Gambusia holbrooki: Pest or Potential Bioindicator for Endocrine Disruption in Victorian Freshwaters, Australia?

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to quality for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed. All procedures involving vertebrate animals (fish) were conducted in accordance with the RMIT University Animal Ethics Committee, under the approved project AEC 0702.

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Signature of Student

Kavitha Chinathamby

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Project Output

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List of Abbreviations

7-ER	7-ethoxyresorufin
AhR	Aryl-hydrocarbon receptor
ANOVA	Analysis of variance
AR	Androgen receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BL	Body length
βNF	Beta-naphthoflavone
CAPIM	Victorian Centre for Aquatic Pollution Identification and Management
CF	Condition factor
CYP1A	Cytochrome P450-1A
DMSO	Dimethysulphoxide
E_1	Estrone
E_2	17β-estradiol
E_2eq	17β-estradiol equivalent concentration
E_3	Estriol
EE_2	17α-ethynylestradiol
EACs	Endocrine active compounds
EDCs	Endocrine disrupting chemicals
ELISA	Enzyme linked immunosorbent assay

ER	Estrogen receptor
EROD	Ethoxyresorufin *O*-deethylase
ES	Total estrogens
GL	Gonopodial length
GL/BL ratio	Gonopodial length/body length ratio
GSI	Gonado-somatic index
HSI	Hepatosomatic Index
I/R	Industrial/Urban
NADPH	Nicotinamide adenine dinucleotide phosphate + H^1
OCP	Organochlorine pesticides
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCDD/F	Poly chlorinated dibenzo dioxins/furans
PNEC	Predicted no effect concentration
POPs	Persistant organic pollutants
PVDF	Polyvinylidene difluoride
R4:6 ratio	Ray 4:6 ratio
R/U	Rural/Urban
S-VtgEq	Salmon vitellogenin equivalent concentration
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SEM	Standard Error of the Mean
TCDDeq	Tetra-chlorinated dibenzo-*p*-dioxin equivalent concentration
Vtg	Vitellogenin
VtgmRNA	Vitellogenin messenger ribonucleic acid
WWTP	Wastewater treatment plant

Abstract

Freshwater aquatic systems are often challenged by harmful anthropogenic contaminants such as sewage effluents, agricultural pesticides, and industrial and urban run-off. Water quality monitoring is necessary to understand effects on aquatic organisms. Biological monitoring tools such as biomarkers are important tools to detect effects in fish and aquatic organisms through cellular, molecular, biochemical, physiological or/and behavioural biomarkers. However, the identification of a suitable fish bioindicator is a challenge because most fish species do not have a wide distribution to enable comparisons between sites or may require a big effort to capture. Furthermore, selecting suitable biomarkers of exposure to toxicants for fish presents another challenge because of the many choices available.

Gambusia holbrooki (mosquitofish) are wide-spread around Australia and are easy to capture with dip nets. Recognized as an invasive species in Australia, acquiring permits for the collection of large numbers is easier than for native species. Furthermore, *G.holbrooki* responses to endocrine active compounds (EACs) have been studied in the laboratory, providing data for comparisons. In this study, the focus is on the evaluation of data gathered from the suite of biomarkers (EROD, Vitellogenin induction and gonopodial measurements) together with chemical analyses of the water (estrogenic and AhR activity) to understand the condition of peri-urban creeks and rivers in Victoria. The suitability of using *G.holbrooki* as a sentinel species for the monitoring of EACs in Australian creeks and rivers based on

the biomarkers chosen for this study is also discussed.

Adult male *G.holbrooki* were collected from February to May between 2007 to 2009 from various land use types such as urban, rural, wastewater treatment plant impacted (WWTP) and reference sites (Ref) around Victoria, using a dip net.

Male *G.holbrooki* gonopodial (anal fin) morphology has been shown to be responsive to endocrine disrupting chemicals (EDCs) in the laboratory and to a limited extent in the field. In the current study, indices assessed were fish body length, gonopodial length:body length ratio, R4: 6 ratio, fish body mass and absence of hooks and serrae. Fish from a number of sites showed various effects in the chosen indices in the three years of sampling. In 2007, GL/BL ratio was largest in fish from Brodies Lake, a reference site. In 2008, fish from Green Lake (Reference) showed significantly smaller gonopodium length: body length ratio, suggesting some effects of endocrine disruption. R4:6 ratio was shortest in fish from Mullum Mullum Creek (WWTP). In 2009, effects were varied. Fish from Brushy Creek (WWTP) had the longest R4:6 ratio. In particular, a small number of adult fish from Gisborne WWTP did not complete full gonopodial development (missing hooks and serrae). However, *G.holbrooki* are also highly plastic and adaptable to a variety of water conditions making interpretations of morphological characteristics complicated. Confounding factors such as temperature, individual site history, predator absence/presence, extreme weather conditions and other factors may have influenced fish gonopodial indices.

Vitellogenin protein induction (a biomarker of exposure to estrogenic EDCs) was analysed in male *G.holbrooki*. This ranged between 4.70 ng/μl S-VtgEq in fish from Watson's Creek (Rural) to the highest at 22.83 ng/μl S-VtgEq in fish from Werribee WWTP. Vtg levels in fish from three out of the four WWTP sites (Gisborne, Brushy Creek and Werribee) were the highest, suggesting that fish from those sites could be exposed to estrogenic (or anti-androgenic) environmental contaminants. In particular, estrogenic activity was highest

at Gisborne WWTP and fish from that site showed high Vtg levels, suggesting that this may be the most impacted site.

EROD activity, a biomarker of exposure to POPs was analysed in male *G.holbrooki*. EROD activity in fish in 2009 ranged from 0.42 pmol/min/mg protein at Campaspe River (Rural) to a high of 10.85 pmol/min/mg protein at Kalparrin Wetland (Urban). As expected, fish collected from urban/industrial sites or sites receiving urban stormwater (Kalparrin Wetland, Kororoit Creek, Narre Warren SD) had generally higher levels of EROD activity.

Estrogenic activity was detected at all sites including the reference sites. The most impacted site was Gisborne WWTP at 12 ng/L EEQ due to a leak in the sewage pipes, while estrogenic activity at the rest of the sites ranged from 0.1-1.7 ng/L EEQ. AhR activity measured at sites in 2009 ranged from 7 ng/L βNF EQ in Brodies Lake (Ref) to 180 ng/L βNF EQ at Narre Warren SD (Rural/Urban). Although water samples from Narre Warren Stormwater Drain had the highest AhR activity, EROD activity in fish was not significantly higher compared to fish from most of the other sites. There was no correlation between EROD activity in fish and AhR activity. There was no consistent pattern observed in the water activity measurements and biomarker results. It was expected that urban/industrial sites would produce moderate to high hepatic CYP1A induction and that rural sites would show moderate Vtg induction from agricultural and dairy effluent run-off. However, this was not always the case in this study. It must be mentioned that one-off grab water sampling for estrogenic and AhR measurements only provide a snapshot of exposure and do not account for temporal variations in concentrations. Ideally, frequent water sampling for temporal variations in estrogenic and AhR measurements are necessary.

Despite the number of challenges that were encountered with the use of *G.holbrooki* for the biomarkers chosen for this study, results obtained were varied and useful for *G.holbrooki* from the wild. Therefore it is concluded that *G.holbrooki* is a suitable bioindicator for

estrogenic EACs for Victorian and Australian creeks and rivers. Although environmental factors may confound the results of some of the biomarkers that were chosen for this study, it should not deter the future use of *G.holbrooki* as a bioindicator. Instead, fish populations from affected study sites should be monitored over time with more frequent sampling to better understand natural variation.

Sites that will benefit from continued monitoring include Brushy Creek (WWTP), Gisborne WWTP, Merri Creek (Industrial/Urban) and Kalparrin Wetland (Urban). Both Merri Creek (Industrial/Urban) and Kalparrin Wetland (Urban) receive effluents from storm water drains and are *G.holbrooki* habitats at point source location. Results from this study should form the basis of an ongoing biomonitoring investigation using *G.holbrooki* at these sites because of the close proximity of the effluent discharge points and the persistence of fish at these sites. Results will contribute to a long term evaluation and risk assessment study of the release and potency of estrogenic contaminants entering Victorian creeks and rivers.

Chapter 1

General Introduction

A growing number of pollutants that affect the endocrine system of aquatic wildlife are frequently released into our creeks and rivers. The two major sources of pollutants are wastewater treatment plant (WWTP) effluents and stormwater (Batty and Lim, 1999). These often contain pollutants such as pesticides, pharmaceuticals and personal care products (PPCPs), heavy metals and persistent organic pollutants (POP) (Scott et al., 2014, Djukić et al., 2016, Roberts et al., 2016). Many of these chemicals often cause disruption by mimicking, interfering with or blocking the natural hormones produced by the endocrine system and are known as endocrine active compounds (EACs) (Barton and Andersen, 1998). Some are defined as endocrine disrupting chemicals (EDCs) when they possess characteristics such as phenolic moieties that structurally mimic natural steroid hormones and interact with steroid hormone receptors of organisms (Gehring et al., 2004, Lorenzetti and Narciso, 2012). In 2007, when the current study began, there was scarce information on endocrine activity in Melbourne's creeks and rivers. Study sites were broadly categorized into land use areas to identify different effects on fish from the different groups of pollutants. Only recently has there been contributions from studies such as Allinson et al. (2011), Andrew-Priestley

et al. (2012), Scott et al. (2014), Allinson et al. (2015) , on levels of estrogenic activity in Melbourne, New South Wales and Australia. In the later stages of the study, some site categories have been taken to be arbitrary because there is no clear definition between land use area and contaminant source. It is now accepted and has been documented that endocrine disruptive compounds (EDCs) and endocrine active compounds (EACs) are present in the Australian aquatic environment including some of the reference sites of this study.

1.1 Endocrine Disruption

The endocrine system is comprised of a set of glands that regulates activities such as body fluid homeostatis, management of stress, reproduction and fertility. The endocrine glands secrete hormones to maintain and regulate functions of the body (Kime, 1998). Hormones communicate using their unique chemical structures which are recognized by specific receptors on their target cells (Nussey and Whitehead, 2001). A wide range of chemicals are known to cause disruption or affect the activity of the endocrine system in teleosts; for full reviews, see van der Oost et al. (2003), Mills and Chichester (2005), Milla et al. (2011). The functioning of these hormone receptors may be interrupted by the binding action of endocrine disrupting chemicals (EDCs) (Ankley et al., 2009, Mnif et al., 2011). The endocrine system is interfered with at least in three possible manners: (i) EACs can act as agonists, antagonists or a selective estrogen receptor modulator (SERM) (compounds wtith agonist or antagonist estrogenic actions depending on the tissue) (Pinto et al., 2014), (ii) Interfere or disrupt production, secretion, transportation, or metabolism of natural hormones, such as estrogen or testosterone, and (iii) Block or alter the production and/or function of hormone receptors (Goksøyr and Male, 2006, Mnif et al., 2011). A full review of the wide range of pathways and processes of the normal function of the endocrine system is not covered in this thesis and can be found in Hoar and Randall (1969) and Kime (1998). Some groups of

pollutants relevant to the Melbourne wetlands, and their effects on the fish endocrine system are reviewed below.

There are two classes of substances which cause endocrine disruption: natural hormones which include estrogen, testosterone, progesterone, and phytoestrogens found in some plants such as soy beans and man-made substances which include synthetically produced hormones such as oral contraceptives (17α-ethinylestradiol), pharmaceutical products, pesticides and plastic additives (Sumpter, 2008, Diamanti-Kandarakis et al., 2009, Mnif et al., 2011). Heavy metals such as cadmium, arsenic and zinc are also known to act as endocrine disruptors (Authman et al., 2015). Some environmental contaminants such as persistent organic pollutants (POPs) are also potential endocrine disruptors that act directly or indirectly via the aryl hydrocarbon receptor (AhR) (Shanle and Xu, 2011). Many of these chemicals including POPs are suspected EACs (Gültekin and Ince, 2007, Snyder and Benoti, 2010, Mnif et al., 2011, Birnbaum, 2013). Because of the vast range of and multiple sources of EACs and the lack of agreement on what chemicals can be labeled as EACs, the process of identifying the sources of EACs is complex (Atkinson, 2014). The persistent nature of EACs means that they may exist in the environment for decades and biomagnify or bioaccumulate in freshwater organisms (Ropero et al., 2006, Marino et al., 2012).

1.2 EACs in the Australian Environment

1.2.1 Pesticides

4.6 million tons of chemicals are reportedly being sprayed annually into the environment on a global scale. These include commonly used conventional pesticides such as glyphosate, simazine, acetochlor, chlorothalonil, 2,4-D and chlorpyrifos (Grube et al., 2011). China has become the world's largest pesticide user (McBeath and McBeath, 2010) with cases

of pesticide overuse reported recently by Zhang et al. (2015). Similarly, overuse of pesticides has been reported in Bangladesh by Dasgupta et al. (2007). Often, pesticide run-off from agricultural lands leach into stormwater drains, creeks and rivers causing widespread contamination of water. This poses significant risks to non-target organisms such as microorganisms, insects, plants, amphibians, fish and birds.

In Australia, pesticides that are currently registered for use has increased to 10000 (APVMA, 2016) from 6000 products in 2002 (Radcliffe, 2002). Atrazine and simazine were reported to be widely used in 2002 followed by 2,4-D and organophosphates which include parathion methyl, chlorpyriphos, and diazinon (Radcliffe, 2002). 2,4-D, diuron and simazine were detected recently at most if not all sites in a study at a Sydney estuary at concentrations between 15-97 ng/L (Birch et al., 2015). Atrazine in particular has been implicated in amphibian declines worldwide yet studies on native amphibian species are lacking (Siddiqua et al., 2010). In Melbourne, atrazine and simazine have been detected in the recent years both in surface waters and sediment of rivers and creeks (Schäfer et al., 2010, Allinson et al., 2015). These pesticides have been reported to cause endocrine disruption such as low induction of plasma vitellogenin in male fish in the laboratory (Vasanth et al., 2015) although other studies have no effects on Vtg induction in male fish (Tyler et al., 2004, Kroon et al., 2014).

Studies have reported on effects of chlorpyrifos, an organophosphate pesticide which is highly toxic to aquatic organisms (John and Shaike, 2015) and a potent neurotoxic at low levels of exposure in fish (Huynh and Nugegoda, 2012). These include affected swimming behaviour in zebrafish hatchings, inhibited acetylcholinesterase (AChE) activity, interference with steriod hormone production and morphological alterations in fish livers and gills; for a full review, see Deb and Das (2013). Chlorpyrifos was detected in sediments and surface waters of wetlands and rivers in Melbourne (CAPIM, Unpublished data 2009).

Another pesticide of concern is glyphosate or glyphosate-containing products that are highly soluble in water and therefore may be bioavailable to aquatic organisms. These have been implicated in several liver histological alterations that might impair normal organ functioning (Langiano and Martinez, 2008) and cause DNA damage in fish (de Castilhos Ghisi and Cestari, 2013).

1.2.2 Industrial chemicals

One of the most investigated estrogenic chemicals released from industries is Bisphenol A (BPA). Bisphenol A is a monomer used in the production of polycarbonate, epoxy resins and as a non-polymer additive in plastics (Geens et al., 2011, Robertson and Farrelly, 2014). It is often used for food and beverage packaging, medical equipment, flame retardants, adhesives and many other products (Robertson and Farrelly, 2014). The main source of BPA has been reported as contributions of effluent discharges of manufacturing plants (Michałowicz, 2014) and other sources include, leaching from landfills, and degradation of plastics in the environment (Staples et al., 1998). Bisphenol A is also prevalent in water samples collected around Victoria and Australia (Scott et al., 2014) and is known to cause Vtg induction in male fish (Hatef et al., 2012).

1.2.3 Pharmaceuticals and personal care products (PPCPs)

Pharmaceuticals and personal care products (PPCPs) have been detected both globally (Hughes et al., 2013) and in the Australian aquatic environment (Khanjani et al., 2006, Birch et al., 2015). Often PPCPs enter surface waters via treated effluents or enter land systems through sewage sludge applied as fertilizer (Overturf et al., 2015). A recent study by Roberts et al. (2016) investigating the removal of micropollutants such as PPCPs by a sewage treatment plant (STP) in Canberra found that removal of most PPCPs in the STP was

incomplete and varied by season and type of compound. Birch et al. (2015) confirms that PPCPs are widely detectable in the Australian environment. In that study, eight pharmaceuticals including codeine, venlafaxine, fluoxetine and carbamazepine, seven pesticides and one food additive were detected above limits of quantification in a Sydney estuary although no wastewater treatment plants (WWTPs) discharge to the Sydney estuary and no relationships could be established between hospitals and pharmaceutical discharge to the area. The most common pharmaceutical that was detected at all sites was paracetamol, followed by iopromide (a contrast agent introduced into a body to enhance the medical imaging of specific organs or tissues) and a food sweetener (acesulframe). It was suggested that domestic wastewater was leaking from the sewage system into the stormwater network in the Sydney estuary area implying that this could be occurring elsewhere in Australia. Paracetemol has been shown to induce multiple endocrine disturbances in both human adult testis (Albert et al., 2013) and in human fetal testes (Jégou, 2015) and could potentially have effects on aquatic wildlife.

The antidepressant fluoxetine (an active ingredient of Prozac), also detected in the Australian aquatic environment (Birch et al., 2015), has potential disruptive effects of neuroendocrine function in teleost fish (Mennigen et al., 2011). The neuroendocrine function modulates processes such as reproduction and food intake. Similarly, triclosan has been implicated as an endocrine disruptor. A study by Ying and Kookana (2007) investigated triclosan (an antibacterial agent) and its fate in effluents, biosolids and surface waters from WWTPs around Australia. The authors found concentrations of up to 75 ng/L in surface waters (outfall, upstream and downstream) from rivers receiving effluent discharge from WWTPs and suggested that this may present risks to aquatic organisms. Tricoslan induced Vtg mRNA expression and decreased sperm count in *Gambusia holbrooki* showing the potential to cause endocrine disruption in the species (Raut and Angus, 2010). In fact, some effects may not be seen immediately as shown in a study by Parrott and Bennie (2009) where fathead minnow were exposed to environmentally relevant concentrations of a mix-

ture of six common pharmaceuticals and one personal care product. No effects were seen in fathead minnow exposed for a life cycle, however, larval deformities were observed in the F1 generation.

1.2.4 Persistant organic pollutants (POPs)

Also of concern are environmental pollutants such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides (DDT, Aldrin, Chlordane, Dicofol and Dieldrin), commonly known as persistent organic pollutants (POPs). Sources of PAHs and dioxins such as PCBs, PCDDs and PCDFs include traffic, industrial waste, forest fires and the release of chemical mixtures during incomplete combustion of wood, garbage, coal and other organic materials (Bosveld et al., 2002). These chemicals eventually make their way into surface waters, collect at wastewater treatment systems and consequently enter into sewage effluent discharges. In a national survey of Australian sewage sludge from 2004 to 2006, Deldrin was the most prevalent OCP detected in all parts of Australia (Clarke et al., 2010). Although OCPs are being phased out in Victoria, these were detected in groundwater (Wightwick and Allinson, 2007) and in sediment in Victorian wetlands (CAPIM, Unpublished data 2009).

1.2.5 *G.holbrooki* as indicators for endocrine disruption

Numerous studies have reported endocrine disrupting effects in fish observed as gene transcription in zebrafish (Bugel et al., 2013), protein expression in rainbow trout (Verslycke et al., 2002), morphological changes in *G.holbrooki* (Angus et al., 2005), behavioral changes (Saaristo et al., 2014) and population-level effects in fathead minnow (Kidd et al., 2007).

Howell et al. (1980) first reported evidence of endocrine disruption in female *G.holbrooki* in Florida. Female fish were found to be strongly masculinized, suggesting exposure to some chemical associated with paper-mill effluents causing an androgenic effect at the effluent discharge site. This was followed by a number of early studies reporting endocrine disruption including the findings of a second population of masculinized female *G.holbrooki* in Fenholloway River (Bortone and Drysdale, 1981). Endocrine disruption in other wild fish were then reported, beginning with the study by Folmar et al. (1996) who reported vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a sewage treatment plant. Soon after, Jobling et al. (1998) reported widespread endocrine disruption in wild roach downstream of sewage effluent treatment sites in the United Kingdom. It appeared that male roach throughout the United Kingdom had a high incidence of intersexuality.

Worldwide, *G.holbrooki* and *G.affinis* have been studied to investigate effects of estrogenic contaminants in the aquatic environment. Of importance are studies describing effects in wild fish such as the study by Toft et al. (2003) where male *G.holbrooki* from a lake contaminated with chemicals showed shorter gonopodia and 32 to 47% fewer sperm cells per milligram testis were found compared to fish from reference sties. Xie et al. (2010) investigated *G.affinis* in the Hanxi River which is heavily contaminated with municipal wastewaters. Fish were analysed for developmental parameters such as total length, liver mass, gonad index and reproductive parameters such as sperm count, viability of males and gonopodial morphology. The study found that *G.affinis* from the Hanxi River were affected by discharged wastewaters and showed significantly decreasing development and reproduction levels compared to fish from reference sites. Using the multiple biomarker approach, a study by Huang et al. (2016b) investigated fish morphology, biochemical indicators and transcriptional processes in *G.affinis* from two urban rivers in South China impacted by municipal effluents. The study reported significant alterations in CYP1A mRNA expression and an increase in EROD activity with a strong correlation between the both. Further-

more, morphological, biochemical and transcriptional responses in fish were affected due to chemical contamination of the two urban rivers.

In Australia, the first evidence of endocrine disruption was reported in *G.holbrooki* as early as 1999 by Batty and Lim (1999) in New South Wales, Australia. Batty and Lim (1999) reported reduced gonopodia (modified anal fin) length in male *G.holbrooki* that were sampled downstream of a sewage treatment plant suggesting the presence of EDCs in the aquatic environment. This was followed by another study by Game et al. (2006) suggesting EDCs in wetlands in Western Australia affect *G.holbrooki* morphological measurements that relate to reproduction. In that study, indices such as gonopodium length/standard body length, pre-anal length/standard body length, the index of elongation and the percentage of male fish with hooks on the gonopodium were used. In the laboratory, Doyle and Lim (2002) quantified the effects of 17β-estradiol (E_2) on the gonopodial development and sexual activity of *G.holbrooki*. The study also found significant effects in gonopodial length and degree of gonopodial elongation in male fish from exposure to (E_2). Furthermore, sexual activity in exposed males also decreased with increasing concentrations of E_2. A later study by Doyle and Lim (2005) further explored the decline of sexual activity in male *G.holbrooki* exposed to E_2. The study found that exposed males were less capable of impregnating females than control males but did not find significant effects in spermatozeugmata production. Most of the early studies on *G.holbrooki* was focused on morphological indices based on the gonopodia. However, Leusch et al. (2005) introduced a method to quantify induction of vitellogenin (Vtg) mRNA in male *G.holbrooki*. The study showed that male fish exposed to the highest concentration of 250 ng/L E_2 induced Vtg mRNA expression in liver. Another study by Leusch et al. (2006b) examined wild *G.holbrooki* living in undiluted treated sewage in Queensland and found that both male and female *G.holbrooki* showed minor elongation forth anal fin ray consistent with androgenic exposure. Rawson et al. (2006) examined the effect of E_2 on the modification of the hemal spines on the vertebrae of male *G.holbrooki* as a potential end point for estrogenic effects. However, this was

not found to be consistent for all end points on a particular spine nor across all spines. Further investigations on skeletal morphology of male *G.holbrooki* captured from two sewage treatment plants in Sydney showed differences in effects on hemal spine morphology with one site suggesting estrogenic effects (Rawson et al., 2008). Rawson et al. (2009) investigated EROD activity in male *G.holbrooki* in a number of wetlands at the Sydney Olympic Park contaminated by POPs before remediation. In that study, one site at the Olympic Park showed elevated EROD activity, however, this was found to be at the lower end of the range found at urban impacted, non-remediated sites. A majority of the studies on *G.holbrooki* are based in New South Wales, followed by Queensland with very few studies conducted on *G.holbrooki* in Victoria.

Effects of estrogenic contamination on male *G.holbrooki* are not limited to the morphological indices of the gonopodia. Recently, Saaristo et al. (2014) found that mating behaviour of male *G.holbrooki* collected from WWTP sites in Victoria were increased compared to those from pristine sites, suggesting the presence of androgenic pollutants in the WWTP effluents that may affect male mating behaviour. Males from WWTP sites spent more time associating with and chasing females. Saaristo et al. (2014) did not find significant effects in gonopodial morphology but the study reported levels of estrogen and androstenedione concentrations at the sites. *G.holbrooki* males have also been used to investigate *in situ* water quality and effects of endocrine disruption at two WWTP in Western Australia where effluents were discharged into open drains (Leusch et al., 2014). Vtg was detected in that study in males upstream and downstream of one WWTP while no significant effects were found in gonopodial length. The study attributed the detection of Vtg in male fish to the presence of estrogenic contaminants upstream and downstream of the WWTP, however, the study did not investigate associations between Vtg in male fish and the water quality parameters that were measured.

An extensive summary of studies in Australia that investigated endocrine disruption

Table 1.1: Summary of studies investigating endocrine disruption in *G.holbrooki* in Australia.

Reference	Location	Species	Influence	Endpoint and Effects
Batty and Lim 1999	New South Wales	*G.holbrooki*	WWTP *in situ*	reduction in gonopodial measurements
Doyle and Lim 2002	New South Wales	*G.holbrooki*	E_2 exposure	reduction in gonopodial measurements and male sexual activity
Doyle and Lim 2005	New South Wales	*G.holbrooki*	E_2 exposure	reduction in male sexual activity no effect on gonopodial measurements
Game et al. 2006	Western Australia	*G.holbrooki*	various	reduction in gonopodial length
Leusch et al. 2006	Queensland	*G.holbrooki*	WWTP *in situ*	increase in gonopodial length
Rawson et al. 2006	New South Wales	*G.holbrooki*	E_2 exposure	delay in gonopodial development
Rawson et al. 2008	New South Wales	*G.holbrooki*	WWTP effluent	decrease in proportion of males no effect on skeletal structures
Rawson et al. 2009	New South Wales	*G.holbrooki*	Industrial	increase in EROD activity
Chinathamby et al. 2013	Victoria	*G.holbrooki*	various	some effect on gonopodial measurements
Leusch et al. 2014	Western Australia	*G.holbrooki*	WWTP *in situ*	some effect on Vtg protein induction in males
Saaristo et al. 2014	Victoria	*G.holbrooki*	WWTP *in situ*	increased mating activity towards females no effect on gonopodial measurements

in *G.holbrooki* is presented in Table 1.1. Most of these studies have either focused on one biomarker only, or do not have parallel water chemistry measurements. Importantly, studies examining estrogenic EDC effects in fish in the wild using multiple biomarkers together with parallel water chemistry studies are lacking in Australia. By testing several biomarkers relevant to pollutant exposure in wild caught *G.holbrooki* together with parallel measurements of water chemistry (estrogenicity and AhR activity), the approach of the current study aims to identify subtle effects of exposure to EACs/EDCs using biomarkers in male *G.holbrooki*.

G.holbrooki are ideal for investigating and demonstrating potential reproductive effects of endocrine disruptors that affect the reproductive system because they are sexually dimorphic. The modified anal fin (gonopodium) in the smaller male, which is used for sperm transfer, develops under the control of the endocrine system (Pyke, 2005). Hooks and spines at the tip of the gonopodium help secure the male fish during transfer of sperm to the female fish (Turner, 1941, Pyke, 2005). In male fish, elongation of the fin rays 3, 4, and 5 of the gonopodium occurs under androgenic control (Turner, 1941). Studies have shown that the gonopodium does not develop fully or develops shorter in response to estrogenic EDCs in the laboratory (Dréze and Monad, 2000, Doyle and Lim, 2002, Toft et al., 2003, Angus et al., 2005). However, once the gonopodium is fully developed, its size cannot be reversed. To this effect, the gonopodia of adult male *G.holbrooki* may not be not a good predictor of immediate endocrine disruption in the wild for effects may have occurred at juvenile stage. Therefore, it is relevant that other biomarkers are included with gonopodia measurements.

1.2.6 Biomarkers of exposure to EDCs

A vast range of biomarkers are available both at the gene and protein level for testing the effects of EDCs on fish. A common biomarker known as vitellogenin (Vtg) has been studied and measured in fish as early as the 1970s (Crim and Idler, 1978, Idler et al., 1979). Vitellogenin is a high molecular weight prospholipoprotein precursor to egg yolk produced in the liver of mature female fish under estrogenic stimulation (Jones et al., 2000). The Vtg gene is present in both sexes and Vtg expression can be induced in male fish exposed to exogenous estrogens (Denslow et al. 1999). The hormone-receptor-complex interacts with specific nucleotide sequences leading to the transcription of Vtg mRNA followed by the secretion of the Vtg protein into the blood stream (Mommsen and Walsh, 1988).

The induction of Vtg has been widely studied in many dose-reponse type laboratory

studies on *Gambusia sp.*. However, fewer studies report induction of Vtg in male *Gambusia spp.* in the wild. Recently, significant levels of hepatic Vtg mRNA were found in both male and female *G.holbrooki* at some sites impacted by municipal wastewaters in South China (Huang et al., 2016a). The study, in fact, showed that Vtg mRNA levels in male fish exceeded levels in female fish. In contrast, Angus et al. (2002) reported no detectable levels of Vtg protein in plasma of effluent-exposed male fish collected downstream of a small southeastern river in the USA. Similarly, a study by Kristensen et al. (2007) reported low levels of Vtg gene expression in all male *G.holbrooki* with no significant differences between the sites. Only one study of Vtg in wild *G.holbrooki* has been reported in Australia and these were from WWTP effluent sites in Western Australia. Leusch et al. (2014) reported Vtg protein induction in male *G.holbrooki* both upstream and downstream of a WWTP site. More studies of Vtg in wild male *G.holbrooki* is necessary Australia wide for baseline levels of Vtg. Previously, Gagnon et al. (2008) and Leusch et al. (2014) have respectively used salmonid Vtg ELISA kits and salmon anti-Vtg antibody for detecting Vtg protein in male *G.holbrooki* while Angus et al. (2002) produced anti-Vtg antibody specific to *G.affinis*. Other commercially available anti-Vtg antibodies have not been tested on *Gambusia.spp.* for affinity. Although, Vtg induction has been studied in *Gambusia spp.*, most of these techniques require the use of sophisticated equipment in the laboratory. A simple method that can be adapted for use in the field has yet to be explored.

Not only does the liver of fish play major roles in sexual reproduction, embryo development and Vtg synthesis, it is also involved in the detoxification of pollutants (Sarasquete and Segner, 2000, Bemanian et al., 2004). In fulfilling its role consistent with xenobiotic metabolism and excretion, the liver is predominantly involved with the expression and induction of cytochrome P4501A (CYP1A) activity (Sarasquete and Segner, 2000). The induction of CYP1A is initiated by the binding of xenobiotic compounds to the AhR (Whyte et al., 2000) and can be measured through CYP1A mRNA, protein and its enzymic activity (Bucheli and Fent, 1995). The enzymic activity of the CYP1A protein and its increased

ability to convert a substrate (7-ethoxyresorufin) to a fluorescent product (resorufin) can be measured by the ethoxyresorufin *O*-deethylase (EROD) assay in fish liver. EROD activity in fish is a classical biomarker and its responses and those of CYP1A to specific contaminants in different fish species have been reviewed in Bucheli and Fent (1995), Goksøyr (1995), Whyte et al. (2000) and van der Oost et al. (2003). EROD appears to be rapidly induced in fish exposed to AhR agonists. EROD activity was significantly induced as early as 24 hours (after exposure to organochlorine compounds) in *G.affinis* (Jakšič et al., 2008) to two days (after exposure to dispersed oil) in Atlantic salmon (Gagnon and Holdway, 1998). Levels remained elevated for eight days in Atlantic salmon (Gagnon and Holdway, 1998) although Danion et al. (2014) reported that EROD activity remained elevated at day 36 after a 21-day exposure to PAHs in sea bass. Some studies have reported that when fish are exposed to high levels of pollution, rapid EROD induction occurs followed by a period of "stabilization" (Kleinow et al., 1987, Vega-López et al., 2009).

AhR agonists include xenobiotic compounds that are hydrophobic (e.g. Polycyclic aromatic hydrocarbons, dibenzodioxins, polychlorinated dibenzofurans, coplanar polychlorinated biphenyls), and pesticides (Goksøyr, 1995, Whyte et al., 2000). Kojima et al. (2010) found that a diverse range of pesticides (11 out of 200 tested pesticides) also induced AhR-mediated transcriptional activity in a cell line called DR-EcoScreen that was constructed in mouse hepatoma cells. Different classes of pollutants can induce or suppress either the AhR or ER. CYP1A is regulated by the AhR and Vtg expression is regulated by the ER. Studies have shown evidence of cross-talk. In a study by (Gräns et al., 2010), combined exposure of BNF (a CYP1A model agonist) and EE_2 on rainbow trout hepatocytes showed a one-way inhibiting AhR-ER cross-talk through the ER mediated Vtg mRNA levels by 40%. Similarly, another study by Bemanian et al. (2004) showed the disruption in the expression of Vtg in fish hepatocytes when the cells were exposed to dioxin (a AhR agonist) while CYP1A expression was enhanced. These results show that the activation of AhR may inhibit the transactivation capacity of the ERα. In contrast, Kirby et al. (2007) showed that E_2

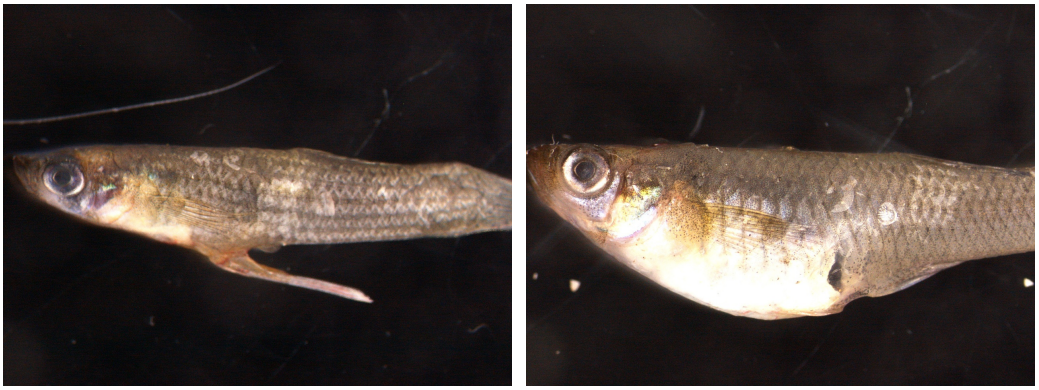
had the ability to suppress EROD in flounder. The study suggested that estrogen-mediated mixed function oxygenase (MFO) suppression may be occurring in wild populations of fish. Many of these studies report cross-talk between the AhR-ER in fish in the laboratory. Studies suggesting cross-talk in fish in the wild are not available to date.

The vast range of pollutants in the aquatic environment presents a new type of challenge of understanding effects of mixture toxicity. Mixtures of EDCs that act through multiple modes of action are not well documented. Most studies focus on dose and response type effects of individual EACs or a combination of two types of EACs on model fish species such as fathead minnow, medaka and zebrafish (Ankley and Johnson, 2004) in the laboratory. Interactions with endogenous endocrine pathways in fish are complex and the intensity of their effects depend on species, type of EAC, concentration, time and duration of exposure (Milla et al., 2011).

Though there are numerous reports about endocrine disruption in the aquatic environment (Sumpter and Johnson, 2005, Sumpter, 2008, Sumpter and Johnson, 2008, Milla et al., 2011), some questions remain unanswered. These include whether measurable effects on aquatic organisms at the individual level can be observed at population levels and whether chemical mixtures cause effects that individual pollutants do not. Effects are further complicated by conditions in the wild. Kleinhenz et al. (2016) reported that a herbicide mixture was more toxic than the herbicide alone on invertebrates in 96 h exposure studies in the laboratory. However, when the study was replicated in the field, the effects of the herbicide applications were shown to be less sensitive to the invertebrates suggesting different factors at play in the field. Finding a causal link for endocrine disruption in fish from the wild is a challenge except in areas of heavy contamination with a clear source of contamination such as studies in Europe in rivers receiving WWTP effluents (Jobling et al., 1998, 2002, Harris et al., 2011). Further studies are required to determine effects of EDCs on fish in the wild. Moreover, inexpensive and more rapid tests are necessary for quick analysis of the effects

of environmental pollution on fish.

1.2.7 *G.holbrooki* as a bioindicator in Australia



(a) Adult male *G.holbrooki.* (b) Adult female *G.holbrooki* with gravid spot.

Figure 1.1: *G.holbrooki* adult male and female

Although *G.holbrooki* has been examined in both field (Batty and Lim, 1999, Game et al., 2006, Gagnon et al., 2008) and laboratory studies in Australia (Doyle and Lim, 2005, Leusch et al., 2005, 2006b, 2014, Saaristo et al., 2014), the suitability of using *G.holbrooki* as a indicator species in the field for biomonitoring of EACs has not been previously discussed. *Gambusia holbrooki*, eastern mosquitofish (*Poeciliidae*) (Figure 1.1), were first introduced in mainland Australia in 1925 from Northern Mexico and the Southern United States of America for mosquito control (Pyke, 2008, Ayres et al., 2010) and has now been declared a noxious species in many states in Australia including Victoria. Figure 1.2 shows the distribution of *G.holbrooki* around Australia.

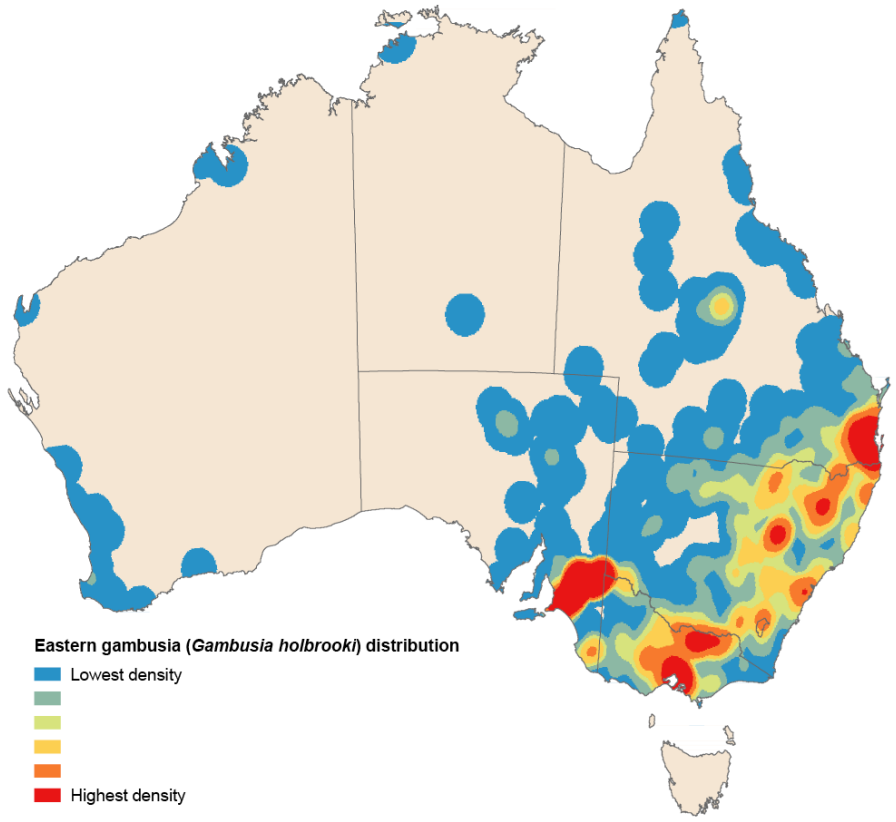


Figure 1.2: Known distribution of *G.holbrooki* in Australia (Source: Atlas of Living Australia).

Fertilization is internal in *Gambusia spp.* and young develop inside the mother until they are born (Pyke, 2005). *G.holbrooki* males and females generally mature at the same age and body length. Growth rates in *Gambusia spp.* have been found to vary with temperature, diet, rate of food consumption, salinity, and density of conspecifics (Pyke, 2005). *G.holbrooki* breed over a period of six months between October and March in temperate parts of Australia (Pen and Potter, 1991). Males died in the same season after participation in reproduction (Singh and Gupta, 2014). Females that overwintered in a temperate Australian river and bred in spring were found to be about one year old when they died (Pen and Potter, 1991). Females are able to fertilize multiple broods by storing sperm from a single mating event (Haynes, 1993). The gestation period is usually around 22 to 25 days (Krumholz, 1948) leading to a productive cycle of 2 to 5 broods per season (Pyke, 2005).

G.holbrooki are carnivorous, their diets consisting of small crustaceans such as littoral cladocerans and chironomid adults (García-Berthou, 1999). They have a wide tolerance to water temperatures and have been found in water with temperatures ranging from 0 to $45°$C (Pen and Potter, 1991). *G.holbrooki* are non-migratory (Pyke, 2005) which makes them ideal for investigating localized pollution. They are usually found in shallow, still or slow-moving waters and can be captured with dip nets without the use of expensive equipment. It is indeed useful that *G.holbrooki* are an invasive species in Australia and are found in bodies of water surrounding different land use types. In contrast, native Australian species are much more sensitive to pollutants and therefore, do not have a wide range of distribution compared to *G.holbrooki*. Furthermore, *G.holbrooki* and the closely related *Gambusia affinis* (western mosquitofish) gonopodia have been shown to be responsive to estrogenic exposure (Angus et al., 2001, Doyle and Lim, 2002, Leusch et al., 2006b, Rawson et al., 2006) which makes the former a good model to investigate its potential as a bioindicator species for Australian aquatic environments.

Endocrine effects on aquatic organisms in Australia is a relatively new area of envi-

ronmental research. Studies that include parallel measurements of estrogenic activity and effects in fish are lacking in Australia. The current study includes measurements of estrogenic and AhR activity of the water from all of the study sites in 2009. The diversity of EACs are enormous and may affect more than one biological signaling system in fish. It is important that studies consider a combination of biomarkers rather than isolated biomarkers to provide a clearer picture of fish health. Information about the effects and interactions of EACs on fish including native species in the Australian environment is hardly known.

1.3 Study Aims

The aim of this thesis was to investigate the effects of estrogenic EDCs on *G.holbrooki* from creeks and rivers around Melbourne, Australia. The thesis also evaluates the suitability of male *G.holbrooki* as a bioindicator species, using the selected biomarkers, for Australian creeks and rivers.

1.4 Chapter Outlines

The thesis investigates three biomarkers of exposure to EDCs that were chosen to be evaluated on male *G.holbrooki*. The individual studies presented in each chapter follows the approach of analyses of morphological indices (gonopodial measurements) as evidence of endocrine disruption in *G.holbrooki*, followed by the addition of two other biomarkers of exposure with parallel water chemistry measurements from the study sites. Sites of concern are identified. The thesis concludes with a discussion on the suitability for the use of *G.holbrooki* as a bioindicator species for Victorian creeks and rivers. The criteria for selection of biomarkers suitable for quick screening of small fish species such as *G.holbrooki* for

biomonitoring contaminated sites is discussed. The chapters of the thesis may have some degree of repetition because chapters were prepared as manuscripts for publication.

Chapter 2 presents gonopodial morphology data from the 3 years of field sampling from sites around Victoria. The chapter correlates estrogenic activity measurements of the sites sampled in the final year (2009) with gonopodial measurements of male *G.holbrooki* captured in that year.

Chapter 3 describes methods for assay development for Vtg induction in male *G.holbrooki* using three commercially available anti-Vtg antibodies. Methods for immunoblots and a semi-quantitative dot blot assay are described in this chapter. Results of Vtg levels in male fish collected from the field sites are discussed in this chapter.

Chapter 4 describes methods for optimization of the EROD assay. Hepatic EROD (CYP1A) activity of male *G.holbrooki* from the field sites were measured and presented in this chapter.

Chapter 5 evaluates the traditional gonadosomatic and hepatosomatic indices (HSI and GSI) and body condition indices for male *G.holbrooki* and describes associations between the three biomarkers (gonopodial measurements, Vtg levels and EROD activity), body indices and estrogenic and AhR activity of the water. The potential for cross-talk between Vtg and EROD activity is investigated here.

Chapter 6 discusses the potential of *G.holbrooki* as a bioindicator species for the Australian aquatic environment. The practicality and validity of the chosen biomarkers for *G.holbrooki* are also discussed here. The chapter concludes the thesis with recommendations for further work on *G.holbrooki* for use as a bioindicator species for the Australian aquatic environment and identifies impacted sites for future monitoring.

Chapter 2

Gambusia holbrooki Gonopodial Indices for Detecting Estrogenic Endocrine Disruption in Melbourne, Victoria

Sections of this chapter have been published.

Chinathamby K, Allinson M, Shiraishi F, Lopata A.L, Nugegoda D, Pettigrove V, and Allinson G. (2013). **Screening for potential effects of endocrine-disrupting chemicals in peri-urban creeks and rivers in Melbourne, Australia using mosquitofish and recombinant receptor-reporter gene assays.** Environmental Science and Pollution Research, 20, 1831-1841.

2.1 Abstract

Male *Gambusia holbrooki* (mosquitofish) were collected from various sites around Victoria for three years to evaluate the performance of gonopodial indices as an estrogenic biomarker for endocrine disruption in Victorian waterways. *G.holbrooki* indices assessed were body length, gonopodial length:body length ratio, R4: 6 ratio, body mass and absence and presence of hooks and serrae. *G.holbrooki* gonopodial indices varied between sites and across

the years. In 2007, GL/BL ratio was largest in fish from Brodies Lake, a reference site. In 2008, Green Lake (Reference) showed significantly smaller gonopodium length: body length ratio compared to some other sites, suggesting some effects of endocrine disruption. R4:6 ratio was shortest in fish from Mullum Mullum Creek (WWTP/Septic) suggesting effects of endocrine disruption. Effects were probably magnified in 2008 at sites receiving effluents because of the severe drought in Victoria, Australia, causing low flows, high evaporation rates and low effluent dilution capacities of the rivers. In 2009, fish from Tourrorong Reservoir (Ref) were the largest. Fish from Merri Creek (Industrial/Urban) had the largest GL/BL ratio and the smallest body size compared to some other sites and fish from Brushy Creek (WWTP) had the largest R4:6 ratio. In 2009, the study was complemented with measurements of estrogenic activity of the water. Measured estrogenic activity was mostly in the range of 0.1 - 1.7ng/L EEQ with the exception of one site (Gisborne WWTP) at 12 ng/L EEQ. GL/BL ratio was not significantly different in fish from Gisborne WWTP although a few adult fish did not complete full gonopodial development. There was no correlation between measurements of estrogenic activity and gonopodium length: body length ratio and body length in 2009. It is recommended that fish populations at Gisborne WWTP, Brushy Creek (WWTP), Kalparrin Wetland (U) and Merri Creek (I/U) are assessed regularly for further analysis of effects of EACs.

It is concluded that *G.holbrooki* gonopodia only fulfills part of the criteria for biomarker selection for screening. It is aninexpensive and easy to perform procedure. However, there is no baseline data at each site to evaluate whether differences observed are a result of natural selection in the population or due to dietary, social differences or estrogenic contaminant exposure at the site.

2.2 Introduction

Organisms in freshwater ecosystems are increasingly subjected to a cocktail of environmental stressors due to anthropogenic pollution. The biggest stressors of water quality decline include discharge of sewage, agricultural, industrial and urban wastewater, fertilizer and agrochemical run-off and leaching of by-products of various industrial and municipal processes (Kungolos et al., 2015). Regular monitoring programs and identification of suitable bioindicator species are necessary to understand the impacts of pollutants on aquatic organisms (Bonacci et al., 2007). However, efforts in environmental monitoring are often challenging. Part of the challenge is the lack of standardized methods and approaches for investigating and assessing impacts such as biological endpoints and the choice of biomarkers and biological assays (Hecker and Hollert, 2011). Another issue is the lack of a combined approach assessing both water samples for hormone activity and biomarkers responsive to contaminants to understand the impacts on organisms as a parallel study (Sumpter and Johnson, 2008).

In Melbourne, different sources of effluents with the potential to cause estrogenic endocrine disruption are discharged into the environment through various land uses. These include agricultural effluents leaching from land and treatment systems, dairy farm effluents, urban and industrial run-off and sewage effluents. When the study was initiated in 2007, information regarding estrogenic activity and concentrations of endocrine active compounds (EACs) in the Victorian aquatic system were scarce. However, recent studies from Scott et al. (2014) and Allinson et al. (2011, 2015) have contributed further to the knowledge of EACs present in creeks and rivers in Victoria, Australia. Although in general, estrogenic activity is low in Victoria, the persistent nature of EACs means that they may exist in the environment for decades and biomagnify or bioaccummulate in freshwater organisms (Ropero et al., 2006, Marino et al., 2012). Mixtures of endocrine disrupting chemicals (EDCs) can act through multiple modes of action and end products of partially degraded EACs may

cause unexpected effects. Interactions with endocrine pathways in fish are complex and the intensity of their effects depend on species, type of EAC, concentration, and time and duration of exposure (Milla et al., 2011).

Fish, as a consumer in the aquatic food chain, is an important bioindicator because effects of contaminants can be measured in terms of biochemical, physiological or behavioural responses (Kumari et al., 2011). However, selecting a suitable species for environmental monitoring can be limiting in terms of abundance and spatial distribution.

G.holbrooki are a noxious species around the world and in Australia, and found in freshwater streams and creeks in Melbourne. Born with an undifferentiated anal fin (gonopodium) which elongates in the male *G.holbrooki* during development, the mature male uses the gonopodium during copulation to transfer sperm into the female (Angus et al., 2001). In female and juvenile male *G.holbrooki*, anal fin rays number 3 through 6 are not different (Figure 2.1). In mature adult males, rays 3, 4 and 5 elongate progressively until twice as long as the other rays in the fin while ray 6 does not elongate. Angus et al. (2001) has reported the ratio between ray 4 and ray 6 at an average of 2.5 times in a normal adult male *G.affinis* and confirms that ray 4:6 ratio does not change once structures at the tip of the gonopodia are visible. Structures at the tip of a fully developed gonopodium consist of a number of serrae and hooks which help the male secure the female during copulation (Figure 2.2). The tip of a developing gonopodium is opaque and the structures are difficult to observe under a microscope. The structures clear up and become clearly visible under a microscope once a gonopodium is developed, signaling permanent cessation of gonopodial elongation and attainment of maturity (Angus et al., 2001). Therefore, sexually mature *G.holbrooki* can be easily identified using a microscope. The development of the gonopodium is under androgenic contol (Turner, 1941). Gonopodial development normally takes between 30 to 50 days to complete (Angus et al., 2005) during which modifications to development and length can occur if fish are exposed to estrogenic EDCs. Importantly, once the gonopodia is

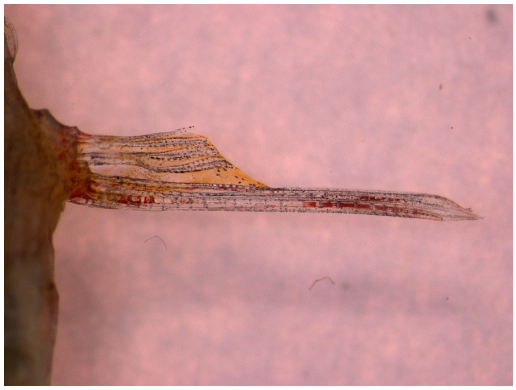
fully developed, exposure to estrogenic compounds does not seem to have an effect (Angus et al., 2005, Doyle and Lim, 2005).

G.holbrooki are ubiquitous and have been used as a bioindicator species for different types of contaminants in many studies all around the world; see Rawson et al. (2010) for review.

Some studies have reported effects to the gonopodia when male fish were exposed to estrogenic chemicals in the laboratory (Dréze and Monad, 2000, Doyle and Lim, 2002, Angus et al., 2005, Rawson et al., 2006), and in the wild (Game et al., 2006, Leusch et al., 2006b) (Table 1.1). While others (Doyle and Lim, 2005, Rawson et al., 2008, Saaristo et al., 2014) have found no effects. Other estrogenic effects reported in *G.holbrooki* include delayed/incomplete gonopodial development, decreased proportion of males in an exposed population, decreased sexual activity, decreased sperm count, and decreased sperm activity and testis size (Batty and Lim, 1999, Dréze and Monad, 2000, Doyle and Lim, 2002, Toft et al., 2003, Angus et al., 2005, Rawson et al., 2008, Edwards and Guillette, 2007, Saaristo et al., 2014). Overall, the hormone-dependant attibutes of the gonopodia, have in part led to *G.holbrooki* being a valuable candidate biomonitor for EDC exposure. Therefore, for quick screening purposes, *G.holbrooki* gonopodia was selected as a cost effective and simple biomarker to detect for the effects of contaminants.

Biomarkers are often considered as early warning systems, however the usefulness of the selected biomarker to trigger further research depends on the suitability of the biomarker (den Besten, 1998, Hanson, 2009).

(a) Adult male *G.holbrooki.*



(b) Fully developed gonopodium showing elongated rays 3, 4 and 5.

(c) Female *G.holbrooki* with gravid spot.

(d) Juvenile fish with undifferentiated gonopodium.

Figure 2.1: a. Adult *G.holbrooki* male, b. Fully developed gonopodium of an adult male, c. Adult *G.holbrooki* female and d. Juvenile fish.

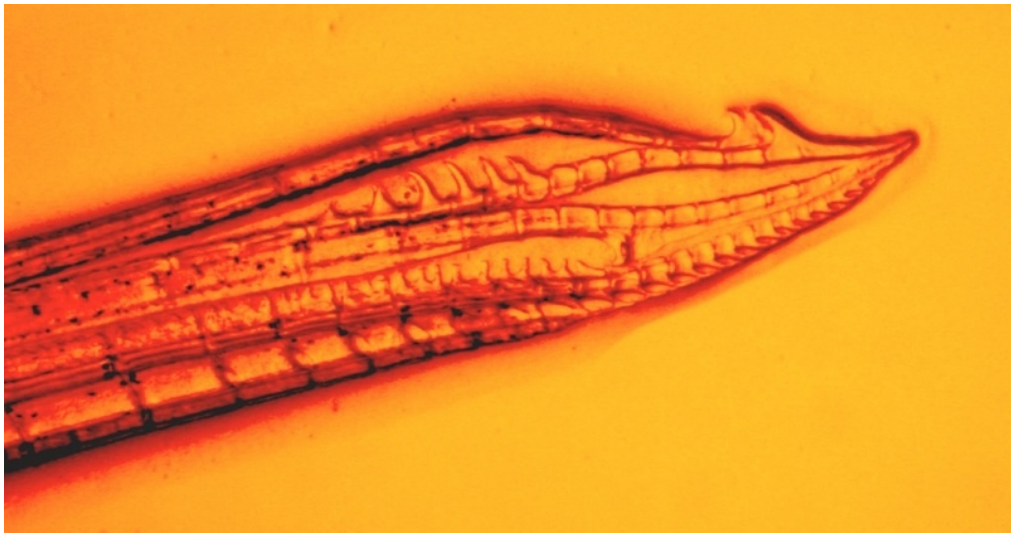


Figure 2.2: Fully developed male *G.holbrooki* gonopodial tip showing serrae and hooks.

van der Oost et al. (2003) describes criteria for biomarker selection:

1. The biomarker should be reliable, relatively cheap and easy to be quantified;
2. In order for the biomarker to be an early warning signal, it should be sensitive to pollutant exposure and/or effects;
3. There should be a baseline data of the biomarker to be able to differentiate between natural variability and pollutant-induced effect;
4. The impact of confounding factors to the response should be clearly understood;
5. ”Dosage and time” mechanisms between biomarker response and pollutant exposure should be established; and
6. Toxicological significance of the biomarker and impact to the organism should be understood;

This study was initiated to assess whether the gonopodia of *G.holbrooki* is a suitable biomarker of exposure for estrogenic contamination. *G.holbrooki* were collected from sites around Melbourne from 2007 to 2009 between February to May. In 2009, the study was complemented with measurements of estrogenic activity in the water at the collection sites. It was hypothesized that impacted areas with high estrogenic activity will be reflected in the traits of the gonopodia of male *G.holbrooki* as previous studies suggest. Sites that may be most impacted by EDCs at the time of the study are also identified.

2.3 Materials and Methods

2.3.1 Study sites

Adult male *G.holbrooki* were collected from February to May between 2007 to 2009 from various land use types such as urban (U), rural (R), wastewater treatment plant impacted (WWTP) and reference sites (Ref.) in Melbourne and Victoria, using a dip net. Figure 2.3 shows the location of sites in 2009. In some cases, land use type grouping is somewhat arbitrary, since some creeks flow through residential and industrial or agricultural land and are likely to contain a combined source of pollutants. A minimum of 6 to a maximum of 34 fish were collected from each site. Factors for site selection were based on accessibility and availability of *G.holbrooki* populations. In 2007, 13 sites were selected out of which one was a reference site (Brodies Lake). In 2008 and 2009, 16 sites were selected with four sites, including Brodies Lake, representing reference sites.

Fish were euthanased (blow to the head), transported on ice to the RMIT University Ecotoxicology laboratory and stored in 70% ethanol for later morphometrical measurements. Sample numbers vary in year two and three because a subset of sampled fish were kept for protein analysis (Chapter 3). In 2008, the severe drought in Victoria affected water flow at the study sites. Some sites were either dry or without fish and were replaced by new sites. The drought continued in 2009 with severe bush fires impacting more sites in the summer of 2009. These were again replaced by other suitable sites. Tables 2.1, 2.2 and 2.3 list the fish collection sites sampled in 2007 to 2009.

Table 2.1: Land use around study sites in **2007.** *Includes site that is industrial. **Site receives septic and greywater discharge.

Reference	Rural	Urban	WWTP
Brodies Lake	Lerderderg River	Kalparrin Wetland	Aikten Creek
	Coliban River	Shankland Wetlands	Brushy Creek
	Deep Creek	Settler's Orchard Reserve	Mullum Mullum Creek**
		Tikalara Park	
		Ruffey Lake	
		Wesgate Park Lake*	

Table 2.2: Land use around study sites in **2008**. *Includes sites that can be classified as both rural and urban or urban and industrial. **Site receives septic and greywater discharge.

Reference	Rural	Urban	WWTP
Brodies Lake	Campaspe River	Kalparrin Wetland	Gisborne
Sanitarium Lake	Deep Creek	Merri Creek*	Sunbury
Green Lake	Lerderderg River	Kororoit Creek*	Werribee
Liverpool Freshwater-	Ryans Creek*		Mullum Mullum
Retarding Basin			Creek**
			Brushy Creek

Table 2.3: Land use around study sites in **2009**. *Includes sites that can be classified as both rural and urban or urban and industrial.

Reference	Rural	Urban	WWTP
Brodies Lake	Campaspe River	Kalparrin Wetland	Gisborne
Sanitarium Lake	Deep Creek	Merri Creek*	Sunbury
Toorourrong Reservoir	Watson's Creek	Kororoit Creek*	Werribee
Liverpool Freshwater-	Woori Yallock Creek*		Brushy Creek
Retarding Basin	Narre Warren-		
	Retarding Basin*		

2.3.2 Water sampling

Water samples were collected as 'grab' or spot samples at the 16 sites in 2009 at the time of fish collections and analysed for estrogenic activity at the Department of Primary Industries Laboratory by Dr. Mayumi Allinson and team. Samples were directly collected in glass bottles, stored on ice, and then at 4°C until processed (generally within 36 hours of collection). Physiochemical measurements of the water were also recorded at the time of sampling.

2.3.3 Methods for estrogenic activity measurements

For each site, an aliquot of the effluent (1L) was extracted for the measurement of receptor (hormonal) activity using a yeast based bioassay. Methods for sample preparation are described in Allinson et al. (2008) and Chinathamby et al. (2013). Briefly, sample preparation included filtration and adding buffer solution to the sample to ensure an acid pH, filtration through GF/C filters to remove particulate matter and then solid phase extraction. The sam-

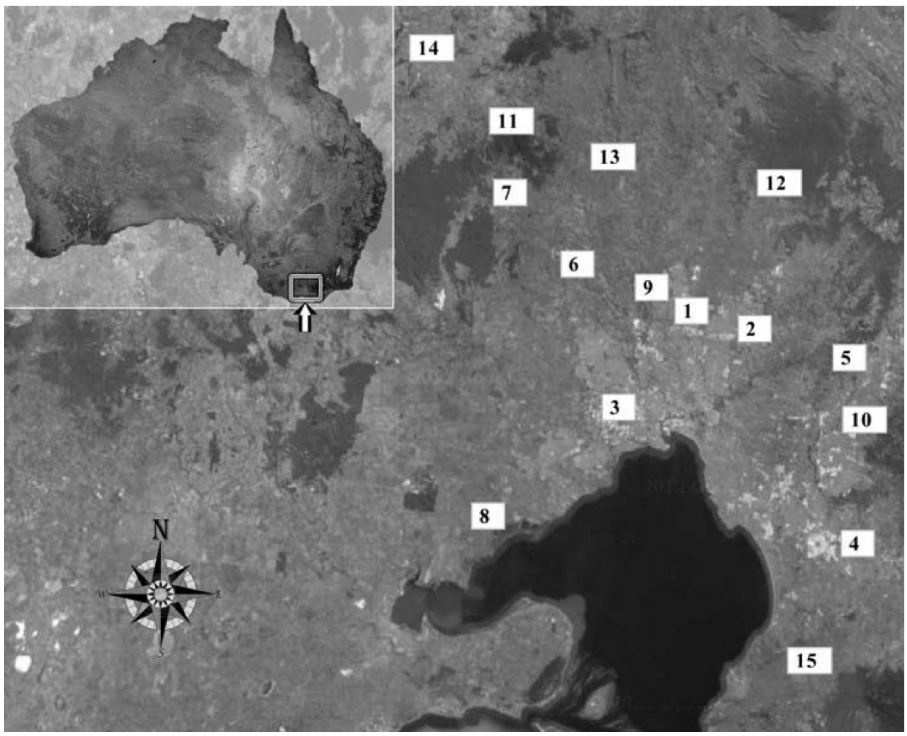


Figure 2.3: Approximate locations of sampling sites (squares with numbers) in 2009 in Melbourne and Victoria. Numbers in map correspond to site numbers in Table 4.3. The figure was generated using Community Walk, an online mapping application (www.communitywalk.com).

ple was re-suspended in a mixture of 3:1 hexane:dichloromethane fraction (A/D) and finally a methanol fraction (MeOH) after elution of analytes with methanol and evaporation. For all samples, elution protocols separated the extract into three fractions, first a 3:1 H/D fraction, second a 1:9 acetone:dicholoromethane fraction (A/D) and finally a methanol fraction (MeOH). The A/D fraction contained the steroid hormones, and the separation was undertaken to minimize the effects of matrix components on the ELISA and bioassay systems.

2.3.4 Fish morphometrics

Adult male fish were examined under a dissecting microscope for structures (presence of hooks and serrae) on the tip of the gonopodium which indicates sexual maturity (Angus et al., 2001). The appearance of the termination complex at the tip of the gonopodium which consists of structures on rays 3,4 and 5, indicates sexual maturity (Turner, 1941). The tip of the gonopodium with the structures are opaque and difficult to observe in a maturing male. However, the structures clear up and become identifiable under a dissecting microscope just before and at maturation (Angus et al., 2001). Sexually mature male fish were selected, based on the presence of the terminal structures, for morphometric measurements.

Body length (BL) was measured using a caliper to the nearest 0.01 mm from snout to caudal peduncle. Gonopodial length (GL) defined as the length from the anterior base of the anal fin to the gonopodial tip was measured under a Wild M3Z Heerbrugg stereozoom microscope using an objective micrometer to the nearest 0.01mm. Gonopodial length/body length ratio (GL/BL) was obtained from the above measurements as described in Game et al. (2006). Game et al. (2006) used body length to normalize the measurements for the length of the gonopodium to account for different sizes of fish. The length of gonopodial rays 4 and 6 were also taken as a measure of the elongation index of the ratio of rays 4:6 (R4:6) as described in Angus et al. (2001) and Doyle and Lim (2002). Ray 6 does not elongate

(Angus et al., 2001). Angus et al. (2001) reports that ratio of rays 4:6 does not change in males after the structures of the termination complex become visible. In fact, the presence of these structures signals the permanent cessation of gonopodial elongation (Angus et al., 2001). Absence of hooks and serrae at the tip of the gonopodia was also noted in males that were larger in length than the smallest sexually mature fish as described in Game et al. (2006). This was to ensure that males that delayed sexual maturity because of endocrine effects from pollutants were not excluded from the study.

Fish whole bodies were weighed with an A&D analytical scale to the nearest 0.001 mg. Fish age was not determined as male *G.holbrooki* are short lived in temperate parts of Australia (Pen and Potter, 1991). Dependent variables included body length (BL), gonopodium length: body length ratio (GL/BL), elongation ratio (R4:6) and body mass (g).

2.3.5 Statistical Analysis

All statistical analyses were performed using PASW 18 for Macintosh (New York, USA). BL was log transformed if necessary and then tested using a single factor analysis of variance (ANOVA) for differences between sites.

Since land-use type classification was somewhat arbitrary (some sites are rural and urban or urban and industrial), land-use type could not be used as a factor in statistical analysis. Therefore, a nested ANOVA could not be performed. All ratio variables were log transformed and then tested using a single factor ANOVA to evaluate differences between sites. A post hoc Dunnet's test was used to identify differences between means. Pearson's correlation coefficient was used to test for differences between estrogenic activity, mean GL/BL ratio and mean BL of fish from the 2009 sites. Data for measured biological endpoints are presented as mean$\pm$standard error (SEM).

A power analysis to estimate adequate sample size was not conducted because meaningful effect size could not be determined (Hoenig and Heisey, 2001).

2.4 Results

2.4.1 Measurements and gonopodial indices of fish collected in 2007

Mass of male fish were significantly different between sites (ANOVA, $F_{12,278}$=22.141, $P < 0.01$). This ranged from a low of 0.075 g at Brodies Lake (Ref) to the highest of 0.224 g at Kalparrin Wetland (U) (Table 2.4). Fish from Kalparrin Wetland were significantly heavier than fish from 6 other sites $P < 0.01$). BL of male *G.holbrooki* differed between sites (ANOVA, $F_{12,277}$=47.116, $P < 0.01$). Brodies Lake (Ref) fish were also significantly smaller in length (14.68 mm) compared to those at all other sites except Shankland Wetlands (U) (15.38 mm) ($P < 0.05$). The largest fish were measured at Aikten Creek (WWTP) at 21.33 mm. Comparisons between GL/BL ratio in fish showed that these were also significantly different between sites (ANOVA, $F_{12,274}$=66.167, $P < 0.01$). The largest GL/BL ratio was in fish from Brodies Lake (Ref) (0.44) and the smallest ratio was in fish from Coliban River (R), Lerderderg River (R) and Kalparrin Wetland (U) (0.33) ($P < 0.05$). Effectively, male fish from Brodies Lake had the largest gonopodia in relation to body size despite being the smallest and lightest. Brodies Lake (Ref) male *G.holbrooki* were also the lightest compared to those from all other sites except for fish from Coliban River (R) at 0.088 g ($P < 0.01$). Similarly, fish from Shankland Wetlands (U) had significantly larger GL/BL ratio (0.41) ($P <0.05$) compared to fish from all other sites but significantly smaller than Brodies Lake fish (Ref) (0.44) (See Appendix A for Brodies Lake site picture).

There were no significant differences between R4:6 elongation ratio in fish from all sites (ANOVA, $F_{12,283}$=1.075, $P > 0.5$).

2.4.2 Measurements and gonopodial indices of fish collected in 2008

There was no consistent pattern when considering significant differences in BL, GL/BL ratio, R4:6 ratio and mass of fish between sites in 2008. BL of male *G.holbrooki* was significantly different between sites (ANOVA, $F_{15,315}$=6.995, $P < 0.01$). The shortest male fish was measured at Ryans Creek (I/R) (18.95 mm) and they were signficantly shorter than fish at Sunbury WWTP (21.89 mm) $P < 0.05$) (Table 2.5).

Mass of male *G.holbrooki* also differed significantly between sites (ANOVA, $F_{15,313}$=9.539, $P < 0.01$). Male fish were lightest at Ryans Creek (0.081 g) and the heaviest fish were from Green Lake (Ref) (0.153 g) and Sunbury WWTP (0.152 g). GL/BL ratio of male *G.holbrooki* was significantly different between sites (ANOVA, $F_{15,315}$=7.898, $P < 0.01$). This also varied among the four reference sites. Fish from Sanitarium Lake (Ref) had significantly larger GL/BL ratio at 0.35 compared to fish from Green Lake (Ref) at 0.30 ($P < 0.05$). Fish from both those sites were also significantly different to both Liverpool FRB and Brodies Lake reference sites at 0.32. This ranged from 0.30 to 0.35 at the four reference sites. Fish from Green Lake (Ref) had smaller GL/BL ratio than Merri Creek (I/U), Kalparrin Wetland (U), Sanitarium Lake (Ref), Deep Creek (R), Lerderderg River (R), and Ryan's Creek (I/R) ($P < 0.05$). There were no significant differences among the four WWTP sites in GL/BL ratio of fish ($P > 0.05$). Both Gisborne WWTP and Werribee WWTP fish also had smaller GL/BL ratios, although this was only marginally significant compared to fish from other sites.

R4:6 elongation ratio of male *G.holbrooki* were also significantly different between sites (ANOVA, $F_{15,315}$=7.524, $P < 0.01$). This ranged from 2.67 at Mullum Mullum Creek (WWTP) to 3.50 at Gisborne WWTP. Fish from Mullum Mullum Creek (WWTP) had significantly smaller R4:6 ratio compared to those from 11 other sites ($P < 0.05$). Fish from Gisborne WWTP had significantly larger R4:6 ratio compared to fish from only three other

sites, Sanitarium Lake (Ref), Ryans Creek (I/R) and Lerderderg River (R) (P <0.05).

2.4.3 Measurements and gonopodial indices of fish collected in 2009

GL/BL ratio of fish differed significantly between sites (ANOVA, $F_{15,223}$=4.214 $P < 0.01$) (Table 2.6). Fish from Merri Creek (I/U) had the largest GL/BL ratio of 0.40, significantly larger than fish from 8 other sites ($P < 0.05$). Although, the smallest GL/BL ratio was measured in fish from Gisborne WWTP, this was not significantly different compared to fish from the other sites because of a high SE. BL of fish differed significantly between sites (ANOVA, $F_{15,223}$=10.852 $P < 0.01$). This ranged from 16.27 mm at Merri Creek (I/U) to 21.47 mm at Tourrorong Reservoir (Ref). Fish from Merri Creek (I/U) were significantly smaller in size than fish from Tourrourong Reservoir (Ref), Werribee WWTP, Watson's Creek (R), Kalparrin Wetland (U), and Kororoit Creek (I/U) ($P < 0.01$). Fish from Tourrorong Reservoir (Ref) were significantly larger than fish from all other sites($P < 0.01$) and were heavier compared to fish from ten other sites ($P < 0.05$).

Male *G.holbrooki* R4:6 elongation ratio differed significantly between sites (ANOVA, $F_{15,223}$= $P < 0.01$). However, the only significant difference was in fish from Brushy Creek (WWTP) which had the longest R4:6 ratio at 2.86 compared to fish from all other sites (See Appendix A for Brushy Creek site picture).

2.4.4 Gonopodium/body length ratio of fish collected from 4 sites in 2007 to 2009

Four out of all the sites were less affected by the drought in 2008 and 2009 and *G.holbrooki* continued to persist at these sites. These were Brodies Lake (Ref), Brushy Creek WWTP, Kalparrin Wetland (U) and Deep Creek (R). In 2007, GL/BL ratios of fish ranged from 0.35

to 0.44 (Figure 2.4). This is because fish from Brodies Lake and Brushy Creek showed larger GL/BL ratios than fish from the other 2 sites. GL/BL ratio of fish from Kalparrin Wetland and Deep Creek remained relatively consistent in the 3 years.

In 2007, fish BL varied considerably from 14.68 mm to 21.14 mm with Brodies Lake fish having the smallest BL (Figure 2.5). Fish BL in general were bigger in 2008 except for Kalparrin Wetland. In 2009, fish BL were slightly smaller compared to 2008.

2.4.5 Estrogenic activity at sites in 2009

Collection sites in 2009 showed levels of estrogenic activity ranging from 0.1 - 1.7 ng/L EEQ except for Gisborne WWTP which was at 12 ng/L EEQ. The other three WWTP sites showed levels between 0.3- 0.9 ng/L EEQ (Table 2.7). Two sites that had levels above 1ng/L EEQ are Liverpool FRB (Ref) and Narre Warren SD (R/U).

GL/BL ratios for fish collected in 2009 were not correlated with estrogenic activity (r = -0.400, n =16, $P > 0.5$). Male BL of fish collected in 2009 were also not correlated with estrogenic activity (r = -0.246, n = 16, $P >$0.5).

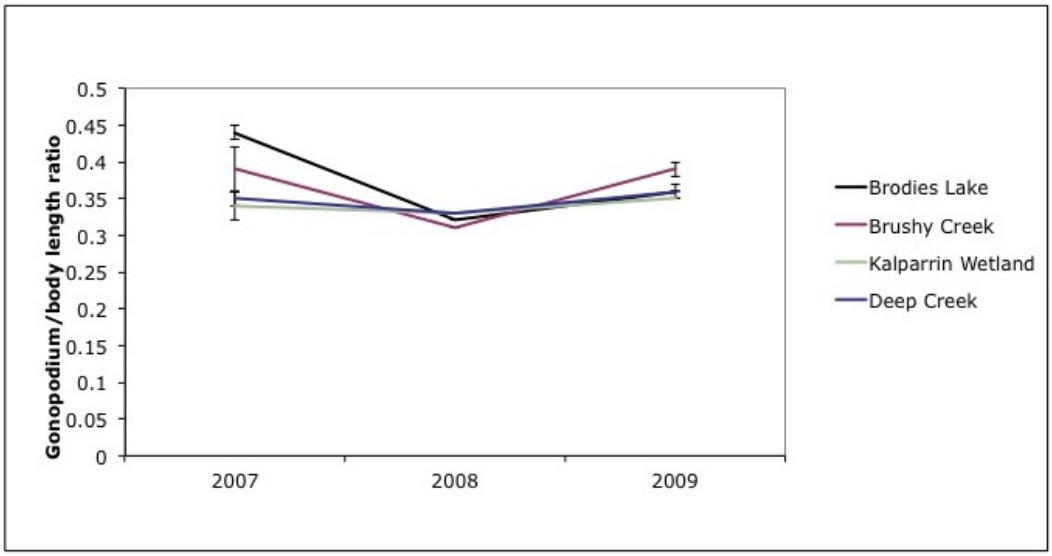


Figure 2.4: Mean (±SEM) gonopodium/body length ratio of male *G.holbrooki* over 3 years.

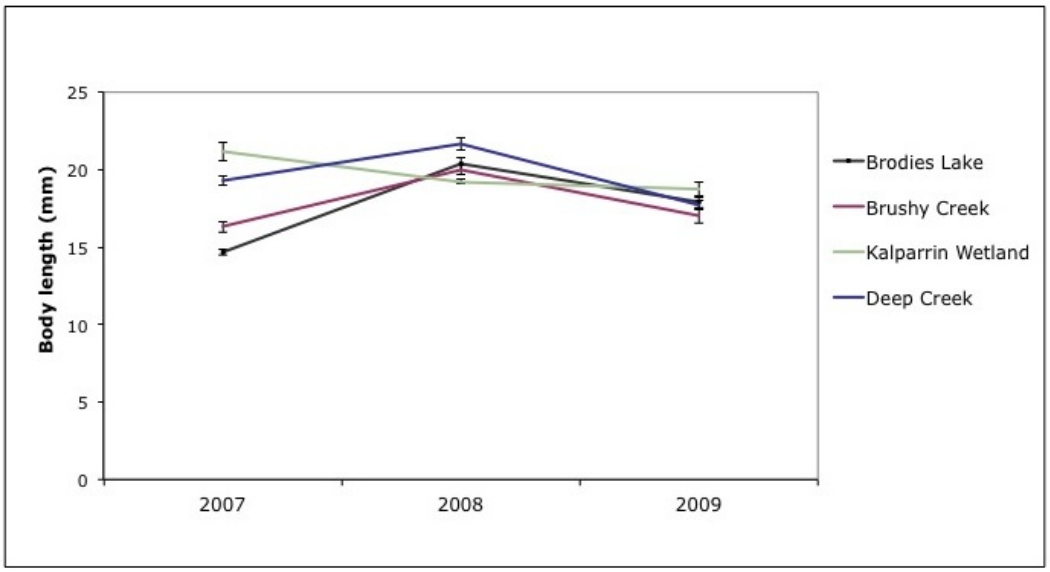


Figure 2.5: Mean (±SEM) *G.holbrooki* male body length over 3 years.

2.5 Discussion

The current study was initiated in 2007 with a "bottoms up" approach of determining if effects could be observed in male *G.holbrooki* in the wild in Victoria before undertaking chemical water analyses of the sites in the final year of study. Sites were grouped into categories representing different land use types such as reference, urban, rural and wastewater treatment plant impacted sites. However, each site is now treated separately because later data (CAPIM, Unpublished data 2009) revealed estrogenic activity and EACs in surface water and sediment of the two reference sites, Brodies Lake and Liverpool FRB, selected for this study. Some other sites were reclassified into more than one land use types because of the various types of land use activities surrounding the area around the sites. Furthermore, Scott et al. (2014) reported the detection of EE_2, traditionally expected in WWTP effluents, in a number of land use types such as agricultural, residential, and industrial areas suggesting that contamination can be widespread and may not be limited to land use types.

Measuring male *G.holbrooki* gonopodia is an inexpensive and easy to perform procedure with a microscope and is suitable for large sample numbers. A wealth of results were obtained in this study. However, results suggest that the selected indices for *G.holbrooki* for the purpose of this study may be sensitive enough to be influenced by natural variability, making interpretation difficult. A number of studies have reported that fish size and gonopodial indices are influenced by a number of factors such as population density and the social environment as well as environmental factors such as temperature. For instance, Meffe (1992) demonstrated that *G.holbrooki* grew faster and larger at 25°C than at 32°C. In 2009, Tourrorong Reservoir (Ref) had the highest temperature at 25°C and also significantly larger fish compared to all other sites. It is likely that fish at the site had the opportunity to

attain a larger size due to the favourable conditions at the reservoir.

The maximum BL in 2007 was measured in male fish at Aikten Creek (WWTP). In 2008, the maximum BL was found in fish from Sunbury WWTP. WWTP effluents are typically rich in organic matter and a good source of nutrients (Subramani et al., 2014). A nutrient-rich diet at those sites may have attributed to the large sizes of fish at both Aikten Creek and Sunbury WWTP. However, not all WWTP sites across the three years showed a trend in larger sized males.

The factors that influence size at which male *G.holbrooki* reach maturity at the sites in this study are ambiguous. Zulian et al. (1995) found that mean size of mature males is dependent on population density. Males tended to attain larger sizes and matured later in high density populations when inter and intra-sexual selection was present. An earlier study by Hughes (1985) suggested similarly that social influence as well as a competition-predation mechanism was a factor in timing of male maturation and body size at maturation in a free-living population of *G.affinis*. In that study, males grew to a larger size when faced with intra-specific competition from other males. However, Bisazza and Marin (1995) found that small males take less time to mature sexually and that small size is favoured in populations with female-biased sex ratios or low density (Zulian et al., 1995). Maturing faster lengthens reproductive life and reduces mortality before reproduction.

In 2007, results showed that fish from Brodies Lake (Ref) were smaller compared to fish from all other sites, followed by fish from Shankland Wetlands (U). It is possible that the population at Brodies Lake and Shankland Wetlands were female-biased, therefore accelerating maturity of males at a smaller size. It is unlikely that further growth in males from the two sites was attained after maturity as male *G.holbrooki* exhibit minimal growth after sexual maturity (Zulian et al., 1993, Livingston et al., 2014). Ultimately, it is unknown as to why male fish at Brodies Lake (Ref) and Shankland Wetlands (U) chose to mature at

a smaller size compard to fish from the other sites. Other unknown confounding environmental and social factors could have also accelerated fish maturity at Brodies Lake (Ref) and Shankland Wetlands (U) in 2007. In a study by Saaristo et al. (2013), male *G.holbrooki* captured from Brodies Lake (Ref) in the summer of 2013 were found to be much larger at approximately 26 mm in length. In subsequent years of sampling (2008 and 2009) in the current study, male fish at Brodies Lake (Ref) were not significantly smaller than fish from the other sites. It is known that after the sampling in the current study was completed, EACs and heavy metals were detected in surface water and sediment at Brodies Lake (CAPIM, Unpublished data 2009). However, it is more likely that social factors contributed to small sizes and large gonopodia at Brodies Lake (Ref) as the fish captured in the later years did not show similar effects.

In this study, male *G.holbrooki* minimum BL varied for each year. In 2007, *G.holbrooki* was 14.68 mm while in 2008, minimum BL was bigger at 18.95 mm. In 2009, fish minimum BL was 16.27 mm. On the other hand, maximum BL of fish remained at an average of around 21 to 22 mm. Fish BL from the current study are comparable to that of Game et al. (2006) where wild male *G.holbrooki* collected from five water-bodies in Western Australia had BL between 20.6 to 21.9 mm. Another study reported size at maturity of wild *G.affinis* (closely related to *G.holbrooki*) at an average of 13.3 mm in southeastern Louisiana (Martin and Leberg, 2011). In yet another study by Zulian et al. (1995), male *G.holbrooki* BL of seven different populations in seven different locations in Italy ranged between 18.6 mm to 20.7 mm which is also within the range of BL of fish in the current study. The natural variation in BL among sites in this study can be attributed to male *G.holbrooki* exhibiting developmental plasticity to take advantage of varying social and environmental conditions (Zulian et al., 1993). Regardless, Locatello et al. (2008) found that BL of male *G.holbrooki* did not correlate with sperm ejaculate quality (such as length, speed, viability and longevity), thus male BL would not reflect their reproductive success.

Developmental plasticity of *G.holbrooki* gonopodia has been shown in a study where male fish exhibited shorter gonopodia in systems with predators. Possessing larger gonopodia seemed to incur a cost of slower burst-swimming speed necessary for anti-predatory behaviour (Langerhans et al., 2005), however, genetic differences among populations could have contributed to the differences as offspring of fish collected in the wild and reared in the laboratory also showed the same results. In contrast, another study showed that high predatory sites were inhabited by male guppies with longer gonopodia (Kelly et al., 2000). A study by (Jennions and Kelly, 2002) similarly showed that *Brachyrhaphis episcopi* (Poecillidae) males with longer gonopodia were found inhabiting sites with more predatory fish. However, in that study, confounding reasons such as water velocity in upstream versus downstream sites have also been proposed.

In 2007, male *G.holbrooki* GL/BL ratio were largest in fish from Brodies Lake (Ref) and Shankland Wetlands (U) at 0.44 and 0.41 respectively. In 2009, fish from Merri Creek (I/U) also had a large GL/BL ratio of 0.40. However, in 2008, GL/BL ratio of fish from all sites did not exceed 0.35. Game et al. (2006) reported a GL/BL ratio of 0.29 to 0.33 in male *G.holbrooki* captured from water-bodies in Western Australia. Similarly, a study by Horth et al. (2010) reported that male *G.holbrooki* gonopodia were 34.9% of BL of melanic males. Brodies Lake is a reference site with a generally healthier aquatic biota and is part of a series of ponds in Greenvale Reservoir. In the current study, the larger GL/BL ratio in fish from Brodies Lake (Ref) may be suggestive of phenotypic differences of the gonopodia of male *G.holbrooki* in response to predators. Shankland Wetlands (U) and Merri Creek (I/U), on the other hand, receive urban and industrial run-off respectively. Merri Creek flows through pastural, urban and then through industrial land where fish were collected at point source of outflow from a drain at an industrial site. It is suggested that androgenic exposure at the sites is likely and may have affected fish gonopodia. Androgenic activity has been previously detected in the Australian aquatic environment. Leusch et al. (2006a) measured androgenic activity in 15 WWTPs in Queensland while other studies (Allinson et al., 2008, Scott et al.,

2014) did not find any evidence of androgenic activity in Australia. Leusch et al. (2006b) found that male *G.holbrooki* living immediately downstream of an effluent discharge site had longer gonopodia which was suggested to be a result of androgenic effects. Leusch et al. (2014) again reported that male *G.holbrooki* downstream of the discharge site were found to have longer gonopodia compared to fish from the reference site although no detectable levels of androgenicity were found at the sites. More importantly, a recent study by Saaristo et al. (2014) suggested the presence of androgenic activity in Victorian aquatic environments as male *G.holbrooki* captured from sites in Victoria were observed to have an increase in mating activity towards females compared to males from the reference site.

Batty and Lim (1999) reported shorter gonopodia in male *G.holbrooki* sampled downstream of a sewage treatment plant in New South Wales. The study also reported gonopodium length as being 25% of body length in fish affected by sewage effluents downstream of the discharge site. In the current study, the shortest gonopodium length was 30% of body length (GL/BL ratio) in fish at Green Lake (Ref) sampled in 2008, although only significantly shorter than in fish from 6 other sites. The observed effects may be suggestive of estrogenic exposure and may be not be as large as observed in the study by Batty and Lim (1999). However, a recent study by Livingston et al. (2014) reported that *G.holbrooki* males that were treated to low food availability for 3 weeks attained the body size of control males after the treatment ceased but developed shorter gonopodia as a result. Green Lake is located in the 100 Acres bushlands Reserve in Park Orchards. The lake was fairly small, shallow and over-populated with *G.holbrooki* in 2008. Fish in Green Lake were likely facing high competition and low food resources and together with a fast drying lake during the drought of 2008, incurred a sexually selected cost of a shorter gonopodia as a result of delaying sexual maturation to attain the ideal body size. Furthermore, Green Lake was originally selected as a reference site because of its location in the reserve, however, the lake receives unsewered wastewater directly from drains servicing the surrounding households around Park Orchards (Philpot and Daniel, 2011). Septic tank systems are utilized to treat domestic

wastewater from the laundry, kitchen and bathrooms. Unmaintained septic tanks increase the probability of highly polluted domestic wastewater discharge into the lake (Philpot and Daniel, 2011). The lake was fairly shallow in 2008 and had dried up in 2009, therefore further sampling at the site was not possible to determine the reasons for the shorter gonopodia in fish at that site.

The lowest GL/BL ratios were measured in fish captured in 2008 from Green Lake (Ref), Gisborne WWTP and Werribee WWTP compared to the other years, although this was only marginally significant in fish from Gisborne and Werribee WWTP compared to other sites. The drought in Victoria in 2008 may have impacted on maturity in fish from those sites. Particularly, Jackson's creek which receives point source effluents from the WWTP at Gisborne had dramatically reduced water levels and low flows in the river compared to 2009. Decreasing flow rates due to a lack of rainfall combined with high evaporation rates during the summer of 2008 and increasing effluent loads may have increased the concentrations of the discharges at both Gisborne and Werribee WWTP. No estrogenic activity measurements were conducted at these sites in 2008, therefore, it is not possible to determine if fish were impacted by EACs at the WWTP sites.

In the three years of fish collections, the lightest fish were from Brodies Lake (Ref) in 2007 at 0.075 g and the heaviest fish were from Kalparrin Wetland (U) at 0.224 g in 2007. The wide range in fish weight may be attibuted to a number of factors such as diet. Meffe and Crump (1987) reported that cannabilistic *Gambusia spp.* grew faster than *Gambusia spp.* raised on various other diets. Fish collected at Kalparrin Wetland (U) were captured at the end of a stormwater drain in a shallow and small pool of water located at the gross pollutant trap which contained urban rubbish such as plastic bags. The small habitat with poor quality water at Kalparrin Wetland (U) may have induced a cannibalistic diet compared to the other habitats such as lakes, creeks and rivers where diets are more varied. This cannibalistic characteristic of *G.holbrooki* was observed in holding tanks at the RMIT University

aquaculture facility. Temporal comparisons between results were not made since fish were sampled only once a year and did not account for seasonal variations.

In 2009, fish from Brushy Creek (WWTP) exhibited significantly longer R4:R6 ratio of 2.86 compared to fish from all other sites, perhaps suggesting exposure to androgenic pollutants at that site. In 2008, fish from Gisborne WWTP had a slightly elongated R4:6 ratio. This is similar to results reported by both Angus et al. (2002) and Leusch et al. (2006b) where fish captured downstream of a treatment plant had longer R4:6 ratios. Angus et al. (2001) reported that R4:6 ratio averages were 2.5 while Leusch et al. (2006b) reported a longer R4:6 ratio of $>$2.60 in male *G.holbrooki* at sites receiving WWTP effluents compared to reference sites (2.54). Brushy Creek (WWTP) may benefit from continued monitoring of fish populations and perhaps measurements of androgenic activity of the water.

Game et al. (2006) reported R4:6 ratio in fish from wetlands in Western Australia at less than 2.2 suggesting endocrine disruption, to approximately 2.6. In contrast, in the current study, the shortest R4:6 ratio was at 2.67 in fish from Mulllum Mullum Creek (WWTP) which is much higher than the minimum reported in the study by Game et al. (2006). Shorter R4:6 ratio was previously reported in male *G.holbrooki* exposed to 100 and 500 ng/L E_2 where fish exposed to the later concentration showed no elongation of the anal fin (Doyle and Lim, 2002).

GL/BL ratio of fish sampled from the 4 sites, Brodies Lake (Ref), Kalparrin Wetland (U), Brushy Creek (WWTP) and Deep Creek (R), over the 3 years varied most in 2007. This could be due to environmental or contaminant effects at Brodies Lake and Brushy Creek in 2007 which may have impacted on GL/BL ratios of fish from those sites.

In 2008, larger sizes were observed in fish from Brodies Lake, Brushy Creek and Deep Creek. It is possible that fish at the sites that year may have delayed sexual maturity until a larger body size was achieved. Livingston et al. (2014) found that sexual maturity was de-

layed in fish that were on a low food diet until optimal body sizes were achieved suggesting that this may be the case for fish at most sites in 2008 because in general GL/BL ratios were smaller and body sizes were larger that year compared to 2007 and 2009. The 4 sites have different hydrologies, therefore, it is hard to suggest why fish at the sites may have delayed maturity until a larger size was achieved. The only suggestion would be that food may have been limited due to the drought at the sites. The fish collected in the three years were new cohorts as male *G.holbrooki* rarely overwinter in temperate waters (Pen and Potter, 1991).

Estrogenic activity was observed at all 4 WWTP effluent impacted sites and in low levels at the reference, rural and urban sites. Gisborne WWTP was the most impacted at 12 ng/L EEQ while the rest of the sites showed levels of estrogenic activity ranging from 0.1 - 1.7 ng/L EEQ . There was a leak in the sewage system at Gisborne WWTP a week before fish were sampled. High estrogenic activity of 12 ng/L EEQ was measured in water samples after post-spillage clean-up was well underway, suggesting that levels during the leak would have been much higher. Although, GL/BL ratio was lowest in fish from Gisborne WWTP, this was not found to be significant. No correlation was found between estrogenic activity in the water and both GL:BL ratios and BL of fish. However, for effects to be observable, juvenile male fish would have to be exposed to estrogenic pollutants during development as the gonopodia, once developed, will not be affected (Angus et al., 2005). As fish were sampled a week after the leak at Gisborne WWTP, it is possible that adult male fish with fully developed gonopodia were captured and effects were not evident at the morphological level. However, it should be mentioned that 5 out of 30 adult male fish at Gisborne WWTP did not have hooks and serrae. Angus et al. (2005) demonstrated that juvenile male *G.affinis* exposed to increasing concentrations of EE_2 in the laboratory failed to complete gonopodial development suggesting that perhaps the affected fish with fully grown gonopodia at Gisborne WWTP failed to attain sexual maturity because of the high estrogenic activity. Furthermore, high acute toxicity may have caused other immediate effects in biomarkers not examined in this study. Saaristo et al. (2014) reported total

estrogen concentrations at Jackson's Creek near the WWTP at 8.97 and 13.19 ng/E_2eq/L in March and May of 2011 respectively, 2 years after the current study. A direct comparison of biological effects between fish in the wild in response to total estrogen and estrogenic activity is not possible because they represent different measures. It is necessary to conduct further and more frequent collections of fish at Gisborne WWTP to identify if fish health may be impacted.

Grab water samples were obtained from the sites at the time of fish collections for measurements of estrogenic activity. Estrogenic activity was not correlated with GL/BL ratios and BL of fish collected in 2009. This interpretation has been made cautiously because of the potential for temporal variation in estrogenic concentrations. Vermeirssen et al. (2008) reported variability in EEQ levels in Swiss river water at one single effluent source over a 48 day sampling period. Therefore, more water samples are required at the sites to estimate variability in estrogenic activity in water.

A number of studies have reported varying effects on fish with corresponding measurements of estrogenic activity in the water. A study conducted in Switzerland by Burkhardt-Holm et al. (2008) reported levels between 0.2 - 2 ng/L EEQ in the rivers and found no direct effects on reproductive and developmental parameters measured in wild brown trout. However, hepatic levels of Vtg mRNA were induced in caged brown trout downstream of the sewage treatment plant while Vtg protein levels (an egg protein found in reproductive females) were elevated in feral brown trout. Another study in Switzerland (Vermeirssen et al., 2005) reported estrogenicity levels in 18 rivers during winter and summer ranging from 0.3 - 2.0 ng/L EEQ and 4.0 - 7.0 ng/L EEQ respectively. In that study, plasma Vtg levels were also elevated in brown trout in 5 of the sites with the highest plasma Vtg levels found at one site with the highest reading of 7.0 ng/L EEQ. However, population effects were evident when levels of 0.19 - 1.9 ng/L EEQ of EE_2 were added to a lake in Canada over a 7 year period causing elevations in Vtg mRNA and protein levels and impacts on go-

nadal development in male fathead minnow and consequently leading to the collapse of the population (Kidd et al., 2007). Although sites selected in this study showed fairly low levels of estrogenic activity, studies mentioned above suggest that even low levels are capable of causing varying levels of effects that may not be morphological in nature.

2.6 Conclusion

In the field, the investigation of gonopodia appear to be a sensitive morphological biomarker of estrogenic exposure if exposure occurs during the period of sexual maturation for male fish. However, *G.holbrooki* are also highly plastic and adaptable to a variety of water conditions making interpretations of morphological characteristics complicated. In this study, confounding factors such as temperature, individual site history, predator absence/presence, extreme weather conditions and other factors may have influenced indices selected for this study. Estrogenicity levels in some of the sites are capable of inducing effects in fish, however effects were variable in this study. It is recommended that baseline data be obtained from the sites to better understand natural variability in the gonopodia of male fish in order for it to be used as a reliable biomarker. It is also recommended that a multi-assessment method be used to examine estrogenic effects in *G.holbrooki*. Periodic measurements of estrogenic activity in water are necessary in order to identify sites that have recurring high levels. It is important to also understand the natural variability of the population at each site if further research is to be conducted. It is necessary to include a suite of biomarkers to properly evaluate effects of endocrine disruption acting on different levels in fish as effects on the gonopodia cannot be seen if adult male *G.holbrooki* with fully developed gonopodia are exposed to estrogenic contaminants. It is recommended that fish populations at Gisborne WWTP, Brushy Creek (WWTP), Kalparrin Wetland (U) and Merri Creek (I/U) are assessed regularly for the effects of EACs.

Table 2.4: Male *G.holbrooki* 2007 morphometrics (mean±SEM). *denotes sites that are significantly different (P >0.5) to Brodies Lake (Reference site). *n* represents total number of fish sampled.

Site type	Description	Sites	(*n*) Body length (mm)	GL (mm)	GL/BL ratio	R4:6	Mass (g)
WWTP	Creek	Brushy Creek	(25) *16.32±0.35	6.26±0.10	*0.39±0.00	2.71±0.05	*0.129±0.01
WWTP/SEPTIC	Creek	Mullum Mullum Creek	(13) *19.92±0.51	6.75±0.16	*0.34±0.01	2.66±0.03	*0.136±0.01
WWTP	Creek	Aikten Creek	(9) *21.33±0.60	7.60±0.33	*0.36±0.01	2.72±0.10	*0.181±0.02
Urban	Wetlands	Shankland Wetlands	(29) 15.38±0.31	6.44±0.08	*0.41±0.01	2.72±0.06	*0.112±0.01
Urban	Wetlands	Settler's Orchard Reserve	(41) *18.38±0.27	6.76±0.07	*0.35±0.00	2.65±0.04	*0.154±0.01
Urban	Wetlands	Kalparrin Wetland	(6) *21.14±0.59	7.11±0.22	*0.33±0.00	2.62±0.07	*0.224±0.01
Urban	Lake	Tikalana park	(22) *19.83±0.23	6.60±0.14	*0.34±0.00	2.57±0.06	*0.136±0.01
Urban	Lake	Ruffey Lake	(25) *19.60±0.22	6.81±0.10	*0.35±0.00	2.57±0.03	*0.121±0.00
Industrial	Lake	Westgate park Lake	(31) *18.77±0.19	6.38±0.04	*0.34±0.00	2.60±0.04	*0.135±0.00
Rural	Creek	Deep Creek	(10) *19.30±0.30	6.72±0.19	*0.35±0.01	2.61±0.06	*0.108±0.01
Rural	River	Coliban River	(25) *18.08±0.26	5.96±0.09	*0.33±0.00	2.59±0.03	0.088±0.00
Rural	River	Lerderderg River	(30) *20.13±0.36	6.64±0.12	*0.33±0.00	2.68±0.03	*0.117±0.01
Reference	Lake	Brodies Lake	(26) 14.68±0.18	6.47±0.08	0.44±0.01	2.63±0.08	0.075±0.00

Table 2.5: Male *G.holbrooki* 2008 morphometrics (mean±SEM). Means not sharing the same letters are significantly different from each other. (*n*) represents total number of fish sampled. *Includes sites that could be classified as both rural and urban or urban and industrial.

Site type	Description	Sites	(*n*) Body length (mm)	GL	GL/BL ratio	Elongation ratio	Mass (g)
WWTP	Creek	Gisborne WWTP	(16) 20.00^{abcd}±0.38	6.06±0.14	0.30^{cef}±0.006	3.50^{bd}±0.10	0.094^{b}±0.00
WWTP	Creek	Brushy Creek	(18) 20.00^{bcd}±0.31	6.28±0.13	0.31^{abce}±0.006	$3.47^{abce\,abd}$±0.12	0.110^{bce}±0.01
WWTP	Creek	Sunbury WWTP	(9) 21.89^{ca}±0.35	6.89±0.21	0.31^{abde}±0.009	2.94^{cbd}±0.12	0.152^{acd}±0.01
WWTP	Wetland	WTP, Werribee	(19) 20.68^{acdef}±0.23	6.22±0.10	0.30^{ce}±0.005	3.29^{ab}±0.09	0.119^{ae}±0.00
WWTP	Creek	Mullum Mullum Creek	(11) 20.00^{abcd}±0.20	6.40±0.07	0.32^{abce}±0.004	2.67^{c}±0.09	0.110^{abc}±0.00
Urban	Wetland/drain	Kalparrin Wetland	(34) 19.24^{b}±0.18	6.25±0.07	0.33^{abc}±0.003	3.24^{abd}±0.06	0.107^{abe}±0.00
Industrial/Urban	Creek	Kororoit Creek*	(17) 20.29^{abcd}±0.38	6.42±0.15	0.32^{abe}±0.007	3.07^{bc}±0.07	0.138^{ade}±0.01
Industrial/Urban	Creek	Ryan's Creek	(19) 18.95^{b}±0.33	6.35±0.04	0.34^{abd}±0.006	3.18^{ab}±0.06	0.081^{eb}±0.01
Industrial/Urban	Creek	Merri Creek*	(12) 20.67^{abcd}±0.41	6.74±0.12	0.33^{bcd}±0.004	3.00^{acf}±0.08	0.140^{ade}±0.01
Reference/Urban	Lake	Green Lake	(35) 21.17^{acd}±0.22	6.41±0.06	0.30^{e}±0.003	3.18^{ab}±0.04	0.153^{d}±0.00
Reference	Lake	Sanitarium Lake	(21) 19.14^{bf}±0.2	6.70±0.1	0.35^{d}±0.006	3.36^{bdf}±0.05	0.114^{abe}±0.00
Reference	Lake	Liverpool FRB	(34) 20.26^{abcd}±0.28	6.44±0.08	0.32^{abce}±0.004	3.13^{ab}±0.05	0.119^{abe}±0.01
Reference	Lake	Brodies Lake	(23) 20.39^{abcd}±0.4	6.43±0.12	0.32^{abce}±0.004	3.01^{ac}±0.05	0.128^{ade}± 0.01
Rural	River	Campaspe River	(20) 20.90^{ade}±0.32	6.75±0.13	0.32^{abe}±0.003	3.23^{abd}±0.08	0.135^{abcd}±0.01
Rural	Creek	Deep Creek	(21) 21.62^{a}±0.39	7.14±0.17	0.33^{abcd}±0.005	3.48^{d}±0.06	0.148^{ad}±0.01
Rural	River	Lerderderg River	(22) 19.55^{be}±0.38	6.47±0.10	0.33^{abdf}±0.004	3.22^{abd}±0.07	0.113^{bce}±0.01

Table 2.6: Summary of Male *G.holbrooki* 2009 morphometrics (mean±SEM). Means not sharing the same letters are significantly different from each other. (*n*) represents total number of fish sampled. *Includes sites that could be classified as both rural and urban or urban and industrial.

Site type	Description	Sites	(*n*) Body length (mm)	GL (mm)	GL/BL ratio	R4:6 ratio	Mass (g)
WWTP	Creek	Gisborne WWTP	(14) 17.33^{bd}±0.37	6.31±0.12	0.34^{bdcef}±0.02	2.68±0.06	0.087^{c}±0.006
WWTP	Creek	Sunbury WWTP	(15) 16.93^{bd}±0.40	6.27±0.17	0.37^{bdcef}±0.01	2.79±0.07	0.093^{c}±0.007
WWTP	Drain	Werribee WWTP	(15) 18.67^{b}±0.35	6.92±0.13	0.37^{bcd}±0.00	2.64±0.05	0.117^{c}±0.006
WWTP	Creek	Brushy Creek	(18) 17.06^{bd}±0.50	6.57±0.11	0.39^{cde}±0.01	2.86^{a}±0.06	0.090^{c}±0.008
Urban	Wetland	Kalparrin Wetland	(10) 18.75^{b}±0.44	6.48±0.15	0.35^{f}±0.00	2.73±0.06	0.124^{b}±0.006
Industrial/Urban	Creek	Merri Creek*	(15) 16.27^{d}±0.32	6.50±0.12	0.40^{e}±0.01	2.72±0.05	0.078^{c}±0.004
Industrial/Urban	Creek	Kororoit Creek*	(14) 18.93^{b}±0.41	6.55±0.16	0.35^{bf}±0.01	2.53±0.08	0.155^{b}±0.007
Reference	Lake	Brodies Lake	(16) 17.88^{bd}±0.33	6.45±0.11	0.36^{bcdf}±0.01	2.71±0.06	0.105^{c}±0.006
Reference	Lake	Liverpool FRB	(15) 17.77^{bd}±0.44	6.11±0.13	0.35^{bdf}±0.01	2.51±0.07	0.101^{c}±0.007
Reference	Lake	Tourrorong Reservoir	(15) 21.47^{c}±0.26	7.61±0.11	0.35^{bf}±0.00	2.65±0.03	0.160^{b}±0.009
Reference	Lake	Sanitarium Lake	(15) 17.87^{bd}±0.5	6.67±0.2	0.37^{bdce}±0.01	2.71±0.05	0.107^{c}±0.009
Rural/Urban	Stormwater drain	Narre Warren SD*	(16) 17.22^{bd}±0.24	6.55±0.10	0.38^{bcde}±0.01	2.67±0.05	0.086^{c}±0.005
Rural	River	Campaspe River	(17) 18.85^{bd}±0.30	6.78±0.14	0.36^{bcdf}±0.01	2.73±0.08	0.118^{b}±0.008
Rural	Creek	Deep Creek	(14) 17.72^{bd}±0.34	6.41±0.13	0.36^{bdef}±0.00	2.60±0.04	0.113^{c}±0.007
Rural/Urban	Creek	Woori Yallock Creek*	(15) 18.37^{bd}±0.36	7.11±0.14	0.39^{ce}±0.00	2.78±0.05	0.118^{b}±0.007
Rural	Creek	Watson's Creek	(14) 19.36^{b}±0.27	6.98±0.09	0.36^{bdf}±0.00	2.55±0.05	0.124^{c}±0.006

Table 2.7: Water quality parameters for sites in 2009. *Includes sites that could be classified as both rural and urban or urban and industrial. Numbers next to sites correspond to numbers in map, Figure 2.3.

Number	Site type	Site	GPS Co-ordinates	Temperature °C	pH	Conductivity (μs/cm)	Estrogenic activity (ng/L EEQ)
1	Industrial/ Urban	*Merri Creek, Campberfield	3767'S; 14497'E	18.8	7.65	1916	<0.1
2	Urban	Kalparrin Wetland, Greensborough	3770'S; 14510'E	18.6	7.45	2369	<0.1
3	Industrial/ Urban	*Kororoit Creek, Altona North	3783'S; 14483'E	20	7.67	3580	<0.1
4	Rural/ Urban	Stormwater Drain, *Narre Warren	3804'S; 14530'E	24.5	6.26	611	1.4
5	WWTP	Brushy Creek, Croyden North	3776'S; 14529'E	19.4	6.79	717	0.3
6	WWTP	Jackson's Creek, Sunbury	3760'S; 14474'E	19.3	8.57	3050	0.9

Number	Site type	Site	GPS Co-ordinates	Temperature °C	pH	Conductivity (s/cm)	Estrogenic activity (ng/L EEQ)
7	WWTP	Jackson's Creek, Gisborne	3749'S; 14461'E	15.3	6.84	813	12
8	WWTP	Drain to Lake Borrie, WTP	380'S; 14456'E	19	7.95	519	0.6
9	Reference	Brodies Lake, Greenvale Reservoir	3764'S; 14490'E	20.6	7.76	2910	<0.1
10	Reference	Liverpool Freshwater Basin, Boronia	3785'S; 14532'E	21.7	7.05	942	1.7
11	Reference	Sanitarium Lake, Mt Macedon	3738'S; 14461'E	18.7	6.04	80	<0.1
12	Reference	Tourourrong Reservoir, Whittlesea	3747'S; 14515'E	25	6.54	164	<0.1

Number	Site type	Site	GPS Co-ordinates	Temperature °C	pH	Conductivity (s/cm)	Estrogenic activity (ng/L EEQ)
13	Rural	Deep Creek, Darraweit Guin	3743'S; 14482'E	20.3	9.05	2006	<0.1
14	Rural	Campaspe River, Kyneton	3726'S; 14445'E	25.1	8.95	1246	0.3
15	Rural	Watson's Creek, Somerville	3820'S; 14517'E	18	6.91	619	0.1
16	Rural	*Worri Yallock Creek, Emerald	3682'S; 14734'E	21.4	7.6	155	<0.1

Chapter 3

Dot Blot Assay for Detecting Vitellogenin in Male *G. holbrooki*

3.1 Abstract

Three commercially available anti-Vtg antibodies (Salmon anti-Vtg antibody, carp anti-Vtg antibody and sea bream anti-Vtg antibody) were tested on *G.holbrooki* (mosquitofish) whole body homogenates. Cross-reactivity was observed with the anti-Vtg antibodies showing varying results depending on reproductive stage of female *G.holbrooki*. A dot blot semi-quantitative assay was developed using a standard curve with salmon protein extract and salmon anti-Vtg antibody for the detection of Vtg protein in wild adult male *G.holbrooki* collected from 16 urban, rural, industrial and reference sites throughout Melbourne and Victoria in 2009. Estrogenic activity was measured in water samples collected from the same sites. Collection sites showed levels of estrogenic activity ranging from 0.1 - 1.7 ng/L EEQ except for a WWTP where elevated levels at 12 ng/L EEQ were found. There was no correlation between estrogenic activity and mean Vtg levels in male *G.holbrooki*. Mean Vtg in male *G.holbrooki* was significantly different at all field sites. The Vtg levels in male *G.holbrooki* from three out of the four WWTP sites (Gisborne, Brushy Creek and Werribee)

were the highest, suggesting that fish from those sites could be exposed to estrogenic (or anti-androgenic) environmental contaminants. In particular, male *G.holbrooki* from Gisborne WWTP had the highest mean Vtg levels and the highest corresponding estrogenic activity, suggesting that this may be the most impacted site.

3.2 Introduction

The city of Melbourne's water supply comprises of major reservoirs and a number of catchments involving a network of rivers and creeks that eventually flow into the ocean. These creeks and rivers flow through agricultural, industrial and urban land, collecting run-off from farming and suburban activities (Wightwick and Allinson, 2007, Lewis et al., 2009, Wightwick et al., 2012). A small number of wastewater treatment plants also discharge treated effluents directly into tributaries that join the rivers (Mispagel et al., 2005, 2009b). Pollutants including endocrine active compounds (EACs) and endocrine disrupting chemicals (EDCs) some of which are heavy metals, aromatic hydrocarbons, new biocides, flame retardants, industrial chemicals, pesticides and pharmaceuticals have the potential of contaminating creeks and rivers and affecting wildlife by altering or disrupting the normal function of the endocrine system (van der Oost et al., 2003, Ankley and Johnson, 2004, Mills and Chichester, 2005, Diamanti-Kandarakis et al., 2009, Barber et al., 2011).

EACs may act via a number of pathways which can be divided into: 1) agonistic and/or antagonistic effects on the estrogenic receptor (ER) and/or the androgenic receptor (AR); 2) disruption of production, transport, metabolism or secretion of natural hormones; and 3) disruption of production and/or function of hormone receptors (Goksøyr et al., 2003, Rotchell and Ostrander, 2006, Ankley et al., 2009).

Endocrine disrupting effects of inputs and run-off on fish is a global occurence. The first

report of endocrine disruption was of female *G.holbrooki* masculinized by pulp mill effluents in the early 1980s in the United States of America (Howell et al., 1980). Subsequently, endocrine disruption has been reported in other male and female fish species in the United Kingdom (Jobling et al., 1998) and countries such as the Netherlands (Vethaak et al., 2005), China (Lu et al., 2011), Korea and Japan (Aoki et al., 2010).

Studies in Australia have established some information of estrogenic activity in waterways. Mispagel et al. (2005) first reported measurements of estrogenic activity in Victorian WWTP effluents (0.3-42 ng/L EEQ) (estradiol equivalent). Other studies have since contributed data on estrogenic activity or measurements of total estrogens in Victorian waterways ranging from 0.1-13.19 ng/L EEQ (Scott et al., 2014) and 7.82-13.9 ng/L EEQ respectively (Saaristo et al., 2014). Bigger sampling events in various water catchments around Victoria (Chinathamby et al., 2013) from 2007 to 2009, have further added to the record of estrogenic activity in the city's rivers. In other states of Australia, measurements of estrogenic activity range from 0.02-6 ng/L EEQ in effluent in Western Australia (Leusch et al., 2014) and 1.32-11.79 ng/L EEQ in Queensland (Ying et al., 2009b).

The estrogenicity of numerous EACs have been studied indepth in laboratories, starting with short term assays to establish effects, followed by long term or full life-cycle experiments for fish that have short life cycles (Hutchinson et al., 2006). One of the biomarkers commonly used to detect estrogenic effects in male fish is vitellogenin (Vtg) induction (Sumpter and Jobling, 1995, Hinck et al., 2008, Oğuz et al., 2015). Vitellogenin is a large glycophospholipid protein (Molecular weight: 250 - 600 kDa) that is synthesized in the liver of female fish, secreted to the plasma and transported to oocytes for uptake in a process involving the female sex steroid hormone 17βestradiol (E_2) (Sumpter and Jobling, 1995, Arukwe and Goksøyr, 2003, Levi et al., 2009). Although E_2 may be present in low levels in male fish, the amounts required to initiate Vtg production is insufficient unless fish are exposed to relevant levels of estrogenic contaminants. Laboratory exposures us-

ing common environmental estrogens have measured induction of Vtg in many fish species including male Japanese medaka (Gronen et al., 1999), rare minnow (Zha et al., 2007), Murray rainbowfish (Woods and Kumar, 2011) and *G.holbrooki* (Tolar et al., 2001, Raut and Angus, 2010).

Vitellogenin is analysed in plasma, liver or whole body homogenates as circulating protein or by hepatic mRNA induction. Vitellogenin mRNA (Vtg mRNA) transcripts are induced rapidly upon estrogenic exposure (Hutchinson et al., 2006) and rapidly reduced after exposure ceases (Craft et al., 2004). This has been thought to be the earliest signal of exposure and is useful if recent exposure to EACs need to be distinguished. Vitellogenin mRNA synthesis results in Vtg protein translation if exposure is high enough (Jones et al., 2000, Hemmer et al., 2002). The Vtg protein, on the other hand, continues to be expressed after recent exposure ceases, and undergoes a higher level of induction compared to the Vtg mRNA (Hemmer et al., 2002, Iguchi et al., 2006) allowing for determination of chronic exposure.

A variety of methods are available to quantify Vtg in fish. These include techniques such as measurement of acid labile phosphorus (ALP) in the blood (Vtg is a phosphorylated licoglycoprotein) (Whitehead et al., 1983, Verslycke et al., 2002), assessment of electrophoretic separation of blood proteins using densitometry (van Bohemen et al., 1981), radioimmunoassay (Idler et al., 1979, Sumpter, 1985) and enzyme-linked immunosorbent assay (ELISA) using Vtg specific antibodies (Parks et al., 1999, Codi King et al., 2008). Assay selection is based on factors such as target animals, availability of equipment, time, cost and assay sensitivity required to meet experimental aims (Hiramatsu et al., 2006). All of the above methods require trained personnel in the laboratory (Vilček et al., 1994, Fredriksen et al., 1999).

The dot blot (immunobinding) assay is a simple method in which a protein extract is di-

rectly applied on to a nitrocellulose membrane for detection of a specific antigen and unlike the immunoblot, the protein samples are not separated using electrophoresis (Sumi et al., 2009). Dot blots have been demonstrated to be useful for antigen-antibody detection in fish virology studies to assess disease in fish (Hsu et al., 1989, Douglas-Helders et al., 2001) and have been previously used to detect the presence of Vtg in the plasma of Mediterranean male swordfish (Desantis et al., 2005). Unlike previously mentioned techniques, a simple dot blot assay without the use of a plate reader can be easily performed by untrained personnel and may be useful when quick interpretation of data is necessary, potentially enabling this method to be used in the field with slight modifications.

Despite widespread reports of Vtg induction in wild fish, not many studies have reported the presence and detection of the Vtg protein in wild male *G.holbrooki* due to estrogenic contaminant exposure. In Australia, estrogenic effects in male *G.holbrooki* were first reported by Batty and Lim (1999). The study reported effects on the anal fin (gonopodia) of wild male *G.holbrooki* collected downstream of a wastewater treatment plant (WWTP) in 1999 in New South Wales followed by a similar observation by Game et al. (2006) of male *G.holbrooki* collected from wetlands in Western Australia. In response to the study by Batty and Lim (1999) on estrogenic effects on the gonopodia of wild male *G.holbrooki*, Tolar et al. (2001) developed an immunoblot assay using anti-Vtg serum specific to *G.holbrooki* to detect the Vtg protein in male *G.affinis*. In that study, Vtg was induced using 17α-ethinylestradiol (EE_2) exposures and identified and purified in the laboratory. This assay was later tested by Angus et al. (2002) on a population of *G.affinis* inhabiting a river in the USA that received treated domestic sewage effluent. In that study, no detectable levels of Vtg were found in the blood of wild *G.affinis* in both effluent-exposed and the control populations. However, the sensitivity of the assay in ng/ml was not determined before field testing was conducted. The assay may not have been sensitive enough to detect low levels of Vtg protein induction in male fish in the wild. Angus et al. (2005) studied the effects of EE_2 on sexual development, in particular, gonopodial development of *G.affinis* and mea-

sured Vtg protein in the blood of *G.affinis* to identify association with other reproductive indices. In that study, the Vtg protein was detectable in the blood of all fish exposed to 1.0 μg or more of EE_2/g food and concentration of Vtg increased dramatically with increasing EE_2 exposure. Angus et al. (2005) concluded that EE_2 exposures high enough to induce Vtg protein expression in *G.affinis* significantly reduced gonopodia elongation and inhibited traits related to reproductive fitness.

Leusch et al. (2005) developed a method to quantify Vtg mRNA in male *G.holbrooki* because of the difficulties in collecting blood from small fish species such as *G.holbrooki*. In that study, Vtg mRNA was expressed significantly in male *G.holbrooki* after a 4 day exposure to 250 ng/L of E_2, however, lower doses did not induce Vtg mRNA expression even after an eight day exposure. Although Vtg mRNA expression has been shown to be a useful biomarker, Vtg mRNA transcripts are induced and rapidly reduced after exposure ceases (Craft et al., 2004, Hutchinson et al., 2006). The Vtg protein, on the other hand, undergoes a higher level of induction compared to the Vtg mRNA (Hemmer et al., 2002, Iguchi et al., 2006) and is more suitable for determination of chronic exposure in the aquatic environment. A recent study by Leusch et al. (2014) investigated water quality at four treatment plants which included the use of *G.holbrooki* from the sites for the detection of plasma Vtg protein as a biomarker for estrogenic exposure. In that study, *G.holbrooki* homogenates were tested on 96-well plates using a salmonid Vtg standard. Plasma Vtg was found in male fish upstream and downstream of one of the wastewater treatment plants but not in fish from the other wastewater treatment plant and reference sites. Leusch et al. (2014) attributed the detection of Vtg in male *G.holbrooki* to exposure of estrogenic compounds both upstream and downstream of the treatment plant outlet. Leusch et al. (2014) reported estrogenicity at less than 4.8 ng/L EEQ in raw sewage and less than 0.02 ng/L EEQ at all other stages of treatment. The focus of that study was on water quality of raw sewage and wastewater treatment at the plants. Studies focusing on the detection of the Vtg protein in male *G.holbrooki* from aquatic environments influenced by various land use activities are

lacking.

One of the challenges of working with small fish such as zebrafish, and *G.holbrooki* is the difficulty in collecting sufficient blood for analysis of Vtg. Instead, whole body homogenates of fish have been used successfully in a number of studies (Holbech et al., 2001, Nilsen et al., 2004, Zhong et al., 2004, Gagnon et al., 2008). Although Vtg antibodies are commercially available for a number of fish species, anti-mosquitofish Vtg antibody is unavailable commercially. Previously, Gagnon et al. (2008) and Leusch et al. (2014) have respectively used salmonid Vtg ELISA kits and salmon anti-Vtg antibody for measuring Vtg in *G.holbrooki* while other studies have produced anti-Vtg antibodies specific to *G.holbrooki*. However, producing anti-Vtg antibodies specific to *G.holbrooki* is a time-consuming process.

Estrogenic effects from both field and laboratory studies worldwide on both male *G.holbrooki* and *G.affinis* include, modifications to the gonopodium size, decreased proportion of males in an exposed population, decreased sperm count and testis size and mRNA Vtg induction (Dréze and Monad, 2000, Doyle and Lim, 2002, Toft et al., 2003, Angus et al., 2005, Leusch et al., 2005, Edwards and Guillette, 2007). *G.holbrooki* is ideal for assessing endocrine disrupting effects because it is sexually dimorphic. It is an introduced species, commonly found in freshwater streams and creeks in Victoria. Its short life cycle makes it ideal for toxicity tests for specific life stages. It prefers and inhabits shallow, still or slow moving water in a wide range of environments making it a suitable species for sampling from a range of river health conditions for comparisions (Pyke, 2008).

This chapter investigates and discusses results of three commercially available anti-Vtg antibodies tested on female *G.holbrooki* whole body homogenates. Such studies comparing the suitability of different commercially available anti-Vtg antibodies on *G.holbrooki* are unavailable. The dot blot semi-quantitative assay was developed using salmon anti-

Vtg antibody for the detection of Vtg in adult male *G.holbrooki* collected from the wild. This chapter reports on the presence of Vtg in male *G.holbrooki* collected from waterbodies throughout various land use areas. Studies involving the use of the dot blot assay for the detection of the Vtg protein in wild caught male *G.holbrooki* have not been described previously. Results of Vtg levels are discussed in parallel with measurements of estrogenic activity in the water. Finally, the suitability of the dot blot assay for field testing is discussed.

The specific aims were:

1. To assess cross-reactivity of three commercially available Vtg antibodies for use in the experimental development of a semi-quantitative dot blot assay for screening Vtg in wild male *G.holbrooki*.
2. To detect the presence of Vtg in male *G.holbrooki* that were collected from sites in 2009.
3. To correlate Vtg and estrogenic activity of water at the collection sites.

3.3 Materials and Methods

3.3.1 Study sites and collection methods

Sixteen sites were selected in 2009 around Victoria, Australia and grouped according to surrounding land use types such as urban (U), rural (R), waste water impacted (WWTP) and reference sites (Ref.), although in some cases the grouping is somewhat arbitrary, since some creeks flow through both residential and industrial or agricultural land and are likely to contain a combined source of pollutants. Approximate locations of sampling sites in 2009 is shown in Figure 2.3 (Chapter 2). Collection methods are described in Chapter 2. Briefly,

mature adult *G.holbrooki* were collected from these sites using dip nets from February to May 2009. In the field, fish were euthanased (blow to the head), transported on ice to the RMIT Ecotoxicology laboratory and sexed under a Wild Heerbrugg M3Z stereozoom microscope. Some of the fish were used for EROD activity measurements (Chapter 4) and the rest were immediately stored in microfuge tubes, frozen in liquid nitrogen and then stored at -80°C for later use.

3.3.2 Fish whole body tissue preparation

Adult male *G.holbrooki* were individually homogenized in ice-cold buffer using a handheld homogenizer from Pathtech (CAT60404-00). Homogenization was completed in 10 ml of 3x buffer stock (pH 6.8), 30 ml 100 uM PMSF (Phenylmethylsulfonyl fluoride) and 20 ml Milli-Q water in 1:4 (w/v) for one minute. The homogenates were centrifuged at 12000 rpm for 98 minutes at 4°C. Pilot studies with the wholebody supernatant clogged the dot blot apparatus. To overcome this, the supernatant was removed after initial centrifugation and centrifuged again for 30 minutes at 12 000 rpm. The supernatant was obtained and stored in aliquots at -80°C until subsequent use.

3.3.3 Measurement of estrogenic activity in the water

Water samples were collected as 'grab' or spot samples from the study sites in 2009 at time of fish collection. Samples were collected in amber glass bottles, stored on ice and then at 4°C until processed and measured for estrogenic activity by Dr Mayumi Allinson (Department of Primary Industries) and team by methods described by Allinson et al. (2011) and Chinathamby et al. (2013). Results are reported as ng/L estradiol equivalent (EEQ).

3.3.4 Statistical analysis

All statistical analyses were performed using PASW 22 and 23 for Macintosh (New York, USA). Significance was set at α=0.05 for all tests. Each set of data was tested for normality and homoscedasity. Data was transformed if assumptions for parametric testing were not met. One-way analysis of variance (ANOVA) was used to test for differences between sites. Vtg Equivalent (VtgEq) values were log transformed before testing. A pot doc Tukey HSD test was performed to identify differences between means. Pearson's correlation coefficient was used to test for differences between estrogenic activity and mean Vtg. Independent-samples t-test was used to test differences in means between male and female *G.holbrooki* Vtg levels.

A power analysis to estimate adequate sample size was not conducted because meaningful effect size could not be determined (Hoenig and Heisey, 2001).

3.3.5 Immunological techniques

Two different immunological techniques were used in this chapter; Part A: Immunoblotting techniques for the anti-Vtg antibody selection and Part B: Dot blot assay which was subsequently used for the final testing of male *G.holbrooki* collected from field sites.

3.3.6 Part A - Optimisation of Immunoblot and ECL Techniques for Selection of the Antibody

3.3.6.1 Denaturing electrophoresis

It was necessary to optimize immunoblot techniques for later testing of the various purchased Vtg antibodies for reactivity to *G.holbrooki* homogenates. Carp liver was used for the optimization. 20 mg each of male and female carp liver tissue were crushed in ice-cold phosphate-buffered saline (PBS) buffer (Sigma-Aldrich, Australia) (1:4 w/v ratio) using a pestle and spun at 10,000 g for 30 minutes at 4°C. Supernatant was obtained and stored in aliquots at -80°C until subsequent use.

Carp liver total protein levels were determined using the Lowry method of protein estimation (Lowry et al., 1951) with the DC Protein assay kit (Biorad, Australia). Liver protein concentration was interpolated using a protein standard curve ranging from 0 to 2 mg/ml bovine serum albumin (BSA) dissolved in Milli Q water. Absorbance was measured at 750 nm with a Thermo Scientific Multiskan Spectrum platereader. Samples were spun for 5 minutes at 10,000 RPM. One μl of protein was diluted with PBS (1:16) and then with 8 μl of sample buffer (3.8 ml Deionized water, 0.5 M Tris-HCL, 0.8 ml Glycerol, 10% (w/v) SDS, 0.4 ml 2-mercaptoethanol, 0.4 ml 1% (w/v) bromophenol to a total volume of 25 μl. Samples were heated for 5 minutes at 95°C and spun again for 5 minutes at 10,000 RPM prior to loading onto the stacking gel for SDS-PAGE (Polyacrylamide gel electrophoresis) analysis. 8% Tris-HCL polyacrylamide gels were determined to be most suitable for resolving protein between 100 to 200 kDa (Results not shown). These were produced a day before use according to methods described in Walker (2002) and Kamath et al. (2014). Each lane of carp liver sample contained approximately 13.5 μg of protein in 25 μl of buffer. Carp Vtg standard containing 0.33 μg of protein was also loaded. 10 μl of a molecular weight marker (Precision Plus Protein Standards Kaleidoscope from Biorad, Australia) was included in

each gel.

Consecutively, another polyacrylamide gel for immunoblotting was loaded with a quarter of the protein concentration that was used for Sodium-dodcylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run in electrophoresis buffer (Appendix B) in a Mini-Protean III Electrophoresis system (Biorad, Australia) at 120 V for 60 min.

3.3.6.2 Immunoblots

Prior to transfer, the PVDF membrane was immersed in 100% methanol for five seconds, then transfered to a container with Towbin transfer buffer containing 10% v/v 10x Towbin solution (25 mM Tris, 192 mM glycine), 20% v/v methanol, and 70% v/v Milli Q water (Appendix B). One of the gels with the separated proteins was retrieved and placed into a container with the Towbin transfer buffer for 10 minutes. The gel was then transferred to the PVDF membrane using the Biorad Transblot SD semi dry transfer cell for 40 minutes. After the transfer, the PVDF membrane was retrieved and completely submerged in 30 ml of 5% Bovine serum albumin (BSA) blocking solution in Tris-Buffered Saline (TBS) (Appendix B) for one hour on a gentle shaker. The membrane was then rinsed five times for 10 minutes each in TBS on a shaker. The membrane was probed with Mouse anti-carp Vtg monoclonal antibody (ND-2D3, Biosense Norway) at a dilution of 1:5000 for two hours at room temperature on a shaker. The membrane was then rinsed in TBS-Tween (1% Tween 20 in TBS) thrice for 15 minutes each. The membrane was incubated in Goat anti-mouse IgG conjugated with Horseradish peroxidase conjugate (HRP) (Biorad, Australia) at a dilution of 1:15000 for 60 minutes at room temperature on a shaker. The membrane was then rinsed in TBS-Tween (Appendix B) twice for 15 minutes each and then in TBS for a final wash of 15 minutes on a shaker. The membrane was stored in TBS until the antibody-Vtg binding was visualized using enhanced chemiluminescence (ECL).

The other gel was placed in a container with ice cold PBS for 10 minutes before Coomassie blue staining. Fresh Coomassie blue stain (Coomassie Brilliant Blue R-250 in 10% methanol, 10% glacial acetic acid, MilliQ water) was prepared prior to gel staining. Gels were place in Coomassie blue stain for one minute and then destained using destaining solution (10% methanol, 10% glacial acetic acid, Milli Q water) over a slow shaker until desired stain was achieved.

3.3.6.3 Enhanced chemiluminescence (ECL) detection system

The proteins on the membrane were visualized using an ECL kit from Amersham. ECL substrate was prepared according to manufacturers recommendations. One ml of the ECL substrate was added to the entire surface of the membrane. The membrane was allowed to develop for 5 minute. Subsequently, the membrane was placed in the dark-room under an x-ray film and into a cassette and closed for a number of exposures for 30 sec, 1 minute, 5 minutes, 10 minutes and 30 minutes and if necessary for one hour. The x-ray film was then placed in the developer solution (ECL kit from Amersham) for one min, washed for 30 sec, then in fixer solution (ECL kit from Amersham) for 60 sec and finally, washed for 60 sec under running water. The x-ray film was air dried at this final stage.

3.3.6.4 Immunoblot using female carp liver

The immunoblot was successful and showed binding to female carp liver homogenates (Figure 3.1). Figure 3.1 (C) shows reactivity of anti-carp Vtg antibody for both female carp liver protein and carp Vtg standard. Carp Vtg appears as a double band at 180 and 150 kDa. Figure 3.2 shows good transfer of protein from gel to PVDF membrane which was stained after the proteins were visualized using ECL. However, background interference is evident in the developed film (Figure. 3.1 (C). This was subsequently reduced with different blocking

solutions, described in the following section.

3.3.6.5 Selection of blocking solution for immunoblots

Further testing with the ECL development technique continued to produce a cloudy instead of a clear background in the x-ray film (See Figure 3.3 (A). This may obscure the visibility of the proteins in blots. The hypothesis was that the blocking solution of BSA was causing a cloudy background. To test this, a different blocking solution of skim milk was applied after SDS-PAGE. Two polyacrylamide gels were loaded with 13.5 μg of female carp liver protein each in two lanes and separated by SDS gel-electrophoresis. Gels were transferred to PVDF membranes using the Biorad Transblot SD semi dry transfer cell for 40 minutes as detailed above. After the transfer, membranes were retrieved and each membrane was completely submerged in one of the two blocking solutions; 1% BSA (in TBS) or 5% skim milk (in TBS) for 1.5 hours and then cut into four parts receiving 4 different treatments of either (1) primary and secondary antibody, (2) secondary antibody only, (3) primary antibody only, or (4) blocking solutions only. Mouse anti-carp Vtg antibody was diluted at 1:5000 and the membranes probed for 2 hours. Membranes were washed with TBST five times, 10 minutes each. Rabbit anti-mouse HRP tagged secondary antibody was diluted at 1:15,000 and the membranes probed for 60 minutes. The membranes were then rinsed in TBST twice for 15 minutes each and then in TBS for a final wash of 15 minutes on a shaker. Membranes were developed using the ECL detection system. Figure 3.2 shows the PVDF membrane (stained with Coomassie blue) with carp liver proteins after ECL detection to demonstrate the protein profile in carp liver and that equal amount of proteins was separated in both lanes.

The two blocking solutions showed different results (see Figure 3.3). Membrane A had a cloudy background when blocked with 1% BSA and treated with both primary and sec-

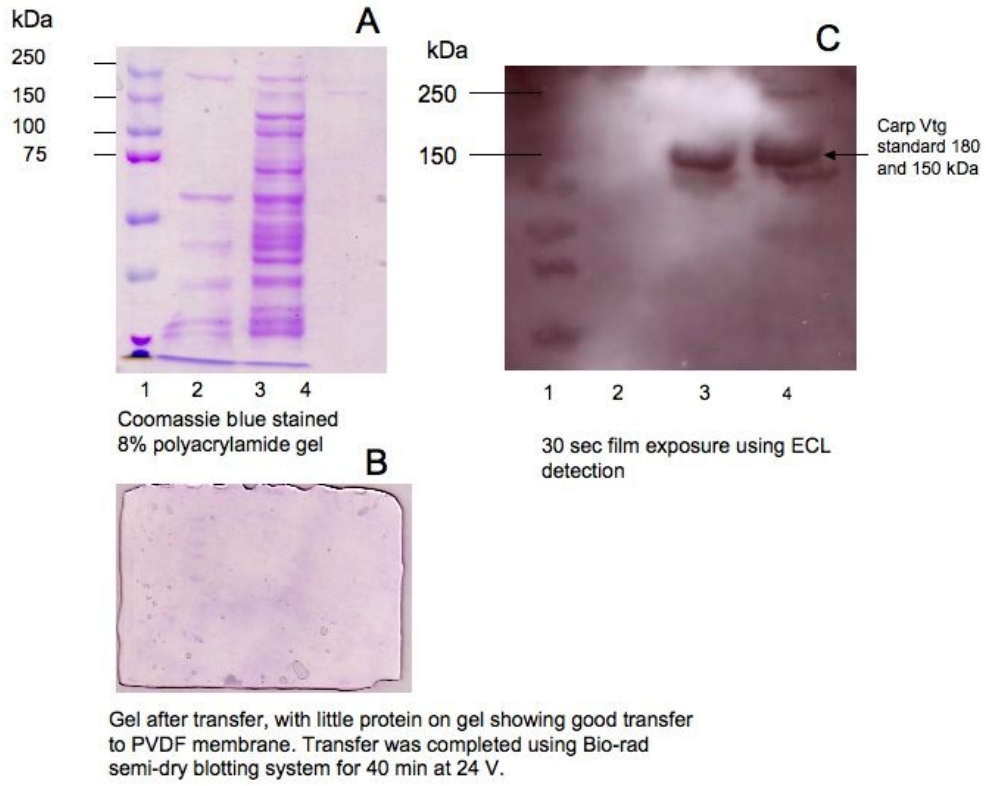


Figure 3.1: SDS gel separation of proteins from carp and carp Vtg standard and subsequent detection using specific antibodies. A. SDS gel showing separation of carp liver proteins. B. demonstrates complete transfer of all proteins. C. Developed x-ray film showing binding to Vtg in female carp liver and carp Vtg standard. Lane 1:Molecular marker, Lane 2: Male carp liver homogenate 13 μg protein, Lane 3: Female carp liver homogenate 13.5 μg protein, Lane 4: Carp Vtg standard 0.3 μg. Note antibody binding to Vtg in female liver and positive control standard in Lane 3 and Lane 4 respectively.

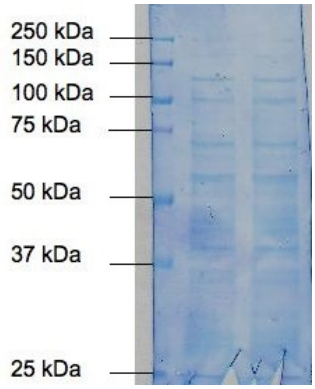


Figure 3.2: PVDF membrane probed with anti-carp Vtg antibody and stained with Coomassie blue after ECL development, showing good transfer of carp protein from gel.

ondary antibodies. Membrane B has a dark background confirming that 1% BSA interferes with the rabbit anti-mouse HRP tagged secondary antibody, but no interference is shown when membrane F is blocked with 5% skim milk and treated with secondary antibody. Membrane E blocked with 5% skim milk and probed with the same treatments had the desired result of no background, but strong signal. Both membranes D and H did not show an interference with the primary antibody. Therefore, 5% skim milk was used henceforth as a blocking solution for all subsequent immunoblots.

3.3.6.6 Testing three Vtg antibodies on *G.holbrooki*

Three different anti-Vtg antibodies, Rabbit anti-salmon Vtg polyclonal antibody (V01402201-00), Rabbit anti-seabream Vtg polyclonal antibody (V01410201-00) and Mouse anti-carp monoclonal Vtg antibody (V01403101) were compared to determine the most appropriate antibody for use on *G.holbrooki* whole body homogenates for the dot blot assay. These were purchased from Biosense Laboratories in Norway. Polyacrylamide gels were prepared and loaded with the same amount of proteins in each lane. These were: adult male mosquitofish

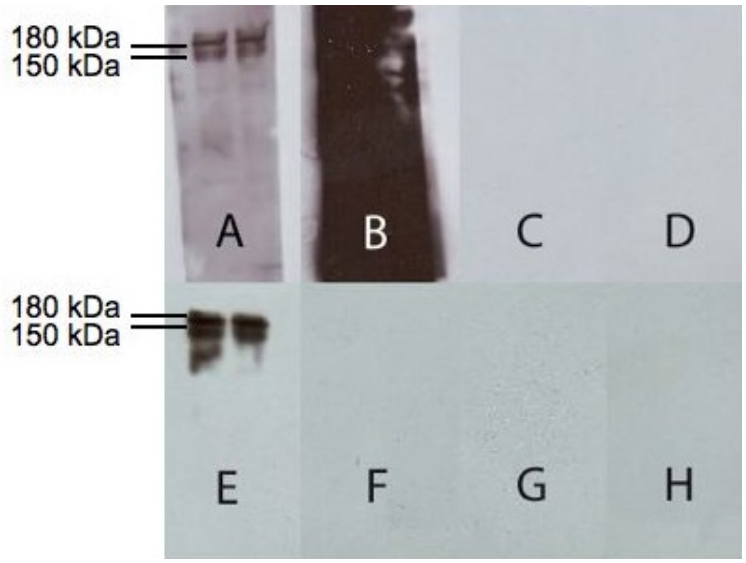


Figure 3.3: Comparing two different blocking solution outcomes on ECL film. Female carp liver protein was separated in 2 lanes (13.5 μg per lane). Treatments A - D received 1% BSA. Treatments E - H received 5% skim milk. A and E: primary antibody, secondary antibody and blocking solution. B and F: Secondary antibody only and blocking solution. C and G: Primary antibody only and blocking solution. D and H: Blocking solution only. Carp Vtg appears at 180 and 150 kDa.

(MM), adult female mosquitofish (FM) with gravid spot, adult female mosquitofish with oocytes (FMY) from Brodies Lake (Ref), adult female mosquitofish from Watson's creek (R) with oocytes (FMY), large female mosquitofish without oocytes but containing plasma (FMP) from Sanitarium Lake (Ref), carp Vtg standard, and female carp liver homogenate.

The dilutions that were used for primary antibodies were: Rabbit anti-salmon Vtg polyclonal antibody (1:2000), Rabbit anti-seabream Vtg polyclonal antibody (1:3000) and Mouse anti-carp monoclonal Vtg antibody (1:5000). Rabbit anti-mouse HRP secondary antibody was diluted at 1:15000 and Goat anti-rabbit secondary antibody was diluted at 1:7500. 5% skim milk was used as a blocking solution. General immunoblot and ECL methods are described in section 3.3.6.1-3.3.6.3 with changes made to the blocking solution and the concentrations of the antibodies as described above. All gels were run in electrophoresis buffer, transferred to PVDF and developed on film at the same time to prevent differences in conditions that may affect results.

3.3.6.7 Selection of commercially available Vtg antibodies for screening Vtg in *G.holbrooki*

Figure 3.4 shows the polyacrylamide gel with male and female *G.holbrooki* proteins in wholebody homogenates. The other proteins such as carp liver and carp Vtg standard, used as positive controls, were loaded in low quantities and therefore not visible in the gel.

All three Vtg antibodies showed different reactivity to adult female *G.holbrooki* at the various reproductive stages. Membrane A shows results of Mouse anti-carp Vtg antibody (see Figure 3.5(A). No reactivity is observed in lane 2 containing MM (male mosquitofish). Lane 3 containing 2 FM (female mosquitofish) with gravid spots unexpectedly had no reactivity to the antibody. However, lanes 4 and 5 containing FMY (female mosquitofish with oocytes) showed some reactivity approximately between 140 and 75 kDa. Some reactivity was also observed in Lane 6 containing FMP (female mosquitofish with plasma) approxi-

mately between 140 and 75 kDa. Lane 7 and Lane 9 shows binding in carp liver and carp Vtg standard respectively. There was however, non-specific binding with bands at lower molecular weights in all of the lanes containing mosquitofish antigen.

Membrane B (see Figure 3.5(B) shows results of rabbit anti-salmon Vtg antibody. Interestingly, Lanes 3, 4 and 5 showed reactivity to the antibody, particularly, Lane 3 containing FM showed reactivity approximately at 200 kDa. Lane 4 and 5 (FMY) showed reactivity at the approximate lower molecular weight of 140 - 100 kDa. No reactivity to the carp whole extract or standard was observed.

Membrane C (see Figure 3.5(C) shows results of rabbit anti-seabream Vtg antibody. Strong reactivity occurs specifically in Lanes 4 and 5 (FMY) approximately at 250 - 200 kDa and approximately at 140 - 75 kDa. Surprisingly, no reactivity in Lane 3 (FM) and Lane 6 (FMP). As expected, no reactivity was observed in Lane 2 (MM). Also, note that no reactivity to carp Vtg was observed.

In a study by Sawaguchi et al. (2005), the various forms of Vtg in estrogen-induced *G.holbrooki* were characterized. These Vtg proteins resolved into 195 and 142 kDa while a major yolk protein resolved into a wider range of bands at 195, 175, 142, 126, 66 and 55 kDa and a few more bands at a lower molecular weight, in SDS-PAGE. In the current study, bands were observed at approximately 250 kDa - 200 kDa and either at 140 - 75 kDa or 100 - 75 kDa. Interestingly, anti-salmon Vtg antibody cross-reacted with female *G.holbrooki* both sexually mature (presumably with circulating Vtg in plasma) and with females containing oocytes though stronger binding was observed at different molecular weights in FM and FMY. This seems to indicate that once Vtg is incorporated into the oocytes, it appears as a band at a lower molecular weight. However, with anti-seabream Vtg antibody, binding occurred at both higher and lower molecular weight bands only with FMY as no reactivity was observed with FM and FMP. Anti-seabream Vtg antibody may be useful if distinction

is required between *G.holbrooki* plasma Vtg and Vtg in the form of yolk protein.

Based on these results, rabbit anti-salmon Vtg antibody was determined to be the most appropriate for screening male *G.holbrooki* in this study. This is important because if Vtg is detectable in male *G.holbrooki*, this would demonstrate that Vtg protein is present in the blood. As male fish do not have ovaries, Vtg tends to accumulate in the blood (Sumpter and Jobling, 1995) and will not show as other forms of incorporated yolk protein. Also, salmon Vtg standard is commercially available making it possible for semi-quantification of Vtg in *G.holbrooki* using a salmon standard curve. There is no seabream Vtg standard available commercially.

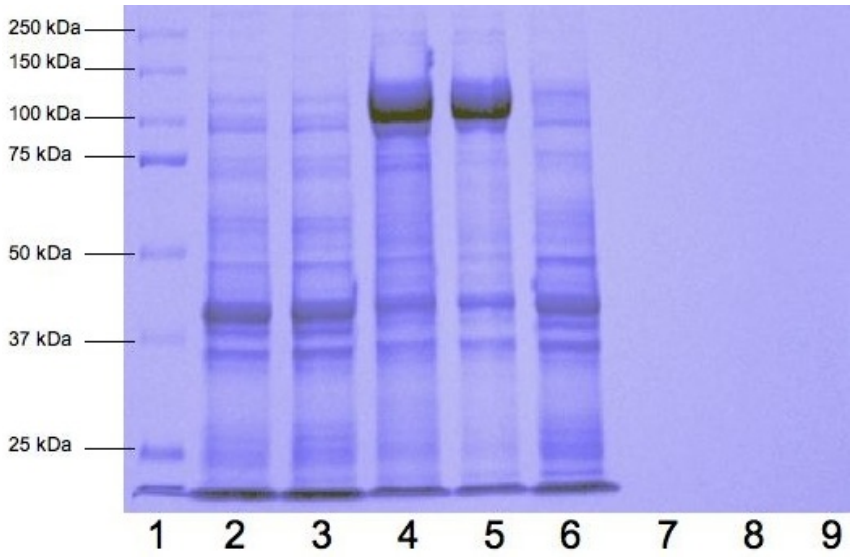
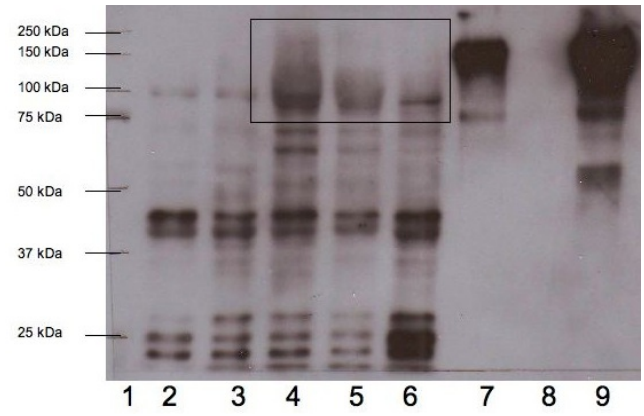
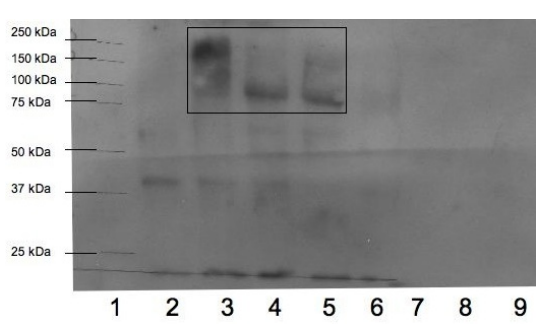


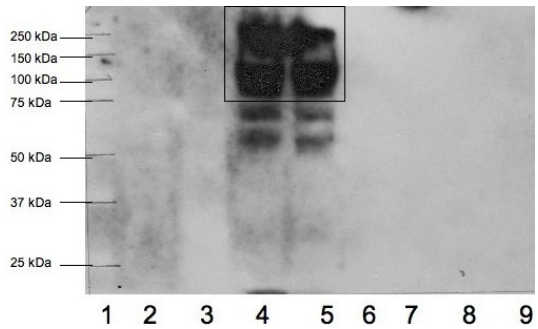
Figure 3.4: Coomassie stained gel showing protein bands in adult male and female *G.holbrooki*; Testing three anti-Vtg antibodies against *G.holbrooki* wholebody homogenates; Lane 1: Molecular weight marker, Lane 2: Male mosquitofish (MM), Lane 3: Two female mosquitofish (FM) with gravid spots pooled, Lane 4: Female mosquitofish with oocytes (FMY) from Brodies Lake, Lane 5: FMY from Watson's Creek, Lane 6: Female mosquitofish with gravid spot and plasma (FMY), no oocytes, Lane 7: Carp Vtg standard 16.65 ng, Lane 8: empty, Lane 9: Carp female liver (positive control) 675 ng.



(A) Mouse anti-carp Vtg antibody, ECL exposure for 20 mins.



(B) Rabbit anti-salmon Vtg antibody, ECL exposure for 30 mins.



(C) Rabbit anti-seabream Vtg antibody, ECL exposure for 30 mins.

Figure 3.5: X-ray film showing results using ECL detection of three anti-Vtg antibodies against *G.holbrooki* wholebody homogenates. Lane 1: Molecular weight marker, Lane 2: Male mosquitofish (MM), Lane 3: Two female mosquitofish (FM) with gravid spots pooled, Lane 4: Female mosquitofish with oocytes (FMY) from Brodies Lake, Lane 5: FMY from Watson's Creek, Lane 6: Female mosquitofish with gravid spot and plasma (FMP), no oocytes, Lane 7: Carp Vtg standard 16.65 ng, Lane 8: empty, Lane 9: Carp female liver (positive control) 675 ng.

3.3.7 Part B - Optimizing Methods for and Applying the Dot Blot Assay to Detect Vtg in Fish Sampled from the Field

3.3.8 Comparing exposed and unexposed male *G.holbrooki* for Vtg induction

Unexposed and exposed male *G.holbrooki* were tested using the dot blot Vtg assay with salmon anti-Vtg antibody to see if Vtg induction could be measured before the assay was tested on field samples. For unexposed fish, whole body homogenates from three male *G.holbrooki* collected from the lake at RMIT University Bundoora (acclimatized for two weeks) and a female *G.holbrooki* with embryos collected from Watson's Creek (R) were analysed.

For exposed fish, acclimatized male *G.holbrooki* received a nominal concentration of 300 ng/L estradiol (E_2) dissolved in ethanol, for 7 days. Exposure was performed in a semi-static system in glass tanks containing 3 litres of dechlorinated, carbon filtered aerated water (henceforth referred to as wet lab water WLW) at a temperature of $25°$C and pH of 7.1. Fish were subjected to a 14 hour light and 10 hour dark photoperiod at a temperature of $25°$C. Fish were fed twice a day with a combination of Tetramin colour range and ground soymeal pallets. On the last day of exposure, fish were euthanased and stored at $-80°$C. Fish were processed according to methods discussed in Section 3.3.2.

All samples were applied in triplicates on nitrocellulose membrane. Assay methods are as discussed in Section 3.3.11. The membrane was developed using ECL on film in the dark room. However, as a Vtg standard curve had not been developed at this stage, data is represented as volume units obtained from the analysis of x-ray film on the Gel doc system (Biorad) using Quantity One image acquisition program (Bio-Rad).

A higher reading of volume units (VU) was observed in the the exposed male mosquitofish sample (MME) as compared to the unexposed male mosquitofish samples (MM) (Table 3.1 and Figure 3.6). The female mosquitofish (MF) sample also showed a higher VU reading compared to MME. However, MM1 (unexposed male mosquitofish 1) also showed a higher VU reading compared to the other two unexposed male fish. It is possible that higher readings of Vtg in MM1 could be because the collection site, RMIT University lake, receives urban stormwater and is a stormwater storage lake. Stormwater drains from the university buildings are connected to the lake. Storm water also drains into the Bundoora East lake and is pumped to the Bundoora West lake for later use such as watering of nearby sports fields (RMIT University, personal communication, 2010). Typically, stormwater runoff contains pollutants (Tang et al., 2013) such as heavy metals and has been shown to induce Vtg mRNA expression in *G.holbrooki* in the laboratory (Huang et al., 2014). A study by Norris and Burgin (2011) found that *G.holbrooki* inhabiting wetlands that receive stormwater directly from the source demonstrated a morphological response consistent with endocrine disruption. Alhough, fish from the RMIT University Lake were acclimatized for a two-week period, a longer period may have been necessary for Vtg protein levels to return to baseline levels. In male fathead minnows, exposure to E_2 induced detectable plasma Vtg protein levels within 16 hours of treatment and remained near maximum levels for at least 18 days (Korte et al., 2000).

Table 3.1: Mean Volume Units (VU) of unexposed and exposed male *G.holbrooki* obtained from Gel Doc System.

Fish	Samples	Mean Volume Units (VU)
Mosquitofish male 1	MM1	30851
Mosquitofish male 2	MM2	24468
Mosquitofish male 3	MM3	28061
Mosquitofish male exposed	MME	34196
Female mosquitofish	MF	37838

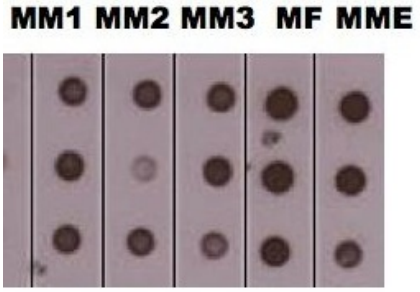


Figure 3.6: Developed x-ray film using ECL detection showing binding of proteins of unexposed and exposed *G.holbrooki*. Mosquitofish male 1 (MM1), Mosquitofish male 2 (MM2), Mosquitofish male 3 (MM3), Mosquitofish male exposed to E_2 (MME), and Mosquitofish female (MF). Note darker dots of MME and MF.

3.3.9 Dot blot Vtg standard curve development

A 5-point standard curve was established using salmon Vtg and expressed as salmon Vtg equivalent concentration (SVtgEq). The standard curve ranged from 3.125 ng to 100 ng/μl, however, the lowest concentration of 3.125 ng was often omitted because of high variabil-

ity. Figure 3.7 shows the dot blot film with the Salmon Vtg standard curve and female rainbow trout liver (10 ug) as a positive control. Manufacturer (Biosense) tests show good cross-reactivity between anti-salmon Vtg antibody and a number of species including rainbow trout, therefore, female rainbow trout liver was used as a positive control. Figure 3.8 is an example of a representative 5 point Vtg standard curve used to interpolate the Vtg concentration in male *G.holbrooki*.

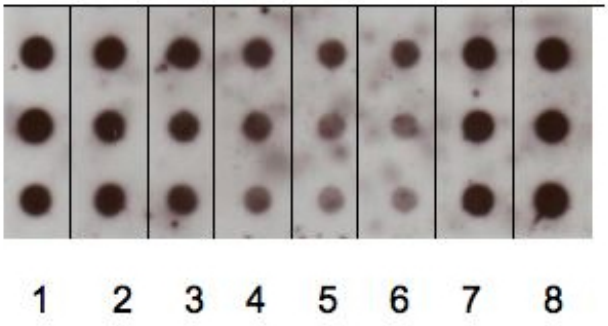


Figure 3.7: Salmon Vtg standard development shown on x-ray film. (1) 100 ng, (2) 50 ng, (3) 25 ng, (4) 12.5 ng, (5) 6.25 ng, (6) 3.125 ng, (7) Rainbow trout female liver (10 μg), (8) Duplicate of 7.

3.3.10 Intra-assay and inter-assay variability

Variability was measured using female rainbow trout liver protein. Intra-assay variablity was measured in one dot blot assay (n = 3) and inter-assay variability was measured using 3 separate dot blots (n=3). This was expressed as coefficient of variation (%CV).

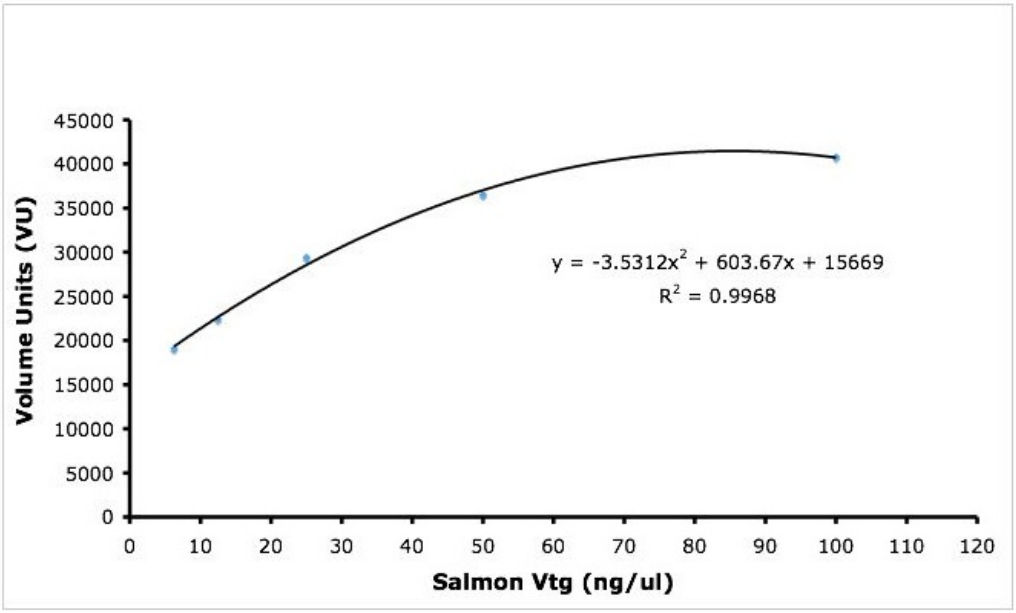


Figure 3.8: Representative standard curve (S-VtgEq) for interpolation of Vtg concentration in wholebody extracts of *G.holbrooki*.

3.3.11 Testing of dot blot assay for semi-quantitive analysis of Vtg in male *G.holbrooki* from field sites

As per manufacturer's instructions, nitrocellulose membrane was recommended instead of PVDF membrane. Nitrocellulose membrane 0.45 μm (Biorad, Australia) was soaked in cold PBS for 10 minutes and then assembled as per manufacturer's instructions into the 96 well Bio-Dot microfiltration apparatus (Biorad, Australia). To ensure a complete seal, vacuum was applied (via a flow valve attached to a vacuum source) while tightening the apparatus. The membrane was reactivated with PBS pipetted into dot blot apparatus.

Approximately 8 to 10 male *G.holbrooki* per site were analysed. Whole body ho-

mogenates, 20 μg in 50 μl of PBS was pipetted into the wells of the apparatus. A female *G.holbrooki* (homogenate) from each site was loaded to check for differences between male and female Vtg levels. Female rainbow trout liver homogenates were included in each assay to assess inter-assay variability and as a positive Vtg control. Male trout liver homogenates were included as a negative control. A Salmon Vtg standard curve was included in each assay.

PBS with 0.5% skim milk was pipetted into empty wells for blocking. Antigen was allowed to filter through gravitationally for 40 minutes and then pulled through with vacuum suction after which each well was washed with 200 μl of TBST, pulled through by vacuum. The membrane was removed after the wash step and blocked in 5% skim milk in TBS for one hour. The membrane was washed twice in TBS for 2 minutes.

Filtered 0.5% skim milk dissolved in PBS was used as a buffer for the antigen. This was centrifuged at 10,000 RPM for 2 minutes to prevent milk particles from clogging the membrane. Fish whole body homogenate was diluted in 0.5% skim milk in PBS and then centrifuged again at 10,000 RPM for 2 minutes prior to pipetting into the apparatus.

The membrane was probed with salmon antibody at a 1:2000 dilution for one hour on a gentle shaker in the warm room at 37°C. After this, the membrane was washed 5 times for 10 minutes each with TBST. The membrane was probed with horse anti-rabbit HRP secondary antibody 1:10,000 for one hour at room temperature on a gentle shaker. The membrane was washed 5 times, 10 minutes each with TBST and then with a final wash of TBS for 10 minutes. The membrane was developed using ECL on film in the dark room. Exposures ranged from 2, 5, 10, 30 minutes with the last exposure lasting 60 minutes. After development, film was scanned on the Gel Doc system (Biorad) and analysed and quantified using Quantity One image acquisition program (Bio-Rad). *G.holbrooki* mean Vtg concentration was semi-quantified using the Salmon Vtg standard curve on each membrane.

Variability between assay runs was tested using rainbow trout male and female liver samples (negative and positive control) on each 96-well plate and expressed as %CV.

3.4 Results

3.4.1 Intra-assay and inter-assay variability

Intra-assay variablity was 0.9% (n = 3) and inter-assay variability for the 3 separate dot blot assays was 2.77% (n=3).

3.4.2 Mean Vtg in male *G.holbrooki* from field sites in the study

Collection sites in 2009 showed levels of estrogenic activity in the water ranging from 0.1 - 1.7 ng/L EEQ except for a WWTP at 12 ng/L EEQ. Two other sites that showed estrogenic activity above 1 ng/L were a reference site (Liverpool FRB) and a rural/urban site (Narre Warren SD).

There was no correlation between estrogenic activity and mean Vtg levels in male *G.holbrooki* for all sites (Pearson's r = .400, n = 16, $P > 0.1$). Gisborne WWTP showed high estrogenic activity (12 ng/L EEQ) and a corresponding high Vtg level in male fish at 16.62 ng/μl.

Figure 3.9 shows the mean Vtg in male and female *G.holbrooki* from the field sites sampled in 2009. Mean Vtg in male *G.holbrooki* was significantly different at all field sites ($F_{15,115}$=11.908, $P < 0.001$). This ranged between 4.70 g/μl S-VtgEq in fish from Watson's Creek (R) to the highest at 22.83 ng/μl S-VtgEq in fish from Werribee WWTP.

Vtg levels in male *G.holbrooki* from the three WWTP sites (Brushy Creek, Gisborne WWTP and Werribee WWTP) were significantly higher than in male fish from the four reference sites ($P< 0.05$), Toorourrong Reservoir, Sanitarium Lake, Brodies Lake, and Liverpool FRB. There were no significant differences in Vtg levels of male *G.holbrooki* from all wastewater treatment plant impacted sites (Brushy Creek, Gisborne and Werribee) ($P> 0.1$) except for fish from Sunbury WWTP, which had significantly lower Vtg than fish from Werribee WWTP ($P< 0.01$). Male *G.holbrooki* from Sunbury WWTP had lower levels of Vtg and therefore were not significantly different to fish from all the other sties ($P> 0.05$) except Watson's Creek (R) ($P> 0.05$). Although male *G.holbrooki* fish from Watson's Creek (R) had the lowest levels of Vtg, this was not significantly lower compared to the reference sites ($P> 0.5$).

There were no significant differences in Vtg levels of *G.holbrooki* males from all the reference sites (Toorourrong Reservoir, Brodies Lake, Sanitarium Lake and Liverpool FRB) (P=1). This ranged from 7.08 to 8.98 ng/μl S-VtgEq. Sites that were not significantly different from the reference sites included Kalparrin Wetland (U), Kororoit Creek (I/U), Merri Creek (I/U), Woori Yallock Creek (R/U), and Deep Creek (R) (P=1). Three male fish from Watson's Creek (R) and two male fish from Sanitarium Lake (Ref) had undetectable levels of Vtg.

Female *G.holbrooki* Vtg levels were higher than in male *G.holbrooki* fish at all sites as expected ($t(30)$=6.667, $P> 0.01$) and varied from site to site. This ranged between 11.38 ng/μl S-VtgEq in the female fish at Merri Creek (I/U) to 38.83 ng/μl S-VtgEq in the female fish at Kalparrin Wetland (U). Mean Vtg levels in female *G.holbrooki* were at 26.89 ng/μl S-VtgEq compared to male *G.holbrooki* at 10.74 ng/μl S-VtgEq.

Inter-assay variability in the total number of plate runs (n=16) was 8.81% for male trout liver negative control samples and 3.14% in female trout liver positive control samples.

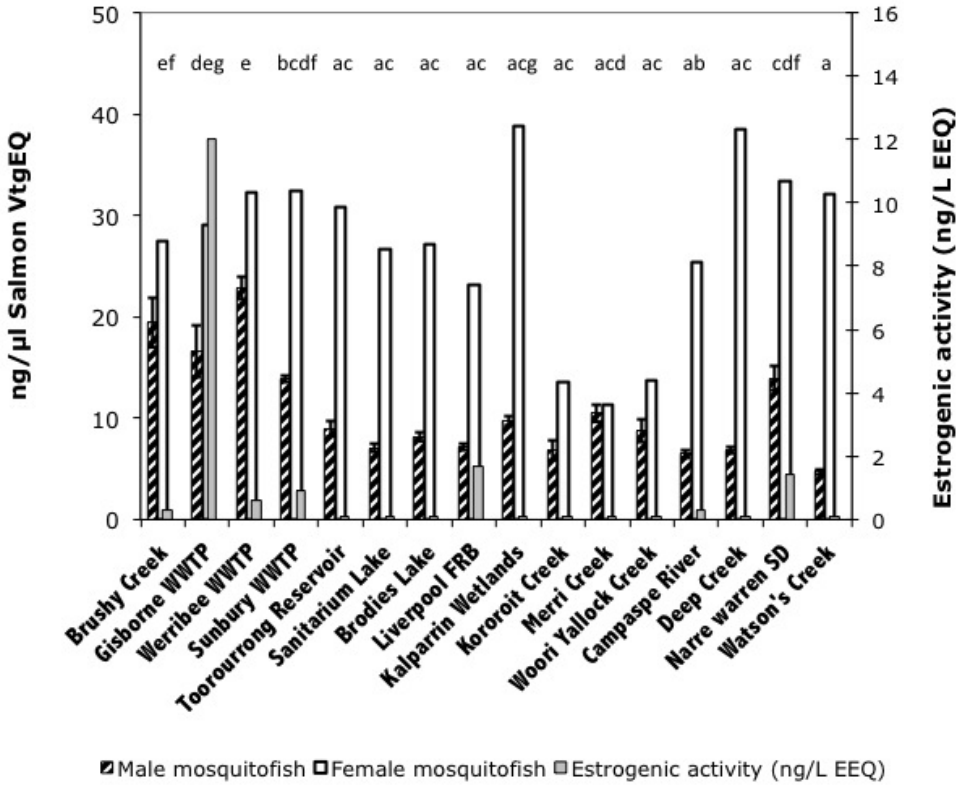


Figure 3.9: Mean (±SEM) Vtg levels (S-VtgEq) in wild male *G.holbrooki* collected from sites in 2009 and estrogenic activity in the water (ng/L EEQ). Female *G.holbrooki* Vtg levels are also shown for comparison with male *G.holbrooki*.

3.5 Discussion

The objective of this study was to develop a screening tool using a dot blot semi-quantitative assay to screen for Vtg in wild male *G.holbrooki* from the selected study sites. After testing of three different anti-Vtg antibodies using immunoblots, the dot blot assay was developed based on salmon Vtg standards and salmon anti-Vtg polyclonal antibody and was successfully tested as a screening tool in this study. Furthermore, contrary to previous knowledge that anti-Vtg antibodies are highly species-specific, in this study, it has been demonstrated using immunoblots that anti-Vtg antibodies of non-related fish species resulted in successful detection of Vtg in female *G.holbrooki* at different reproductive stages. Due to time and animal ethics approval limitations, the experimental testing of the dot blot assay on unexposed and exposed male mosquitofish was simplified. The Vtg assay would benefit from further testing using a robust experiment with more replicates of exposed and unexposed male mosquitofish and female mosquitofish to better estimate variability in background levels and induction of Vtg. The data obtained in the assay testing were in mean volume units and could not be converted to S-VtgEq because of the lack of a Vtg standard curve during the initial testing stages. In future, an inclusion of the salmon Vtg standard curve during the testing of the assay on exposed and unexposed male mosquitofish would improve the quality of the data providing comparisons with both volume units and S-VtgEq.

The use of salmon anti-Vtg antibody and salmon Vtg standards to semi-quantify *G.holbrooki* Vtg is not ideal because of the multiplicity and different responses of Vtg to estrogenic contaminants in different fish species (Hiramatsu et al., 2006). However, the assay was adequate for the purposes of the detection of Vtg for this study without the expense of time and costs for specific anti-Vtg production. There is potential for the current method to be improved and modified into a simple dot blot assy for detecting Vtg for field-testing. Small fish present difficulty in collecting sufficient plasma to test for circulating Vtg in male fish. The use of whole body homogenates of fish is an alternative, however, unlike immunoblots where de-

natured proteins are used and relevant proteins can be identified by molecular weight, whole body homogenates will contain non-specific proteins that may appear in the dot blot.

To achieve the objective of quick analysis in the field using dot blot assays, ultra purification filters may be a solution by potentially separating proteins of lower molecular weights from the samples. Unfortunately, this could not be tested within the timeframe and scope of the present study.

The dot-blot apparatus is a device that produces consistent results, however, assembly of the device is time-consuming. It is crucial that the device is screwed together at all corners with equal pressure, otherwise leading to inconsistent areas of protein absorption on the membrane.

Vtg was detected in most male *G.holbrooki* from all sites including the reference sites. This was unexpected but not surprising because estrogenic activity was also detected in the water of the reference sites in the range between 0.1 to1.7 ng/L EEQ.

Consistent with this study, it was previously reported that male fish from reference sites also showed detectable levels of Vtg. Cline (2002) reported Vtg in caged male fathead minnows at all sites including reference sites at 6μg/ml from rivers of North Georgia in America. Seasonal levels of Vtg in adult wild male fathead minnow were reported at reference sites (60% of the time in spring and 20% of the time in the fall) in the study (Cline, 2002). Similarly, Gagnon et al. (2008) reported low levels of Vtg in male *G.holbrooki* from all sites sampled in the drains of the Swan-Canning estuary, Western Australia, and attributed the presence of Vtg to background levels in male *G.holbrooki*. Background levels were also reported in male caged cod in a study by Scott et al. (2006) where Vtg levels were reported to be less than 0.01 - 1.35 μg/ml in reference sites of the North Sea. In the current study, Vtg levels found in male *G.holbrooki* at the reference sites (mean= 7.83 ng/μl) and a number of the other sites in this study could simply be background levels. A longer study over time of

Vtg levels in male *G.holbrooki* at reference sites is necessary to ascertain background levels of Vtg.

The lowest mean Vtg was in male *G.holbrooki* from Watson's Creek (R) (4.7 ng/μl) with three male fish having undetectable levels of Vtg. Two male fish from Sanitarium Lake (Ref) similarly had undetectable levels of Vtg. A study by Oğuz et al. (2015) similarly showed that Vtg was also undetectable in some male Lake Van fish and not others from the same site. The highest levels of Vtg was found in male *G.holbrooki* from Werribee WWTP, 5-fold higher than Watson's Creek (R) and approximately 2.7 fold higher than the reference sites. Vtg levels in male *G.holbrooki* from three (Gisborne, Brushy Creek and Werribee) out of the four WWTP sites were the highest, suggesting that male *G.holbrooki* from those sites could be exposed to estrogenic (or anti-androgenic) contaminants. Gisborne WWTP in particular, had 5 out of 30 adult male fish that did not show hooks and serrae development in their gonopodia (Discussed in Chapter 2). Angus et al. (2005) demonstrated that juvenile male *G.holbrooki* exposed to increasing concentrations of EE_2 via food in the laboratory failed to complete gonopodial development after 150 days, suggesting that the small number of male fish at Gisborne WWTP may be affected due to the high estrogenic activity from the sewage leak or/and from unknown events. Estrogenic activity at 12 ng/L EEQ at the affected site was measured a week post-spillage after clean-up was well underway and alarmingly, fish at Gisborne WWTP were most likely exposed to even higher elevated levels of estrogenic activity at the time of spillage.

Published studies report varying levels of Vtg in different species of wild fish making comparisons difficult. Assay protocol differences such as antibody type and Vtg standards in the different studies (Cline, 2002) mean that relative differences are more important than differences in reported values. In a study by Puy-Azurmendi et al. (2013), Vtg in plasma was observed in most male thicklip grey mullets captured from Basque estuaries with background levels of between 100 to 300 ng/ml Vtg in both male and juvenile fish not exposed to

estrogenic compounds. In comparison to the above study, the lowest Vtg levels in the current study would equate to 4700 ng/ml, which is higher than in the study by Puy-Azurmendi et al. (2013). Vtg in male fish exposed to estrogenic contaminants in the above study was however up to 57,500 ng/ml and up to 20,000 ng/ml in intersex fish. The highest levels of Vtg detected in male *G.holbrooki* in the current study would equate to 22830 ng/ml which is comparable to levels in the intersex fish in the above study. A recent study by Oğuz et al. (2015) reported levels of Vtg in plasma of male Lake Van fish up to 26.35 μg/ml which is similar to levels in the current study.

The expression of different Vtg genes and ER function in different species of fish is poorly understood and complex. A study by Miyagawa et al. (2014) of guppies, zebrafish, roach, and other species of fish ERα showed differing responses to synthethic estrogenic contaminants such as BPA and 4-nonylphenol (NP). Similarly, Petit et al. (1995) reported that in the rainbow trout ER, a fivefold lower affinity was demonstrated for diethylstilbestrol (a nonsteroidal estrogen) compared with its affinity for E_2, making extrapolations of reponses to EACs across species difficult. Some fish species may have Vtg genes that show different sensitivities to estrogenic contaminants, therefore, it is more difficult to explain differences between sample sites without an understanding of all chemical contaminants in the water. This was shown in a study by Ferreira et al. (2013) where Vtg gene I had a higher response to EE_2 than Vtg gene II in male *Lipophrys pholis*. This was found to be 475-fold higher than Vtg II at 15 ng/L EE_2 and 13-fold higher at 5 ng/L EE_2. Sawaguchi et al. (2004) found that exposure to E_2 upregulated Vtg A and B mRNAs but did not affect Vtg C expression in the plasma of female *G.holbrooki*.

An analysis of estrogenic activity of the water in 2009 was incorporated into this study. The most impacted was Jackson's Creek in Gisborne WWTP at 12 ng/L EEQ. The high estrogenic activity at Gisborne WWTP is likely to be attributed to a leak in the sewage system at the site a week before the sampling event or/and other events that may have occurred

prior to sampling. WWTPs often have variable flows, which may differ seasonally, and water quality should continue to be checked to ensure acceptable levels of estrogenic activity. Since 2009 was a drought impacted year in Victoria with creeks and rivers experiencing reduced water flow, it may be that the capacity for the creek to dilute effluents and the leak at the same time was severely reduced.

The other sites showed estrogenic activity ranging from 0.1 - 1.7 ng/L EEQ . This is comparable to values reported by other studies in Australia. Scott et al. (2014) reported EEQ values below 0.1 ng/L EEQ while 2% of samples had values above 1 ng/L EEQ in waterbodies in Australia. The highest estrogenic activity was detected downstream of a WWTP (6.5 ng/L EEQ) in that study. Mispagel et al. (2009a) reported estrogenic activity from 12 WWTPs in Southern Australia, tested using two different assays, at a maximum of 7.9 ng/L EEQ (hERα assay) and at much higher values of between 0.7 to 44.5 ng/L EEQ using the medERα assay.

Estrogenic activity higher than 1 ng/L EEQ was also detected at a reference site (Liverpool FRB) and an rural/urban site (Narre Warren SD). EACs and estrogenic activity were detected in surface water and sediment of Liverpool FRB (CAPIM, Unpublished data 2009) after the current study was conducted. The reference site, Liverpool FRB, had higher estrogenic activity, at least 2-fold higher compared to the other reference sites, however, mean Vtg in male fish from this site was not significantly higher than the other reference sites. Estrogenic activity at Narre Warren SD (R/U) on the other hand was as high as the other three WWTP sites (Brushy Creek, Sunbury WWTP and Werribee WWTP). Narre Warren is a semi-rural suburb with market gardens and farms. It is likely that pesticide run-off may be entering the creek. Male *G.holbrooki* from Narre Warren SD (R/U) had comparably higher Vtg although not significantly higher compared fish from the other reference, rural and urban sites except Watson's Creek (R).

A study by Saaristo et al. (2014) reported total estrogen (ES) concentrations (E_1, E_2, EE_2 ng/L) two years later at two of the sites in the current study, Brodies Lake (Ref) and Jackson's Creek (WWTP). Total estrogen concentrations at Brodies Lake on the 15th of February 2011 and 22nd of March 2011 were reported at 7.82 and 6.52 (ES) ng/E_2eq/L respectively, showing unexpected elevated levels at the reference site. Values at Jackson's Creek were at 8.97 and 13.19 (ES) ng/E_2eq/L on the 25th of March 2011 and the 16th of May 2011 respectively. The latter ES levels are surprisingly similar to ES values in the current study at 12.5 ng/L EEQ (Results not shown) but a direct comparison of ES measurements is difficult because ES measurements from the current study are from E_1, E_2 and E_3 measurements (Chinathamby et al., 2013). The high values of estrogenic activity and total estrogens reported in both studies more likely reflect higher estrogenic activity in general at Jackson's Creek as it receives discharges from two WWTPs located in close proximity to each other. It is necessary in future studies to obtain temporal changes in estrogenicity at all sites in parallel with fish biomarker tests for a better understanding of estrogenic effects in fish and the aquatic community.

A characterization of EACs in fish bile would be necessary and useful in explaining bioaccumulation in fish. Many EACs may not breakdown into ER ligands but may nonetheless have a toxic breakdown product. Although Vtg induction in this study may only be suggested at one or two sites, it is not possible to suggest that the fish populations in the other sites are not exposed to other contaminants that may induce other biomarkers.

A number of studies have reported varying effects on fish with corresponding measurements of estrogenic activity in the water. A study conducted in Switzerland (Burkhardt-Holm et al., 2008) reported levels between 0.2 - 2 ng/L EEQ in the rivers and found no direct effects on reproductive and developmental parameters measured in wild brown trout, however, hepatic levels of Vtg mRNA were induced in caged brown trout downstream of the sewage treatment plant while Vtg protein levels were elevated in feral brown trout. Another

study in Switzerland (Vermeirssen et al., 2005) looked at estrogenic activity in 18 rivers during winter and summer and found levels ranging from 0.3 - 2.0 ng/L EEQ and 4.0 - 7.0 ng/L EEQ during winter and summer respectively. Consequently, plasma Vtg levels were also elevated in brown trout in five of the sites with the highest plasma Vtg levels found at one site with the highest reading of 7.0 ng/L EEQ.

There was no correlation between estrogenic activity in the water and mean Vtg levels in male *G.holbrooki*. This may not reflect on internal and external exposures since grab samples are only a snapshot and not reflective of variability in the water. A study investigating EEQs in Swiss river water at one single effluent source showed variability over the 48 day sampling period (Vermeirssen et al., 2008). Estrogenic activity varies according to events such as increases in stormwater or surface run-off and effluent discharge. In the case of WWTPs, estrogenicity varies with discharge rates and times of effluents. It is unclear whether in-stream attenuation occurs and for how long a period fish and the aquatic community are exposed for during their life cycle in between discharge of effluents. More importantly, the Vtg protein is a long term biomarker and may remain elevated for a month or more after exposure depending on exposure concentrations and species sensitivity (Cline, 2002), therefore Vtg levels in fish may not be indicative of estrogenic activity in the water at time of fish capture. Furthermore, degraded products and some environmental estrogens can have different biological potencies that are not reflected in bioassays for estrogenic activity testing. Therefore, low estrogenic activity may be assumed to be of low risk to fish and the aquatic community unless individual hormonal activity measurements are conducted, as in the case of EE_2 which is 10x more potent than E_2 in vivo and was detected above the PNEC of 0.1 ng/L in a quarter of the samples of Australian river water recently (Scott et al., 2014). Frequent sampling events to measure temporal variations in Vtg levels in fish and estrogenic activity should be conducted in future.

Anti-androgenic activity in the water was not tested in this study and its effects cannot

be excluded. Anti-androgens bind to the androgen receptor (AR) and have the potential to block the action of androgens. This causes demasculinisation of male fish under laboratory conditions, in turn materializing into feminizing effects such as Vtg induction (Jensen et al., 2004) and reduced sex characteristics (Filby et al., 2007, Sebire et al., 2008) in fish. Response is also subjective depending on the presence of other antagonistic activity that may inhibit Vtg expression. Recently, a study by Huang et al. (2014) showed metal (Cd, and Pb) induced Vtg mRNA expression, while Zn inhibited the expression of Vtg mRNA in the liver of adult female *G.affinis* after just 1, 3 and 8 days of exposure. Although, the sites tested in this study were of freshwater sources, there is potential for stormwater drain catchment sites such as Narre Warren SD to accumulate heavy metals in its road run-off for interference with Vtg protein expression in fish due to heavy metal exposure. Therefore, regardless of total estrogenic activity in the water, biological effects may not correspond. Anti-androgenic pathways are complex and act through multiple modes of action (Martinović-Weigelt et al., 2011) and its investigation is beyond the scope of this thesis.

Inappropriate induction of Vtg leads to diversion of vital proteins (Carragher and Sumpter, 1991), alterations in kidney development and disruptions in kidney function (Herman and Kincaid, 1988). Jobling et al. (2002) found that in wild-caught intersex fish that were severely feminized, sperm motility was reduced by up to 50% and fertilization success reduced by up to 75% compared with less severely intersex fish. Difficulty in undertaking studies looking at population effects in wild fish makes predicting implications of intersex fish hard. However, population effects were evident when levels of 0.19 - 1.9 ng/L EEQ of EE_2 were added to a lake in Canada over a 7 year period causing elevations in Vtg mRNA and protein levels and impacts on gonadal development in male fathead minnow and consequently leading to the collapse of the population (Kidd et al., 2007). Although sites selected in this study showed fairly low levels of estrogenic activity, studies mentioned above suggest that even low levels are capable of causing varying levels of effects although further research needs to be done on species sensitivity to the different estrogenic contaminants.

3.6 Conclusion

Results from this study show elevated levels of Vtg in male *G.holbrooki* from all of the WWTP sites. In particular, fish from Gisborne WWTP showed elevated levels of Vtg and a correspondingly high estrogenic activity of the water suggesting exposure to estrogenic contaminants. However, fish collections were grab samples and it is not possible to determine whether the fish populations at Gisborne WWTP are exposed to low level chronic exposures of estrogenic contaminants or whether effects in fish were from the one-off exposure due to the sewage leak at the WWTP a week before fish collections. Vitellogenin protein was induced in *G.holbrooki* exposed to 6.6 ng/L EEQ for 7 days in the laboratory (Scott et al., 2017) which is lower than levels found at Gisborne WWTP.

It is necessary to continue monitoring Vtg in *G.holbrooki* populations at the affected site and some of the other WWTP sites showing elevated Vtg in male *G.holbrooki* to understand the capacity of the receiving waterbodies to dilute and assimilate effluents. It would be beneficial to obtain regular readings of estrogenic activity to understand the variability of estrogenic activity at the sites and the tolerance of local fish populations.

Although, it was useful obtaining results of estrogenic activity of the water at the same time as fish collections, the Vtg protein biomarker measured in the current study is a long term biomarker and may remain elevated for several months after exposure depending on exposure concentrations and species sensitivity, therefore Vtg levels in fish may not be indicative of estrogenic activity in the water at time of fish capture. It may be necessary to include measurements of Vtg mRNA levels in future studies as evidence for short-term estrogenic exposure for a better correlation with estrogenic activity.

EAC effects on signalling pathways in fish and localization of Vtg in fish are complex processes and vary in different species of fish and types of EACs. Some EACs may cross-

talk with both the AR and ER signaling pathways, potentially suppressing or elevating levels of Vtg. This has to be considered and investigated to interpret biomarker-monitoring data if population effects of EACs on fish are to be understood.

Chapter 4

Hepatic EROD Activity (CYP1A) in Male *Gambusia holbrooki* in Victorian Rivers, Australia

4.1 Abstract

The Induction of cytochrome P4501A (CYPIA) in fish (measured using ethoxyresorufin-*O*-deethylase (EROD) activity) is a common biomarker for assessing environmental exposure to aryl hydrocarbon receptor (AhR) ligands. While certain chemicals have been identified as AhR ligands, the common inducers are exogenous aromatic compounds (eg., polychlorinated dibenzodioxins (PCDDs), polychlorinated biphenyls (PCBs), and organochlorine pesticides (OCPs). These are commonly known as persistent organic pollutants (POPs). The EROD assay was optimized for male *G.holbrooki* in the laboratory. Male *G.holbrooki* were collected from sites in 2008 and 2009 to evaluate hepatic EROD activity from a number of land use types such as rural, urban, reference and WWTP impacted sites. AhR activity of the water ranged from ranged from 7 ng/L βNF EQ in Brodies Lake (Ref) to 180 ng/L βNF EQ at Narre Warren SD (R/U). Fish from Narre Warren SD (R/U) did not show high EROD activity induction corresponding to high AhR levels of the water. There was no correlation between EROD activity in fish and AhR activity of the water. There were significant differ-

ences in EROD activity of fish from the different sites. In 2009, this ranged from a low of 0.42 pmol/min/mg protein at Campaspe River (R) to a high of 10.85 pmol/min/mg protein at Kalparrin Wetland (U). As expected, EROD activity in fish from industrial/urban sites or sites receiving urban stormwater such as Kalparrin Wetland (U), Kororoit Creek (I/U), Narre Warren SD (R/U) were generally higher. Hepatic EROD activity for fish collected in 2008 ranged from 0.94 to 7.30 pmol/min/mg protein and were not significantly different from each other. The presence of water AhR activity in all of the sites, Narre Warren SD drain in particular, is an important indicator that levels need to be monitored. The lack of a correlation between water AhR activity and EROD activity in fish represents the need for an understanding and a reconciliation between AhR measurements in water and bioavailability of contaminants to fish.

4.2 Introduction

4.2.1 AhR agonists in freshwater systems in Victoria

A vast number of environmental pollutants end up in our waterways. These include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides such as DDT, Aldrin, Chlordane, Dicofol and Dieldrin, commonly known as persistent organic pollutants (POPs) (Jones and de Voogt, 1999, Rawson et al., 2009, Zhou et al., 2010). These POPs are potential endocrine disruptors that act directly or indirectly via the aryl hydrocarbon receptor (AhR) (Shanle and Xu, 2011). Sources of PAHs and dioxins such as PCBs, PCDDs and PCDFs include traffic, industrial waste, forest fires and the release of chemical mixtures during incomplete combustion of wood, garbage, coal and other organic materials (Bosveld et al., 2002). These chemicals eventually make their

way into surface waters, collect at wastewater treatment systems and consequently enter sewage effluent discharges. Although, in Victoria, organochlorine pesticides (OCPs) have been mostly deregistered or are being phased out, residues are persistent in the environment and were detected in Victorian groundwater in 2007 (Wightwick and Allinson, 2007) and in sediment in Victorian wetlands (CAPIM, Unpublished data 2009). In a national survey of Australian sewage sludge from 2004 to 2006, Deldrin was the most prevalent OCP detected in all parts of Australia (Clarke et al., 2010). In addition to that, low concentrations of dioxin-like compounds were detected in sewage sludge spot samples collected in a survey from fourteen wastewater treatment plants around Australia (Clarke et al., 2008), confirming the persistence of these contaminants in the Australian aquatic environment.

POPs are generally 'fat loving' and 'water-hating' and tend to partition to organic matter, avoiding the aqueous phase (Jones and de Voogt, 1999) and ultimately ending up in sediments (Akkanen et al., 2012). They persist in the environment because of their hydrophobic nature and eventually bioaccumulate in fatty tissues of animals and humans (Kland, 1988, Jones and de Voogt, 1999). However, bioaccumulation in organisms depends on a number of complex factors such as environmental conditions, properties of chemicals (Barron et al., 2004, Zhang et al., 2004), characteristics and feeding patterns of organisms, and interactions of organisms with the environment (Akkanen et al., 2012).

4.2.2 CYP1A protein and EROD activity

Organic pollutants taken up by organisms undergo some sort of biotransformation in their bodies before excretion or carcinogenesis occurs. This adaptive response of biotransformation is mediated by the AhR together with the aryl hydrocarbon nuclear translocator (ARNT). The AhR is a ligand-dependent transcription factor that regulates the expression of several genes such as the cytochrome P4501A (CYP1A) group. The CYP1A subfamily

is part of the large Cytochrome 450 family of heme proteins responsible for the oxidative metabolism of substrates such as drugs, aromatic hydrocarbons, fatty acids, pesticides, and steriods (Goksøyr, 1995).

In the presence of AhR ligands, binding to the AhR first occurs in the cytoplasm of the cell. Upon binding, the receptor translocates to the nucleus and heterodimerizes with the ARNT for transcription, where it then binds to the xenobotic response elements (XRE) sequence in the promoter regions of target genes such as the cytochrome P4501a genes (Barouki et al., 2007, Zhou et al., 2010) for activation or suppression (Whyte et al., 2000). Once induced, initiation of the CYP1A gene transcription occurs, followed by enhanced CYP1A activity (Sarasquete and Segner, 2000). Finally, biotransformation of the xenobiotic compounds occur either by detoxification and excretion or to carcinogenesis (Sarasquete and Segner, 2000, Bemanian et al., 2004, Uno et al., 2012). The AhR mediated CYPIA response can be quantified by measuring CYPIA mRNA or protein content using catalytic assays such as 7-ethoxyresorufin-*O*-deethylase (EROD) activity (Sarasquete and Segner, 2000).

See Whyte et al. (2000) for an extensive review on EROD activity in fish. In short, EROD assays measure the enzymatic activity of CYP1A to convert 7-ethoxyresorufin (substrate) to a fluorescent product, resorufin. The more CYP1A present in the tissue sample, the higher the catalytic activity towards the substrate. EROD activity is expressed as a concentration of resorufin produced per mg protein per sample per minute of reaction time (mol/mg/min). In fish, numerous studies report hepatic EROD activity but studies have also shown EROD induction in gills (Jönsson et al., 2002, Abrahamson et al., 2007), kidney (Ortiz-Delgado et al., 2008) and brain (Li et al., 2016). Hepatic EROD induction in fish is rapid within 24 hours of exposure (Jakšič et al., 2008) and up to 8 weeks for recovery (Behrens and Segner, 2001).

4.2.3 EROD activity in fish as a biomarker of exposure to pollutants

EROD activity in fish has been extensively studied and widely used as a biomarker for xenobiotics (Sarasquete and Segner, 2000) providing a baseline for comparison between species. Numerous studies have shown the relevance of the EROD assay as a monitoring tool in the field for many purposes. Most recently, a study by Habila et al. (2017) showed induction of EROD activity in both liver and gills of barbel captured at a water reservoir in Algeria which receives wastewater. The study showed both temporal and spatial variations in EROD activity in fish sampled a number of times from different locations of the reservoir. Similarly, EROD activity in fish was found useful in a study by Debrowska et al. (2017) where moderate positive correlations were found between EROD activity in flounder and the concentrations of organic and metal contaminants in the southern Baltic Sea coastal area. A study by Sturve et al. (2017) to evaluate the environmental health of the North and Baltic Seas, that receive extensive anthropogenic activities, showed highest levels of EROD activity in dab captured in an area with extensive oil drilling in the in North and Baltic Seas.

More importantly, the toxicology mechanism of POPs is complex and depends on the mediation of the AhR pathway in fish. Type, number and expression pattens of AhR pathway genes may contribute to differences in contaminant toxicity and CYP1A induction within fish species (Zhou et al., 2010) with further inter-species differences between EROD basal activity and the extent of induction (Whyte et al., 2000).

A number of studies using EROD induction in fish as a biomarker have been conducted in Australia on species that include pink snapper, yellowfin bream, sand flathead, Perth herring, sea mullet, yellowtail trumpeter, black bream, bluethroat wrasse, sixspine leatherjacket, pikey bream, and carp (Ahokas et al., 1994, Holdway et al., 1994, Cavanagh et al., 2000, Smith and Gagnon, 2000, Gagnon and Holdway, 2002, Webb and Gagnon, 2002, Shaw et al., 2004, Gagnon and Bakhtyar, 2013). Early studies were conducted by Ahokas

et al. (1994) on carp exposed to highly treated pulp mil effluent in Lake Coleman in southern Victoria. In that study, significantly elevated EROD levels in carp from the lake were reported compared to reference fish and correlations with low levels of PCDD/PCDFs were measured in carp tissue. Ahokas et al. (1994) concluded that EROD activity in carp represented a good biomarker to low levels of organochlorine contaminants from pulp mill effluent. In the same year, Holdway et al. (1994) reported EROD levels in sand flathead collected from Port Phillip Bay in Victoria. In that study, high induction was observed in fish collected in regions closest to highly industrial and urbanized areas of the bay. Furthermore, EROD activity in sand flathead from a zone in the bay was positively correlated with total freshwater input mainly from the Yarra River, suggesting AhR inducers in the input. Soon after, Smith and Gagnon (2000) conducted a study on sand flathead, bluethroat wrasse and sixspine leatherjacket to identify the most suitable bioindicator species, native to Australia, for marine environmental monitoring. The study proposed the sand flathead as the most suitable native species for its high induction as induction did not occur in bluethroat wrasse while sixspine leatherjacket had a limited distribution. Further studies by Gagnon and Holdway (2002) on sand flathead collected at six stations in Port Phillip Bay showed the highest EROD activity in fish collected near a station closest to Melbourne city while fish from stations in non-urbanized and non-industrialized part of the Bay showed lower EROD activity. Of all fish species studied in Australia, most are marine or estuarine. Studies on EROD activity in freshwater fish species are limited. The most recent study on EROD activity in freshwater fish was by Rawson et al. (2009) who reported induction in *G.holbrooki* in New South Wales, Australia in response to POPs. The study also found that increased catchment size was correlated with increased EROD activity and concluded that *G.holbrooki* are an appropriate species to investigate POP exposure in freshwater wetlands using EROD activity.

Although EROD activity is more often used as a biomarker of contaminant exposure, studies have reported an association between EROD measurements in fish and biological

damage such as an increase in hepatic lipofuscin/ceriod and possible cytological damages in immature *Solea ovata* (Au and Wu, 2001) and an increase in non-neoplastic lesions in subadult English sole (Myers et al., 1998). While an increase in EROD activity has not been causally linked to traditional EDCs, many xenobiotics such as organochlorinated pesticides, industrial chemicals, plastics and plasticizers potentially affect the endocrine and reproductive system of fish via the AhR (Whyte et al., 2000, Diamanti-Kandarakis et al., 2009, Shanle and Xu, 2011). Sites in the current study include areas that receive industrial effluent, hence it was considered of interest to evaluate EROD activity in *G.holbrooki* in Victoria.

G.holbrooki has a wide distribution due to its tolerance to a varied range of water conditions (Pyke, 2005) allowing for the suitability of this species for the purpose of this study.

The aims of this study are to evaluate basal hepatic EROD activity of male *G.holbrooki* from the various land use types around Melbourne and to compare EROD activity of fish against measured AhR activity in the water. The suitability of this biomarker for *G.holbrooki* as a screening tool for exposure to organic contaminants is further discussed. It is hypothesized that EROD activity would be higher in sites that receive urban and industrial pollutants than rural areas because industrial run-off often contains xenobiotics that may act as AhR agonists (Whyte et al., 2000, Rawson et al., 2009).

4.3 Materials and Methods

4.3.1 Study sites

Sites selected in 2008 and 2009 for this study were grouped according to surrounding land use types such as urban (U), rural (R), waste water impacted (WWTP) and reference sites

(Ref), although in some cases the grouping is somewhat arbitrary since some creeks flow through both residential and industrial or agricultural land and is likely to contain a combined source of pollutants. Some of these sites had dried up in 2009 because of severe drought conditions in Victoria in 2008 and 2009, therefore new sites were selected. Approximate locations of sampling sites in 2009 in Victoria, Australia is shown in Figure 2.3 (Chapter 2).

4.3.2 Fish collection and liver processing methods

Adult male *G.holbrooki* were collected from these sites using dip nets from February to May 2008 and 2009. In the field, fish were euthanased (blow to the head), transported on ice to the RMIT Ecotoxicology laboratory and sexed under a Wild Heerbrugg M3Z stereozoom microscope. Fifteen male fish from each site were weighed individually to the nearest 0.001 mg, measured for body length (BL) using a caliper to the nearest 0.01 mm, and then dissected for liver and gonad tissue immediately on the day of capture. Rawson (2008) reported unreliable measurements of EROD activity in *G.holbrooki* gill and gonadal tissue, however, gill and gonadal tissue was added to liver samples because of low tissue quantities in the study by Rawson (2008). Liver tissue alone was analysed for the current study without the addition of gills and gonad, the reasons being that if CYP1A was absent or undetectable in the other tissues, the addition of these tissues may dilute CYP1A content in the liver leading to lower readings of EROD activity. Therefore, due to the small amount of liver tissue on its own from each fish (approximately 0.001 - 0.004 g), the excised livers were pooled into groups of five, yielding a total of three sets (replicates) for each site. These were stored in microfuge tubes, frozen in liquid nitrogen and then stored at -80°C for later use.

4.3.3 Water AhR activity

Water samples were collected as 'grab' or spot samples from the study sites in 2009. Samples were collected in amber glass bottles, stored on ice and then at $4°$C until processed by Dr Mayumi Allinson (Department of Primary Industries) and team by methods described by Allinson et al. (2011) and Chinathamby et al. (2013) and measured for estrogenic (see Chapter 3) and AhR activity (in βNF EQ). Briefly, The transcriptional agonistic activities of compounds to the AhR were measured with a reporter assay using yeast cells (YCM3) carrying the response element for the AhR complex, XRE5. Agonist activity was measured as EC x 10. The assay was performed using a chemiluminescent reporter gene method employing a 96-well culture plate. Beta-naphthoflavone (βNF) was used as a positive control. A solvent (vehicle) control (dimethylsulfoxide) was also used. The bioassay method limits of reporting was 0.5 ng/L βNF EQ.

4.3.4 EROD assay optimisation

The assay was adapted from Holdway et al. (1993) by Sue Codi King, previously from the Australian Institute of Marine Science, and optimized for use on *G.holbrooki*. The initial aim was to standardize the protocol to enable comparisons with different fish species using a similar protocol. Only adult male *Gambusia holbrooki* were used for this study as Rawson et al. (2009) previously showed an interaction between fish developmental stage and sex (female fish) in EROD activity in *G.holbrooki*.

Forty four sexually mature male *G.holbrooki* were collected from the RMIT University Lake, Bundoora, using dip nets, for the optimisation of the assay. The fish were processed according to methods described in Section 4.3.2 and all forty four livers were pooled into a single sample for sufficient tissue for the optimisation of the assay. Methods for sample

preparation are described in Section 4.3.6. The assay was tested on the fluorescent plate reader at temperatures of 20°C, 25°C, 30°C, and 35°C. Phosphate buffer for the assay was also tested at a pH of 6.5, 7, 7.4 and 7.8 as enzymic activity often differs at various pH levels. In addition, liver homogenate (S9) volumes of 25 μl and 50 μl on the microplate were tested to evaluate whether S9 volumes yielded different results.

4.3.5 Exposure of Male *G.holbrooki* to Beta-naphthoflavone

Male *G.holbrooki* were exposed to concentrations of Beta-naphthoflavone (βNF) for 72 hours following exposure methods stated in Aubry et al. (2005). This part of the study is similar to the study of Aubry et al. (2005) on *G.holbrooki* hepatic EROD activity with the same nominal exposures of βNF to *Gambusia holbrooki* using a different assay method and a different plate reader at RMIT University Bundoora. The aim was to see if similar induction levels could be achieved between these two studies.

Adult male *G.holbrooki* were obtained from the lake at RMIT University Bundoora campus and acclimatised for 17 days in the aquaculture facility in WLW at a temperature of 25°C and pH of 7.1. Fish were exposed to a nominal concentration of 150 μg/l βNF dissolved in acetone (0.1% total volume) for 72 hours. Fish were also exposed to the solvent as a control and a control without solvent. For the experiment, fish were subjected to a 14 hour light and 10 hour dark photoperiod at a temperature of 25°C. Exposure to βNF was performed in a semi static system in glass tanks containing 3 litres of WLW water. Forty fish were allocated to each treatment with 20 fish per replicate in a separate tank (20 x 2 replicates). Fish were fed twice a day with a combination of Tetramin colour range and ground soymeal pellets. βNF (5, 6-benzoflavone) was purchased from Sigma-Altrich Australia.

On the last day of the experiment, the fish were euthanased and then livers were re-

moved and pooled into sets of five resulting in a total of eight groups of liver (n=2 for each treatment), except for the control group which had a total of four groups of liver as there was a loss of liver during storage.

4.3.6 *G.holbrooki* EROD assay

Liver tissue was thawed on ice and homogenized (1:4 w/v) in 0.1 M ice-cold potassium phosphate buffer at pH 7.4 containing 1mM DTT, 1mM EDTA, 0.1 M KCL and 0.1 M Phenanthroline. Livers were homogenized in 0.75 μl glass micro tissue grinders with micropestles and the homogenates were centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant (S9 fraction) was removed and transferred to chilled microfuge tubes, then stored at -80°C until analysis. EROD activity assay is described in the appendix (Appendix C).

On the day of analysis, S9 fractions were diluted 1:10 in phosphate buffer at a pH of 7.1 and pipetted in triplicate to black 96 well plates. 50 μl of sample volume was used in each well along with 200 μl 7-ER (0.2mM) calibrated at 450 nM using a cuvette on a Thermo Scientific Multiskan Spectrum (P97263, Pathtech, Australia). A resorufin standard curve run was included in each plate. A resorufin solution at 0.64 μM was prepared on the day of the assay run from 1mM Resorufin stock in DMSO and calibrated at 572 nM. The resorufin standard curve was then prepared from serial dilutions of the 0.64 μM resorufin solution on the same day. Phosphate buffer without liver samples (blanks) was also added to the plate as a check for contamination or unusual activity. Fish liver samples used for the earlier assay optimisation were added as a control on each plate to ensure repeatability. Appendix C, Figure C.1 shows the layout of the assay plate. The samples were retested if the control readings were more than 10% of values obtained during optimization of samples. Prior to reading plates on the fluorescent reader, 25 μl of NADPH (2.4 mM) was added to each well of the sample and blanks to start the reaction. The assay was read at a buffer pH of 7.1

at 35°C on a Perkin Elmer Wallac Victor2 Fluorescent plate reader (excitation: 531 nm, emission: 590 nm).

The EROD assay is based on the conversion of 7-ER into resorufin by the enzymatic activity of CYP1A in the liver. The more CYP1A present in the tissue sample (as a response to xenobiotic exposure), the higher the catalytic activity towards the substrate. EROD enzyme activities were normalized to protein content determined by the Lowry Method using the DC protein assay kit (Biorad, Australia). Protein content was measured as absorbance at 750 nm using a Thermo Ascent Multiskan plate reader. The reading from each well is expressed as the maximum rate of resorufin production (moles of resorufin produced per minute per mg protein in the liver sample represented as pmol res/min/mg protein).

4.3.7 Statistical analysis

Statistical analyses were performed using PASW 21 for Macintosh (New York, USA). Level of significance was set at ($P< 0.05$). EROD data for βNF exposures were tested using one-way analysis of variance (ANOVA). EROD data for *G.holbrooki* in 2009 and 2008 did not meet assumptions of normality, therefore the Kruskal-Wallis non-parametric analysis of variance with pairwise comparisions was used to test for differences in means between sites. Studies apply adjustments such as the Bonferroni correction to adjust for an inflated type 1 error when interpreting statistical results of multiple comparisons (post hoc tests) however, this was not applied in the current data analysis because adjustments have been reported as unnecessary and inaccurate as controlling for a Type I error tend to inflate the Type II error (Perneger, 1998, Rothman, 1990). Spearman's rank correlation coefficients were measured between the two variables, EROD activity in 2009 and AhR activity at the sites.

A power analysis to estimate adequate sample size was not conducted because meaningful effect size could not be determined (Hoenig and Heisey, 2001).

4.4 Results

4.4.1 EROD assay optimisation for male *G.holbrooki*

Preliminary investigations carried out in male *G.holbrooki* liver homogenates using volumes of 50 μl on the plate wells produced stable readings unlike volumes of 25 μl (results not shown). Maximum velocity was observed in the pooled liver samples at a temperature of 35°C with the phosphate buffer between the pH of 7 and 7.4. When interpolated on a graph, phosphate buffer with a pH of 7.1 was optimal (Figure 4.1). Therefore, it was decided that an assay protocol with liver homogenate volumes of 50 μl using buffer pH of 7.1, read at a temperature of 35°C would give acceptable results for *G.holbrooki* liver.

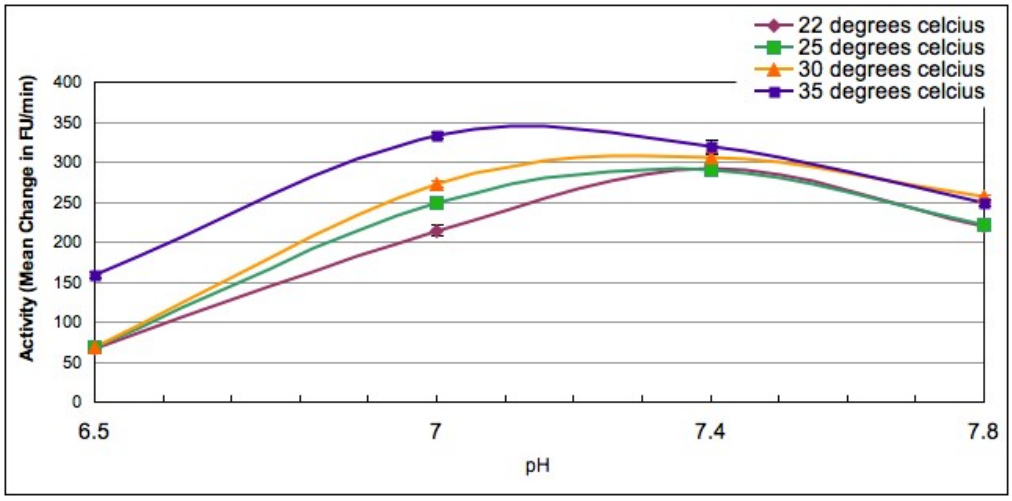


Figure 4.1: Optimisation of Hepatic EROD Activity in Relation to pH and Temperature showing (Mean ± SEM) Change in Fluorescent units per Minute).

4.4.2 Exposure of male *G.holbrooki* to βNF

Hepatic EROD activity in *G.holbrooki* induced by 150 μg/l of waterborne βNF in a 72 hour exposure was measured at 13.18 pmol/min/mg (Figure 4.2) and significantly higher than activity in both control and solvent vehicle groups ($P < 0.01$). Exposure to the solvent vehicle acetone did not induce EROD activity, however, fish in the control group had a significantly higher level of EROD activity (6.56 pmol/min/mg protein) than fish in the solvent group (2.21 pmol/min/mg protein) ($P < 0.04$).

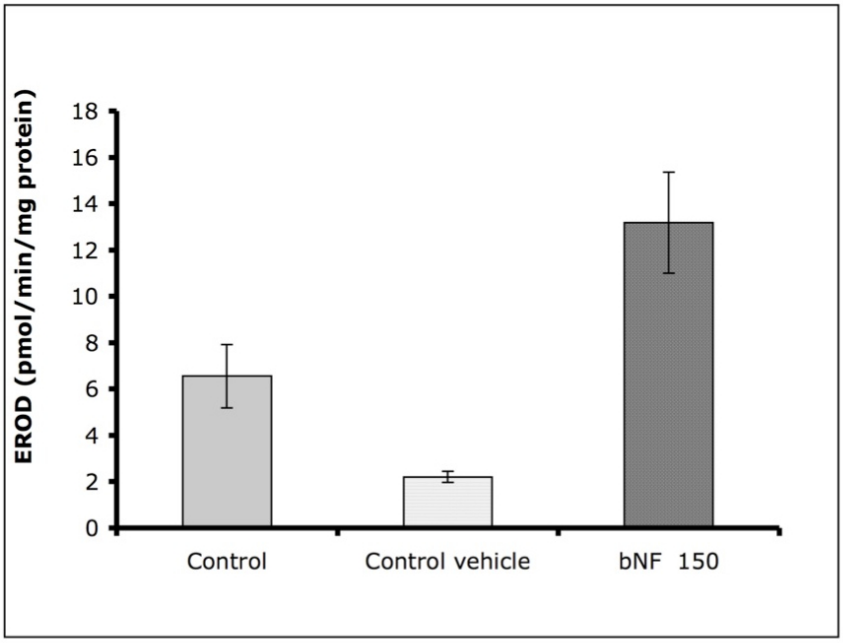


Figure 4.2: EROD hepatic activity in male *G.holbrooki* exposed to 150 μg/l βNF (Data are the mean $\pm$ SEM). Means are significantly different from each other.

4.4.3 AhR levels in water samples in 2009

AhR activity measured at sites in 2009 ranged from 7 βNF EQ in Brodies Lake (Ref) to 180 ng/L βNF EQ at Narre Warren SD (R/U). Although water samples from Narre Warren Stormwater Drain had the highest AhR activity, EROD activity in fish was not significantly higher than in fish from any other sites except Campaspe River (R) ($P< 0.037$).

There was no correlation observed between EROD activity in fish and AhR activity in water collected from sites in 2009 (Pearson's r =-0.026, $P> 0.5$) (Figure 4.3 and Table 4.1).

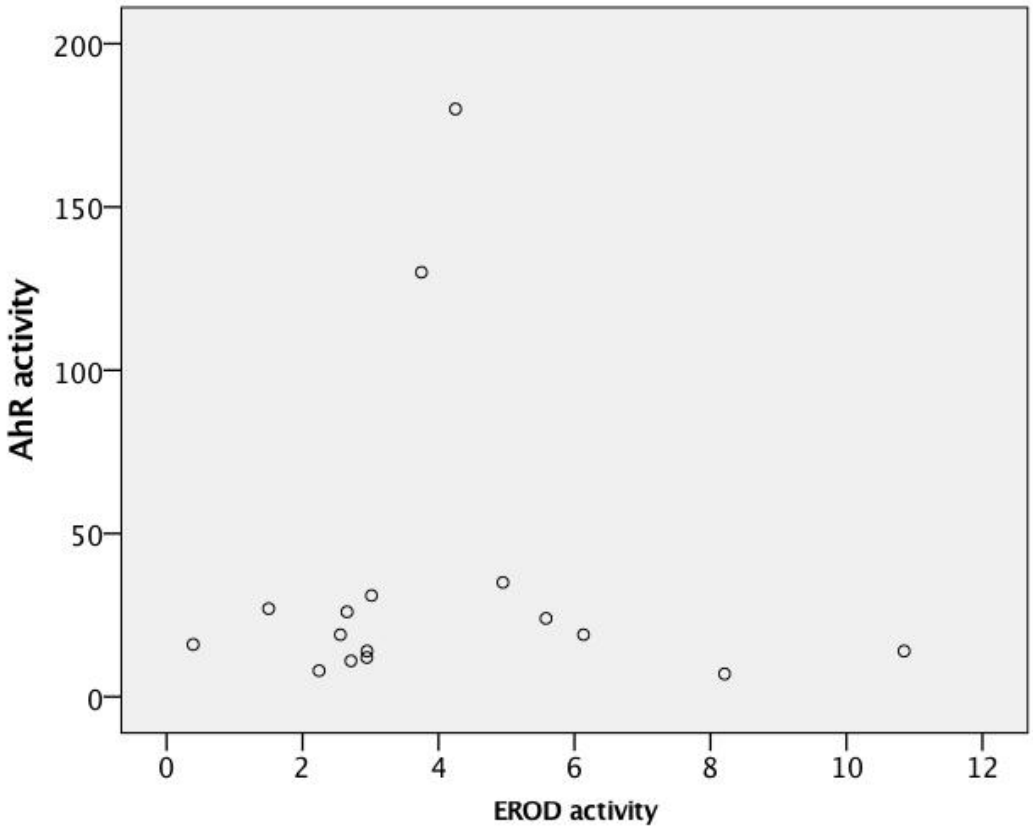


Figure 4.3: Scatterplot showing associations between AhR water activity and EROD activity in fish.

Table 4.1: Pearson's Correlation showing relationship between AhR water activity and EROD activity in fish.

Pearson Correlation

		EROD activity	AhR activity
EROD activity	Pearson Correlation	1	-.026
	Sig. (2-tailed)		.924
	N	16	16
AhR activity	Pearson Correlation	-.026	1
	Sig. (2-tailed)	.924	
	N	16	16

4.4.4 EROD activity in *G.holbrooki* collected in 2008

Hepatic EROD activity for *G.holbrooki* collected in 2008 ranged from 0.94 to 7.30 pmol/min/mg protein and were not significantly different from each other ($P > 0.05$, Kruskal-Wallis H = 24.38, df = 15) (Table 4.2). Again, due to the small sample sizes (n=3) and high variability in EROD activity for some of the sites, results should be interpreted cautiously. In 2008, fish from Gisborne WWTP had the lowest EROD activity at 0.94 pmol/min/mg protein and fish from Sunbury WWTP were the highest at 7.30 pmol/min/mg protein, followed by fish from Kororoit Creek (I/U) at 5.70 pmol/min/mg protein. EROD activity in fish from the four reference sites ranged from 1.04 to 3.04 pmol/min/mg protein and averaged 2.25 pmol/min/mg protein.

4.4.5 EROD activity in *G.holbrooki* collected in 2009

Results should be interpreted conservatively due to small sample subsets (n=3). EROD activity in fish collected in 2009 were significantly different from each other ($P < 0.018$,

Table 4.2: EROD activity (pmol/min/mg protein) (±SEM) in male *G.holbrooki* collected in 2008. Means were not significantly different from each other *Includes sites that could be classified as both rural and urban or urban and industrial. ** Site receives septic and greywater discharge.

Site type	Site	EROD activity (pmol/min/mg protein)
Ref.	Brodies Lake	3.04±2.43
	Liverpool FRB	1.04±0.08
	Green Lake	2.52±0.35
	Sanitarium Lake	2.30±0.12
Rural	Lerderderg River	1.90±0.68
	Campaspe River	1.18±0.06
	Deep Creek	1.12±0.10
	Ryans Creek*	1.56±0.24
Urban	Kalparrin Wetland	1.45±0.24
	Merri Creek*	2.65±0.38
	Kororoit Creek*	5.70±2.25
WWTP	Gisborne	0.94±0.16
	Sunbury	7.30±3.90
	Werribee	3.20±0.86
	Brushy Creek	2.77±0.50
	Mullum Mullum Creek**	2.32±1.27

Kruskal-Wallis H = 28.65, df = 15) and ranged from a low of 0.42 pmol/min/mg protein in fish from Campaspe River (R) to a high of 10.85 pmol/min/mg protein in fish from Kalparrin Wetland (U). There was no consistent pattern observed in EROD activity in fish from the sites based on land use, for example, high EROD activity was found in fish from reference sites and low activity in fish from urban sites (Table 4.3).

Interestingly, in 2009, EROD activity in fish from the four reference sites were inconsistent. Fish from Sanitarium Lake (Ref) and Liverpool FWB (Ref) had an EROD activity of 2.71 and 2.65 pmol/min/mg protein respectively. However, higher EROD activity (3-4 fold) was observed in fish from Tourrourong Reservoir (Ref) (6.13 pmol/min/mg protein) and Brodies Lake (Ref) (8.21 pmol/min/mg protein) although these results were not significantly higher than the other two reference sites.

As expected, fish collected from industrial/urban sites or sites receiving urban stormwater (Kalparrin Wetland, Kororoit Creek, Narre Warren SD) had generally higher levels of EROD activity except for Merri Creek (I/U), although this was not significantly higher compared to the reference sites (Sanitarium Lake and Liverpool FWB). The highest EROD activity was in fish from Kalparrin Wetland (U) at 10.85 pmol/min/mg protein which was significantly higher than fish from Campaspe River (R) ($P<$ 0.00), Merri Creek (I/U) ($P<$ 0.006), Deep Creek (R) ($P<$ 0.024), Gisborne WWTP ($P<$ 0.035), Liverpool FRB (Ref) ($P<$ 0.037), Sunbury WWTP ($P<$ 0.037), and Sanitarium Lake (Ref) ($P<$ 0.046). Despite high EROD activity in fish from Kalparrin Wetland (U), AhR activity was a moderate 14 ng/L βNF EQ.

Fish from Campaspe River (R) had the lowest EROD activity at 0.42 pmol/min/mg protein, significantly lower than fish from Narre Warren SD (R/U) ($P<$ 0.037), Brushy Creek (WWTP) ($P<$ 0.032), Kororoit Creek (I/U) ($P<$ 0.006), Werribee WWTP ($P<$ 0.003), Tourrorong Reservoir (Ref) ($P<$ 0.002), Kalparrin Wetland (U) ($P<$ 0.000), and Brodies Lake

(Ref) ($P < 0.002$).

Table 4.3: EROD activity (pmol/min/mg protein) (±SEM) in male *G.holbrooki* collected in 2009 and corresponding water AhR activity. Means not sharing the same letters are significantly different from each other *Includes sites that could be classified as both rural and urban or urban and industrial. Numbers next to sites correspond to numbers in Figure 2.3.

Site type	Site	EROD activity (pmol/min/mg protein)	AhR (βNF EQ in ng/L)
Ref.	9. Brodies Lake	$8.21^{be} \pm 2.68$	7
	10. Liverpool FRB	$2.65^{ae} \pm 1.04$	26
	11. Toorourrong Reservoir	$6.13^{bde} \pm 0.49$	19
	12. Sanitarium Lake	$2.71^{ae} \pm 1.60$	11
Rural	4. Narre Warren SD*	$4.25^{bcde} \pm 1.92$	180
	14. Campaspe River	$0.39^{a} \pm 0.07$	16
	13. Deep Creek	$2.24^{ad} \pm 0.64$	8
	16. Woori Yallock Creek*	$2.95^{ab} \pm 0.57$	14
	15. Watson's Creek	$2.75^{ab} \pm 0.29$	12
Urban	2. Kalparrin Wetland	$10.85^{b} \pm 4.49$	14
	1. Merri Creek*	$1.50^{ac} \pm 0.20$	27
	3. Kororoit Creek*	$4.95^{bde} \pm 0.48$	35
WWTP	7. Gisborne	$2.56^{ae} \pm 0.97$	19
	6. Sunbury	$3.01^{ae} \pm 2.11$	31
	8. Werribee	$5.58^{bde} \pm 0.86$	24
	5. Brushy Creek	$3.75^{bcde} \pm 0.16$	130

Figure 4.4: Rural/Urban collection site - Narre Warren Stormwater Drain.

Figure 4.5: Industrial/Urban collection site - Merri Creek.

Figure 4.6: Urban collection site - Kalparrin Wetland Stormwater Drain with Dr Vincent Pettigrove (Melbourne Water and CAPIM).

Figure 4.7: Rural collection site - Campaspe River, Kyneton.

4.5 Discussion

EROD induction levels of exposed *G.holbrooki* in the current study were lower by a factor of 100 compared to the study by Aubry et al. (2005) using the same nominal concentrations and duration of exposure of βNF in male *G.holbrooki*. Aubry et al. (2005) reported EROD activity in control groups of male *G.holbrooki* at approximately 100 pmol/min/mg protein. The highest induction in the study by Aubry et al. (2005) was at 700 pmol/min/mg proteins, 7 fold higher than basal levels of EROD activity. In contrast, the highest induction in EROD hepatic activity in *G.holbrooki* in the current study was at 13.18 pmol/min/mg protein. However, EROD activity was 6 to 7 fold higher than in fish exposed to the solvent (acetone) in the current study which is comparable to induction rates reported by Aubry et al. (2005). These results highlight the variabilities in assays between laboratories.

However, EROD activity in the control group was significantly higher than in the solvent vehicle group. The reasons may be that possible contamination during the exposure experiment might have occurred, otherwise, acetone may have repressed basal levels of EROD activity in fish leading to much lower levels than in the control group. David et al. (2012) showed that DMSO and methanol commonly used as solvent vehicles in studies significantly reduced EROD activity in zebrafish larvae at 0.1% and 0.05% v/v respectively.

AhR activity (βNF EQ in ng/L) reported in the current study at Kalparrin Wetland (U) (site with the highest EROD activity in fish) at 14 ng/L βNF EQ is very low in comparision to the βNF exposure of 150μg/l in the laboratory that induced the highest EROD activity of 13.18 pmol/min/mg in laboratory fish. However, the highest EROD activity between the laboratory fish and wild fish are within range at 13.18 and 10.85 pmol/min/mg protein respectively. The low measurement of AhR activity of 14 ng/L βNF EQ in the field eliciting a similar induction as the exposure of 150 μg/L βNF in the laboratory could be due to combined effects of more potent AhR agonists present at Kalparrin Wetland, on hepatic

EROD activity of fish. The lack of a corresponding difference in EROD activity between both the laboratory and wild fish at Kalparrin Wetland (U) when there is a big difference in exposures needs to be reconciled. To date, there are no studies exploring these differences in EROD activity in fish in the laboratory and fish in the wild exposed to similar levels of AhR activity or concentrations of βNF.

Water samples tested in 2009 during fish capture showed detectable levels of AhR activity in all of the samples. AhR activity ranged from 7 to 35 ng/L βNF EQ with the exception of the two outliers at 130 and 180 ng/L βNF EQ and is within the range of the levels found in a study by Allinson et al. (2011) (10 - 25 ng/L βNF EQ) in 2008 and (19 - 27 ng/L βNF EQ) in 2009 in locations along the Yarra River.

Both the industrial/urban sites (Kororoit Creek and Merri Creek) did not show an unusually high level of AhR activity in water compared to the other sites. This could be because AhR activity in this study was measured only in the dissolved phase. Activity in suspended solids was not assessed. Aqueous passive sampling would have been useful in the current study. This is an *in situ* technique that uses devices that sequester bioavailable organic contaminants from the aquatic environment. Without additional data (eg.passive sampling and sediment data), AhR activity in water may only reflect the presence of more water soluble POPs as the majority of hydrophobic chemicals avoid the aqueous phase (Jones and de Voogt, 1999), ultimately ending up in sediments (Akkanen et al., 2012), and later becoming bioavailable through desorption. Dagnino et al. (2010) reported that AhR activity (up to 80 percent) was carried in the suspended solid phase and not the dissolved phase in wastewater effluents in the study and suggest analyzing AhR activity in the suspended solid phase in addition to the dissolved phase. In addition, Zhang et al. (2004) reported higher concentrations of total PAHs, PCBs and OPs in sediment compared to water.

The two extreme outliers of AhR activity in water were Narre Warren Stormwater Drain

(R/U) (180 ng/L βNF EQ) and Brushy Creek (WWTP) (130 ng/L βNF EQ). Narre Warren is a semi-rural suburb with market gardens and farms and had been urbanized with new housing developments during the time of sampling (Figure 4.4). It is expected that water samples from Narre Warren SD would be high in AhR levels. Stormwater drains are used for collection, and storage of run-off from urban areas such as roads, and hard surfaces and would typically carry pollutants containing heavy metals and AhR agonists such as run-off from spillages, sediments and particles from construction sites (Tang et al., 2013). Tang et al. (2013) similarly reported low levels of TCDD-EQ (0.71 and 4.12 ng/L) (CAFLUX assay) collected from stormwater at an industrial site and high levels at a commercial site with high road traffic (>10 ng/L TCDD-EQ) in a study sampling stormwater in various Australian capital cites.

Brushy Creek (WWTP) is a small tributary of the Yarra River in the urban outskirts of Melbourne that passes through rural land. In addition to urban and rural run off, a nearby sewage treatment plant also discharges treated effluent into Brushy Creek. It is possible that the WWTP discharging into Brushy Creek might not be removing dioxin-like chemicals effectively. Jálová et al. (2013) found significant seasonal variability during the year from 13 to 90 % in the efficiency of treatment of a highly efficient WWTP for compounds with dioxin-like potency. It is difficult to suggest the source of contaminants in the creek as the sources of influents into Brushy Creek are rather complex. Perhaps illegal sources of pollutants are being discharged into this creek. Further investigation and monitoring are required for this site.

AhR activity comparisons across studies are difficult because of the differences in bioassays and reporting units (eg. βNF EQ compared to TCDD equivalents). Therefore, comparisons of the results of the current study with previous studies reporting in TCDD-EQ units may not be accurate. However, Allinson et al. (2011) cites Kamata et al. (2009) and reports comparisons between βNF and TCDD numerical values (using the YCM3 cell assay) as

approximately the same when comparing low concentrations. Using this comparison, AhR levels in the current study are estimated to be much higher than that of Rawson et al. (2009) where lower levels up to 0.032 ng/L TCDD-EQ were reported in water samples in creeks and rivers in Sydney. Continuous monitoring of these sites for AhR activity is necessary, in the long run. Macova et al. (2011) similarly showed much lower levels of TCDD-EQ of raw sewage in Queensland, Australia at less than 2 ng/L.

There were no significant differences in EROD activity in fish in 2008. The highest levels were found in fish from Sunbury WWTP, however, results were insignificant because of the high standard error in EROD activity.

EROD activity in fish in 2009 ranged from 0.42 pmol/min/mg protein at Campaspe River (R) to a high of 10.85 pmol/min/mg protein at Kalparrin Wetland (U) which is 5 fold higher than levels at two of the reference sites. Although this is comparable to the study by Aubry et al. (2005), other published data have shown different levels of induction in *Poeciliidae*. The lowest hepatic EROD activity in a study by (Jakšič et al., 2008) in wild populations of *G.affinis* (western mosquitofish) was 7.5 pmol/min/mg protein. In that study, the highest induced levels of EROD activity exposed to Lindane were approximately 10 fold higher than fish in control groups. In contrast, Rawson et al. (2009) reported high basal EROD activity for wild *G.holbrooki* at 2308 pmol/min/mg in the wetlands of Sydney.

EROD activity in fish from sites near industrial or urban land use or sites receiving urban run-off (except Merri Creek) were generally higher although not significantly higher compared to some of the reference sites, suggesting that fish populations in those sites were exposed to contaminants that were bioavailable. Low levels of EROD activity in fish populations in sites surrounded by industrial and urban land use may suggest a chronic exposure to contaminants as low induction may be an acquired resistance while high induction levels may mean a sudden and acute exposure (Elskus, 2001).

Fish from Merri Creek (I/U) showed low EROD activity compared to the other urban sites. Merri Creek flows into pastoral, urban, industrial, and then again through residential areas. Fish were collected in a heavily industrialized site, near a stormwater drain flowing into the creek (Figure 4.5). It could also be suggested that the fish population at Merri Creek may be affected by other contaminants that can suppress AhR induction (eg. estradiol) (Havelková et al., 2008).

Fish from Kalparrin Wetland (U) had significantly higher EROD activity than those from a number of the other sites. Kalparrin Wetland receives stormwater from the surrounding area. The fish were caught at the end of the drain in a shallow pool of water located at the gross pollutant trap. Urban litter such as plastic bags and plastic bottles collect at the gross pollutant trap where fish were captured (Figure 4.6). As discussed earlier in the case of Narre Warren Stormwater Drain (R/U), stormwater contains high levels of urban pollutants. In this case, the fish were localized in a small shallow pool at the end of the stormwater drain. It is possible that the fish had a higher exposure to the pollutants in the stormwater because of their habitat. Similarly, Rawson et al. (2009) reported higher EROD activity in fish from wetlands fed by highly urbanized catchments.

On the other hand, Campaspe River (R) had the lowest EROD activity in fish in 2009 regardless of the presence of AhR activity at the river. This river is located in Kyneton and passes through farmland (Figure 4.7). Compared to a lake, a river system presents different types of conditions for the bioavailability of POPs (eg. flow rates, sediment dynamics and kinetics) (Akkanen et al., 2012). The low induction in EROD in fish from Campaspe River suggest that, at the time of sampling, the fish were exposed to minimal levels of AhR inducers.

Basal levels of EROD activity in wild populations of *G.holbrooki* in Melbourne are difficult to determine. Whyte et al. (2000) reports a large variance in published data on basal

EROD activity and just in the common carp, this ranges from 0 - 4600 pmol/min/mg protein. In 2009, EROD activity in fish from the four reference sites were inconsistent. *G.holbrooki* from Tourrourong Reservoir (Ref) and Brodies Lake (Ref) had 3 to 4 fold higher EROD activity than those from the other two reference sites (although not significantly higher). Brodies Lake was chosen as a reference site because of its location in Greensvale Reservoir, yet a number of pesticides were detected both in surface water and sediment at Brodies Lake in 2009 (CAPIM, Unpublished data 2009). This site requires future monitoring with further collections of fish for EROD activity measurements. It is difficult to find pristine reference sites in the field (Whyte et al., 2000) as contamination could occur through unexpected sources such as wildfires in the case of Tourrourong Reservoir which was severely burnt a week before fish collections.

In 2008, Victoria faced a long dry spell due to a lack of rainfall (BOM, 2008). The drought persisted into the summer of 2009 with the lowest precipitation and highest summer temperature recorded in the months of January (BOM, 2009*b*) and February of 2009 (BOM, 2009*a*) leading to wildfires. Fires occurred around the Toorourrong catchment area on February 7, 2009 and burnt 100 percent of the area, although the reservoir was declared safe and unaffected by the fire. The reservoir, a reference site, was sampled for fish and water on April 2, 2009. Water AhR activity (19 ng/L βNF EQ) was not elevated compared to the other sites, however, EROD activity was 3 fold higher, although not significant, compared to the other two reference sites. The higher EROD activity in fish from Toorourrong Reservoir may be due to influxes from the wildfires in the area. The heavy use of brominated flame retardants at the site (Hayden, S, personal communication, December 21, 2009) may also have contributed to higher EROD activity in the fish. Rinne (1996) found that toxic slurry or ash flows were fatal to a high percentage of salmonids immediately after a fire and suggest that survivors become physiologically stressed. In addition to ash flows, fire-suppressant foams were found to be most toxic to some early life stages of rainbow trout, indicating that accidental input of fire-fighting chemicals into aquatic environments

could affect fish populations (Gaikowski et al., 1996).

It is known that forest fires (or any large combustion) are a notorious source of PAHs (Simoneit, 1999). AhR activity in water at Toorourrong Reservoir was not elevated as the sampling event took place two months after the fires and contaminants from the fire may have adsorbed to organic matter in sediments (Schäfer et al., 2010, Jones and de Voogt, 1999). An earlier study by Allinson et al. (2011) showed higher AhR activity in sites around the Yarra River in 2009 compared to 2008 and attributed the higher levels to the wildfires in the upper part of the Yarra catchment. Another study undertaken at around the same time in 2009 following the wildfires by Schäfer et al. (2010) suggested an input of ash slurry due to elevated levels of PAHs using passive samplers in sites adjacent to the burnt areas close to Toorourrong Reservoir although no short-term toxic effects were seen on the macroinvertebrate communities.

The aqueous solubility of POPs, in particular, PAHs, varies according to its chemical structure. Solubility of PAHs decreases for each additional ring (Masih et al., 2010). Zhang et al. (2004) analysed levels of 16 PAHs, 12 PCBs and 18 OCPs in water and sediment samples in a river in China and reported that 2-ringed PAHs were dominant in water samples while 4-ringed PAHs in sediment. Kafilzadeh et al. (2011) found the highest concentrations of 3-ring PAHs in water and 4-ring PAHs in sediments in a river in Iran. Petrogenic PAHs are characterized by 2-3 rings and are contributions from contamination of petroleum products and pyrogenic PAHs are composed of above 4-ring PAHS and are a source of forest fires and incomplete combustion of organic matter (Zadeh et al., 2010). In the current study, industrial sites such as Kororoit Creek (I/U) and Merri Creek (I/U) are likely to contain petrogenic PAHs characteristic of industrial contamination while pyrogenic PAHs would be present in the sediment of Toorourrong Reservoir after the wildfires.

A lack of correlation between EROD and AhR activity in this study is not unexpected

due to the complex nature of AhR agonists, their bioavailability and fate in the water, different responses in the mediating systems in fish and sampling methods for AhR activity used in this study (water vs sediment). The topic of bioavailability of POPs in the aquatic environment is complex and multidisciplinary in itself and beyond the scope of this thesis, however, it is a crucial link in explaining the relationship between EROD activity in fish in this study and AhR levels in water samples at the time of fish capture. Semple et al. (2004) and Akkanen et al. (2012) define and describe bioavailability as that of a chemical which is freely available (freely dissolved concentration) to be taken up by an organism and bioaccessibility as a portion of a chemical which is available to an organism (after desorption) if the organism has access to the chemical. Several conditions of the aquatic environment and qualities of a contaminant affect the freely dissolved concentration and the bioaccessible fraction of the chemical to organisms (Akkanen et al., 2012). Akkanen et al. (2012) describe these conditions as aqueous solubility, sediment structure, partitioning between water and solid or sediment, chemical properties of organic contaminants, and the origin of compounds in the case of PAHs (pyrogenic or petrogenic). It is necessary to note that one off grab water samples were obtained in this study for measurements of AhR activity because of the high cost of the assay at the time of the study. Ideally, more frequent measurements of water AhR activity are necessary at the sites to understand temporal variation.

To add to the chemical complexity, some organochlorine pesticides that are AhR agonists such as DDT and Endosulphan II were found dominant in water while Dieldrin and DDE were mostly in sediment (Zhang et al., 2004). Barron et al. (2004) compiled published studies on CYP1A induction and AhR binding in teleost, avian, and mammalian systems and reported that PAHs with 4-6 rings containing fluoranthene or phenanthrene structures with an exposed bay region seemed to have a higher potency to the AhR in fish than the 2-3 ringed unsubstitued PAHs which were inactive in fish systems. If 2-3 ringed unsubstituted PAHs or other similar chemicals that are inactive in fish systems were dominant and consequently detected in the water surfaces, it might explain the lack of correlation in this study

between AhR levels and EROD activity in fish.

Behaviour of the organism, such as feeding patterns and movement habits in water, are important considerations when understanding exposure to POPs. Exposure is likely via these routes: through dietary sources (eg. food or sediment ingestion), through skin and gills (aqueous exposure) or through dissolved contaminants in sediment pore water (Akkanen et al., 2012, Whyte et al., 2000). A study comparing aqueous uptake versus dietary uptake of dioxins in copepods and fish showed that dietary accumulation was the primary pathway for dioxin accumulation in black bream and copepods (zooplankton) (Zhang et al., 2011). Trophic transfer of dioxins was predominant because of the high bioaccumulation potentials of dioxin in phytoplankton. In most habitats, *G.holbrooki* are littoral and a predominantly planktivorous species (Blanco et al., 2004) showing a preference for cladocerans supplemented with algae, terresterial insects and detritus (Specziár, 2004, García-Berthou, 1999). Their feeding behaviour is closely linked to the water surface (García-Berthou, 1999), therefore dietary exposure through contaminated phytoplankton in the water column is more likely to be the primary route of contamination than aqueous as particles would have sorbed to the sediment rapidly. This further supports the lack of a correlation between water AhR levels and EROD activity in fish. AhR levels in grab samples are a snapshot of the immediate situation at the time of sampling. There could be an underestimation of chronic contaminant exposure to organisms through desorption and through dietary sources. Furthermore, accumulation in body tissues or excretion of contaminants may have already occurred in the fish prior to water sampling and liver analyses. In fact, different biomarkers in addition to EROD activity are necessary to understand contaminant exposure. In a study by Andersson et al. (2010), three-spined sticklebacks, exposed to synthetic and natural sources of humic substances, showed EROD activity in gills but not in liver of fish.

The trouble with the AhR is that it has been reported to be fairly promiscuous because of its ability to bind and be activated by a diverse range of chemicals (Denison and Nagy,

2003), some of them endocrine disrupters. Kojima et al. (2010) reported eleven out of two hundred tested pesticides induced AhR-mediated transcriptional activity using an assay called DR-assay. These are again a diverse range, Acifluorfen-methyl, Bifenox, Chlorpyrifos, Isoxanthion, Quinalphos, Chlorpropham, Diethofencarb, Propanil, Diuron, Linuron, and Prochloraz. A recent study by Huang et al. (2014) showed that heavy metals, in particular, Zinc, Cadmium and Lead, could induce CYP1A mRNA expression levels in *G.holbrooki*. Although this does not mean CYPIA protein in liver was consequently induced, the suggestion that heavy metals (that are not the classical inducers of AHR) modulate CYP1A mRNA expression is a concern and an important implication in interpreting AhR effects in fish. Furthermore, AhR inducers have been shown to cause estrogenic, anti-estrogenic or anti-androgenic effects and potentially cross-talk with a number of systems, specifically the estrogen receptor (ER) (Aubry et al., 2005, Kirby et al., 2007), from alterations in hormone synthesis to reductions in hormone (estrogen in particular) responsiveness. This presents a further complication. Other factors that were not investigated, may also have modulated EROD activity in this study. The effects of rainfall and catchment size were not examined in this study and affected EROD activity in wild *G.holbrooki* in the study by Rawson et al. (2009).

4.6 Conclusion

It is important to note that basal levels of EROD activity in wild populations of male *G.holbrooki* in Victoria cannot be determined due to the differences in EROD activity in fish from the reference sites. In this study, EROD activity was determined in wild populations of fish as well as in the laboratory through a single βNF exposure to fish. This has provided a means of comparisons between EROD induction in laboratory fish and wild populations of *G.holbrooki* in Victoria. The lack of a correlation between water AhR activ-

ity and EROD activity in fish represents the need for an understanding and reconciliation between measurements in water and combined and complex effects of AhR inducers in the wild to fish. EROD activity data cannot be extrapolated to reflect the health of the water due to a number of limitations. AhR inducers have been shown to cause estrogenic, anti-estrogenic or anti-androgenic effects and potentially cross-talk with a number of systems, specifically the ER. Therefore, EROD induction in *G.holbrooki* in this study may be an underestimation. Also, differences in EROD activity between species of fish (different AhR affinity between species to contaminants) make comparisons to other species of fish and aquatic wildlife difficult. Excising liver of male *G.holbrooki* is a time consuming effort due to the size of fish (approximately between 18 to 24 mm). This is an important consideration when rapid methods of analyses are necessary for quick assessment of water health. The presence of water AhR activity in all of the sites in the current study presents a necessity to monitor AhR activity at the sites over time to gain a better understanding of EROD activity and temporal variations in AhR activity.

Chapter 5

Analysis of Condition Indices and Biomarkers for *G.holbrooki* in Victorian Freshwaters

5.1 Abstract

A suite of biomarkers, EROD activity, Vtg induction, gonopodial length/body length (GL/BL) ratio , condition factor (CF), gonadosomatic index (GSI), and hepatosomatic index (HSI), were evaluated in sexually mature male *G.holbrooki* collected from sites in Melbourne and Victoria. These sites were grouped according to surrounding land use types such as urban (U), rural (R), reference (Ref) and wastewater treatment plant (WWTP) impacted sites. No strong associations were evident among the biomarkers selected for this study. A number of sites showed confirmation of anthropogenic influences which was evident from the high AhR and estrogenic activity of the water, however, physiological responses of fish were complex with no consistent pattern. Mean HSI in *G.holbrooki* ranged from a low of 1.35 at Campaspe River (R) to a high of 2.24 and 2.23, respectively at Sunbury WWTP and Brushy Creek (WWTP). Mean GSI in fish ranged from a low of 2.21 at Liverpool FRB (Ref) to a high of 4.40 at Kalparrin Wetland (U) followed by Merri Creek (I/U) at 4.27 and Deep Creek (R) at 4.26. Some sites have been identified as impacted sites based on the results of

the selected indices and are recommended for further monitoring This multi-biomarker approach improved the understanding of the effects of exposure to pollutants and the complex responses of fish at the different sites.

5.2 Introduction

Freshwater ecosystems are among the most vulnerable habitat types in the world (Masese et al., 2013) constantly challenged by the input of anthropogenic pollutants into the aquatic environment through consumer activities, municipal waste disposal, agricultural and industrial run-off and accidental releases of chemicals (Manning, 2005, Barber et al., 2011). Anthropogenic pollutants that have been identified as potential EACs and EDCs include heavy metals, industrial chemicals such as Bisphenol A and surfactants, and persistent organic pollutants (Jones and de Voogt, 1999, Manning, 2005).

Although EACs and estrogenic activity (Allinson et al., 2011, Chinathamby et al., 2013, Allinson et al., 2015) have been detected in Victorian and Australian rivers, with some at levels exceeding PNEC values (Scott et al., 2014), factors affecting the fate and bioavailability of the pollutants to fish and aquatic organisms are complex and impossible to predict using simple partitioning models (van der Oost et al., 2003). The use of physical and chemical analysis is useful for managing and monitoring water quality, but the information is inadequate for analysing bioavailability or toxicity to fish and aquatic organisms (Chapman, 2006). Biological monitoring tools such as biomarkers are therefore necessary to detect effects or changes in fish and aquatic organisms through cellular, molecular, biochemical, physiological or/and behavioural biomarkers (Facey et al., 2005, Sweidan et al., 2015). Biomarkers are of great relevance because they can be used as early warning signals of possible toxicity in the aquatic ecosystem and provide an understanding of the mode of toxicity of pollutants (van der Oost et al., 2003). The growing challenge in understanding

the effects of mixture toxicity on fish and other organisms means that a suite of biomarkers is necessary for a greater understanding of effects on target organs (van der Oost et al., 2003).

Many questions have been identified following reports of EACs in the water. These include whether the current exposures are high enough to cause significant effects in fish and aquatic organisms, whether mixtures cause effects that individual pollutants do not and whether the reported effects are related to disruption of the organism's endocrine system or some other mechanisms are in play (van der Oost et al., 2003, Sumpter, 2010).

Of interest are studies on interactions between fish biological systems, the AhR and ER, also known as "cross-talk", that may influence each other when stimulated by a mixture of contaminants (Kirby et al., 2007, Kawahara et al., 2009, Celander, 2011). Cross-talk between two classical biomarkers, EROD induction and Vtg expression, used often in risk assessments, have been investigated in a number of studies (Aubry et al., 2005, Kirby et al., 2007, Kawahara et al., 2009) and as early as 2004 by Bemanian et al. who reported disruptions between Vtg synthesis and enhancements in CYP1A expression in primary cultured salmon hepatocytes. The results are conflicting and the studies suggest either an underestimation of published EROD monitoring data because of estrogen-mediated MFO suppression (Kirby et al., 2007) or an underestimation of estrogenic activity because of suppression by Ah-R ligands in the aquatic environment (Kawahara et al., 2009). This is because ER-AR interactions are complex and vary depending on exposure time and the individual chemical concentrations.

The AhR and the ER are both ligand-activated transcription factors that can interact during exposure to different classes of compounds that include both AhR and ER inducers. Effects on AhR signaling pathways have been seen as transcriptional changes of AhR-subtypes such as AhRα, AhRβ, ARNT, and CYP1A (Mortensen and Arukwe, 2007). In a

study by Bemanian et al. (2004), the AhR pathway had contradictory effects on the molecular functions of the ERα in the liver cells of salmon. The ability of ERα to initiate transcription of the Vtg gene was inhibited and the auto-regulatory loop of the ERα gene expression was blocked. It has also been shown that active AhR can redirect ER target genes to AhR target genes, suggesting that AhR can moderate ERα protein levels and consequently, estrogenic responses (Matthews et al., 2005). In yet another study, activation of the AhR2 was shown to directly inhibit expression of Vtg genes in zebrafish *in vivo* (Bugel et al., 2013).

There is still no unanimity on the best suite of biomarkers or their sensitivity and reliability as this is very much species and pollutant specific (Sanchez and Porcher, 2009). However, in addition to classical biomarkers such as EROD activity, measurements such as condition factor (CF), gonadosomatic index (GSI) and hepatosomatic index (HSI) have been useful for determining the effects of pollution in a number of studies (Facey et al., 2005, Hauser-Davis et al., 2012, Liebel et al., 2013, Sadekarpawar and Parikh, 2013). The CF value is a measure of various conditions, both ecological and biological such as degree of fitness, gonad development and the suitability of the environment in relation to the feeding condition (MacGregoer, 1959). The HSI is a commonly used biomarker because the liver is an important organ for the detoxification of pollutants (Dogan and Canan, 2011). The GSI is used to determine reproductive maturity (West, 1990). These indices assist in determining the fitness and well being of fish.

Studies such as Sanchez et al. (2007) and He et al. (2012) have reviewed and reported results favoring the use of an integrated suite of biomarkers. Recently, Gagnon and Rawson (2016) reported significant results from using an integrated suite of biomarkers to assess fish health in ports in Western Australia. However, one of the challenges in environmental monitoring is the lack of a combined approach using chemical measurements of water quality and biomonitoring tools as a parallel study (Sumpter and Johnson, 2008).

In this study, the focus is on the evaluation of data gathered from the suite of biomarkers (EROD activity, Vtg induction and gonopodial measurements) from previous chapters (Chapters 2, 3 and 4) together with chemical analysis of the water to understand the condition of peri-urban creeks and rivers in Melbourne and Victoria. Different land use areas have different characteristics of contaminants. Wastewater treatment plant sites in general, may have elevated levels of estrogenic activity in the effluents because of estrogenic hormones such as E_1, E_2 and EE_2 (Mispagel et al., 2005, 2009a, Ying et al., 2009a, Scott et al., 2014). Rural areas are used for livestock farming, market gardens or vineyards. Pesticides applied to grape vines (Rose et al., 2009) and irrigated agricultural land can often be transported off-site via air, soil and water via drainage systems to rivers and waterbodies (Wightwick and Allinson, 2007). Urban areas including wetlands and stormwater catchment areas contain a range of pollutants including heavy metals from contaminated sediments, contaminated road run-off and pesticides from weed management (Boxall and Maltby, 1995, Rawson et al., 2009, Allinson et al., 2015). The study sites were grouped according to general land use types to identify different responses in the fish in relation to the contamination found at the sites.

In addition, fish organosomatic indices (GSI and HSI) and Fulton's condition factor are included in the analyses. It is hypothesized that male *G.holbrooki* from sites showing elevated EROD activity, Vtg induction and impacted GL/BL ratio may show effects in body condition indices. Furthermore, the potential of cross-talk in male *G.holbrooki* between the biomarkers EROD activity and Vtg induction is investigated. The study sites were broadly grouped into the different land use areas focusing on rural, urban, WWTP-impacted and reference sites to identify potentially different effects on *G.holbrooki* communities inhabiting the different land use areas.

G.holbrooki are a suitable candidate to evaluate local contamination because of their small home range (Pyke, 2005). Although noxious species such as *G.holbrooki* are in gen-

eral more tolerant of poor water quality (Lloyd et al., 1986, Arthington, 1991), this study will provide information that may assist with understanding impacts and effects of EACs on native fish populations that may be more sensitive to the pollutants. Sites that are impacted may be identified for future risk assessments.

5.3 Materials and Methods

5.3.1 Study sites

Sixteen sites were selected in 2009, the third year of the project, and grouped according to surrounding land use types such as urban (U), rural (R), waste water impacted (WWTP) and reference sites (Ref) (Table 2.3), although in some cases the grouping is somewhat arbitrary, since some creeks flow through both residential and industrial or agricultural land and may contain a combined source of pollutants. Approximate locations of sites in 2009 in Melbourne and Victoria is shown in Figure 2.3 (Chapter 2).

5.3.2 Fish collection and processing

Sexually mature male *G.holbrooki* were collected from the sites around Melbourne and Victoria using dip nets during February to May 2009. In the field, the fish were euthanased (blow to the head), and transported on ice to the RMIT University Ecotoxicology laboratory. Fifteen male fish from each site were weighed individually and measured for body length (BL) using a caliper to the nearest 0.01 mm, and then dissected for liver and gonad immediately on the day of capture. Liver and gonad were removed and weighed to the nearest 0.1 mg immediately after dissection.

5.3.3 EROD activity and Vtg induction in male *G.holbrooki*

Briefly, the extracted livers from 15 male fish per site were pooled together for sufficient liver tissue for analysis of hepatic EROD activity (Chapter 4). Fish liver weighed approximately 0.001 - 0.004 g, therefore five livers were pooled together into three sets (replicates). Liver samples was analysed using 96 well plates. Protein content was measured as absorbance at 750 nm using a Thermo Ascent Multiskan plate reader. EROD activity is expressed as pmol res/min/mg protein.

The remaining male fish (approximately 15 per site) were immediately stored in microfuge tubes and at a later time, fish wholebody homogenates were analysed for Vtg induction using a 96 well Bio-Dot microfiltration apparatus (Biorad, Australia). A salmon Vtg standard curve was used to semi-quantify *G.holbrooki* Vtg protein, therefore Vtg is expressed as ng/μl Salmon VtgEQ (Chapter 3).

5.3.4 Male fish condition indices

5.3.4.1 Hepatosomatic index (HSI), Gonadosomatic index (GSI) and Fulton's K Condition Factor (CF

The HSI index is often used to estimate the condition of the liver which is associated with liver energetic reserves and the energy status of the fish (Chellappa et al., 1995). The HSI index was calculated according to Htun-Han (1978).
HSI = (Liver weight (g)/ body weight (g)) x 100.

The gonadosomatic index (GSI) is a common metric of reproduction capacity and gonadal development (Gunderson, 1997). GSI index was calculated according to Htun-Han (1978) and Strum (1978).

GSI = (Gonad weight (g)/ body weight (g) x 100.

Condition factor (K) is used for comparing the condition and well being of the fish (Chellappa et al., 1995). Fulton's condition index was calculated as $K = 100(W/L^3)$ according to Htun-Han (1978) where W is the weight of the fish in grams, L is the length of the fish in centimeters and 100 is a factor to bring the value of K closer to unity.

5.3.5 Fish morphology

The body length (BL) of sexually mature adult male *G.holbrooki* was measured using a caliper to the nearest 0.01 mm. Gonopodial length (GL) defined as the length from the anterior base of the anal fin to the gonopodial tip was measured under a Wild Heerbrugg M3Z stereozoom microscope using an objective micrometer to the nearest 0.01 mm (Figure 2.2, Chapter 2). This is expressed as GL/BL ratio.

5.3.6 Chemical analysis of the water

Water samples were collected as 'grab' or spot samples from the sites in 2009 at the time of fish collections and analysed for estrogenic and AhR activity by Dr Mayumi Allinson and team (Department of Primary Industries). The analytical methods are detailed in Allinson et al. (2011) and Chinathamby et al. (2013). In addition, water temperature, pH and conductivity data were collected.

5.3.7 Statistical analysis

Multivariate analysis, which is often based on linear models such as PCA and factor analysis, is not suitable for use on this data set because of the small sample sizes of fish (n=15) per site. Small sample sizes do not provide enough statistical power for large linear models. Tabachnick and Fidell (1996) recommends a minimum of 300 cases or subjects for multivariate linear models. In addition, fish livers were pooled into groups of three (n=3) per site for the measurement of EROD activity because of insufficient material and as a result, combined multivariate analysis with the other variables was not possible because of uneven sample sizes. Bivariate data analyses were conducted using a scatterplot matrix to identify associations between the 8 variables in the total number of sites (n=16). The 8 variables that were analysed in this chapter are: GL/BL ratio, EROD activity, Vtg levels, HSI, GSI, CF, and AhR and estrogenic activity of the water. Data collected from male *G.holbrooki* captured in 2009 (see chapters 2, 3 and 4) were analysed in this chapter.

Further data exploration was carried out to examine the possibilities of linear and non-linear relationships in the bivariate analyses using Pearson's and Kendell's tau-b rank correlation respectively.

Statistical analyses were performed using PASW 23 for Mackintosh (New York, USA). Level of significance was set at $P< 0.05$). Differences between sites in HSI, GSI and CF values were tested using one-way analysis of variance (ANOVA). Results are expressed as mean$\pm$standard error.

5.4 Results

Estrogenic activity was mostly in the range of 0.1 - 1.7 ng/L EEQ with one site (Gisborne WWTP) having higher levels at 12 ng/L EEQ (Table 5.1). Two sites that had levels above 1 ng/L EEQ are Liverpool FRB (Ref) and Narre Warren SD (R/U). Gisborne WWTP also had the lowest water temperature of $15.3°$C with the rest of the sites showing temperatures between $18°$C to the highest temperatures of close to $25°$C at both Campaspe River (R) and Toorourrong Reservoir (Ref) (Chapter 2, Table 2.7). Sanitarium Lake (Ref) had the lowest pH of 6 and the highest pH of close to 9 were found at the rural sites of Deep Creek (R) and Campaspe River (R). Sites with the highest conductivity were Brodies Lake (Ref), Kororoit Creek (I/U) and Sunbury WWTP. These ranged from 2910 to 3580 μS/cm (Table 2.7).

Most of the sites showed low levels of estrogenic activity except for moderate and high estrogenic activity at a reference site, Liverpool FRB, and Gisborne WWTP respectively (Table 5.1). However, AhR activity varied among land use areas. One reference site (Liverpool FRB) had moderate AhR activtity, followed by a rural/urban site (Narre Warren SD) and a wastewater effluent impacted site (Brushy Creek). Biomarker responses were generally low except at three out of four WWTP sites for fish Vtg induction, and one urban site (Kalparrin Wetland) where fish showed high EROD activity. Similarly, fish from a few sites showed morphological effects. Fish from Gisborne WWTP had small GL/BL ratios and fish from Merri Creek (I/U) and Brushy Creek (WWTP) had large GL/BL ratios.

Table 5.1: Mean biomarker data (±SEM) in male fish collected in 2009. The variables included in the bivariate analysis are, EROD activity (pmol/min/mg protein), AhR activity (βNF EQ), GL/BL ratio, Vtg levels (ng/μl Salmon VtgEQ) , Estrogenic activity (ng/L EEQ), GSI, HSI, CF. Organosomatic indices means not sharing the same letters are significantly different from each other. *Includes sites that could be classified as both rural and urban or urban and industrial. Numbers next to sites correspond to numbers in Figure 2.3.

Site type	Site	EROD activity	AhR activity	GL/BL ratio	Vtg	Estrogenic activity	GSI	HSI	CF
Ref	9. Brodies Lake	8.21±2.68	7	0.36±0.01	8.14±0.13	<0.1	2.41^{acg}±0.38	2.04^{ec}±0.16	1.76±0.06
	10. Liverpool FRB	2.65±1.04	26	0.35±0.01	7.11±0.30	1.7	2.21^{ag}±0.31	1.96^{de}±0.18	1.83±0.07
	11. Toorourrong Reservoir	6.13±0.49	19	0.35±0.00	8.98±0.79	<0.1	2.54^{ae}±0.18	1.95^{de}±0.06	1.77±0.04
	12. Sanitarium Lake	2.71±1.60	11	0.37±0.01	7.08±0.45	<0.1	3.28^{abf}±0.43	1.71^{de}±0.12	1.79±0.06
Rural	4. Narre Warren SD*	4.25±1.92	180	0.38±0.01	13.94±1.18	1.4	2.35^{ae}±0.23	1.89^{de}±0.11	1.70±0.05
	14. Campaspe River	0.39±0.07	16	0.36±0.01	6.50±0.41	0.3	3.52^{abd}±0.15	1.35^{d}±0.10	1.87±0.06
	13. Deep Creek	2.24±0.64	8	0.36±0.00	6.87±0.36	<0.1	4.26^{bf}±0.40	1.72^{de}±0.12	1.77±0.05
	16. Woori Yallock Creek*	2.95±0.57	14	0.39±0.00	8.70±1.11	<0.1	3.71^{bce}±0.27	1.66^{de}±0.10	1.83±0.07
	15. Watson's Creek	2.75±0.29	12	0.36±0.00	4.70±0.31	0.1	2.49^{ae}±0.17	1.40^{bd}±0.11	1.85±0.05
Urban	2. Kalparrin Wetland	10.85±4.49	14	0.35±0.00	9.73±0.44	<0.1	4.40^{bd}±0.36	1.99^{ebc}±0.10	1.81±0.05
	1. Merri Creek*	1.50±0.20	27	0.40±0.01	10.47±0.82	<0.1	4.27^{bf}±0.41	2.12^{ec}±0.14	1.78±0.06
	3. Kororoit Creek*	4.95±0.48	35	0.35±0.01	6.79±0.97	<0.1	2.30^{ag}±0.14	2.12^{ec}±0.12	1.95±0.05
WWTP	7. Gisborne	2.56±0.97	19	0.34±0.02	16.62±2.57	12.0	3.43^{defg}±0.20	1.51^{dc}±0.15	1.69±0.06
	6. Sunbury	3.01±2.11	31	0.37±0.01	13.87±0.31	0.9	3.08^{abf}±0.26	2.24^{e}±0.17	1.79±0.06
	8. Werribee	5.58±0.86	24	0.37±0.00	22.83±2.42	0.6	3.18^{abg}±0.26	1.78^{de}±0.12	1.87±0.05
	5. Brushy Creek	3.75±0.16	130	0.39±0.01	19.42±2.43	0.3	2.80^{ae}±0.16	2.23^{e}±0.16	1.84±0.06

Table 5.2: ANOVA table showing significance for CF in fish.

ANOVA

CF_ratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.016	15	.068	1.441	.129
Within Groups	10.621	226	.047		
Total	11.637	241			

Mean CF ranged from 1.69±0.06 in fish from Gisborne WWTP to 1.95 ±0.05 in fish from Kororoit Creek I/U. There were no significant differences in CF measured in fish at all sites (ANOVA, ($F_{15,226}$= 1.44, $P>$ 0.1) (Table 5.2).

Mean HSI in fish ranged from a low of 1.35±0.37 at Campaspe River (R) to a high of 2.24±0.67 and 2.23±0.60, respectively at Sunbury WWTP and Brushy Creek (WWTP) (Table 5.1). Mean HSI in fish were significantly different among sites (ANOVA, ($F_{15,226}$= 4.58, $P<$ 0.001) (Table 5.3). Fish from both Brushy Creek (WWTP) and Sunbury WWTP had the largest mean HSI, only larger than fish from Campaspe River (R), Watson's Creek (R) and Gisborne WWTP $P<$ 0.05).

Table 5.3: ANOVA table showing significance for HSI in fish.

ANOVA

HSI_ratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.523	15	1.168	4.577	.000
Within Groups	57.686	226	.255		
Total	75.210	241			

Mean GSI in fish ranged from a low of 2.21±1.2 at Liverpool FRB (Ref) to a high of 4.40±1.37 at Kalparrin Wetland (U) followed by Merri Creek (I/U) at 4.27±0.4 and Deep Creek (R) at 4.26±1.5 (Table 5.1). Mean GSI in fish were significantly different among sites (ANOVA, ($F_{15,220}$= 6.72, $P<$ 0.001) (Table 5.4). Fish from Kalparrin Wetland (U), Merri Creek (I/U) and Deep Creek (R) had significantly higher GSI than fish from three out of the four reference sites by a factor of two.

Table 5.4: ANOVA table showing significance for GSI in fish.

ANOVA

Dependent Variable: GSI_ratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	119.926	15	7.995	6.722	.000
Within Groups	261.656	220	1.189		
Total	381.582	235			

An analysis of bivariate relationships between all variables did not reveal any clear evidence of significant associations, potentially because of the small sample set of 16 sites. (Figure 5.1).

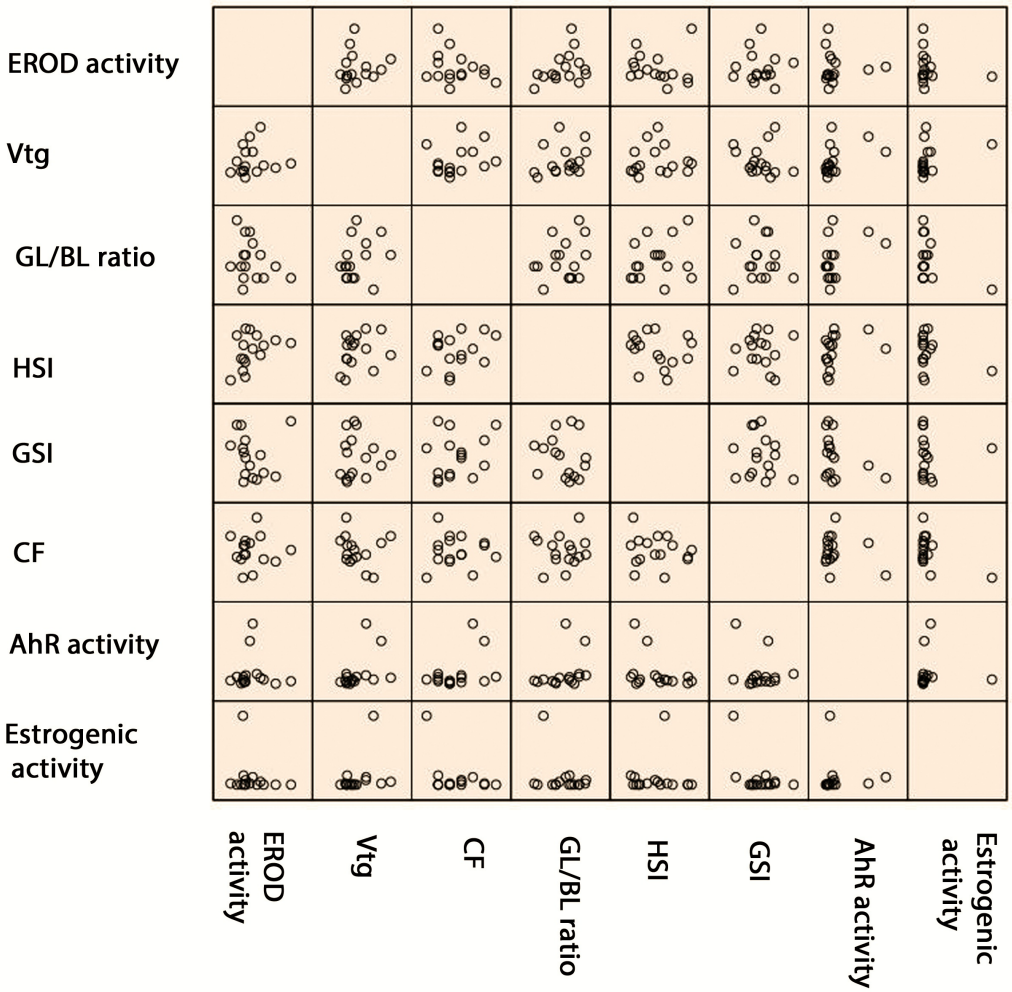


Figure 5.1: Scatterplot matrix showing associations between selected variables in fish.

Pearson's correlation coefficient revealed a negative association of significance between estrogenic activity and CF (Pearson's r = -0.502, $P = 0.047$) (Table 5.5). However, this was

because of the influence of a high leverage point in the estrogenic activity data (Gisborne WWTP). The association was not significant when the outlier was removed (Pearson's r = -0.100, P>0.5). There was no association between GL/BL ratio and estrogenic activity (Pearson's r = -0.299, P> 0.1) and between Vtg levels and estrogenic activity (Pearson's r = -0.177, P> 0.5) (Figure 5.5). There was no association between EROD activity and Vtg levels in male *G.holbrooki* (Pearson's r = -0.106, P> 0.5). This association was further explored in a scatterplot and no trends were observed (Figure 5.2).

Table 5.5: Pearson's Correlation table showing relationships between variables in fish.

Correlations

		EROD activity	Vtg	GL/BL ratio	HSI	GSI	CF	AhR activity	Estrogenic activity
EROD activity	Pearson Correlation	1	.106	-.299	.381	-.066	-.016	-.026	-.177
	Sig. (2-tailed)		.697	.260	.145	.807	.953	.923	.512
	N	16	16	16	16	16	16	16	16
Vtg	Pearson Correlation	.106	1	.266	.260	.015	-.170	.444	.340
	Sig. (2-tailed)	.697		.319	.330	.955	.530	.085	.197
	N	16	16	16	16	16	16	16	16
GL/BL ratio	Pearson Correlation	-.299	.266	1	.273	.225	-.010	.414	-.400
	Sig. (2-tailed)	.260	.319		.307	.403	.972	.110	.125
	N	16	16	16	16	16	16	16	16
HSI	Pearson Correlation	.381	.260	.273	1	-.161	.041	.318	-.300
	Sig. (2-tailed)	.145	.330	.307		.552	.880	.230	.259
	N	16	16	16	16	16	16	16	16
GSI	Pearson Correlation	-.066	.015	.225	-.161	1	-.141	-.347	.027
	Sig. (2-tailed)	.807	.955	.403	.552		.603	.188	.920
	N	16	16	16	16	16	16	16	16
CF	Pearson Correlation	-.016	-.170	-.010	.041	-.141	1	-.224	-.502*
	Sig. (2-tailed)	.953	.530	.972	.880	.603		.405	.047
	N	16	16	16	16	16	16	16	16
AhR activity	Pearson Correlation	-.026	.444	.414	.318	-.347	-.224	1	-.011
	Sig. (2-tailed)	.923	.085	.110	.230	.188	.405		.968
	N	16	16	16	16	16	16	16	16
Estrogenic activity	Pearson Correlation	-.177	.340	-.400	-.300	.027	-.502*	-.011	1
	Sig. (2-tailed)	.512	.197	.125	.259	.920	.047	.968	
	N	16	16	16	16	16	16	16	16

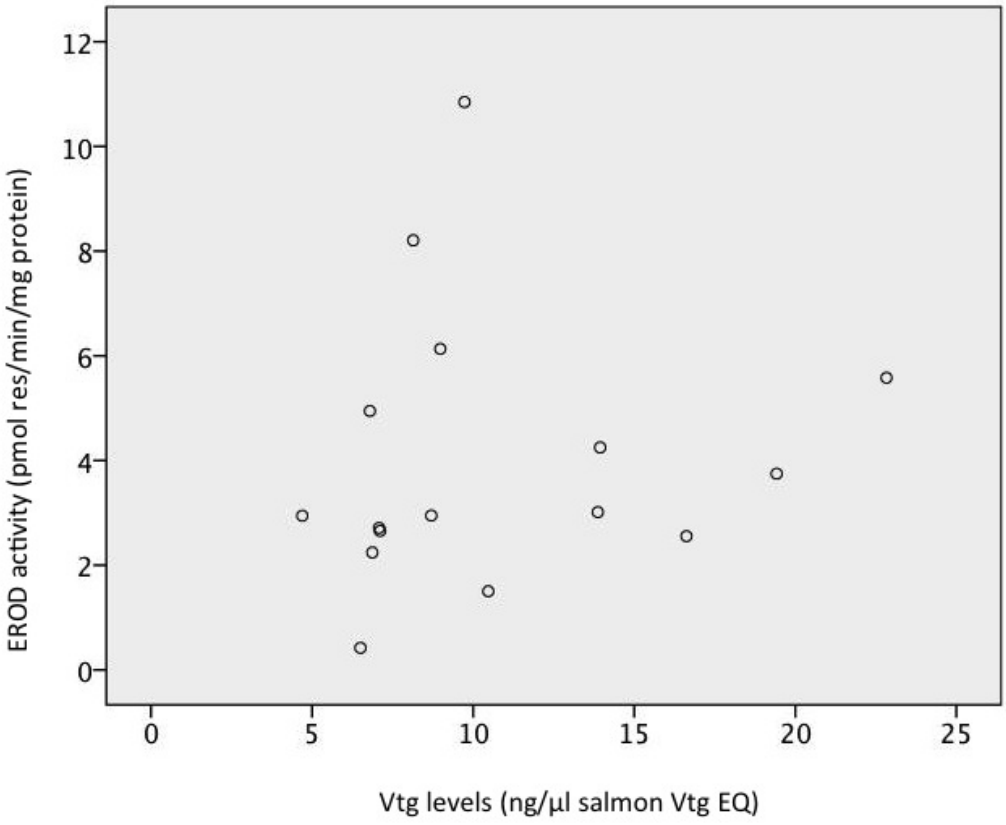


Figure 5.2: Scatterplot showing relationship between EROD activity and Vtg (S-VtgEq) in male *G.holbrooki*.

Kendall's tau-b rank correlation showed moderate non-linear associations between estrogenic activity and AhR activity in the water (Kendall's tau = 0.424, $P< 0.05$) and AhR activity and Vtg (Kendall's tau = 0.387, $P< 0.05$) (Table 5.6). However, these bivariate relationships did not show any strong associations when further explored in a scatterplot matrix (Figure 5.3).

Table 5.6: Kendall's tau-b correlation table showing relationships between variables in fish.

			EROD activity	Vtg	GL/BL ratio	HSI	GSI	CF	AhR activity	Estrogenic activity
Kendall's tau_b	EROD activity	Correlation Coefficient	1.000	.233	-.107	.267	-.283	.017	.067	-.170
		Sig. (2-tailed)	.	.207	.580	.150	.126	.928	.718	.396
		N	16	16	16	16	16	16	16	16
	Vtg	Correlation Coefficient	.233	1.000	.197	.267	.083	-.220	.387*	.371
		Sig. (2-tailed)	.207	.	.311	.150	.653	.240	.038	.064
		N	16	16	16	16	16	16	16	16
	GL/BL ratio	Correlation Coefficient	-.107	.197	1.000	.125	.161	.027	.144	-.054
		Sig. (2-tailed)	.580	.311	.	.519	.407	.890	.460	.799
		N	16	16	16	16	16	16	16	16
	HSI	Correlation Coefficient	.267	.267	.125	1.000	-.150	-.034	.336	.010
		Sig. (2-tailed)	.150	.150	.519	.	.418	.857	.071	.960
		N	16	16	16	16	16	16	16	16
	GSI	Correlation Coefficient	-.283	.083	.161	-.150	1.000	-.051	-.269	-.230
		Sig. (2-tailed)	.126	.653	.407	.418	.	.786	.149	.250
		N	16	16	16	16	16	16	16	16
	CF	Correlation Coefficient	.017	-.220	.027	-.034	-.051	1.000	.154	-.082
		Sig. (2-tailed)	.928	.240	.890	.857	.786	.	.415	.688
		N	16	16	16	16	16	16	16	16
	AhR activity	Correlation Coefficient	.067	.387*	.144	.336	-.269	.154	1.000	.424*
		Sig. (2-tailed)	.718	.038	.460	.071	.149	.415	.	.035
		N	16	16	16	16	16	16	16	16
	Estrogenic activity	Correlation Coefficient	-.170	.371	-.054	.010	-.230	-.082	.424*	1.000
		Sig. (2-tailed)	.396	.064	.799	.960	.250	.688	.035	.
		N	16	16	16	16	16	16	16	16

*. Correlation is significant at the 0.05 level (2-tailed).

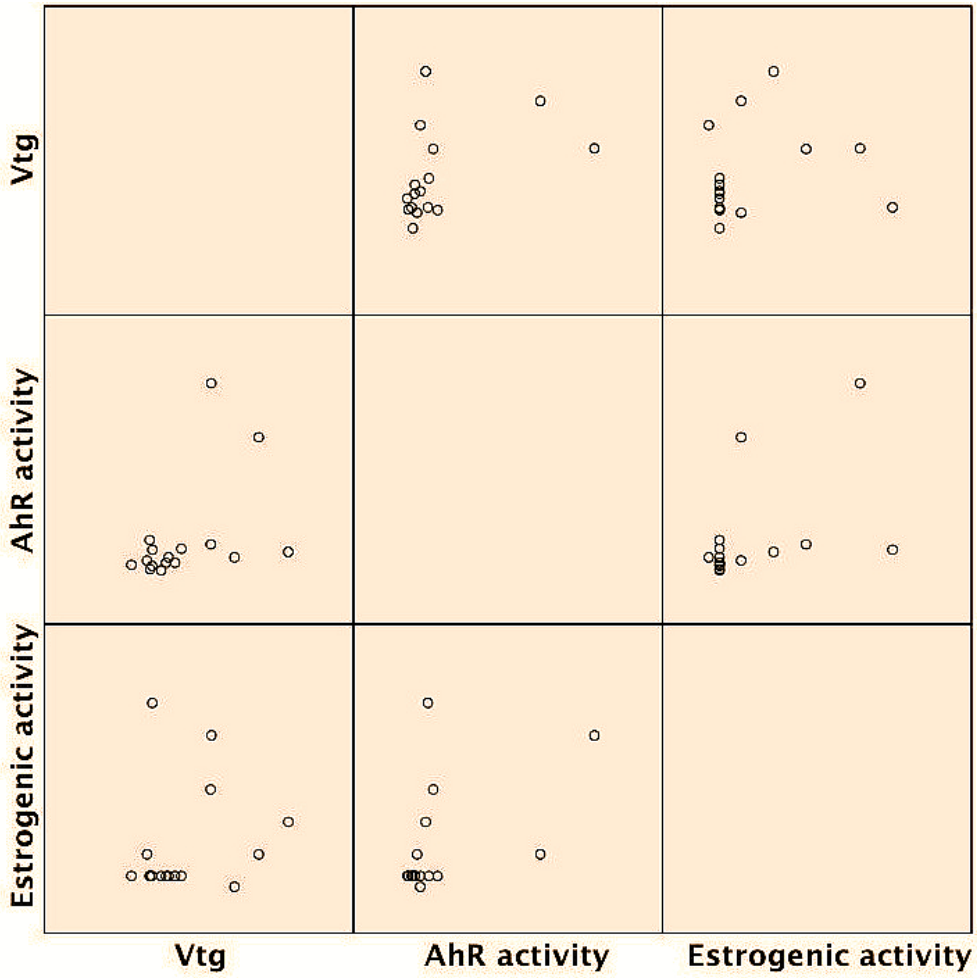


Figure 5.3: Scatterplot showing relationships between AhR activity, estrogenic activity, and Vtg levels in male *G.holbrooki*.

5.5 Discussion

In retrospect, general land use area classifications selected for this study, such as urban, rural, reference and WWTP effluent sites may not have been as useful as expected as evidenced by the unexpected moderate estrogenic activity at a reference site and the results of AhR activity at all of the sites. It was expected that urban sites would produce moderate to high hepatic EROD activity induction because of urban and industrial pollutants and that rural sites would show moderate Vtg induction from agricultural and dairy effluent run-off. However, the results showed no consistent pattern observed in the AhR and estrogenic activity measurements and biomarker results. Similarly, a study by Scott et al. (2014) showed no clear land-use activity associated with high estrogenic activity. High activity was found at a number of sites including residential, agricultural and industrial sites in the study by Scott et al. (2014).

The low estrogenic activity of <0.1 ng/L EEQ observed in surface water at 9 out of 16 sites is indicative of low estrogenic contamination in general in Victoria and Australia and lower than levels reported in the study by Scott et al. (2014) of estrogenic activity in Australian rivers. Three out of the four WWTP sites showed levels below 1 ng/L EEQ indicating that Victorian WWTP are generally effective in the removal of estrogenic contaminants. Other factors such as temperature and pH may have had an effect on the fish biomarkers selected for this study. The exception is Gisborne WWTP where high estrogenic activity is suggested to have contributed to the high Vtg levels in male *G.holbrooki* from that site.

The CF value is based on the hypothesis that heavier fish of a given length are in better condition and well being (Bagenal and Tesch, 1978). van der Oost et al. (2003) reported that CF value may be affected if food resources are limited or if food consumption of the fish is impaired due to stress factors. WWTP effluents are typically rich in organic matter and a

good source of nutrients (Subramani et al., 2014) for organisms that thrive in such habitats. Fish living in effluent receiving sites would have access to a nutrient rich diet, compared to urban sites, which may in turn affect CF values. In the current study, CF values ranged from a low of 1.69 to a high of 1.95 and were not statistically significant across all sites, similar to the studies considered in the review by van der Oost et al. (2003). *G.holbrooki* persist in environments of low water quality and are known to be opportunistic feeders, therefore, food resources may not be a limiting factor (Pyke, 2005). In a study by Northington and Hershey (2006), fish gut contents revealed a diverse diet of terrestrial and aquatic insects at forested sites but mostly Chironomidae were found in the gut content at urban sites, showing that *G.holbrooki* have opportunistic diets.

The HSI is a measurement of fish energy reserves and has been validated as a useful biomarker for monitoring the effects of pollution such as the impacts of sewage water (Mdegela et al., 2010, Al-Ghais, 2013) and metal toxicity as shown in a study by Kumari et al. (2014) where Rohu, a common carp in India, exposed to chromium showed elevated HSI values. Both Mdegela et al. (2010) and Al-Ghais (2013) demonstrated that fish exposed to treated effluents had higher HSI values. In the current study, fish from two of the four WWTP sites, Brushy Creek (WWTP) and Sunbury WWTP, had significantly higher HSI values than fish from Campaspe River (R), Watson's Creek (R) and Gisborne WWTP, although, not significantly higher than fish from the reference sites. Brushy Creek (WWTP) also had the highest AhR activity of 130 βNF EQ after Narre Warren SD (R/U), the highest Vtg induction at 19.42 ng/μl Salmon VtgEQ after Werribee WWTP and the highest R4:6 ratio (see chapter 2) which is indicative of an androgenic response (Hou et al., 2011). Brushy Creek is a small tributary of the Yarra River in the urban outskirts of Melbourne that passes through rural land. In addition to receiving urban and rural run off, a nearby wastewater treatment plant discharges treated effluent into Brushy Creek. The sources of effluents at Brushy Creek (WWTP) are complex and the high AhR activity at the site cannot be explained. It can be suggested that fish at Brushy Creek may have been exposed to

AhR inducers, increasing liver detoxification and consequent significantly higher HSI values. AhR inducers at the site may be ER agonists subsequently causing Vtg induction in fish from Brushy Creek. Further investigation is necessary at this site.

Similarly, Sunbury WWTP had significantly high HSI values but moderate AhR activity of 31βNF EQ. It is important to note that single grab samples of the water were obtained for AhR activity measurements. The condition of the river would vary depending on the frequency of effluent release from the treatment plant. Therefore, AhR activity may not correlate with HSI. Elevated HSI values at the two WWTP sites are not indicative of chronic effects and the HSI of *G.holbrooki* may return to normal if river health improves. A study by Al-Ghais (2013) showed that tilapia raised in treated sewage water had a significantly greater HSI than control fish which returned to normal following depuration for a period of 6 weeks. More importantly, some AhR ligands partition to the sediment rather than the water column depending on their water solubility and may become bioavailable through leaching from the sediment or during natural events such as fast flowing river water (Zhang et al., 2004) leading to acute effects on fish liver that cannot be correlated to AhR activity of the water. Concentrations of organic pollutants in the sediment were not investigated in this study. Liver is the target for metabolism of toxicants in the fish body, therefore the most affected organ during the process of detoxification (Sadekarpawar and Parikh, 2013). An increase in liver size indicates the increased capacity of the liver to metabolize pollutants (Kumari et al., 2014).

Studies often use GSI and HSI values simultaneously in determining effects of pollutants on fish such as a study by Abdel-Hameid (2007) which reports the effects of phenol, commonly present in industrial waste waters and in non-specific pesticides, herbicides and fungicides, inducing an increase in HSI and a reduced GSI in exposed tilapia. This was not evident in the current study as fish from both Brushy Creek (WWTP) and Sunbury WWTP which had higher HSI values did not show corresponding low GSI values.

GSI is often used in fisheries biology as a measure of reproductive allocation and reproductive condition (Sedeño-Díaz and López-López, 2012). Lower GSI values in male fish have been associated with exposure to pollution in a number of fish including thinlip grey mullet (Tancioni et al., 2015) and in male Mozambique tilapia (Sadekarpawar and Parikh, 2013). In the current study, significantly higher GSI values were found in fish from Kalparrin Wetland (U), Merri Creek (I/U), and Deep Creek (R) compared to three of the reference sites (Brodies Lake, Liverpool FRB and Toorourrong Reservoir). A study by Morley et al. (2010) similarly found a higher GSI value in chub from a polluted site compared to an unpolluted site, suggesting an androgenic effect on gonadal development. Orlando et al. (2007) similarly observed higher testis masses in *G.holbrooki* exposed to pulp and paper mill effluent receiving river. Fish in Kalparrin Wetland (U) was captured at the end of a stormwater drain in a shallow and small pool of water located at the gross pollutant trap which contained rubbish such as plastic bags and bottles. It is suggested that the presence of androgenic pollutants in the stormwater drain in the highly urbanized area is likely, causing the high GSI values in the fish. Although, EROD activity was also increased in fish from Kalparrin Wetlands, HSI was not affected in the fish. Similarly, fish were captured at Merri Creek (I/U) near a stormwater drain flowing into the creek. Merri Creek is located in a heavily industrialized site. Androgenic pollutants may have been present at the site contributing to the high GSI value in fish. Fish from Deep Creek (R) may also be impacted by pollutants that increased gonadal development, however, it is difficult to speculate the sources of contaminants that may have been present in Deep Creek at Darraweit Guim at the time of the current study. A recent report with data gathered from 2004 to 2009 has reported poor water quality at Deep Creek (DSE, 2013).

Changing environmental parameters such as temperature may influence effects of EACs on fish. It has been shown in the laboratory that higher water temperatures simultaneously increased the concentration of Vtg protein levels, from EE_2 and E_2 exposure respectively in the plasma of brown trout (Kömer et al., 2008) and Atlantic salmon (Anderson et al.,

2012). In the current study, sites with the highest water temperatures did not show elevated Vtg levels in fish. Fish with high Vtg levels were captured at Gisborne WWTP which had the lowest water temperature, although the other two WWTP sites with elevated Vtg levels in fish were not much different in water temperature compared to the other sites. Similarly, environmental parameters affect EROD activity in fish. In Mozambique tilapia, water temperature of 35°C increased hepatic EROD activity while lower EROD activity was observed at a pH of 6 which increased as pH increased. No consistent pattern relating environmental factors such as water temperature, pH and salinity to measured biomarkers in fish was observed in the current study.

There was no association between GL/BL ratio and estrogenic activity and between Vtg protein levels and estrogenic activity. There was also no correlation between GL/BL ratio and Vtg protein levels. A study by Brockmeier et al. (2013) reported a lack of correlation between androgen-exposed female *G.holbrooki* and female *G.affinis* anal fin elongation and Vtg mRNA levels suggesting other molecular pathways affecting endocrine response to fin alterations. This may also be the case for male fish fin morphology. GL/BL ratio is a morphological biomarker that shows effects of EDC exposure if fish were affected before full gonopodial development (Angus et al., 2005) and therefore may not reflect potential effects of estrogenic measurements of the river. Estrogen receptor activity measured in one-off grab water samples in this study does not take into account the temporal variations and therefore, correlations with Vtg levels and the other biomarkers in fish cannot be expected. One off grab water samples only provide a ”snapshot” of the condition of the river at time of fish capture and periodic water chemical analyses are necessary for a better understanding of river health.

Studies such Sanchez et al. (2007) and He et al. (2012) have reviewed and reported results favouring the use of an intergrated set of biomarkers, but often not all of the chosen biomarkers show evidence of contaminant effects. Sadauskas-Henrique et al. (2011)

chose a number of biomarkers such as condition factor and blood variables, including erythrocyte lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in two fish species *Astyanax fasciatus* and *Pimelodus maculatus* and found that condition factor was not directly affected in the assessment of the effects of contaminants which included organochlorines in the water. A recent study by Gagnon and Rawson (2016) examined fish using a suite of biomarkers and found an increase in LSI and greater oxidative DNA damage that was thought to compromise the health of all fish in Fremantle port, Western Australia. This was evident only when all data on biomarkers were analysed using multivariate statistics as compared to assessment using individual biomarkers.

Studies have shown evidence in cross-talk between ER and AhR activity in fish. This had been demonstrated in Gräns et al. (2010) where one-way inhibition of the AhR-ER in primary cultures of rainbow trout hepatocytes was observed when ethinylestradiol (ER agonist) was Co-administered with βNF (AhR agonist), resulting in a 40% reduction of Vtg mRNA levels. In a different study investigating the interaction between two biomarkers (EROD and Vtg induction) (Kirby et al., 2007), flounder was exposed to EROD inducers and estrogenic chemicals. It was found that E_2, on the other hand, had the ability to suppress EROD. In that study, it was hypothesized that published EROD monitoring data may be an underestimation because of estrogen-mediated MFO suppression in the wild. In the current study, no association was found between EROD activity and Vtg levels in fish. Larger sample numbers are required to study this effect in fish in the wild. Due to multiple stressors present in the aquatic environment, the indirect interactions between EROD activity at the enzymic level and Vtg protein induction may not be straightforward. It is necessary to investigate EROD activity and interactions of Vtg at the mRNA level to understand initial effects.

It is important to clarify that the results in the current study only represent the health of

the fish that were captured. The small sample sizes in the current study are not adequate for extrapolation to the rest of the population of fish inhabiting the sites. However, given the detectable levels of estrogenic and AhR activity of the water at the time of fish capture, it is suggested that fish at the affected sites may have been subjected to pollutants. To understand this further, a larger sample size is required at the affected sites.

5.6 Conclusion

The biomarkers analysed in this study demonstrate the complexity of the physiological responses of fish exposed to pollutants in their natural environment. No strong associations were found among the biomarkers analysed in the current study. There was no association between EROD activity and Vtg levels in *G.holbrooki*. There was no association between GL/BL ratio and estrogenic activity as expected and between Vtg levels and estrogenic activity. Both GSI and HSI can be used to assess water quality. Elevated GSI and HSI values were observed in fish from some of the sites that are highly disturbed and in poor condition. Fish condition factor, on the other hand, was not affected at any of the sites. Although the sites were classified according to land use types, this is somewhat arbitrary because each site has different characteristics, such as river sediment type and potential unknown pollutants at the site that may not be obvious. As such, fish showed effects of pollution in some sites belonging to a land use type such as WWTP impacted sites while fish from the other sites in the same land use type did not show any effects in the biomarkers that were selected.

In conclusion, this multi-biomarker approach improved the understanding of the effects of exposure to pollutants and the complex responses of fish at the different sites. Sites that will benefit from continued monitoring include Brushy Creek (WWTP), Gisborne WWTP, Merri Creek (I/U) and Kalparrin Wetland (U). Both Merri Creek (I/U) and Kalparrin Wetland (U) receive effluents through stormwater drains and are *G.holbrooki* habitats at point

source locations. Monitoring of fish from these sites over a period of time is relevant and will be valuable because of the close proximity of the discharge of effluents to fish. It is recommended that baseline data of all biomarkers be obtained from these sites to further understand the complexity of pollution impacts on fish physiology and health.

Chapter 6

General Discussion and Conclusions

This study discusses the suitability and potential of using *Gambusia holbrooki* (mosquitofish) as a sentinel species for the monitoring of EACs in Victorian and Australian creeks and rivers based on the biomarkers chosen for this study. Although *G.holbrooki* has been used for both field (Batty and Lim, 1999, Game et al., 2006, Gagnon et al., 2008, Rawson, 2008, Leusch et al., 2014) and laboratory studies (Doyle and Lim, 2005, Leusch et al., 2005, 2006b, Saaristo et al., 2014), the suitability of *G.holbrooki* as an indicator species in the field for biomonitoring freshwater environments with multiple input sources has not been evaluated previously. This study was initiated to determine the effects of EDCs using *G.holbrooki* gonopodial indices (Chapter 2) as a cost effective and simple biomarker for quick screening purposes. Fish gonopodial indices were shown to be a useful biomarker in a number of laboratory based studies (Doyle and Lim, 2002, Angus et al., 2005, Doyle and Lim, 2005, Leusch et al., 2006b) and field studies on fish collected downstream of WWTP effluent receiving sites (Angus et al., 2002, Batty and Lim, 1999). In addition, this study assesses the usefulness of the selected biomarkers, gonopodial indices of male *G.holbrooki* (Chapter 2), presence of Vtg (Chapter 3), EROD activity (Chapter 4), HSI, GSI, and CF factor (Chapter

5) in fish collected from sites with multiple land use input in Victoria, Australia with parallel measurements of estrogenic and AhR activity of the water.

6.1 Male *G.holbrooki* as a bioindicator species for EACs

The aim of this study on *G.holbrooki* was to evaluate the potential of this invasive fish species as a sentinel organism for *in situ* monitoring of aquatic pollutants in creeks and rivers in Victoria and Australia. *Gambusia spp.* are ubiquitous and have been used as a bioindicator in many studies around the world for detecting the effects of different types of contaminants such as EACs/EDCs (Batty and Lim, 1999, Angus et al., 2002, Game et al., 2006) and POPs (Rawson et al., 2009).

There are several advantages in using *G.holbrooki* as a bioindicator species. *G.holbrooki* were first introduced in mainland Australia in 1925 from Northern Mexico and the Southern United States of America for mosquito control and now have been declared a noxious species in Victoria (Pyke, 2008). As an invasive species in Australia, it is easier to obtain permits for collection of large numbers unlike native fish species. Sexual dimorphism enables morphological effects to be analysed easily with simple equipment such as a dissecting microscope. They are small in size and can be captured with dip nets without the use of expensive equipment and methods such as electrofishing and with boats. Their short life cycle makes them an ideal candidate for toxicity tests. *G.holbrooki* are a widely studied species, therefore, laboratory toxicity data is available for comparisons with field studies.

However, there were a number of challenges identified in this study when working with this introduced species. Although, the fish thrive throughout the warmer months, the potential of using *G.holbrooki* as a bioindicator during winter is not possible in temperate parts of Australia. *G.holbrooki* have been observed as having a breeding period from October

to March in a temperate Australian river with high mortality during winter months (Pen and Potter, 1991). Using *G.holbrooki* for the detection of Vtg remains a challenge. Although *G.holbrooki* is a commonly studied fish species, there is no commercial anti-Vtg antibody available for *G.holbrooki*. Producing specific *G.holbrooki* anti-Vtg antibody is a time-consuming process. Vtg is commonly measured in fish plasma because increased concentrations of the precursor protein circulate in plasma (Jones et al., 2000). For small fish models, extracting sufficient plasma is a challenge. In this study, whole body homogenates were used to overcome this issue but again, this meant that additional time-consuming steps were required to obtain a clean sample for use in the dot blot assay for the analysis of the Vtg protein.

The small size of the fish means that they are easy to maintain in aquaria. However, this is a disadvantage when obtaining sufficient tissue for assays such as EROD. Special care and excessive time were required when extracting organs that easily disintegrate. This was encountered with *G.holbrooki* livers that weighed only up to 4 mg in weight. Unlike working with larger fish where dissections are carried out in the field, *G.holbrooki* have to be transported to the laboratory because of the requirements for a dissecting microscope. On the other hand, *G.holbrooki* have a high tolerance to a wide range of water conditions and therefore can be obtained from reference sites to disturbed sites (Lloyd, 1987) enabling comparisons of river health, which may not be possible with native fish species. The tolerance of the species to a wide range of pollutants from organic wastes to phenols, pesticides, and heavy metals has been reviewed by (Lloyd, 1987). The question of insensitivity to pollutants because of this tolerance remains and has to be further investigated in *G.holbrooki*. Although the species have been reported to tolerate degraded aquatic environments and toxins (Lloyd, 1987, Jester et al., 1992, Pyke, 2005), a study by Franssen (2009) reported otherwise. Franssen (2009) observed *G.affinis* living in mine outflow waters contaminated by heavy metals at both population and individual levels compared to fish from a non-contaminated site. The study found that metal-contaminated sites had reduced pro-

portions of both male and reproductively active female fish and altered male fish population size structures. The study also reported individual-level effects in *G.affinis* such as smaller livers, low body and gonad weights and smaller clutch sizes in female fish. In a study by Kamata et al. (2011), *G.affinis* were exposed to βNF (an AhR agonist), E_2 and BPA (both ER agonists) in the laboratory. The study concluded that *G.affinis* sensitivity to βNF was comparable to that of other fish species and that *Gambusia spp.* were suitable for detecting *in vivo* AhR and ER effects.

It is crucial that a baseline or natural variation of responses of the sentinel organism be determined before a thorough evaluation of the usefulness of the sentinel organism is conducted (Sedeño-Díaz and López-López, 2012). In the current study, results show that *G.holbrooki* can be used as a bioindicator for EACs for Melbourne and Australian creeks and rivers. Biomarker responses were seen in fish captured from a number of sites such as Gisborne WWTP, Brushy Creek (WWTP) and Kalparrin Wetland (U). Gonopodial indices can be used for quick screening of general effects of contamination. Elevated HSI and GSI were observed in *G.holbrooki* from some of the highly disturbed sites. Although environmental factors and effects of exposure to pollutant mixtures may confound the results of some of the biomarkers that were chosen for this study, it should not deter from the future use of *G.holbrooki* as a bioindicator species. Instead, fish from affected study sites should be monitored over time with more frequent sampling to better understand natural variation and effects of multiple pollutants. Furthermore, the potential of less invasive biomarkers such as behavioural endpoints and biomarkers using whole bodies of fish instead of target organs should be investigated. These may prove to be better suited to small fish like *G.holbrooki*.

6.2 Estrogenic and AhR activity in Melbourne's creeks and rivers

Estrogenic activity in surface waters was found at all sites, including reference sites. This ranged from 0.1-1.7ng/L EEQ with the exception of one site (Gisbourne WWTP) at 12 ng/L EEQ. In comparison, estrogenic activity was lower than the values reported by Scott et al. (2014) which was in the range of 1-6.5 ng/L EEQ in rivers across mainland Australia, including Victoria. Another study by Allinson et al. (2010) reported no activity to 73 ng/L EEQ in effluent samples from 45 wastewater treatment plants in Victoria, Australia between 2006 and 2007.

Estrogenic activity at the three WWTP sites ranged from 0.3 -0.9 ng/L EEQ with an exception of a fourth site (Gisborne WWTP) at 12 ng/L EEQ where a sewage leakage had occurred a week prior to sampling. However, two years after the current study Saaristo et al. (2014) similarly found high levels of total estrogens at 8.97 and 13.19 ng/L EEQ at Jackson's Creek, 100 m downstream of Gisborne WWTP. It is possible that high estrogenic activity may be recurrent at the creek and of concern to fish and other aquatic organisms inhabiting the creek. Further investigation is necessary at this site. It is important to note that grab water sampling does not represent temporal variations in estrogenic activity measurements and it is not possible to conclude whether this is a persistent issue unless repeated sampling is conducted at this site.

Recently, estone (E_1) has been reported as the most abundant hormone measured in water samples from Victorian and Australian rivers (Scott et al., 2014). Scott et al. (2014) did not detect the presence of E_2 in the water samples. This was attributed to the short aquatic half-life of E_2 which quickly degrades to E_1 (Zhang et al., 2013). Most recently, it was suggested that E_1 does not biodegrade easily and has a longer half time than the other estrogens (Bradley et al., 2009). Similarly, Saaristo et al. (2014) also found higher levels of E_1 compared to both E_2 and EE_2 in water samples from two sites in Victoria. It is

possible that E_1 may be the most dominant estrogenic hormone present in the sites of the current study. Dammann et al. (2011) found an increase in Vtg in fathead minnow in the laboratory when fish were exposed to both E_1 and E_2 separately, suggesting that E_1 is also capable of affecting reproduction in fish. The concentration of E_1 (15 ng/L) used in the study of Dammann et al. (2011) was within the range of maximum values of 10 to 57 ng/L (E_1) found by Scott et al. (2014) in Australian rivers. Interestingly, a study by Nakamura et al. (2015) reported multigenerational effects on Japanese medaka after exposure to E_1. This was evident only in the second generation with declines in egg production and fertility at an exposure of 91.4 ng/L (E_1) and in the third generation at exposures of both 47.1 and 91.4 ng/L (E_1) together with the appearance of testis-ova obesrvations in the second and third generation. The evidence that no effects on reproduction were observed in the first generation in that study highlights the importance of research on subsequent generations of fish, which are very scarce. E_1 measurements may be a better indicator than E_2 for future studies of estrogenic hormone levels in water samples.

It is important to consider the different water systems (lentic vs lotic) and effects on variability in estrogenic activity measurements in surface waters. A study by Rohr and McCoy (2010) found 2.5-10 times higher atrazine than the maximum concentrations in lentic systems compared to lotic stystems because water is often not replenished in lentic systems. Chemicals can concentrate as evaporation occurs. In the current study, it could be expected that river/stream systems would have more variable levels of estrogenic activity because of the water flow.

AhR activity was observed in all water samples in this study in the range of 7-180 ng/L βNF EQ (highest at a rural/urban site and a WWTP site). There was no consistent pattern in the level of AhR activity relating to land use types. The current study did not find any correlations between EROD activity in fish and AhR water activity. It is however important not to underestimate the complex nature of AhR agonists, their bioavailability and fate in

the water and different responses in the mediating systems in fish. Several conditions of the aquatic environment and qualities of the pollutant affect the freely dissolved concentration and the bioaccessible fraction of the chemical to organisms (Akkanen et al., 2012), which may explain why AhR activity may not always correlate with EROD activity in fish. Furthermore, AhR activity was only measured in the water and not in the sediment. Some AhR ligands are expected to be found in higher concentrations in sediments than in the water column (Zhang et al., 2004), therefore, may bioaccumulate in sediment associated organisms which may be the prey of fish or desorb into the water column at a later time and impact on fish (Jones and de Voogt, 1999). *G.holbrooki* are surface feeders (Pyke, 2008), therefore, the likelihood of direct exposure to contaminated sediments is unlikely, however, desorption from sediment has to be considered together with the dissolved phase in water when considering bioavailability of AhR ligands to fish. Measurements in sediment need to be included in future studies for a comprehensive analysis.

6.3 Validity of selected biomarkers for *G.holbrooki*

Interest in the effects of EACs on wild fish in Australia was in its early stages at the start of the current study in 2007 shortly after evidence of estrogenic activity in creeks and rivers was beginning to emerge in Victoria, Australia in 2005 (Mispagel et al., 2005) indicating that aquatic wildlife were already exposed to EACs. However, studies have mainly focused on wastewater effluents (Mispagel et al., 2009a, Allinson et al., 2010).

In 2006, Game et al. investigated gonopodial indices in male *G.holbrooki* collected from five south west Australian wetlands and reported evidence of endocrine disruption at some of the sites. However, it is always a challenge to implement a combined approach using chemical measurements of contaminants as a parallel study. Furthermore, due to the vast number of pollutants present in the aquatic environment at any one time, no single

biomarker is useful on its own (Sanchez and Porcher, 2009), therefore, using a battery of biomarkers to assess fish and river health is necessary but provides its own challenges because of the great choice of biomarkers available. In 2007, a "bottoms up" approach of determining if effects were observed in *G.holbrooki* (gonopodial indices) in Victoria (Chapter 2), was initiated before the analyses of Vtg (Chapter 3) and EROD activity (Chapter 4) and before undertaking expensive chemical analyses of water from the sites in 2009. The current study is the first comprehensive study reporting estrogenic and AhR activity in sites from various land use types (urban, rural, WWTP and reference) in Victoria. This contributes to the current literature on estrogenic activity in creeks and rivers in Victoria, combined with an assessment of biomarkers in wild *G.holbrooki* to detect if the effects of EACs/EDCs are a concern. Selecting suitable biomarkers for fish can be perplexing and depends on the focus of the study.

Results in the current study show that fish gonopodial indices only fulfills part of the criteria described by van der Oost et al. (2003) for biomarker selection (see Chapter 2). Male fish gonopodial indices varied between sites and suggest that the selected indices for *G.holbrooki* for the purpose of this study may be sensitive enough to be influenced by natural variability and other environmental factors, confounding results and making interpretation difficult. Studies have shown that *Gambusia spp.* may exhibit developmental plasticity and are adaptable to a variety of water conditions making interpretations of morphological characteristics complicated. A number of studies have reported that *G.holbrooki* gonopodial indices are influenced by population density (Zulian et al., 1995) and the social environment as well as environmental factors such as temperature (Meffe, 1992). In particular, it was found that small size is favoured in populations with female-biased sex ratios or low density (Zulian et al., 1995). In the current study, the body length of sexually mature male *G.holbrooki* ranged widely from 16.27 to 21.47 mm suggesting the possibility of environmental or other factors such as food availability influencing body size which in turn affect sexual maturity and consequently GL/BL ratio.

Furthermore, for effects to be observable, juvenile male fish would have to be exposed to estrogenic pollutants during development as the gonopodia, once developed, will not be affected (Angus et al., 2005). Gonopodial development normally takes between 30 to 50 days to complete in the laboratory (Angus et al., 2005), therefore, it is not possible to conclude whether effects are indicative of acute or chronic exposure if assessing gonopodial indices alone. Measuring *G.holbrooki* gonopodia is inexpensive, easy to perform, can be measured with a microscope in the field and fulfills the requirements for a quick screening assay. However, in the current study, there is no comprehensive baseline data at each site to evaluate whether differences observed are a result of estrogenic exposure at the site or due to environmental factors. Although gonopodial indices may only fulfill part of the criteria for biomarker selection, continued assessment of fish gonopodial indices over time may provide more useful information of the variability affecting this biomarker.

A single fish biomarker alone is insufficient when assessing pollution in an environment that is exposed to a cocktail of pollutants. Therefore, the presence of the Vtg protein was measured in male *G.holbrooki* whole body homogenates (Chapter 3) to examine for effects of EACs; as gonopodial indices are not sufficiently indicative of the immediate state of river health.

In the present study, a semi-quantitative assay was developed to measure the egg-yolk precursor protein, Vtg, in male *G.holbrooki* whole body homogenates using the Biorad 96 well Bio-Dot microfiltration apparatus. The dot blot is a simple technique in which antigen is directly applied on to a nitrocellulose membrane for the detection of proteins. This technique is widely used in fish virology studies to detect disease in fish (Hsu et al., 1989, Douglas-Helders et al., 2001) and has been used to detect Vtg in the plasma of Mediterranean male swordfish (Desantis et al., 2005). However, the current study is the first to use the bio-dot microfiltration apparatus to detect Vtg in the whole body tissues of fish. Results from the use of anti-Vtg antibodies from salmon, sea bream and carp for comparisons

of cross-reactivity against female *G.holbrooki* in various reproductive stages have not been previously presented. Contrary to previous knowledge that anti-Vtg antibodies are highly species specific, the current study demonstrated, using immunoblots, that anti-Vtg antibodies of other fish species could result in successful detection of Vtg in female *G.holbrooki* at different reproductive stages. Salmon anti-Vtg antibody was used for the assay because good cross-reactivity was observed in female fish homogenates with gravid spots (indicating reproductive maturity) but without oocytes. This indicates that salmon anti-Vtg antibody is likely to detect circulating Vtg in plasma (as is expected in male fish) that is yet to be incorporated into oocytes. This was the case with sea bream anti-Vtg antibody which showed binding to female *G.holbrooki* with oocytes but not in females with gravid spots. This aspect of Vtg specificity could be used in further studies investigating the time course of Vtg incorporation and chemicals affecting this process.

This assay was then applied to male *G.holbrooki* collected from sites in Victoria. Presence of Vtg was detected in male fish from all of the sites including reference sites at varying levels. Similarly, Vtg in male fish from reference sites was also reported in other fish species (Cline, 2002, Scott et al., 2006) and in male *G.holbrooki* (Gagnon et al., 2008). It is likely that the low levels of Vtg found in male fish at some of the sites are background levels and a long-term study is necessary at the reference sites to gather baseline levels and variations in Vtg in male fish.

Vtg in *G.holbrooki* partially fulfills the criteria for biomarker selection proposed by van der Oost et al. (2003). Developing a Vtg assay is not easy and anti-Vtg antibodies are expensive. There is no baseline data for *G.holbrooki* available for this biomarker from the sites in this study and the impacts of confounding factors in the field on fish are yet to be understood. Sensitivity of the Vtg biomarker depends very much on the type of pollutant.

Biomarker responses differ in fish species as found by Palace et al. (2009) in a three

year study of EE_2 exposure added to a lake containing four wild fish species. Results showed differences in Vtg induction and histopathological damage in the four species. Often biomarkers examined in the laboratory are deemed useful following exposures to single contaminants, however in the wild, the interplay and interactions between mixture toxicity of pollutants (Celander, 2011) and potential "cross-talk" between different signaling systems in the organism (Kirby et al., 2007, Kawahara et al., 2009) are complex. Ecotoxicological data in the laboratory may not translate similarly into effects in the wild (van der Oost et al., 2003). A recent study by Kleinhenz et al. (2016) showed results of herbicide toxicity to invertebrates in the laboratory, however, no detrimental impacts found in the field after spray treatment of the pesticide replicating laboratory toxicity concentrations. The authors suggest that animals living in the environment of concern can have quite different levels of sensitivity in terms of ecological toxicity and laboratory toxicity tests. In the field, buffering effects through biodegradation, desoption to sediment and other environmental factors have to be considered.

While the dot blot assay system was successfully used to detect Vtg, the assay relies on salmon primary antibody and salmon Vtg standards. Ideally, purifying and characterizing *Gambusia spp.* Vtg molecule to generate a specific anti-Vtg antibody would have been preferred. However, for the purposes of this study for quick detection of the presence of Vtg in wild *G.holbrooki*, this semi-quantitive assay using salmon anti-Vtg antibody was deemed sufficient, cost-effective and less time-consuming.

The Biorad apparatus presented a number of technical challenges during usage for the dot-blot assay. Maintaining a tight seal when assembling the apparatus was an issue and this caused leakage when buffer was applied into the wells. The whole body homogenates often clogged the wells, which meant that the samples did not diffuse through the nitrocellulose membrane. The samples had to be spun in the centrifuge a number of times to remove fine particles that were clogging the wells. This issue with using whole body homogenates may

not have occurred with a cleaner sample such as plasma. While dot blots have been used in a number of studies, mostly using rudimentary techniques with a pipette, the BIORAD dot blot instrument provided an easy means of analyzing sufficient samples that would fit into a 96 well plate and produced consistent dots that could be quantified using an image acquisition program. However, running the whole assay took 16 hours until visualization in the dark room using the ECL detection system. This method requires further optimization to shorten the assay time. There is potential for the method to be adapted for use in the field for quick screening of fish. Dots can be applied using a pipette as in some studies such as in Desantis et al. (2005) to detect for the presence of the Vtg protein in male fish and to identify affected sites. This can be followed by thorough analyses at a later stage at if the protein is found to be present in male fish.

EROD hepatic induction in male *G.holbrooki* was analysed (Chapter 4) to investigate for the effects of AhR inducers in Melbourne's creeks and rivers. Recent studies have shown that EACs that are persistent organic pollutants (POPs) act directly or indirectly via the AhR (Shanle and Xu, 2011).

The current study reports induction of hepatic EROD activity in male fish collected from the sites. Results in this study showed a wide range of EROD activity from a low of 0.42 pmol/min/mg protein at Campaspe River (R) to a high of 10.85 pmol/min/mg protein at Kalparrin Weland (U). There was no consistent pattern observed in this study for both water AhR activity and EROD activity in fish. For example, low activity was found in fish from the urban/industrial sites and higher activity was found in fish from reference sites.

Hepatic EROD activity also partially fulfills the criteria by van der Oost et al. (2003) for a candidate biomarker in *G.holbrooki*. Although EROD activity is ideal for assessing the current health of rivers because it occurs rapidly in fish within 24 hours of exposure (Jakšič et al., 2008), there have been problems reported with the AhR that it is fairly promiscuous

because of its ability to bind and be activated by a diverse range of chemicals (Denison and Nagy, 2003). Kojima et al. (2010) reported a diverse range (eleven out of 200) of tested pesticides induced AhR-mediated transcriptional activity. A recent study by Huang et al. (2014) showed that heavy metals, in particular, zinc, cadmium and lead, could induce CYP1A mRNA expression levels in *G.affinis*. Trace metals such as zinc and iron were detected in sediment of some of the sample sites of a current study by Allinson et al. (2015). Although this does not mean CYPIA protein in liver is consequently induced, the suggestion that heavy metals (that are not the classical inducers of the AhR) modulate CYP1A mRNA expression is a concern and an important implication in interpreting EROD data. Other factors that were not investigated may also have modulated EROD activity in this study. Furthermore, the effects of rainfall and catchment size were not examined in this study and were shown to have affected EROD activity in wild *G.holbrooki* in the study by Rawson et al. (2009). Water temperature and liver pathology have also been shown to affect EROD activity (Whyte et al., 2000, Khan and Payne, 2002, Amutha and Subramanium, 2010). The AhR's ability to bind to many compounds and be affected by environmental factors may mean that it may be sensitive to confounding factors. A lack of baseline data from the study sites means that confounding factors have not been clearly understood. Further studies are recommended at a single site to evaluate and understand the factors that influence EROD activity in wild fish.

The criteria described by van der Oost et al. (2003) (see below in bold) for biomarker selection may be suitable for long-term studies, however, for short-term studies, the criteria is rigid and unrealistic. Validation of biomarkers in fish is difficult in the field compared to laboratory based studies and may have to undergo rigorous testing to pass the criteria proposed by van der Oost et al. (2003). The current study discusses the author's proposed criteria:

1. **The biomarker should be reliable, relatively cheap and easy to perform.**

In reality, most biomarkers, especially classical biomarkers such as Vtg and EROD assays are not cheap. Anti-Vtg antibodies are costly and obtaining specialized plate readers for kinetic measurements for EROD assays are expensive. Optimizing biochemical assays is time consuming and not easy as often an assay requires lengthy troubleshooting before it is ready for use in the field.

2. **In order for the biomarker to be an early warning signal, it should be sensitive to pollutant exposure and/or effects.**
   In the field, it is difficult to determine how sensitive a biomarker is to pollutant exposure. This may be established in the laboratory however, the combination of a number of pollutants in the field makes it hard to determine sensitivity to each pollutant. In a study by (Kamata et al., 2011), *G.affinis* were exposed separately to βNF (an AhR agonist), E_2 and BPA (both ER agonists) in the laboratory and the transcription of the CYP1A gene was analysed along with Vtg gene transcription. The study concluded that *G.affinis* are insensitive to E_2. However, in the field, E_2 seldom exists on its own and often is present with other hormones. The presence of many ER agonists and hormones make it difficult to pinpoint the exact pollutant that may have caused the sensitive biomarker response or effect.

3. **There should be a baseline data of the biomarker in order to differentiate between natural variability and pollutant-induced effect.**
   It is necessary to obtain baseline data and consistent long-term observations to understand natural variability. However, for a short-term project, this is not realistic. Baselines often take years to establish and most habitats have been subjected to pollutant exposure as was evident in the sites chosen as ”reference sites” in the current study. Therefore, understanding natural variability in the field is difficult in the short-term.

4. **The impact of confounding factors to the response should be clearly understood.**

In the field, confounding factors will always be present and cannot be avoided. In reality, confounding factors such as the impact of severe droughts, and catastrophic fires may not be clearly understood.

5. **"Dosage and time" mechanisms between biomarker response and pollutant exposure should be established.**

   "Dosage and time" mechanisms may be easily established in the laboratory but in the field, the biomarker can behave differently in response to the various types of pollutants and often additive responses may occur without a clear linear relationship between dosage and biomarker response. This was reported in a study where herbicide toxicity in invertebrates was shown in the laboratory but the same experiment conducted in the field did not show similar sensitivities, suggesting environmental factors mediating the toxicity (Kleinhenz et al., 2016). It is also important to understand the potential of cross-talk to occur in the field containing multiple contaminants making it difficult to understand relationships between pollutant exposure and biomarker response.

6. **Toxicological significance of the biomarker and impact to the organism should be understood.**

   Some studies have found that a biomarker may affect the organism generations after and effects may not be clearly seen in the current generation. This was shown in a study by Nakamura et al. (2015) where no effects on reproduction were observed in the first generation but appears in the second and third generation of Japanese medaka after exposure to E_1. Therefore, in reality, this is difficult to determine in every biomarker study.

The current study alternatively proposes the following criteria for biomarker selection for studies involving quick screening of pollution in fish.

1. Available time, dosage and sensitivity of biomarker data that is currently available in other studies should be used for assessing suitability of candidate biomarker. There is a vast number of studies assessing biomarker responses in a wide range of fish species in the current literature. Reviewing the literature should provide sufficient information on the suitability of the candidate biomarker. This would save time and costs. However, an allowance has to be made for differences in response of biomarker in the laboratory compared to biomarker responses in the wild. Differences in biomarker responses among fish species should also be taken into consideration.

2. To understand variability, a quick analysis of the candidate biomarker in wild caught fish over a few months should be carried out. For a quick analysis, fish should be collected from a reference site to establish temporal variability in data.

3. Avoid using assays that require specific equipment and time for optimization before use in the field but consider simple biomarkers such as fecundity, and organosomatic indices. It may be useful adding biomarkers of effect such as histopathology to identify if exposure indeed causes organ damage. While specific proteins like the presence of Vtg in male fish may indicate exposure to estrogenic EDCs, the time and expense involved is a concern. Limited use of such biomarkers in field monitoring programs at targeted sites only, which are known to contain EDCs, is recommended.

6.4 Conclusions and Future Research Directions

Comprehensive studies measuring both estrogenic and (anti-androgenic) activity in river water and effects in wild populations of fish are scarce in Victoria and Australia. This is

particularly difficult to evaluate as wild populations of fish are exposed to numerous natural stressors such as unfavourable temperatures, limited food availability, high water velocity, and types of natural episodic variables (Sedeño-Díaz and López-López, 2012). The multi-biomarker approach used in this study improved the understanding of the effects of exposure to EACs/EDCs and the complex responses of fish at the different sites. In addition, this study contributed to current data of estrogenic activity and AhR activity measurements of creeks in Victoria. No strong associations were observed between estrogenic and AhR activity and the selected biomarkers. This is indicative of the generally low estrogenic activity in Victorian creeks and rivers.

The biomarkers selected for this study yielded varied and useful results in *G.holbrooki* from the wild, therefore it is concluded that *G.holbrooki* is a suitable bioindicator species for estrogenic EACs for Victorian and Australian creeks and rivers. This study demonstrates the complexity of the physiological responses of *G.holbrooki* to the mix of pollutants in the environment. Unlike laboratory based dose-response studies, relationships between biomarker responses and chemical exposure are not strictly linear as demonstrated in this study. This is due to the different modes of action (MOAs) of the pollutants to target organisms and, which effects may be additive, synergistic or antagonistic depending on concentrations (Kaviraj et al., 2014). Such responses are expected as numerous factors are at play. Although, there was no association between hepatic EROD activity and Vtg levels to indicate cross-talk between biomarkers, it is necessary to investigate this in individual fish from the wild for possible cross-talk between AhR and ER pathways. This was not possible in the current study as the whole body of fish was used for the Vtg dot blot assay which meant that EROD activity could not be measured in the same fish. This study does not take into account anti-androgenic effects in fish and this needs to be investigated in future studies.

Sites that will benefit from continued monitoring include Brushy Creek (WWTP), Gisborne WWTP, Merri Creek (I/U) and Kalparrin Wetland (U). Both Merri Creek (I/U) and

Kalparrin Wetland (U) receive effluents through storm water drains and are *G.holbrooki* habitats at point source location. These results should form the basis of an ongoing biomonitoring study for *G.holbrooki* at these sites because of the close proximity of the effluent discharge points to the fish. It is necessary that a bigger sample size be obtained in a study such as this where multiple biomarkers are investigated, for significant effects to be seen. Furthermore, temporal sampling for estrogenic and AhR activity of the water at the sites is necessary to identify impacted sites.

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Appendices

Appendix A

Site Pictures

Figure A.1: Brushy Creek, Croydon North. Site receives WWTP discharge.

Figure A.2: Brodies Lake, a reference site at Greensvale Reservoir.

Figure A.3: Lake Sanitarium, a reference site at Mount Macedon.

Figure A.4: Merri Creek at an industrial area inCampbellfield.

Figure A.5: Watson's Creek in Somerville, a rural site.

Appendix B

Mosquitofish Immunoblot Assay

List of buffers and solutions:

Transfer buffer

10x Towbin Buffer, 0.5 L.

30.3 g Tris base (25mM Tris)

144 g Glycine (192 mM Glycine)

Dissolve in 500 ml Milli Q water

Add 10% v/v 10x Towbin solution

20% v/v methanol

70% v/v Milli Q water to make Transfer buffer.

Electrophoresis Buffer

3 g Tris

14.4 g Glycine

1 g SDS

Add Milli Q water to make 1 L.

0.5M Tris-HCL pH 6.8

Dissolve 12.1 g of Tris base in 150 ml of Milli Q water. Adjust pH to 6.8 using HCL. Make up the final volume to 200 ml with Milli Q water.

10% (w/v) SDS

Dissolve 5 g of SDS in 50 ml of Milli Q water.

10x TBS

24.23 g Tris base

80.06 g NaCl

Adjust pH to 7.6 with HCl

Make up to 1 L with Milli Q water.

1x TBS

Take 100 ml of 10x TBS and add to 900 ml of Milli Q water.

To make TBS-Tween, add 1% Tween 20 in TBS.

Appendix C

EROD Activity Assay

Method: *G.holbrooki* hepatic EROD activity assay

Equipment:

Fluorescense plate reader

Sterile individually wrapped 96 well plates

Multi-channel Pipettor

Single Pipettor: 10ul, 100ul, 1000 ul

Sterile reservoirs: 25ml and 100 ml

Sterile pipette tips

Vortex mixer

Stock Buffers:

0.1M KH_2PO_4

0.1 M $K_2H_2PO_4$

Adjust pH to 7.1

7-Ethoxyresorufin stock (0.2 mM) in DMSO

NADPH solution (2.4 mM) in 0.1 M Potassium-phosphate buffer

Resorufin stock (1mM) in DMSO

Assay method on day of test run:

*Prepare Potassium-phosphate buffer at pH of 7.1

*Prepare resorufin standards: 0.64 uM, 0.32 uM, 0.16 uM, 0.08 uM, 0.04 uM, 0.02 uM, 0.01 uM from 1 mM Resorufin stock. Keep standards away from light. Calibrate 0.64 uM Resorufin at 572 nm with a cuvette absorbance reader

*Prepare 7-Ethoxyresorufin reagent by adding 0.75 ml into 50 ml of potassium-phosphate buffer. Keep away from light.

*Calibrate 7-Ethoxyresorufin reagent at 450 nm with a cuvette absorbance reader

*Dilute S9 fractions in 1:10 in phosphate buffer on ice at a pH of 7.1.

*Pipette 50 μl of diluted samples on to the plate in triplicate wells.

*Pipette 275 μl resorufin standards (0.65 to 0.01 uM) in triplicate wells on plate

*Pipette 50 μl of blank (Potassium-phosphate buffer) in triplicate wells on plate

*Pipette 200 μl of 7-Ethoxyresorufin into all the samples

*Finally, prepare NADPH solution and add 25 μl of NADPH to all sample and blank wells and read immediately on platereader at 35°C (excitation: 531 nm, emission: 590 nm)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.01	0.02	0.04	0.08	0.16	0.32	0.64	BB	BB		CS
B	0	0.01	0.02	0.04	0.08	0.16	0.32	0.64	BB	BB		CS
C	0	0.01	0.02	0.04	0.08	0.16	0.32	0.64	BB	BB		CS
D												
E												
F	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
G	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
H	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12

Figure C.1: Example of an EROD assay plate.

CS: control samples, SI-S12: S9 samples to be tested, Cells A1 to C10 are Resorufin standards, BB: Potassium-phosphate buffer.

DC Protein Assay

*96 well microplates

*Sterile reservoirs

*Pipettors

*Pipette tips

Prepare protein standard curve using BSA dissolved in Milli Q water, ranging from 0 to 2.0 mg/ml using serial dilutions. Pipette 10 μl of standards, samples and blanks in triplicates into a sterile clear 96 well plate. Add 25 μl of Reagent A into each well. Add 200 μl of Reagent B into each well. Incubate plate for 15 minutes, then read absorbance on platereader at 750 nm.