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The use of Poecilliids to Assess the Endocrine Disrupting Capacity of Waters with Reference to the Mosquitofish Inhabiting Water Bodies in the Sydney Olympic Park, Homebush Bay.

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Abstract:

A number of chemical pollutants have the ability to disrupt endocrine function and cause developmental reproductive abnormalities in humans and animals. They are commonly referred to as endocrine disrupting compounds (EDCs). Fish species have been used to assess waterways for their endocrine disrupting capacity. The use of Poecilliid species, the guppy (*Poecilia reticulata*) and the mosquitofish *Gambusia affinis* and *Gambusia holbrooki* as bioindicator species for EDCs is examined with reference to the mosquitofish inhabiting water bodies in the Sydney Olympic Park, Homebush Bay.

Sydney Olympic Park (SOP), located at Homebush Bay has an extensive history of contamination due to the dumping of 9 million cubic metres of domestic, industrial and commercial waste. The site, now remediated, features sporting, residential and commercial development, as well as extensive parklands and it is therefore important that potential hazard issues at the site are monitored. Monitoring of the containment mound leachate has identified a number of potential EDCs, including dioxins, phenolics and polyaromatic hydrocarbons (PAHs). The male and female mosquitofish (*Gambusia holbrooki*) were used to assess the endocrine disrupting capacity of water bodies within Sydney Olympic Park. It was found that the use of sexual behaviour and reproductive morphology of the mosquitofish was useful in assessing the study sites for the presence of EDCs. As a result there is initial evidence to suggest that two of the sites within Sydney Olympic Park may contain EDCs that elicit an estrogenic or anti-androgenic response in the male fish.

INTRODUCTION

In multicellular organisms, the endocrine system regulates homeostasis, development, behaviour and reproduction through the use of hormones. Endocrine disrupting compounds (EDCs) are those compounds that alter the function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) population (Vos et al. 2000). EDCs can have a feminising effect in males, that is they are oestrogen mimics and/or androgen blockers, or a masculinizing effect in females, by mimicking natural androgens or blocking oestrogen. A number of studies have linked behavioural and morphological affects and population declines in a variety of wildlife species to the exposure of EDCs found in sources including, sewage effluent, detergents, pesticides and industrial chemicals (Colborn et al. 1996).

The world's oceans, rivers and wetlands have become sinks for thousands of chemicals. Fish inhabiting these water bodies, therefore spend their entire life cycle in intimate contact with potential EDCs, this makes fish ideal bioindicators for the identification of EDCs and understanding their effects in the environment. Many fish are also appropriate for use in the laboratory, because, they are easy to breed, have relatively short life cycles and are not high maintenance (Bolis et al. 2001). For these reasons a number of fish species have been used to study endocrine disruption, both in the field and the laboratory, these include, the goldfish, rainbow trout, Japanese Medaka, fathead minnow, the guppy and mosquitofish.

The guppy and the mosquitofish are live bearing fish species that belong the Poecilliidae family. *Poecilia reticulata* (guppy) and *Gambusia affinis* and *Gambusia holbrooki* (mosquitofish) have been used worldwide as indicator species in the study of EDCs both in the field and the laboratory. They are an appropriate species to use in studies of endocrine disruption, because they are sexually dimorphic. The male has a modified anal fin, the gonopodium, which is used as an intromittent organ for internal fertilization. Development of the gonopodium is paralleled by the maturation of the testis, which are both under androgenic control (Turner 1947). A sexually mature male is signified by the presence of terminal elements (hooks and serrae) on its gonopodium, which act as a holdfast device to transfer sperm to the female (Turner 1947). The Poecilliid species have distinctive sexual behaviour, which is strongly linked to reproductive success and is easily quantified under laboratory conditions, it provides a biomarker for affected reproductive capability and hence effects at the population level (Bayley et al. 1999). *Poecilia reticulata* males exercise two distinctive sexual behaviours, the first being the performance of a sigmoid display, which the male uses to stimulate the female into consensual copulation. During this display the body of the male assumes an S or C shape, and vibrates whilst swimming sideways and displaying an orange yellow coloration (Bayley et al. 2002). The second action is one that is performed by the male guppy and mosquitofish, the gonopodial thrust. This involves the male positioning itself under the female and rapidly thrusting its gonopodium forward 180 degrees in and attempt to make contact with her genital pore.

Sexual activity of the male guppy (*Poecilia reticulata*) is significantly reduced upon exposure to a number of estrogenic compounds including 17 β-estradiol and 4-tert Octylphenol (Bayley et al. 1999) and anti-androgenic compounds including, vinclozolin, *p,p'*-DDE and flutamide (Bayley et al. 2002). The male mosquitofish has similarly been found to have reduced sexual activity when exposed sewerage effluent (Gray et al. 2002) and 17 β-estradiol (Doyle and Lim 2002). Exposure of the male guppy and mosquitofish to estrogenic and anti-estrogenic compounds has also resulted in morphological affects, which include, reduced gonopodia length (Batty and Lim 1999, Doyle and Lim 2002, Gray et al. 2002) delayed or inhibited development to sexual maturity (Doyle and Lim 2002 and Bayley et al. 2002), inhibited testes growth and reduced sperm count (Bayley et al. 2002, Toft and Baatrup 2003). Studies have also detected affects in the behaviour and morphology of female Poecilliids when exposed to androgenic compounds (Larsson et al. 2002, Angus et al. 2001).

The Mosquitofish (both male and female) has recently been used to assess the endocrine disrupting capacity of waters within the Sydney Olympic Park (SOP), Homebush Bay. SOP is located 15 kilometres west of Sydney CBD. An estimated 9 million cubic metres of industrial, commercial and domestic waste was remediated, largely by consolidation into waste mounds, prior to the Sydney 2000 Olympics. The site now features sporting, residential and commercial development, as well as extensive parklands. Previous studies at the site (The Environmental Geology Group 2001, Stauber et al. 2000, The Ecology Lab 2001) have identified numerous organic contaminants, such as dioxins, phenolics and PAHs, which are suspected endocrine disruptors. Therefore, although most waste is now contained, managers at SOP wish to study the effectiveness of remediation and the current land management practices by identifying areas of potential concern, including the presence of EDCs.

This paper aims to show how Poecillid species can be used as bioindicators of EDC exposure to identify sites of potential concern with reference to the study using the mosquitofish (*Gambusia holbrooki*) at SOP.

METHOD

Sampling:
Due to their small size Poecilliids can easily be caught in the field using a dip net after which they can be transported back to the laboratory live.

The study at SOP is different to other field studies, which have used the guppy or mosquitofish, because sampling usually occurred downstream of the potential EDC source, for example studies of sewage effluent and pulp mill effluent releases. In these cases the control site is often located upstream of the discharge site. At SOP it was not known whether EDCs were present in the water bodies or their source. This made the allocation of a control site difficult. One option was to source reference sites off site, however, due to extensive land use within the Sydney Basin, many of the water bodies have the potential of containing EDCs. In addition to this the control site/s should not be too geographically removed from the test sites, or environmental and genetic differences between the populations could be confounding factors. For this reason two control sites were selected within SOP. They were selected based on information provided about their histories and were considered to have a low potential of EDC contamination. There were six study sites, two of these being the control sites and four sites considered to potentially exposed to EDCs because of their proximity to a waste mound and/or due to a prior history of contamination.

Fish collected from SOP were transported back to the laboratory after which, the males were separated from the females. The fish were left to acclimatise to laboratory conditions for 24 hours.

There were 2 methods selected for studying EDCs for this project:

1. Sampling fish from the selected sites and studying sexual behaviour and reproductive morphology in the laboratory, and
2. Set up a laboratory experiment using test and control water on the same population of fish.

Sexual Activity
The behavioural tank set up used to measure sexual behaviour in Poecillid species varies depending on the study and the species being used. This is because the guppy and the mosquitofish use two different sexual behaviours when initiating copulation with the female. The male guppy (*Poecilia reticulata*) uses the sigmoid display to entice the female into consensual copulation. Therefore, interaction with the female is required and thus, the male is

placed in the observation tank with the female. The endpoints measured include the males posturing behaviour, number and duration of the sigmoid displays and the males colouration (Bayley et al. 1999, Toft and Baatrup 2003). For the majority of the time, copulation in the mosquitofish, however, is not based on a consensual agreement between the male and female. Often the mating is forced upon the female through sneaky mating. For this reason the female is separated from the male in the observation tank to prevent her from influencing the behaviour of the male through her resistance to copulation.

The methodology that was used to assess the sexual behaviour of the male mosquitofish sampled from SOP is consistent to the method employed by Doyle and Lim (2002). Briefly, two 400mL beakers were suspended in either end of a 20 L glass tank filled with filtered Sydney mains water. In one beaker was placed two mature females and in the other beaker was placed five juvenile males. A mature male was selected randomly from the collected fish, placed into the centre of the tank, left to acclimatise for three minutes and observed for a period of ten minutes. The number of approaches and copulatory attempts made by the male toward the females was recorded. An approach was defined as a lunge by the male toward the female so that its snout touched the glass. A copulatory attempt was defined as a thrust of the gonopodium greater than 90 degrees forward at the females. Following behavioural assessment, each male was euthanised by immersion in 400mg/L of benzocaine and preserved in 70% alcohol for morphological assessment.

Reproductive Morphology

There are many morphological endpoints that have been used in both the guppy and the mosquitofish to quantify affects of exposure to EDCs. The primary focus of measurement for reproductive morphology has been centred on the gonopodium, due to its central role in copulation. Morphological endpoints include, the degree of gonopodial elongation and thickness, gonopodium length and presence of hooks on the gonopodium. In measuring the degree of gonopodium elongation, the length of the fourth ray and sixth rays of the anal fin are measured. This is because, while the 3rd, 4th, and 5th rays of the anal fin in males elongate to form the gonopodium, the other rays do not, therefore, the 4th to 6th ray ratio facilitates an index of elongation (Angus et al. 2001). The degree of thickening of the gonopodium is a measure of the addition of new bone that is added to the third anal fin ray that occurs as the gonopodium develops. By measuring the thickness of rays three and four, the degree of thickening can be calculated (Angus et al. 2001, Doyle and Lim 2002). The degree of elongation and thickness of the gonopodium have been used as morphological endpoints by Angus et al. (2001) and Doyle and Lim (2002) in their studies on reproductive morphology of the mosquitofish.

Two morphological endpoints were measured on the male mosquitofish sampled from SOP, these were gonopodium length and the presence of hooks on the tip of the gonopodium. The males were sexed by the presence of an elongated anal fin, only males greater than 21.00mm were used, as this was the minimum size that sexually mature males (males with hooks on their gonopodia) were found to occur. Each male was photographed using a Leica DC 100 digital microscope camera mounted on a stereo-microscope. Measurements were taken from the images using the Leica Qwin Processing and Analysis System to the nearest 0.001mm. The total length of both males was measured as the longest distance from the tip of the snout to the end of the tail fin. Gonopodium length was measured as the length from the first segment of the third anal fin ray to its distal tip. The presence or absence of hooks on the distal tip of the gonopodium was recorded and the proportion of sexually mature males calculated.

Laboratory Exposure

To verify the behavioural and morphological results obtained from the field sampling at SOP, and eliminate some of the confounding factors associated with field sampling, a laboratory exposure experiment was run. Juvenile male mosquitofish sampled from a reference

population were exposed until maturity to water from one of the sampling sites at SOP that showed evidence of the presence of EDCs in the behavioural and morphological endpoints measured.

Juvenile fish were sampled from a reference site using a dip net and transported back to the laboratory where they were anesthetised in 100mg/L of benzocaine and sexed. Fish were sexed by observing the length of the first segment of the 3rd fin ray. In males the first segment of the 3rd fin ray is longer than that of females (Rosa-Molinar et al. 1998). The laboratory set up comprised of eight 15L tanks in a temperature-controlled room, four allocated to the SOP treatment and four to a control. Water was collected from the SOP site using a belge pump and transported back to the laboratory in 3 x 25L plastic containers, the containers were left to stabilize to room temperature. The water used in the control was dechlorinated Sydney tap water filtered through sand and carbon filters and was adjusted to the same salinity as the water from SOP. There were ten mosquitofish in each replicate giving a total of forty fish per treatment, allocation of the juvenile males was done randomly. Water was renewed weekly and fish were fed twice a day with a predetermined amount of fry fish food. The exposure ran for a period of 12 weeks, after which time the sexual behaviour was quantified and morphological parameters measured, using the method described above.

Use of the Female Mosquitofish
Measurements were taken on the anal fin of female mosquitofish sampled from sites within SOP; to determine weather there was any evidence of androgenic or anti-estrogenic EDCs in the water bodies at each site. The total body length and gonopodium length were measured using the same method described for the male mosquitofish.

RESULTS AND DISCUSSION

Although no actual results are presented here, a brief summary of results follows:

Preliminary analysis of the six study sites demonstrated that mosquitofish sampled from one of the control sites showed evidence of endocrine disrupting affects. For this reason, this site was no longer deemed fit as a control and therefore statistical comparisons for all endpoints measured where only performed against the one control site. This highlights the difficulties of selecting appropriate reference and control sites and the need for preliminary investigation either of the water and/or the fish population for evidence of EDCs. One method that may prove useful as a screen when selecting sites is the recombinant yeast oestrogen screen. This assay, detects the presence of estrogens in a water sample due to a reaction at occurs with a genetically introduced human oestrogen receptor within the yeast (Routledge and Sumpter 1996).

Sexual Activity
The study of sexual activity of the male mosquitofish inhabiting sites within SOP found male fish sampled from three sites exhibited reduced sexual activity compared to that of males from the control site. Reduced sexual activity has been found to occur in both the guppy and mosquitofish when exposed to estrogenic or anti-estrogenic compounds (Bayley et al. 2002, Doyle and Lim 2002). The sexual activity results of males sampled from SOP therefore suggest that EDCs may be present at the sites in which the males sampled demonstrated reduced sexual activity. As sexual behaviour of both the guppy and the mosquitofish has been linked to reproductive success, the effects measured in sexual activity in the male mosquitofish at some of the sites with in SOP are probably representative of an endocrine disrupting effect on the population. One of the sites demonstrating an affect was a site that was originally thought of as a possible control site. This perhaps indicates that this site may not have been exposed to contaminants from past activities within the area, however, is being exposed to EDCs by current land management practices.

Reproductive Morphology

Male populations sampled from two of the three sites that had previously demonstrated affected sexual behaviour had significantly reduced gonopodia and significantly less sexually mature males when compared with the males sampled from the control population. This consistency between behavioural and morphological endpoints further supports the likelihood that EDCs are present at the sites where the males are demonstrating these affects. A consistency between endpoints that measure different processes within an organism, may suggest the EDCs are having an affect on more than one of the individual's hormonal processes. This is demonstrated in previous studies by the affects that have been measured in the Poecillid species when exposed to 17 ß-estradiol, these affects include: significantly reduced sexual behaviour (Bayley et al. 1999, Doyle and Lim 2002), inhibited testes growth and fewer offspring output following the exposed males copulation with females (Toft and Baatrup 2001), blocked spermatogonial mitosis (Kinnerg et al 2003), female biased sex ratio and reduced sperm count (Toft and Baatrup 2003) and reduced gonopodium length, degree of elongation and overall percentage of mature males (Doyle and Lim 2003). When a study demonstrates an affect on an organism after exposure to an EDC for one or two endpoints, it is possible that other endpoints that were not measured are being affected within the individual. Therefore, the male mosquitofish populations exhibiting behavioural and developmental affects sampled from the various sites within SOP may also be experiencing a number of other affects that have not been quantified in this study.

Laboratory Exposure

There were differences observed in the sexual behaviour and reproductive morphology of male mosquitofish exposed to water from the SOP site compared to males exposed to the control water, however, these differences were not significant. A significant difference was detected between the two treatments using the end point of sexual development. This inhibition of sexual development in males may be due to the presence of EDCs in the water, Bayley et al. (2002) found that the maturation of juveniles exposed to anti-androgens resulted in a delay in maturation of the males by up to four weeks and Doyle and Lim (2003) reported that none of the mosquitofish exposed to the highest concentration of 17 β-estradiol during a laboratory exposure developed hooks on their gonopodia, compared to over fifty percent of the males in the control treatment.

Female morphology

Anal fin measurements taken on females inhabiting the water bodies within SOP were not different from the anal fins of females inhabiting the control site. Previous studies have demonstrated that females exposed to androgenic compounds have developed gonopodia. Following exposure to methyltestosterone the female guppy (*Poecilia reticulata*) was reported to exhibit male colouration, a gonopodium like structure, induced male sexual behaviour and failed to reproduce (Larsson et al. 2002). Similarly gonopodium like structures were induced in the female mosquitofish after exposure of kraft mill effluent and testosterone (Botone and Cody 1999 and Angus et al. 2001 respectively). Female guppies and mosquitofish therefore can be useful bioindicators of androgenic and/or anti-estrogenic EDCs. Thus, based on the morphological results of the females sampled from the various sites at SOP, it appears that any EDCs present in the water bodies are not androgenic or anti-androgenic in their nature.

Limitations

Affects measured in behavioural and morphological endpoints may not representative an affect exclusively caused by EDCs. There are a number of factors for example that may influence behaviour, and morphological differences may be due to natural differences between populations. The study at SOP has been effective in identifying areas that appear to be showing affects caused by the presence of EDCs and these affects are consistent for all measured endpoints. It is recommended that further research is conducted at these sites into their endocrine disrupting capacity. Endpoints such as histological analysis of testis and/or

measurement of vitellogenin a female specific protein (Angus et al. 2002) which have been measured in Poecilliids and are more directly correlated with EDC exposure could be used.

CONCLUSION

Poecilliid species, including the guppy and the mosquitofish, have proved to be useful bioindicators in identifying compounds that elicit an endocrine disrupting effect demonstrated by previous field and laboratory studies and the recent study conducted at SOP. Due to their distinctive sexual behaviour and reproductive morphology, EDC effects are easily quantified in the individual and the results from these endpoints can be linked to affects on the population. Behavioural and morphological endpoints measured on the male mosquitofish sampled from the various sties within SOP, suggested that there was evidence of EDCs present at some of the study sites. The results from the endpoints measured on the male and the female mosquitofish also suggest that any EDCs present are eliciting an estrogenic and/or anti-androgenic affect. Weather these affects are due to the past history of contamination within SOP or due to current land management activities is yet to be determined. Overall the mosquitofish (*Gambusia holbrooki*) was an effective indicator species for the initial assessment of the endocrine disrupting capacity of the waters at SOP, highlighting the areas that require further study.

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