An Investigation into the Effects of Reproductive Endocrine Disrupters on the Sexual Behaviour and Morphology of Male Mosquitofish, *Gambusia* sp.

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ABSTRACT

It has recently been acknowledged that effluents from sewage treatment plants (STPs) are one potential source of endocrine disrupting chemicals (EDCs). This diverse group of chemicals can include environmental oestrogens or exoestrogens, substances that mimic or antagonise gonadal hormones. These are of particular concern because they are known to impair the sexual development and reproductive success of vertebrates, including fish. It is well established that reproductive behaviours are regulated by endogenous hormone concentrations, yet there has been very little work conducted on the effects of these EDCs on behaviour. The experiments described in this thesis investigate the reproductive behaviour and morphology of adult male mosquitofish (*Gambusia* sp.) which have been exposed in the laboratory to low levels of oestrogens, an oestrogen mimic and sewage effluent and also exposed under natural conditions through inhabiting two sewage contaminated rivers in NSW, Australia. All males were observed for reproductive behavioural characteristics in a standard observation procedure, and several reproductive characteristics were also recorded: gonopodium length (GPL), gonadosomatic index (GSI: testis to body weight ratio), testis area (TA mm^3), body condition index (BCI: using Fulton's condition factor - body weight x 100 / body length^3), and number of spermatozeugmata, or SPZ (visible sperm packets).

Adult male mosquitofish were exposed to 0.4, 2 and 10ng/l diethylstilbestrol (DES), 20, 100 and 500ng/l 17β-oestradiol (E2) and 2, 10 and 50μg/l octylphenol (OP) for 8-10 weeks in the laboratory. These experiments demonstrated significant reductions in the reproductive behaviour of exposed males compared to controls, but no consistent treatment effects on the other reproductive characteristics of the fish were found.

Exposure of adult male mosquitofish to 25, 50 and 100% treated sewage effluent for 8-10 weeks in the laboratory yielded variable results in reproductive behaviour. Males collected from an urban polluted river in 1999 and 2000 exposed to 50% and 100% sewage effluent demonstrated a significant reduction in approach behaviour but not in duration in female zone or mate attempt. However, no significant differences in

reproductive behaviour were observed in exposed males collected from a 'pristine' river. No differences in reproductive morphological characteristics were found between treatment groups.

When wild adult males were sampled 10km downstream of St. Marys sewage treatment plant (STP) outfall in 1999 and 2000 and observed in the laboratory, they did not show any differences in the reproductive behaviour or morphology compared to upstream fish. However, males collected 5km downstream of the same outfall in 2002 were suggestive of a reduction in reproductive behaviour, compared to upstream males. In addition, they had significantly reduced GPL, TA and GSI compared with upstream. Similar reductions were found when wild adult males were sampled from another sewage-contaminated river in NSW, downstream of Quakers Hill STP in 2000 and 2002. Laboratory examination of males sampled from a site 50m downstream from this sewage outfall showed significantly reduced levels of reproductive behaviour over both years compared to upstream. Males collected in 2002 had significantly reduced BCI, TA and suggested decrease GSI compared to upstream fish, but this was not apparent in the 2000 sample. There were no significant differences in GPL and SPZ counts in fish between up and downstream sites of this STP outfall.

The overall pattern of results is consistent with a hypothesis of reproductive endocrine disruption, potentially impacting populations of fish inhabiting sewage-contaminated rivers.

ACKNOWLEDGEMENTS

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List of Abbreviations

ANCOVA	analysis of covariance
ANOVA	analysis of variance
AP	alkylphenol
APEO	alkylphenol polyethoxylate
BCI	body condition index
BL	total body length
BOD	biological oxygen demand
DES	diethylstilbestrol
E2	17β-oestradiol
EE2	ethynyloestradiol
EDC	endocrine disrupting chemical
ER	oestrogen receptor
GP	gonopodium
GPL	gonopodium length
GSI	gonadosomatic index
GtH	gonadotropin
GtRH	gonadotropin releasing hormone
HPG	hypothalamus-pituitary-gonadal (axis)
HSD	honest significant difference
KME	kraft mill effluent
NP	nonylphenol
OP	octylphenol
RED	reproductive endocrine disrupter
SE	sewage effluent
SPZ	spermatozeugmata (sperm packets)
STP	sewage treatment plant
TA	testis area
UTS	University of Technology, Sydney
VTG	vitellogenin
WWTP	wastewater treatment plant

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CHAPTER 1

General Introduction

1.1 ENDOCRINE DISRUPTER HYPOTHESIS – THE BEGINNING

Nearly 40 years ago, Rachel Carson's book *Silent Spring* identified the urban use of pesticides (primarily DDT) as the cause of a noticeable decline of birds singing in the US and also the cause of mass songbird mortalities (Carson, 1962). While direct exposure to DDT is not highly toxic to birds, heavy and repetitive use of the pesticide is. The bioaccumulation of DDT in non-target species of earthworms resulted in levels that were high enough that songbirds (which ingested the earthworms) received lethal doses of the pesticide, resulting in large losses of urban birds. Carson emphasised that pesticides (e.g., organochlorines and DDT) and other synthetic chemicals (e.g., PCBs) were collecting in and contaminating water, soil, wildlife and even humans. These chemicals, she warned, were causing severe health problems (cancer and death) in wildlife, especially in species at the top of the food chain that eat other contaminated animals and accumulate the most chemicals. Subsequent research has identified other pesticides and industrial chemicals that cause mortality and reproductive impairment in vertebrates (fish, birds, and mammals), (Leatherland, 1992; Bowerman *et al*, 2000; De Guise *et al.*, 1994a), and scientists are beginning to understand the processes of how these substances affect health (for a review refer to Kavlock *et al*, 1996).

A decade after the publication of *Silent Spring*, scientists were discovering that reproductive abnormalities were evident in male and female offspring whose mothers were administered the drug diethylstilbestrol (DES). DES is a synthetic oestrogen that was widely used in the 1950s and 1960s to prevent miscarriages and enhance growth in livestock. It was banned from use in the 1970s because it was recognised for its carcinogenicity in relatively high doses during pregnancy (effects of this discussed in more detail in section 1.2.5). The parallel between the similar reproductive changes observed in DES children and the reproductive effects observed in wildlife led to the hypothesis that many pollutants have endocrine disrupting effects (Colborn *et al.*, 1993).

Researchers are concluding that a wide range of chemicals (in addition to chemicals in some pesticide formulations) are contaminating the environment and altering the endocrine system (hormone levels and sexual development), which could severely limit the ability to reproduce (Colborn and Clement, 1992; Kavlock *et al.*, 1996; EC, 1997).

1.2 BACKGROUND TO ENDOCRINE DISRUPTING CHEMICALS

This section will summarise what is meant by the 'endocrine system', and summarise the physiological effects of some of the hormones directly associated with reproduction. The evidence that polluting compounds can effect the action of the endocrine system will be reviewed.

A wide variety of physiological processes are carried out by the endocrine system through chemical messengers called hormones. The endocrine system is a collection of glands that produces hormones, which are necessary for physiological regulation. All vertebrate animals (fish, amphibians, reptiles, birds and mammals, including humans) have an endocrine system with many similarities, including the release of similar or identical hormones (the function of these may however differ). Hormones control a wide range of physiological processes such as reproduction, sexual development, metabolism, and growth.

There is considerable scientific, regulatory and public concern regarding the possible adverse affects of environmental pollutants capable of disrupting endocrine function in humans and wildlife (e.g., Colborn and Clement, 1992; EC, 1997; Cooper and Kavlock, 2001). Although routes of exposure are diverse for these endocrine disrupting chemicals (EDCs), it has been suggested that the dangers are greatest for aquatic animals, which are constantly exposed to low concentrations in the environment (White *et al.*, 1994). This section will cover the source and chemical nature of EDCs, the mechanism of action, the main routes and effects of human exposure and the scope of the environmental problem, in particular the effects reported on fish.

1.2.1   What are Endocrine Disrupting Chemicals?

Endocrine disrupting chemicals (EDCs) have been variously defined as "exogenous agents that interfere with the production, release, transport, metabolism, binding action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock *et al.*, 1996) or "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function" (EC, 1997).

The above definitions include a broad spectrum of chemicals inducing a variety of endocrine effects; most attention so far has been given to those substances that mimic the natural female steroid hormone 17ß-oestradiol (E2). This endogenous steroid plays a central role in the hormonal pathways involved in the regulation and development of gametes, sexual phenotype, and sex-specific behavioural characteristics in vertebrates (Arcand-Hoy and Benson, 1998).

1.2.2   Source of Reproductive Endocrine Disrupters (REDs)

A wide diversity of chemicals, natural and man-made, have been found capable of disrupting normal endocrine function. There are many kinds of EDCs, but one area of increasing concern, and the focus of this study, is the impact of certain pollutants on gonadal hormone function, which is critical for reproduction. These so-called reproductive endocrine disrupters (REDs) are a sub-group of EDCs that imply the impact of contaminants on reproductive hormones (e.g. oestrogens and androgens).

A few well-documented cases of high-level exposure (usually from accidental spills or the pre-existing residues of highly bioaccumulative substances) that have affected wildlife reproduction lead to the discovery of their hormone-like activity. Some of these REDs include: natural dietary phytoestrogens (from plants); mycoestrogens (from fungi); natural

and synthetic hormones; organochlorine pesticides; polychlorinated biphenyls (PCBs); dioxins; alkylphenol polyethoxylates (APEOs) (used in detergents); phthalates (used in plastics); chlorotriazine herbicides; fungicides and Bisphenol-A (used in dental sealants and the lining of food cans). For a detailed review of sources of endocrine disrupters refer to Kavlock *et al.*, (1996).

1.2.2 The Vertebrate Reproductive Endocrine System

Reproductive hormones are not necessary for life, but are essential for reproduction. Oestrogens are steroid hormones made primarily in the female ovaries and the male testes in humans and other vertebrates. Known as the female hormones, oestrogens are found in greater amounts in females than males. These essential molecules influence growth, development and behaviour, regulate reproductive cycles (e.g., menstruation, pregnancy) and affect many other body parts (e.g., bones, skin, arteries, the brain, etc.).

The hypothalamus, through the influence of environmental signals (photoperiod, temperature, feeding and social factors), controls the synthesis and release of hormones and chemical messengers resulting from neural stimulation from the central nervous system (CNS). The gonads, adrenal cortex, and placenta secrete steroid hormones. Negative feedback regulates the secretion of almost every hormone. Reproductive cycles in vertebrates are mediated by the hormones of the hypothalamus (gonadotropin releasing hormone; GnRH) and of the pituitary (gonadotropin; GtH), which in turn act on the gonads to stimulate synthesis of steroid hormones. For example, the oestrogen oestradiol is regulated through a complex series of chemical indicators that involve at least three glands: the hypothalamus, the pituitary and the ovary. In most vertebrate taxonomic groups, the process starts when the hypothalamus responds to a low level of oestradiol in the blood by releasing the hormone GnRH, which then stimulates the pituitary gland to synthesise and release two more hormones:

- LH (luteinizing hormone) and

- FSH (follicle-stimulating hormone)

Unique to female mammals, LH and FSH stimulate the ovaries to secrete oestradiol and progesterone, which stimulates the growth of the egg-producing ovarian follicle and prepares the uterus for pregnancy. In males, LH and FSH stimulate the testes to secrete the androgen testosterone, which then stimulates sperm production. The ovaries (and testes) make and release oestradiol (and testosterone) until a certain level is reached in the bloodstream. The hypothalamus and pituitary then stop secreting GnRH, LH and FSH hormones. This causes the ovaries to stop releasing oestradiol and progesterone (or the testes to stop releasing testosterone). In all vertebrates, the liver also influences this feedback loop; as part of its normal cleaning function, the liver degrades some of the hormone molecules, removing them from the blood and lowering the amounts found in the system.

Two forms of GtH have been isolated form fish (Kawauchi et al., 1989; Swanson et al., 1991). GtH I and II are analogous to mammalian follicle stimulating hormone (FSH) and luteinising hormone (LH), respectively. GtH I is involved in gametogenesis and steroidogenesis, whereas GtH II is involved in the final maturation stages of gametogenesis. Generally, the gonadotropins are responsible for stimulating the sex steroids (androgens, oestrogens and progestins) which in turn act on target tissues to regulate gametogenesis, reproduction, reproductive phenotype and behavioural characteristics.

The concepts of organisation versus activation have been useful in explaining the role of hormones in the differentiation of vertebrate sexual dimorphism, whether morphological, physiological or behavioural (Arnold and Breedlove, 1985). Mechanisms of hormone action have traditionally been divided into effects that occur early in development and are permanent (i.e., organisational) versus those that occur later in development and are transitory because they depend upon the presence of circulating hormones (i.e.,

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activational). For example, the testis synthesises and releases testosterone early in development (pre-pubertal) and induces the differentiation of the Wolffian duct (part of the male internal reproductive duct system). This change is irreversible, is an organisational effect. In contrast, the pubertal stimulation of reproductive duct growth and secretion by testosterone is activational since it can partly be reversed if testosterone ceases to be released.

The concepts of organisational versus activational roles of hormones are central to understanding the effects of environmental EDCs. Effects of hormones in adulthood are typically transient (activational effects), and exposure to an environmental chemical that interferes with signalling molecules can modulate the functioning of systems while the chemical is present, but the effects disappear when the chemical is not present (assuming exposure levels were not high enough to cause permanent damage to cells).

1.2.4 Mechanism of Action of Reproductive Endocrine Disrupters (REDs)

The specific mechanisms by which steroids (and REDs) elicit organisational effects on the endocrine system are lacking for most wildlife. The endocrinology of only a small percentage of bony fish (teleosts) has been studied to any significant degree, therefore, many of the mechanisms leading to disruption of endocrine function in fish is unknown (Leatherland, 1992; Arcand-Hoy and Benson, 1998).

A wide diversity of chemicals has been shown to mimic or antagonize the action of endogenous hormones through a receptor-mediated mechanism, although indirect effects may occur through actions on the pituitary and hypothalamus (Soto et al, 1992). Endocrine disrupters alter hormonal functions by several means; these substances can mimic or partly mimic the sex steroid hormones such as oestrogens and androgens by binding to hormone receptors or influencing cell signalling pathways. Those that act like oestrogen are called environmental oestrogens. They can also block, prevent and alter hormonal binding to hormone receptors or influencing cell signalling pathways.

Chemicals that block or antagonise hormones are labelled anti-oestrogens or anti-androgens. They have the ability to alter production and breakdown of natural hormones, and/or modify the making and function of hormone receptors. Environmental oestrogens are the most studied of all the endocrine disrupters because of their widespread use and detection levels in fish, rivers and food (e.g., Sumpter and Jobling, 1995; Desbrow *et al.*, 1998 and Guenther *et al.*, 2002).

There are many kinds of REDs, and at first glance, their structures may appear dissimilar (Figure 1.1). As mentioned previously, most attention so far has been given to those substances that mimic the natural female steroid hormone 17ß-oestradiol, which can disturb reproductive function. 17ß-oestradiol is an 18-carbon steroid with a phenolic A ring (illustrated in Figure 1.1). The phenolic A ring is the structural component responsible for high-affinity binding to the oestrogen receptor (ER), (Arcand-Hoy *et al.*, 1998). Most environmental oestrogens possess a *para*-substituted phenolic group (Jordon *et al.*, 1985). The presence of more than one phenolic group can render a compound more oestrogenic (Nimrod and Benson, 1996). When the phenolic ring binds to the ER, the rest of the molecule plays an important role in determining whether the compound will act as an agonist or an antagonist. A more in depth explanation of structural features that distinguish oestrogen agonists and antagonists can be found in Duax and Griffin (1987).

Figure 1.1 displays the chemical structures of natural and synthetic oestrogens, which, apart from 11-ketotestosterone, have been used in this study. Although not usually an environmental contaminant, diethylstilbestrol (DES) is used in many studies as a 'model' oestrogen in investigative studies due to its high potency and relatively slow degradation. DES is included in this study as a 'model' oestrogen (Figure 1.1).
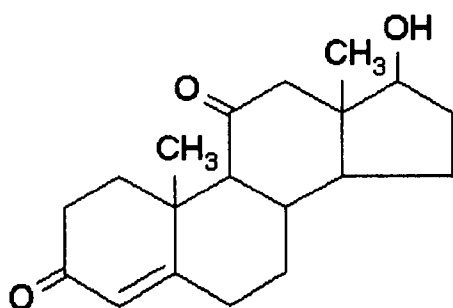
11-ketotestosterone (11-kT)
In addition to testosterone (T), 11-kT
is a major androgen in male fish.
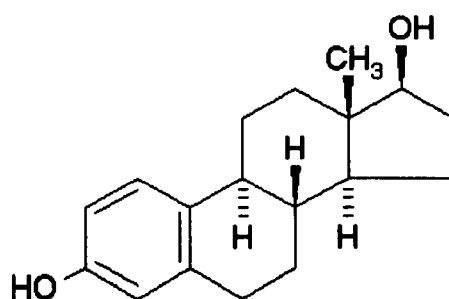
17ß-oestradiol (E2)
The most potent natural oestrogen
in vertebrates.

Natural
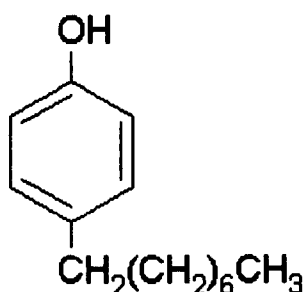
Diethylstilbestrol (DES)
A pharmaceutical oestrogen banned
from use in the 1970s. DES is a well
known potent oestrogen mimic.
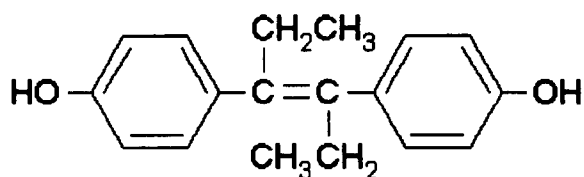
Octylphenol (OP)
A breakdown product of detergents that are
widely used in household products,
agricultural and industrial applications, and
in plastics manufacturing. OP has been
demonstrated to induce weak oestrogen
effects in fish.

Synthetic

Figure 1.1 Chemical structures of steroids analysed (except 11-kT) – they include

natural and synthetic hormones (E2 and DES) or chemicals that can induce hormone-like

effects (OP).

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1.2.5 Human Exposure

The observed changes in wildlife reproductive health may parallel changes observed in humans (Harrison *et al.*, 1997). Although there are recognised adverse trends in some aspects of reproductive health in humans, the causes of these trends are largely unknown, but they all have a component of sensitivity towards REDs. The human aspect of exposure to REDs is outside the scope of this research, but effects in humans for which links with exposure to REDs will be reviewed in this section.

One of the situations that most clearly indicate the extent to which synthetic chemicals can harmfully affect humans involves the drug diethylstilbestrol (DES), a synthetic oestrogen, which was prescribed to many women in an effort to prevent miscarriages in the 1950s and 1960s. Not only did DES not prevent miscarriages, but it also had many harmful effects on the children of many of these women. This drug caused increased rates of vaginal cancer (Herbst *et al.*, 1971), deformities of the uterus and abnormal pregnancies (Palmlund *et al.*, 1993), and immune-system dysfunction in daughters born to women who took the drug (Blair, 1992). In addition, both sons and daughters exhibit reduced fertility (Wingard and Turiel, 1988), and daughters are more likely to have had premature births, spontaneous pregnancy losses, or ectopic pregnancies than unexposed women (Kaufman, 2000). Despite these major problems in the children, the mothers who took DES were not apparently affected. Unlike the infamous drug, thalidomide, which caused gross malformations of limbs in 8000 children in 46 countries, DES's more subtle, but potentially more harmful effects escaped notice for years. Unless the effects are physically apparent or severe, signs of hormone disruption may not be noticed for many years in humans. For example, a major symptom of the DES daughter syndrome is the occurrence of vaginal clear cell carcinoma in young women (Herbst *et al.*, 1971). This phenomenon only occurs after puberty when plasma oestrogen concentrations are elevated and stimulate reproductive tract growth.

A concern has been raised that exposures to ambient levels of oestrogenic compounds in the environment may also be having adverse effects on development and fertility of people as well as increasing the rates of certain types of cancers. One of the first reports, and many that followed, proposed that the cause of the falling sperm counts in some countries (Sharpe and Skakkebaek, 1993; Auger *et al.*, 1995; Van Waeleghem *et al.*, 1994) and increased reproductive abnormalities in men was increased exposure to oestrogens in the womb (Sharpe and Skakkabaek, 1993; Giwercman and Skakkabaek, 1992; Sharpe, 1993). They cited the DES experience and laboratory studies as support for this hypothesis, noting that prenatal exposure to elevated levels of synthetic or natural oestrogens resulted in reduced sperm counts and an increase in the incidence of undescended testicles, hypospadias, and possibly testicular tumours in male offspring (Sharpe and Skakkebaek, 1993). There is evidence to suggest that increases in the rates of disorders of the male reproductive tract, like hypospadias and cryptorchidism associated with higher rates of testicular cancer (in adulthood), lower sperm counts and lower fertility, are concentrated in more affluent nations or highly industrialised countries (Paulozzi, 1999).

The changes induced by *in utero* exposure of rats to phthalate esters are similar to the testicular abnormalities noted on the increase in trend analyses of humans. Studies in several laboratories demonstrated that several, but not all of the phthalate esters, have 'anti-androgenic' activity (Mylchreest *et al.*, 2000; Li *et al.*, 2000; Gray *et al.*, 1999b). However, indirect evidence will have to suffice in evaluating the possible link between rodent chemical exposure and human male reproductive disorders (Toppari *et al.*, 1996). More significant, a recent study of Puerto Rican girls (Colon *et al.*, 2000) has provided new evidence that suggests a possible association between these phthalate plasticisers and the cause of premature breast development in the human female population. The phthalates that they identified have been classified as endocrine disrupters.

Analysis of human trend data reveals that over the last 50 years the rate of breast cancer in women living in the developed world has increased (Kohlmeier *et al.*, 1990; Hoel *et*

al., 1992). In 1993, a group of researchers working on these trends proposed the theory that hormonally active synthetic chemicals may be causal in both the rise in breast cancer incidence and deaths among older women by increasing their overall oestrogen exposure (Davis *et al.*, 1993). They also hypothesise that prenatal exposure to oestrogens may predispose a woman to breast cancer later in life through an "imprinting" process that sensitises her to oestrogen exposure. This study and theory is one of many (Pujol *et al.*, 1994; Dewailly *et al.*, 1994; Wolff *et al.*, 1993; Krieger *et al.*, 1994), but because of our poor understanding of what causes breast cancer and significant uncertainties about exposure, it may take some time to satisfactorily test the hypothesis and evaluate whether synthetic chemicals are contributing to rising breast cancer rates.

Endometriosis (a reproductive abnormality in women) is an oestrogen-dependent disease characterised by the presence of endometrial glands and stroma outside the uterine cavity. The etiology of this disease remains elusive, but is clearly influenced by genetic, immune, and endocrine factors. The potential role of exposure to environmental toxicants, such as PCBs and dioxins, and the pathophysiology of the female endometriosis have been met by many studies (Rier *et al.*, 1993, 1995; Pauwels *et al.*, 2001). Rier *et al.*, (1993) studying the long-term reproductive effects of the dioxin TCDD, found a dose-dependent relationship between dioxin and endometriosis in the rhesus monkey. Animals with more exposure were more likely to develop the disease, and the greater a female monkey's exposure to dioxin, the greater the severity of the disease. Only one of 7 animals exposed to 25 parts per trillion dioxin was free of endometriosis. Experimental work has now afforded additional clues; in mice and rats, exposure to dioxin increases the size of endometriotic sites that are experimentally induced (Rier *et al.*, 1995). However, Pauwels *et al.* (2001) showed no significant association between exposure to dioxin-like compounds and the occurrence of endometriosis in infertile women (although after adjusting for body mass index and alcohol consumption, the risk increased slightly).

The realisation in recent decades that a substantial number of environmental chemicals possess oestrogenic activity has understandably raised concern as to whether or not these

could affect humans and whether this might explain the increase in incidence of reproductive disorders outlined above.

1.2.6 Environmental Problem of Reproductive Endocrine Disrupters

The original hypothesis concerning effects of exposure to endocrine disrupters came from studying the reproduction of natural populations of animals living around the Great Lakes in North America (Colborn et al., 1993).

Organochlorine pesticides represent one of the better-studied groups of REDs. A frequently cited example of environmental effects of the pesticide DDT is the work done by Guillette et al. (1994; 1995) on the alligator population in Lake Apopka, Florida. Severe reproductive problems in male alligators were noted 10 years after a chemical spill of DDT and other pesticides (chlorinated and organophosphate insecticides, and a copper-based fungicide). Although the water was found not to contain any measurable concentrations of pesticides, the alligators and their eggs had detectable levels of endocrine disrupting pesticides. They provided detailed evidence of higher than normal mortality among eggs and newborn alligators; juvenile females had severe ovarian abnormalities (an increase in polyovular follicles and polynuclear oocytes) and had blood oestrogen levels two times higher than normal and; the male juvenile alligators were de-masculinised (or feminised), that is, they had smaller than normal penises, had abnormal testes and had higher oestrogen levels and lower testosterone levels in their blood than normal males of the same age.

Similarly, Crews et al. (1995) found that contamination on Apopka has also taken effect on its red-eared turtle (*Trachemys scripta elegans*) population. Unlike the carnivorous alligators, which are top predators and therefore potentially exposed to higher levels of contaminants that have become more concentrated in the food web, the red-eared turtles eat plants - a dietary habit that should expose them to less pollution. Nevertheless,

researchers found the gonads of many turtles showed characteristics of both sexes, termed intersex, i.e., presence of ovary and testis (ova-testis). However, it should be noted that while the observations with alligators and turtles are consistent with exposure to a hormonally active substance, the phenomenon appears to be mainly restricted to Lake Apopka, and it represents a sudden considerable exposure and is not quantitatively comparable to the more diffuse exposure to ambient levels of environmental contaminants elsewhere.

The negative effects of REDs in many wildlife species have been consistent with those effects demonstrated in laboratory exposures to specific pollutants. For example, Park *et al.* (2001) discovered that exposure to extremely low levels of a commonly used pesticide, endosulfan, interferes with reproduction in the red-spotted newt (*Notophthalmus viridescens*) by disrupting the development of glands that synthesize a pheromone used in female-male communication (exposed females had smaller glands). Affected females produced less pheromone and were less likely to attract mates than unaffected females, which could lead to lower mating success. Their experiments reveal an impact at 5 parts per billion (ppb), which was the lowest concentration they used. The US EPA recommends that the amount of endosulfan in lakes, rivers, and streams should not be more than 74 ppb, almost 15 times higher than the level reported by Park *et al* (2001). This report is important because it shows that biologically significant effects can easily be missed by traditional toxicological testing, at levels far beneath those targeted by regulations.

A study by Willingham *et al.* (2000) demonstrated that different REDs could alter sex ratios via different biochemical mechanisms. They exposed red-eared slider turtles (*Trachemys scripta elegans*) to PCB mixture Arochlor 1242, trans-nonachlor and chlordane during foetal development and found more females were hatched in the groups treated by the three compounds than expected (based on the strongly male-biased sex ratio of the unexposed eggs). Arochlor and chlordane both suppressed testosterone levels in hatchling males, as did chlordane in hatchling females. Chlordane treatment also

suppressed progesterone levels in hatchling females. Willingham *et al.* interpret details of the results to suggest that chlordane's impact on sex ratio is a result of anti-androgenic activity by the compound, whereas trans-nonachlor is working as an oestrogen mimic.

There has been few studies investigating the potential RED impacts on the invertebrate phyla, with the exception of the occurrence of imposex (male reproductive characteristics in female genitalia) in several species of marine gastropods following exposure to organotin compounds (Bryan *et al.*, 1986), and a study by Oehlmann *et al.* (2000). This latter study reports that bisphenol A and octylphenol create "superfemale" snails at extremely low levels (of 1 µg/L). The authors exposed two species of snail, one from freshwater habitats, the ramshorn snail (*Marisa cornuarietis*), the other from salt water, the dogwhelk (*Nucella lapillus*) to varying concentrations of bisphenol A and octylphenol (1 µg/L to 100 µg/L), and examined the consequences of exposure on morphology and reproduction in the two snails. Exposed adults of both species responded even to the lowest level of exposure with the female genital system malformed while spawning mass and egg production were increased. Oehlmann *et al.* (2000) called these "superfemales." In *Marisa*, they observed dramatic excessive growth (hypertrophy) of the two female glands and enhanced production of spawning mass (eggs and fluid), which was so extreme that it ruptured the pallial oviduct in 4% of cases, resulting in the animal's death. Rupturing occurred at all levels of BPA exposure (above 0), irrespective of the applied concentration. The dose-response for *Marisa* treated with octylphenol revealed a non-monotic form: intermediate levels of exposure produced a much greater change in spawning masses per female and eggs per female than did lower or higher exposures. *Nucella* females also developed the "superfemale" phenotype, with an increase in egg production and expansion of the pallial female sex glands. No rupturing occurred, however, because of a difference in the anatomy of *Nucella* compared with *Marisa*. *Nucella* males were also affected while *Marisa* males were not. Fewer males had sperm in the seminal vesicle, and penis and prostate glands were reduced in size.

Although many classes of chemicals are known to have oestrogenic effects, this study will concentrate on some of those oestrogenic compounds that have been identified in sewage effluent.

1.2.7 Oestrogenic Contaminants Identified in Sewage Effluent

REDs may be introduced into the aquatic environment by means of discharge from industrial effluents, from municipal sewage treatment facilities, and as run-off from agricultural waste products. Sewage treatment plants (STPs), receiving both industrial and domestic wastewaters, can release a complex mixture of hormonally active chemicals into the aquatic environment. Oestrogenic compounds identified in some sewage effluents include natural oestrogens, 17β-oestradiol, oestrone and oestriol, ethynylestradiol, and xenoestrogens (chemicals that are structurally dissimilar to oestrogens but have oestrogenic properties) such as alkylphenols, pthalates and some pesticides (Desbrow *et al.*, 1998; Korner *et al.*, 2000).

Alkylphenol polyethoxylates (APEOs) have been in use for over 40 years, they are used as detergents, emulsifiers, wetting agents, and dispersing agents in household products and in agricultural and industrial applications. Alkyphenols (APs) in STPs are mainly a result of the biodegradation of APEOs. Description of the aerobic and anaerobic biotransformation pathways of APEOs shows that the degradation is initiated by sequentially cleaving ethoxylate units (Ahel *et al.*, 1994).

Their surfactant activity is derived from the alkylphenol hydrophobe and a *para*-substituted long chain of repeating ethylene oxide units as the hydrophilic moiety. The ethoxylate chain may have one to 100 repeating units; the longer the chain, the more water-soluble the compound. The alkyl group is typically a branched nonyl, octyl, or dodecyl chain. While the mechanism of toxicity of non-ionic surfactants to aquatic organisms is unclear, the chemical structure is predictive of a detrimental effect (Nimrod

and Benson, 1996). The toxicity of APs to aquatic organisms increases with a decreasing number of ethylene oxide units and increasing hydrophobic length. Therefore, the toxicity of the parent surfactants is less than the degradation products. The chemical structure of OP is illustrated in Figure 1.1.

Compounds such as APs, for example, octylphenol (OP), a breakdown product of APEOs, have been shown to possess oestrogenic activity by mimicking the action of 17β-estradiol (Jobling and Sumpter, 1993; White *et al.*, 1994; Sharpe *et al.*, 1995; Routledge and Sumpter, 1996; Jobling *et al.*, 1995, 1996). A variety of *in vitro* approaches (e.g., oestrogen receptor assays, fish hepatocyte vitellogenin synthesis, MCF-7 cell proliferation) have been used to confirm that APEOs (mainly nonyl- and octyl-) have oestrogenic properties. For example, the binding of APEO degradation products measured by White *et al.* (1994) demonstrated that both OP and nonylphenol (NP) were able to compete with oestradiol for liver oestrogen receptor (ER) binding sites in rainbow trout, i.e., OP and NP was interacting at the same site of the ER as oestradiol.

As mentioned previously, one potential source of environmental oestrogens in the aquatic environment is through the discharge of sewage. An investigation by Desbrow *et al.* (1998) demonstrated that the synthetic oestrogen ethynylestradiol, as well as natural oestradiol and oestrone (a derivative of oestradiol), significantly contributed to the oestrogenic activity in selected sewage effluents. These oestrogenic compounds are used extensively in oral contraceptive formulations and hormone replacement therapy. In addition, conjugates of natural oestradiol and oestrone are excreted daily by women of reproductive age. Concerns regarding potential environmental exposure to pharmaceutical oestrogens arise because of their greater potency in relation to well-studied environmental oestrogens. For example, APs and some organochlorine compounds are relatively weak oestrogens, NP has been shown to bind to the ER with a potency approximately 10^{-4} to 10^{-5} times that of E2 (White *et al.*, 1994; Tremblay and Van Der Kraak, 1998).

Women excrete a conjugated form of endogenous oestradiol daily, and the amount depends on age, reproductive status, and stage of the ovarian cycle. For example, a normal female typically excretes 25-100 μg/day of oestradiol at ovulation and 10-80 μg/day during the luteal phase. After menopause, the amount of oestradiol excreted drops to 5-10 μg, whereas a pregnant woman may release as much as 30 mg. Men also excrete approximately 2-25 μg of oestradiol daily (Arcand-Hoy *et al.*, 1998).

Another source of oestrogens in sewage effluent is from sulphate conjugates of 17ß-oestradiol and its derivatives often used in hormone replacement therapy, HRT. It has been estimated that of the 40 million postmenopausal US women in 1995, 5 to 13 million are prescribed hormone replacement drugs (Andrews, 1995). The regimen varies depending on the individual, but typically involves daily intake of 0.625 mg of conjugated oestrogens for 25 consecutive days followed by 5 days with no drug treatment (Arcand-Hoy *et al.*, 1998).

Oestrogens from the contraceptive pill are also a major contributor to the oestrogenic activity of sewage effluent. The contraceptive pill contains synthetic hormones such as ethynylestradiol and mestranol, synthesised from 17ß-oestradiol. The concentration of ethynylestradiol in the contraceptive pill ranges from 20 to 50 μg, with 35 μg/day being most commonly prescribed. The regimen is similar to that of HRT.

Several studies have demonstrated that wild populations of male fish inhabiting rivers receiving treated sewage effluent have been affected by oestrogenic contaminants in the UK (Harries *et al.*, 1996, 1997; Purdom *et al.*, 1994) the US (Folmar *et al.*, 1996), and in Australia (Batty and Lim, 1999); these will be reviewed in later sections.

1.3 EFFECTS OF REDS ON FISH

Detecting endocrine disruption *in situ*, and provision of early warning of potentially ecologically relevant effects might be achieved using biomarkers. Biomarkers are defined as biological responses that can be measured in tissue samples, excreta, or at the level of whole organisms, which signal exposure to or adverse effects of anthropogenic chemicals (Taylor *et al.*, 1999). The potential threat to reproduction posed by the presence of endocrine disrupters in the aquatic environment has been met by an intensive effort to identify and develop fast and reliable cause and effect biomarkers of exposure and effect, i.e., markers have been developed to measure the effects and the level of exposure of EDCs, *in vitro* and *in vivo*. Effect biomarkers have been specifically developed to assess the potential endocrine-disrupting activity of chemical compounds and complex mixtures. These methods include recombinant yeast cells with the human oestrogen receptor (Routledge and Sumpter, 1996) and the oestrogen-inducible MCF-7 breast cancer line (White *et al.*, 1994). Higher-level effect biomarkers will be discussed in the next section. Similarly, but as an *in vivo* exposure biomarker of oestrogen and EDCs, vitellogenesis induction in fish hepatocytes has proven to be a sensitive method of detection (Sumpter and Jobling, 1995; Jobling *et al.*, 1996).

Vitellogenin (VTG) induction has received much attention as an endpoint potentially indicative of exposure to endocrine disrupters. VTG is a protein precursor of yolk, which serves as a food reserve for developing embryos, and is synthesised by the liver. VTG is a specific oestrogenic response - expression of the VTG gene is not normally found in male fish due to the concentration of circulating oestrogens being too low to trigger expression (Copeland *et al.*, 1986). However, if presented with an exogenous source of oestrogens, males can synthesise VTG in quantities approaching those of mature females (Sheahan *et al.*, 1994; Purdom *et al.*, 1994). In males there is almost no natural oestradiol, so VTG induction in male fish is an excellent marker of exposure to low levels of environmental oestrogens (it is not however, a marker for anti-androgens). Using caged rainbow trout, Harries *et al.* (1996 and 1997), have shown that some UK rivers downstream of sewage

treatment plants (STPs) discharges are oestrogenic (as indicated by VTG induction) for several kilometres, although the effect declines with increasing distance from the source due to dilution and other processes. The direct consequences of VTG production in males are poorly understood, but can include reduced calcium in the scales and skeleton, liver hyperplasia/hypertrophy, and kidney damage (Herman and Kincaid, 1988). In addition, VTG production represents a substantial waste of energy to the male and female fish, and thus the production will almost inevitably reduce reproductive fitness.

The use of bioassays to detect the oestrogenicity of a chemical, or mixture of chemicals, have proven useful in assessing the potential endocrine-disrupting activity of chemical compounds and, particularly in the case of VTG, have been central in indicating the presence of low levels of oestrogenic chemicals in the environment (Jobling and Sumpter, 1993; Harries et al., 1996, 1997). However, they do not provide evidence of negative effects on animal reproduction itself. For that purpose it is necessary to develop biomarkers at higher levels of biological organisation with endpoints that more directly relate to the reproductive fitness of the individual with links to population-level effects.

1.3.1 Effects of REDs on Fish Morphology

Although VTG is a reliable and very sensitive indicator of the presence of REDs in the environment, the measure of VTG alone would not suffice in determining the impact of endocrine disrupters on reproductive success. Other characteristics that are hormonally dependant can easily be quantified and used to measure the impact of REDs.

Previous to the knowledge of environmental oestrogens, studies on male fish have shown a decrease in testicular growth following exposure to many pollutants, for example cadmium and the pesticide carbaryl (Sehgal and Pandey, 1984; Kulshrestha and Arora, 1984). One of the most common measures of the effects of pollutants on reproduction in male fish is the gonadosomatic index, GSI (gonad weight expressed as a percentage of

body weight). A decrease in GSI is generally accepted as a negative reproductive consequence.

The effect of exposure of fish to oestrogenic compounds or to oestrogen mimics has been investigated in several laboratories. One set of experiments demonstrated that caged male rainbow trout (*Oncorhynchus mykiss*) downstream of a sewage outfall expressed increased VTG levels along with with lower testis weights (or GSI) and liver enlargement (measured by the hepatosomatic index: HSI) compared to control fish (Harries *et al.*, 1997). Similarly, the induction of VTG has been associated with decreased testicular growth in male rainbow trout (*Oncorhynchus mykiss*) following exposure to alkylphenolic chemicals (Jobling *et al.*, 1996) and in male fathead minnows (*Pimpephales promelas*) following exposure to oestrogens (Panter *et al.*, 1998). The latter study exposed fathead minnows to oestradiol and oestrone and demonstrated a concentration-related response for plasma VTG level accompanied by an associated decrease in the rate of testicular growth (the levels of plasma VTG and inhibition of testicular growth were greater with oestradiol, suggesting that oestradiol is more potent than oestrone). However, it is important to note here that Jobling *et al.* (1996) found that the sensitivity of GSI to oestrogenic substances in rainbow trout was dependent on the stage of gonad maturation in rainbow trout and it is realistic to expect greater sensitivity by using fish that are undergoing rapid gonadal growth (summer for recrudescent fish). However, mosquitofish reproduce all year round (have continuous spermatogenesis) and therefore this effect will not impact the results of the current study.

Histological studies of the stage of spermatogenesis in testes of male fish can also provide useful information, and is commonly used to investigate potential reproductive impairment. Kinnberg *et al.* (2000), exposed adult male platyfish (*Xiphophorus maculatus*) to nonylphenol (NP) and demonstrated an increase in plasma VTG levels in parallel with a reduction in GSI as well as marked effects of testis morphology - the formation of cysts was impaired and there was an increase in the amount of hypertrophied

Sertoli cells present which could likely result in impaired formation of spermatozeugmata (sperm packets), and release of free spermatozoa into the efferent duct system.

Länge et al. (2001) conducted a fish full life-cycle (FFLC) study for exposure to ethinylestradiol (EE2) using the fathead minnow, Pimephales promelas. Newly fertilised embryos were exposed to five concentrations of EE2 and they found at 56-day post-hatch a higher female-to-male (F: M) sex ratio and presence of ovatestis was observed in exposed fish (controls and <1ng/L exposed fish had 50:50 with no ovatestes, whereas > or = 4 ng/L exposed fish had a F: M sex ratio of 84:5 with ovatestes in 11% of fish). At 172-day post-hatch, no testicular tissue was observed in any fish exposed to EE2 at 4.0 ng/L. At the same time point, plasma VTG levels were significantly higher in fish exposed to EE2 at 16 ng/L. On the other hand, assumed females exposed to > 4 ng/L of EE2 were still able to breed when paired with males that had not been exposed to EE2.

These previous studies outlined above accept decreased GSIs and changed gonad morphology as a negative reproductive consequence. A number of studies have reported a variety of developmental effects associated with gonadal differentiation in fish following exposure to natural, synthetic, and/or some environmental oestrogens. Many developmental changes include complete sex reversal where the phenotype is different from the genetic composition, and intersex conditions (ovatestis). The fact that sex determination in fish is rather labile has been confirmed in studies in the 1940s in which sex reversal has been induced by the treatment of juveniles or larvae with sex hormones (Berkowitz, 1941; Yamamoto, 1953; Tang et al., 1974). Gonadal differentiation can be affected by the administration of the steroid sex hormones during critical stages of development. Endogenous androgens given to genetic females direct the developing gonad to become a testis, and oestrogens given to fry may cause genetic males to develop ovaries and become functional females as adults (Liley and Stacey, 1983; Piferrer, 2001). Thus, in some fish species, both the androgenic and oestrogenic steroid hormones are capable of redirecting sexual development in a direction opposite to that of the genetic sex of the individual.

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Gimeno *et al.* (1996) exposed genetic male carp (*Cyprinus carpio*) during a period of sexual differentiation to an alkylphenol (4-*tert*-pentylphenol, TPP), and observed oviducts in all male fish exposed for 60 days. They also found that exposure of male carp to oestradiol for 90 days produced phenotypic females with no testicular tissue. Gray and Metcalfe (1997) also demonstrated an intersex condition in Japanese medaka (*Oryzias latipes*) exposed to the alkylphenol, 4-nonylphenol (NP), from hatch to 3 months of age. In fish exposed to 50 μg/L and 100 μg/L of NP, 50 and 86% of the male fish developed ovatestis, respectively. The ratio of male to female fish was 1:2, compared to 2:1 in the control population.

Recent research in wild roach (*Rutilus rutilus*) has shown that this oestrogenic exposure is accompanied in most instances by the presence of intersex conditions (Jobling *et al.*, 1998). In some cases, 100% of the male fish contain oocytes in their testes (ovotestis), and effects are more marked below STP discharges in comparison with upstream stretches (which were partially isolated by weirs). No UK roach populations appear to be totally free of ovatestis, but it is not known whether background levels of this condition are natural or due to the absence of completely pristine surface waters.

1.3.2 Effects of REDs on Live-Bearing Poeciliidae

Live-bearing fish of the family Poeciliidae may serve as valuable indicators of exposure to REDs as they exhibit hormone-dependent sexual dimorphism. Particularly in males that possess a gonopodium (GP), a modified anal fin that is used for sperm transfer, may the effects of REDs be evident. The development of the GP has been demonstrated to be under androgenic control, with development normally occurring in males as the testes begin to produce androgens at the time of sexual maturation (Turner, 1947). Exposure during foetal and juvenile periods would affect this male sexual character, and alterations in natural populations exposed to REDs have been observed. Batty and Lim (1999)

reported smaller gonopodium lengths in males living in sewage contaminated rivers, and development of gonopodia was observed in female poeciliids inhabiting rivers contaminated with paper mill effluent (Bortone *et al.*, 1989; Howell *et al.*, 1980; Davis and Bortone, 1992), these studies will be reviewed in more detail later in this chapter.

In another study, exposure of adults to E2 did not affect gonopodium length; Toft and Baatrup (2001) demonstrated that short-term exposure (30 days) of adult male guppies (*Poecilia reticulata*) to OP and 17β-oestradiol (E2) did not reduce GP length. They did however find that exposure reduced the area and colour intensity of the sexually attractive orange spots, unexpectedly increased the number of sperm cells in the ejaculates and inhibited testis growth. The concentrations of OP and E2 used in this study were as much as three orders of magnitude higher than those normally found in rivers and estuaries (Blackburn and Waldock, 1995; Desbrow *et al.*, 1998; Ternes *et al.*, 1999).

1.3.3 Effects of REDs on Fish Behaviour

An additional important aspect of environmental oestrogen exposure is their potential behavioural impact (referred to as ethotoxicology). Colborn *et al.* (1993) recognised both the lack and current need for the behavioural impact of endocrine disrupters to be investigated. Since, there been an increase in research examining reproductive behaviour as another RED endpoint in fish (Bayley *et al.*, 1999; Gray *et al.*, 1999a; Bjerselius *et al.*, 2001).

The development of the nervous and endocrine systems of vertebrates shows similarities across the various classes of vertebrates. Animals with a common phylogeny show numerous similarities in morphological, physiological, and behavioural traits, which share common adaptive functions, and it is assumed that these traits have been subjected to similar selection pressures. For example, during foetal life, endogenous steroid hormones, such as testosterone and oestradiol, have marked effects on the development of

the brain and reproductive organs in all vertebrates (Vom Saal *et al.*, 1992). Across a wide variety of vertebrate species, testosterone influences aggression and sexual behaviour in males, while oestradiol influences sexual behaviour in females. What has been less clear is the degree to which testosterone influences the normal development, subsequent regulation of organ function, and expression of behaviour in females, and the degree to which oestradiol influences the normal development, subsequent regulation of organ function, and expression of behaviour in males (Vom Saal *et al.*, 1994).

Androgens are important regulators of reproductive behaviours in a variety of vertebrate species including fish (Liley and Stacey, 1983; Borg, 1994). In male fish, the gonadotropins stimulate the proliferation of spermatogonia as well as the synthesis of androgens required for gametogenesis and development of secondary reproductive characteristics. Androgen synthesis typically takes place in the Leydig cells. The type of androgen synthesised is dependent upon the species and developmental stage but may include testosterone, 11-ketotestosterone and/or androstenedione (Kime, 1995). The most important androgen in fishes are testosterone (T) and its derivative, 11-ketotestosterone (KT). In poeciliids, testosterone is present in much higher concentrations in male than in female guppies (Borg, 1994; Bayley *et al.* (2002). Kime (1993) offers a good review of reproductive steroids in fish, but the work cited shows that there is an enormous diversity in the nature of teleost steroids and that we can no longer consider only the 'classical' teleost hormones. For example, in *Poecilia latipinna* (Poeciliidae) testes, both 3α- and 3β-hydroxy-5β-products were the major *in vitro* metabolites of testosterone (Kime and Groves, 1986), and in plasma, 11-ketotestosterone was not detectable while testosterone and 11β-hydroxytestosterone levels rarely exceeded 1ng/ml and did not correlate with gonadal weight, suggesting that the reduced metabolites might play more of a role than the 'classical' androgens. Although it is unclear exactly which androgenic steroids play a role in male reproductive behaviour, it is acknowledged that they are of primary importance in the expression of it.

If the ability to perform sexual behaviour is negatively affected by chemicals, reproductive success is potentially reduced and, subsequently, population structure and dynamics also can be affected. Therefore it is important to evaluate the effects of REDs on the reproductive behaviour of fishes. A study by Gray *et al.*, (1999a), exposed male Japanese Medaka (*Oryzias latipes*), from one day to 6 months post-hatch, to OP and demonstrated that increasing concentrations decreased the sexual activity made by males toward females, and observed a decrease in the fertilisation rate following exposure. In a similar study, Bayley *et al.* (1999) demonstrated that E2 and OP caused a reduction in the sexual display of the male guppy (*Poecilia reticulata*) (an ovoviviparous species where the males show characteristic courtship behaviour). However, the concentrations of chemicals used in this latter study were very high in comparison with levels reported in contaminated river systems.

Bjerselius *et al.*, (2001) demonstrated that male goldfish reproductive behaviour and physiology are severely affected by exposure to environmentally relevant concentrations of E2. Following exposure to E2 via food and water (separately), for a period of 24-28 days, they demonstrated that sexual activity of the goldfish was significantly reduced. In addition, they demonstrated significantly reduced GSI, milt production and spawning tubercles of exposed males compared to controls. Both behavioural and physiological measurements were reduced in a dose-dependant manner.

In the same way that hormone effects can be activational or organisational in terms of morphological effects, the same distinction can apply to behavioural development. Although it must be recognised that many fish species show greater adult behavioural plasticity than do, for example, mammals and birds. Fish in the wild are exposed to compounds from fertilisation onwards and changes in brain development or morphology may occur during 'sensitive' periods, but little is known as to whether these effects will be permanent or transient (if and when exposure ceases), i.e., if they really are sensitive periods. Currently, the behavioural studies on fish have examined the effects of endocrine disrupters in adult male model systems (reviewed above), but the various changes in fish

behaviour produced have not been examined in terms of activational effects (i.e., when exposure ceases does behaviour or morphological effects revert back to pre-exposure?).

1.4 MOSQUITOFISH GENERAL BIOLOGY

The mosquitofish is a member of the livebearer family: the Poeciliidae. Native to America, this freshwater fish was distributed throughout much of the world in the 1920s for intended biological control of mosquitoes. The poeciliid genus *Gambusia* is represented by one of 30 known species in Australia, *G. holbrooki*, mostly inhabiting tropical fresh and brackish waters. Their use as a biological control did not last long in Australia, where they are now considered a pest species. They are a surface dwelling species that prefer shallow, slow moving water where they can escape predation from larger fish (Krumholz, 1948). They are omnivorous, feeding on algae, detritus, small invertebrates and young fish. The normal life expectancy of mosquitofish is less than one year and fish seldom live for more than 3 years (Krumholz, 1948). Mosquitofish are viviparous and the period of gestation varies from 16 to 80 days (Krumholz, 1948; Hubbs, 1962). The females can produce several broods during a breeding season, which generally extends through the warmer spring and summer months (Krumholz, 1948). They are characterised by a marked sexual dimorphism in size, males being considerably smaller than females. A smaller size in males is not uncommon among teleost fishes, but dimorphism is often extreme and the males are among the smallest living vertebrates. One study by Bisazza (1993), found the ratio of the maximum female to male length about 1.7 but the largest female outweigh the largest male by 5 times. Typically, mature males range from 20-30mm, and females range from 30-50mm in total body length (see fig. 1.3). The anal fin of the adult male is modified into a long intromittent organ, the gonopodium (GP). The GP is used to transfer sperm to the female and is the main character employed as a basis for the taxonomy and classification of the genus (Rosen and Tucker, 1961).

Figure 1.2 Male mosquitofish *Gambusia affinis*, approximately 25 mm total body length.

Both juvenile males and females have an anal fin that is essentially the same in structure. In males, the anal fin and the spinal column undergo a series of hormone-dependent changes that result in the development of the GP. The development of the GP is androgen dependent, i.e., it grows as a result of rising blood testosterone concentrations during maturation (Turner, 1947 and 1960; Rosa-Molinar *et al.*, 1994). The GP is an elongation and modification of anal fin rays 3, 4 and 5. The elongation of fin rays 3-5 is under androgenic control: castration of males results in cessation of gonopodial development, and replacement therapy with ethynyl testosterone restores the normal developmental pattern (Turner, 1947). In addition, exposure of juvenile females to ethynyl testosterone induces the development of the GP (Turner, 1942a and 1942b). The GP rays have evolved a functional independence from the more posterior rays and the male can extend the GP at various angles and directions from the body to facilitate copulation. The GP tip is equipped with barbs and spines, which serve as holdfast devices during GP insertion

into the female genitalium (Rosen and Gordon, 1953). Female fin rays are unmodified and they continue to grow throughout their lifetime while males have a slower rate of growth after the development of the GP is complete (Turner, 1947; Krumholz, 1948).

1.4.1 Reproductive Behaviour

Male poeciliids are sexually very active and their behaviour is easily observed in laboratory and in the field. Male *Gambusia sp.* have been shown to demonstrate two mating tactics. The male can court the female to obtain her cooperation during mating, or he can bypass female acceptance and attempt a forced insemination. The action by which males achieve a forced copulation has been given various names such as rape (Farr, 1980) and sneak copulation (Endler, 1983); the term gonopodial thrust is the most often used and will be adopted here. In *G. holbrooki*, gonopodial thrust is the only male mating tactic observed (Bisazza and Marin, 1991), whereas in *G. affinis*, gonopodial thrusts are always preceded by a courtship display (Hughes, 1986).
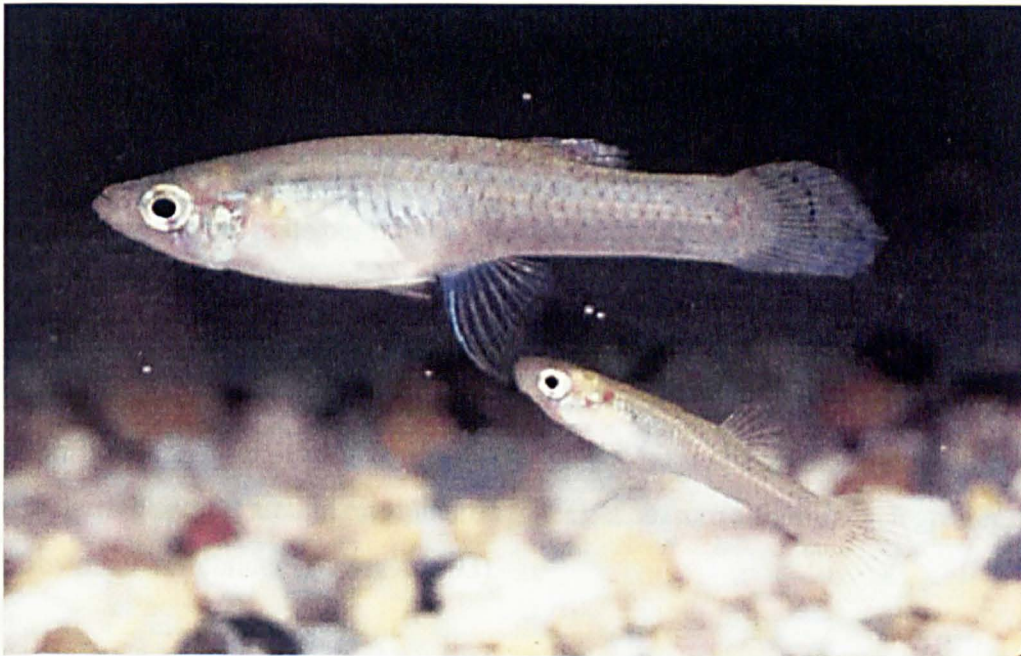
Figure 1.3 Male mosquitofish, *G. affinis*, about to copulate with a female.

Two distinct displays (frontal display and lateral display) can occur during the courtship of *G. affinis*. Frontal display is much more conspicuous than lateral display and occurs when a male moves directly in front of or anterolaterally to the head of a female and orients his body more or less at 90° to the axis of her body. With his median fins partly folded and body in sigmoid posture, he first vibrates or quivers his body for as long as several seconds and then quickly swims in a tight arc to approach the female from behind. After reaching a point below the females anal area, the male vigorously and repeatedly thrusts his GP towards the females genitalia. Lateral display is performed slowly and inconspicuously with the male facing in the same direction as the female. During this display the male drifts slowly and with a stiff posture towards a stationary female until he is at the same level but approximately 5 to 10mm from her eye. After maintaining this position for several seconds, he suddenly swims backward below the female and thrusts his GP upwards towards the female genitalia (Peden, 1972).

Females can avoid copulation by changing their orientation, slapping the male with their tail, moving away from him, folding their anal and caudal fins over the genital opening or position their tail close to a solid object when harassed by males (Rosen and Tucker, 1961).

The frequency of mate attempts is very high; in natural populations of *G. holbrooki* there is about one act per male per minute (Martin, 1975; Bisazza and Marin, 1991). However, approximately only one in every 30 copulatory thrusts results in a contact between genitalia and analysis of slow motion video recordings suggests that only a small minority of these contacts involves a complete intromission of the GP. Using a conservative estimate of one insemination every 1000 attempts, one male would mate (in the sense of transferring sperm) around one hundred times per season, a figure much greater than in many other vertebrates (Bisazza, 1993).

1.4.2 Previous RED Effect Studies with *Gambusia* sp.

Previous studies by Batty and Lim (1999) have revealed morphological changes consistent with exposure to oestrogens or oestrogen mimics in male *G. holbrooki* inhabiting sewage-contaminated waters in New South Wales, Australia. Males sampled over two seasons showed a reduced GP length at sites downstream from a sewage effluent discharge point, compared to upstream and additional reference sampling sites. This is consistent with oestrogen exposure, since gonopodium development in males is impaired in the presence of oestrogens (Doyle and Lim, 2002).

A recent study by Doyle and Lim (2002) demonstrated that exposure of male *G. holbrooki* over the period of sexual differentiation (post-hatch to 12 weeks) to E2 had negative effects on gonopodial development. The development of the GP was determined by examination of the degree of gonopodial elongation (defined as ray 4 to ray 6 length ratio of 1.00), and the presence of hooks on the gonopodial tip. Juvenile males exposed to 100, and 500ng/l E2 showed smaller GP, lower ray 4 to ray 6 ratios, and reduced sexual activity compared to controls. No gonopodial or behavioural effects of exposure were observed at the lowest concentration of 20ng/l E2.

Studies of female *Gambusia* sp. have demonstrated abnormal reproductive physiology in fish exposed to pulp and paper effluents (or kraft mill effluent, KME). The preparation of wood pulp for paper and cellulose manufacture separates cellulose fibres and lignin from the sugars, saps and other components of tree stems. Although most components of KME are not known to be persistent or bioaccumulative, they are released continuously and in vast quantities. Basic to all the pulping processes is separation and discharge of sugars, lipids, resins and fatty acids that are the by-products of kraft-mill operations. They receive bacteriological treatment analogous to sewage treatment processes. Plants, especially pine trees, are potentially rich sources of phytosterols; phytosterols in tall (pine) oil can be microbially converted to C-19 sterols (Conner *et al.*, 1976). The treatment processes appear not to destroy phytosterols, in addition, the chemical processes within a mill (e.g.,

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chlorine, chlorine dioxide, or oxygen bleaching) may add to the complexity of the effluent.

Microbial degradation of plant sterols such as sitosterols and stigmastanol, abundant in the resin fraction of coniferous trees used in the pulping industry, was suspected as the source of androgenic steroids responsible for altering the secondary sexual characteristics of mosquitofish (Davis and Bortone, 1992; Howell *et al.*, 1980). KME-induced male secondary sex characters in female mosquitofish occur under both field and laboratory conditions (Bortone *et al.*, 1989; Drysdale and Bortone, 1989; Howell *et al.*, 1980; Davis and Bortone, 1992). These studies describe the abnormal expression of modified secondary sexual characteristics in populations of mosquitofish, *G. affinis* and *G. holbrooki*, in streams receiving KMEs in the southeastern United States. Bortone *et al.* (1989), found masculinised female fish as far as 4 miles downstream, but not upstream, of two KME discharge sites; females were strongly masculinised showing both the presence of a fully developed GP and reproductive behaviour characteristic of the male of this species (Bortone *et al.*, 1989). In addition, the masculinsation of females occurring downstream of paper mills discharging KME, has also been found in the least killifish, *Heterandria Formosa*, and the sailfin molly, *Poecilia latipinna* (Bortone and Drysdale, 1981).

Laboratory studies demonstrated that female mosquitofish experimentally exposed to plant sterols, particularly β-sitosterol and stigmastanol, in the presence of *Mycobacterium* developed gonopodial characteristics in 6 days (Denton, 1985). These characteristics did not regress when the transformed fish were removed to an environment free of plant sterols. Bortone *et al.* (1989) found that male reproductive behaviour was not detectable among KME-masculinised female mosquitofish after they had been acclimatised for two months to water lacking KME. Another study by Bortone and Davis, (1994) demonstrated that the degree of masculinisation of behaviour among female fish (when compared with normal male behavioural traits) decreased when fish were captured from the field and placed in aquaria free of KME. Furthermore, gonopodial and other morphological

features showed no further development. An earlier study by Hunsinger *et al.* (1988) confirmed that KME masculinsed mosquitofish could produce viable offspring after having been placed in aquaria free of KME. In summary, these studies demonstrated that, after acclimatizing in KME free water; masculinised behaviour of exposed females normalised (Bortone *et al.* 1989; Bortone and Davis, 1994); females could produce viable offspring (Hunsinger *et al.* 1988); and gonopdial development in females did not regress (Denton, 1985; Bortone and Davis, 1994). These findings are of profound importance to the organisational versus activational concept of EDC exposure – a source of androgens has had both an organisational effect (females have permanent masculinised morphology) and an activational effect (when exposure ceases, masculinised behaviour ceases), which together does not affect the ability of the animal to reproduce. Currently, there are no data on transient effects (activational) on behaviour of environmental oestrogen exposure.

1.4.3 Why Use Mosquitofish for this Study?

The use of mosquitofish in field and laboratory studies offers several advantages. Mosquitofish are small and can easily be bred and raised in aquaria at low cost. Their life cycle is relatively short (the fish reach adulthood about two months after birth), and reproductively active females can produce a brood of fry approximately once a month (Constanz, 1989). It has a high reproductive rate and an ability to attain high population densities. A genetic study by Feder *et al.* (1984), demonstrated that mosquitofish exhibit a high degree of site tenacity, and therefore are a useful sentinel of exposure to pollution. For purposes of comparison and integration, there is a wealth of background information on the Poeciliidae (guppy family), including studies on their reproduction, development genetics, and behaviour (see Meffe and Snelson, 1989). One important feature is that their mating behaviours have been extensively studied, and these behaviours are very obvious and can be readily recorded under laboratory conditions. Another advantage is that mosquitofish have a relatively wide salinity tolerance. They are found abundantly in fresh and brackish waters, and thus can be used as bioindicators in coastal and inland

environments. In addition, they are a hardy fish that tolerate stressful conditions, and can thus be used for monitoring waters of variable quality.

Mosquitofish are a particular suitable sentinel for examining reproductive effects of endocrine disrupters because they exhibit hormone-dependent sexual dimorphism. As stated previously, differences in external morphology include body size (males being smaller than females), and the size and shape of anal fin (males have an elongated anal fin, the gonopodium: GP, and in females it is rounded). The androgenic gland produces the hormones involved in the development of this secondary sexual characteristic. Disruption of these hormone systems through castration of the GP and replacement therapy, and exposure to natural plant sterols, is known to affect the development of the GP (Turner, 1947; Drysdale and Bortone, 1989). This suggests that the morphology of this secondary sexual characteristic may respond to disruption of the hormone systems through exposure to REDs. The size of the GP can be used as an endpoint in assessing the impact of REDs.

The selected end-points chosen to investigate the effects of REDs in this report are: male spermatozeugmata count (cellular level), testis weight and gonopodial index (organ level), and reproductive behaviour (organismal level). These characteristics are important for the reproductive success of mosquitofish and can be easily quantified (which make them potential *in vitro* effect biomarkers of REDs).

1.4 AIMS

The aim of this report is to:

- Investigate reproductive behaviour and morphological characteristics of adult male mosquitofish exposed under laboratory conditions to:

  oestrogens

  oestrogen mimics

  sewage effluent

- Investigate reproductive behaviour and morphological characteristics of adult male mosquitofish inhabiting sewage-contaminated waters

CHAPTER 2

General Materials and Methods

2.1 EXPERIMENTAL FISH: HOUSING AND MAINTENANCE

Males of two closely related species of mosquitofish, *Gambusia* sp. have been utilised for this study - one *Gambusia affinis* was laboratory reared at Napier University in Edinburgh - and *Gambusia holbrooki* were obtained from rivers near Sydney in Australia. These two species are very similar in appearance and differ only by speckles on the surface skin (*G. affinis* are not speckled whereas *G. holbrooki* are). Taxonomic differences are made by microscopic examination of the gonopodium (GP); the hooks on the GP of *G. affinis* are larger than in *G. holbrooki*. There are slight differences in reproductive behaviour; as mentioned previously in section 1.4.1, gonopodial thrust is the only male mating tactic observed in *G. holbrooki* (Bisazza & Marin, 1991a; 1991b), whereas in *G. affinis*, gonopodial thrusts are always preceded by a courtship display (Hughes, 1985; 1986).

The study in chapter 3 reports on two separate laboratory based experiments using both *G. affinis* and subsequent experiments using *G. holbrooki*, experiments in chapter 4 and 5 use only *G. holbrooki*.

2.1.1 Experiments Utilising *Gambusia affinis*

(i) Source
A breeding group of mosquitofish (*Gambusia affinis*) was established at the laboratory in Napier University with fish which originated from the Camargue region in Southern France. The laboratory population was bred for 2 years prior to any experiments being conducted.

(ii) Maintenance

The breeding group of mosquitofish were housed in large aquaria measuring 700 x 600 x 600 mm (H x W x L) of mixed sex and similar age. They were maintained at a temperature range of 22-29°C on a 16:8 light: dark photoperiod and were fed daily with commercial fish food (*tetra fin*).

Prior to the initiation of experiments, male fish were taken out of the breeding tanks and kept in single sex groups of approximately 10 individuals and allowed to acclimatise for at least 72 hours. All test males were housed in glass aquaria measuring 225 x 230 x 300 mm (H x W x L) holding aerated water. Fish were fed daily and any dead fish were removed from the tank. All aquaria were emptied and manually scrubbed weekly with water (no detergents or cleaning products used). During cleaning, fish were transferred to holding aquaria.

2.1.2 Experiments Utilising *Gambusia holbrooki*

(i) Source

The mosquitofish, *G. holbrooki*, were obtained from the Hawkesbury River system in Sydney, Australia. The fish were collected by fishing from the river bank with a hand-net, and placed in a large cool box of water to be transported live back to the laboratory. Sampling was undertaken during the summer months; Dec – Mar 1999; Jan - Mar 2000 and Jan – April 2002 at specific sampling locations as indicated for each experiment. Upon arrival at the University of Technology, Sydney (UTS) laboratory, the fish were sexed and housed in glass aquaria containing separate male and female groups.

(ii) Maintenance

For experiments involving a number of males in each test tank, fish were kept in single sex groups of approximately 15 individuals and allowed to acclimatise for at least 72 hours prior to the initiation of experiments. All fish were housed in glass aquaria (225 x

230 x 300 mm - H x W x L) holding aged water and maintained at 25 ± 2°C on a 16:8 light: dark photoperiod. Fish were fed daily and any dead fish were removed from the tank. All aquaria were emptied and manually scrubbed weekly with water (no detergents or cleaning products used). During cleaning, fish were transferred to holding aquaria.

For experiments on individual males collected at up- and downstream sites of the STPs in 2000 and 2002 (detailed in chapter 5), upon arrival back at the laboratory, males were separated into 500ml beakers holding aged water maintained at the light and temperatures stated above.

2.2 Characteristics of the study sites and STPs

The study area is within the middle section of the Hawkesbury River system (Fig. 2.1), which is located on the central coast of New South Wales (NSW), Australia. The system extends approximately 22,000 km², and can be divided into fluvial and estuarine sections, with the mainstream receiving inflows from 14 tributaries (Sydney Water, 1996). Although it is tidally influenced, the study area remains freshwater throughout the year. Maximum summer temperatures average between 26 and 28°C, whereas minimum winter temperatures average between 2.6 and 5.9°C (Sydney Water, 1991). Throughout spring and summer, rain falls over the region about 10 to 11 days per month, but decreases to approximately 6 days per month in the winter. Annual rainfall averages between 650 and 750 mm (Sydney Water, 1991). More than 60% of the Hawkesbury-Nepean River system catchment is forested and includes sections of nine national parks. Agricultural land comprises approximately 30% of the area and supports cattle and sheep grazing, dairy farms and irrigated horticultural crops, and intensive pig and poultry production. Less than 10% of the total catchment area is developed for urban and industrial use (NSW EPA, 1993).
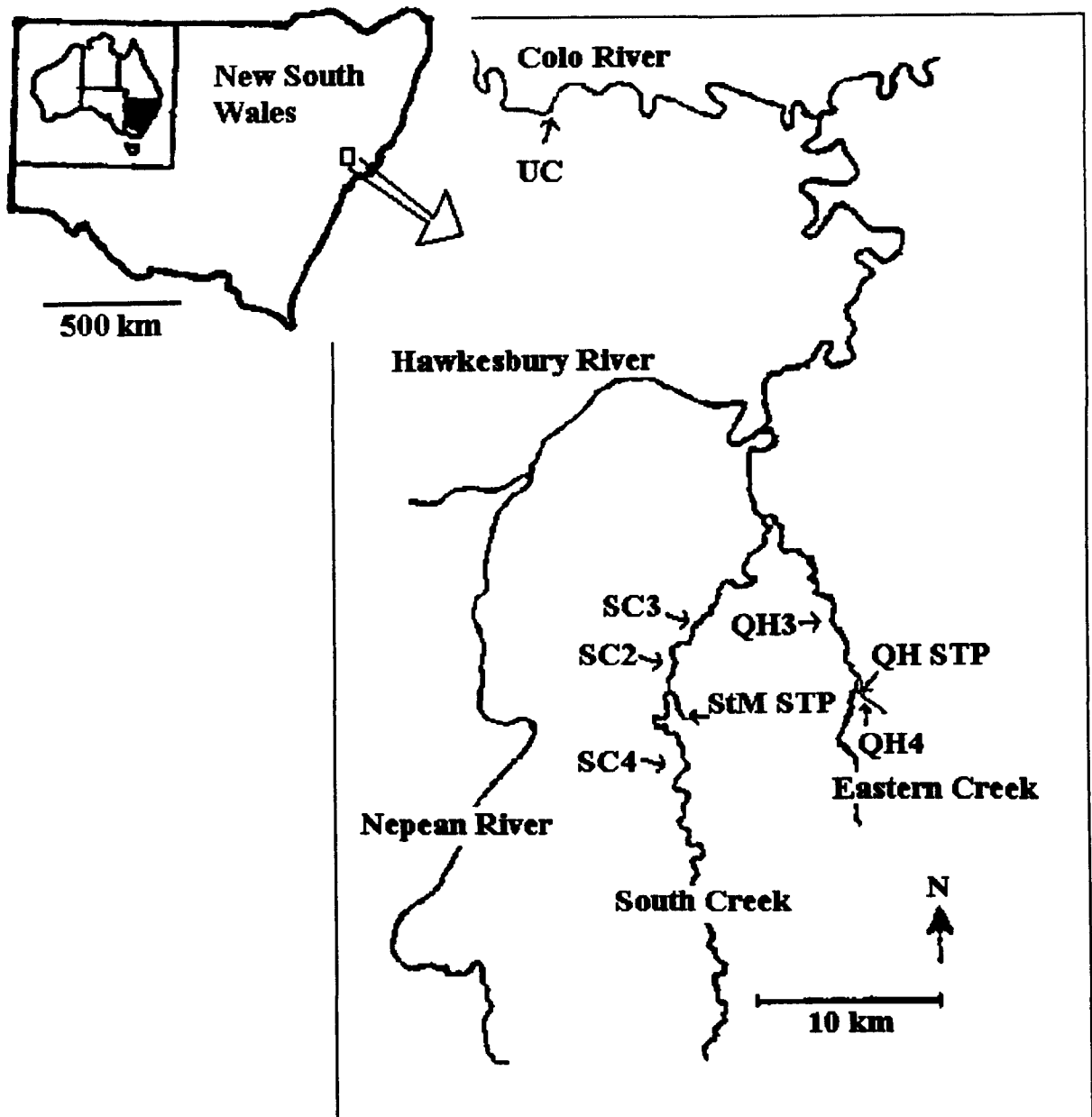
Figure 2.1 Map of study area showing sewage treatment plants (STPs) and sample sites.

UC	Upper Colo
QH STP	Quakers Hill STP
QH4	Quakers Hill Upstream
QH3	Quakers Hill Downstream
StM STP	St Marys STP
SC4	St Marys Upstream
SC3	St Marys Downstream
SC2	St Marys Downstream

Two STPs situated in the western suburbs of Sydney have been utilised for this study: St Marys and Quakers Hill. Industrial development located within the sewage catchments are served by both STPs. Sydney Water regulates industrial discharges to the sewer system; current regulations ban any discharge of organochlorine and organophosphorus pesticides, PCBs, and dioxins into the sewage system.

(i) St Marys STP

St Marys is the largest plant discharging into the Hawksbury Nepean River system. This catchment contains both residential and industrial areas, serving an area of 7,200 hectares including nineteen townships with a total population of 140,800 people. The STP is a tertiary treatment facility, processing domestic and industrial sewage, with additional nitrogen and phosphorus removal and disinfection by chlorination (and dechlorination implemented in 2001). St Marys STP typically discharges effluent with a biological oxygen demand (BOD) of 2-5mg/l, and Sydney Water boasts the effluent is the "best quality produced by any STP in the world". It is consented to discharge 35 million litres per day into Ropes Creek, a tributary of South Creek (SC) (where sampling was conducted), and finally to the Hawkesbury River. Three sites on South Creek were utilised, one upstream of St Marys STP and two downstream of the outfall:

Upstream of STP: Site SC4 is located on South Creek, upstream of St. Marys STP. SC4 is located at Kingsway Bridge, just outside the township of St. Marys. The surrounding land is suburban, with a school and leisure and shopping centre close by. The site is subject to urban pollution, it is part of a picnic area, although not ever busy with people and on first appearance appears clean (no litter), but with closer inspection one can find objects such as shopping trolleys and indeed a car in the deeper parts of the water. There is little shade; there are no trees or vegetation overhanging the river. The exact location of sampling is what appears to be a lagoon (but the river flows in and out of this very slow moving area of water). The fish like the sunny, shallow and stagnant part of this site - on approaching the riverbank, one can see the fish move from this spot to the area of reeds that grow in a deeper part of the site for protection.

Downstream of STP: Site SC2 is located on South Creek, at the only bridge on Eighth Avenue, downstream of St. Marys STP which discharges both treated domestic and industrial effluent. It is approximately 5km from the sewage effluent outfall at St Marys STP. The surrounding land is suburban with little agricultural usage. The river is slow moving, wide (about 10 meters at the widest point) deep in some parts of the sampling area (too deep to test) and with little shade (not many trees).

Downstream of STP: Site SC3 is located at Stoney Creek Bridge on South Creek, it is approximately 10km from the sewage effluent outfall at St Marys STP. The surrounding land is agricultural, with cattle being kept in fields surrounding. The river is slow moving, wide (about 10 meters at the widest point) deep in some parts of the sampling area (too deep to test) and quite shaded by trees. Fish are easier caught in shallower, sunnier parts of the riverbank.

(ii) Quakers Hill (QH) STP

The sewage catchment is around 9,700 hectares and is primarily a residential, serving a population of approximately 110,000 people. The STP is a tertiary treatment facility, processing domestic and industrial sewage, with additional nitrogen and phosphorus removal and disinfection by chlorination (and dechlorination implemented in 2001 – not affecting this study), and has a low BOD of < 2mg/l. It is consented to discharge 32 million litres per day into Breakfast Creek (where sampling was conducted), 760 m upstream of Eastern Creek, which subsequently flows into South Creek, which then enters the Hawkesbury River. Two sites were sampled on Breakfast Creek, one upstream of Quaker's Hill STP and one downstream of the outfall:

Upstream of STP: Site QH4 is located on Breakfast Creek, upstream of Quaker's Hill STP, just outside the township of Quaker's Hill. The surrounding area is suburban, with a school and hospital nearby. There is little shade; there are no trees or vegetation overhanging the river. The river is relatively narrow (3 metres at widest point), shallow

(1 metre at deepest point) and relatively fast-flowing in some parts. The fish can be found in abundance at the river edge, hiding amongst the reeds that grow at the riverbank.

Downstream of STP: Site QH3 is located on Breakfast Creek, downstream of the Quaker's Hill STP outfall which discharges treated domestic and industrial effluent. The effluent outfall can be seen about 50 metres upstream from the sampling area, there is a distinct smell of chlorine when closer to the outfall. The surrounding land is suburban. There is little shade; there are no trees or vegetation overhanging the river. The river is relatively narrow (5 metres at widest point), shallow (1 metre at deepest point) and relatively fast-flowing in some parts. The fish can be found in abundance at the river edge, hiding amongst the reeds that grow at the riverbank.

(iii) Upper Colo (UC) river

Site UC represents an uncontaminated reference site as it is located in an area remote from industry, and is within a national park. The river is very wide (approximately 100 metres), shallow (0.5 metres), and relatively fast-flowing. The riverbed is coarse sand, and the water very clear with little suspended matter.

2.3 EXPOSURE TO HORMONES AND SEWAGE EFFLUENT: TREATMENT AND PROCEDURES

2.3.1 Chemical Preparation

Exposure to three specific endocrine disrupting chemicals was undertaken. The oestrogens [diethystilbestrol (DES) and 17β-oestradiol (E2)] were purchased from Sigma Chemical Company and the oestrogen mimic [4-*tert*-octylphenol (OP)] was purchased from Aldrich Chemical Company. They were all stated to be a minimum of 97% pure. They were prepared for use by means of a carrier solvent. It was noted after the first few experiments that using alcohol as the carrier solvent produced a bacterial growth on the surface of the water and so acetone was used for later experiments. The use of alcohol, however, did not have a more negative effect on mortality in control groups more so than the use of acetone.

Diethylstilbestrol (DES)

Three stock solutions were made of DES using analar ethanol (for experiments using *G. affinis*) or analar acetone (for experiments using *G. holbrooki*) as a carrier solvent. 27mg DES was dissolved in 27ml solvent for stock A. To make stock B, 0.25ml of stock A was added to 25ml solvent. 0.4ml of stock B was then added to 25ml solvent to make working stock C (to make concentration of 160 μg/l DES). The full procedure was carried out in a fume cupboard using plastic gloves and pipettes. In all cases, tanks were filled with 8L of water.

Three final concentrations of DES were required:

0.4ng/l DES - 0.02ml stock C added to the 8 L tank

2ng/l DES - 0.1ml stock C added to the 8 L tank

10ng/l DES - 0.5ml stock C added to the 8 L tank

Solvent control - 0.5ml ethanol/acetone added to the 8 L tank

17β-oestradiol (E2)

2 mg E2 was weighed and dissolved in 250ml analar acetone to make the working stock (8 mg/l). Then the following volumes were added to the tanks:

20 ng/l E2 - 0.02 ml stock added to 8 L tank

100 ng/l - 0.1 ml stock added to 8 L tank

500 ng/l - 0.5 ml stock added to 8 L tank

Solvent control - 0.5 ml acetone added to 8 L tank

Octylphenol (OP)

40mg OP was weighed and dissolved in 50ml analar acetone to make the working stock (800mg/l). Then the following volumes were added to the tanks:

2 μg/l OP - 0.02 ml stock added to 8 L water tank

10 μg/l OP - 0.1 ml stock added to 8 L water tank

50 μg/l OP - 0.5 ml stock added to 8 L water tank

Solvent control - 0.5 ml acetone added to 8 L water tank

2.3.2 Chemical Administration Procedure

The fish were exposed by immersion. Experimental groups were exposed under a semistatic regime with weekly replacement of test media. The fish were exposed to each chemical at several nominal concentrations and controls were exposed to the solvent carrier only. Chemicals were pipetted into the tank during tank water replacement, to ensure even distribution. Experiments conducted with *G. affinis* used ethanol as the carrier and experiments conducted with *G. holbrooki* used acetone as the carrier solvent. The control was treated in the same manner as that described above except acetone or ethanol only were used, at a concentration equivalent to the maximum solvent addition in control tanks (i.e., maximum = 0.5 ml / 8L).

2.3.3 Sewage Effluent Collection and Administration Procedure

Treated sewage effluent samples were collected from St Marys STP in 1999 and 2000 and refrigerated during the exposure period. A 24-hour composite final effluent sample was collected from the plant – this consisted of a uniform volume of effluent collected every 15 minutes over a 24-hr period in a 208 L heavy duty, high density polyethylene drum. The effluent was vigorously mixed and divided into the aquaria for exposure

The fish were exposed by immersion. Experimental groups were exposed under a semistatic regime with weekly replacement of test media. The fish were exposed to each concentration of sewage effluent at several concentrations and controls were exposed to water only.

2.4 SELECTION OF FISH

For all laboratory exposures (detailed in chapters 3 and 4), fish were sampled from South Creek, upstream of St. Marys sewage treatment plant (SC4). This site was chosen because it is particularly abundant with mosquitofish and has good road access to the bank of the river. Although not pristine, this section of the river is free of any discharges, but still subject to urban pollution.

Fish were allocated into groups such that as far as possible, each experimental group was matched for body size. In addition, males were pre-tested for reproductive behaviour to ensure activity was equivalent between all experimental groups. Pre-testing was conducted using the behavioural protocol detailed in the following section (2.5). The sample size for each experimental group was between 10-16 individuals.

2.5 BEHAVIOURAL MEASUREMENTS

Two separate methods for measuring the reproductive behaviour were used. A general overview of reproductive behaviour has been given in section 1.4.1. The initial test procedure allowed free interaction between males and females in a large observation tank. After the experiments were conducted, it was apparent that females and males showed some aggressive responses to one another, which would clearly impact behaviour. To get around this problem, a more restricted test procedure was devised. Sometimes, but not in all experiments, the behavioural observations were reviewed blind (to ensure no bias of male behaviour in the different treatments). All fish were transferred between tanks using a small hand-net.

2.5.1 Unrestricted Method

The method used for *G. affinis* involved a test male and female freely interacting, hence named the "unrestricted method". An observation tank was used measuring 225 x 250 x 450 mm (H x W x L) and filled with 20L of aged water. The observation tank had only one side exposed to the observer, and the remainder of the tank was covered in black paper to reduce disturbance of the fish.

Before the observation began, a single female fish with a clear gravid spot was selected and placed into the observation tank before the male was introduced. When the male was introduced into the tank, the 10-minute observation period began immediately and the following observations were recorded:

- Pursuit time - This is defined as the time the male spends following the female so that the male remains within two body lengths of the female.
- Gonopodial Thrust - These are defined as the rapid approach of the male together with the movement of the gonopodium 180° toward the female gonopore for insemination.

- <u>GP Stretch</u> - This personal observation was noted in preliminary experiments although no references to it can be made in the published literature, it is assumed that this forms part of the courtship display. The GP stretch is defined as a slow movement of the GP 180° forward, but not directed toward the female's gonopore, but in front of the female, as if a frontal display of the males fins. The GP stretch is essentially the same action as a GP thrust, but in slow motion and not directed toward the female for insemination. This tactic is not used in the data analysis of this report partly because of the questionable reason for it and partly because it is not imperative for successful copulation.

2.5.2 Restricted Method

The behaviour of the male *G. holbrooki* was investigated using a more structured environment. The experimental set-up is shown in Figure 2.2. The observation tank measured 225 x 250 x 450 mm (H x W x L) and was filled with 20L of aged water. The observation tank had only one side exposed to the observer, and the remainder of the tank was covered in black paper to reduce disturbance of the fish as in the previous method. A line was drawn on the outside of the tank separating the tank into two equal sections.

Before the observation began, two female fish with clear gravid spots were selected and contained within a beaker at one end of the test tank and a small group of juveniles (4-5 individuals) placed at the other. Females were chosen to represent a reproductive motivation and juveniles were chosen as an 'alternative', to represent activity (males were not chosen to represent activity as they may stimulate aggressive motivation). These beakers were suspended in the observation tank so that the water level in the beakers matched that in the tank. When the male was introduced into the tank, the 5-minute observation period began immediately and the following observations were recorded:

- Duration in female zone - The time the male spends in the left side of the tank (inside the female zone).

- Approach - Measured by the numbers of times the male attempts to approach the female. This is defined as an approach of the male so that his snout touches the glass.

- Mate attempt - Measured by movement of the GP 180° towards the female in the beaker.
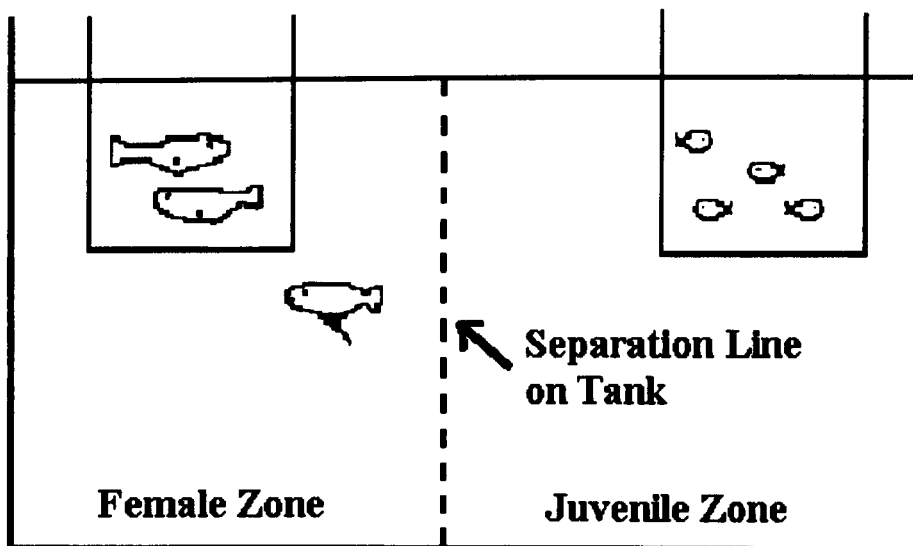
Figure 2.2 Experimental set-up of behavioural observation tank

2.6 MORPHOLOGICAL MEASUREMENTS

Immediately following completion of behavioural observations, fish were euthanised by immersion in carbon dioxide saturated water. They were then dried on tissue paper for a set time of 5 seconds and body weight measured. The following additional measurements and observations were made using a Leica DC100 digital camera attached to a stereomicroscope using the imaging program Leica Qwin (Leica Microsystems, North Ryde, NSW, Australia).

2.6.1 Gross Morphology

Precise measurement points are indicated in Figure 2.3. The following measurements were taken accurately (to nearest 0.01 mm) with the image analysis software:

Total body length (BL) – defined as the longest straight line distance between the snout and the caudal fin

Gonopodium length (GP) - distance from the base to the tip of the anal fin
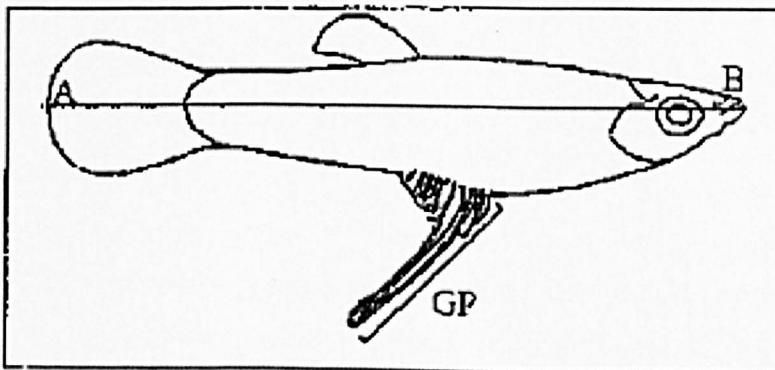
Figure 2.3 Morphological measurements taken. Line A-B: BL (total body length) and GP: gonopodium length.

2.6.2 Testis and Spermatozeugmata Measurements

Spermatozeugmata count (SPZ) - In addition to morphological measurements, the number of visible spermatozeugmata (sperm packets) was also recorded. This was measured by pressing, with blunt forceps, anterior to the anal fin on the ventral surface and squeezing out the white spherical sperm packets. Their abundance was classified according to a simple grading in groups of 100; 0-100, 101-200; 201 - 300; 301 – 400; 501 – 600 and 600+

Testis area (TA) - The testis were then removed and the visual area measured using the Leica image analysis set-up described above.

Testis Weight (TW) - The testis weight was also measured in some fish (sampled in 2000 and 2002) using an AT261 DeltaRange Mettler Toledo electronic balance set to 10µg tolerance.

2.7 DATA ANALYSIS

2.7.1 Preliminary Behavioural Measurements

Before the restricted method was developed, it was obvious that both male and female fish were aggressive to one another. The male would bypass female acceptance and attempt a forced insemination, and the female, being harassed by the male, would try to avoid copulation by slapping him with her tail. It was decided that consequent experiments (which happened to be all experiments using *G. holbrooki*) would include the restricted method of observation (described earlier).

Preliminary studies on the behaviour of *G. affinis* were conducted at Napier University, in order to determine the protocol for future experiments. This involved observing males (which had been isolated from females for about a week) freely interacting with females (using the unrestricted method, described earlier), for 20-minute periods. It was found that males courted females less persistently after a 10-minute period, the majority of attempted copulations occurring during the first 5-minutes. Consequently, sequences of behaviour were recorded only for the first 10-minutes using the unrestricted method and for 5-minutes using the restricted method. No acclimatisation time was given for the test male because reproductive behaviour began immediately when the male was introduced to the test tank.

In addition, preliminary studies were conducted to test if males had a preference of size of female (small or large) and if he preferred gravid females (as marked by a black spot in the anal area). Males were placed in a tank with a small and a large female, and using the unrestricted method, observations were recorded and determined that the males preferred large females. Peden (1972) demonstrated that the dark pigment (gravid spot) provided a cue for orientation during thrusting of the GP (copulation attempt). Males were placed in a tank with a gravid and a non-gravid female, and using the unrestricted method, males

were observed to determine which female the male responded to more. It was found that the male had a preference for gravid females; all experiments consistently used large (> 40mm) gravid females.

2.7.2 Statistical Tests Employed

Reproductive behaviour

As stated previously, two separate methods for measuring the reproductive behaviour were used, with several characteristics of behaviour being observed. The measures analysed were GP thrust, and pursuit time for *G. affinis* (unrestricted method), and approach, duration in female zone, and number of mate attempts for *G. holbrooki* (restricted method).

The data was analysed using a variety of statistical tests. All behavioural data and normality tests were analysed using the computer software package Statistica ® for Windows (Statsoft, USA). Behavioural observations of pre-exposed fish in each experiment in chapter 3 and 4 were tested for normality using the Kolmogrov-Smirnov normality test. The rationale for using 'unexposed' fish was to avoid time (of exposure) and treatment having any effect on the distribution. If the data was normally distributed, a repeated measures two-way analysis of variance (ANOVA) test was used to determine treatment and test differences. Where significant differences were observed, *post-hoc* comparisons between groups were conducted using the Tukey Honest Significant Difference (HSD) for unequal sample sizes test. A Spearman Rank correlation test was used to test for significant behavioural dose-response relationships: the values measured on an ordinal scale contain information about their relationship to other values in terms of whether they were "greater than" or "less than" other values (but not in terms of "how much greater" or "how much smaller").

Behavioural observations of upstream and downstream fish for each year (and size class) were tested for normality using the Kolmogrov-Smirnov normality test (chapter 5). If both upstream and downstream data was normally distributed, differences between males collected from up- and downstream sites were tested using a parametric repeated measures ANOVA test. If data failed the normality tests, the non-parametric Mann-Whitney 'U' test for independent samples was used. The Mann-Whitney 'U' test were two-tailed tests to reduce the risk of committing a type I or II error.

To determine whether the level of mating attempts varied between groups a Fisher Exact Test was used to compare the proportion of fish that attempted to mate or not.

Pearson's Product Moment test (for parametric data) and Spearman's Rank correlation test (for non-parametric data) were used to determine if the behavioural measures were correlated with each other.

Morphology

Morphological data of control fish (in chapter 3 and 4) and up- and downstream fish (in chapter 5) were tested for normality using the Kolmogrov-Smirnov normality test. Although similar in appearance, the morphological measurements of *G. affinis* were not pooled with measurements of *G. holbrooki*. Using this same data (control or upstream fish), Pearson's Product Moment test (for parametric data) and Spearman's Rank correlation test (for non-parametric data) were used to determine if the measures were correlated with body size (length or weight).

Gonopodium lengths (GPL) and testes area (TA) were compared between treatments or sites using analysis of covariance (ANCOVA) to adjust for differences in size using the software package SPSS (Microsoft). ANCOVA was first conducted to determine whether GPL and TA measures needed to be corrected for body length. If body length accounted for a significant component of the variance, then group means were adjusted for body length. For gonopodium length, total length was the covariate (and likewise with TA). In

an ANCOVA, each value of the dependent variable (y) is adjusted along a common regression line to the overall mean covariate value for all groups combined. If body length did not count for a significant component of the variance, then the data were re-analysed by ANOVA, and group means were not adjusted for length.

In order to be able to compare fish of different body weight, the relative testes weight was calculated by dividing it by body weight (so it is independent of body weight). The resultant gonadosomatic index (GSI) was tested again for normality using the Kolmogorov-Smirnov normality test. Effective control of body weight as a variable was confirmed by determining the relationship between the GSI and body weight. If data was normally distributed, the Pearson product-moment correlation test was used, and if data was not normally distributed, the Spearman Rank correlation test was employed.

The body length and weight data were used to calculate Fulton's condition factor or body condition index (BCI) using the formula below:

$$\frac{BW\ (g) \times 100}{BL\ (mm)^3} = BCI$$

If data was normally distributed, differences between treatments were analysed using the parametric one-way ANOVA test. Where significant differences were observed, the post-hoc Tukey HSD for unequal sample sizes test was used to determine which treatments differed significantly from each other. If data was not normally distributed, the non-parametric Kruskal-Wallis one-way ANOVA test was performed to determine if there were significant differences between the test treatments. Where significant differences were observed, the Mann-Whitney U test was used to determine which treatments differed significantly from each other.

Morphological differences between males collected from up- and downstream sites were tested for normality using the Kolmogrov-Smirnov normality test (chapter 5). If both

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upstream and downstream data was normally distributed, differences between males collected from up- and downstream sites were tested using a parametric t-test for independent samples. If data failed the normality tests, the non-parametric Mann-Whitney 'U' test for independent samples was used. The t-test and Mann-Whitney 'U' test were two-tailed tests.

The level of statistical significance was selected to be at $p < 0.05$ and the data is presented as the mean ± standard error, unless otherwise stated.

CHAPTER 3

An Investigation of the Effects of Oestrogenic Chemical Exposure on Reproductive Behaviour and Morphology of Adult Male Mosquitofish (*Gambusia* sp.)

3.1 Introduction

Exposure to oestrogens affects various aspects of reproduction in a number of fish species and the main findings are reviewed in section 1.3. In order to investigate the impact of REDs on male mosquitofish (*Gambusia* sp.) from a source of contamination in the field (for example, sewage effluent discharge), it is first important to investigate what the effects of known oestrogens on reproductive behaviour and morphology. This series of experiments investigates the effects of two oestrogens (diethylstilbestrol, DES and 17ß-oestradiol, E2) and an oestrogen mimic (octylphenol, OP) on the reproductive behaviour and morphology of adult male *Gambusia* sp.

3.2 Effects of diethylstilbestrol (DES) exposure on reproductive behaviour and morphology of *Gambusia* sp.

3.2.1 Introduction

Diethylstilbestrol (DES) is a potent synthetic oestrogen (in relation to endogenous 17ß-estradiol) that was used by physicians to prevent spontaneous abortions in women from 1948 until 1971, when its use for this purpose was banned. There is a substantial literature documenting the detrimental effects of exposure to DES, but it is primarily recognised for its carcinogenicity in female offspring whose mothers were administered it as a drug (described in section 1.2.5). Its use is now limited to the treatment of prostrate cancer. Although not usually an environmental contaminant, DES is used in many studies as a 'model' oestrogen in investigative studies due to its high potency and relatively slow degradation. DES was included in this study as a 'model' oestrogen.

3.2.2 Methods

Three separate experiments on the effects of DES were carried out. Housing and maintenance of fish, as well as chemical exposures, were as described in the general methods. Experiments 1a and 1b were conducted within three weeks of each other using *G. affinis* at Napier using the unrestricted interaction method for recording reproductive behaviour. Experiment 1c was conducted using *G. holbrooki* at UTS using the restricted interaction method.

The treatment groups in experiment 1a were solvent control and DES (2ng/l and 10ng/l) exposure for 8 weeks. The treatment groups in experiment 1b were solvent control and DES (0.4ng/l, 2ng/l and 10ng/l) exposure for 6 weeks. The groups in experiment 1c were

the same as experiment 1b with exposure of 8 weeks. Measures of reproductive behaviour over the exposure period were recorded weekly in experiments 1a and 1b, and at weeks 1, 2, 4, 6 and 8 in experiment 1c. Morphological measurements were made within two days of the final behavioural observations. The behavioural and morphological measurement protocols were as described in the general materials and methods.

Males were not pre-tested for reproductive behaviour before the treatments in experiments 1a and 1b, but were conducted prior to experiment 1c. The number of males examined in each treatment group prior to exposure was as follows:

Experiment 1a -: solvent control 7, DES 2ng/l: 7, 10ng/l: 7
Experiment 1b -: solvent control: 16, DES 0.4ng/l: 10, 2ng/l: 10, 10ng/l: 10
Experiment 1c- solvent control: 15, DES 0.4ng/l: 15, 2ng/l: 15, 10ng/l: 15

3.2.3 Results

Mortality

In all three experiments there was high mortality in both control and experimental groups (Table 3.1). Mortality in the control groups ranged from zero to 44%, and was not related to exposure to DES.

Table 3.1 Sample size and mortality rate of males in experiment 1 a, b and c.

Treatment	Experiment 1a: Week 1	8-week exposure of *G. affinis* Week 4	Week 8	% Mortality
Control	7	7	7	0
2ng/l DES	7	7	5	29
10ng/l DES	7	7	1	86

Treatment	Experiment 1b: Week 1	6-week exposure of *G. affinis* Week 4	Week 6	% Mortality
Control	16	12	9	44
0.4ng/l DES	10	10	10	0
2ng/l DES	10	10	10	0
10ng/l DES	10	8	8	11

Treatment	Experiment 1c: Pre-Exposure	8-week exposure of *G. holbrooki* Week 4	Week 8	% Mortality
Control	15	10	10	40
0.4ng/l DES	15	13	10	33
2ng/l DES	15	14	11	27
10ng/l DES	15	7	4	73

Reproductive Behaviour

As stated previously, males of two closely related species of mosquitofish (*G. affinis* and *G. holbrooki*) have been utilised for exposure to DES. Two separate methods for measuring several reproductive behaviour characteristics were used (for a description of the behavioural protocol and reasons for preferred choice of protocol refer to section 2.5). The aim was to examine reproductive behaviour, and all the measures were chosen to reflect this.

The data on reproductive behaviour recorded in experiments 1a and 1b (and the results of fish exposed to an additional concentration of DES in experiment 1b) have been pooled together (1ab) up to week 6, since the protocols were identical. Results of experiment 1c are presented separately as the effects were conducted on a different species, *G. holbrooki*. Since the mortality in all groups increased considerably from week 6 onward in experiment 1a, only behavioural data up to weeks 6 was included in the results analysis.

Experiment 1ab (a and b pooled)

The measurements taken of males in the control groups of experiments 1a and b at week 1 were pooled and tested for normality. Unless stated otherwise, all measurements were normally distributed.

Gonopodial Thrusts

Two-way ANOVA of gonopodial thrusts (repeated measures of weeks 1 - 6) revealed significant treatment effects ($F_{(3, 54)}$ = 5.54, p < 0.003) and test effects ($F_{(5, 270)}$ = 6.03, p < 0.001). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in gonopodial thrusts of males exposed to 2ng/l and 10ng/l DES compared to controls (p < 0.004 and p < 0.03 respectively). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in gonopodial thrusts at weeks 4 and 5 compared to

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weeks 1, 2 and 3 ($p < 0.02$). The mean GP thrusts observed over the 6-week exposure period in experiment 1ab are shown in Figure 3.1a.

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-6 of testing) and were plotted in relation to exposure dose in Figure 3.4a on page 73. There was no significant correlation between treatment and GP thrusts, i.e., no dose response relationship (Spearman rank r = -0.8, $n = 4$, $p = 0.2$).

Pursuit Time

The mean pursuit time is shown in Figure 3.1b. From this figure, it can clearly be seen there were differences between treatments; from week 2, the pursuit time of all three exposed groups was consistently lower than the control group. Parametric two-way ANOVA of pursuit time (repeated measures of weeks 1 - 6) revealed significant treatment effects ($F_{(3, 54)} = 10.34$, $p < 0.001$), but no test effects ($F_{(5, 270)} = 0.67$, $p = 0.4$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in pursuit time of males exposed to 2ng/l and 10ng/l DES compared to controls and the lowest exposure dose of 0.4ng/l DES (controls: $p < 0.02$ and $p < 0.001$ respectively and 0.4ng/l DES: $p < 0.02$ and $p < 0.001$ respectively).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-6 of testing) and tested for a correlation (Spearman rank). There was no significant correlation between treatment and duration in female zone, i.e., no dose response relationship (Spearman rank r = -0.8, $n = 4$, $p = 0.2$).

GP thrusts and pursuit time of control males recorded in week 1 of experiments 1a and b were pooled and tested for correlations; the two behaviours were positively correlated (r = 0.40, $p < 0.05$, n = 23), i.e., both measures were representative of reproductive motivation.
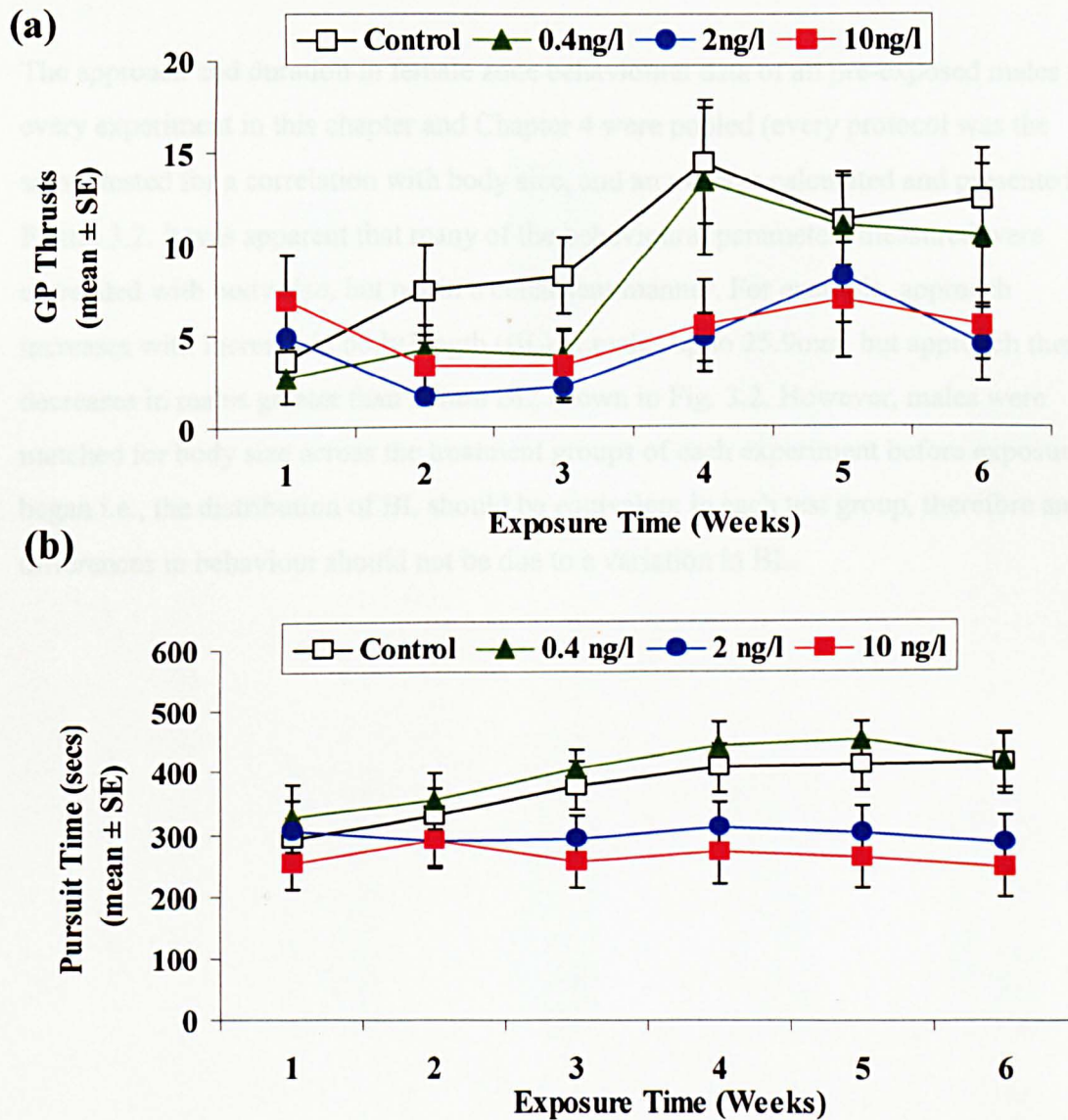
Figure 3.1 Mean (± standard error) of (a) GP thrusts and (b) pursuit time of male *G. affinis* exposed to three concentrations of diethylstilbestrol (DES). Experiment 1a and b data pooled; 10-minute observation period (unrestricted method). Two-way ANOVA of GP thrusts and pursuit time (repeated measures of weeks 1 - 6) revealed significant treatment effects ($p < 0.003$ and $p < 0.001$ respectively): Tukey of GP thrust revealed: control > 2ng/l and 10ng/l DES and Tukey of pursuit time revealed: control and 0.4ng/l > 2ng/l and 10ng/l DES.

Additional Data Analysis

The approach and duration in female zone behavioural data of all pre-exposed males from every experiment in this chapter and Chapter 4 were pooled (every protocol was the same) tested for a correlation with body size, and an average calculated and presented in Figure 3.2. It was apparent that many of the behavioural parameters measured were correlated with body size, but not in a consistent manner. For example, approach increases with increase in body length (BL) in males up to 25.9mm, but approach then decreases in males greater than 26mm BL, shown in Fig. 3.2. However, males were matched for body size across the treatment groups of each experiment before exposure began i.e., the distribution of BL should be equivalent in each test group, therefore any differences in behaviour should not be due to a variation in BL.
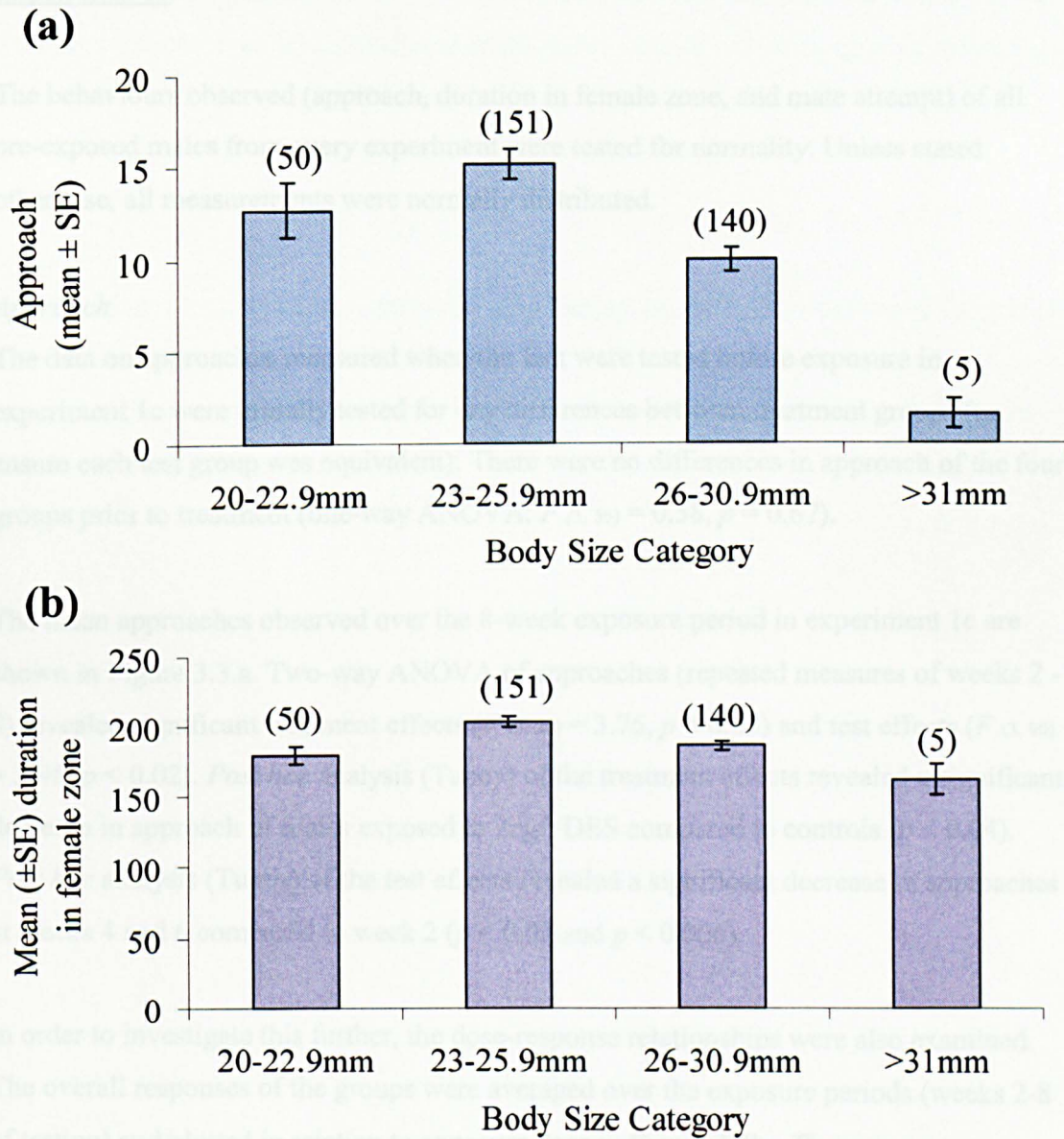
Figure 3.2 Mean (± standard error) of (a) approach and (b) duration in female zone of male *G. holbrooki*. All pre-exposed males (in chapter 3 and 4) have been pooled and separated into body size (mm) categories. Sample size in brackets.

<u>Experiment 1c</u>

The behaviours observed (approach, duration in female zone, and mate attempt) of all pre-exposed males from every experiment were tested for normality. Unless stated otherwise, all measurements were normally distributed.

Approach

The data on approaches measured when the fish were tested before exposure in experiment 1c were initially tested for any differences between treatment groups (to ensure each test group was equivalent). There were no differences in approach of the four groups prior to treatment (one-way ANOVA: $F_{(3, 56)} = 0.58$, $p = 0.67$).

The mean approaches observed over the 8-week exposure period in experiment 1c are shown in Figure 3.3.a. Two-way ANOVA of approaches (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 30)} = 3.76$, $p < 0.03$) and test effects ($F_{(3, 90)} = 3.98$, $p < 0.02$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in approach of males exposed to 2ng/l DES compared to controls ($p < 0.04$). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in approaches at weeks 4 and 6 compared to week 2 ($p < 0.02$ and $p < 0.006$).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and plotted in relation to exposure dose in Figure 3.4b. There was no significant correlation between treatment and approach, i.e., there was no dose-response relationship (Spearman rank r = -0.8, $n = 4$, $p = 0.2$).

Duration in Female Zone

The duration in female zone observed over the 8-week exposure period in experiment 1c are shown in Figure 3.3b. Two-way ANOVA of duration in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 30)} = 10.97$, $p <$

0.001) but no test effects ($F_{(3, 90)} = 6.64$, $p = 0.59$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in duration in female zone of males exposed to 2ng/l and 10ng/l DES compared to controls ($p < 0.003$ and $p < 0.006$, respectively) and the lowest exposure dose of 0.4ng/l DES ($p < 0.007$ and $p < 0.02$, respectively).

In order to investigate any dose-response relationship, the overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing). There was a dose-response effect: increasing exposure to DES was associated with decreased duration in female zone (Spearman rank r = -1.0, n = 4, $p < 0.05$).

(a)
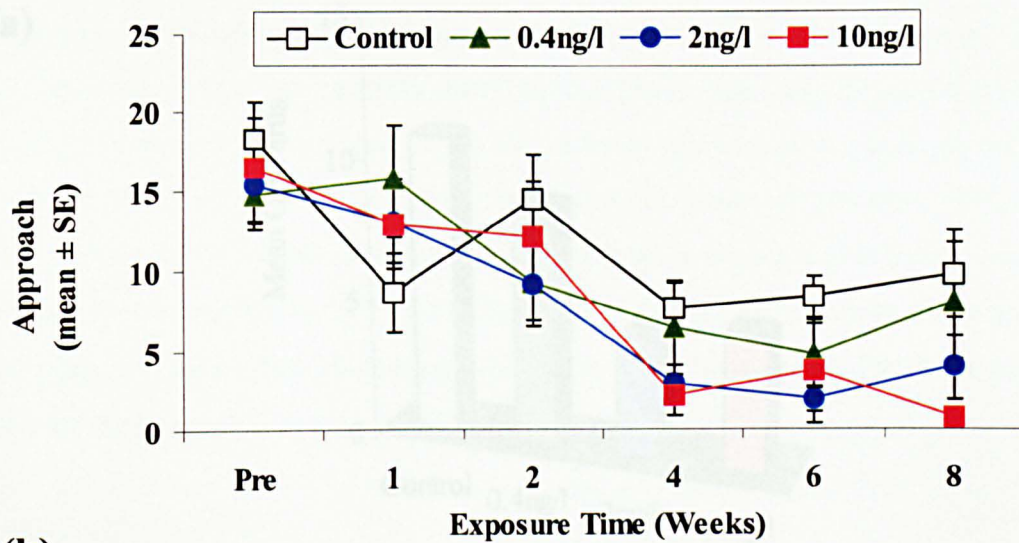
(b)
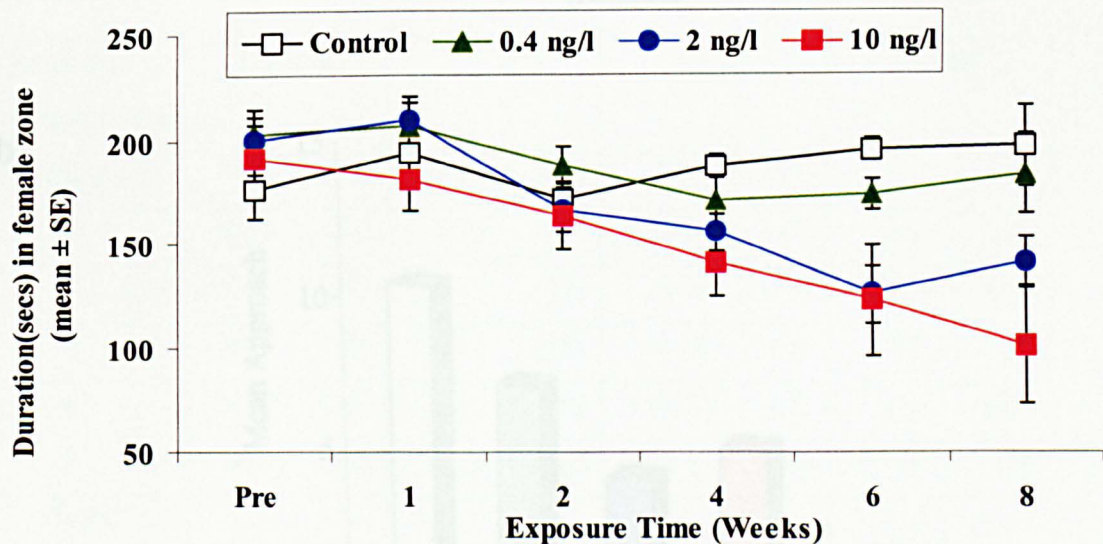
Figure 3.3 Mean (± standard error) of (a) approach and (b) duration in female zone of male *G. holbrooki* exposed to three concentrations of diethylstilbestrol (DES). Experiment 1 c; 5-minute observation period (restricted method). Two-way ANOVA of approach and duration in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($p < 0.03$ and $p < 0.001$ respectively): Tukey of approach revealed: control > 2ng/l and Tukey of duration time revealed: control and 0.4ng/l > 2ng/l and 10ng/l DES.

(a)
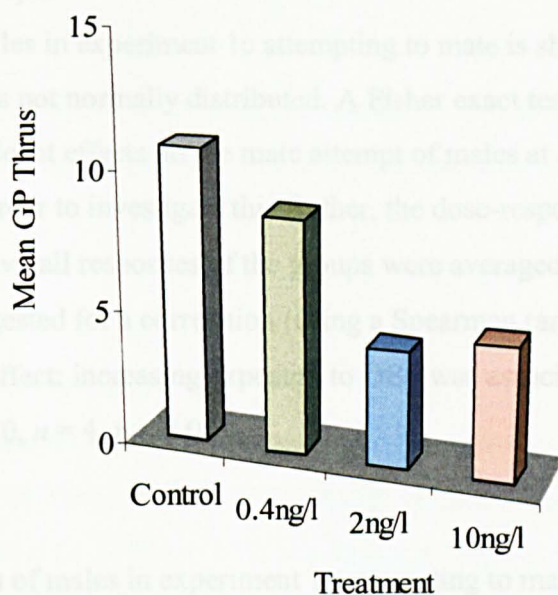
(b)
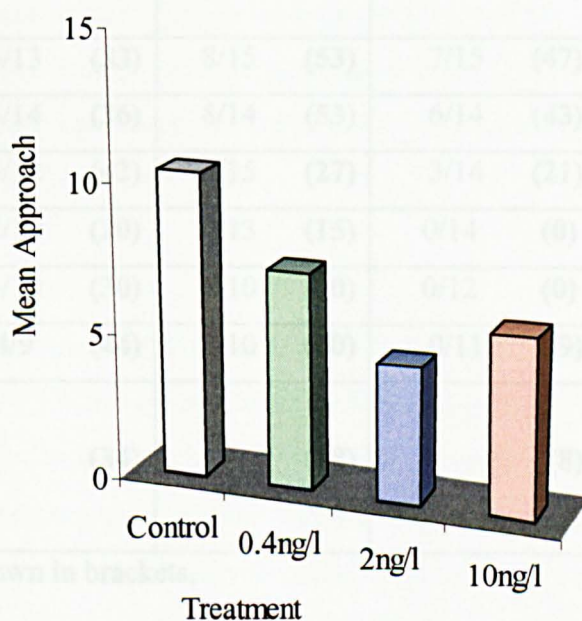
Figure 3.4 Mean of mean (a) GP thrust of *G. affinis* and (b) approaches of *G. holbrooki*
exposed to three concentrations of diethylstilbestrol (DES). Data was pooled across the
exposure period for each treatment group.

Number of Mate Attempts

The proportion of males in experiment 1c attempting to mate is shown below in Table 3.2. Mate attempt was not normally distributed. A Fisher exact test revealed that exposure to DES had no significant effects on the mate attempt of males at any week during the exposure period. In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over weeks 2–8 of the exposure period and tested for a correlation (using a Spearman rank test). There was a clear dose-response effect: increasing exposure to DES was associated with decreased mate attempts ($r = -1.0$, $n = 4$, $p < 0.05$).

Table 3.2 Proportion of males in experiment 1c attempting to mate with females[1]

Treatment/ Exposure Time	Control		0.4 ng/l		2 ng/l		10 ng/l	
Pre-exposure	5/13	**(33)**	8/15	**(53)**	7/15	**(47)**	7/15	**(47)**
1	5/14	**(36)**	8/14	**(53)**	6/14	**(43)**	5/13	**(38)**
2	5/12	**(42)**	4/15	**(27)**	3/14	**(21)**	1/10	**(10)**
4	2/10	**(20)**	2/13	**(15)**	0/14	**(0)**	0/7	**(0)**
6	3/10	**(30)**	1/10	**(10)**	0/12	**(0)**	1/6	**(17)**
8	4/9	**(44)**	2/10	**(20)**	0/11	**(9)**	0/4	**(0)**
Mean Response (Week 2-8)		**(34)**		**(18)**		**(8)**		**(7)**

[1] Percentages are shown in brackets.

Morphology

The morphological measurements of *G. affinis* cannot be pooled with measurements of *G. holbrooki*, so results of experiment 1a and b were analysed separately from experiment 1c.

Experiment 1ab (a and b data pooled)

The morphological data from experiments 1a and 1b have been pooled together (1ab), since the protocols were identical. Data on the morphological measurements taken after the 6-8 week exposure of *G. affinis* to DES are shown in Table 3.3.

Gonopodial Length (GPL)

There was a significant positive correlation between GPL and BL of control males ($r = 0.81$, $n = 16$, $p < 0.001$). Results comparing adjusted mean GPL in male mosquitofish between treatment groups are shown in Table 3.3. ANCOVA revealed no significant differences in adjusted means ($p = 0.54$).

Body Condition Index (BCI)

The BCI was calculated (as described in section 2.7.2). The BCI was positively correlated with body length ($r = 0.67$, $n = 16$, $p < 0.005$), which suggests that in reality, this conventionally used index may not be an appropriate measure of body condition for this species. However, as experiment 1a and b was the only experiment detailed in this report using *G. affinis*, the results of normality tests and correlations (between body size and measurements) taken will have a large margin of error because of the small sample size.

Kruskal Wallis revealed no significant differences in BCI between any of the treatment groups after exposure of male *G. affinis* to DES ($H_{(3, n=50)} = 2.9$, $p = 0.48$).

Table 3.3 Morphological measurements of male *G. affinis* in experiment 1ab exposed to DES for 6-8 weeks[1]

Treatment	*n*	BL	BCI	[a]GPL
Control	16	26.64 ± 0.89	0.081 ± 0.003	6.99 ± 0.18
0.4ng/l DES	10	27.67 ± 0.60	0.083 ± 0.003	6.90 ± 0.23
2ng/l DES	14	25.69 ± 0.82	0.082 ± 0.002	6.85 ± 0.20
10ng/l DES	9	26.88 ± 0.55	0.087 ± 0.003	7.29 ± 0.25

[1] Table entries represent mean or [a]adjusted mean (± standard error). No significant treatment effects were found.

Experiment 1c

To test for normality, the control morphological data was pooled between all the experiments in this chapter and chapter 4 (that used *G. holbrooki*). Unless stated otherwise, all measures were normally distributed.

Gonopodium Length (GPL)

There was a significant positive correlation between GPL and BL of control males ($r = 0.36$, n = 89, $p < 0.001$). Results comparing adjusted mean GPL in male mosquitofish between treatment groups are shown in Table 3.4. ANCOVA revealed no significant differences in adjusted means ($p = 0.48$).

Body Condition Index (BCI)

The BCI was not correlated with body length ($r = 0.07$, $p > 0.5$). There were no treatment effects on BCI (One-Way ANOVA: $F_{(3, 31)} = 0.53$, $p = 0.58$).

Spermatozeugmata Count (SPZ)

SPZ counts were not normally distributed (K-S d = 0.33, n = 87, $p < 0.01$). The SPZ count was positively correlated with body length (Sp $r = 0.3$, $p < 0.005$). There were no significant treatment effects on the SPZ Count (Kruskal Wallis ANOVA: $H_{(3, n=31)} = 0.87$, $p = 0.83$).

Testes Area (TA)

There was a significant positive correlation between TA and BL of control males ($r = 0.36$, n = 89, $p < 0.001$). Results comparing adjusted mean TA of male mosquitofish between treatment groups are shown in Table 3.4. ANCOVA revealed significant differences in adjusted means ($p = 0.03$); TA was smaller in males exposed to DES compared to controls. A Post-hoc test (Tukey) did not reveal any significant differences between treatments.

Table 3.4 Morphological measurements of male *G. holbrooki* in experiment 1c exposed to DES for 8 weeks[1]

Treatment	*n*	[a]GPL	[a]TA*	BCI	SPZ Count
Control	10	6.65 ± 0.12	4.19 ± 0.23	0.073 ± 0.005	600
0.4ng/l DES	10	6.91 ± 0.12	3.19 ± 0.25	0.068 ± 0.002	450
2ng/l DES	11	6.87 ± 0.10	3.51 ± 0.19	0.071 ± 0.002	500
10ng/l DES	4	6.68 ± 0.17	3.28 ± 0.33	0.077 ± 0.011	550

[1] Table entries represent mean or [a]adjusted mean (± standard error) for normally distributed data or median values for non-normal data. * Indicates significant treatment effects using ANCOVA ($p < 0.05$); Control TA > exposed males (no significant differences found using post-hoc tests).

3.2.4 Summary of DES Exposure

Mortality

- Mortality rate was variable (in control groups: 0 – 44%).
- Mortality not related to DES exposure.

Reproductive Behaviour

Experiment 1ab (G. affinis):

- Significant reduction in GP thrust behaviour revealed using a repeated measures test, but no significant dose response relationship (when data was pooled).
- Significant reduction in pursuit time revealed using a repeated measures test, but no significant dose response relationship observed (when data was pooled).

Experiment 1c (G. holbrooki):

- Significant reduction in approach behaviour revealed using a repeated measures test, but no significant dose response relationship (when data was pooled).
- Significant reduction in duration in female zone revealed using a repeated measures test, and a significant dose response relationship observed (when data was pooled).
- No significant effects on mate attempt revealed on a test-to-test basis, but a significant dose-response relationship was found (when the data was pooled).

Morphology

- No significant effects on any of the morphological measurements.

3.3 Effects of 17β-oestradiol (E2) exposure on reproductive behaviour and morphology of *Gambusia holbrooki*

3.3.1 Introduction

A standard means of examining the oestrogenic potency of a substance is to compare it to 17β-oestradiol (E2), which is the principal natural oestrogen in fish and mammals (Nimrod and Benson, 1996). The relative E2 potency of a compound is determined by examining its competitive displacement of E2 from the oestrogen receptor (ER). E2 plays a major role in VTG production (Crisp *et al.*, 1998) and is responsible for the development of secondary sex characteristics. As described in section 1.2.7, E2 has been detected in sewage effluent and in some rivers that are contaminated with sewage effluent. Larsson *et al.* (1999) found concentration of E2 at 1.1ng/l in effluent water from a Swedish STP receiving mainly domestic wastewater. Thomas *et al.* (2001), found a concentration of 19ng/l E2 in sewage effluent, but levels too low for detection (<15ng/l) in water samples collected from two estuaries in the UK. Detection limits of E2 in rivers reported in the UK vary, concentrations of E2 recorded downstream of STPs have been reported in the range of 1 – 2ng/l in one study (Williams *et al.*, 1999), and 0.2ng/l E2 in other UK rivers (Desbrow *et al.*, 1998). More recently, Sheahan *et al.* (2002), measured E2 concentration in treated effluent at 23ng/l and at 2ng/l E2 in a UK river 0.4km downstream of the sewage effluent outfall. The maximum recorded concentration of E2 recorded in rivers receiving sewage effluent is 64ng/l E2, associated with STP in Canada (Ternes *et al.*, 1999). However, not all studies have found concentrations of E2 in treated sewage effluent (Fawell *et al.*, 2001). E2 has been used an oestrogen in a few studies on fish, for a review of the findings refer to section 1.3.

3.3.2 Methods

Two separate experiments on the effects of E2 were carried out. Housing and maintenance of fish, as well as chemical exposures, were as described in the general methods. Experiments 2a and b were conducted using *G. holbrooki* at UTS using the restricted interaction method for recording behaviour.

The treatment groups in experiment 2a were solvent control and E2 (20ng/l and 100ng/l) exposure for 6 weeks. The treatment groups in experiment 2b were solvent control and E2 (20ng/l, 100ng/l and 500ng/l) exposure for 10 weeks. Measures of reproductive behaviour over the exposure period were recorded at weeks 2, 4 and 6 in experiments 2a, and at weeks 1, 2, 4, 6, 8 and 10 in experiment 2b. Morphological measurements were made within two days of the final behavioural observations. For fish in experiment 2a, an additional measurement of testes weight was recorded. The behavioural and morphological measurement protocols were as described in the general methods.

Males were pre-tested for reproductive behaviour before the treatments in both experiments. The number of males examined in each treatment group prior to exposure was as follows:

Experiment 2a -: solvent control: 14, E2 20ng/l: 14, 100ng/l: 14.
Experiment 2b -: solvent control: 15, E2 20ng/l: 15, 100ng/l: 15, 500ng/l: 15.

3.3.3 Results

Mortality

In both experiments there was some mortality in both control and experimental groups (Table 3.5). Mortality in the control groups ranged from zero to 14%, mortality was not related to exposure to E2.

Table 3.5 Sample size and mortality rate of males in experiment 2 a and b

| Treatment | Experiment 2a: 6-week exposure | | | |
	Pre-Exposure	Week 4	Week 6	% Mortality
Control	14	13	12	14
20ng/l E2	14	11	10	29
100ng/l E2	14	12	11	21

| Treatment | Experiment 2b: 10-week exposure | | | |
	Pre-Exposure	Week 4	Week 10	% Mortality
Control	15	15	15	0
20ng/l E2	15	14	12	20
100ng/l E2	15	15	15	0
500ng/l E2	15	14	13	13

Reproductive Behaviour

As described earlier, the behaviour observations from all pre-exposed groups from each experiment were tested for normality: all data was normally distributed, unless stated otherwise.

The data on behaviour recorded in experiments 2a and 2b have been pooled together (2ab), since the protocols were identical. Since the approaches decreased in all four treatment groups at week 10 in experiment 2b (due to unknown factors), only behavioural data up to week 8 was included in the results analysis.

Experiment 2 (a and b pooled)

Approach

The mean approaches observed over the exposure period in experiment 2 are shown in Figure 3.5a. The data on approach measured when the fish were tested before exposure in experiment 2 were initially tested for any differences between treatment groups (to ensure each test group was equivalent). There were no differences in approaches of the four groups prior to treatment (ANOVA: $F_{(3, 98)} = 1.01$, $p = 0.39$).

Two-way ANOVA of approaches (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 54)} = 16.86$, $p < 0.001$) and test effects ($F_{(3, 162)} = 7.48$, $p < 0.001$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in approach of males exposed to 20ng/l and 500ng/l E2 compared to controls ($p < 0.002$ and $p < 0.001$ respectively) and the intermediate dose of 100ng/l E2 ($p < 0.03$ and $p < 0.001$ respectively). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in approaches at week 8 compared to week 2 and 4 ($p < 0.001$ and $p < 0.003$, respectively).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and are plotted in relation to exposure dose in Figure 3.6. There was no significant correlation between treatment and approach, i.e., there was no dose-response relationship (Spearman rank r = -0.8, n = 4, p = 0.2).

Duration in Female Zone

The average time the male spends in the female zone observed over the exposure period in experiment 2 are shown in Figure 3.5b. Two-way ANOVA of duration in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 53)}$ = 16.42, p < 0.001) and test effects ($F_{(3, 159)}$ = 8.68, p < 0.001). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in duration in female zone of males exposed to 500ng/l E2 compared to controls (p < 0.001), 20ng/l E2 (p < 0.001) and 100ng/l E2 (p < 0.001). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in duration in female zone at week 6 and 8 compared to week 2 and 4 (week 6: p < 0.001 and p < 0.001, respectively; and week 8: p < 0.006 and p < 0.006, respectively).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing). There was no significant correlation between treatment and duration in female zone, i.e. there was no dose-response relationship (Spearman rank r = -0.8, n = 4, p = 0.2).
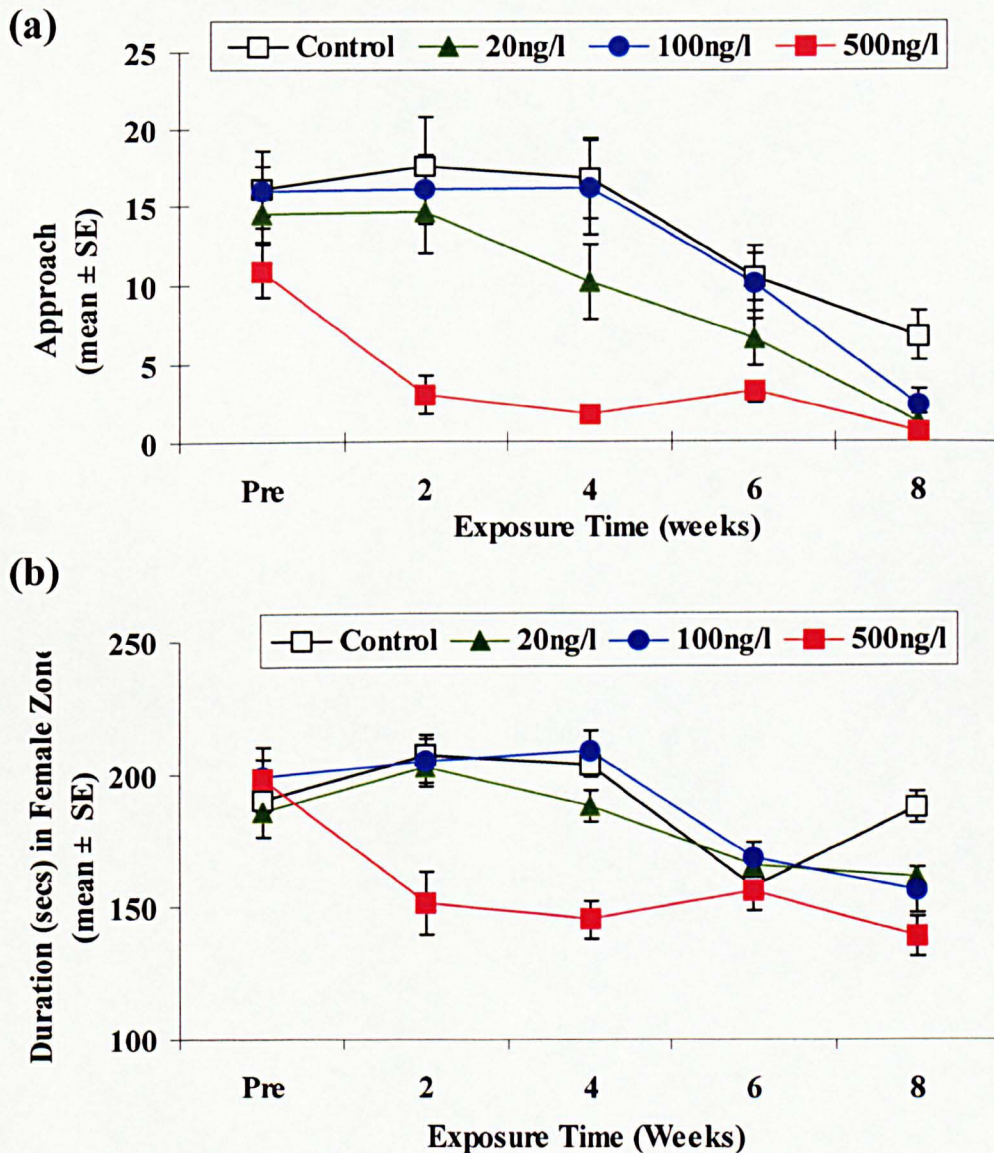
Figure 3.5 Mean (± standard error) of (a) approach and (b) duration in female zone of male *G. holbrooki* exposed to three concentrations of 17β-oestradiol (E2). Data from experiment 2a and b were pooled. Two-way ANOVA of approach and duration time in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($p < 0.001$ and $p < 0.001$ respectively): Tukey of approach revealed: control and 100ng/l > 20ng/l and 500ng/l E2 and Tukey of duration time revealed: control, 20ng/l and 100ng/l > 500ng/l E2.
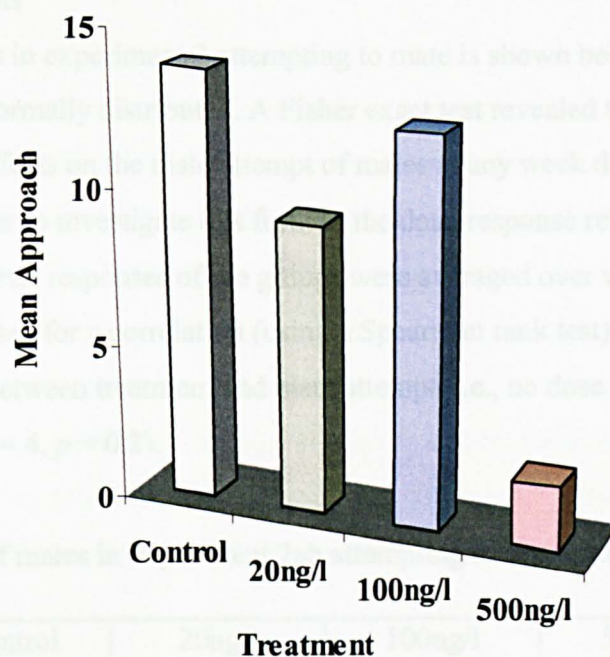
Figure 3.6 Mean of mean approach of male *G. holbrooki* exposed to three concentrations of 17β-oestradiol (E2). Data from experiment 2a and b were pooled over the 2-8 week exposure period. No significant dose-response relationship was found (using Spearman Rank test).

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Number of Mate Attempts

The proportion of males in experiment 2 attempting to mate is shown below in Table 3.6. Mate attempt was not normally distributed. A Fisher exact test revealed that exposure to E2 had no significant effects on the mate attempt of males at any week during the exposure period. In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over weeks 2–8 of the exposure period and tested for a correlation (using a Spearman rank test). There was no significant correlation between treatment and mate attempt, i.e., no dose response relationship ($r = -0.8$, $n = 4$, $p = 0.2$).

Table 3.6 Proportion of males in experiment 2ab attempting to mate with females[1]

Treatment/ Exposure Time	Control		20ng/l		100ng/l		500ng/l	
Pre-exposure	10/29	(35)	7/29	(24)	11/29	(38)	4/15	(27)
2	6/28	(21)	5/26	(19)	7/28	(25)	1/14	(0)
4	7/28	(25)	1/25	(4)	5/27	(19)	0/14	(0)
6	5/27	(19)	3/24	(9)	4/26	(15)	0/14	(0)
8	2/15	(13)	0/14	(0)	0/15	(0)	0/14	(0)
Mean Response (Weeks 2-8)		(20)		(9)		(15)		(2)

[1] Percentages are shown in brackets.

Morphology

The control data was pooled between all the experiments in this chapter and Chapter 4 (that used *G. holbrooki*) in order to test for normality of the morphological measurements. The GPL, TA and BCI were normally distributed and the GSI and SPZ count were not normally distributed. The GPL, SPZ count, and TA were positively correlated with BL.

The results of morphological measurements of exposed fish in experiment 2a and b cannot be pooled (unlike the behavioural data) because a different exposure period was conducted for each (6 weeks and 10 weeks for experiment 2a and b, respectively), i.e., the exposure period may have had different effects on morphology.

Experiment 2a

The morphological measurements taken after the 6-week exposure of *G. holbrooki* to E2 are shown below in Table 3.7.

Table 3.7 Morphological measurements of male *G. holbrooki* in experiment 2a exposed to E2 for 6-weeks[1]

Treatment	*n*	[a]GPL	[a]TA	BCI	SPZ Count
Control	12	6.61 ± 0.08	4.35 ± 0.25	0.069 ± 0.002	350
20ng/l E2	10	6.61 ± 0.09	5.06 ± 0.28	0.071 ± 0.003	400
100ng/l E2	11	6.39 ± 0.09	4.52 ± 0.28	0.071 ± 0.001	600

[1] Table entries represent mean or [a]adjusted mean (± standard error) for normally distributed data or median values for non-normal data. No significant treatment effects were found.

<u>Experiment 2b</u>

The morphological measurements taken after the 10-week exposure of *G. holbrooki* to E2 are shown in Table 3.8.

ANCOVA revealed significant differences in adjusted mean TA ($p = 0.002$). Tukey analysis revealed that fish exposed to 100ng/l and 500ng/l E2 had a significantly greater TA average compared to the control fish ($p < 0.001$ and < 0.02, respectively), in addition, fish exposed to 100ng/l E2 had a significantly greater TA compared to the lowest dose of 20ng/l E2 ($p < 0.02$).

Kruskal Wallis ANOVA of SPZ counts and GSI revealed significant differences between treatment groups ($H_{(3, n=55)} = 30.75$, $p < 0.001$ and $H_{(3, n=54)} = 29.3$, $p < 0.001$, respectively). A Mann Whitney U test revealed fish exposed to 100ng/l and 500ng/l E2 had a significantly greater mean GSI compared to the control fish ($p < 0.001$). A Mann Whitney U test revealed fish exposed to 500ng/l E2 had a significantly lower SPZ count compared to the control fish ($p < 0.001$). There were no significant treatment effects on GPL or BCI.

Table 3.8 Morphological measurements of male *G. holbrooki* in experiment 2b exposed to E2 for 10-weeks[1]

Treatment	n	aGPL	aTA	GSI	BCI	SPZ Count
Control	15	8.01 ± 0.07	3.63 ± 0.21	0.096	0.072 ± 0.001	600
20ng/l E2	12	7.92 ± 0.07	3.39 ± 0.23	0.096	0.074 ± 0.001	600
100ng/l E2	15	7.89 ± 0.07	4.46 ± 0.20*	0.138*	0.074 ± 0.001	600
500ng/l E2	13	7.82 ± 0.07	4.29 ± 0.22*	0.128*	0.069 ± 0.002	200*

[1] Table entries represent mean or aadjusted mean (± standard error) for normally distributed data or median values for non-normal data. Differences in treatments were analysed using the Kruskal-Wallis test (for non-normal data) or parametric one-way ANOVA or ANCOVA (for normally distributed data). Where significant differences were observed, treated groups were compared with the control group using the Mann Whitney U or Tukey test. * Indicates $p < 0.05$ relative to control.

3.3.4 Summary of E2 Exposure

<u>Mortality</u>

- Mortality rate was not high (of control groups: 0 – 14%).
- Mortality not related to E2 exposure.

<u>Reproductive Behaviour</u>

Experiment 2ab:

- Significant reduction in approach behaviour revealed using a repeated measures test, but no significant dose response relationship (when data was pooled).
- Significant reduction in duration in female zone revealed using a repeated measures test, but no significant dose response relationship observed (when data was pooled).
- No significant effects on mate attempt revealed on a test-to-test basis, and no dose-response relationship was found (when the data was pooled).

<u>Morphology</u>

Experiment 2a (6-week exposure):

- No significant effects on any of the morphological measurements.

Experiment 2b (10-week exposure):

- Males exposed to 100ng/l and 500ng/l E2 had a significantly greater TA and GSI than controls.
- Males exposed to 500ng/l produced significantly less SPZ than controls.

3.4 Effects of octylphenol (OP) exposure on reproductive behaviour and morphology of *Gambusia holbrooki*

3.4.1 Introduction

The environmental contaminant, octylphenol (OP), a breakdown product of APEOs, is an oestrogen mimic commonly reported to be found in sewage effluent. All the environmental oestrogens discovered to date are relatively weak oestrogens (Nimrod and Benson, 1996). Nevertheless, they appear to possess full activity and interact with the oestrogen receptor in exactly the same manner as the natural ligand E2 (Jobling and Sumpter, 1993). OP has been reported to have oestrogenic activity in both *in vitro* and *in vivo* studies (White *et al.*, 1994; Sharpe *et al.*, 1995; Sonnenschein *et al.*, 1995; Jobling *et al.*, 1996; Nagel *et al.*, 1997) described in section 1.2.7.

Effluent from STPs contain hundreds of micrograms per litre (μg/l) of APs, whereas river-water concentrations of the major degradation products are in the tens of μg/l range or less. Even drinking water contains detectable amounts of these environmental oestrogens (Clark *et al.*, 1992). An extensive study by Jobling *et al.*, (1996) on the oestrogenic potential of various alkylphenols in rainbow trout revealed that threshold concentrations above which a significant elevation in the vitellogenin (VTG) synthesis was observed, were in the range of 10 μg/l and 3 μg/l for nonylphenol (NP) and OP, respectively. In the same study, there was shown to be a coincidence between the elevated VTG levels and inhibited spermatogenesis in developing male rainbow trout exposed to alkylphenolic compounds.

Doses used were based on detection limits found in rivers reported in the literature. The doses chosen for this study were 2 μg/l, 10 μg/l and 50 μg/l OP. OP has been used to

demonstrate endocrine disruption in many fish studies, for reviews of the findings refer to section 1.3.

3.4.2 Methods

Three separate experiments to investigate the effects of OP were carried out. Housing and maintenance of fish, as well as chemical exposures, were as described in the general methods. All experiments were conducted using *G. holbrooki* at UTS using the restricted interaction method for behaviour.

The treatment groups in experiment 3 a, b, and c were solvent control and OP (2μg/l, 10μg/l and 50μg/l) exposure for 8, 10 and 5 weeks respectively. Measures of reproductive behaviour over the exposure period were recorded at weeks 2, 4, 6 and 8 in experiment 3a, at weeks 1, 2, 4, 6, 8 and 10 in experiment 3b, and weekly in experiment 3c. Morphological measurements were made within two days of the final behavioural observations. The behavioural and morphological measurement protocols were as described in the general methods.

Males were pre-tested for reproductive behaviour before the treatments in all three experiments. The number of males examined in each treatment group prior to exposure was as follows:

Experiment 3a -: solvent control: 14, OP 2μg/l: 14, 10μg/l: 14, 50μg/l: 14
Experiment 3b -: solvent control: 15, OP 2μg/l: 15, 10μg/l: 15, 50μg/l: 15
Experiment 3c -: solvent control: 10, OP 2μg/l: 10, 10μg/l: 10, 50μg/l: 10

3.4.3 Results

Mortality

In all three experiments there was some mortality in both control and experimental groups (Table 3.9). Mortality ranged from zero to 21% in the control groups, and was not related to exposure to OP.

Table 3.9 Sample size and mortality rate of males in experiment 3 a, b and c.

Treatment	Experiment 3a: 8-week exposure Pre-Exposure	Week 4	Week 8	% Mortality
Control	14	13	11	21
2µg/l OP	14	13	9	36
10µg/l OP	14	11	8	43
50µg/l OP	14	12	10	29

Treatment	Experiment 3b: 10-week exposure Pre-Exposure	Week 6	Week 10	% Mortality
Control	15	13	13	13
2µg/l OP	15	12	8	47
10µg/l OP	15	13	12	20
50µg/l OP	15	11	11	27

Treatment	Experiment 3c: 5-week exposure Pre-Exposure	Week 3	Week 5	% Mortality
Control	10	10	10	0
2µg/l OP	10	10	9	10
10µg/l OP	10	10	10	0
50µg/l OP	10	10	10	0

Reproductive Behaviour

The data on behaviour recorded in experiments 3 a, b and c have been pooled together, since the protocols were identical up to week 8. Since mortality in the lowest exposure concentration group ($2\mu g/l$) of OP, increased considerably from week 8 onwards in experiment 3b, only behavioural data up to week 8 is included in the results analysis. As experiment 3c was conducted for only 5 weeks, this data was pooled with week 6 of experiment 3a and b.

Experiment 3 (a, b and c pooled)

Approach

The data on approach measured when the fish were tested before exposure in experiment 3 was initially tested for any differences between treatment groups (to ensure each test group was equivalent). There were no differences in the approach of the four groups prior to treatment (ANOVA: $F_{(3, 152)} = 0.66, p = 0.57$).

The mean approaches observed over the 8-week exposure period in experiment 3 are shown in Figure 3.7a. Two-way ANOVA of approaches (repeated measures of weeks 2 - 8) revealed significant test effects ($F_{(3, 240)} = 15.54, p < 0.001$) but no treatment effects ($F_{(3, 80)} = 2.00, p = 0.12$). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in approaches at weeks 6 and 8 compared to week 2 and 4 (week 6: $p < 0.003$ and $p < 0.001$, respectively; and week 8: $p < 0.001$ and $p < 0.001$, respectively).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and are plotted in relation to exposure dose in Figure 3.8. There was a suggestive dose-response relationship: an increase in exposure to OP was associated with reduced approach (Spearman rank $r = -1.0$, $n = 4, p < 0.05$).

Duration in Female Zone

The average time the male spends in the female zone observed over the 8 week exposure period in experiment 3 are shown in Figure 3.7b. Two-way ANOVA of duration in female zone (repeated measures of weeks 2 - 8) revealed significant test effects ($F_{(3, 237)}$ = 10.17, $p < 0.001$), but no significant treatment effects ($F_{(3, 79)}$ = 1.73, $p = 0.17$). *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in duration in female zone at week 8 compared to weeks 2, 4 and 6 ($p < 0.001$, $p < 0.001$, and $p < 0.03$, respectively).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and tested for a correlation (using Spearman Rank). There was a suggestion of a dose-response relationship: an increase in exposure to OP was associated with reduced duration in female zone (Spearman rank r = -1.0, $n = 4$, $p < 0.05$).
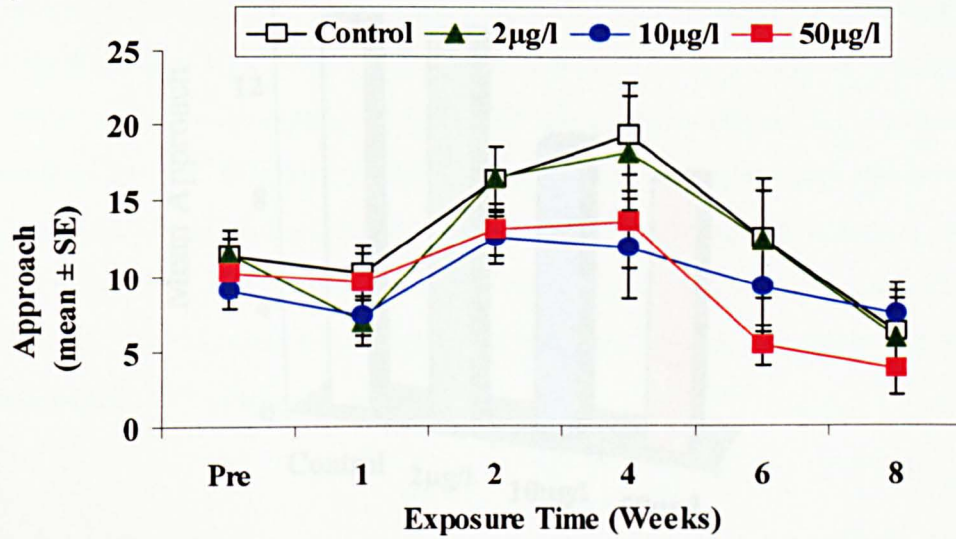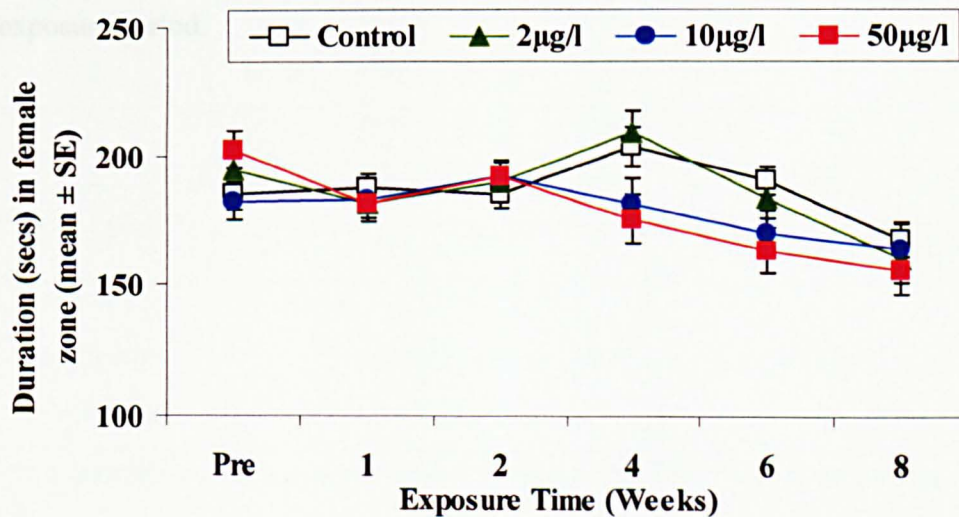
Figure 3.7 Mean (± standard error) of (a) approach and (b) duration in female zone by male *G. holbrooki* exposed to three concentrations of octylphenol (OP). Data from Experiment 3a, b and c is pooled. Two-way ANOVA of approach and duration time in female zone (repeated measures of weeks 2 - 8) revealed no significant treatment effects.
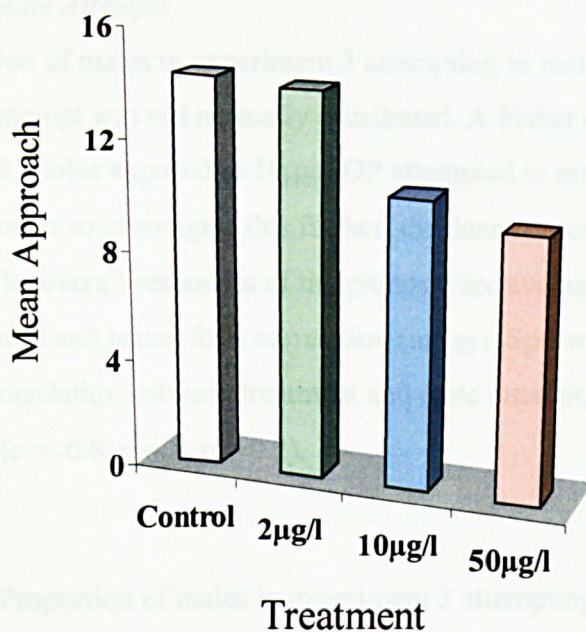
Figure 3.8 Mean of mean approach of male *G. holbrooki* exposed to three concentrations of octylphenol (OP). Data from experiments 3a, b, and c is pooled over the 2–8 week exposure period.

Number of Mate Attempts

The proportion of males in experiment 3 attempting to mate is shown below in Table 3.10. Mate attempt was not normally distributed. A Fisher exact test revealed that, at week 2 and 4, males exposed to 10µg/l OP attempted to mate significantly less than controls. In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over weeks 2–8 of the exposure period and tested for a correlation (using a Spearman rank test). There was no significant correlation between treatment and mate attempt, i.e., no dose response relationship ($r = -0.8$, $n = 4$, $p = 0.2$).

Table 3.10 Proportion of males in experiment 3 attempting to mate with females[1]

Treatment/ Exposure Time	Control		2µg/l		10µg/l		50µg/l	
Pre-exposure	12/39	(31)	15/39	(39)	13/39	(33)	12/39	(31)
2	13/39	(33)	11/36	(31)	* 5/38	(13)	11/37	(30)
4	10/37	(27)	9/35	(26)	* 2/32	(6)	5/34	(15)
6	7/35	(20)	4/32	(13)	6/31	(19)	3/33	(9)
8	2/23	(9)	2/19	(11)	2/20	(10)	1/21	(5)
Mean Response (Weeks 2-8)		(22)		(20)		(12)		(15)

[1] Percentages are shown in brackets. * Indicates $p < 0.05$ relative to control.

Morphology

Males in experiment 3a and b were exposed to OP for a similar exposure period (8 and 10-weeks respectively) so therefore these two experiments were pooled and analysed simultaneously. Experiment c however, was conducted for a shorter exposure period of 5-weeks, and therefore was analysed separately.

Experiment 3ab (a and b pooled)

The morphological measurements taken after the 8-10 week exposure of *G. holbrooki* to OP are shown in Table 3.11. No significant treatment effects were found.

Table 3.11 Morphological measurements of male *G. holbrooki* in experiment 3ab exposed to OP for 8-10 weeks[1]

Treatment	*n*	[a]GPL	[a]TA	GSI	BCI	SPZ Count
Control	24	7.01 ± 0.06	3.80 ± 0.25	0.89	0.074 ± 0.002	600
2µg/l OP	17	6.93 ± 0.07	4.32 ± 0.30	0.99	0.071 ± 0.002	600
10µ/l OP	20	6.95 ± 0.07	4.02 ± 0.28	0.90	0.071 ± 0.001	600
50µ/l OP	21	7.08 ± 0.07	4.00 ± 0.27	1.03	0.071 ± 0.002	400

[1] Table entries represent mean or [a]adjusted mean (± standard error) for normally distributed data or median values for non-normal data. Data from experiment 3a and b have been pooled. No significant treatment effects were found.

Data on the morphological measurements taken after the 5-week exposure of *G. holbrooki* to OP are shown in Table 3.12. No significant treatment effects were found.

Table 3.12 Morphological measurements of male *G. holbrooki* in experiment 3c exposed to OP for 5 weeks[1]

Treatment	*n*	[a] GPL	[a] TA	GSI	BCI	SPZ Count
Control	10	0.261 ± 0.003	0.170 ± 0.009	0.0156	0.076 ± 0.004	600
2μg/l OP	9	0.262 ± 0.003	0.163 ± 0.012	0.0195	0.072 ± 0.001	600
10μ/l OP	10	0.266 ± 0.004	0.143 ± 0.006	0.0158	0.069 ± 0.001	600
50μ/l OP	10	0.266 ± 0.005	0.174 ± 0.009	0.0156	0.071 ± 0.001	600

[1] Table entries represent mean or [a] adjusted mean (± standard error) for normally distributed data or median values for non-normal data. No significant treatment effects were found.

3.4.4 Summary of OP Exposure

<u>Mortality</u>

- Mortality rate was moderately high (of control groups: 0 – 21%).
- Mortality not related to OP exposure.

<u>Reproductive Behaviour</u>

Experiment 3abc:

- No significant differences between treatments in approach behaviour using a repeated measures test, but a significant dose response relationship was found (when data was pooled).
- No significant differences between treatments in duration in female zone revealed using a repeated measures test, but a significant dose response relationship was found (when data was pooled).
- Significant reduction in mate attempt revealed on a test-to-test basis, but no dose-response relationship was found (when the data was pooled).

<u>Morphology</u>

Experiment 3ab (8-10 week exposure):

- No significant effects on any of the morphological measurements.

Experiment 3c (5-week exposure):

- No significant effects on any of the morphological measurements.

3.5 SUMMARY

Table 3.13 Summary of behavioural responses of *Gambusia* sp. exposed to DES, E2 and OP (relative to controls)

		DES		E2	OP
		(*G. affinis*)	(*G. holbrooki*)		
Mean approach	Repeated measures test	↓	↓	↓	NS
	Dose-response relationship: data pooled over tests	NS	NS	NS	↓
Mean duration in female zone	Repeated measures test	↓	↓	↓	NS
	Dose-response relationship: data pooled over tests	NS	↓	NS	↓
Proportion attempting to mate	Individual test analysis	-	NS	NS	↓
	Dose-response relationship: data pooled over tests	-	↓	NS	NS

↓   Indicates a statistically significant reduction

NS   Indicates no statistically significant difference

2-6   Indicates week of exposure differences found

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3.6 DISCUSSION

Reproductive Behaviour

The experimental results described show clear reductions in reproductive behaviour of adult male mosquitofish exposed to the synthetic oestrogen diethylstilbestrol (DES), and the natural oestrogen 17β-oestradiol (E2), but no consistent significant effects of exposure to the oestrogen mimic octylphenol (OP).

Reproductive behaviour was measured using several measures: initial studies exposing males to DES utilised pursuit time and GP thrust, but subsequent tests utilised duration in female zone, approach, and mate attempt. It was clear from the first two experiments (using *G. affinis*) that chasing with possible aggressive motivation could not be distinguished from sexual, so the second method was more controllable when the structured experimental protocol was adopted. In addition, the behaviour of the female (e.g. aggression) in pheromones released by them, may have affected the responses of the males. The fact that the fish were separated by the beaker, not only eliminated the aggressiveness of the male and female interaction, but also eliminated any olfactory cues from the fish that may have had an influence on the effects of the hormone treatments administered.

It is well established that the movement of the GP 180° toward the female is the male attempting to transfer sperm to the female, hence the name, mate attempt. However, the number of approaches towards the female and duration in female zone is harder to identify as reproductive behaviour characteristics. However, since these behaviours were significantly correlated with mate attempt strongly suggests they are types of reproductive behaviour.

There were periodic variations in behavioural response of control fish throughout the exposure period. Animals often exhibit a considerable natural variability in behaviour (e.g., Sutherland, 1995 and references therein) and this can be enhanced by stress of

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handling when housed in a laboratory. However, the fact that behavioural effects of treatment were established in all the experiments in this chapter demonstrates the methods are repeatable.

These experiments have demonstrated clear behavioural effects of exposure to known oestrogens, the most pronounced effect seen with exposure to DES. The observed threshold concentration for a reduced reproductive behaviour in all the observations of males recorded in these three experiments was 2ng/l DES. As mentioned previously, DES was chosen as a 'model' oestrogen because of its known potent oestrogenic effects; it has been shown to bind to the human oestrogen receptor (hER), and have greater oestrogenic potency than the natural oestrogen E2. The greater potency of DES compared to E2 was demonstrated in the current study: throughout the exposure period a concentration of 20ng/l E2 was enough to elicit a significant reduction response in behaviour of mosquitofish, i.e. the results found DES to be ten-fold more potent than E2. To the author's knowledge, no studies have investigated the effects of DES on the reproductive behaviour of fish.

The clear reductions in reproductive behaviour of fish exposed to E2 were in agreement with previous studies. Bayley *et al.*, (1999) demonstrated a significant reduction in the sexual display of the adult male guppy (*Poecilia reticulata*) at a concentration of 10μg/l E2. However, this is a twenty-fold greater exposure concentration than that required to induce a behavioural reduction in mosquitofish in this study. More recently, Bjerselius *et al.*, (2001) demonstrated a significant decrease in sexual behaviour of the goldfish (*Carassius auratus*) at concentrations of 1μg/l and 10μg/l E2, this is a two- and twenty-fold greater exposure concentration than that required to induce a behavioural reduction in mosquitofish in this study.

The dose response pattern evident among fish exposed to DES and E2 was variable. A linear dose-response was not demonstrated: the most pronounced effect seen with exposure to DES was observed in males exposed to the intermediate dose of 2ng/l DES.

This pattern was observed in all three experiments utilising both *G. affinis* and *G. holbrooki*. Similar variable effects of treatment were consistently found in the dose response of E2. No significant treatment effects on behaviour were demonstrated in males exposed to the intermediate concentration of 100 ng/l E2. However, a significant reduction was observed when males were exposed to the lowest and highest dose, 20ng/l and 500ng/l E2, and this was demonstrated in both experiments conducted.

The concept a non-linear shape of behavioural dose-response curves and differential behavioural responses depending on sex, species, endpoint, dose, timing and route of exposure is discussed by Barlow (1999). Examples in the literature of non-linear dose response curves include *hormesis*, a response that has been reported for low levels of stressors as diverse as pollutants and radiation (Calabrese and Baldwin, 2001, and references therein). This dose-response relationship is characterised by low dose stimulation and high dose inhibition, and is an adaptive dose-response relationship that occurs as a result of a compensatory mechanism following a disruption in homeostasis (Calabrese and Baldwin, 2001). This dose response has been demonstrated in insect models; toxicity tests of the blowfly response to cadmium exposure (Nascarella *et al.*, 2003) and behavioural effects of *Drosophila* exposed to low levels of lead (Hirsch *et al.*, 2003). However, none of the responses of male mosquitofish exposed to low doses were higher than that of the controls in the current study i.e., no stimulation at low doses. There is no explanation for the variability in dose-response of males exposed to DES and E2 at this stage of the investigation.

Males exposed to OP however, demonstrated a significant dose-dependant reduction in reproductive behaviour of exposed males in each of three experiments. However, it should be noted that because of the small sample size, the power of a Spearman rank correlation test is questionable (values measured on an ordinal scale contain information about their relationship to other values only in terms of whether they are "greater than" or "less than" other values but not in terms of "how much greater" or "how much smaller"). Although suggestive of reduction in behaviour, the more powerful repeated measures

ANOVA test found no significant treatment effects. Overall, the effects of OP were not as pronounced as with exposure to the oestrogens, DES and E2.

There have been a couple of studies published that demonstrate negative effects on fish reproductive behaviour consistent with the effects of OP in this study. Bayley *et al.*, (1999) demonstrated a significant reduction in the sexual display of the adult male guppy (*Poecilia reticulata*) at a concentration of 150µg/l OP after 4 weeks. Unfortunately, this was the only dose administered in this study. A study by Gray *et al.*, (1999a) demonstrated that the number of approaches of male Japanese Medaka (*Oryzias latipes*) toward females were significantly reduced only at the highest concentration of 50µg/l OP when compared to the control group (other doses they administered were 10- and 25µg/l OP). However, the males in this study by Gray and co-workers were exposed for a long period of time, from one day post-hatch to 6 months post-hatch, i.e., long-term exposure during 'sensitive periods' may have a more marked permanent effect than experiments described herein (which exposed adults).

Morphology
With respect to the effects of treatment on the morphological measurements, very few significant effects were observed. Males exposed to the highest doses of 100ng/l and 500ng/l E2 for 10-weeks demonstrated significantly greater testes size (TA and GSI), and lower SPZ count compared to controls. However, no differences between treatments were observed in males exposed to E2 for 6 weeks, which suggests this exposure time was insufficient.

In contrast to the results of the present study, other laboratory exposure studies have shown that chemicals with oestrogenic properties can inhibit testis growth (Bjerselius *et al.*, 2001; Toft and Baatrup, 2000; Harries *et al.*, 1997). Bjerselius *et al.* (2001) demonstrated a decrease in GSI of adult male goldfish (*Carassius auratus*) exposed to 1µg/l and 10µg/l E2 via water and a significant decrease in males exposed to 10µg/l and

100μg/l E2 via food for 4 weeks. Toft and Baatrup (2000) also demonstrated a decrease in GSI of adult male guppies (*Poecilia reticulata*) exposed to 0.03 μg/l E2 (but not to 0.1 and 1 μg/l E2) in the laboratory for 4 weeks. There are clear discrepancies between effects seen in GSI between these studies and the current one; a possible explanation could be differences in species-susceptibility.

The finding that males exposed to the highest dose of E2 showed a significantly reduced SPZ count is however in agreement with other studies. Bjerselius *et al.* (2001) demonstrated a significant reduction in the presence of milt in male goldfish exposed to 100 μg/l E2 (via food, but not via water) compared to controls. Another study by Bayley *et al.* (2002) demonstrated a reduction in sperm count in the adult male guppy (*Poecilia reticulata*) exposed as juveniles to DDE, flutamide, and vinclozolin (a testosterone antagonist).

Overall, there were no consistent significant effects of exposure to the REDs used in the present study on the reproductive morphology of males. This may reflect a lack of response to these compounds, or may be due to insufficient exposure time. In addition, there may be 'sensitive' periods of development which were outwith the exposure time of the current study. A recent study by Doyle and Lim (2002), the results of which are reviewed in section 1.4.2, suggests this latter hypothesis may apply to the current results. This report is of particular relevance to the current study because of the similarities in experimental protocol (fish were collected from the same site, exposed to the same concentrations of E2, and tested for behaviour using a similar assessment of behaviour utilised in the current study). They demonstrated juvenile males exposed to 100, and 500ng/l E2 had smaller GP, lower ray 4 to ray 6 ratios, and reduced sexual activity of E2 exposed males compared to controls. This discussion will be developed in greater detail in chapter 6.

The mortality rates in the present study were not related to treatment effects because the mortality rates of exposed fish were no greater than in controls. There is a general lack of

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data concerning the toxicity (i.e. LC50 tests) of endocrine disrupters in fish. However, a pilot experiment by Toft and Baatrup (2001) estimated the acute toxicity concentration (96-h LC50) of OP in adult guppies (*Poecilia reticulata)* to be between 1400 and 3700μg/L. In this same study, a toxic effect was demonstrated following a one-month exposure to 900 ug/L OP (they found 60% mortality the guppy). Although this was a 40-fold greater exposure concentration of OP than that required to induce a behavioural reduction in mosquitofish, response of lower exposures over a longer period of exposure may potentially induce a general toxic response which was not evident at the time of recording in this study (e.g. disturbances in equilibrium, or hypo-/hyperactivity).

CHAPTER 4

An Investigation of the Effects of Sewage Effluent Exposure on Reproductive Behaviour and Morphology of Adult Male Mosquitofish, *Gambusia holbrooki*

4.1 INTRODUCTION

The discovery of hermaphrodite roach (*Rutilus rutilus*) in stretches of river close to sewage outfalls in the UK triggered the beginning of research into the oestrogenic activity of river systems receiving effluent inputs. Caged male fish held downstream of many STPs produced the egg yolk protein VTG, indicating the presence of oestrogenic substances in the water (Purdom *et al.*, 1994; Harries *et al.*, 1996; 1997). The compounds responsible for this activity are believed to be primarily steroidal oestrogens, both natural (oestrone and 17ß-oestradiol) and synthetic (ethinyloestradiol), and the environmental oestrogens, the APs (e.g., Desbrow *et al.* 1998; Harries *et al.*, 1997). However, many other chemicals known to possess weak oestrogenic activity (e.g., pthalates, pesticides and dieldrin) are frequently present in sewage effluents (e.g., Harris *et al.*, 1997; Singh and Kime, 1995; Soto *et al.*, 1994). What is of concern is that some of these compounds that have been shown to possess oestrogenic activity are at concentrations readily detectable in river water (e.g., Blackburn and Waldock, 1995; Sheahan *et al.*, 2002), sewage effluent (e.g., Larsson et al., 1999; Ternes *et al.*, 1999) and occasionally in drinking water (Clark *et al.*, 1992).

The aim of this part of the study was to assess the effect of treated sewage effluent on the reproductive behaviour and morphology of the adult male mosquitofish, *G. holbrooki*. This was a accomplished by exposing fish to sewage effluent at different concentrations for an 8-10 week period and measuring the reproductive behaviour throughout the exposure period and morphological characteristics at the end of the exposure time. As no chemical analysis was conducted on the effluent, it is the intention to determine whether similar response patterns occurred in fish exposed to known REDs as demonstrated in the previous chapter.

A typical sewage effluent contains many thousands of chemicals, only a small proportion have been identified. It is likely that some, if not many, of these unidentified compounds

will possess oestrogenic activity, and fish living in waters contaminated with sewage effluent are therefore probably exposed to a mixture of oestrogenic chemicals. The actions of these chemicals are not mutually exclusive, because studies have shown that an additive oestrogenic response can be observed with mixtures of related and unrelated oestrogenic chemicals (Sumpter and Jobling, 1995). This is not surprising, as most REDs probably act via the same receptor. This is significant because many oestrogenic chemicals that are present in sewage effluent at individually negligible concentrations could still exert oestrogenic effects on organisms inhabiting the body of water. In addition to chemicals that are hormonally active, untreated sewage effluent can contain a range of pollutants that are toxic, for example trace metals, ammonia, nitrogen and phosphorus. The levels of these toxic substances entering the rivers receiving the effluent will depend on the level of treatment of the processing facility.

The effluent was obtained from St Marys STP, the largest inland STP in Sydney, Australia. It is a tertiary treatment facility: with nitrogen and phosphorus removal and disinfection by chlorination (and dechlorination implemented in 2001 – not affecting this study), and processes domestic and industrial sewage. The treatment process of St Marys is very advanced when compared to other STP facilities in other developed countries, indeed a beaker of 100% treated effluent is as clear as a glass of tap water. However, some studies have demonstrated that additional treatment, like secondary biological degradation, can breakdown non-oestrogenic compounds into chemicals that have weak oestrogenic properties, for example, APEOs to NP and OP (Ahel et al., 1994). In addition, the majority of oestrogenic material excreted from humans and wildlife, and therefore released into sewers, is in a conjugated form. However, the finding of "free" oestrogens in sewage effluent suggests that these metabolites are somehow converted back into an active form, at some stage of the sewage treatment process. For example, a study by Panter et al. (1999) demonstrated that the inactive metabolite, oestradiol-3-glucuronide, broke down into a more potent oestrogen by laboratory simulated microbial degradation. Another serious implication of advanced STPs is the addition of chlorine

(for disinfection), which has been shown to add to the toxicity of sewage effluent, through the formation of disinfection by-products.

4.2 METHODS

For background information on St Marys STP (service, process, and discharge) refer to general materials and methods. In summary, the daily discharge is 35 million litres per day, and the flow directly below the outfall should consist on an annual average, of approximately 50% effluent (Sydney Water, 2000).

Housing and maintenance of fish, as well as effluent exposure procedures, were as described in the general methods. Concentrations of sewage effluent administered were 25%, 50% and 100% effluent mixed with aged water. Three separate experiments on the effects of sewage effluent (SE) were carried out. All experiments (1, 2 and 3) were conducted using *G. holbrooki* (at UTS using the restricted interaction behavioural method).

Experiments 1 and 3 were conducted using fish sampled (several times) from a quite polluted river collected in summer of 1999 and 2000: South Creek (upstream of St Marys STP). Experiment 2 was conducted using fish sampled from a 'pristine' river: the Upper Colo, which flows through a national park. For detailed descriptions of sites and refer to the general methods.

The treatment groups in experiment 1 were control and sewage effluent (SE) (50% and 100%) exposure for 10 weeks. The treatment groups in experiment 2 were the same as experiment 1 with exposure of 10 weeks. The treatment groups in experiment 3 were control and SE (25%, 50% and 100%) exposure for 8 weeks. All experiments utilised treated sewage effluent collected from St Marys STP. Experiment 1 and 2 were conducted in 1999 (using the same effluent) and experiment 3 was conducted the following year.

Measures of reproductive behaviour over the exposure period were recorded at weeks 2, 4, 6, 8 and 10 in experiment 1, at weeks 1, 2, 4, 6, 8 and 10 in experiment 2, and at weeks 1, 2, 4, 6 and 8 in experiment 3. Morphological measurements were made within a few days of the final behavioural observations. The behavioural and morphological measurement protocols were as described in the general methods.

Males were not pre-tested for reproductive behaviour before the treatments in experiments 1 and 2, but were conducted prior to experiment 3. The number of males examined in each treatment group prior to exposure was as follows.

Experiment 1 -: Control - 19; 50% SE - 19; 100% SE - 18
Experiment 2 -: Control - 15; 50% SE - 12; 100% SE – 16
Experiment 3 -: Control - 13; 25% SE - 11; 50% SE - 11; 100% SE - 11

4.3 RESULTS

4.3.1 Mortality

In all three experiments there was high mortality in both control and experimental groups (Table 4.1). Mortality in the control groups ranged from 54% to 67%, but was not related to exposure to sewage effluent.

Table 4.1 Sample size and mortality rate of experiment 1, 2 and 3; 8-10 week exposure of male *G. holbrooki* to treated sewage effluent

Treatment	Experiment 1: 10-week exposure			% Mortality
	Week 2	Week 4	Week 10	
Control	19	19	7	63
50%	19	19	9	53
100%	18	18	8	58
Treatment	Experiment 2: 10-week exposure			% Mortality
	Week 1	Week 4	Week 10	
Control	15	8	5	67
50%	12	12	2	83
100%	16	12	2	88
Treatment	Experiment 3: 8-week exposure			% Mortality
	Pre-Exposure	Week 4	Week 8	
Control	13	10	6	54
25%	11	9	8	27
50%	11	11	7	36
100%	11	9	7	36

4.3.2 Reproductive Behaviour

The behaviour observations from all pre-exposed groups of each experiment were tested for normality: all data was normally distributed, unless stated otherwise.

Since the mortality in all groups increased considerably from week 8 onwards in experiment 1 and in experiment 2, only the behavioural data recorded up to week 8 was included in the results analysis.

Experiment 1

Approach

The mean approaches observed over the 8-week exposure period in experiment 1 are shown in Figure 4.1a. Parametric two-way ANOVA of approaches (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(2, 31)}$ = 3.78, $p < 0.04$), but no test effects ($F_{(3, 93)}$ = 0.06, $p = 0.97$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in approach of males exposed to 100% sewage effluent compared to controls ($p < 0.04$).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and are plotted in relation to exposure dose in Figure 4.4 on page 125. There was a clear dose-response effect: increasing exposure to the sewage effluent was associated with decreasing approach behaviour (Spearman rank r = -1.0, $n = 3$, $p < 0.05$).

Duration in Female Zone

The mean time the male spends in the female zone observed over the 8-week exposure period in experiment 1 are shown in Figure 4.1b. Two-way ANOVA (repeated measures of weeks 2 – 8) revealed that exposure to sewage effluent had no significant treatment or test effects on the duration in female zone.

In order to investigate any dose-response relationship, the overall responses of the groups were averaged over weeks 2-8 and tested for a correlation (using the Spearman rank test). There was no significant correlation between treatment and duration in female zone ($r = -0.5$, $n = 3$, $p = 0.67$).

Number of Mate Attempts

The proportion of males in experiment 1 attempting to mate is shown below in Table 4.2. A Fisher exact test revealed that at week 4, males exposed to 50% SE attempted to mate more than control fish ($p < 0.01$). In order to investigate any dose-response relationship, the overall responses of the groups were averaged over the exposure period (weeks 2-8 of testing) and shown in the table below. Inspection of the mean response indicates there is no dose-response relationship (i.e., the proportion of males attempting to mate does not decrease with increasing dose).

Table 4.2 Proportion of males in experiment 1 attempting to mate with females[1]

Treatment/ Exposure Time	Control	50%	100%
2	12/19 **(63)**	9/19 **(47)**	8/18 **(47)**
4	6/19 **(32)**	*14/19 **(74)**	6/18 **(33)**
6	2/13 **(15)**	5/15 **(33)**	2/12 **(17)**
8	1/10 **(10)**	3/12 **(25)**	1/11 **(9)**
Mean Response (Week 2-8)	**(30)**	**(45)**	**(27)**

[1] Percentages are shown in brackets. * Indicates Fisher exact test, $p < 0.01$ relative to control.
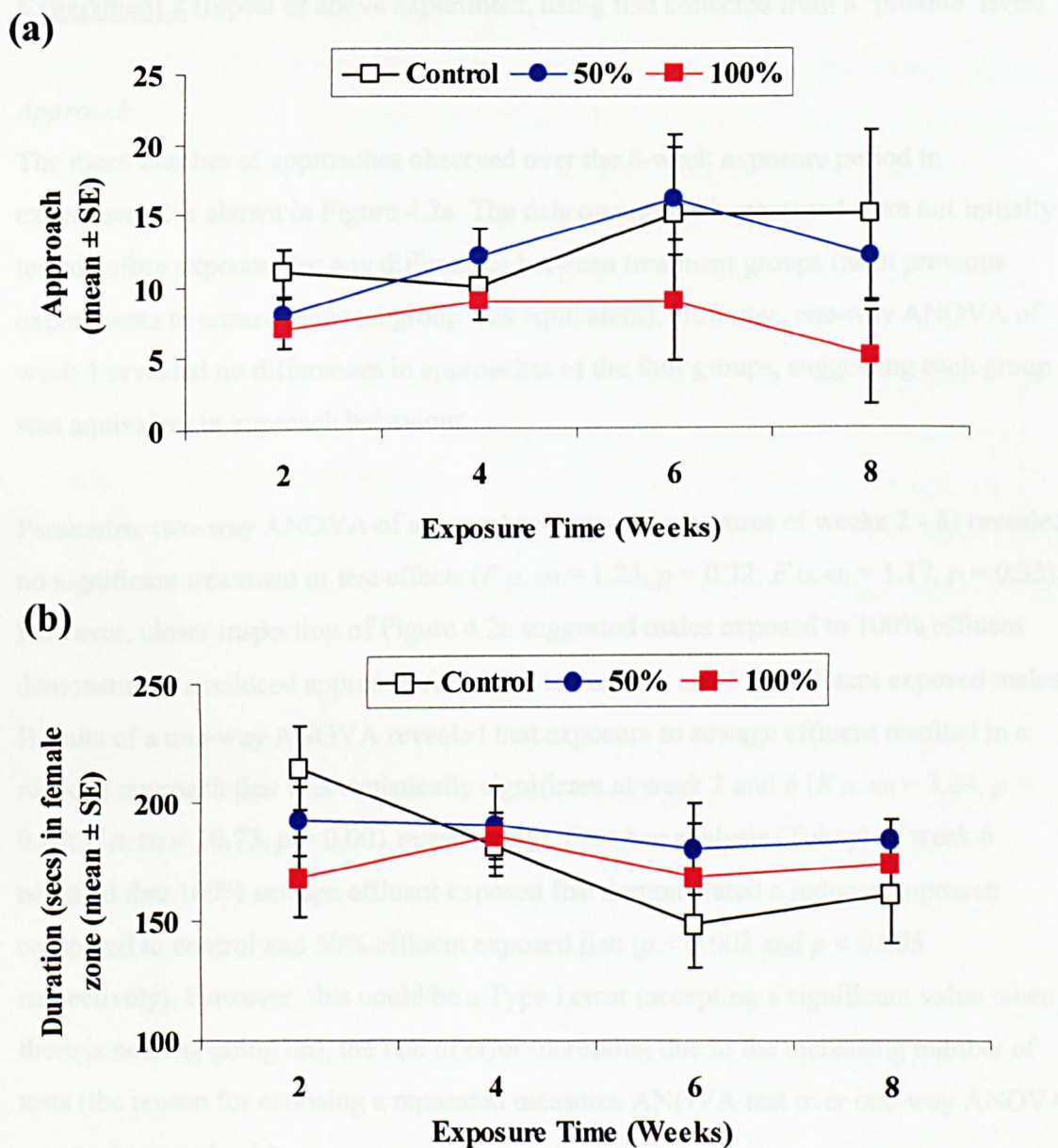
Figure 4.1 Experiment 1: Mean (± standard error) (a) approach and (b) duration time in female zone of male *G. holbrooki* exposed to three concentrations of sewage effluent (SE) over 8-weeks. Exposure using fish originally from a quite polluted site, using SE collected from St Marys STP in 1999. Two-way ANOVA of approach (repeated measures of weeks 2 - 8) revealed significant treatment effects ($p < 0.04$), but no differences between treatments in the duration in female zone. Tukey analysis of approach revealed: control > 100% SE.

Experiment 2 (repeat of above experiment, using fish collected from a 'pristine' river)

Approach

The mean number of approaches observed over the 8-week exposure period in experiment 2 is shown in Figure 4.2a. The data on approach measured were not initially tested before exposure for any differences between treatment groups (as in previous experiments to ensure each test group was equivalent). However, one-way ANOVA of week 1 revealed no differences in approaches of the four groups, suggesting each group was equivalent in approach behaviour.

Parametric two-way ANOVA of approaches (repeated measures of weeks 2 - 8) revealed no significant treatment or test effects ($F_{(2, 15)}$ = 1.23, p = 0.32; $F_{(3, 45)}$ = 1.17, p = 0.33). However, closer inspection of Figure 4.2a suggested males exposed to 100% effluent demonstrated a reduced approach compared to controls and 50% effluent exposed males. Results of a one-way ANOVA revealed that exposure to sewage effluent resulted in a reduced approach that was statistically significant at week 2 and 6 ($F_{(2, 40)}$ = 3.24, p = 0.05; $F_{(2, 25)}$ = 10.73, p = 0.001 respectively). *Post hoc* analysis (Tukey) of week 6 revealed that 100% sewage effluent exposed fish demonstrated a reduced approach compared to control and 50% effluent exposed fish (p < 0.002 and p < 0.005 respectively). However, this could be a Type I error (accepting a significant value when there is nothing going on), the rate of error increasing due to the increasing number of tests (the reason for choosing a repaeated measures ANOVA test over one-way ANOVA on a test-to-test basis).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure period (weeks 2-8 of testing) and are plotted in relation to exposure dose in Figure 4.4 on page 125. There is a clear dose-response effect: increasing exposure to the sewage effluent was associated with decreased approach behaviour (Spearman rank r = -1.0, n = 3, p < 0.05).

Duration in Female Zone

The mean time the male spends in the female zone observed over the 6-week exposure period in experiment 3 are shown in Figure 4.2b. The data on duration in female zone measured were not initially tested before exposure for any differences between treatment groups. One-way ANOVA of week 1 revealed significant treatment effects ($F_{(2, 40)} = 7.2$, $p < 0.002$). *Post hoc* analysis (Tukey) of week 1 revealed that fish exposed to 100% effluent spent a reduced amount of time in the female zone compared to control and 50% effluent exposed fish ($p < 0.04$ and $p < 0.005$ respectively). Due to this difference early on in exposure, and because no tests were recorded pre-exposure, it is impossible to conclude whether this difference is solely due to exposure or whether the reduced behaviour of the 100% effluent group (compared to the controls and 50% effluent group) was present before exposure began. Caution should be used when interpreting the results of this experiment.

The more statistically powerful repeated measures (of weeks 2-8) two-way ANOVA test of duration in female zone revealed significant treatment effects ($F_{(2, 15)} = 4.54, p < 0.03$), and significant test effects ($F_{(3, 45)} = 3.11, p = 0.04$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in duration in female zone in males exposed to 100% effluent compared to the 50% effluent exposed males ($p < 0.05$), but no difference between the control fish. *Post hoc* analysis (Tukey) of the test effects revealed a significant decrease in duration in female zone at week 8 compared to week 2 ($p < 0.04$).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and tested for a correlation (Spearman rank). There is no dose-response effect: increasing exposure to the sewage effluent is not associated with decreasing duration in female zone (r = -0.5, $n = 3, p = 0.67$).
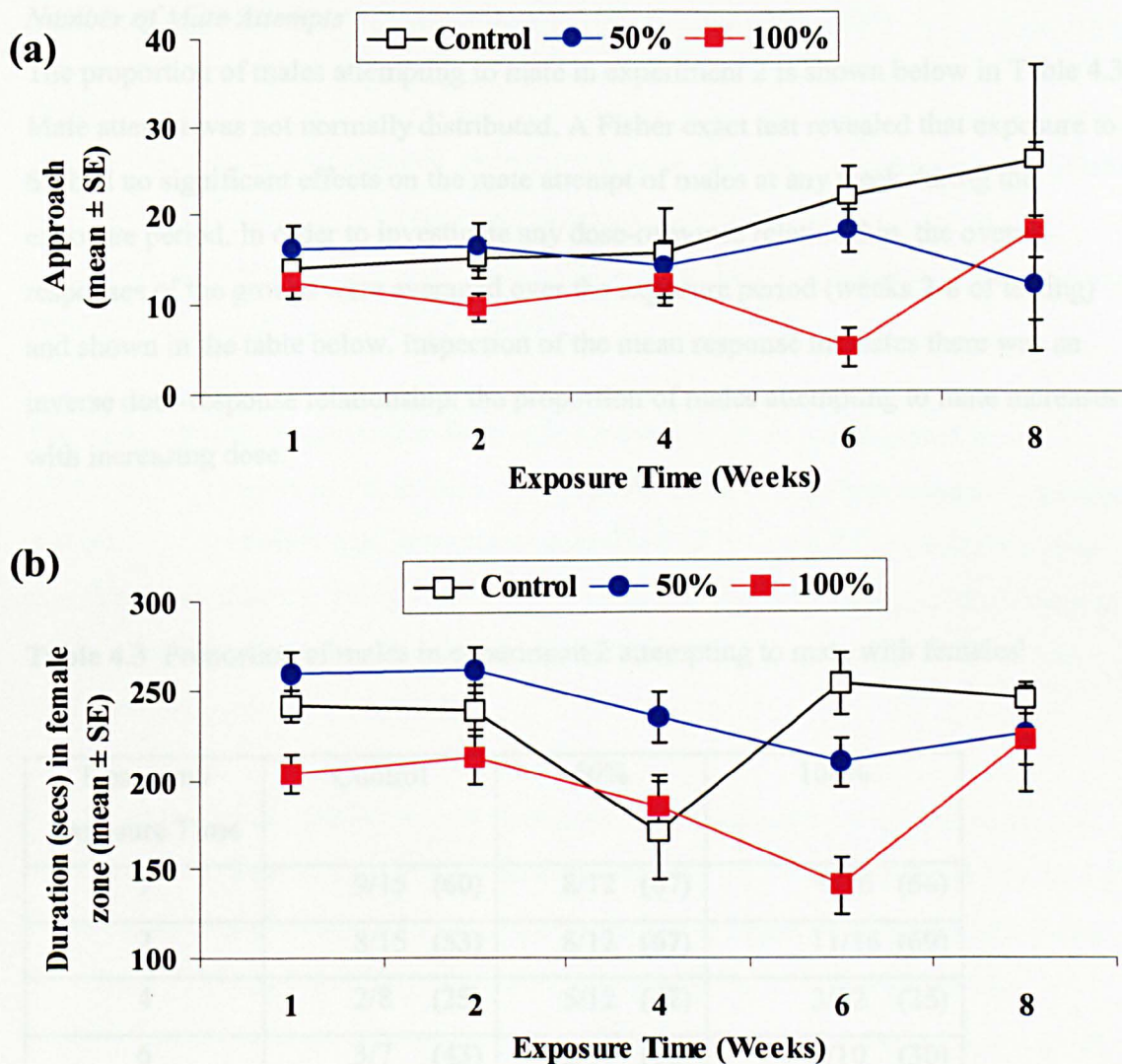
Figure 4.2 Experiment 2: Mean (± standard error) (a) approach and (b) duration in female zone of male *G. holbrooki* exposed to two concentrations of sewage effluent (SE) over 8-weeks. Exposure using fish originally from an apparently pristine river (using the same batch of SE as used in Experiment 1: from St Marys STP in 1999). No significant treatment effects were found when the approach data were analysed using the repeated measures ANOVA test, but duration in female zone was significant; 100% effluent group < 50% effluent group. * Indicates one-way ANOVA, $p < 0.05$ and ** $p < 0.001$. Significant treatment effects on approaches at week 2 and 6. Tukey test of approaches reveals that at week 6, control and 50% SE > 100% SE ($p < 0.002$ and $p < 0.005$, respectively). Tukey test of duration in female zone reveals that at week 1, control and 50% SE > 100% SE ($p < 0.04$ and $p < 0.005$, respectively); Tukey test, week 6, control and 50% SE > 100% SE ($p < 0.002$ and $p < 0.02$, respectively).

Number of Mate Attempts

The proportion of males attempting to mate in experiment 2 is shown below in Table 4.3. Mate attempt was not normally distributed. A Fisher exact test revealed that exposure to SE had no significant effects on the mate attempt of males at any week during the exposure period. In order to investigate any dose-response relationship, the overall responses of the groups were averaged over the exposure period (weeks 2-8 of testing) and shown in the table below. Inspection of the mean response indicates there was an inverse dose-response relationship: the proportion of males attempting to mate increases with increasing dose.

Table 4.3 Proportion of males in experiment 2 attempting to mate with females[1]

Treatment/ Exposure Time	Control	50%	100%
1	9/15 **(60)**	8/12 **(67)**	9/16 **(56)**
2	8/15 **(53)**	8/12 **(67)**	11/16 **(69)**
4	2/8 **(25)**	5/12 **(42)**	3/12 **(25)**
6	3/7 **(43)**	2/11 **(18)**	3/10 **(30)**
8	0/5 **(0)**	1/8 **(13)**	2/5 **(40)**
Mean Response (Week 2-8)	**(30)**	**(35)**	**(41)**

[1] Percentages are shown in brackets.

Experiment 3 (repeat of experiment 1, conducted the following year)

Approach

The data on approaches measured when the fish were tested before exposure was initially tested for any differences between treatment groups (to ensure each group was equivalent). One-way ANOVA revealed no differences in the approach of the four groups prior to treatment ($F_{(3, 42)} = 0.32, p = 0.87$).

The mean number of approaches observed over the 8-week exposure period in experiment 3 is shown in Figure 4.3a. Two-way ANOVA of approach (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 24)} = 4.87, p < 0.009$), but no significant test effects ($F_{(3, 72)} = 9.4, p = 0.4$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in approach in males exposed to 100% effluent compared to the controls ($p < 0.007$).

In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing) and are plotted in relation to exposure dose in Figure 4.4 on page 125. There was a clear dose-response effect: increasing exposure to the sewage effluent was associated with decreased approach behaviour (Spearman rank r = -1.0, $n = 4, p < 0.05$).

Duration in Female zone

Two-way ANOVA of duration in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($F_{(3, 24)} = 16.68, p < 0.001$), but no significant test effects ($F_{(3, 72)} = 0.36, p = 0.78$). *Post hoc* analysis (Tukey) of the treatment effects revealed a significant decrease in duration in female zone in males exposed to 50% and 100% sewage effluent compared to the controls ($p < 0.001$ and $p < 0.001$, respectively) and the lowest concentration of 25% effluent ($p < 0.04$ and $p < 0.003$, respectively)

In order to investigate any dose-response relationship, the overall responses of the groups were averaged over the exposure periods (weeks 2-8 of testing). There was a clear dose-

response effect: increasing exposure to the sewage effluent was associated with decreased duration in female zone (Spearman rank r = -1.0, n = 4, *p* < 0.05).
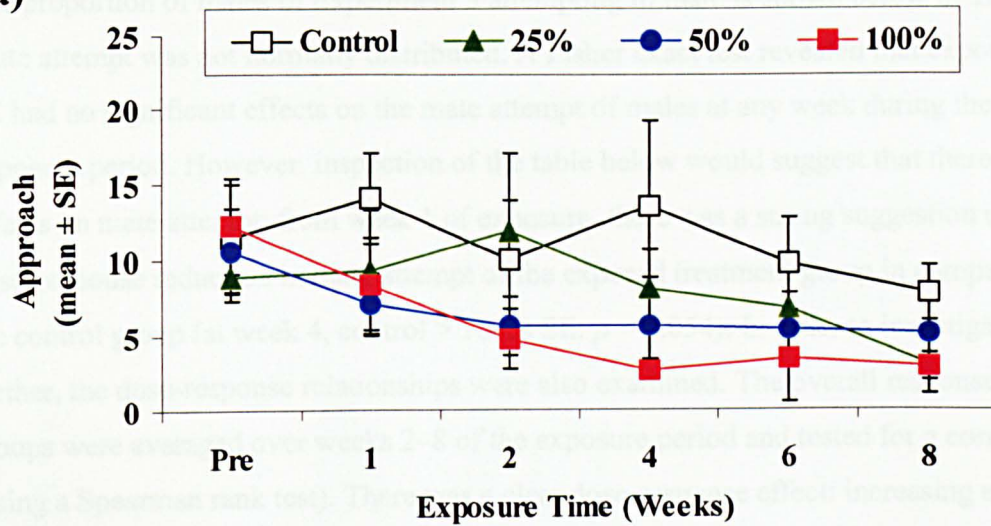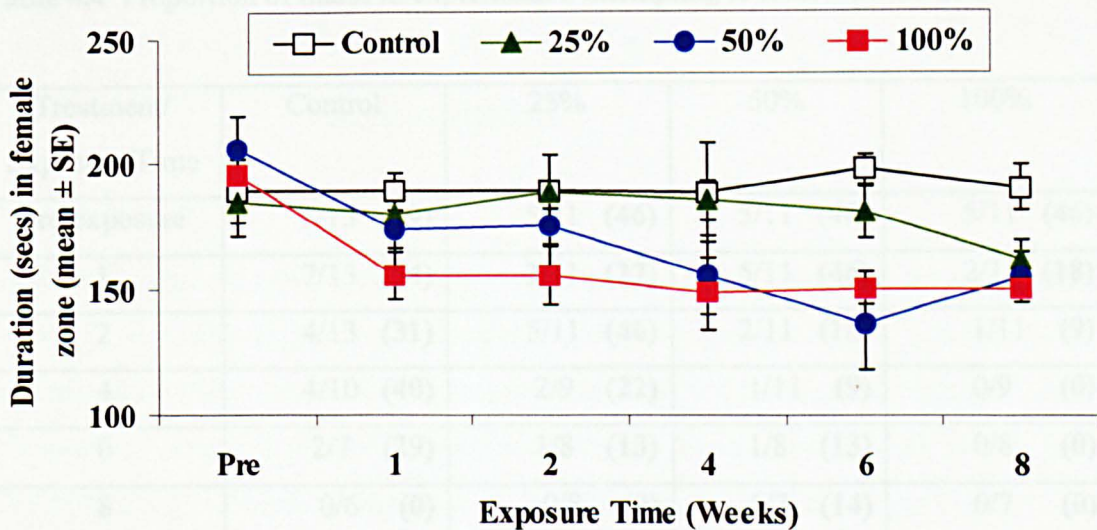
Figure 4.3 Experiment 3: mean (± standard error) (a) approach and (b) duration in female zone of male *G. holbrooki* exposed to three concentrations of sewage effluent (SE) over 8-weeks. Exposure using fish from a quite polluted site, using SE collected from St Marys STP in 2000. Two-way ANOVA of approach and duration in female zone (repeated measures of weeks 2 - 8) revealed significant treatment effects ($p < 0.04$ and $p < 0.04$ respectively). Tukey analysis of approach revealed: control > 100% SE. Tukey analysis of duration in female zone revealed: control and 25% SE > 50% and 100% SE.

Number of Mate Attempts

The proportion of males in Experiment 3 attempting to mate is shown below in Table 4.4. Mate attempt was not normally distributed. A Fisher exact test revealed that exposure to SE had no significant effects on the mate attempt of males at any week during the exposure period. However, inspection of the table below would suggest that there were effects on mate attempt; from week 1 of exposure, there was a strong suggestion of a dose-response reduction in mate attempt of the exposed treatment group in comparison to the control group (at week 4, control > 100% SE: $p = 0.054$). In order to investigate this further, the dose-response relationships were also examined. The overall responses of the groups were averaged over weeks 2–8 of the exposure period and tested for a correlation (using a Spearman rank test). There was a clear dose-response effect: increasing exposure to SE was associated with decreasing mate attempts ($r = -1.0$, $n = 4$, $p < 0.05$).

Table 4.4 Proportion of males in experiment 3 attempting to mate with females[1]

Treatment/ Exposure Time	Control	25%	50%	100%
Pre-exposure	5/13 **(39)**	5/11 **(46)**	5/11 **(46)**	5/11 **(46)**
1	7/13 **(54)**	3/11 **(27)**	5/11 **(46)**	2/11 **(18)**
2	4/13 **(31)**	5/11 **(46)**	2/11 **(18)**	1/11 **(9)**
4	4/10 **(40)**	2/9 **(22)**	1/11 **(9)**	0/9 **(0)**
6	2/7 **(29)**	1/8 **(13)**	1/8 **(13)**	0/8 **(0)**
8	0/6 **(0)**	0/8 **(0)**	1/7 **(14)**	0/7 **(0)**
Mean Response (Week 2-8)	**(25)**	**(20)**	**(14)**	**(2)**

[1] Percentages are shown in brackets.

Figure 4.4 Mean of mean approach (averaged across week 2-8 for each treatment group) of male *G. holbrooki* exposed to three concentrations of sewage effluent. Experiment 1: exposure using fish collected from a polluted site in 1999. Experiment 2: exposure using fish collected from an (apparently) pristine river using the same batch of SE as used in (1). Experiment 3: Exposure using fish collected from same polluted site as (1), but conducted the following year. There was a clear dose-response effect in all experiments: increasing exposure to the sewage effluent is associated with decreasing approach behaviour (Spearman rank $r = -1.0$, $p < 0.05$).

4.3.3 Morphology

As detailed in chapter 3 (p.68), to test for normality, the control morphological data was pooled between all the experiments in this chapter and Chapter 3 (that used *G. holbrooki*).

Experiment 1

Data on the morphological measurements taken after the 10-week exposure of *G. holbrooki* to sewage effluent in 1999 are shown below in Table 4.5.

There were no significant effects on any of the morphological measurements taken (GPL: ANCOVA ($p = 0.7$); TA: ANCOVA ($p = 0.51$); BCI: One-Way ANOVA ($F_{(2, 20)} = 0.42$, $p = 0.68$; and SPZ Count: Kruskal Wallis ANOVA ($H_{(2, n=24)} = 0.74$, $p = 0.63$).

Table 4.5 Morphological measurements of male *G. holbrooki* in experiment 1 exposed to sewage effluent for 10-weeks[1]

Treatment	n	[a]GPL	[a]TA	BCI	SPZ Count
Control	7	6.35 ± 0.095	3.14 ± 0.27	0.069 ± 0.005	300
50%	9	6.29 ± 0.083	3.16 ± 0.24	0.073 ± 0.002	200
100%	8	6.25 ± 0.085	2.81 ± 0.24	0.072 ± 0.004	400

[1] Table entries represent mean or [a] adjusted mean (± standard error) for normally distributed data or median values for non-normal data. No significant treatment effects were found.

Experiment 2

The morphological measurements taken after the 10-week exposure of *G. holbrooki* (sampled from a pristine river) to sewage effluent in 1999 are shown below in Table 4.6. The sample size was too small for comparisons between treatments to be made.

Table 4.6 Morphological measurements of male *G. holbrooki* in experiment 2 exposed to sewage effluent for 10-weeks[1]

Treatment	*n*	[a]GPL	[a]TA	BCI	SPZ Count
Control	5	0.281 ± 0.007	0.158 ± 0.013	0.0732 ± 0.0018	400
50%	2	0.302 ± 0.007	0.164 ± 0.009	0.0635 ± 0.0031	225
100%	2	0.315 ± 0.014	0.191 ± 0.055	0.0766 ± 0.0031	500

[1] Table entries represent mean or [a]adjusted mean (± standard error) for normally distributed data or median values for non-normal data.

Experiment 3

The morphological measurements taken after the 8-week exposure of *G. holbrooki* to sewage effluent in 2000 are shown below in Table 4.7. There were no significant effects on any of the morphological measurements taken (ANCOVA: GPL ($p = 0.49$); TA: ($p = 0.29$); BCI: One-Way ANOVA ($F_{(3, 24)} = 0.89$, $p = 0.46$; and SPZ Count: Kruskal Wallis ANOVA ($H_{(3, n=28)} = 0.99$, $p = 0.87$).

Table 4.7 Morphological measurements of male *G. holbrooki* in experiment 3 exposed to sewage effluent for 8-weeks[1]

Treatment	n	[a]GPL (adjusted mean ± SE)	[a]TA (adjusted mean ± SE)	BCI (mean ± SE)	SPZ Count (median)
Control	6	6.54 ± 0.11	0.131 ± 0.014	0.0761 ± 0.0035	600
25%	8	6.35 ± 0.09	0.145 ± 0.014	0.0890 ± 0.0140	600
50%	7	6.52 ± 0.10	0.158 ± 0.016	0.0706 ± 0.0029	600
100%	7	6.42 ± 0.10	0.172 ± 0.015	0.0750 ± 0.0030	600

[1] Table entries represent mean or [a]adjusted mean (± standard error) for normally distributed data or median values for non-normal data. No significant treatment effects were found.

4.4 SUMMARY

<u>*Mortality*</u>

- Mortality was high in all control groups (54 – 67%).
- Mortality was not related to SE exposure.

<u>*Reproductive Behaviour*</u>

Experiment 1:

- Significant reduction in approach revealed using repeated measures test and a significant dose-dependent reduction found (when data was pooled).
- No significant effects on duration in female zone.
- At week 4, the proportion of males exposed to 50% SE attempting to mate was significantly greater than the control group. No significant dose-response relationship was found when the data was pooled.

Experiment 2:

- No significant effects on approach behaviour revealed using a repeated measures test, but a significant dose-dependent reduction observed when data was pooled.
- Significant reduction in duration in female zone revealed using a repeated measures test, but no significant dose-response relationship found when data was pooled.
- No significant effects on mate attempt.

Experiment 3:

- Significant reduction in approach revealed using repeated measures test, and a significant dose-dependent reduction was found when the data was pooled.

- Significant reduction in duration in female zone revealed on a test-to-test basis, and a significant dose-dependent reduction found when data was pooled.

- No significant effects on mate attempt on a test-to-test basis, but significant dose-dependent reduction found when data was pooled.

Morphology

- No significant effects on any of the morphological measurements.

Table 4.8 Summary of behavioural responses of _G. holbrooki_ exposed to sewage effluent (relative to unexposed controls)

		Experiment 1	Experiment 2	Experiment 3
Mean approach	Repeated measures test	↓	NS	↓
	Dose-response relationship: data pooled over tests	↓	↓	↓
Mean duration in female zone	Repeated measures test	NS	↓	↓
	Dose-response relationship: data pooled over tests	NS	NS	↓
Proportion attempting to mate	Individual test analysis	↑ (Week 4)	NS	NS
	Dose-response relationship: data pooled over tests	NS	↓	↓

↓ Indicates a statistically significant reduction

↑ Indicates a statistically significant increase

NS Indicates no statistically significant difference

4.5 DISCUSSION

The overall findings revealed a consistent reduction in reproductive behaviour in sewage effluent exposed males. Although approach behaviour was significantly reduced in males exposed to SE in experiment 1, carried out in 1999, the duration in female zone was not different between treatments. The results of the follow-up experiment in 2000 revealed a more marked reduction in both approach and duration in female zone in exposed males.

One reason for more marked effects between years could be a difference in the nature of the substances in the sewage effluent. There was no difference between years in processing of the effluent or consents to discharge, although daily discrepancies within the STP processing could be responsible for contamination (e.g. insufficient processing time, a late cleavage of ethoxylate units which will not initiate degradation of APEOs). Collection and storage of the sewage effluent did not vary between the two years; it was refridgerated at all times except during transportation (which could potentially lead to toxicity binding inactivation). A comparison of reproductive behaviour levels between mosquitofish populations begins to address the question of sensitivity to sewage effluent within species.

Contrary to expectation, fish sampled from the 'pristine' river demonstrated little effect in reproductive behaviour compared to fish sampled from the urban 'polluted' river. The differences in reproductive behaviour between years and between sites suggest that variations in population-susceptibility exist. However, other studies demonstrate apparent pollutant tolerance, for example it is well accepted that fish can become tolerant to elevated concentrations of heavy metals by, for example, detoxification of metals by binding to induced metallothionein, decreased uptake of water, and induced excretion of metals (e.g., Hodson, 1988 and references therein). In addition, a number of authors (Prince and Cooper, 1995; Armknecht *et al.*, 1998) have shown that fish living in environments contaminated by PAHs and PCBs frequently have elevated or altered levels of the phase I (cytochrome P450 monooxygenases) or phase II (conjugating enzymes)

enzymes which can aid in the detoxification and excretion of lipophilic chemicals (Goksoyer and Forlin, 1992). If the phase I and/or phase II enzyme systems were induced in *G. holbrooki* collected from the polluted site, higher biotransformation and excretion rates of the contaminants might result in less of the compound being available for endocrine disruption. If this phenomenon applied to the current study, one would expect males from the 'polluted' river to have a higher tolerance to pollutants than fish collected from the 'pristine' river (or that fish from the 'pristine' river are more sensitive to pollution than fish from the 'polluted' river).

However, further analysis of the 'pristine' river data suggested that approach and duration in female zone of control fish were significantly greater than 100% effluent exposed males in the first 2 weeks of exposure. As reproductive behaviour was not recorded pre-exposure, it is likely that differences between treatment groups existed before exposure began, and therefore it is difficult to make conclusions on effects without further experimental work.

An important aspect of any behavioural study is to compare the results with those of previous studies. Unfortunately only one study (to the authors knowledge) has examined the reproductive behaviour of fish exposed to sewage effluent in the laboratory. Schoenfuss *et al.* (2002) conducted two 10-week laboratory exposure experiments exposing male goldfish (*Carassius auratus*) to 100% treated sewage effluent. They conducted two trials, one in the winter and one in the summer (when the effluent was chlorinated). Five- and 10-weeks after the exposure they measured "nudging behaviour", spawning frequency, sperm motility volume, and sperm quality. They demonstrated no seasonal (and effluent quality) difference in spawning behaviour: exposure to chlorinated and non-chlorinated sewage effluent induced small, but not significant, decreases in the frequency of male spawning behaviour.

The results of the present study demonstrated clear reductions in reproductive behaviour of male mosquitofish exposed to sewage effluent. One hypothesis to explain the

reductions in reproductive behaviour is that REDs, such as oestrogens and oestrogen mimics, may have induced changes in the hormonal status of the males; they may have directly impaired behaviour through, for example, binding at specific hypothalamus sites. Although the effluents utilised in this study were not chemically analysed for REDs, there is much evidence that sewage effluents contain such compounds, including EMs (for a review of references refer to section 1.2.7). In addition, the behavioural characteristics described here are consistent with the observations of laboratory induced endocrine disruption described in chapter 3. However, a fundamental question of this study is whether the observed behavioural changes relate to an endocrine disruption effect (i.e., a result of the action of the exposures on the oestrogen receptors), or whether the observed changes were as a result of other toxic effects (i.e., a non-specific toxic effect). The likelihood of this effluent containing oestrogenic chemicals is very high, but treated sewage effluent also contains a range of other contaminants, so a non-specific toxic response cannot be excluded.

The treated sewage effluent used in the current study was chlorinated. Many studies have shown that different disinfectants, including chlorine, can react with natural organic substances (humic and fulvic acids) present in surface waters to give rise to numerous by-products with mutagenic and/or carcinogenic activity (Dolara *et al.*, 1981; Gauthier *et al.*, 1989; Blatchley *et al.*, 1997; and Monarca *et al.*, 2000). Some of these by-products are known to pose potential health risks while the residual chlorine in the effluent may be acutely lethal to fish and other aquatic life (McHugh Law *et al.*, 1998). In most previous studies, it has been difficult to determine whether residual chlorine or other substances contributed to observed effects on aquatic life (Rutherford *et al.*, 1993 and Schoenfuss *et al.*, 2002). Rutherford *et al.* (1993) encountered this problem when assessing the impact of chlorinated effluent discharges on an aquatic ecosystem. Total residual chlorine (TRC) was responsible for some, but not all of the observed effluent toxicity as indicated by the toxicity tests run on dechlorinated samples. Marine bivalve larvae were found to be particularly sensitive, the alga *Selenastrum capricornutam* was found to be not sensitive, in fact the effluent samples were stimulatory to the growth of the alga, and changes in

macroinvertebrate community structure were observed, but it was not possible to determine whether residual chlorine or other substances contributed to observed effects.

One indication of a general non-specific toxic response would be reduced food consumption. Physiological or behavioural stress can result from a reduction in food consumption or food assimilation (Lett *et al.*, 1976), and from increased metabolic costs associated with detoxification and homeostasis during chronic, sublethal exposures (Dixon and Sprague, 1981). However, although not systematically measured, no apparent reduction in feeding activity in any of the treatments was observed in this study. One published study on ammonia toxicity in rainbow trout, (*Oncorhynchus mykiss*) indicates that elevated ambient ammonia reduces growth rate because of reduced food consumption (Wicks and Randall, 2002). If the fish exposed to the effluent were feeding at a reduced rate, then a reduction in body condition (BCI) might be expected. However, this was not the case in any of the laboratory exposures. There was no consistent change in BCI of males exposed to any of the treatments in any of the experiments in this chapter. In addition, the general appearance of the fish swimming in the tank did not appear to be any different between treatment groups.

Although toxic effects are not always lethal, the mortality rate in all the experiments in this chapter was high, but this was not related to the treatment. One explanation for the high mortality rate in all treatment groups could be due to increased aggression between males in the exposure tanks. The density of males in the treatment tanks could influence the behavioural responses observed. In a study carried out by Farr and Herrnkind (1974), the courtship activity of the guppy, *Poecilia reticulata*, was recorded as a function of population density. They demonstrated an increase in aggression with increasing density, and in ten pair (female and male) populations, there was a significant positive correlation between amount of aggression initiated and amount of courtship activity of specific individuals. They concluded that this was perhaps due to changes in social organisation (e.g., a breakdown in hierarchy) or reflected other changes in motivational state of the individual males under the stress of increased density. These same reasons will apply to

the present study; the high density of males in the tanks may have led to increased aggression (which went unnoticed) and this may explain the high mortality rate of males in all treatment groups in chapter 3 and 4.

There were no treatment effects of sewage effluent exposure on reproductive morphology of the fish. This lack of effect was consistent with the results described in chapter 3, which found no significant effect of exposure to oestrogenic chemicals on reproductive morphology. This may be due to insensitivity to the compounds in sewage effluent (i.e. they are not compounds which have any effect on morphology of this species), or they may affect development, but exposure in these adult fish was outwith a responsive period (i.e. a critical period). This discussion will be developed in greater detail in chapter 6.

In conclusion, exposure to high concentrations of sewage effluent reduces the reproductive behaviour of adult males, but appears to have no effect on reproductive morphological characteristics. From the data presented, a dilution of 50% effluent (which is the permitted quantity in the rivers receiving effluent at the STP studied) may reduce the reproductive behaviour of mosquitofish, and could be potentially much more significant when exposure occurs throughout the lifetime of the fish. The impact of lifetime exposure to sewage effluent is investigated in the next chapter.

CHAPTER 5

An Investigation of Reproductive Behaviour and Morphology of *Gambusia holbrooki* Inhabiting Sewage-Contaminated Rivers in NSW, Australia

5.1 INTRODUCTION

Very little research has been conducted on wild fish populations inhabiting rivers receiving treated sewage effluent. A stimulus for this study was the finding of Batty and Lim (1999) that male mosquitofish, *Gambusia holbrooki*, inhabiting some sites downstream of a sewage effluent outfall had reduced gonopodium (GP) lengths compared to males living upstream and at control sites. It was suggested that exposure to endocrine disrupters in the STP effluent may have inhibited the androgen-dependent development of the GP.

The rationale of previous chapters was to establish whether *Gambusia* sp. are responsive to endocrine disruption by assessing the reproductive behaviour and morphology of adults exposed to oestrogenic chemicals and sewage effluent in the laboratory. In chapter 3 it was established that prolonged exposure to low levels of oestrogenic chemicals reduce reproductive behaviour in adult male mosquitofish, *Gambusia* sp. Further, chapter 4 clearly demonstrated that exposure to high concentrations of treated sewage effluent, also reduced aspects of reproductive behaviour in the adult male. In these previous experiments there were no effects of exposure on the reproductive morphology of the adult male fish, such as that demonstrated in the study by Batty and Lim (1999). The objectives of this part of the study were to determine if there was any evidence of reproductive behavioural and morphological disruption in wild populations of male *G. holbrooki* living below two major inland STPs in NSW, Australia. Knowledge of the behavioural responses established in laboratory exposures in chapter 3 and 4 may provide information that can be used when interpreting results of field studies. This should enable a 'weight of evidence' approach to be used to evaluate whether endocrine disruption is occurring and to determine its significance with respect to reproductive fitness.

Two STPs situated in the Western Suburbs of Sydney, NSW, have been utilised for this study. The first half of this chapter will evaluate fish sampled up- and downstream of St Marys STP, and the second half will evaluate fish sampled around Quakers Hill STP. Both STPs are similar in terms of size and output (detailed in section 2.2).

5.2 Reproductive behaviour and morphology of male *Gambusia holbrooki* inhabiting sewage-contaminated waters downstream of St Marys sewage treatment plant, NSW, Australia

5.2.1 Introduction

The objective of this study was to investigate a population of male mosquitofish, *G. holbrooki*, living below St. Marys STP. This population was chosen because a previous study had demonstrated reduced gonopodial lengths of this species living below this sewage outfall (Batty and Lim, 1999). Specifically, a comparison of male *G. holbrooki* from a population 5km and 10km downstream of the sewage outfall was made with fish from an upstream population (where exposure was expected to be minimal). The reproductive behaviour, length of the gonopodium, testes weights and sizes, quantity of sperm packets, and body condition were examined and compared.

5.2.2 Materials and Methods

St Marys STP is the largest inland processing plant in NSW. For a detailed description of the study sites and STP refer to section 2.2.

Reproductively mature male *G. holbrooki* were sampled (over several days) during the summer months of 1999, 2000, and 2002 up- and downstream from St Marys STP. If a wet weather event occurred within 24 h before sampling, sampling was rescheduled to ensure that samples were representative of dry weather operating conditions. Due to difficulty in catching fish downstream in 2000, a different downstream site was sampled in 2002; the downstream site sampled in 1999 and 2000 was 10km from the effluent outfall and in 2002; the downstream site was 5km from the outfall. The housing and

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maintenance of fish, as well as a detailed description of study sites, were as described in the general materials and methods, section 2.1.2.

Upon arrival at the UTS laboratory in 1999, the fish were sexed and housed in glass aquaria containing separate male and female groups. The behaviour of males sampled in 1999 was recorded once after 24hrs acclimatisation in the laboratory. The procedure for housing and behavioural testing was improved in 2000 and 2002 so that a much better measure of behaviour was achieved for each individual; upon arrival at the laboratory, males were separated into individual 500ml beakers holding aged water and the behaviour of each individual was recorded every 24, 36, and 72hrs after they were caught. Standard morphological measures, as described in section 2.6, were then recorded. Behavioural and morphological measurement protocols were as described in the general materials and methods (section 2.5 and 2.6). For fish sampled in 2000 and 2002, additional measures of testis weight and body weights were recorded.

Data Analysis

From the laboratory studies in chapter 3 and 4, it was established that many of the behavioural and morphological parameters measured were correlated with body size. For example, the approach behaviour increases with increase in body length (BL) in males up to 25.9mm, but approach then decreases in males greater than 26mm BL (Fig. 3.2 on page 69). Therefore, for comparison of behavioural characteristics the male fish were divided into four size classes: 20-22.9mm, 23-25.9 mm and 26-28.9 mm and >31mm BL.

Some difficulties were encountered in the data analysis of this chapter because of the small number of fish caught at some sites; this meant there was a large variability in body size distribution between sites and years (which will in turn affect the distribution of some of the measured parameters). For these reasons, it was not appropriate to pool data between sites and years and unfortunately it was only possible to measure a small number of fish in some of the data presented in this chapter. This created additional problems when testing for normality, therefore in some cases, data was assumed to be normal as

was seen in previous data sets (with the exception of some cases where non-parametric techniques have been used). Unless stated otherwise, measurements of both up- and downstream males were normally distributed.

5.2.3 Results

Sample Year: 1999 (downstream site 10km from outfall)

The effort required to catch fish at the downstream site was considerably greater than at the upstream site; it took approximately 2hrs to collect a total of 41 males upstream, whereas it took slightly over 2hrs to collect a total of only 18 males from the downstream site. Three males from the downstream site died in transit, thus only some morphological measurements were made for these individuals.

Total Body Length

The body size distribution of males caught up- and downstream is shown in Table 5.1. The mean BL of males sampled in the river below the STP was 24.05 (± 0.38) and the mean BL of the fish sampled upstream was 23.93 (± 0.24).

Table 5.1 Size distribution of males sampled up- and downstream of St Marys STP in 1999

Body Length (mm)	Upstream	Downstream
21	2	0
22	4	3
23	10	4
24	13	6
25	5	1
26	5	3
27	1	0
28	1	1
n-value	41	18

Reproductive Behaviour

Only males ranging from 23 - 25.9mm were used for data analysis of the 1999 sample (because there were too few downstream fish to separate the data into all the size categories). The reproductive behaviour was measured once, 24hrs after they were caught.

Approach

The approach of upstream and downstream males ranging from 23 – 25.9mm BL sampled in 1999 was normally distributed (upstream: K-S d = 0.17, n = 28, p > 0.15, mean = 11.7, median = 8.0). However, the mean and median approach varied quite considerably, which suggested that the distribution was not normal (which is in contrast to previous data in chapter 3 and 4, which established that approach was normally distributed). Therefore, parametric and nonparametric analyses were conducted. The results of both parametric and non-parametric analysis revealed no significant difference in approach between up and downstream fish (U = 78, p = 0.087 and t = -1.66 (df = 35), p = 0.18). To be consistent with other data presentation, the mean approaches observed are shown in Figure 5.1a.

Duration in female zone

A two-tailed t-test revealed no significant differences between sites (t = -0.4 (df = 35), p = 0.68). The average duration in female zone observed for these males are shown in Figure 5.1b.

(a)

(b)

Figure 5.1 Mean (± standard error) (a) approach and (b) duration (secs) in female zone of male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 1999. The downstream site is approximately 10km from the sewage outfall. Males were tested once 24hrs after capture. Males of a body length range of 23 - 25.9mm were pooled. No significant differences were found between fish from up- and downstream sites.

<u>Mate attempt</u>

The number of mate attempts of upstream males ranging from 23 – 25.9mm BL sampled in 1999 was not normally distributed (K-S d = 0.52, n = 28, p < 0.01). The proportion of males attempting to mate is shown below in Table 5.2. A Fisher exact test revealed no significant differences in fish between sites.

Table 5.2 Proportion of males sampled up- and downstream of St Marys STP in 1999 attempting to mate with females[1]

Size Category	Upstream	Downstream
23 – 25.9mm	3/28 **(11)**	3/9 **(33)**

[1] Percentages are shown in brackets.

The approach was positively correlated with duration in female zone and mate attempt (r = 0.5, p < 0.005 and r = 0.48, p < 0.005, respectively), but duration time and mate attempt were not correlated (r = 0.29, p = 0.089), i.e., approach is representative of sexual motivation but the impetus behind duration in female zone is less clear.

Sample Year: 2000

(Note: same up- and downstream sites as previous sample date)

The effort required to catch fish at the downstream site was considerably greater than at the upstream site (and the previous year); it took approximately 3 hrs to catch a total of 6 males downstream, whereas it took less than 1 hr to catch 15 males at the upstream site.

Total Body Length

The body size distribution of males caught is shown in Table 5.3. Previous data revealed BL was normally distributed (the small sample size of this years data was too small for normality tests). The mean BL of males sampled in the river below the STP was 25.29 ($\pm$ 1.2) and the mean BL of the fish sampled upstream was 28.15 ($\pm$ 0.6).

Table 5.3 Size distribution of males caught up- and downstream of St Marys STP in 2000

Body Length (mm)	Upstream	Downstream
22	0	2
23	0	0
24	0	1
25	1	2
26	5	0
27	3	0
28	2	0
29	1	0
30	0	1
31	1	0
32	1	0
33	1	0
n-value	15	6

Since BL influenced behaviour (see p.69), the data was separated into body size categories for statistical analysis. However, due to the small number of fish caught at the downstream site (and the lack of large males), the most appropriate course of comparison

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was to match BL between sites. Large males (≥ 28mm) were taken away from the upstream group so that the mean was similar between sites; the mean BL of upstream males was 26.52 (± 0.3) and the mean of downstream males was 25.29 (± 1.2).

Reproductive Behaviour

The protocol was modified for this experiment so that, upon arrival to the laboratory, males were separated into beakers and behaviour recorded 24, 48 and 72 h after.

The very small sample size meant that it was difficult to test for normality. As there was a clear doubt whether the distribution was normal, parametric and nonparametric analysis was conducted.

Approach

The results of both parametric and non-parametric analysis revealed a significant increase in approach of downstream males compared with upstream fish (parametric two-way ANOVA repreated measures: $F_{(1, 13)} = 16.67$, $p < 0.002$ and Mann-Whitney test: at tests 1: $U = 3.5$, $p < 0.01$ and test 2: $U = 5.0$, $p < 0.01$). To be consistent with other data presentation, the mean approaches observed over the three tests are shown in Figure 5.2a.

Duration in female zone

The results of both parametric and non-parametric analysis revealed no significant differences in duration in female zone between fish from the up- and downstream site. To be consistent with other data presentation, the mean duration times recorded over the three tests are shown in Figure 5.2b.

Mate attempt

No upstream males attempted to mate over the three-day test period (0%). One male of the downstream group attempted to mate once on the second day of testing (17%).

The behaviours observed in 2000 were not correlated with each other, i.e., this suggests approach and duration in female zone were not indicative of sexual motivation.
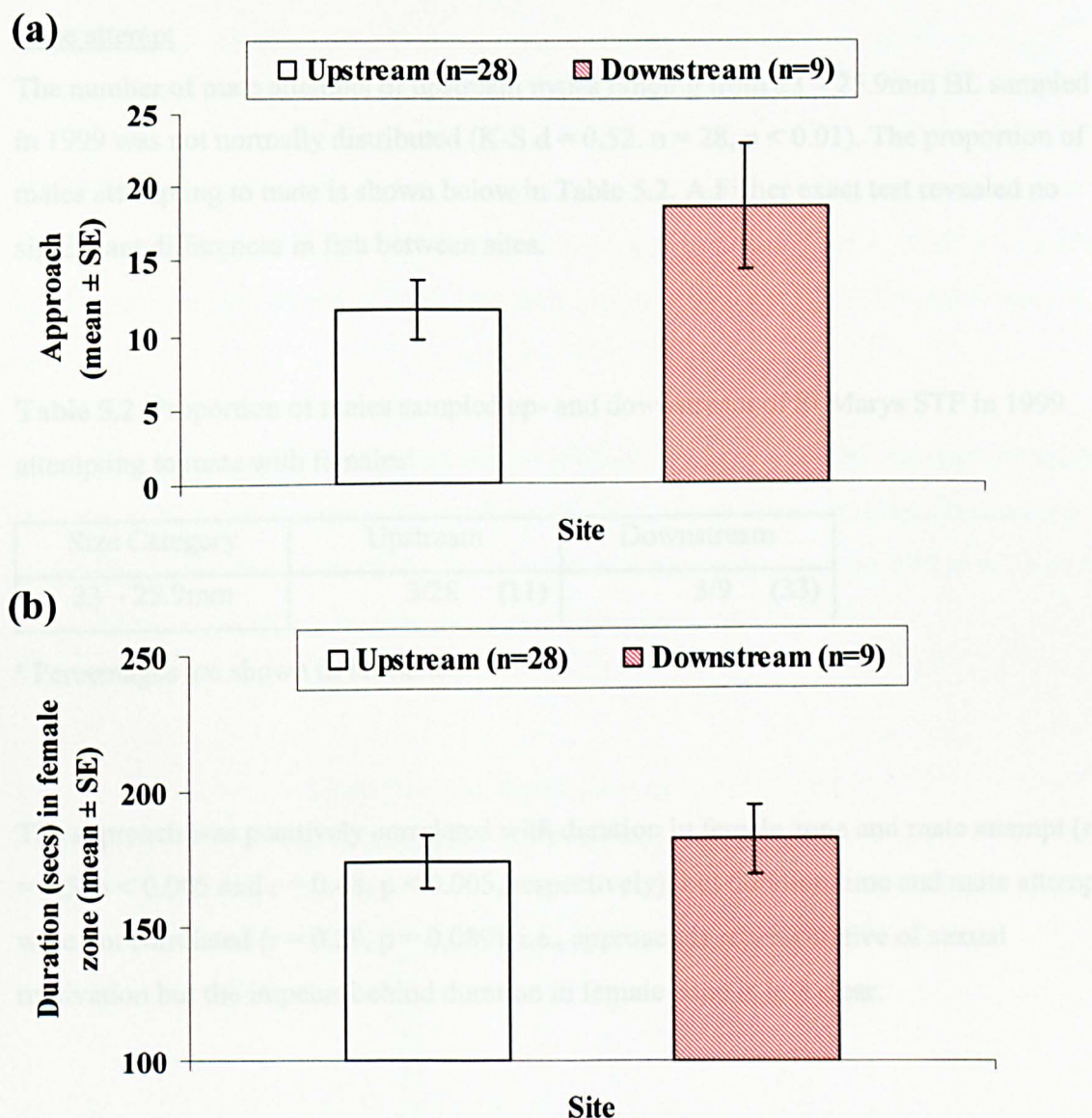
Figure 5.2 Mean (± standard error) (a) approach and (b) duration (secs) in female zone of male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 2000. The downstream site is approximately 10km from the sewage outfall. Males were tested every 24hrs for 3 consecutive days. Data is all males excluding ≥ 28mm BL. Two-way ANOVA repreated measures test on approach data revealed significant differences between fish from the two sites: upstream > downstream (*p* < 0.002).

Sample Year: 2002

(Note: different downstream site from previous samples - 5km from outfall)

The effort required to catch fish at downstream site was considerably greater than at the upstream site; it took approximately 3 hrs to collect a total of 48 males upstream whereas it took approximately 8 hrs to catch 29 males at the downstream site. One fish died in transit, thus only some morphological measurements were taken of it.

Total Body Length

The body size distribution of males caught is shown in Table 5.4. The mean BL of males sampled in the river below the STP was 26.14 ($\pm$ 0.38) and the mean BL of the fish sampled upstream was 25.52 ($\pm$ 0.26).

Table 5.4 Size distribution of males caught up- and downstream of St Marys STP in 2002

Body Length (mm)	Upstream	Downstream
21	3	0
22	2	3
23	6	2
24	4	2
25	11	5
26	15	7
27	6	5
28	0	3
29	0	1
30	0	1
31	1	0
n-value	48	29

Reproductive Behaviour

The data was separated into two size categories, 23 – 25.9mm BL and 26 - 30.9mm BL. The mean approach and duration in female zone recorded over the three tests are presented in Figure 5.4 and 5.5.

Approach

From Figure 5.3a and 5.3b it can clearly be seen there was a consistent reduction in approach of downstream males compared with upstream at all a three behaviour tests, however, significance was not reached (small males: $F_{(1, 27)} = 1.11$, $p = 0.3$, larger males: $F_{(1, 36)} = 0.51$, $p = 0.5$).

Duration in female zone

There were no significant differences in duration in female zone of males between fish from the two sites (small males (Fig 5.4b): $F_{(1, 27)} = 0.1$, $p = 0.75$, and larger males (Fig. 5.4a): $F_{(1, 36)} = 3.47$, $p = 0.07$).
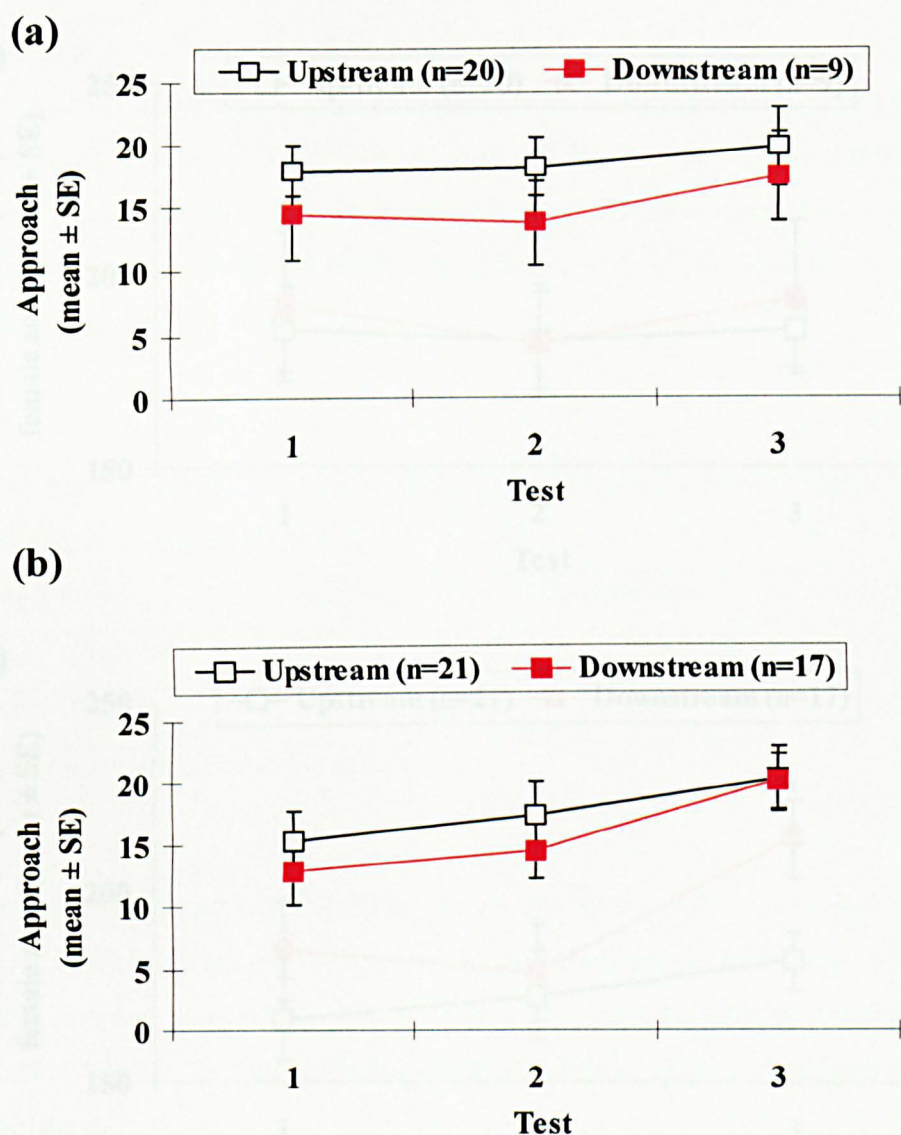
Figure 5.3 Mean (± standard error) approach of a) 23 - 25.9mm and b) 26 - 30.9mm male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 2002. The downstream site is approximately 5km from the sewage outfall. Males were tested every 24hrs for 3 consecutive days. No significant differences in approach were found between sites using a repeated measures ANOVA test.
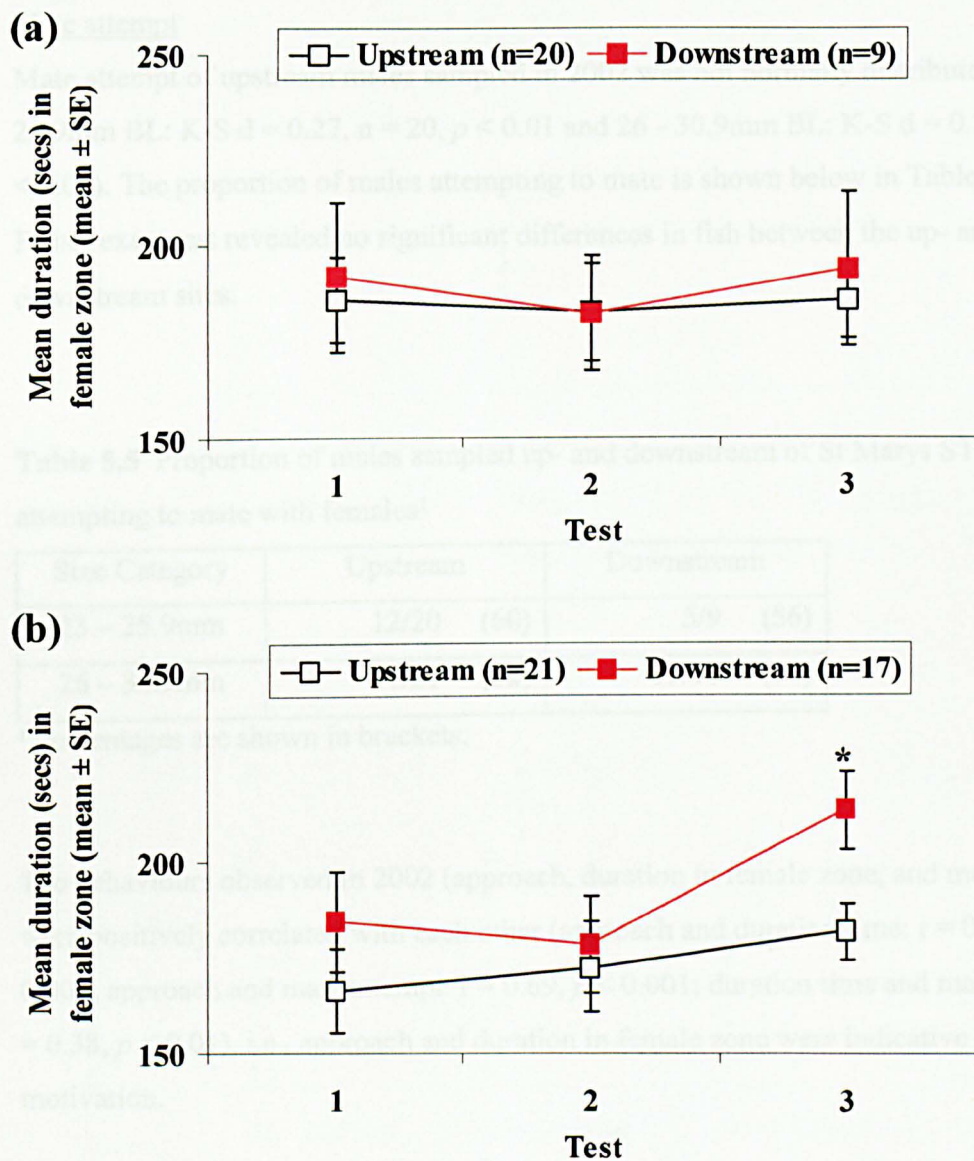
Figure 5.4 Mean (± standard error) duration in female zone of a) 23 - 25.9mm and b) 26 - 30.9mm male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 2002. The downstream site is approximately 5km from the sewage outfall. Males were tested every 24 hrs for 3 consecutive days. No significant differences in duration in female zone were found between sites using a repeated measures ANOVA test.

<u>Mate attempt</u>

Mate attempt of upstream males sampled in 2002 was not normally distributed (23 – 25.9mm BL: K-S d = 0.27, n = 20, p < 0.01 and 26 - 30.9mm BL: K-S d = 0.29, n = 21, p < 0.05). The proportion of males attempting to mate is shown below in Table 5.5. A Fisher exact test revealed no significant differences in fish between the up- and downstream sites.

Table 5.5 Proportion of males sampled up- and downstream of St Marys STP in 2002 attempting to mate with females[1]

Size Category	Upstream		Downstream	
23 – 25.9mm	12/20	**(60)**	5/9	**(56)**
26 – 30.9mm	11/21	**(52)**	13/17	**(76)**

[1] Percentages are shown in brackets.

The behaviours observed in 2002 (approach, duration in female zone, and mate attempt) were positively correlated with each other (approach and duration time: r = 0.55, p < 0.001; approach and mate attempt: r = 0.69, p < 0.001; duration time and mate attempt: r = 0.38, p < 0.01), i.e., approach and duration in female zone were indicative of sexual motivation.

In addition to presenting the mean behaviour of the three behaviour trials (in 2000 and 2002 only), the average for each individual (over the three behaviour trials) was calculated to obtain a more representative measure of the individual's behaviour, this was then averaged for each year and presented as a summary in Figure 5.5. In 2000, there was a significant increase in approach of downstream males compared with upstream fish (t = -4.1 (df = 13), p < 0.005) (Fig. 5.5a). This increase was not reflected in the duration in female zone (Fig. 5.5b). There were no significant differences in approach or duration in female zone between fish from the two sites in 2002.
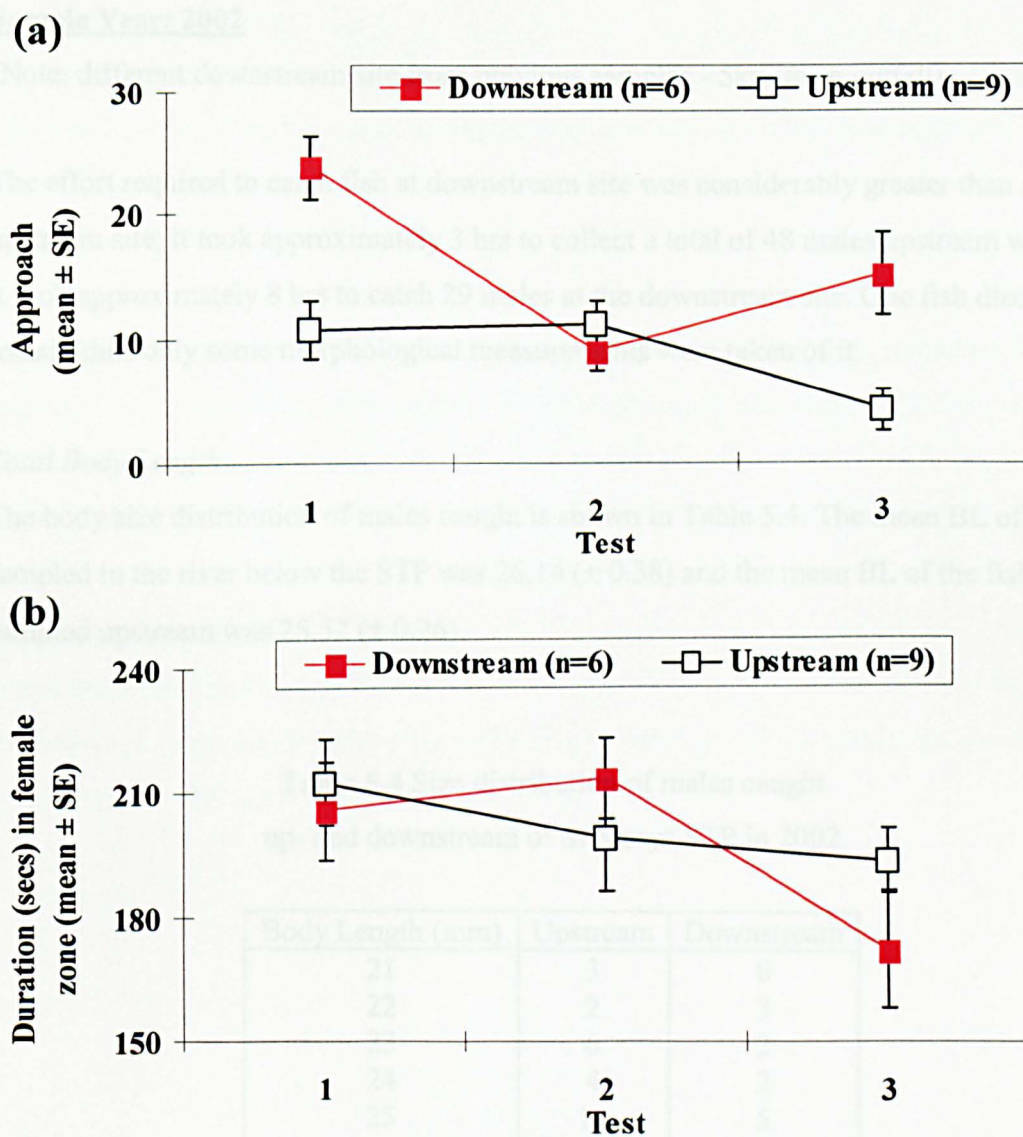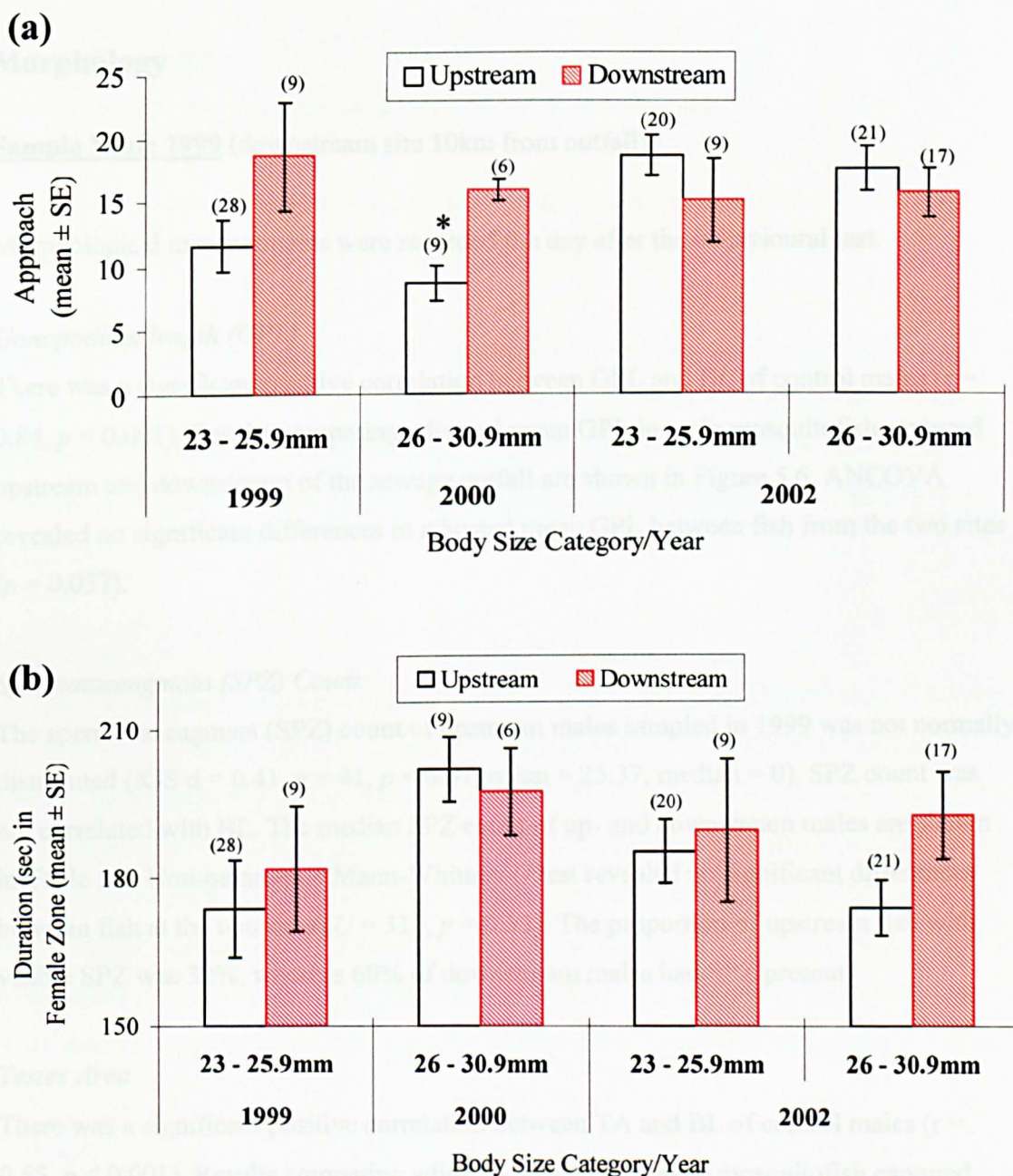
Figure 5.5 Mean (± standard error) (a) approach and (b) duration in female zone of male *Gambusia holbrooki* sampled up- and downstream of St Marys sewage treatment plant in 1999, 2000 and 2002. The downstream site in 1999 and 2000 was approximately 10km from the sewage outfall, and 5km downstream in 2002. Males ranging from 23 – 25.9mm and 26 - 30.9mm BL were analysed separately. Behavioural values for 2002 were averaged for each individual over three tests, and then averaged to get an overall value for the group. Sample size is in brackets. * $p < 0.05$; downstream > upstream, using the student's t-test for two independent samples.

Morphology

Morphological measurements were recorded the day after the behavioural test.

Gonopodium length (GPL)

There was a significant positive correlation between GPL and BL of control males (r = 0.84, $p < 0.001$). Results comparing adjusted mean GPL in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Figure 5.6. ANCOVA revealed no significant differences in adjusted mean GPL between fish from the two sites ($p = 0.057$).

Spermatozeugmata (SPZ) Count

The spermatozeugmata (SPZ) count of upstream males sampled in 1999 was not normally distributed (K-S d = 0.41, n = 41, $p < 0.01$, mean = 25.37, median = 0). SPZ count was not correlated with BL. The median SPZ count of up- and downstream males are shown in Table 5.6. Non-parametric Mann-Whitney U test revealed no significant differences between fish at the two sites ($U = 310$, $p = 0.33$). The proportion of upstream fish with visible SPZ was 32%, whereas 60% of downstream males had SPZ present.

Testes Area

There was a significant positive correlation between TA and BL of control males (r = 0.55, $p < 0.001$). Results comparing adjusted mean TA in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Table 5.6. Results of ANCOVA comparing adjusted mean TA between the up- and downstream males was not significant ($p = 0.9$).

<u>**Sample Year: 2000**</u>

(Note: same up- and downstream sites as previous sample date)

Morphological measurements were recorded on the day of the last behavioural test.

Gonopodium Length (GPL)

There was a significant positive correlation between GPL and BL of control males (r = 0.84, $p < 0.001$). Results comparing adjusted mean GPL in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Figure 5.6. ANCOVA revealed no significant differences in adjusted mean GPL between the two sites ($p = 0.21$).

Body Condition

The BCI of upstream males sampled in 2000 was not normally distributed (K-S d = 0.45, n = 15, $p < 0.01$, mean = 0.089, median = 0.079). However, results of other data in this chapter established BCI was normally distributed. Therefore, parametric and non-parametric analysis was conducted. Both tests revealed different results: a two-tailed t-test revealed no significant differences between up- and downstream fish ($t = -0.45$ (df= 19), $p = 0.66$), but the non-parametric Mann-Whitney U-test revealed a significant increase in BCI of downstream males compared with upstream fish ($U = 12, p = 0.01$). To be consistent with other data presentation, the mean BCI recorded for these males are shown in Table 5.6.

Spermatozeugmata (SPZ) Count

The spermatozeugmata (SPZ) count of upstream and downstream males sampled in 2000 was not normally distributed. SPZ was not correlated with BL. Median SPZ count values for these males are shown in Table 5.6. Non-parametric Mann-Whitney U test revealed no significant differences between fish from the two sites ($U = 32, p = 0.31$). 100% of males sampled both up- and downstream had visible SPZ present.

Testis Area

There was a significant positive correlation between TA and BL of control males (r = 0.68, p < 0.01). Results comparing adjusted mean TA in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Table 5.6. Results of ANCOVA comparing adjusted mean TA between the up- and downstream males was not significant (p = 0.68).

Sample Year: 2002

(Note: different downstream site from previous samples - 5km from outfall)

Morphological measurements were recorded on the day of the last behavioural test.

Gonopodium Length (GPL)

There was a significant positive correlation between GPL and BL of upstream males (r = 0.65, p < 0.001). Results comparing adjusted mean GPL in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Figure 5.6. The adjusted mean GPL was significantly greater in males sampled upstream compared to downstream (p < 0.05).

Body Condition

The mean BCI recorded for these males are shown in Table 5.6. A two-tailed t-test revealed no significant differences between fish from the two sites (t = -1.9 (df = 74), p = 0.064).

Spermatozeugmata (SPZ) Count

The spermatozeugmata (SPZ) count of upstream males sampled in 2002 was not normally distributed (K-S d = 0.25, n = 47, p < 0.01, mean = 219, median = 99). Upstream SPZ was not correlated with BL. Median SPZ count values for these males are shown in Table

5.6. Non-parametric Mann-Whitney U test revealed no significant differences between fish from up- and downstream sites ($U = 499.5$, $p = 0.082$). The proportion of upstream fish that had visible SPZ present was 57%, whereas 79% of downstream males had SPZ present.

Testis Area

The testes area (TA) of upstream males sampled in 2002 was not normally distributed (K-S d = 0.22, n = 47, $p < 0.05$, mean = 5.47, median = 4.55). TA was not correlated with BL (Sp r = -0.13, $p = 0.38$). However, results of other data in this chapter established TA was normally distributed; therefore parametric and non-parametric analysis was conducted. Results of both a non-parametric Mann-Whitney U test and a two-tailed t-test revealed a significant reduction in TA of fish downstream of the effluent outfall compared upstream ($U = 430$, $p < 0.01$ and $t = 2.59$ (df = 75), $p < 0.01$). To be consistent with other data presentation, the mean TA recorded for these males are shown in Table 5.6.

Gonadosomatic Index (GSI)

The testes weight (TW) of upstream males sampled in 2002 was not normally distributed (K-S d = 0.19, n = 47, $p < 0.05$, mean = 0.0027, median = 0.0022). TW was correlated with body weight (Sp r = 0.29, $p < 0.05$); therefore this data was transformed into the gonadosomatic index (GSI), detailed in section 2.6. GSI was not correlated with BL or BW, i.e., GSI was an appropriate index (length and weight factors are removed). The GSI of upstream males sampled in 2002 was not normally distributed (K-S d = 0.22, n = 47, $p < 0.05$, mean = 0.022, median = 0.017). However, results of other data in this chapter established GSI was normally distributed, therefore parametric and non-parametric analysis was conducted. Results of both a non-parametric Mann-Whitney U test and a two-tailed t-test revealed a significant reduction in GSI of fish downstream of the effluent outfall compared to upstream ($U = 443$, $p < 0.02$ and $t = 2.0$ (df = 75), $p = 0.048$). To be consistent with other data presentation, the mean GSI recorded for these males are shown in Figure 5.7.
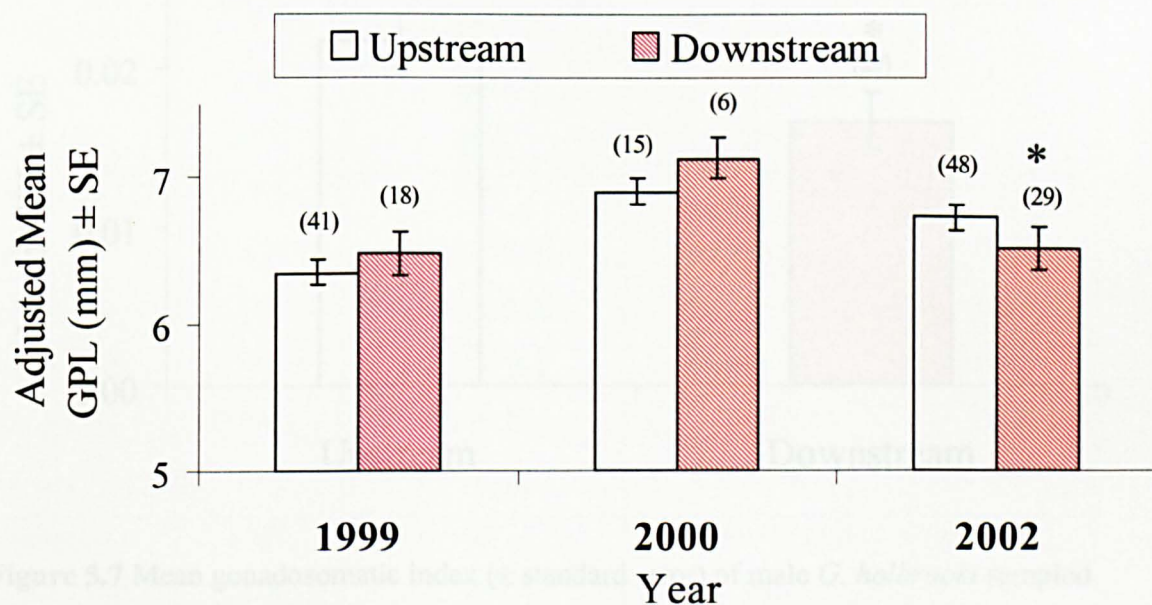
Figure 5.6 Adjusted mean gonopodium length (± standard error) of male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 1999, 2000 (10km downstream) and 2002 (5km downstream). Sample size is in brackets. * $p < 0.05$ Indicates GPL upstream > downstream using the Student's t-test for two independent samples.

Figure 5.7 Mean gonadosomatic index (± standard error) of male *G. holbrooki* sampled up- and downstream of St Marys sewage treatment plant, year 2002. The downstream site is approximately 5km from the sewage outfall. Sample size is in brackets. * *p* < 0.05 Indicates GSI upstream > downstream using the Student's t-test for two independent samples.

Table 5.6 St Marys STP: Other measurements taken of male *G. holbrooki* sampled in 1999, 2000 and 2002 up- and downstream of the sewage effluent outfall[1]

Measurement	Year	Sample Site		*p*-value
		Upstream	Downstream	
Sample Size	1999	(41)	(18)	-
	2000	(15)	(6)	-
	2002	(48)	(29)	-
Mean ± SE BCI	1999	-	-	-
	2000	0.080 ± 0.001	0.096 ± 0.004	0.68
	2002	0.075 ± 0.001	0.077 ± 0.001	0.064
Mean ± SE TA (mm²)	[a]1999	6.17 ± 0.2	6.16 ± 0.3	0.97
	[a]2000	4.26 ± 3.61	3.98 ± 2.81	0.68
	2002	5.51 ± 0.28	4.46 ± 0.24	< 0.01*
Median SPZ Count	1999	0	40 (15)	0.33
	2000	100	450	0.31
	2002	99	225	0.082

[1] The upstream site was 1 km from St Marys effluent outfall. The downstream site sampled in 1999 and 2000 was 10km from the effluent outfall and in 2002; the downstream site was 5km from the outfall. SE = Standard Error. Sample size represented in parentheses. Differences between sites were analysed using the Mann-Whitney *U*-test (for non-normal data) or parametric two-tailed t-test (for normally distributed data). [a] Indicates adjusted means. * Indicates t-test, $p < 0.01$; males living 5 km downstream of the effluent outfall had significantly reduced TA compared to fish sampled upstream.

5.2.4 Summary of results (St Marys STP)

Males sampled 10 km downstream of the sewage effluent outfall of St Marys STP in 1999 showed no evidence of a reduction in the reproductive behaviour in comparison to males sampled upstream. However, males sampled the following year at the same downstream site demonstrated a significant increase in approach at tests 1 and 3 compared to upstream males. This was confirmed when the data of each individual was averaged (over the three behaviour trials) for each year; a significant increase in approach of downstream males compared with upstream fish. This increase in approach was not reflected in the other measures of reproductive behaviour recorded (duration in female zone and mate attempt).

Males sampled 5 km downstream of the sewage effluent outfall of St Marys STP in 2002, showed a significant increase in duration time of larger downstream males compared to the upstream fish at test 3. However, the approach of downstream males was consistently lower (over three tests) than that of upstream males and this was apparent in males of both body size categories. The mate attempt was not different between sites. No significant differences in approach or duration in female zone between fish from the two sites were found when the data of each individual was averaged. Overall, there were no marked effects on reproductive behaviour.

Males sampled 10 km downstream of the sewage effluent outfall of St Marys STP in 1999 showed no significant differences in morphology compared to upstream fish. This was supported by the lack of effects at the same site the following year (2000). This lack of effects between up- and downstream populations was in contrast with the findings of a previous study by Batty and Lim, 1999, which found a decrease in GP length in male mosquitofish living at this site between 1995-1997.

Males sampled 5 km downstream of St Marys STP in 2002, however, demonstrated a significantly reduced GP, TA and GSI compared to upstream males. The BCI and SPZ were not different between up- and downstream sites.

5.3 Reproductive behaviour and morphology of male *Gambusia holbrooki* inhabiting a sewage-contaminated river downstream of Quakers Hill sewage treatment plant, NSW, Australia

5.3.1 Introduction

The objective of this study was to investigate a population of male mosquitofish, *G. holbrooki*, living below Quakers Hill STP, NSW. This population was chosen because the STP discharges into a very small river. Thus, during the dry weather of the summer months (when many fish are maturing and mating), the river has minimal capacity to dilute the effluent. A comparison of male *G. holbrooki* from a population 50m below the sewage effluent outfall was made with fish from an upstream population (where exposure is expected to be minimal) for; reproductive behaviour, length of the gonopodium, testes weights and sizes, quantity of sperm packets, and body condition.

5.3.2 Materials and Methods

Quakers Hill STP is the second largest inland processing plant in NSW. For a detailed description of the study sites and STP refer to section 2.2.

The area surrounding the Quakers Hill STP outfall was investigated for suitable mosquitofish habitats so that samples were sampled in such an area immediately downstream of the outfall. Reproductively mature male *G. holbrooki* were sampled (over several days) during the summer months of 2000 and 2002 up- and downstream from Quakers Hill STP. If a wet weather event occurred within 24 h before sampling, sampling was rescheduled to ensure that samples were representative of dry weather operating conditions. The housing and maintenance of fish, as well as a detailed description of study sites, were as described in the general materials and methods (section 2.1).

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Fish were taken back to the laboratory and separated into individual 500ml beakers holding aged water. The behaviour of each individual was recorded every 24, 36, and 72 h after they were caught. Standard morphological measurements recorded on the day of the last behavioural test. The behavioural and morphological measurement protocols were as described in the general materials and methods (section 2.5 and 2.6).

Data Analysis

From the laboratory studies in chapter 3 and 4, it was established that many of the behavioural and morphological parameters measured were correlated with body size. For example, the approach behaviour increases with increase in body length (BL) in males up to 25.9mm, but approach then decreases in males greater than 26mm BL (p. 69). Therefore, for comparison of behavioural characteristics the male fish were divided into two size classes: 23-25.9 mm and 26-28.9 mm BL.

5.3.3 Results

Sample Year: 2000 (*note*: downstream site 50m from outfall)

The effort required to catch fish at the downstream site was considerably greater than at the upstream site; it took a little over 2hrs to catch a total of 37 males upstream, whereas it took approximately 2hrs to collect 16 males from the downstream site.

Total Body Length

The body size distribution of males caught up- and downstream is shown in Table 5.7. The mean BL of males sampled in the river below the STP was 27.34 (± 0.38) and the mean BL of the fish sampled upstream was 27.76 (± 0.37).

Table 5.7 Size distribution of males caught up- and downstream of Quakers Hill STP in 2000.

Body Length (mm)	Upstream	Downstream
23	1	0
24	4	1
25	4	1
26	6	4
27	5	6
28	4	2
29	7	1
30	5	1
31	0	0
32	1	0
n-value	37	16

Reproductive Behaviour

Only males ranging from 26 - 30.9mm BL were used for data analysis in this year's sample (because there were too few fish to separate the data into all the size categories).

Approach

The mean approaches observed at each behaviour test are shown in Figure 5.8a. The approach was significantly reduced in downstream males compared with upstream fish ($F_{(1, 39)} = 5.52, p < 0.03$).

Duration in female zone

The mean duration in female zone observed at each behaviour test is shown in Figure 5.8b. There were no significant differences in duration in female zone of males between the two sites ($F_{(1, 39)} = 1.03, p = 0.31$).

Mate attempt

One upstream male attempted to mate over the three-day test period (4%). No males of the downstream group attempted to mate.

The approach was positively correlated with duration in female zone and mate attempt (r = 0.45, $p < 0.05$ and r = 0.66, $p < 0.001$, respectively), however, the duration time and mate attempt were not correlated (r = 0.19, $p = 0.38$). This indicates approach and duration in female zone were indicative of sexual motivation.

(a)
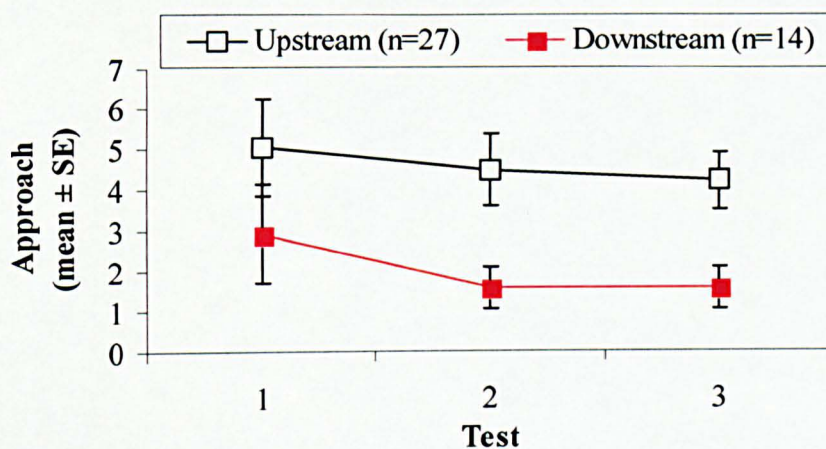
(b)
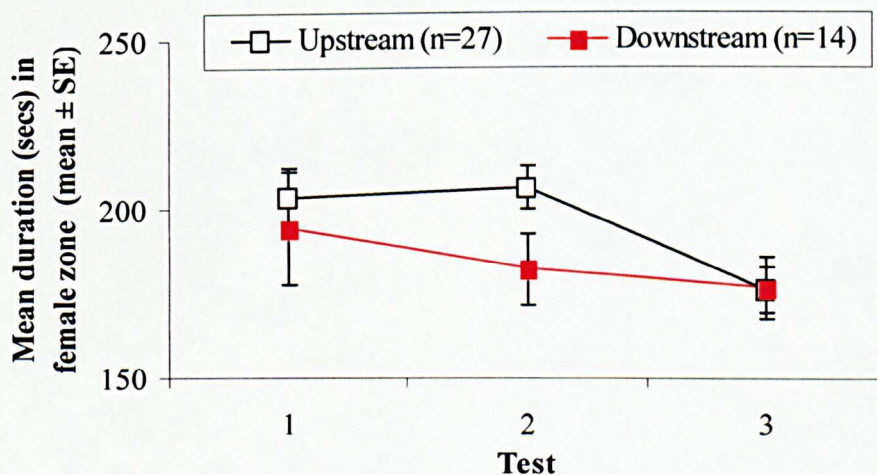
Figure 5.8 Mean (± standard error) (a) approach and (b) duration in female zone of male *Gambusia holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant in 2000. The downstream site was approximately 50m from the sewage outfall. Males of a body length range of 26 - 30.9mm were pooled. Males were tested every 24 hrs for 3 consecutive days. Two-way ANOVA repreated measures test on approach data revealed significant differences between fish from the two sites: upstream > downstream ($p < 0.03$).

Sample Year: 2002

(*note*: same up- and downstream sites as previous sample date)

The effort required to catch fish at the downstream site was greater than that required at the upstream site; it took approximately 2 hrs to catch a total of 36 males upstream, and 6 hrs to collect 36 males at the downstream site.

Total Body Length

The body size distribution of males caught is shown below in Table 5.8. The mean BL of males sampled in the river below the STP was 27.43 ($\pm$ 0.37) and the mean BL of the fish sampled upstream was 26.61 ($\pm$ 0.37).

Table 5.8 Size distribution of males caught up- and downstream of Quakers Hill STP in 2002

Body Length (mm)	Upstream	Downstream
20	1	0
21	0	1
22	1	0
23	2	2
24	4	2
25	4	2
26	5	6
27	12	8
28	3	7
29	2	4
30	2	3
31	0	0
32	0	1
n-value	36	36

Only males ranging from 23 - 25.9mm and 26 - 30.9mm BL were used for data analysis in this sample (because there were too few fish to separate the data into all the size categories).

Approach

Males of the smaller body size category (23 – 25.9mm BL) were compared in their approach behaviour at each behaviour test, shown in Figure 5.9a. The approach was significantly reduced in downstream males compared with upstream fish ($F_{(1, 14)} = 4.27$, $p < 0.01$).

The mean approach observed at each behaviour test of these males (26 – 30.9mm BL) is shown in Figure 5.9b. The approach was suggestive of a reduction in downstream males compared with upstream fish, but this was only marginally significant ($F_{(1, 50)} = 3.35$, $p = 0.073$).

Duration in female zone

Males of the smaller body size category (23 – 25.9mm BL) were compared in their duration in female zone at each behaviour test, shown in Figure 5.10b. The duration in female zone of downstream males was significantly reduced compared with upstream fish ($F_{(1, 14)} = 5.39$, $p < 0.04$).

The mean duration in female zone observed at each behaviour test of these males (26 – 30.9mm BL) is shown in Figure 5.10b. No significant differences between fish from the up- and downstream sites were found ($F_{(1, 50)} = 0.48$, $p = 0.49$).

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Mate Attempt

Mate attempt of upstream males sampled in 2002 was not normally distributed (23 – 25.9mm BL: K-S d = 0.37, n = 10, $p < 0.01$ and 26 - 30.9mm BL: K-S d = 0.23, n = 24, p

< 0.01). The proportion of males attempting to mate is shown below in Table 5.9. A Fisher exact test revealed a significant reduction in attempt of large males living downstream compared to upstream. Although no significant differences were revealed between up- and downstream of the smaller males, upstream males attempted to mate twice the amount than downstream fish.

Table 5.9 Proportion of males sampled up- and downstream of Quakers Hill STP in 2002 attempting to mate with females[1]

Size Category	Upstream	Downstream	p
23 – 25.9mm	9/10 **(90)**	3/6 **(50)**	0.12
26 – 30.9mm	22/24 **(92)**	14/28 **(50)**	0.002*

[1] Percentages are shown in brackets. * Indicates significant difference using Fisher exact test (up > downstream).

The behaviours observed in 2002 (approach, duration in female zone, and mate attempt) were positively correlated with each other (approach and duration time: r = 0.67, p < 0.001; approach and mate attempt: r = 0.73, p < 0.001; duration time and mate attempt: r = 0.45, p < 0.05), i.e., approach and duration in female zone were indicative of sexual motivation.
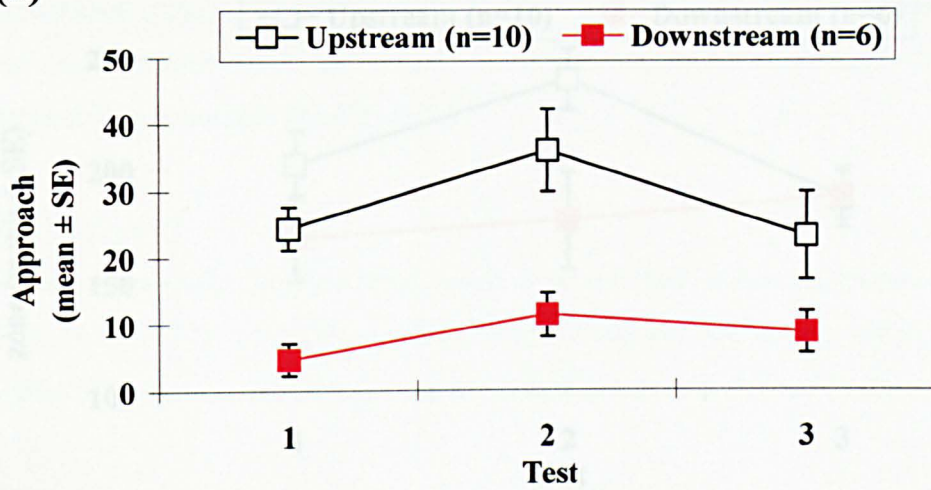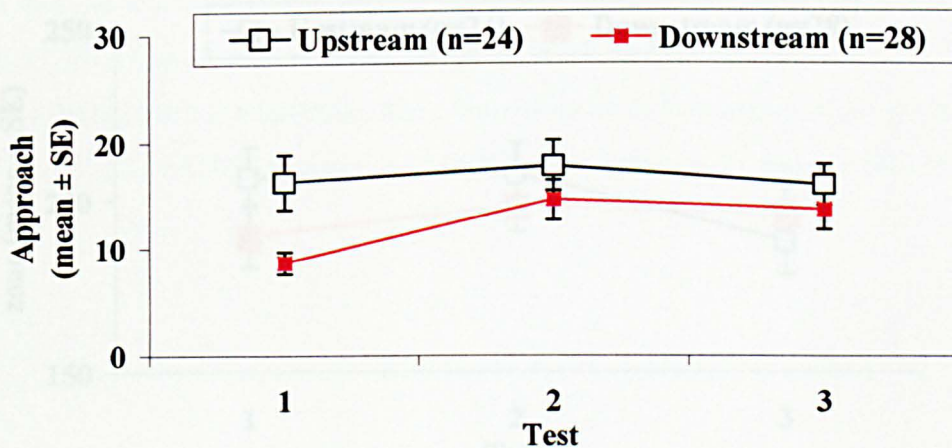
Figure 5.9 Mean approach (mean ± standard error) of a) 23 - 25.9mm and b) 26 - 30.9mm BL male *G. holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant, year 2002. The downstream site is approximately 50m from the sewage outfall. Males were tested every 24 hrs for 3 consecutive days. Two-way ANOVA repeated measures test on approach data revealed significant differences between smaller-sized fish from the two sites: upstream > downstream ($p < 0.01$).
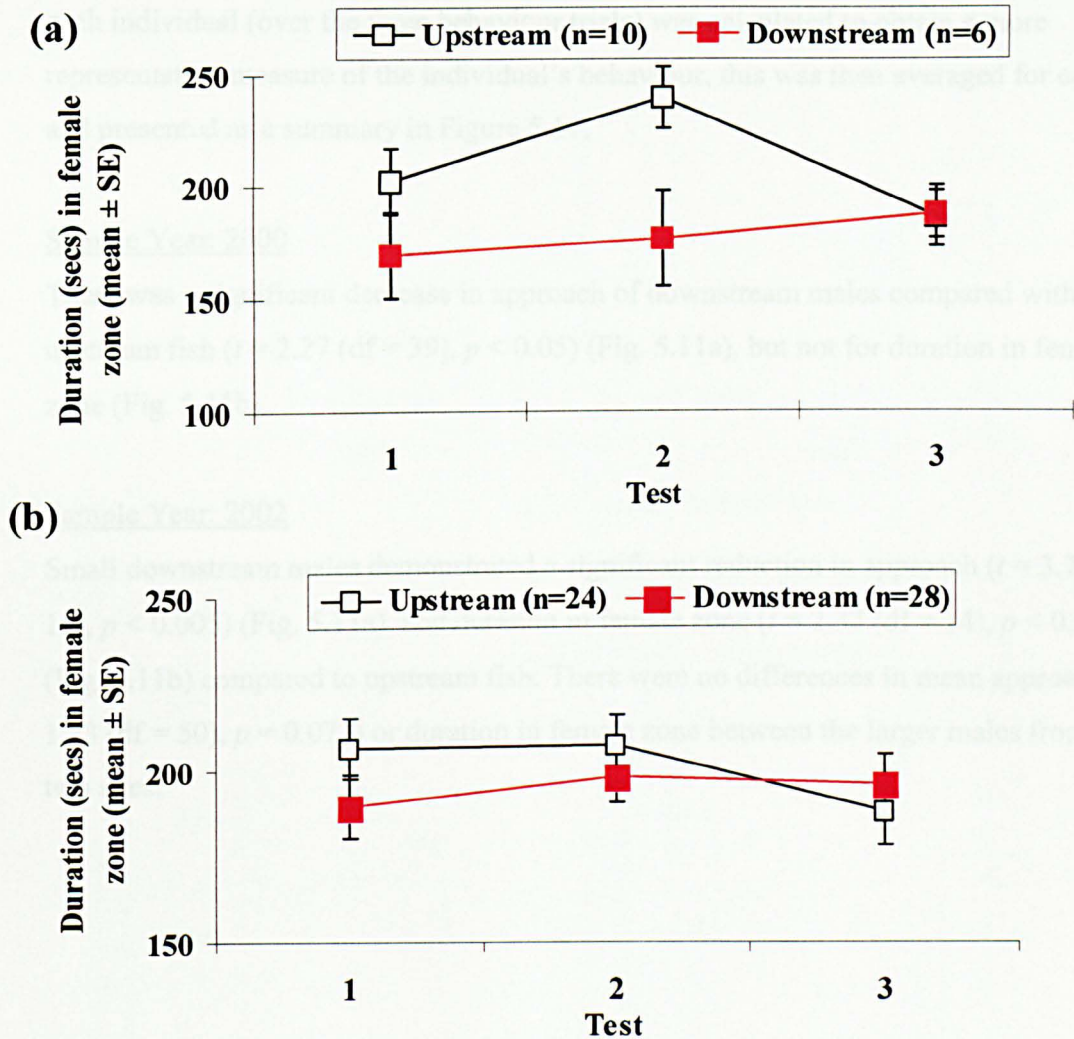
Figure 5.10 Mean (± standard error) duration in female zone of a) 23 - 25.9mm and b) 26 - 30.9mm BL male *G. holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant, year 2002. The downstream site is approximately 50m from the sewage outfall. Males were tested every 24 hrs for 3 consecutive days. Two-way ANOVA repreated measures test on duration in female zone data revealed significant differences between smaller-sized fish from the two sites: upstream > downstream (*p* < 0.03).

In addition to presenting the mean behaviour of the three behaviour trials, the average for each individual (over the three behaviour trials) was calculated to obtain a more representative measure of the individual's behaviour, this was then averaged for each year and presented as a summary in Figure 5.11.

Sample Year: 2000

There was a significant decrease in approach of downstream males compared with upstream fish ($t = 2.27$ (df = 39), $p < 0.05$) (Fig. 5.11a), but not for duration in female zone (Fig. 5.11b).

Sample Year: 2002

Small downstream males demonstrated a significant reduction in approach ($t = 3.7$ (df = 14), $p < 0.005$) (Fig. 5.11a), and duration in female zone ($t = 2.32$ (df = 14), $p < 0.05$) (Fig. 5.11b) compared to upstream fish. There were no differences in mean approach ($t = 1.83$ (df = 50), $p = 0.073$) or duration in female zone between the larger males from the two sites.

(a)
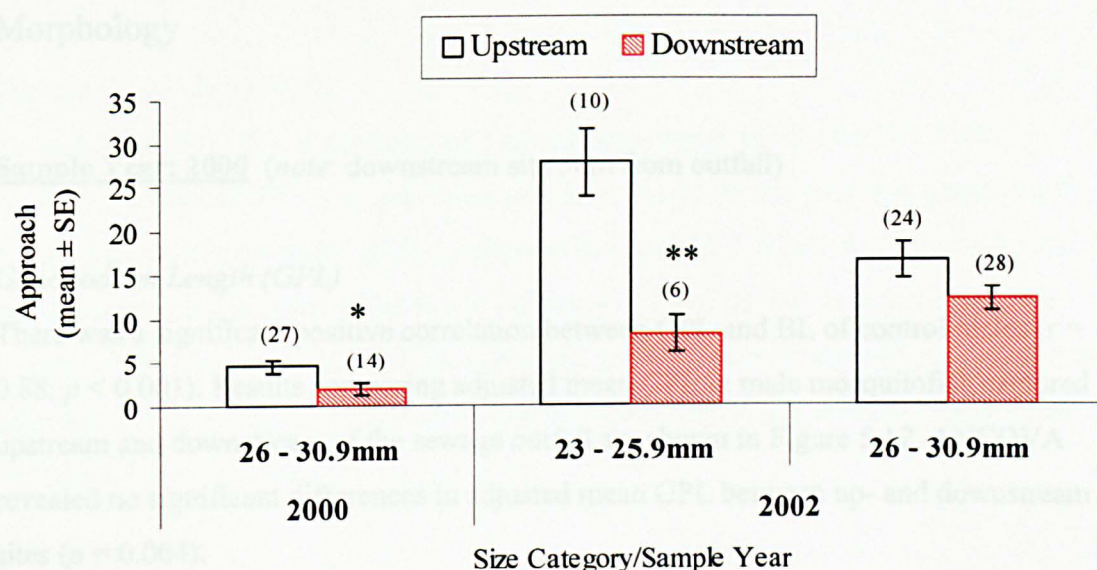
(b)
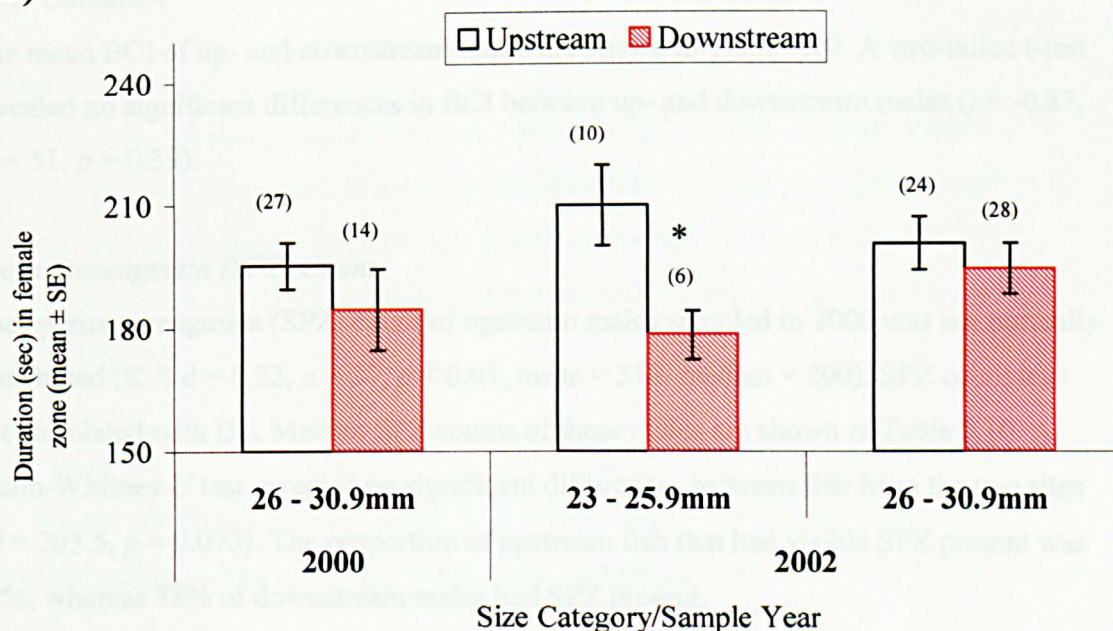
Figure 5.11 Mean (± standard error) (a) approach and (b) duration in female zone of male *G. holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant in 2000 and 2002. The downstream site was approximately 50m from the sewage outfall. Males ranging from 23 – 25.9mm and 26 - 30.9mm BL were analysed separately. Behavioural values were averaged for each individual over three tests and then averaged to get overall for group. Sample size is in brackets. * Indicates $p < 0.05$ and ** $p < 0.005$; upstream > downstream using a student's *t*-test for two independent samples.

Morphology

Gonopodium Length (GPL)

There was a significant positive correlation between GPL and BL of control males (r = 0.88, p < 0.001). Results comparing adjusted mean GPL in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Figure 5.12. ANCOVA revealed no significant differences in adjusted mean GPL between up- and downstream sites (p = 0.064).

Body Condition

The mean BCI of up- and downstream males are shown in Table 5.10. A two-tailed t-test revealed no significant differences in BCI between up- and downstream males (t = -0.87, df = 51, p = 0.39).

Spermatozeugmata (SPZ) Count

The spermatozeugmata (SPZ) count of upstream males sampled in 2000 was not normally distributed (K-S d = 0.22, n = 37, p < 0.01, mean = 318, median = 200). SPZ count was not correlated with BL. Median SPZ counts of these males are shown in Table 5.10. A Mann-Whitney U test revealed no significant differences between fish from the two sites (U = 203.5, p = 0.073). The proportion of upstream fish that had visible SPZ present was 92%, whereas 88% of downstream males had SPZ present.

Testis Area

There was a significant positive correlation between TA and BL of control males (r = 0.84, p < 0.001). Results comparing adjusted mean TA in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Table 5.10. Results of ANCOVA comparing adjusted mean TA between the up- and downstream males was not significant (p = 0.19).

Gonadosomatic Index (GSI)

The testes weight (TW) of upstream and downstream males sampled in 2000 was normally distributed. TW was correlated with BW; therefore this data was transformed into the gonadosomatic index (GSI), described in section 2.7. The GSI of upstream and downstream males sampled in 2000 was normally distributed. GSI was not correlated with BL or BW (r = 0.099, $p < 0.6$ and r = -0.16, $p < 0.4$ respectively). Mean GSI of these males are shown in Figure 5.13. There were no significant differences in GSI between sites ($t = 1.1$, df = 51, $p = 0.27$).

Sample Year: 2002 (*note*: same up- and downstream sites as previous sample date)

Gonopodium Length (GPL)

There was a significant positive correlation between GPL and BL of control males (r = 0.89, $p < 0.001$). Results comparing adjusted mean GPL in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Figure 5.12. ANCOVA revealed no significant differences in adjusted mean GPL between up- and downstream sites ($p = 0.22$).

Body Condition

BCI was not correlated with BL. Mean BCI of these males are shown in Table 5.10. BCI was significantly reduced in downstream males compared to upstream ($t = 2.7$, df = 69, $p < 0.01$).

Spermatozeugmata (SPZ) Count

The spermatozeugmata (SPZ) count of upstream males sampled in 2002 was not normally distributed (K-S d = 0.21, n = 36, $p < 0.01$). Upstream SPZ was not correlated with BL. Median SPZ counts of these males are shown in Table 5.10. Non-parametric Mann-Whitney U test revealed no significant differences between sites ($U = 557.5$, $p = 0.4$). The

proportion of upstream fish that had visible SPZ present was 60%, whereas 80% of downstream males had SPZ present.

Testis Area

There was a significant positive correlation between TA and BL of control males ($r = 0.40$, $p < 0.05$). Results comparing adjusted mean TA in male mosquitofish captured upstream and downstream of the sewage outfall are shown in Table 5.10. Results of ANCOVA revealed males living downstream had significantly smaller (adjusted mean) TA compared to upstream males ($p < 0.01$).

Gonadosomatic Index (GSI)

TW was correlated with BW; therefore this data was transformed into the gonadosomatic index (GSI). The GSI of upstream and downstream males sampled in 2002 was normally distributed. GSI was not correlated with BL or BW ($r = 0.07$, $p = 0.76$ and $r = 0.02$, $p = 0.89$). The mean GSI of these males are shown in Figure 5.13. This figure suggests GSI was reduced in downstream fish compared to upstream, although this was not significant ($t = 1.77$, $df = 67$, $p = 0.074$).
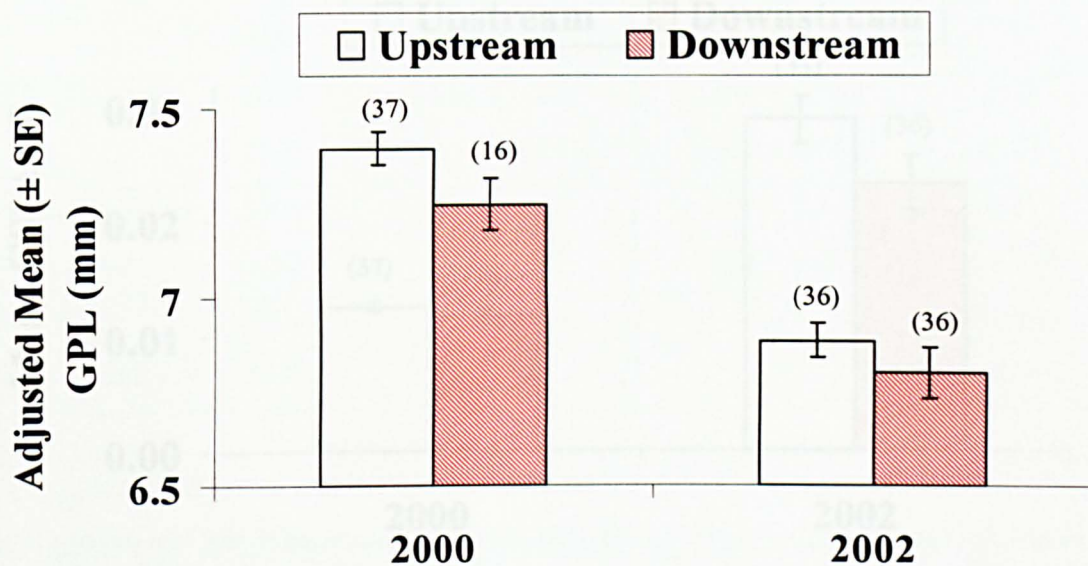
Figure 5.12 Adjusted mean gonopodium length (± standard error) of male *G. holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant, sampled in 2000 and 2002. Sample size in parentheses. No significant differences between sites were found using ANCOVA.

Figure 5.13 Mean (± standard error) gonadosomatic index of male *G. holbrooki* sampled up- and downstream of Quakers Hill sewage treatment plant in 2000 and 2002. Sample size in parentheses. No significant differences between sites were found using a two-tailed t-test.

Table 5.10 Quakers Hill STP: Other measurements taken of male *G. holbrooki* sampled in 2000 and 2002 up- and downstream of the sewage effluent outfall[1]

| Measurement | Year | Sample Site | | *p*-value |
		Upstream	Downstream	
Sample Size	2000	(37)	(16)	-
	2002	(36)	(36)	-
Mean (± SE) BCI	2000	0.078 ± 0.002	0.079 ± 0.002	0.39
	2002	0.086 ± 0.001 (35)	0.082 ± 0.001	< 0.01*
Mean (± SE) [a] TA (mm²)	2000	3.95 ± 0.082	3.75 ± 0.13	0.19
	2002	7.35 ± 0.39	5.79 ± 0.39	< 0.01*
Median SPZ Count	2000	200	100	0.073
	2002	80	200	0.4

[1] The upstream site was less than 1 km from Quakers Hill effluent outfall, and the downstream site was 50m from the outfall. SE = Standard Error. Sample size represented in parentheses. Differences between sites were analysed using the Mann-Whitney U test (for non-normal data) or parametric two-tailed t-test (for normally distributed data).

[a] Indicates values are adjusted mean, and differences analysed using ANCOVA.

* Indicates *p* < 0.01: males sampled downstream of the effluent outfall had significantly reduced BCI and TA compared to fish sampled upstream.

5.3.4 Summary of results (Quakers Hill STP)

Significant differences in reproductive behaviour were found between fish from sites in the 2000 sample. The approach was significantly reduced in downstream males compared with upstream fish. This reduction was confirmed when the data of each individual was averaged (over the three behaviour trials). There were no differences in mate attempt or duration in female zone between fish from the two sites.

Significant differences in reproductive behaviour were also found between fish from sites in the 2002 sample. Downstream males (23 – 25.9mm BL) demonstrated a significant reduction in approach and duration in female zone compared to upstream fish. This reduction was confirmed when the data of each individual was averaged (over the three behaviour trials) for approach and duration in female zone. There was a suggestion that mate attempt was reduced, but this was not quite significant ($p = 0.06$).

The behavioural results of larger males partly supported the reductions observed in smaller males: downstream males showed a significant reduction in mate attempt compared to upstream males. The approach was significantly reduced in downstream males compared with upstream fish at test 1. The behavioural results of larger males were suggestive of a decrease in mean approach of downstream fish compared with upstream, although significance was not reached. No differences in duration in female zone between males from the two sites were found on a test-to-test basis or when the data for each individual was averaged. The proportion of males attempting to mate was significantly reduced in all males (of both body size ranges) collected from downstream compared to upstream.

There was a suggestion that GPL was reduced in downstream males compared to upstream in 2000, but this was not significant ($p = 0.06$). There were no differences between sites in GPL in males sampled in 2002. There were no significant differences between sites in the BCI of males sampled in 2000, but males sampled downstream in 2002 showed a significant decrease in BCI compared to upstream. There were no

significant differences between sites in SPZ count at either year. The TA did not differ between sites in 2000, but there was a significant reduction in TA in downstream males compared to upstream of the 2002 sample. GSI and TA are strongly correlated, so it is not surprising there was a suggestion GSI was also reduced in downstream males compared to upstream ($p = 0.07$). The GSI of males sampled in 2000 did not differ between fish from the two sites.

5.4 SUMMARY

Table 5.11 Reproductive behaviour and morphology of *G. holbrooki* inhabiting two rivers receiving treated sewage effluent (compared to unexposed upstream males)

		St. Marys STP			Quakers Hill STP	
		1999	2000	2002	2000	2002
Mean approach	Repeated measures test	-	↑	NS (S and L)	↓	↓ (S and L)
	Individual	NS	↑	↓ (S and L)	↓	↓ (S and L)
Mean duration in female zone	Repeated measures test	-	NS	NS (S and L)	NS	↓ (S) NS (L)
	Individual	NS	NS	NS (S and L)	NS	↓ (S and L)
Proportion attempting to mate		NS	NS	NS (S and L)	NS	NS (S) ↓ (L)
Mean GP		NS[a]	NS[a]	↓[a]	NS[a]	NS[a]
Mean BCI		-	NS	NS	NS	↓
Median SPZ		NS	NS	NS	NS	NS
Mean TA		NS[a]	NS[a]	↓	NS[a]	↓[a]
Mean GSI		-	-	↓	NS	NS

↓   Indicates a statistically significant reduction downstream

↑   Indicates a statistically significant increase downstream

NS   Indicates no statistically significant differences between fish from the two sites

[a]   Adjusted (for length factors)

S   Small males (ranging from 23-25.9mm BL)

L   Large males (ranging from 26-30.9mm BL)

5.5 DISCUSSION

The experiments described in this chapter examined the adult male reproductive behaviour and morphology of wild populations of *Gambusia holbrooki* inhabiting rivers contaminated with STP effluent. Differences were found in fish within sites over the sampling years, between up- and downstream sites, and between the two STPs utilised in the study and these will be discussed in the following paragraphs.

Reproductive Behaviour

Before discussing the impact of sewage effluent on behaviour, the variations between sampling years at Quakers Hill STP will be discussed. Interestingly, it was apparent in both up- *and* downstream fish that reductions in approach, duration in female zone and number of mate attempts in the 2002 sample were more marked compared to the 2000 sample. For example, in the 2000 sample, only 14% of larger males from the upstream site attempted to mate compared to 96% of large males in the upstream 2002 sample. The reason why the behaviour of fish observed in 2000 was significantly less than the behaviour of fish observed in 2002 is unclear.

There are numerous physical and chemical properties of water that will influence aquatic organisms, including food availability, and subsequently may explain the differences in reproductive behaviour of fish between the sample years. Chemicals from, for example, diffuse pollution, can arise from many sources and can impact all surface waters by leaching through soil and surface run-off (it is mainly related to the way land and soil is managed). Individually the sources may be small, but their collective impact can be damaging. Diffuse pollution may include:

- Nutrients such as nitrogen and phosphorus from over-application of fertilisers and manures;
- Faecal and other pathogens from livestock and from overloaded and badly connected drainage systems;

- Soil particles from arable and livestock farming, upland erosion, forestry, urban areas and construction and demolition sites;

- Pesticides, veterinary medicines and biocides from industrial, municipal and agricultural use, poor storage and handling, and run-off;

- Organic wastes (slurries, silage liquor, surplus crops, sewage sludge, and industrial wastes) that are poorly stored or disposed of and spread to land;

- Oil and hydrocarbons from car maintenance, disposal of waste oils, spills from storage and handling, road and industrial run-off;

- Chlorinated solvents from industrial areas where the use of solvents are ubiquitous;

- Metals, including iron, acidifying pollutants and chemicals from atmospheric deposition, industrial processes etc.

Diffuse water pollution can have significant effects on aquatic organisms, these effects include: nutrient enrichment, oxygen depletion, and direct toxicity. Any of those parameters above may vary from year to year and may affect fish living upstream and downstream. In addition, physical characteristics, such as temperature, turbidity and erratic rainfall patterns (which are common in Sydney), could account in some part for the differences in results between years. Unfortunately it is not possible to determine the exact causes of differences observed in upstream fish between years.

However, differences between up- and downstream fish behaviour within the sample years are most likely to be due to material in the effluent since there were no other apparent sources of pollutants. The effects of laboratory exposure to sewage effluent of males in 1999 and 2000 (chapter 4), demonstrated that 50% effluent was sufficient to induce a significant reduction in behaviour. There were no significant differences in any of the measures of reproductive behaviour of males sampled 10 km downstream of St Marys STP compared to upstream fish in 1999. Males sampled downstream at this site in 2000 however, demonstrated a significantly increased approach compared to upstream males. This increase in reproductive behaviour of fish living in sewage-contaminated

water was in contrast with the results of experiments exposing fish to sewage effluent in the laboratory. This suggests that other factors in the wild may account for different effects (e.g., dilution, receiving water quality, within species susceptibility), because males were exposed to effluent from the same STP and the same years. It may be that this increase demonstrated in the field was solely due to the smaller sample size in downstream fish (n=6) and that the reproductive behaviour would be similar between sites if sample sizes were comparable.

There was a consistent reduction in approach (at all three behaviour tests) of males sampled 5km downstream compared to upstream fish, but this was not significant. There were no significant differences between up- and downstream males in the duration in female zone and proportion attempting to mate. Overall it would seem there were no differences in reproductive behaviour between up- and downstream males at the St Marys STP sampling sites used in this study.

The reproductive behaviour of males living 50m downstream from the sewage effluent outfall of Quakers Hill STP was however, significantly reduced (compared to males living upstream). Over both years of sampling, 2000 and 2002, the approach, duration in female zone and proportion of males attempting to mate were all significantly reduced in males living downstream compared to upstream fish.

While diffuse pollution may have accounted for some of the differences between up- and downstream fish between years, changes in the treatment procedures of the effluent between the years may have additionally affected the nature of the effluent. Sydney Water (the dischargers for St Marys and Quakers Hill STPs) reported that at the end of 2000, results indicated that aluminium, residual chlorine and chloramines posed a potential risk to aquatic environments (Sydney Water, 2000). Aluminium occurs naturally in most soils within the Sydney area, thus aluminium is present in dissolved or suspended form in river and creek beds. Aluminium has also been detected as a trace impurity in chemicals used in sewage treatment to achieve a high level of solids and nutrient removal. In addition, 11

discharge permits were issued to discharge aluminium from commercial and industrial sources via Quakers Hill STP. The discharge of aluminium from the major source was supposedly reduced by the end of 2000. Despite this reduction in aluminium levels, the difference in behaviour of downstream males is only slightly greater than the differences between the two upstream samples, for example, the difference between 2000 and 2002 overall mean approach for upstream = 12.0 and difference between downstream years = 10.2 (Fig. 5.13a).

In addition to aluminium levels being reduced in 2001, improvements in the treatment of effluent at St Marys and Quakers Hill STPs also occurred. In 2001, the treatment plants changed from using chlorine gas to liquid chlorine. The use of chlorine and sodium hypochlorite in sewage treatment to kill pathogens produces free residual chlorine as well as disinfection byproducts such as bromoform, chloroform, dibromochloromethane and chloramines (Sydney Water, 2000). Furthermore, this was also when a dechlorination facility was added: residual chlorine was removed using sodium bisulphate. A tentative hypothesis is that chlorination of the effluent added to the toxicity therefore causing a reduction in reproductive behaviour of fish sampled in 2000 (compared to 2002). In most previous studies, it has been difficult to determine whether residual chlorine or other substances contributed to observed effects on aquatic life (Rutherford *et al.*, 1993 and Schoenfuss *et al.*, 2002); this uncertainty applies to the present study. Despite dechlorination and reductions in aluminium levels, the reproductive behaviour was still significantly reduced in downstream males compared to upstream.

Morphology

A previous investigation by Batty and Lim (1999) demonstrated that some samples of male mosquitofish sampled below St Marys STP in 1995 and 1996 had significantly shorter gonopodium length (GPL) than those from upstream sites. One of the same downstream sites in this study was adopted in the present study (SC3, 10km from outfall). There were no significant differences in GPL between fish from the up- and downstream site in the current study. Possible reasons for the differences between these two studies

are differences in protocol or changes in the nature of the effluent. However, fish sampled 5 km downstream of this STP in 2002 demonstrated very different results. Male mosquitofish demonstrated a significantly reduced GPL compared with upstream, which is consistent with the findings of Batty and Lim (1999).

Although the huge variation in treatment protocols used by different STPs and conditions of receiving water makes it difficult to extrapolate the results of this study to potential impacts of other STPs, a study by Angus *et al.* (2002), also investigated the effects of sewage effluent on the GPL of *G. holbrooki*. Angus and co-workers studied a population of male mosquitofish inhabiting a river immediately downstream of a wastewater treatment plant (WWTP) outfall in America. They found no significant differences in GPL of males living downstream compared with upstream fish. Despite minor differences in treatment processes between the WWTP investigated in Angus's study and the present study, the volume of discharge is similar. However, the flow below the WWTP in the Angus *et al* study consisted, on an annual average, of approximately 5.7% effluent; both STPs in this study are licensed to have a 50% flow of effluent in the river below the outfall, i.e., the current study has shown an increased concentration of effluent was required to induce reductions in GPL of male mosquitofish.

In addition to significantly shorter GPL, male mosquitofish sampled 5km downstream of St Marys STP outfall in 2002 have significantly reduced gonadosomatic index (GSI) compared to upstream fish. There is a strong positive correlation between GSI and testis area (TA), so it is not surprising to find some parallel trends in these measurements between the study sites. This significant decrease in GSI of downstream males of St Marys STP was reflected by a significant decrease in TA compared with upstream males. However, there were no significant differences in GSI and TA of males sampled up and downstream in previous years (1999 and 2000) in this study.

Failure to find an effect at a greater distance downstream in 1999 and 2000 may be due to dilution. Harries *et al.* (1997) found out of a total of 18 field sites, that seven sites had

fish with mean GSIs significantly smaller than those of upstream controls after field exposure in cages for three weeks. The majority of these sites were closest to the effluent discharges. In the same study Harries *et al.* (1997), demonstrated that VTG levels in the caged fish decreased with greater distance downstream of a sewage effluent outfall in the UK. In two of the rivers studied, the VTG response had diminished considerably within 1.5 km downstream of the STP and had completely disappeared by 13 km.

The relationship between increasing distance sampled downstream of the sewage outfall and decrease in testes size (TA and GSI) and GPL was less clear in males living directly below Quakers Hill STP. The TA was significantly reduced in downstream males compared with upstream males sampled in 2002. A decrease in GSI of downstream males however, just reached marginal significance ($p = 0.07$) in the 2002 study. In addition, there were no significant differences in GPL between males from the up- and downstream site (although a marginal significant reduction in GPL of downstream fish was reached in 2000).

The significance of 'body condition index' (BCI) as an indicator of fish 'health' has been subject to much discussion (e.g., Adams and Huntingford, 1997). BCI can be an indication of the health or somatic condition of the individual, and any alteration in this could be indicative of an adverse impact on the fishes energy reserves e.g. food availability. For this reason, if effects of contamination were to generate a reduction in energy spent on feeding, one would expect the BCI to be higher upstream than downstream. However, there were no differences in BCI between sites of St Marys STP. This implies that, while fish living downstream can have reduced GP lengths and testis size, the one aspect of general fitness, in other words the time spent on feeding and ability to assimilate energy, might not necessarily be affected. The BCI of fish sampled downstream from Quakers Hill STP in 2002 however, was significantly reduced compared with upstream fish, although no BCI effects were observed in 2000. This suggests that other factors in addition to REDs may be impacting the fish.

Very few studies on possible endocrine disruption measure the overall fish condition by BCI, except Hemming *et al.* (2001) who demonstrated that the BCI in male fathead minnows (*Pimephales promelas*) exposed to domestic wastewater effluent had a reduced BCI compared to controls. They hypothesised that the lower BCI values recorded in effluent exposed fish may have been a result of oestrogenic stimulation of excessive vitellogenesis; VTG levels increased with a decrease in BCI. High levels of VTG can cause physiological stress, leading to kidney and liver damage and necrosis because males cannot remove the VTG from its blood supply (unlike females who sequester the VTG into developing eggs). Further experiments would be necessary to ascertain whether the reduced BCI in downstream fish of this study was due to an endocrine mediated response resulting in the excessive production of VTG, or whether the toxicity of the effluent resulted in a reduction in feeding or due to other factors.

There were no significant differences in the spermatozeugmata (SPZ) count between up- and downstream fish sampled from either of the STPs in this study. This lack of effect is consistent with results of previous chapters that demonstrated exposure to known oestrogenic chemicals and treated sewage effluent had no effect on the SPZ production. To the author's knowledge, no other study has investigated the SPZ count in wild fish.

The underlying causes for the observed reductions in GPL and GSI in this study are not known, however, given that GP and testis growth are endocrine dependent processes, it is possible that some kind of endocrine change is involved. If REDs reduce the levels of circulating androgens, then there would be inhibition of the expression of male secondary reproductive characteristics, like growth of the GP and testes. Oestrogens present in sewage effluent could also potentially inhibit the growth of the testis in the mosquitofish in this study, an effect that has been demonstrated in other fish species (Harries *et al.*, 1996; 1997).

On the other hand, one must always consider other possible factors that could account for the differences in reproductive morphology between up- and downstream males.

Reductions in GSI of fish have been demonstrated after exposures to metals such as cadmium (Sehgal and Pandey, 1984), and pesticides such as carbaryl (Arora and Kulshrestha, 1984), suggesting that non-specific toxic effects can also impair testis growth. In addition, differences in food availability between sites could also affect testes size, although the pattern of little effect on BCI would suggest this is not the case.

One possible explanation for the BCI differences between sites in the 2002 sample is that some contaminants in the effluent were having a toxic effect on the fish and therefore reducing the energy spent on feeding. Changes in food availability, due to pollutants or differences in habitat type not apparent, could have resulted in changes in the energy budget, which would impact the BCI. This does not however explain why these differences were not observed in the fish sampled in 2000. One would expect dechlorination (which was implemented in 2001) to reduce the toxicity of the effluent and consequently the BCI might be expected to be similar between up- and downstream sites.

Since the fish used in this study were wild, one might speculate that those sampled below Quakers Hill STP could have avoided long-term exposure to the effluent by moving upstream of the outfall. A genetic study by Feder *et al.* (1984), demonstrated that mosquitofish exhibit a high degree of site tenacity. Thus to avoid effluent exposure, the fish would have to leave their local home range and migrate through sections of the river that provide little protection from predators. The lifespan is unclear for wild populations of mosquitofish, although findings of a study by Krumholz (1948) in America suggested they rarely survive more than a year in nature, and few successfully over winter. For these reasons, it is probable that fish utilised in this study were born during the spring and had grown to reproductive maturation near the same locations where they were sampled in the late summer months, i.e., they are probably an isolated population. However, climatic conditions may vary in Australia, which means that larger males (> 28mm) sampled may have lived for 2 years.

The experiments in this chapter have demonstrated significant differences in reproductive morphology of mosquitofish living 5km (but not 10km) downstream of St Marys STP compared to upstream males. The reductions in GPL and testes size (TA and GSI) of downstream males suggests that contaminants in the treated effluent could be causing an adverse effect on reproductive development. No apparent differences in reproductive behaviour were found between up- and downstream fish, suggesting these are not contaminants that affect behaviour, or the contaminants were not in a concentration high enough to elicit behavioural effects. However, males sampled 50m downstream from the effluent outfall of Quakers Hill STP in both 2000 and 2002 demonstrated a reduction in all measures of reproductive behaviour compared to males living upstream. This supports the hypothesis that contaminants in the effluent are in sufficient concentrations to significantly reduce reproductive behaviour. The reproductive morphology results between up- and downstream males of Quakers Hill were not as clear. The results of males sampled downstream in 2002, but not 2000, demonstrated a significant reduction in TA compared to upstream males (which reflects a marginal significant reduction in GSI observed). However, there were no significant differences in GPL between males at up- and downstream sites. In addition, the BCI was reduced in fish sampled in 2002, which suggest that contaminants in the sewage effluent have variability in effects. The possible reasons for differences between years and sites sampled could be due to differences in:

1) Dilution
2) Contaminant levels
3) Changes in STP processing
4) Receiving water quality
5) Food availability
6) Within species susceptibility

The fact that reproductive behaviour and morphology was still significantly reduced in males exposed to effluent even after dechlorination and aluminium reductions, suggests that other compounds in the effluent, for example, metals, chlorinated organics, and

oestrogens could have caused these effects. Since numerous studies have detected the presence of oestrogens in STP effluent, it is possible that these chemicals may have played a role in the differences observed in fish between sites of the current study.

CHAPTER 6

FINAL DISCUSSION

6.1 Introduction

The aim of this thesis was to investigate the potential impact of prolonged exposure to reproductive endocrine disrupters (REDs) on reproductive behaviour and morphology in adult male mosquitofish (*Gambusia* sp.). The approach has been to evaluate the possible impact of oestrogens associated with sewage effluent, and the impact of sewage effluent both in the laboratory and the field on behavioural and morphological measures. The first step of this thesis was to test the response of adult male mosquitofish to REDs by exposing them to oestrogens and an oestrogen mimic (EM) under controlled laboratory conditions and known doses. This stage of investigation was aimed at detecting effects on several aspects of reproductive behaviour and morphology that may impact reproductive success, through the use of a range of endpoints. The next stage was to investigate the effects of a potential source of REDs by exposing adult males to different concentrations of sewage effluent in the laboratory. The final set of experiments was an investigative field study which was carried out to assess whether mosquitofish collected from sewage-contaminated rivers demonstrated the reproductive effects which had been observed with laboratory exposure to REDs and sewage effluent.

The results of individual experiments have been discussed in the relevant chapters; therefore this discussion concentrates on the overall significance of the findings of the study. A brief overview of the results will initially be given, followed by a section on variables that may have influenced the results. The reliability and reproducibility of the test battery employed in this study will be discussed and suggestions for future experiments made. The possible underlying mechanisms of RED exposure will then be discussed in relation to the findings of this study. Finally, the wider implications of the findings of this study will be discussed.

6.2 Overview of Findings

These experiments have shown that exposure to three known REDs clearly reduces reproductive behaviour in adult male mosquitofish. The most marked behavioural effects induced were in order of oestrogenic potency; DES, E2, and OP. When males from the same population were exposed to different concentrations of treated sewage effluent over two consecutive years, they also demonstrated a reduction in reproductive behaviour. Males collected from an urban polluted river demonstrated a reduced behaviour when exposed to 100% sewage effluent. Contrary to expectation, this reduction was not evident in another experiment conducted the same year but using males collected from a 'pristine' river. It was concluded that further experimentation was required due to the lack of adequate controls. The behavioural response was variable in all experiments, and when males were exposed to OP and sewage effluent the response followed a dose-dependent reduction, but when exposed to the oestrogens, DES and E2, this pattern did not occur. After the laboratory exposures, the morphology and some other reproductive characteristics were examined. Few differences were found between fish in the treatment groups.

It was demonstrated in chapter 5 that wild males living below two STPs in NSW have reduced reproductive behaviour and morphological characteristics. Overall, males sampled 5 and 10km downstream of St Mary STP outfall demonstrated no consistent trends in reproductive behaviour over the three years of study, however, males sampled 5km downstream had significantly reduced GPLs, smaller testes size and testes weights compared with upstream fish. Males collected 50m downstream of Quakers Hill outfall demonstrated a significant reduction in reproductive behaviour over both years of study, but effects on morphological characteristics were less clear: there was a significant reduction in body condition index and testis size, but only a suggestive decrease in GPLs and testes weights compared to fish living upstream.

6.3 Battery of Tests Employed

The control of variables and ease of observation are two of the main reasons that ethological studies on fishes are usually conducted in the laboratory. An inherent risk of the laboratory approach however, is the possible exclusion of events and stimuli found under natural conditions. For example, a study by Martin (1975) demonstrated that field reproductive behaviour of male *G. holbrooki* consisted of chasing, whereas there was no chasing involved in the repertoire of laboratory fish. Martin's study also demonstrated that aggression is non-existent in wild *G. holbrooki* compared to fairly high levels observed in the laboratory. Thus it is often important to complement laboratory behaviour studies with fieldwork. Unfortunately *in situ* behavioural observations were beyond the scope of this research project, but to reduce any factors that could affect the response, males were separated in beakers to reduce aggression and enable individual identity to be retained, and tested for three consecutive days so a more behaviourally representative average was determined. The fact that differences in behaviour levels between field and laboratory results highlights the influence of the testing protocol.

A sensitive and specific tool for measuring oestrogenicity in fish is measurement of hepatic or plasma VTG concentrations. Unfortunately no study to date has measured VTG induction in combination with reproductive behaviour, but comparisons with the doses administered in other studies and the current study suggests that reproductive behaviour is more sensitive to oestrogens (but not a specific indication of exposure to oestrogens). The only study to measure the impact of DES on fish is that by Folmar *et al.*, (2000). They reported that a concentration of 20ng/l DES was enough to significantly induce plasma VTG levels in male sheepshead minnow (*Cyprinodon variegates*) after a 16-day exposure (they did not get significant VTG increase at 0.2ng/l and 2ng/l DES). This is tenfold the concentration required to induce a significant reduction in behavioural responses in male mosquitofish of the current study. This study by Folmar *et al.*, (2000) also found that 100ng/l E2 and 100ng/l EE2 (ethinyloestradiol) was required for the same level of plasma VTG induction when exposed to 20ng/l DES, i.e. the concentration of E2

required to induce VTG in sheepshead minnow is five-fold the concentration required to induce a significant reduction in behaviour of mosquitofish. A laboratory study of the effects of E2 on the hepatic VTG levels of Japanese medaka (*Oryzias latipes*) exposed for 3-weeks demonstrated a significant induction at 55.7ng/l E2 (Joon Kang *et al.*, 2002). Still greater than the concentration required to inhibit reproductive behaviour in male mosquitofish. The results of OP exposure in the current study suggest that the male mosquitofish behaviour is at least 2 times less sensitive than laboratory exposed Japanese medaka since a significant VTG response was found in the males at an exposure level of 20μg/l OP after 3-weeks (Groonen *et al.*, 1999), while for mosquitofish an exposure level of 50μg/l OP was still insufficient to inhibit reproductive behaviour.

Separation of male and female fish by the beaker prevented genital contact therefore the results cannot determine the consequence of the decreased behaviour in terms of reproductive success. However, it was established throughout this report that the behaviour characteristics measured are correlated with mate attempt, i.e. a decrease in approach would be indicative of a decrease in mate attempts and in turn reproductive success.

While the study of reproductive behaviour is important, use of it alone would be insufficient to provide an overall assessment of reproductive health. The use of image analysis to measure additional parameters of reproductive morphology is a useful additional tool. It is highly accurate in measuring lengths (e.g. GPL was measured to nearest 0.01mm), and can also be used to measure the TA, not documented as a parameter in any previous studies. Initial reservation of whether TA was truly an indication of size was felt, because the testes are a 3-dimensional object, which image analysis may not accurately represent. However, the results are consistently in agreement with GSI (testes area and weight are correlated), and considering the testes are very small and difficult to remove, indicates that the method of measurement can be a useful additional tool to verify effectiveness of GSI.

6.4 What Factors May Have Influenced The Results?

Several factors can influence the findings of a behavioural study and the variables that may have influenced the findings of this study will be discussed. Certainly, the findings demonstrated adult male mosquitofish reproductive behaviour was sensitive to exposure to REDs and sewage effluent (both in the laboratory and in wild fish) but a number of factors must be considered when interpreting the results. For example, a decrease in behavioural response over time, which was evident in most laboratory exposures, could be a reflection of habituation to the beaker, i.e., the fish learn there is no point approaching because of the barrier.

Where the reproductive morphology of males exposed to sewage effluent (and REDs) in the laboratory did not differ, there were significant reductions in some of the measures taken in the field, suggesting the fish respond differentially different to possible hormonal constituents of the effluent, i.e. failure to observe a response in the laboratory exposed males suggests the experimental protocol (exposure in these sub-adult or adult fish) was outwith a critical period of development (males had reached maturation prior to exposure). Several studies would be in agreement with the hypothesis that morphological and behavioural effects of a chemical agent can be influenced by the stage of development when exposure occurs (e.g., Gray *et al.*, 1999a; Doyle and Lim, 2002). Of particular significance to the current study, Doyle and Lim (2002), discussed in section 1.4.2, found significant reduction in approach and mate attempt behaviours in addition to retardation of gonopodial growth in juvenile male mosquitofish exposed to E2 (although this could be interpreted as a delay in growth, as it was only a 12 week exposure). However, it does imply that this chemical (which is commonly found in sewage effluent) can affect morphological development at a critical stage of development, which was missed by the laboratory-exposed males to E2 in the current study.

6.5 Possible Mechanisms of REDs Action

In the general introduction, evidence that gonadal androgens stimulate male reproductive behaviours (Liley and Stacey, 1983) and morphological development (Turner, 1947; Angus *et al.*, 2001) in teleost fish was considered. In addition, the effect of REDs on reproductive function was reviewed and it was evident that environmental oestrogens can impair some reproductive processes in fish (e.g., Länge *et al.*, 2001; Gimeno *et al.*, 1996; Gray and Metcalfe, 1997). However, the exact mechanisms by which REDs affect reproductive behaviour and other reproductive characteristics are currently unknown.

Currently, risk assessments related to the endocrine disrupting effects of environmental oestrogens are based, primarily, on relative potencies derived for ER binding and ER-mediated responses *in vitro* (Ankley *et al.*, 1998) (e.g., breast cancer cell proliferation assays such as MCF-7 and VTG induction in fish hepatocytes). Although *in vitro* approaches provide a measure of the direct-acting potency of environmental oestrogens, they cannot easily model the potential indirect effects on endocrine homeostasis in a whole organism. The complex regulation of steroid hormone levels through the HPG-axis, via GtH and both positive and negative feedback among multiple tissues has not been adequately modeled *in vitro*. Even at the level of a single tissue, *in vitro* approaches will miss endocrine effects caused by metabolites or effects caused by interference with steroid synthesis and metabolism. Furthermore, the various endocrine pathways in intact animals are extremely complex and interdependent (e.g. Cyr and Eales, 1996), so effects in an *in vitro* assay will not necessarily be replicated *in vivo*.

An insult to testis function could potentially explain the morphological differences observed in mosquitofish living downstream of the sewage outfalls in the present study. Gonopodium growth in mosquitofish is paralleled by the progressive maturation of the testes, with the stage of gonopodial development being indicative of the degree of gonadal maturation (Bisazza *et al*, 1996). This suggests that there is a possible direct effect of RED and sewage effluent exposure on testicular development. Several studies

have reported an inhibition of testicular development in male fish following exposure to exogenous oestrogens (Panter *et al.*, 1998; Gimeno *et al.*, 1998). Furthermore, it is possible that there was a direct effect of exposure on testicular development and/or androgen levels which triggered a cascade of disturbances in neuroendocrine function to have significant effects on reproductive behaviour and/or gonopodial development. The synthesis of oestrogen in the brain plays a key role in reproductive behaviour.

Secondary sexual characteristics and body markings in males may also not develop properly, leading to abnormal or absent reproductive behaviour. It is generally accepted that reproductive behaviour of fish is reduced when exposed to androgen-competing hormones (Chizinsky, 1968) and EDCs (Bayley *et al.*, 1999; 2002; and Bjerselius *et al.*, 2001). Some sewage-derived chemicals may be both oestrogenic and androgenic in action: for example, nonyl- and octylphenols bind to both oestrogen receptors (ER) and androgen receptors (AR), as ER agonists and AR antagonists (Gray, 1996). Oestrogen exposure has been shown to inhibit androgen synthesis in goldfish (Trudeau *et al.*, 1993) and reduce the synthesis of gonadotropins in salmon, which could cause reduced androgen synthesis (Xiong *et al.*, 1994). Previous studies have demonstrated that E2 implants significantly reduce serum levels of the major male androgens testosterone and 11-ketotestosterone in goldfish (*Carassius auratus*) (Trudeau *et al.*, 1993) and in black porgy (*Acanthopargus schlegeli*) (Chang and Lin, 1998). The observed changes in this study may have resulted from alterations in normal hormonal pathways, by reducing the amount of circulating androgens.

Androgen reductions may both cause and reflect differences in behaviour and hormone dependent secondary sexual characteristics. In summary, contaminants can directly affect the brain and subsequent behaviour or the pollutants may affect the physiology that affects the testes, and ultimately behaviour.

6.6 Future Research

Pollutants may affect virtually all aspects of behaviour and morphology in aquatic organisms, and behavioural monitoring is a promising additional tool for screening and differentiating chemicals according to their mode of action (Drummond and Russom, 1990). As is often the case with such research, this study has posed several questions which could be investigated in future studies.

In order to provide conclusive evidence that the reproductive behaviour and morphological variations observed in this study are a consequence of environmental endocrine disruption, a more extensive survey specific to detecting environmental oestrogens must be carried out (e.g., VTG levels).

Currently, there are no data on transient effects on behaviour of environmental oestrogen exposure. This same effect (behaviour effects of exposure being transient and morphological effects of exposure being organisational) demonstrated in mosquitofish by Bortone and co-workers (1989 and 1994), could apply to this study. It has been confirmed in this study that reproductive behaviour is reduced in wild fish exposed to high concentrations of sewage effluent throughout life and in adult males after maturation exposed to REDs and sewage effluent in the laboratory. However, the question remains as to whether these reductions in reproductive behaviour are permanent (organisational) or transient (activational), i.e., when exposure ceases does behavioural effects revert back to pre-exposure? An interesting study would be to examine behaviour and morphology of males acclimatised to an environment free of effluent for some time.

6.7 What is the Ecological Significance of this Study?

Although not usually an environmental contaminant recorded because of its restricted use (now only used for treatment of prostrate cancer), Solé *et al.*, (2000) found concentrations of DES at approximately 40ng/l in the raw influent of STPs in Spain. This is twenty-fold the concentration required to induce a reduction in behavioural responses in male mosquitofish of the current study. As detailed in section 3.3.1, although not all studies have detected concentrations of E2 in treated sewage effluent (Fawell *et al.*, 2001), the majority of studies in the literature have (Desbrow *et al.*, 1998; Larsson *et al.*, 1999; Ternes *et al.*, 1999; Thomas *et al.*, 2001; Sheahan *et al.*, 2002). Detection limits reported in these studies range from 0.2ng/l - 64ng/l. This is three-fold the concentration required to induce reductions in reproductive behaviour in mosquitofish. Given that concentrations of OP recorded in rivers (receiving sewage effluent) are in tens of μg/l range (refer to section 3.4.1 for examples of references), the reproductive behaviour could be potentially inhibited by long-term exposure to this chemical in wild populations. The comparison of previous recordings of levels reported and results of the study herein suggests that when establishing a criterion for persistent oestrogen levels in receiving waters, the concentration which does not damage reproduction of biota may be missed by traditional screening methods.

This study has demonstrated that the male mosquitofish provides a sensitive 'model' of reproductive behaviour and morphology to oestrogenic and sewage effluent exposure. As is often the case with such research, this study has posed several questions that require further investigation.

It is likely that any decrease in mate attempts of male *Gambusia* sp. would result in lower reproductive success. Previous studies have demonstrated that male *G. holbrooki* normally attempt to mate at a very high rate, but with only a small proportion (one in every 30 attempts) resulting in contact between genitalia and only a small minority of these contacts involves complete intromission of the gonopodium (Bisazza and Marin,

1991). In addition, under natural conditions, there would be an intense competition between several males for the copulation of females (Bisazza, 1993), thus any reduction in reproductive characteristics observed under controlled conditions is likely to be enhanced by natural dynamics of reproductive success. Very few studies have been made to determine the overall ecological implications of REDs at a fish population level. Although a cause-effect relationship between REDs and the effects observed in the field is inconclusive, the increased catch effort required to catch fish at downstream sites suggests that population size was reduced compared to upstream sites.

The cause-effect relationship between the reduction in behavioural and morphological abnormalities observed in this study and reduction in population size remains unclear, but is strongly suggestive of an impact of sewage effluent, which may be mediated through hormonal disruption.

The results in this study are in agreement with earlier studies that have shown that REDs and sewage effluent exposure will reduce reproductive behaviour (Schoenfuss *et al.*, 2002; Bjerselius *et al.*, 2001; Bayley *et al.*, 1999) and impair secondary sexual characteristics (for example, Batty and Lim, 1999; Hemming *et al.*, 2001). The long-term effects of exposure to these oestrogens or oestrogen mimics on adult fish and their offspring can at present only be the subject of speculation. The fact that many of the oestrogen mimics identified to date are lipophilic and have a tendency to accumulate in organisms that are continuously exposed (Ahel *et al.*, 1993) add to the potential problem. Larsson *et al.*, (1999) demonstrated that the bile of Rainbow Trout caged downstream of a STP contained oestrogenic substances at concentrations 104-106 -fold greater than water levels. This shows that exposure to different environmental oestrogens results in the accumulation of prominent amounts of these substances. The current study of mosquitofish exposed to oestrogenic chemicals does not take into account bioaccumulation or the metabolic changes that may occur in the whole animal with the effect of changing bioavailabilty and potency. Chemicals that appear to be weak

oestrogens *in vitro* may be more potent *in vivo* (the opposite of course may hold true). Long-term studies could reveal more widespread chronic effects.

The list of chemicals that can act as endocrine disrupters is expanding as more chemicals are tested in the laboratory. The stable and versatile physical properties of REDs (lipophillic nature and chemical stability) has favoured their widespread use in industry. However, this also means they have the ability to bioaccumulate. Indeed, humans are exposed to oestroegnically active chemicals through the environment and the food chain (Guenther *et al.*, 2001). A recent study in Germany by Guenther *et al.* (2001) found high concentrations of nonylphenol (a breakdown product of APEOs, used in detergents) in a range of foods, and not only found in fatty food like e.g. butter (14.4 µg/kg), lard (10.2 µg/kg), or liver sausage (13.0 µg/kg) but also in non-fatty food like e.g. marmalade (7.3 µg/kg), apples (19.4 µg/kg), or tomatoes (18.5 µg/kg). Using data on the average food consumption patterns of Germans, the authors were able to calculate an expected average daily intake of nonylphenol as 7.5 µg/day for adults and between 0.2-1.4 µg/day for infants (for babies exclusively breast-fed).

As oestrogenically active substances are routinely detected throughout the ecosystem (Perez *et al.*, 1998), their impact on natural populations of animals and humans needs to be more fully investigated.

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