesis or metabolism of hormones, and (c) a modulator of biochemical and molecular events, including cell signalling pathways and gene expression (McKinney and Walter 1998; Phillips and Foster 2008; Roy *et al.* 1997). All of these impacts disrupt the major functions of natural steroids, such as estrogens and androgens, and effect sex determination, maturation, and development (Tyler *et al.* 1998; Witorsch 2002).

A major source of exposure of wildlife (terrestrial and aquatic) to endocrine disrupting compounds is through contact with contaminated surface waters. Pollution sources may include animal (estrogens) and vegetal (phytoestrogens) contaminants, and a range of compounds of synthetic origins (e.g., phthalate plasticisers, surfactants, polychlorinated bi-phenyls [PCBs], and polycyclic aromatic hydrocarbons [PAHs]; Rodriguez-Mozaz *et al.* 2004). Two major sources of pollutants causing endocrine disruption occur in sewage effluent discharge (e.g., Batty and Lim 1999; Chapman 2003; Ying *et al.* 2002) and urban stormwater (e.g., Dillon 2000; Manning 2005).

The responses of aquatic-dependent wildlife (e.g., amphibians, Carey 2000; fish, Jobling and Tyler 2003; marine invertebrates, Depledge and Billinghurst 1999), feral species (e.g., Batty and Lim 1999; Diniz *et al.* 2005a, b; Todorov *et al.* 2002), and the mussel *Elliptio complanata*, (Gagné *et al.* 2004) to treated sewage effluent have been widely reported. Another source of endocrine disrupting contaminants, stormwater, contains chlorinated pesticides, poly-aromatic hydrocarbons (PAHs), and polychlorinated compounds. These all rank highly in risk assessment of stormwater runoff, particularly PAHs (Baun *et al.* 2006). Many of these chemicals are considered to be at levels that are a risk to wildlife associated with stormwater effluent (Eriksson *et al.* 2007). However, endocrine disrupting contaminants, even at concentrations that are not individually harmful to wildlife, may act additively. They may also exhibit non-classical concentration - response relationships at levels of low exposure (Singleton and Khan 2003).

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Current testing routines for endocrine disrupting chemical contamination in aquatic systems have not been designed specifically to assess endocrine disruption properties or, alternatively methods tend not to be fully developed for such purposes (Matthiessen and Johnson 2007). An alternative approach to assessing endocrine disruption in aquatic systems is to use an indicator species. Mosquito fish *Gambusia holbrooki*, considered to be a useful sentinel species (Pyke 2005), has been shown to respond to estrogens in laboratory studies of 17βEstradiol (Doyle and Lim 2002) and 17βEthynylestradiol (Angus *et al.* 2005). They have also been observed to be an indicator of endocrine disruption in aquatic systems (Batty and Lim 1999). Exposure to endocrine disruption impacts in *G. holbrooki* results in a reduction in the length of the male gonopodium (as segment of the modified anal fin used by live-bearers during copulation) and the length of the anal fin; see Figure 1). Natural systems receiving treated effluent have been a common test scenario for environmental exposure (Batty and Lim 1999; Leusch *et al.* 2006; Toft *et al.* 2003), and as a tool in the initial assessment of contaminated lakes (Game *et al.* 2006). However, there has been no study that has compared the effect of treated sewage effluent and stormwater from the same source (township) to determine the comparative effects of the two sources of endocrine disruption.

We used *G. holbrooki* as a surrogate for native aquatic species in the assessment of endocrine disruption in wetlands used for the storage of stormwater, wetlands used for treated sewage effluent, and adjacent wetlands used for watering stock (farm dams). Our null hypothesis that there would be no statistically significant difference in the indicators (level of endocrine disruption) between 1) fish from wetlands that received effluent (sewage or stormwater) from the adjacent township, and fish from farm dams that received water only from precipitation and ground water seepage, and 2) fish from the first in the chain of effluent wetlands (difference between the fish from the wetland that initially received effluent and those from wetlands that subsequently received waters directly from the previous wetland in the chain), and fish from farm dams.

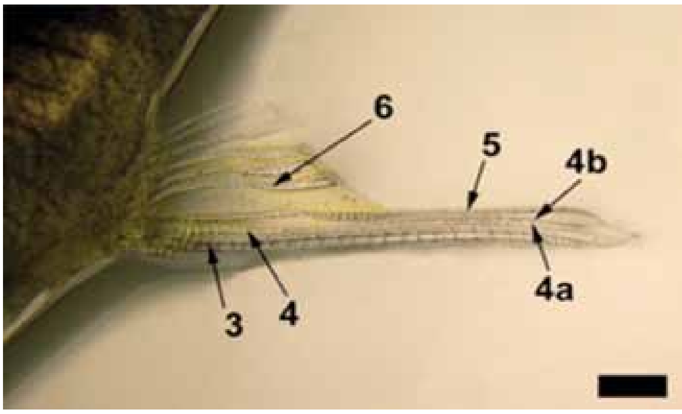

Figure 1. Anal fin and gonopodium of adult male *Gambusia holbrooki.* Rays 3-5 are under androgenic control and make up the gonopodium, while ray 6 represents the longest non-elongated ray of the anal fin; bar = 1 mm (Source: Figure 2b, Leisch *et al.* 2006).

Materials and Methods

Site description

The study was carried out on the Hawkesbury campus of the University of Western Sydney, located approximately 50 km northwest of the Sydney CBD, Australia. The site has been used for agricultural teaching purposes for over a century (White and Burgin 2002). Between 2008 and 2009 the mean monthly maximum temperature varied between 17.0°C (July, 2008) and 32.0°C (January, 2009), and the mean monthly minimum temperature varied between 1.7°C (August, 2008) and 18.3°C (February, 2009). In 2008 the annual precipitation was 1,051 mm, while in 2009 it was 800 mm. Mean monthly precipitation during the period varied between 2.8 mm (May, 2008) and 255.6 mm (February, 2008) (http://www.bom.gov.au/climate/data).

Since the 1960s treated sewage effluent from the neighbouring township of Richmond (population 2006 census = 5,560, ABS 2008) has been collected in purpose-built wetlands to supplement potable water for irrigation on the campus. More recently, the scheme has been upgraded and expanded to include wetlands constructed to store stormwater collected from the local township to supplement on-campus non-potable use, and to contribute to environmental flows into nearby Rickabys Creek, a tributary of the Hawkesbury – Nepean River. The treated sewage effluent and stormwater wetlands receive water from their respective sources in a specific sequence of movement through the wetland system. This system has a design working volume of 188.5ML (stormwater) and 267.3 ML (treated sewage effluent). The timing of the movement of water through the systems is based on the requirement to maintain water levels as they are depleted for irrigation and other uses, and is not based on specific retention time (Anderson 2009). Elsewhere on the University farmlands, there is a network of small wetlands (farm dams) originally developed as part of the agricultural operation. These do not receive supplementary water and the water levels are not manipulated.

Sampling protocols

Sampling was undertaken in the first three treated effluent and stormwater wetlands (beyond this point water from these two sources is sometimes mixed), and three farm dams.

Between September 2008 and March 2009, mosquito fish were sampled at the edge of the nine wetlands using a 30 cm x 40 cm dip net on a 2.4 m extendable pole. Wetlands were sampled by progressively moving around the edge of the wetland until approximately 30 adult male fish had been captured, or until it was determined that mosquito fish were apparently not present (approximately 100 sweeps). As sweeps with the net were taken and the contents investigated, native fish captured in the net were immediately released, together with female and immature (<10 mm in length) mosquito fish. These were returned to the point of capture. Male mosquito fish were placed in a 20 L container with ~ 10 L of water from the body of water where the fish were collected, and kept in a cool shaded place until appropriate numbers of male fish had been captured. They were then transported to the laboratory where they were euthanized by immersion in 400 mg/L benzocaine (*cf.* Doyle and Lim 2002; Rawson *et al.* 2008).

After male fish had been euthanized they were viewed under a dissecting microscope at 40X magnification to determine if the terminal hooks on the gonopodium had developed, reported by Doyle and Lim (2002) to be evidence of maturity. Each mature male was then weighed, and again using 40X magnification, and a 50 mm glass slide rule with 0.1 mm divisions, the gonopodium rays 4 and 6 (ray 4 is under androgenic control, ray 6 is not, see Figure 1) were measured as the basis of establishing if endocrine disruption was occurring (*cf.* Batty and Lim 1999).

Statistical analysis

A two-way Nested Analysis of Variance was used to determine differences between the measurements of the gonopodium length (ray 4 minus ray 6) and anal fin length (ray 4). Where statistically significant differences were identified at one of the levels within the nested analysis, a one-way Analysis of Variance was used as the basis for testing the specific differences using the Bonferroni Posthoc Test. To reduce the risk of experiment-wide error (the probability of at least one Type 1 error), the results of the Bonferroni Posthoc Test were considered significant at the 0.025 level of significance.

Results

Thirty mature male *Gambusia holbrooki* were collected from six wetlands: two that received treated sewage effluent, two that relied on stormwater, and two farm dams. None of the other wetlands sampled had resident mosquito fish.

There was no statistically significant differences observed in fish gonopodium length ($F_{2,174}$ = 0.861, p = 0.507), or the anal fin length ($F_{2,174}$ = 0.591, p = 0.608) among water source types: treated sewage effluent, stormwater and farm dams. Conversely, there was a significant difference among the six wetlands sampled for gonopodium length (Figure 2; $F_{3,174}$ = 9.305, p = <0.001), and fin length (Figure 3, $F_{2,174}$ = 0.608, p = <0.001). Significant differences were observed between farm dams A and B (controls) and the first of the sequence of treated effluent wetlands (Figure 2 & 3, gondopodium length, p(A) = 0.005, p(B) = <0.001; anal fin length, p(A) = <0.001, p(B) = <0.001) and stormwater (gondopodium length, p(B) = 0.004 ; anal fin length, p(B) = <0.001). These results are consistent with endocrine disruption in the first in the sequence of treated sewage effluent and stormwater wetlands. There was a significant difference in gonopodium length (Figure 2) and anal fin length (Figure 3) which was also consistent with endocrine disruption between treated sewage effluent storages (gondopodium length, p = 0.002; anal fin length, p = <0.001). Exposure to stormwater showed the same trend, however, it was not significantly different (gondopodium length, p = 0.129; anal fin length, p = 0.099).

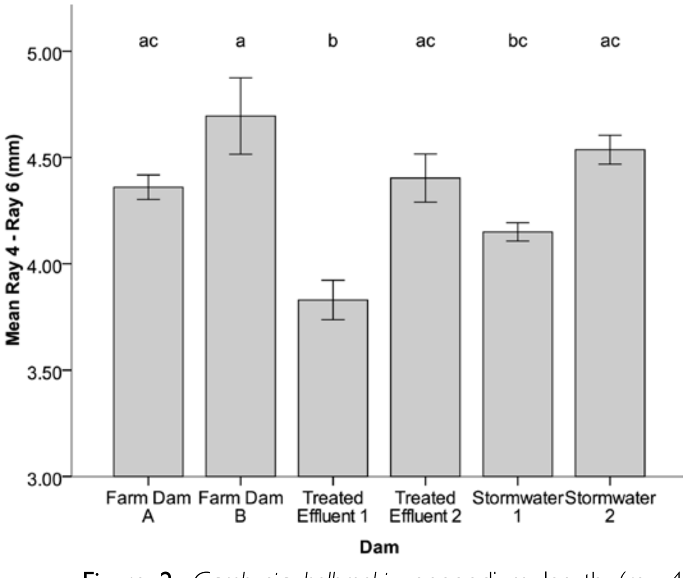

Figure 2. *Gambusia holbrooki* gonopodium length (ray 4 minus ray 6), measured from fish collected from wetlands at Richmond (New South Wales). Water was sourced from treated sewage effluent (Treated effluent 1 and 2), stormwater (Stormwater 1 and 2), or from farm dams with natural precipitation and ground water seepage without supplementation or cross contamination (Farm dam A and B) which acted as controls for the study (1 = initial waterbody in the sequence of wetlands receiving pollution, 2 = received water from waterbody 1; A and B = unique identification of wetlands without implied sequence; error bars = +/- 1 SE; n = 30 fish per dam; a – c identify significant differences [p > 0.05] among wetlands based upon the Bonferroni post-hoc test; Farm Dams A, B, Treated Effluent 2, and Stormwater 2 are not significantly different).

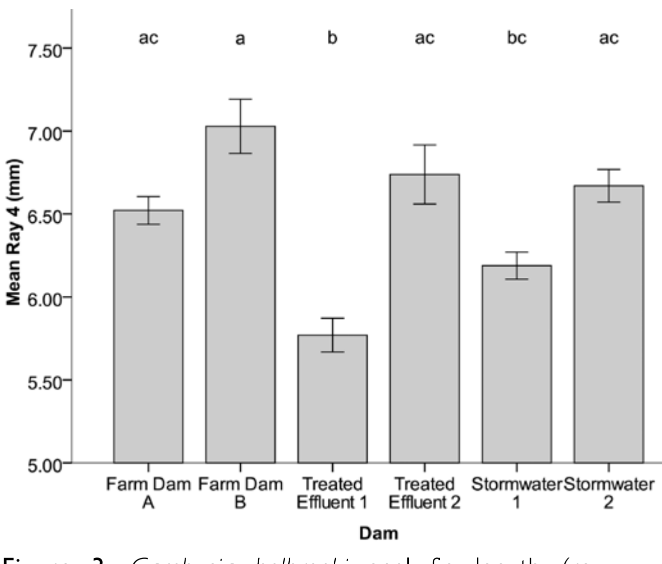

Figure 3. *Gambusia holbrooki* anal fin length (ray 4), measured from fish collected from wetlands at Richmond (New South Wales). Water was sourced from treated sewage effluent, stormwater, or from farm dams with filled with natural precipitation and ground water seepage without supplementation or cross contamination (Farm dam A and B) which acted as controls for the study (1 = initial waterbody in the sequence of wetlands receiving pollution, 2 = received water from waterbody 1; A and B = unique identification of wetlands without implied sequence; error bars = +/- 1 SE; n = 30 fish per dam; a – c identify significance amongs [p > 0.05] among wetlands based upon the Bonferroni post-hoc test; Test Treated Effluent 1, and Stormwater 1 are not significantly different).

The pattern of deformities in fish was not significantly different between the first wetlands in the sequence of stormwater and treated sewage effluent (Figure 2, 3 gondopodium length, $p = 0.437$; anal fin length, $p = 0.271$), and the second wetlands in the sequence (gondopodium length, $p = 1.000$; anal fin length, $p = 1.000$). There was also no significant difference between farm dams A and B (controls) and the second wetlands in the sequence for either treated sewage effluent (Figure 2 & 3, gondopodium length, $p(A) = 1.000$, $p(B) = 0.697$; anal fin length, $p(A) = 1.000$, $p(B) = 1.000$) or stormwater (gondopodium length, $p(A) = 1.000$, $p(B) = 1.000$; anal fin length, $p(A) = 1.000$, $p(B) = 0.636$). The first in the sequence of dams, particularly treated sewage effluent, had measures of gonopodium and anal fin lengths that were consistent with endocrine disruption. However, the second in the sequence of storage wetlands did not. The fish in these wetlands had gonopodium and anal fin lengths that were equivalent to the gonopodium and anal fin lengths of fish from farm dams.

Discussion

Fish were collected from six of nine sampled wetlands, while no fish were collected from other less sampled wetlands. For example, the third in the sequence of receiving waters for both stormwater and treated sewage effluent did not contain *Gambusia holbrooki.* We selected this species, in part, because it is an introduced species in many countries. In Australia, it is considered to be a ubiquitous pest species, capable of surviving in polluted waters (Pyke 2005). The lack of fish within some wetlands in our studies does not correspond with poor water quality (unpubl. data). It is due to the lack of dispersal of mosquito fish to these wetlands. We had even more difficulty finding mosquito fish in farm dams that had not been subject to urban pollution. This is potentially a disadvantage of using mosquito fish as a surrogate for native wildlife for studies of endocrine disruption.

The gonopodium of mosquito fish from wetlands that were the first in the sequence of wetlands to receive effluent (treated sewage effluent or stormwater) showed evidence consistent with endocrine disruption. These data are also consistent with previous investigations of the effects of endocrine disruption on fish from waters receiving sewage effluent (Batty and Lim 1999; Diniz *et al.* 2005a, b; Gagné *et al.* 2004; Todorov *et al.* 2002) and stormwater (Baun *et al.* 2006; Eriksson *et al.* 2007). Our data therefore confirm that even with tertiary treated effluent and stormwater from a township without secondary industries, fish may suffer endocrine disruption within receiving waters. This indicates that native wildlife resident in these waters, or those that visit them (turtles – *Chelodina longicollis*; ducks – *Anas castanea*, *A. gracilis*, *A. superciliosa*; cormorants – *Phalacrocorax melanoleucos*; frogs – *Limnodynastes peroni, L. tasmaniensis*; Burgin 2010), are potentially at risk.

There are no previous reports showing that the impact of endocrine disruption diminishes with retention time of effluent. Although the management of the wetlands precludes quantification of the time the water is retained within wetlands, there is constant movement between them, when water from the wetland with longest water storage time is used for irrigation or environmental flows, and the level is 'topped up' from recycled water from a previous wetland in the sequence. These water levels are checked, and adjusted if necessary, on a daily basis. Our study was undertaken during a prolonged dry period and the wetlands were therefore not substantially supplemented with water from precipitation. The observation that the fish within the second in the sequence of wetlands, either stormwater or treated sewage effluent, had a gonopodium length/proportion consistent with fish from farm dams that were not contaminated with these effluent sources indicates that over time the potency of endocrine disruptors such as sex steroids and/or their mimics in both pollution types had diminished. This may have been a result of breakdown of the offending compounds. However, Sumpter and Johnson (2005) reported that up to 40% of compounds typically described as endocrine disrupting can survive sewage treatment. There is also evidence that pollutants in both treated sewage effluent and stormwater may continue to play a role in endocrine disruption in the process of breakdown. Some metabolites may actually be more toxic than the original compound. Alklphenol polyethoxylates (APEs) used as surfactants in industrial and household applications, is biodegraded in activated sludge to a range of shorter chain ethoxylates. Some of these breakdown products, (e.g., 4-*tert* isomers of nonyl and octylphenol) have been demonstrated to be estrogenic to fish. It is therefore unlikely that breakdown was a major factor in the apparent loss of potency in the second in the sequence of wetlands for the two effluent types.

Such compounds may also be removed due to uptake by animals and plants within wetlands and/or animals visiting the wetlands. In a discussion of the properties, actions and routes of exposure of farm animals to endocrine disruption, Rhind (2002) noted that although largely unstudied, there was a potential risk of 'significant bioaccumulation'. We consider that the apparent loss of endocrine disruption compounds from the water is more likely to have been due to sedimentation processes. This would have the potential result of increasing the concentration of endocrine disruptors in the first in the sequence of wetlands used to store each of the effluent types, and a decrease in concentration in subsequent wetlands. Tyler *et al.* (1998) reported that many of the compounds identified as having a role in endocrine disruption are persistent and accumulate in the environment. This indicates that the change in potency of endocrine disruptors, such as sex steroids and/or their mimics, had diminished between the two wetlands in both treated sewage effluent and stormwater was due to accumulation in the sediments in the first in the sequence of wetlands.

Conclusion

The extrapolation from surrogate, in this case a pest species, to the potential impacts on wildlife more generally needs to be treated with caution. This is because as Sumpter and Johnson (2005) recorded 'one animal's poison may not be another's'. However, *G. holbrooki* has been widely used in this role (e.g., Angus *et al.* 2005; Batty and Lim 1999; Doyle and Lim 2002).

This mosquito fish model showed that there was a similar pattern of endocrine disruption contamination within wetlands used to store both stormwater and treated sewage effluent. Based on these data, it could be detrimental for wildlife in contact with the first in the sequence of recycling (stormwater or treated sewage effluent). However, in the second in both sequences of wetlands, endocrine disrupting compounds had diminished in strength, and associated impacts on wildlife would also be lessened. Since all waters are moved through at least three wetlands before being used for irrigation, or being released into Rickabys Creek as environmental flows into the Hawkesbury – Nepean River, the impact on wildlife is expected to be minimal beyond the first in the sequence of wetlands.

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