WESTERN PORT TOXICANT STUDY: Stage 3 – Fish Health Assessment

Kathryn Hassell, Sara Long, Rhianna Boyle, Jessica French, Craig Sherman and Vincent Pettigrove

March 2019

RMIT UNIVERSITY

AQUEST

AQUATIC ENVIRONMENTAL STRESS RESEARCH GROUP

How to cite this report: Hassell, K., Long, S., Boyle, R., French, J., Sherman, C., and Pettigrove, V. (2019), WESTERN PORT TOXICANT STUDY Stage 3 – Fish Health Assessment. Aquatic Environmental Stress Research Group (AQUEST), Technical Report No. 3, RMIT University, Victoria, Australia.

Revision	Date issued	Reviewed by	Approved by	Date approved	Revision type
Draft or final	Date	Name	Name	date	Internal/external
Draft	15/12/2018	Sara Long	Kathryn Hassell	18/12/2018	Internal
Draft	15/01/2019	Vincent Pettigrove	Vincent Pettigrove	31/01/2019	External
Final	08/02/2019	Rhys Coleman	Rhys Coleman	15/03/19	External

Printed:	15/03/2019
Last saved:	15/03/2019
File name:	Technical report APPRG#3 WP toadfish study Mar 2019 - FINAL
File saved location	
Author:	Kathryn Hassell, Sara Long, Rhianna Boyle, Jessica French, Sherman, C. and Vincent Pettigrove
Project manager:	Kathryn Hassell (Fish health assessment)
Name of organisation:	Aquatic Environmental Stress Research Group
Name of project:	WESTERN PORT TOXICANT STUDY Stage 3
Name of document:	Assessment of Fish Health
Document version:	No. 1
Project number:	Stage 3

Table of Contents

Table of Figures

List of Tables

Acknowledgements

Thanks to Rhys Coleman (Melbourne Water) and Vin Pettigrove for establishing the study and to Melbourne Water for providing funding. Field sampling and laboratory work was completed with the assistance of Jessica French, Montse Sole, Sara Long, Jackie Myers, Rhianna Boyle and Craig Sherman. Thanks to Simon Sharp for assistance in generating maps. All field collections and lab work were done through CAPIM at the University of Melbourne. The report was completed through the Aquatic Environmental Stress Research Group (AQUEST) at RMIT University. All fish were collected under Victorian Fisheries Research Permit RP1141, following procedures approved by the University of Melbourne Animal Ethics Committee, Project Number AEC 1613886.1.

1 Executive Summary

1.1 Background

Research conducted by CAPIM since 2012 has identified multiple pesticides in marine, estuarine and freshwater sections of catchments that drain into Western Port, and some pesticides have been detected at concentrations known to cause biological effects in fish, aquatic invertebrates and plants (Melbourne Water, 2018). Based on these findings, a research program was developed to assess the health of fish living within Western Port. The species chosen was the Smooth toadfish (*Tetractenos glaber*), which is an endemic species that inhabits estuarine and shallow coastal areas throughout south eastern Australia. Their wide distribution and abundance, yet limited movement and high site fidelity, along with demersal feeding habits are characteristics that deemed them a suitable model species for detecting pollution impacts.

The first phase of the fish health assessment included two sampling periods: winter, Round 1 (16-26th June 2015); and spring, Round 2 (16-26th November 2015). In Round 1, fish were sampled from 3 sites (n=83) and from 5 sites (n=150) in Round 2. Differences were observed in general biological measurements of fish between sampling sites and rounds, such as differences in sex ratios, size and age. There was no evidence of the toadfish being exposed to endocrine disruptors (based on vitellogenin levels and gonad histology), however some histological changes were observed in toadfish livers. The histological changes observed included a low incidence of pre-cancerous and cancerous growths, as well as the presence of nematode parasites and other infectious agents. Although, the results were inconclusive in terms of identifying impacts from specific waterways, since environmental stress was observed in fish from all sites (including reference sites).

Based on the findings of the first phase of the study, a series of recommendations were produced, as follows:

Recommendations from the initial study (2015)

1. Complete other analyses – biomarkers, otoliths, tissue contaminants. Tissue biomarkers are more sensitive than coarse tissue indices such as gonadosomatic index (GSI) and condition factor (CF), therefore examination of tissue biomarkers would provide valuable information on specific biochemical changes in fish between sites, which may assist in determining the types of pollutants present in Western Port. Biomarkers such as glutathione-S-transferase (GST) (to measure generalised stress response) as well as other more specific biomarkers, such as ethoxyresorufin-o-deethylase (EROD) activity (indicative of exposure to organic toxicants) are proposed. Ageing of fish using otoliths is particularly important as it enables a comparison of fish growth rates between sites, as well as comparisons of tissue indices and histological changes based on fish age, as opposed to size (which can be quite variable). Tissue contaminant analysis would enable identification of which contaminants are actually bioavailable to toadfish and accumulating in their tissues. This information would assist in interpretation of other findings and identification of specific contaminants responsible for any adverse biological effects observed. While these three components are acknowledged as being important, and samples were preserved for this purpose, the analyses were beyond the scope of the original study.

2. Expand to more catchments, in particular more external reference sites.

3. Consider sampling fish in the upper estuary (as opposed to the lower estuary), since the sourcing component of Stage 3 of the Western Port Toxicants Study has reported that much of the pollution is coming from upstream. Candidate species is the blue spot goby due to their abundance in these areas and extensive use in previous projects (eg. Sharley et al., 2013).

4. Repeat the study in the future, to see if fish health has changed over time.

These recommendations formed the basis of the next stage of the project, which commenced in late 2016.

1.2 Objectives

This project will help Melbourne Water gain a better understanding of the current health status of demersal fish in Western Port, through comparison of physiological indicators in smooth toadfish, (*Tetractenos glaber*) collected from within Western Port as well as from multiple external reference sites. The initial study showed some differences in fish from each site, but there was only one external reference site and it was unclear how much movement there may have been in toadfish between sites within Western Port. In the present study, toadfish were again selected as the study species (to allow comparisons to 2015 data), and two of the sites were (coastal) external reference sites (EXT) – Anderson Inlet and Shallow Inlet; two sites were within Port Phillip (PPB) – Williamstown and Blairgowrie; and one site was within Western Port (WP) – Bass River. These sites were selected based on some knowledge of surrounding land use, accessibility and known presence of smooth toadfish. The fishing was done using similar methods to the previous study, and were sampled in November/December 2016, to allow comparisons to the fish that were sampled in the previous spring (November, 2015). A full list of the fish health indicators used by the AQUEST for pollution assessment can be found in the Appendices (Table A1).

1.3 Key Findings

- Smooth toadfish collected from different sites within Western Port displayed some differences in physiological and histological endpoints compared to toadfish collected from Port Phillip and two external reference sites (Andersons and Shallow Inlets).

- In general, fish from the external reference sites tended to be ranked most highly (most impacted), but it was variable between indicators, suggesting that fish from all 10 collection sites displayed some changes indicative of environmental stress.

- When all indicators are combined, the overall ranks for sites (most impacted to least impacted) were EXT=PPB>WP for female toadfish and EXT>WP>PPB for male toadfish.

- In some locations, more than 80% of fish were infected with external parasites (such as anchorworm, *Lernaea sp*.), but overall the incidence, across all sites was relatively low (22.0%; 66/300). Incidence rates were higher in fish collected in 2016 than 2015, whilst in contrast, skin lesions and scarring was more prevalent in 2015. There was no correlation between lesion and parasite rates and body condition in the toadfish in this study.

- There were significant differences in tissue condition indices (GSI, HSI and CF) between fish from different field sites. These are general indicators that are influenced by factors such as food availability, nutrient enrichment, reproductive state, disease status and toxicant exposure, and all of these factors may influence energy allocation.

- Energy allocation was assessed through measuring differences in liver glycogen and lipid content between sites, and the profiles were variable both across and within sites. No correlations were observed between fish size or GSI with energy markers, whilst a weak correlation was seen between condition factor and lipid content ($r^2 = 0.193$) and a stronger correlation was observed between HSI and lipid content ($r^2 = 0.381$).

- Total lipid and glycogen concentrations in female fish from Bass River were lower than levels in fish from all the other sites, indicating a reduced energy capacity (and hence likely lower growth rates and lower fecundity). These fish also displayed correspondingly low HSI values, as well as low EROD and catalase activity levels.

- In this study EROD activity, glycogen and lipid content were more sensitive indicators compared to catalase activity and lipid peroxidation (LPO). Therefore, we would

recommend measuring these responses in future studies assessing toadfish health as they are cost effective and sensitive biomarkers.

- Fish from the Bass River were the highest ranked (most affected) for external lesions and parasites, and liver histology scores. The liver scores at this site were the highest for the study, due to the presence of parasites, granuloma and foci of cellular alteration. Yet no fish from Bass River displayed benign or malignant liver tumours.

- There was no evidence of endocrine disruption-related gonadal changes (testis-ova, oocyte atresia) in fish from any sites, whilst there were some differences in female-specific and male-specific gonadal changes between sites.

- Female fish from Western Port showed higher numbers of gonad macrophage aggregates (MMCs) compared to other sites, whilst the number of atretic follicles and post-ovulatory follicles was variable between sites. MMCs are often associated with ageing, and gonad MMCs numbers were correlated with total length in males (r^2=0.384), and to a lesser extent in females (r^2=0.206).

- Male fish showed higher levels of gonad changes than females, with individual scores of up to 8 (of a possible 12) observed in some fish from Western Port. Median male gonad scores were highest (6) for fish from Blairgowrie (PPB) and Shallow Inlet (EXT).

- The size, colour and general appearance of smooth toadfish livers was quite variable between fish from all sites. The overall presence of liver parasites was low, at just 3.67% (11/300) across the entire population of smooth toadfish sampled, and similarly, the presence of granuloma was only 8.67% (26/300).

- One interesting observation was the presence of two ectopic oocytes associated with pancreatic tissue in the liver of a female fish from Williamstown (PPB). The significance of this rare finding is unknown.

- Across all toadfish sampled, a small number of fish were observed to have pre-cancerous (pre-neoplastic), or cancerous lesions in their livers. The overall presence of pre-cancerous changes of 7.67% (23/300) and just 1.0% (3/300) for cancerous changes, including both benign and malignant tumours.

- Three fish displayed benign or malignant liver tumours, and all of them were from PPB sites (Edwards Point, Blairgowrie and Williamstown). These fish were not the largest (or oldest) fish collected during the study.

- Whilst the overall levels of pre-cancerous and cancerous liver changes are low, they were observed in toadfish from multiple sites, especially PPB. Further investigation is required to determine if these levels represent ‘natural’ baseline levels or if they are the result of exposure to carcinogenic contaminants at some point during their lifetime.

- Low levels of genetic structuring were observed between populations (indicating strong mixing of individuals and gene flow between study populations). Whilst not tested, the mixing is more likely to be due to dispersal during the early life stages, rather than movement in adults. If this is the case, then adult toadfish may still be a useful indicator for assessing site-specific pollution impacts.

1.4 Recommendations

Based on the findings of this study there are several recommendations for further investigations to better understand the potential impacts of toxicants on fish health (in Western Port and other bays and estuaries):

1. Contaminant analysis of remaining carcass and liver samples to see if there has been any bioaccumulation of toxicants;
2. Have all remaining otoliths aged – this will assist interpretation of other findings, and may be useful for determining how long the fish may have been exposed to persistent bioaccumulative toxicants;
3. Further investigation into liver tumours in fish from Port Phillip sites;
4. Further investigation into the Bass River catchment to better understand why fish from this location showed higher levels of impact for some indicators than toadfish from other collection sites;
5. Focus future biomarker work with toadfish on EROD activity, glycogen and lipid content, which were more sensitive indicators compared to catalase and lipid peroxidation (LPO).
6. Further investigation into the importance of diet in determining energy status in toadfish, through a better understanding of food sources at the different field sites.
7. Thorough site characterisation to identify differences in substrate type (muddy or sandy), types of aquatic vegetation (i.e. seagrass and mangroves), tidal influence and levels of suspended solids, which would assist in understanding differences in food sources at different sites.
8. Catchment mapping and more detailed chemical analysis at each site to better characterise the differences in land use between these sites, which would drive differences in the types of contaminants present.
9. Examine the health of fish collected from upper estuary sites, since it is in these areas that toxicant concentrations have been found to be highest (Melbourne Water, 2018).

Priority 1 and 2: Contaminant analysis of remaining carcass and liver samples; have all remaining otoliths aged:

Measuring contaminant concentrations in the remaining tissue and carcasses of all of the toadfish used in the study will help establish if there are any site-based differences in contaminant exposure. This will also help clarify if the high levels of genetic mixing are due to dispersal in the early life stages, or dispersal as adults (i.e. strong site-based differences would be expected if dispersal was during early life stages only; weak site-based differences would be expected if dispersal occurs in adults). Other studies have observed accumulation of environmental contaminants in toadfish tissues, including metals (Alquezar et al., 2006) and dieldrin (Mat Piah, 2011). The otoliths for all fish collected in 2016 have been preserved and are available for age estimation. Contaminant analysis and age estimation could be valuable to determine the extent of contamination with legacy, bioaccumulative toxicants such as DDT and dieldrin, which have been detected in sediments or water samples from different locations around Western Port. This knowledge will help us interpret the findings of the present study (i.e. carcinogens) and will allow us to determine the levels of bioaccumulative substances in some of these fish which are up to 20 years old (and those collected from 2016 potentially even older), to potentially reconstruct exposure histories.

Priority 3: Further investigation into liver tumours in fish from Port Phillip sites:

Whilst the overall levels of pre-cancerous and cancerous liver changes are low, further investigation is required to determine if these levels represent ‘natural’ baseline levels or if

they are the result of exposure to carcinogenic contaminants at some point during their lifetime. Assessment of other long-lived fish species from Port Phillip would be valuable to determine if the prevalence of pre-cancerous and cancerous changes is restricted to smooth toadfish or is present in other species as well.

Priority 4: Further investigation into the Bass River catchment:
Smooth toadfish from the Bass River displayed low HSI and EROD values, low energy stores but high liver scores and a high presence of lesions and parasites. This indicates the fish are affected by stressors that may be compromising health and immunity. Toxicant screening and potentially further ecological assessment (algae, invertebrates and fish) within the Bass River catchment may assist in identifying what is driving this. In particular, further sampling to capture any seasonal differences would be worthwhile.

Priority 5: Focus future biomarker work with toadfish on EROD activity, glycogen and lipid content, which were more sensitive indicators compared to catalase and lipid peroxidation (LPO).
For any future projects that utilise smooth toadfish, we recommend using EROD activity and energy allocation markers in fish health assessments.

Priority 6 and 7: Further investigation into the importance of diet in determining energy status in toadfish.
Given how different some of the waterways were that were used in this study, the available food sources across sites was likely quite variable. For example, some collection sites were sandy, beach sites with no mangroves and low levels of suspended solids (BLTF, EPTF, ADTF, SPTF), whilst others were muddy, very tidal estuarine sites with mangroves and high levels of suspended solids (WLTF, CHTF, WCTF, WDTF, BRTF, BNTF). Future work should incorporate assessments of food web interactions, since some toxicants may not have serious direct effects on fish, but may have strong indirect effects on their food sources. For example, some herbicides are considered relatively non-toxic to fish, however if they affect phytoplankton, then zooplankton and other invertebrate prey may be affected, leading to poor food availability for the fish (and reduced resilience to other environmental stressors).

Priority 8: Catchment mapping and more detailed chemical analysis at each site to better characterise the differences in land use between these sites, which would drive differences in the types of contaminants present.
Toadfish from all 10 collection sites showed some indications of environmental stress, yet no particular sites showed strong patterns of impact across all indicators. Since toadfish are a long-lived species (>20 years), it is possible that their health may be influenced by toxicant exposure that occurred a long time ago. A better understanding of current (and legacy) issues within specific catchments may assist in identifying specific contaminants that might be contributing to the impacts that were observed.

Priority 9: Examine the health of fish collected from upper estuary sites, since it is in these areas that toxicant concentrations have been found to be highest (Melbourne Water, 2018).
Other studies conducted by AQUEST (formerly CAPIM), have shown that toxicant concentrations, in particular some herbicides and fungicides are highest in the upper catchments, as opposed to the marine bay areas of Western Port. Therefore, fish inhabiting the upper catchments may be at higher risk of exposure to toxicants than fish in the lower estuary/bay areas, so a study to examine the health of fish inhabiting those sites would be valuable to determine the extent of any impacts caused by toxicants.

2 Introduction

Research conducted by CAPIM since 2012 has identified multiple pesticides in marine, estuarine and freshwater sections of catchments that drain into Western Port, and some pesticides have been detected at concentrations known to cause biological effects in fish, aquatic invertebrates and plants (Melbourne Water, 2018). Smooth toadfish collected in 2015 from different sites within Western Port displayed some differences in physiological and histological endpoints compared to toadfish collected from Port Phillip (Edwards Point). However, there were no strong and consistent results to indicate any one particular site was most affected, but rather fish from all 5 collection sites, including the external reference site – Edwards Point, displayed some changes indicative of environmental stress. Between sites there were significant differences in tissue condition indices: gonadosomatic index (GSI); hepatosomatic index (HSI); and condition factor (CF), indicating differences in energy allocation of fish sampled from each field site, however these are general indicators, and cannot be specifically linked to toxicant exposure. There was no evidence of endocrine disruption-related changes in the gonads of toadfish from any sites, nor was there any evidence of vitellogenin (VTG) induction in blood or surface mucus (VTG induction is indicative of exposure to endocrine disruptors).

For the sampling that was done in 2015, some individual fish were observed to have pre-cancerous (pre-neoplastic), or cancerous lesions in their livers. The rates were low, however their presence suggests the fish may have been exposed to something during their lifetime that has triggered this change. Liver tumours in flatfish such as flounder have been classified as direct indicators of chemical exposure, and as such histological assessment of livers has been widely used in marine environmental monitoring programmes worldwide (Cefas, 2007; Stentiford et al., 2009). The toadfish that were observed to have benign or malignant liver tumours were not the largest (or oldest) fish collected during the study, and were observed in both a Western Port site (Watsons Creek) and the external reference site (Edwards Point). There is very limited information available on smooth toadfish ecotoxicology, and no information on the natural rates and incidence of liver tumours in this species. Given that we observed these neoplastic changes in fish from impact and reference sites, further investigation of additional samples was recommended from the 2015 study to establish baseline information for this species.

The lack of any strong site-specific impacts in toadfish may indicate that they are pollution tolerant or that we require more sensitive bioindicators, or that the pollution within Western Port is low, or fairly homogenous and evenly distributed throughout the bay. It also raised the question of toadfish connectivity, and whether or not the fish may be moving around between sites. In order to adequately address these uncertainties, further sampling, and in particular further sampling of multiple external reference sites was needed, as well as an assessment of additional sensitive biomarkers (EROD, catalase, energy allocation markers) and genetics analysis. These considerations formed the basis of the present study, to address the aims as listed below.

Aims

- To assess the spatial health status of toadfish from Western Port using morphological, biochemical and histological markers of fish health.
- To compare the health of toadfish within Western Port to toadfish from external reference sites (Port Phillip, Anderson Inlet and Shallow Inlet).
- To determine if the health and condition of fish collected from different locations can be related to differences in aquatic pollution.
- To determine the genetic structure of toadfish populations in order to establish knowledge on patterns of connectivity, levels of genetic diversity and tests for signatures of adaptive variation, which may be relevant to understanding any influences of aquatic pollution.

3 Methods

3.1 Fish Collection

Fishing was carried out between 25th November and 13th December 2016. Fish were collected using fyke nets set overnight or seine nets, dissected within 2 days of capture and tissue samples were retained for different bioassays. Fish were sampled from two coastal external reference sites – Anderson Inlet and Shallow Inlet; two sites within Port Phillip – Williamstown and Blairgowrie; and one site within Western Port – Bass River (Figure 1). Since some of the analyses conducted in this round of reporting were based on samples collected in 2015, all data from the Spring 2015 sampling are presented again in this report to allow comparisons from both years (Table 1; Figure 1). Specific details of sample collection and analysis from 2015 can be found in the technical report of Hassell et al. (2016). For each site a total of 30 fish (mixed sex) were tested.

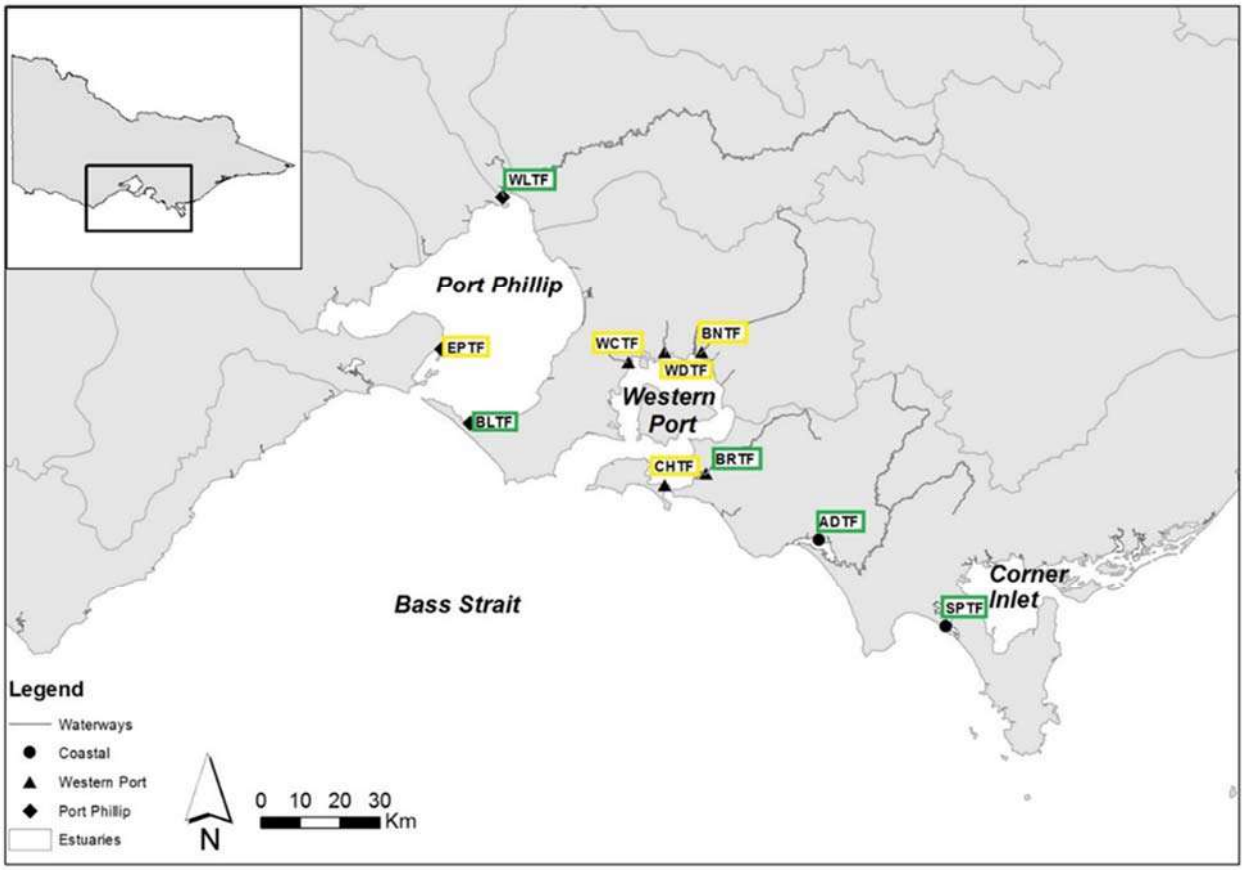

Figure 1. Smooth toadfish (*Tetractenos glaber*) sampling locations within Western Port, Port Phillip and external coastal reference sites. Sites in yellow were sampled in November 2015 and sites in green were sampled in November/December 2016.

Table 1. Details of the ten sampling locations for smooth toadfish (*Tetractenos glaber*) used in the Fish Health Assessment component of the Western Port Toxicants Study 2015-2017. Samples were collected in November 2015 or November/December 2016.

Sampling Location	Sample ID	Year	Catchment	Site Classification	Predominant Landuse or catchment type	Latitude	Longitude
Shallow Inlet (Sandy Point)	SPTF	2016	West Gippsland	External reference	Dryland pastures	38°50'31.41"S	146° 9'16.71"E
Anderson Inlet (Mahers Landing)	ADTF	2016	West Gippsland	External reference	Dryland pastures/dairy	38°38'19.17"S	145°47'26.41"E
Blairgowrie	BLTF	2016	Port Phillip	External reference	Residential	38°21'44.77"S	144°47'15.11"E
Edwards Point	EPTF	2015	Port Phillip	External reference	Pastures/cropping	38°11'35.83"S	144°42'40.63"E
Williamstown (Greenwich reserve)	WLTF	2016	Port Phillip	External reference	Residential/industrial	37°50'53.92"S	144°53'51.58"E
Churchill Island (Phillip Island)	CHTF	2015	Westernport	Internal reference	Dryland pastures	38°30'33.47"S	145°20'46.76"E
Bass River	BRTF	2016	Westernport	Impact site	Dryland pastures/dairy	38°29'6.76"S	145°28'1.55"E
Bunyip River	BNTF	2015	Westernport	Impact site	Horticulture	38°12'44.46"S	145°27'33.66"E
Watsons Creek	WCTF	2015	Westernport	Impact site	Pastures/ horticulture	38°13'55.53"S	145°14'51.20"E
Western Contour Drain	WDTF	2015	Westernport	Impact site	Pastures/ horticulture	38°12'36.89"S	145°21'15.12"E

General markers of fish health, such as condition factor (CF), gonadosomatic index (GSI) and hepatosomatic index (HSI) were measured, as well as several tissue biomarkers (EROD, GST, CAT, LPO, LIP, GLY) and liver and gonad histopathology. Liver samples were also analysed to determine genetic structure and patterns of connectivity. See Appendix Table A1 for a complete list of all fish health indicators that A3P AQUEST (formerly CAPIM) uses for pollution assessment in waterways.

3.2 General Indicators

Simple observations and measurements of the external appearance of fish when they are first captured can be valuable general indicators of fish health (Goede & Barton, 1990). Observations of scale, skin or fin damage, as well as the presence of lesions and parasites may indicate stress associated with disease or infection. Similarly, measurements of length and weight can be used to determine growth rates and condition indices. Observations and measurements of internal organs can be used to determine if the vital organs appear 'normal' and to calculate tissue indices.

The condition factor is an index used to describe the relative size and weight of each fish. This information is useful for comparing different groups of fish and provides some indication of general health status. **[Total body weight (g) / Fork length (cm)3] x 100.**

The GSI is an index used to describe the gonad weight relative to the overall weight of each fish. This information is useful for comparing different groups of fish and provides some indication of reproductive status and general health condition.

[Gonad weight (g) / Total body weight (g)] x 100.

The HSI is an index used to describe the liver weight relative to the overall weight of each fish. This information is useful for comparing different groups of fish and provides some indication of nutritional and reproductive status, as well as general health condition.

[Liver weight (g) / Total body weight (g)] x 100.

Otoliths are calcified structures that lay down annual growth layers and can be counted to estimate age. Toadfish are long lived (>5 years) so it is important to know how old the fish are to provide context for other biological findings. An accurate age estimation enables calculation of growth rates too. All otolith analysis was done by Fish Ageing Services, Queenscliff.

3.3 Tissue Biomarkers (Liver)

Biomarker and energy reserve analyses

The following biomarkers were measured: catalase activity and lipid peroxidation (LPO - MDA) concentration (both are measures of oxidative stress which occurs when organisms are exposed to chemical pollutants or under physiological stress such as disease or aging), Ethoxyresorufin-o-deethylase (EROD) activity (a marker of exposure to organic chemicals such as pesticides, polycyclic aromatic hydrocarbons and PCBs), lipid and glycogen content (provide a functional measure of energy reserves within the tissue).

General sample preparation:

Sub-samples of the minced liver were used for biomarker analyses. Samples were prepared and analysed randomly to remove any bias in the analysis. Livers were prepared following standard methods of homogenisation and centrifugation, described briefly below. The resulting supernatant was used for determining enzyme activity, and total protein was determined through the method of Lowry et al. (1951) adapted for the microplate reader using the Bio-Rad DC reagent protocol. All enzyme activities, energy reserves and protein concentrations were measured using a Synergy 2 plate reader (Biotek Instruments). Blanks and commercial standards (if available) were run for each enzyme. Each sample, blank and standard was run in triplicate. Lipid, glycogen, total protein and MDA concentrations were determined using a standard curve with commercial vegetable oil, D-glucose, Bovine Serum Albumin (BSA) and tetraethoxypropane (TEP) as standards, respectively.

3.3.1 Liver glycogen (GLY) and Lipid (LIP)

Lipid and glycogen analyses followed the method of Van Handel (1985 a & b) and were modified for use in a microplate reader. The volume of solution in each well was 60 µL; absorbance was measured at 490 nm for lipid and 625 nm for glycogen.

3.3.2 Liver Ethoxyresorufin-o-deethylase (EROD)

EROD activity and MDA concentration were prepared and measured following the methods of Edge et al. (2013). Briefly, liver was homogenised (1:25 weight:volume) in phosphate buffer (pH 8.0) using a mixermill, centrifuged at 10,000g for 20 min at 4^{o}C and the S9 fraction was collected for analysis. EROD activity was measured following the addition of 7-ethoxyresorufin and NADPH to the microplate (containing S9 fraction and phosphate buffer). Activity was measured over time, with excitation/emission wavelengths of 530/585 nm. Resorufin was used as the standard to allow activity to be expressed as pmol resorufin produced/min/mg protein.

3.3.3 Liver Lipid Peroxidation (LPO - MDA)

MDA concentration were prepared and measured following the methods of Edge et al. (2013). Tissue was homogenised in phosphate buffer (4:1 weight: volume) at 13,000g for 15 min at 4^{o}C. Butylated hydroxytoluene (BHT) and thiobarbituric acid (TBA) were added to each sample, heated at 100^{o}C for 15 min, centrifuged and absorbance was measured at 532 nm.

3.3.4 Liver Catalase (CAT)

Livers were homogenised (1:4 weight:volume) in phosphate buffer (pH 6.5) using a mixermill and glass beads to disperse the tissue. Following homogenisation, samples were centrifuged at 14000 rpm for 5 min. The reaction was started with the addition of 50mM hydrogen peroxide and activity was measured at 240 nm over time.

3.4 Gonad and Liver Histology

Following dissection, gonad and liver samples were fixed in Bouin's fixative for 48 hours, then preserved with 70% ethanol. Up to three portions were prepared from each toadfish gonad, consisting of a 3-5 mm transverse section from the anterior, mid and posterior parts of the gonad. Liver samples were prepared in a similar way. The samples were dehydrated in an ethanol series, cleared in histolene, and embedded in paraffin wax. Transverse sections were prepared at 5 µm, mounted on slides and stained with haematoxylin and eosin (H&E), then examined by light microscopy. Male gonadal sections were examined for the presence of testicular oocytes (testis-ova), altered spermatogenesis and increased testicular degeneration (interstitial cell hyperplasia, syncytial cells, apoptotic germ cells and eosinophilic granules). Female gonadal sections were examined for the presence of post-ovulatory follicles, oocyte fragmentation, atresia, oocyte membrane folding and disorganised appearance. All histological features were assessed as described in the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (Johnson et al., 2009; Dietrich and Krieger, 2009). A semi-quantitative scoring system was used to categorise different histological features, where 0 = no incidence, 1 = low incidence, 2 = moderate incidence, 3 = high incidence. The highest possible score using this semi-quantitative scoring system was 12 in males and 12 in females. The reproductive stage of all gonads was determined using staging criteria modified from Johnson et al. (2009) and Dietrich and Krieger (2009) (Appendix Table A2).

Liver sections were examined for generalised tissue damage and the presence of macrophage aggregates. A semi-quantitative scoring system was used to categorise different histological features, where 0 = no incidence, 1=low incidence, 2 = moderate incidence, 3 – high incidence. The overall liver score comprised 4 different markers (hepatocyte alterations, macrophage aggregates, pancreatic duct alterations and other general alterations), with the highest possible score being 12.

3.5 Genetics Analysis

A small portion of liver from each fish was used to extract high quality DNA for population genetics analysis (in collaboration with Dr Craig Sherman, Deakin University). The DNA was extracted using Qiagen DNeasy® Blood & Tissue kits and sample purity checked by agarose electrophoresis, then quantified by QUBIT analysis. All samples were standardised to 10 ng/ul before being sent for library preparation at the Monash University Genomics Facility (Malaysia). A total of 252 samples (19-30 individuals from each location) were sequenced (paired end) over three lanes of the Illumina HiSeq4000 platform by MACROGEN (Seoul, Korea). Sequences were first processed using the FASTX-Toolkit program (http://hannonlab.cshl.edu/fastx_toolkit/) by trimming the raw reads to 80 bp length and discarding all reads that had a Phred score below 20. Three samples were excluded from further analysis due to poor sequencing quality. The de novo program from Stacks 2.0 (Catchen et al. 2013) was used to create a catalogue of SNPs and genotypes for all individuals with a minor allele frequency of 0.01 and minimum representation for a SNP to be retained at 70% across the data set. This resulted in 13217 SNPs being retained for further analysis. Estimates of neutral population genetic structure were calculated in the R package DiveRsity and included global FST, pairwise FST and several genetic diversity estimates (allelic richness, % polymorphic loci, expected heterozygosity, and observed heterozygosity). A discriminant analysis of principal components (DAPC) was used to visualise any genetic

clusters within the dataset and was calculated using the find clusters function in the R package Adegenet. A total of 150 PC's were retained which represented approximately 70% of the variation.

3.6 Statistical Analysis

All general indicator, biomarker and energy reserve data were $\log_{10}$ transformed prior to statistical analysis to meet the assumptions of parametric analyses. One-way analysis of variance (ANOVA) were carried out to determine if there were significant differences in responses between sites ($\alpha = 0.05$); males and females were considered separately as there may be sex-related differences in responses. Data were checked to ensure they conformed to the assumptions of ANOVA, if they didn't conform, a non-parametric Kruskal Wallis test was carried out to determine site differences. To determine which sites responded differently a Tukey-Kramer HSD post hoc test was carried out. All analyses were carried out using JMP 12.0.1 (SAS, 2017) statistical analysis software.

4 Results

Data from 2015 has been reported previously (Hassell et al., 2016), but to aid in the interpretation of findings, all data from both spring sampling runs: first (2015); second (2016) are presented here.

4.1 General Indicators

4.1.1 General Condition

Smooth toadfish showed a variable incidence of external parasites and lesions (Figure 2), and for both males and females, fish from BRTF displayed the highest incidence of external parasites (81-100%). Excluding BRTF fish, the incidence across all sites ranged from 0-39%. The most commonly observed external parasite was the copepod crustacean, *Lernaea sp.* (anchor worm) (Figure 2).

Male fish from BNTF (WP) displayed the highest incidence of external lesions (57%), whilst fish from all other sites ranged between 0-35%. The most commonly observed lesions were reddening of the pectoral and caudal fins, raised nodules on the head, red patches on the ventral surface (belly) and healing bite marks along the body (Figure 2).

Figure 2. Examples of some of the external parasites and lesions that were observed on Smooth toadfish throughout the study.

4.1.2 Length and Weight

Differences were observed in the length (ANOVA $F_{(9, 299)} = 22.126$, $p < 0.001$) and weight (ANOVA $F_{(9, 299)} = 29.870$, $p < 0.001$) of smooth toadfish between sites. The largest, heaviest fish were females from the two reference sites (ADTF and SPTF), whilst the smallest were females from BNTF and EPTF. In general, male and female fish from Western Port had lower mean length and weight values compared to fish from the external reference sites or Port Phillip (Table 2) (EXT REF>PPB>WP).

4.1.3 Sex Ratio

Thirty fish were sampled from each field site (10 in total), resulting in 194 female and 106 male fish in total. Sex ratios were variable, and there did not seem to be any obvious patterns occurring across sites. The number of females in each sample ranged from 6 (20%) to 28 (93.3%) (Table 2; Figure 3).

4.1.4 Gonadosomatic Index (GSI)

Differences were observed in GSI values for males and females across different sites (Kruskal-Wallis, $p < 0.001$), and in general, females tended to have higher values than males (Table 2). The highest GSI value observed was 20.4%, in a female toadfish from ADTF. The lowest GSI value was 0.2% in a male fish from EPTF. The overall pattern in GSI values in females was EXT REF=PPB>WP, whilst for males there were no distinct patterns across sites (EXT REF=PPB=WP).

4.1.5 Hepatosomatic Index (HSI)

There were significant differences in the HSI values between females (ANOVA $F_{(9,192)} = 6.5847$, $p < 0.001$) and males (ANOVA $F_{(9,104)} = 6.1649$, $p < 0.001$) from each field site (Table 2). Females tended to have higher values than males, and fish from Port Phillip and the external reference sites displayed higher values than fish from Western Port (except for females from the BNTF). The highest HSI value was 9.5% in a female from BNTF, and the lowest HSI value was 1.7% in a female from BLTF. The overall pattern in HSI values for female fish was EXT=PPB>WP, and for males was PPB >WP>EXT REF.

4.1.6 Condition Factor (CF)

There were significant differences in the condition factor values between females (ANOVA $F_{(9,193)} = 9.6056$, $p < 0.001$) and males (ANOVA $F_{(9,105)} = 5.8598$, $p < 0.001$) from each field site (Table 2). Mean values were around 2%, with the smallest values of 1.2% and 1.3% in fish from WDTF and the highest values of 2.6% and 2.8% in fish from EPTF and BNTF. The overall pattern in condition factor values for females was EXT REF>PPB>WP, and for males was PPB>EXT REF>WP.

4.1.7 Age (and growth)

The estimated age range of Smooth toadfish collected during this study was 1 to 20 years, with a median of 3 years. There was some difference between sexes, with the females tending to be larger than males of the same age (Figure 4). Growth, measured as Total length/Age (mm/year), was different between females and males from each site. Fish from EPTF (PPB) had the fastest growth rates, of 43.5 mm/year in females, and 51.7 mm/year in males. The slowest growing fish were from CHTF, with growth rates in females of 22.6 mm/year, and in males, 17.4 mm/year. Age and growth data are only available for fish sampled in 2015.

When a subset of the data was re-assessed, using only fish of ages 3, 4, or 5 years old (for which there was sufficient data between sites to allow comparisons), differences were still observed in growth rates between sites (ANOVA, $F_{(4,84)} = 2.5848$, $p = 0.0432$). Fish from EPTF had significantly greater growth rates (32.07 mm/year) than WDTF (27.12 mm/year). There was a significant difference between females and males, with females having a mean growth rate of 31.18 mm/year, and males having a mean growth rate of 28.01 mm/year.

Table 2. Biological data (Mean ± SEM) for smooth toadfish (*Tetractenos glaber*) sampled from ten locations across Western Port (WP), Port Phillip (PPB) and external reference (EXT REF) sites during November 2015 and November/December 2016.

Site	Catchment	Sex	n	Total Length (mm)	Weight (g)	GSI (%)	HSI (%)	CF (%)	External Parasites		Skin lesions and scarring	
SPRING 2015									n	%	n	%
Watsons Creek (WCTF)	WP	M	20	120.0 ± 2.60	34.83 ± 1.41	4.71 ± 0.20	4.33 ± 0.24	1.94 ± 0.04	2	10.0	7	35.0
		F	10	109.2 ± 5.16	25.24 ± 4.37	2.06 ± 1.06	4.19 ± 0.29	1.92 ± 0.05	1	10.0	3	30.0
Western Contour Drain (WDTF)	WP	M	24	116.2 ± 2.84	27.23 ± 1.66	2.38 ± 0.21	3.99 ± 0.21	1.70 ± 0.05	0	0.0	4	16.7
		F	6	107.2 ± 9.43	22.41 ± 5.75	2.64 ± 0.22	3.59 ± 0.40	1.78 ± 0.16	0	0.0	1	16.7
Churchill Island (CHTF)	WP	M	6	121.0 ± 4.56	34.19 ± 2.90	2.93 ± 0.38	4.22 ± 0.46	1.87 ± 0.06	0	0.0	2	33.3
		F	24	115.3 ± 3.35	28.83 ± 2.47	2.02 ± 0.38	4.17 ± 0.19	1.86 ± 0.04	1	4.2	1	4.2
Bunyip River (BNTF)	WP	M	14	112.3 ± 2.66	28.95 ± 1.49	2.57 ± 0.23	5.04 ± 0.27	1.97 ± 0.07	0	0.0	8	57.1
		F	16	104.6 ± 4.05	21.80 ± 2.92	2.06 ± 0.26	3.92 ± 0.47	1.89 ± 0.06	0	0.0	3	18.8
Edwards Point (EPTF)	PPB	M	7	107.0 ± 5.23	29.52 ± 2.90	1.71 ± 0.84	6.45 ± 0.28	2.29 ± 0.10	0	0.0	0	0.0
		F	23	97.0 ± 3.54	21.46 ± 2.69	1.81 ± 0.20	6.48 ± 0.26	2.31 ± 0.05	0	0.0	3	13.0
SPRING 2016												
Bass River (BRTF)	WP	M	9	108.2 ± 3.01	26.21 ± 1.82	3.45 ± 0.44	3.51 ± 0.32	2.06 ± 0.08	9	100.0	1	11.1
		F	21	118.4 ± 4.27	34.91 ± 3.14	3.60 ± 0.44	3.96 ± 0.27	2.03 ± 0.05	17	81.0	3	14.3
Blairgowrie (BLTF)	PPB	M	3	130.3 ± 3.67	41.23 ± 4.50	3.80 ± 0.12	4.49 ± 0.56	1.84 ± 0.06	0	0.0	0	0
		F	27	136.9 ± 3.21	52.62 ± 4.06	4.64 ± 0.38	4.73 ± 0.26	1.97 ± 0.04	10	37.4	2	7.4
Williamstown (Greenwich Reserve) (WLTF)	PPB	M	3	125.7 ± 8.41	45.14 ± 7.16	3.10 ± 0.68	5.20 ± 0.11	2.26 ± 0.12	1	33.3	0	0
		F	27	124.1 ± 3.19	42.82 ± 2.95	4.27 ± 0.49	5.59 ± 0.29	2.15 ± 0.03	7	25.9	1	3.7
Shallow Inlet (Sandy Point) (SPTF)	EXT REF	M	2	129.0 ± 9.00	44.19 ± 9.84	2.39 ± 0.99	4.09 ± 0.71	2.02 ± 0.03	0	0.0	0	0
		F	28	139.8 ± 2.22	59.20 ± 2.77	3.06 ± 0.14	5.64 ± 0.33	2.13 ± 0.04	7	25.0	1	3.6
Anderson Inlet (Mahers Landing) (ADTF)	EXT REF	M	18	134.6 ± 2.20	49.55 ± 2.27	3.61 ± 0.45	3.87 ± 0.17	2.02 ± 0.04	7	38.9	2	11.1
		F	12	144.1 ± 5.24	64.52 ± 5.77	7.08 ± 1.81	5.20 ± 0.50	2.11 ± 0.06	4	33.3	0	0
*median (range)												

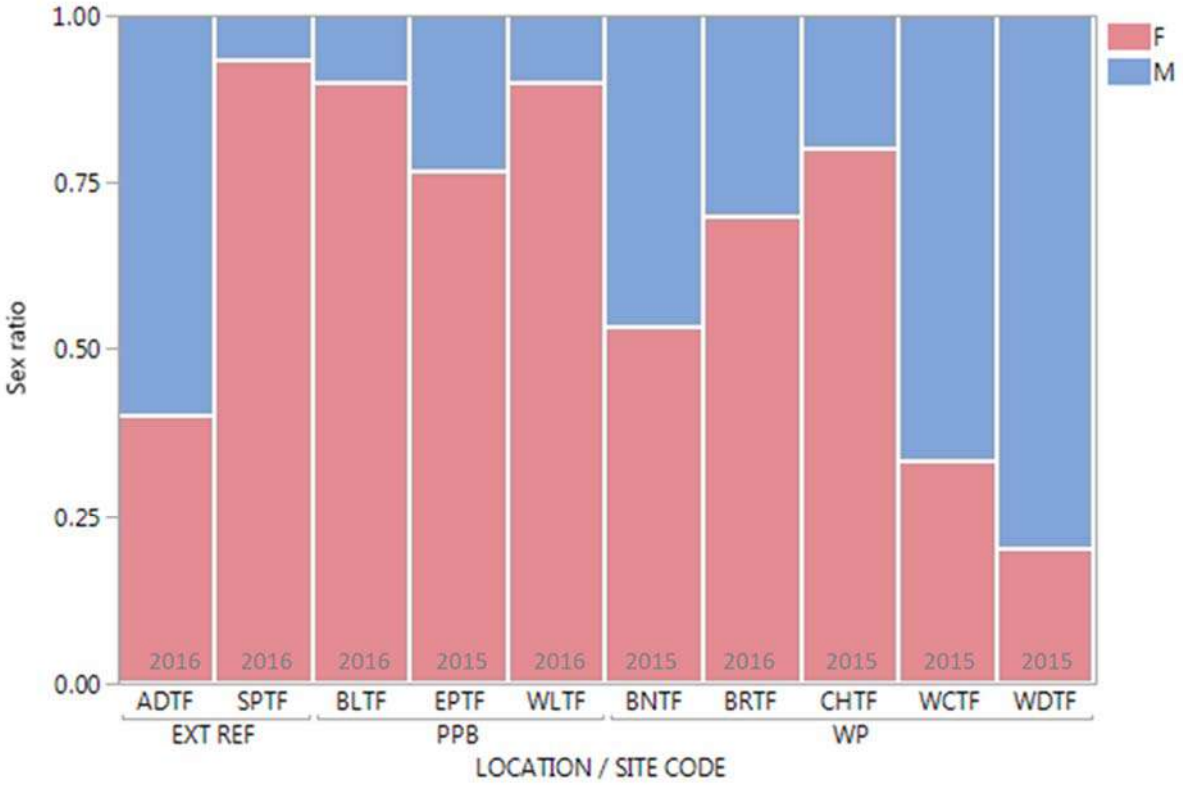

Figure 3. Sex ratios of smooth toadfish (*Tetractenos glaber*) sampled from 10 different locations within Western Port, Port Phillip and two external coastal reference sites. Blue – male; red – female, n=30/site.

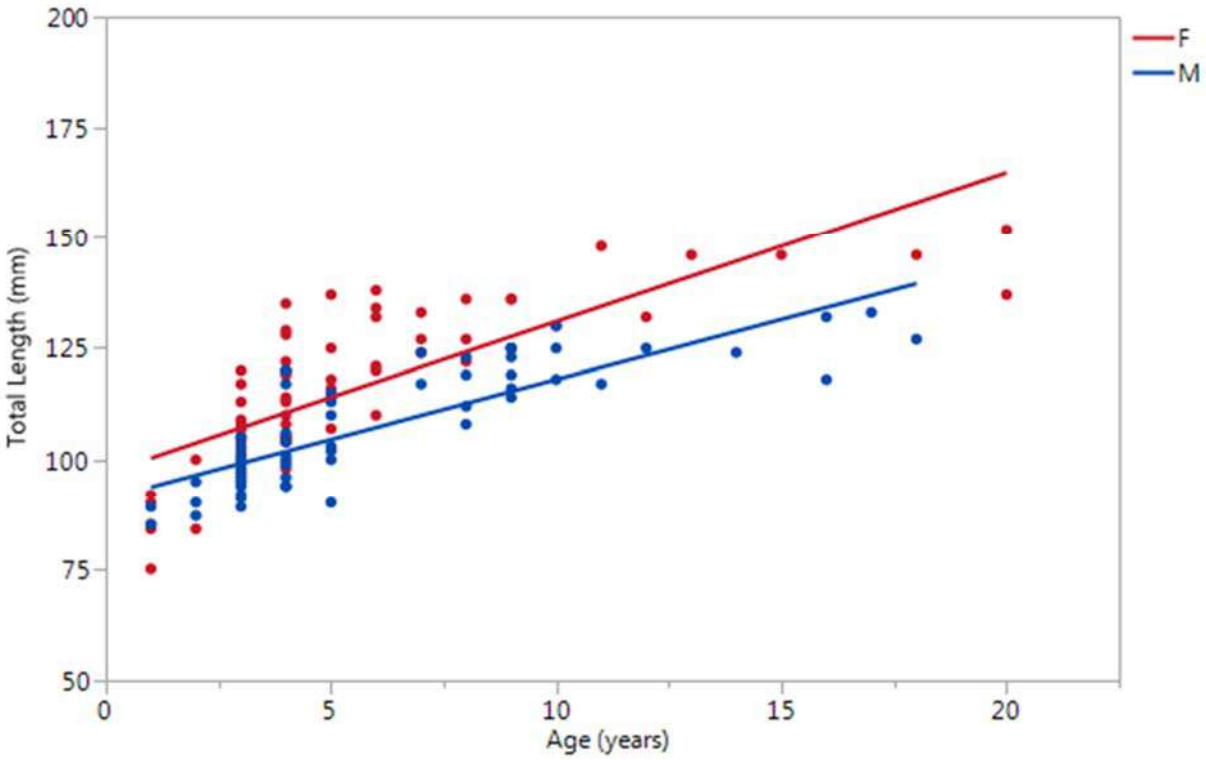

Figure 4. Length-age relationship in male (blue) and female (pink) toadfish sampled throughout the study (n=229).

4.2 Tissue Biomarkers

The following biomarkers were assessed in livers of toadfish collected during this study: catalase, lipid peroxidation (by analysing MDA concentration) and EROD activity. The liver was used for these analyses as it is the primary site of detoxification of organic chemicals, including pesticides and hydrocarbons, and is where anti-oxidant responses to chemicals occur.

4.2.1 Liver glycogen (GLY) and Lipid (LIP)

Energy reserves

Toadfish livers (from the Spring 2016 sampling) were assessed for concentrations of total lipid and total glycogen to see if there were any differences in these reserves between the sites.

Total glycogen (GLY)

There were significant differences in total glycogen concentration in female fish between the sites (ANOVA $F_{(4,29)} = 8.3943$, $p < 0.001$). The Tukey-Kramer HSD post-hoc test showed that total glycogen concentration in female fish from BRTF and WLTF were significantly lower than fish from the reference sites (ADTF and SPTF) and glycogen concentration in fish from BRTF was lower than in fish from BLTF (Figure 5). Interestingly, although total glycogen concentrations in male fish from BRTF and WLTF were lower than in male fish from the reference sites, it was not significantly different at $\alpha = 0.05$ (ANOVA $F_{(4,11)} = 2.7911$, $p = 0.08$) (Figure 5). In general, therefore, fish from BRTF and WLTF had lower total glycogen concentrations than fish from the other sites.

Total lipid (LIP)

There were significant differences in total lipid concentrations in female fish between the sites (ANOVA $F_{(4,29)} = 5.0394$, $p < 0.05$) (Figure 6), with lower lipid concentrations in females from BRTF compared to the other sites, except ADTF. There were between-site differences in total lipid concentrations in male livers, although the differences were not significant (ANOVA $F_{(4,10)} = 1.8381$, $p > 0.05$). Males from BRTF had the lowest lipid concentration of all sites, including the references sites ADTF and SPTF; however, males from WLTF had elevated concentrations of lipid. These results also show that fish from BRTF and WLTF had different total lipid concentrations than fish from the other sites within Western Port.

4.2.2 Liver Ethoxyresorufin-o-deethylase (EROD)

EROD activity

EROD activity was assessed in toadfish collected in spring 2015 and spring 2016 (Figure 7) and activity was detected in fish at all collection times. There were significant differences in EROD activity in female fish between sites (ANOVA $F_{(9,41)} = 3.892$, $p = 0.0014$), with fish from BRTF having significantly lower activity compared to fish from WLTF, EPTF, BLTF, SPTF, BNTF and CHTF (Figure 7). There were no significant differences in EROD activity in male fish between sites (ANOVA $F_{(9,21)} = 1.116$, $p > 0.05$) (Figure 7).

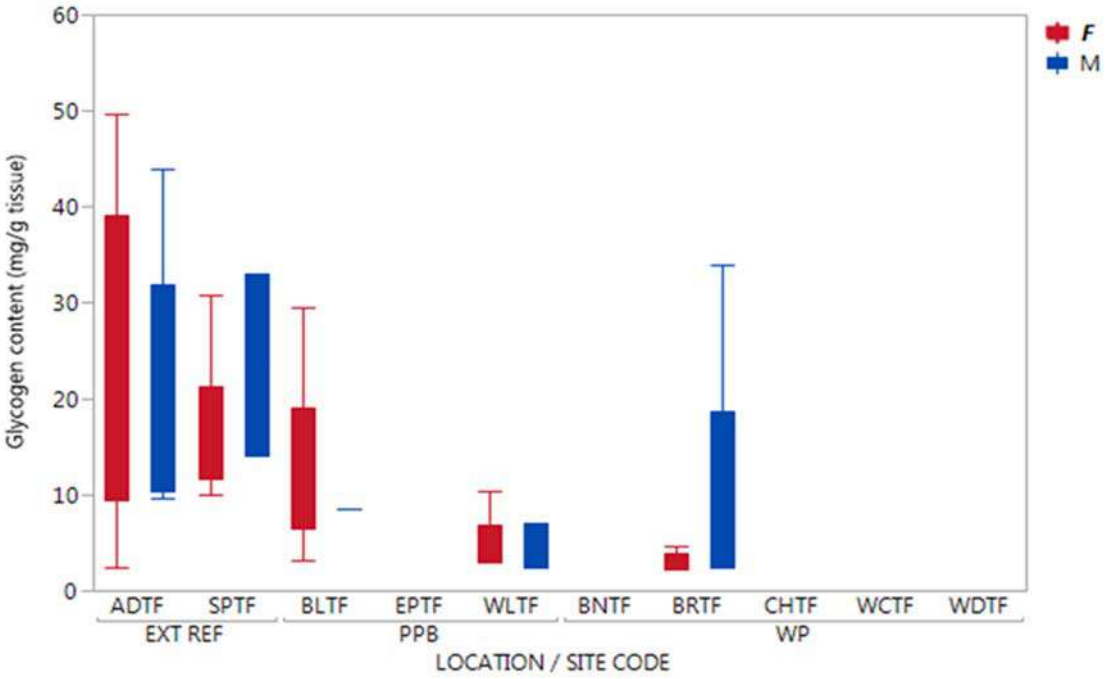

Figure 5. Box plots showing median values for liver glycogen content in female and male Smooth toadfish collected from reference sites (EXT REF), sites within Port Phillip (PPB) and sites in Western Port (WP). For this biomarker only fish collected in spring 2016 were used.

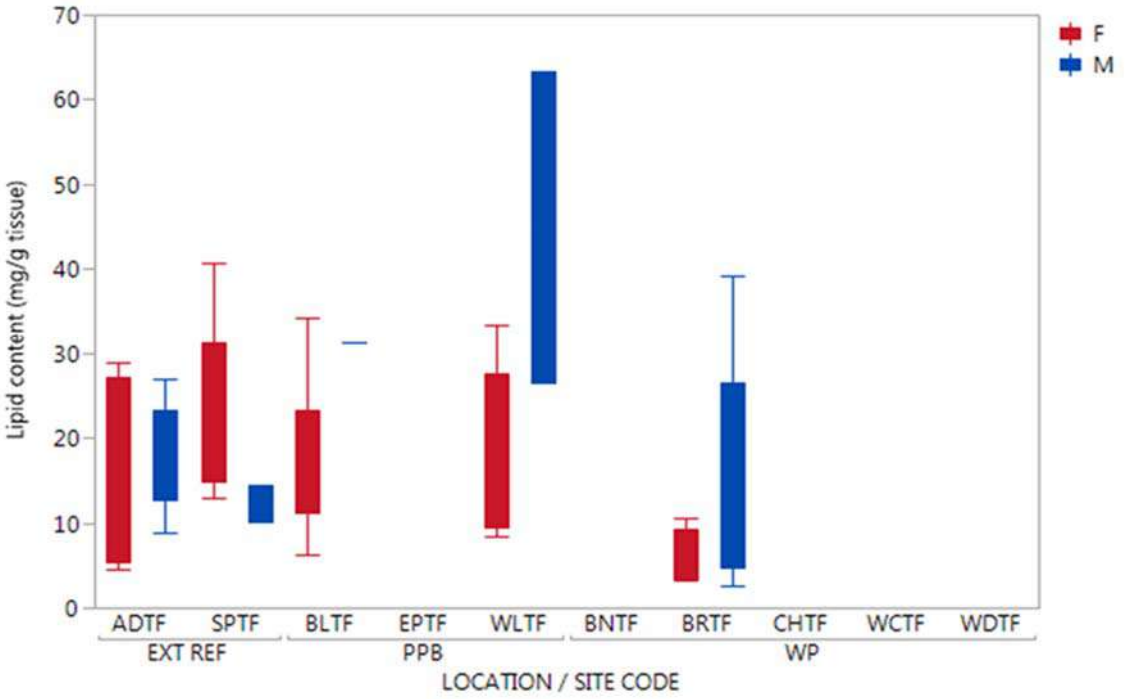

Figure 6. Box plots showing median values for liver lipid content in female and male Smooth toadfish collected from reference sites (EXT REF), sites within Port Phillip (PPB) and sites in Western Port (WP). For this biomarker only fish collected in spring 2016 were used.

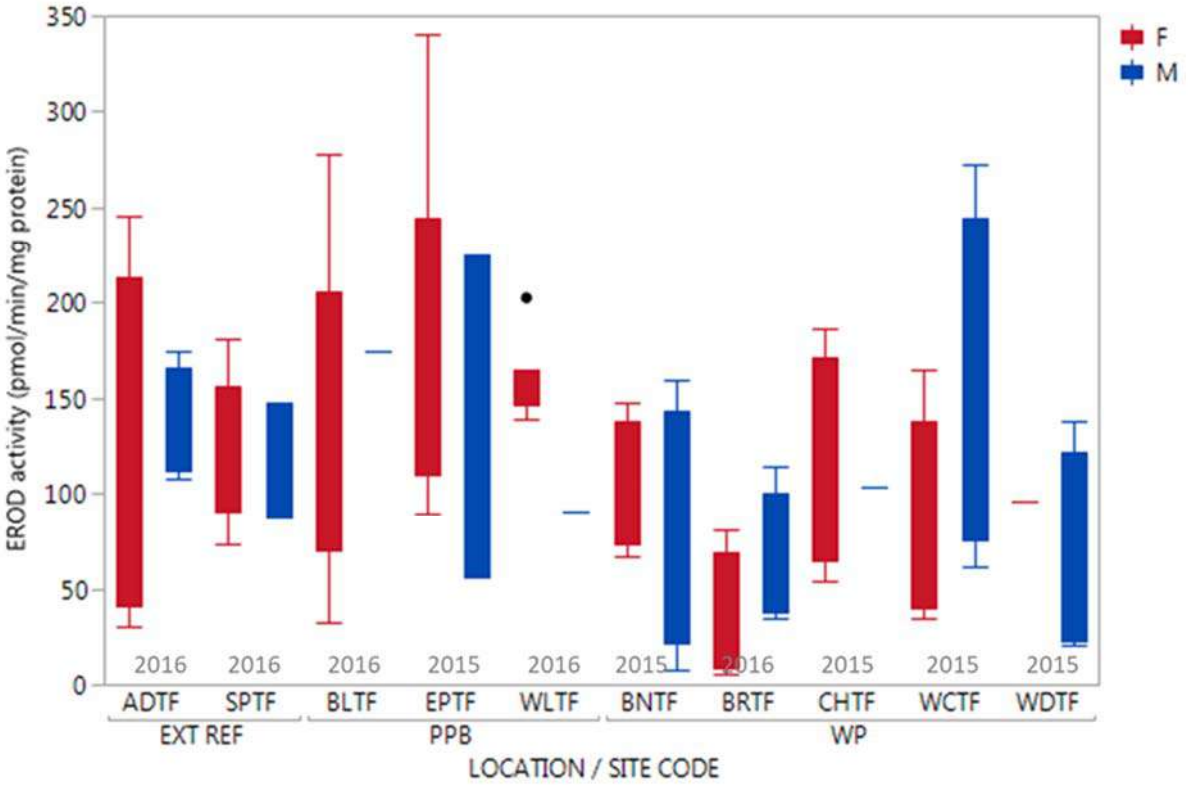

Figure 7. Box plots showing median values for liver EROD activity in female and male Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

4.2.3 Liver Lipid Peroxidation (LPO)

LPO activity

Lipid peroxidation activity was assessed using malondialdehyde activity (MDA) in fish from the Spring 2016 collections. Females at site WLTF had higher MDA concentrations compared to females from the other sites (Figure 8) with the mean MDA concentration approximately double those from the reference sites. In contrast, MDA concentrations in males from WLTF were lower than males from all the other sites (Figure 8). Interestingly, WLTF was the only site where there was clear separation in MDA concentrations between males and females, this is mainly due to the elevated concentration in the female fish from this site.

4.2.4 Liver Catalase (CAT)

Catalase activity

Catalase activity was only assessed in fish from the spring 2016 collections. There were no significant differences in catalase activity in female fish between sites from Western Port or Port Phillip bays (ANOVA $F_{(4,28)} = 1.1499$, $p > 0.05$) (Figure 9), although catalase activity in females from BRTF were lower than the other sites it was not significantly different. Furthermore, there were no significant differences in catalase activity in male fish between sites (ANOVA $F_{(3,7)} = 1.5727$, $p > 0.05$) (Figure 9). In general, there was no difference in catalase activity between male and female fish from all sites, with mean activity ranging from approximately 75 - 150 nmol/min/mg protein for all fish.

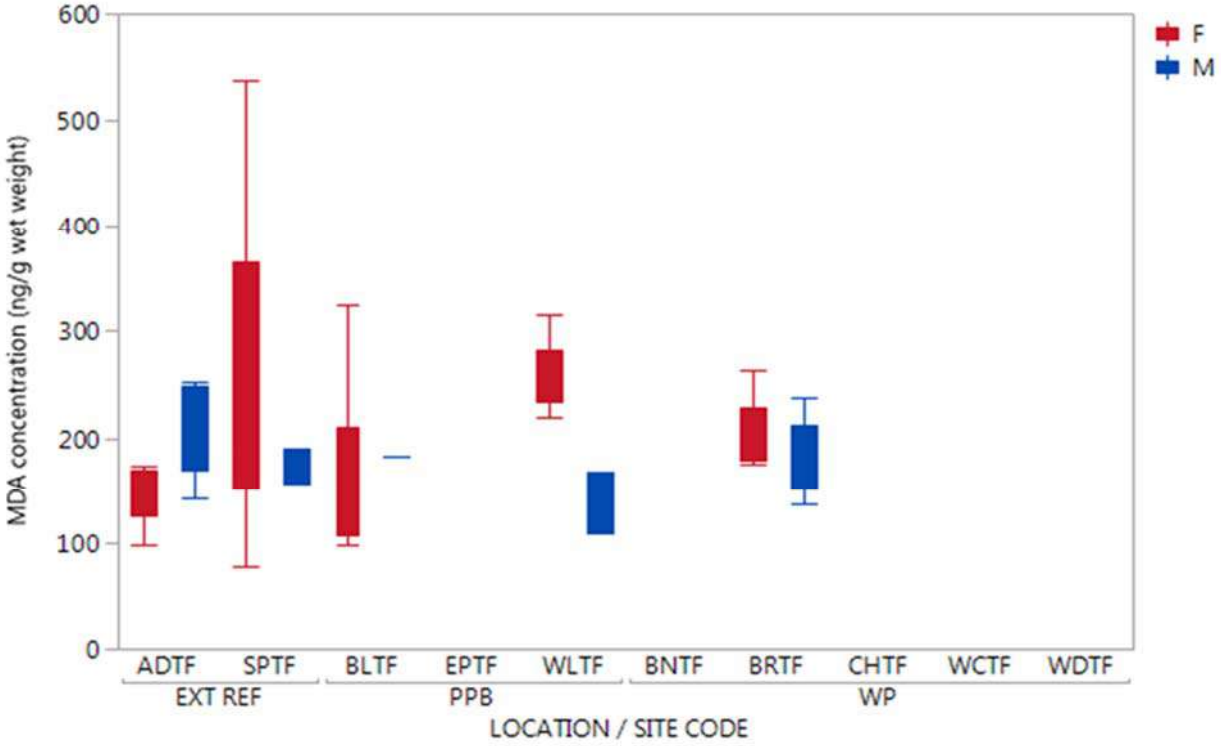

Figure 8. Box plots showing median values for liver lipid peroxidation, measured as MDA concentration in female and male Smooth toadfish collected from two reference sites (EXT REF), Port Phillip (PPB) and Western Port (WP) in spring 2016.

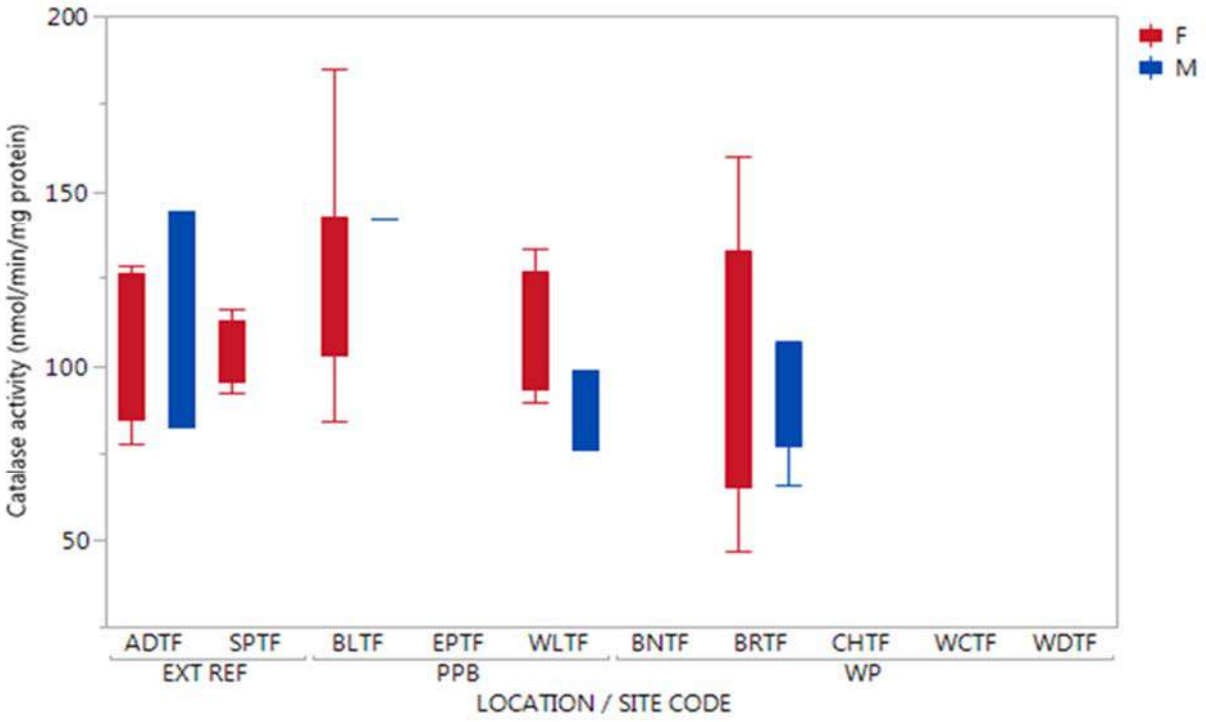

Figure 9. Box plots showing median values for liver catalase activity in female and male Smooth toadfish collected from two reference sites (EXT REF), Port Phillip (PPB) and Western Port (WP) in spring 2016.

4.3 Histology

4.3.1 Gonad Histology

Gonad staging

All fish were sampled in the same season (spring) of either 2015 or 2016 to minimise seasonal based differences in reproductive maturity. Despite this, there were some differences in the maturity of gonads in fish from different sites, based on gonad staging (Table 3). In females, the median ovarian maturity stage for fish from most sites was Stage 5 (post-ovulatory), except for BLTF and WLTF, where the median gonad stage was Stage 3 (late development) and Stage 2 (mid development), respectively (Table 3; Appendix Table A2). In males, the median testis maturity stage was Stage 3 (late spermatogenic) in fish from all sites (Table 3; Appendix Table A2). These results indicate recent or active spawning was occurring in fish from all sampling locations.

Gonadal macrophage aggregates

Macrophage aggregates, or melanomacrophage centres (MMCs) were observed in the ovaries and testes of most fish that were examined (Table 3). In female fish, the mean number of MMCs per unit of ovarian area (mm^2) was highest in fish from CHTF and WDTF, and the general pattern across all sampling locations was WP>EXT REF>PPB (Figure 10). For male fish, the mean number of MMCs per unit of testis area (mm^2) was more variable than in females, and for some sites there were as few as 2 samples. The pattern observed for males was EXT REF>WP>PPB (Figure 11).

Female-specific indicators

Gonadal change scores were quantified for all female fish, and median values across sites varied from 2 to 5 (Table 3). In general, the values were quite low, with a maximum score of 5 observed (of a total possible of 12), and values in fish from Western Port were generally lower than other sites (EXT=PPB>WP) (Table 3). Oocyte atresia, the presence of atretic oocytes within the ovary, was observed in female fish from all sites (Table 3). There was no distinct pattern in the mean number of atretic folicles per unit of ovarian area (mm2) across locations, with much variability in mean values between sites (EXT=PPB=WP) (Figure 12; Table 3). The maximum number of atretic follicles counted within an ovarian section was 214. Post-ovulatory follicles are what remain in the ovary after an oocyte has been released (ovulated). The presence of post-ovulatory follicles indicates recent spawning, and fish from all sites displayed some (Figure 13). There was no distinct pattern in the mean number of post-ovulatory follicles per unit of ovarian area (mm^2), with much variability between sites (EXT=PPB=WP) (Figure 13). The maximum number of post-ovulatory follicles counted within an ovarian section was 136.

Male-specific indicators

Gonadal change scores were quantified for all male fish, and median values ranged from 0-6 across sites (Table 3). There were no patterns between sites, with much variability due to low sample numbers in some cases (n=2) (EXT=PPB=WP). Testis-ova (male) were not observed in male fish from any site (Table 3).

Table 3. Gonad histology scores for smooth toadfish (*Tetractenos glaber*) sampled from five locations within Western Port, three locations within Port Phillip and two external reference sites (EXT REF) during spring (November/December) 2015-2016. Gonad staging criteria are listed in Appendix Table A2. A semi-quantitative scoring system was used to categorise different histological features, where 0 = no incidence, 1 = low incidence, 2 = moderate incidence, 3 = high incidence. Gonadal change scores comprised 4 different markers for each sex, with the highest possible score being 12.

Site	Catchment	Sex	n	Gonad stage*	Macrophage aggregates*	Testis-Ova*	Male gonadal changes*	Atresia*	Female gonadal changes*
Watsons Creek (WCTF)	WP	M	20	3 (2-4)	1 (0-2)	0	2 (1-6)		
		F	10	5 (5-5)	1 (0-3)			1 (1-3)	3 (3-3)
Western Contour Drain (WDTF)	WP	M	24	3 (3-4)	1 (0-3)	0	2 (0-8)		
		F	6	5 (5-5)	1.5 (1-3)			1 (1-2)	3 (3-3)
Churchill Island (CHTF)	WP	M	6	3 (3-3)	1 (1-2)	0	1.5 (0-6)		
		F	24	5 (3-5)	2.5 (0-3)			3 (0-3)	3 (3-3)
Bunyip River (BNTF)	WP	M	14	3 (3-3)	1 (0-2)	0	2 (0-8)		
		F	16	5 (0-5)	2 (0-3)			1 (0-3)	2 (1-4)
Edwards Point (EPTF)	PPB	M	7	3 (0-3)	0 (0-1)	0	1 (0-4)		
		F	23	5 (0-5)	1 (0-3)			3 (0-3)	3 (3-3)
SPRING 2016									
Bass River (BRTF)	WP	M	9	3 (3-3)	1 (0-2)	0	2 (1-3)		
		F	21	5 (1-5)	1 (0-3)			1 (0-3)	5 (4-5)
Blairgowrie (BLTF)	PPB	M	3	3 (2-3)	1 (1-1)	0	6 (6-7)		
		F	27	3 (0-5)	0 (0-2)			3 (0-3)	4 (4-5)
Williamstown (Greenwich reserve) (WLTF)	PPB	M	3	3 (3-3)	1 (0-2)	0	0 (0-0)		
		F	27	2 (0-5)	0 (0-2)			1 (0-3)	5 (2-5)
Shallow Inlet (Sandy Point) (SPTF)	EXT REF	M	2	3 (2-3)	1.5 (1-2)	0	6 (6-6)		
		F	28	5 (1-5)	1 (0-3)			3 (1-3)	4 (4-5)
Anderson Inlet (Mahers Landing) (ADTF)	EXT REF	M	18	3 (2-3)	2 (1-3)	0	2 (1-5)		
		F	12	5 (4-5)	1 (0-3)			2 (0-3)	4 (1-5)
*median (range)									

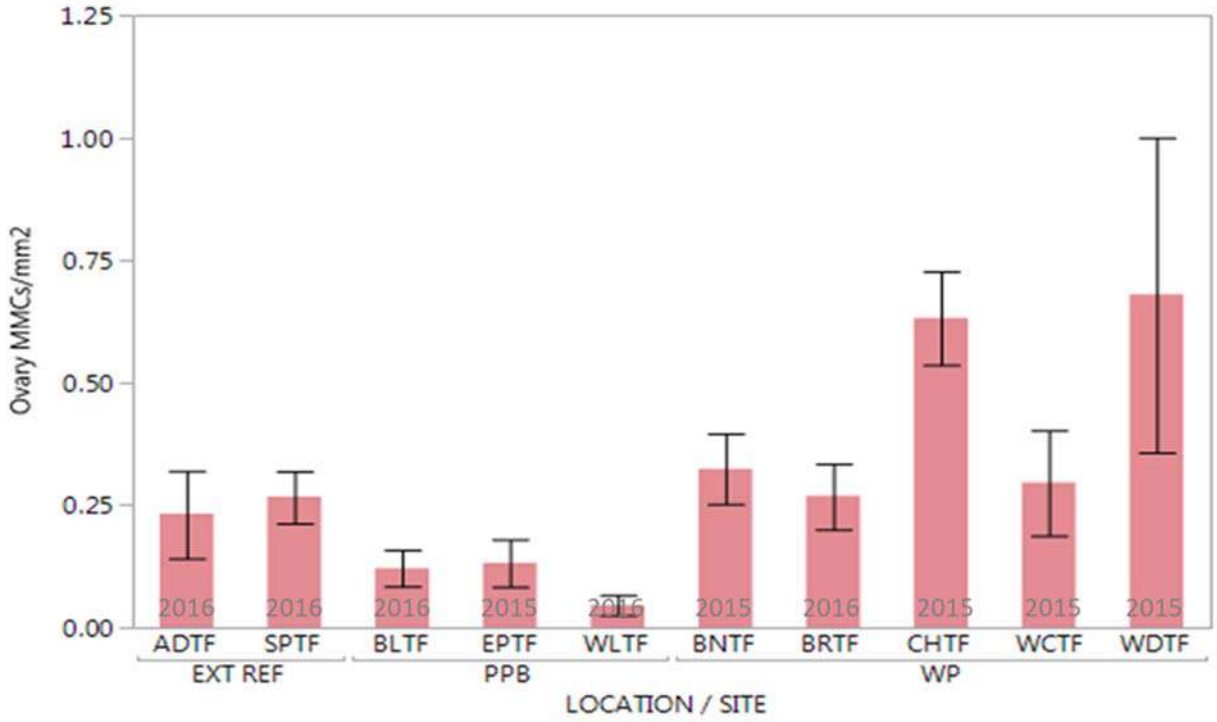

Figure 10. Mean (±SEM) number of melanomacrophage centres (MMCs) per unit area of ovary in female Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

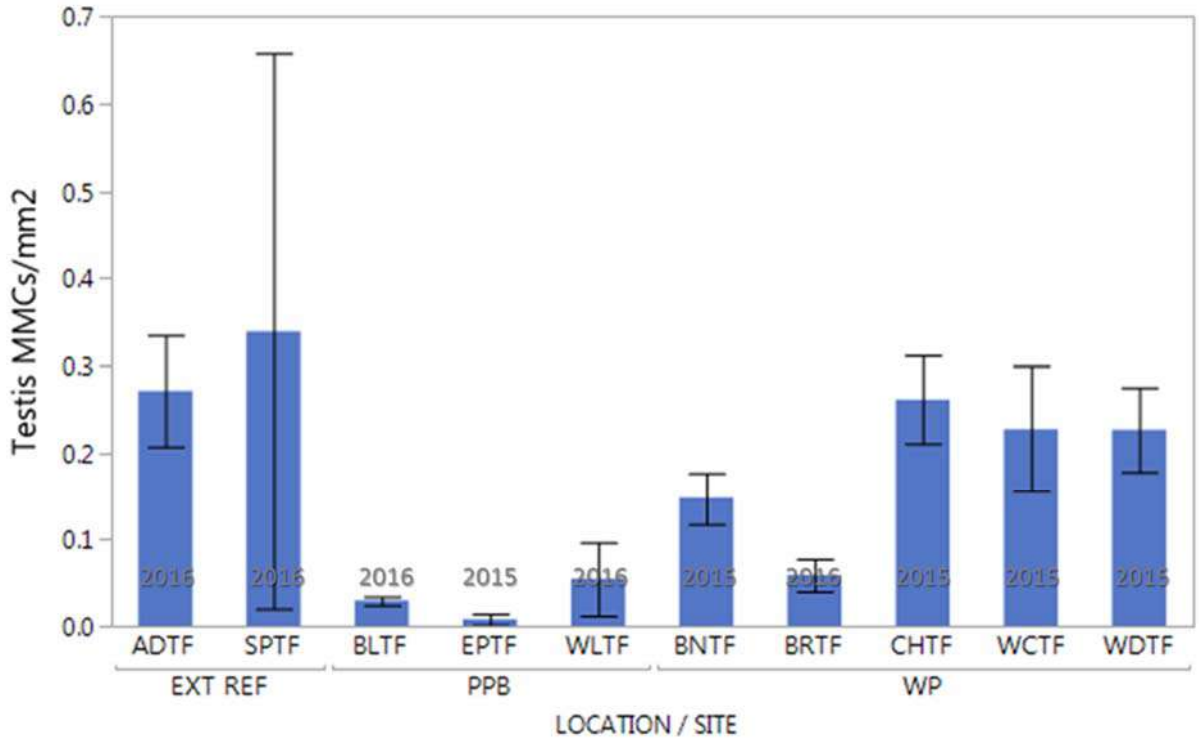

Figure 11. Mean (±SEM) number of melanomacrophage centres (MMCs) per unit area of testis in male Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

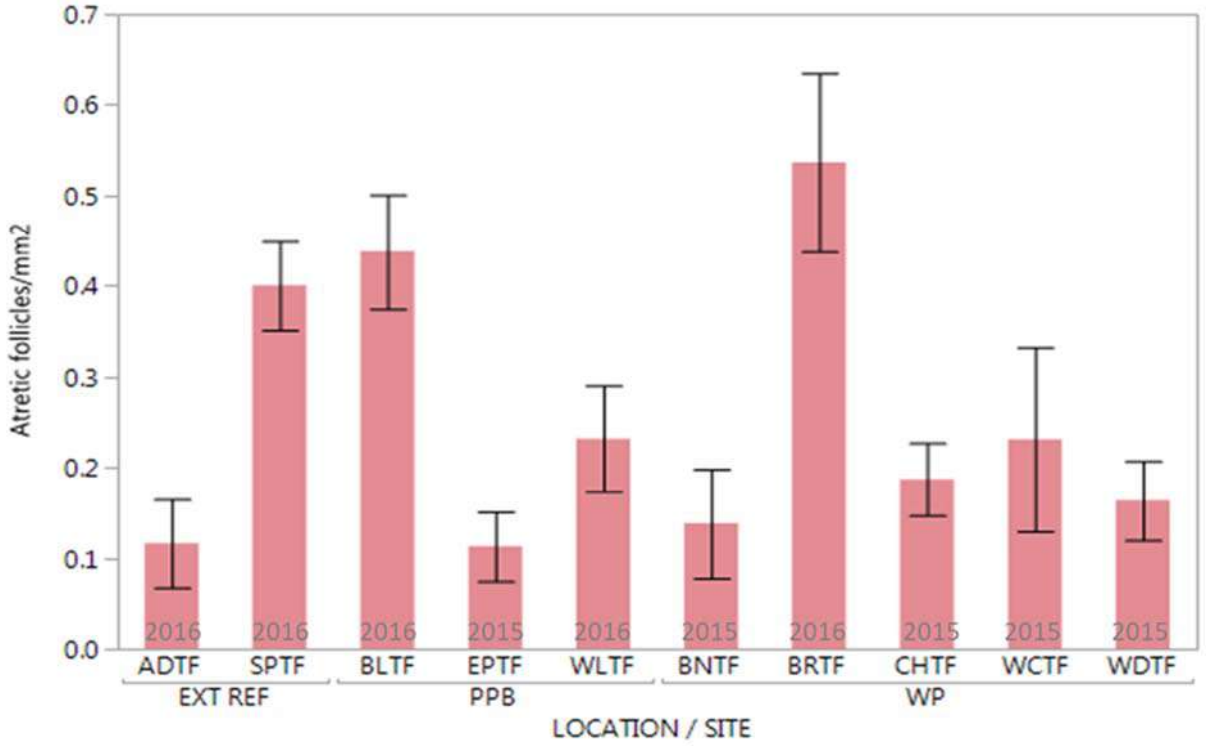

Figure 12. Mean (±SEM) number of atretic follicles per unit area of ovary in female Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

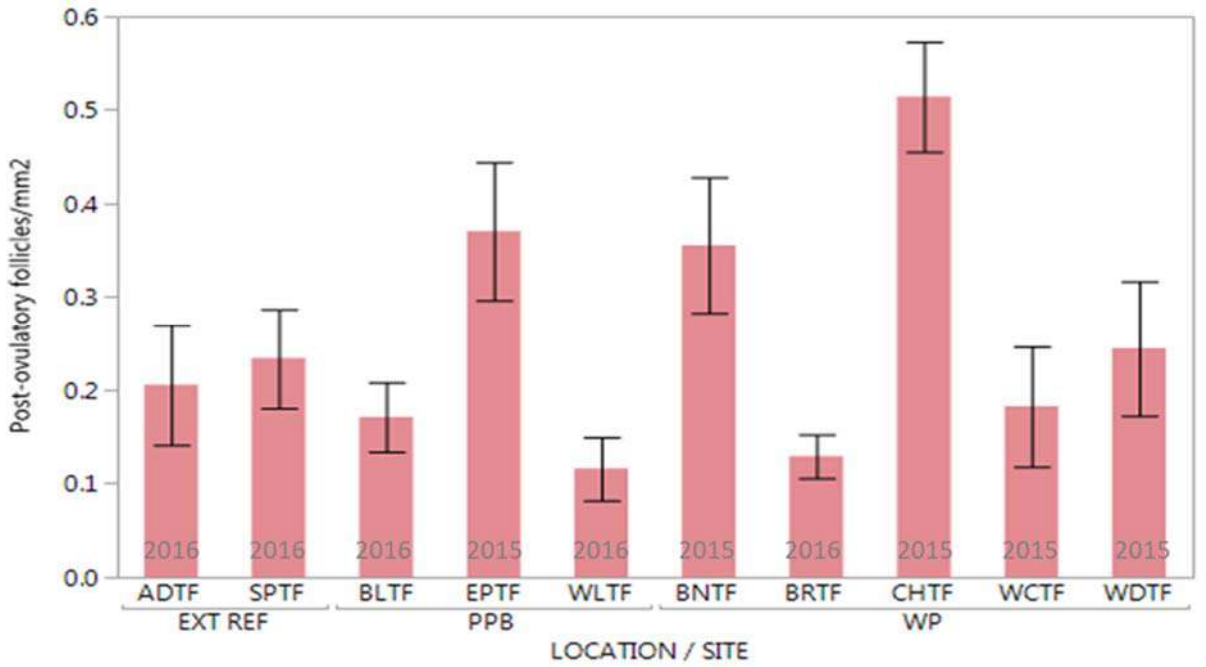

Figure 13. Mean (±SEM) number of post-ovulatory follicles per unit area of ovary in female Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

4.3.2 Liver Histology

Liver scores and macrophage aggregates

Toadfish livers showed much variation in size and colour across sites, and median liver scores ranged from 2 to 6 (Figure 14; Table 4). The liver scores are based on 4 different indicators of damage and degenerative changes, with a highest possible score of 12. Some individual fish from BRTF had scores of 11 or 12, whilst fish from other sites generally had scores ≤9 (Table 4). The general patterns for female fish were WP>PPB>EXT and for male fish were WP>EXT>PPB. Liver macrophage aggregates were observed in some fish from all sites except males from EPTF (Table 4). The values were variable but with a pattern of EXT>WP>PPB across sites, and in general male fish displayed higher values than females (Figure 15). Older and larger fish tended to have higher MMC scores than smaller/younger fish.

Liver parasites, granuloma, non-neoplastic and neoplastic changes

Parasites were observed in the livers of fish from 5 different sites and were more prevalent in females than males (Table 4). Some parasites were observed in hepatic tissue associated with blood vessels (Figure 16) and others were observed within bile ducts (Figure 17). No effort was made to identify what the specific types of parasites were. The general pattern, for both female and male fish was EXT=WP>PPB. Other changes that were commonly observed were granuloma formation and foci of cellular alteration (FCA). Granulomas form as part of an inflammatory response to foreign bodies/injury and FCAs are pre-cancerous changes. At least some fish from all sites displayed granulomas, whilst FCAs were observed in only some (Table 4). Granulomas were more prevalent in female and male fish from Western Port than other locations, and FCAs were variable across sites. One interesting observation was the presence of two ectopic oocytes associated with pancreatic tissue in the liver of a female fish from WLTF (Figure 18). The significance of this rare finding is unknown. A low number of benign and malignant tumours were observed in toadfish livers (Table 4). One male fish from WLTF had a hepatocellular adenoma (benign tumour), whilst one female from EPTF had a large hepatocellular carcinoma (malignant tumour), and one female from BLTF had a large hepatocellular carcinoma that contained adipocytes, necrotic tissue and pleomorphic cells (Figure 19). Whilst the incidence was low, the presence of neoplastic lesions (tumours) was higher in toadfish from PPB than other locations and was not related to fish size (or age) (PPB>EXT=WP).

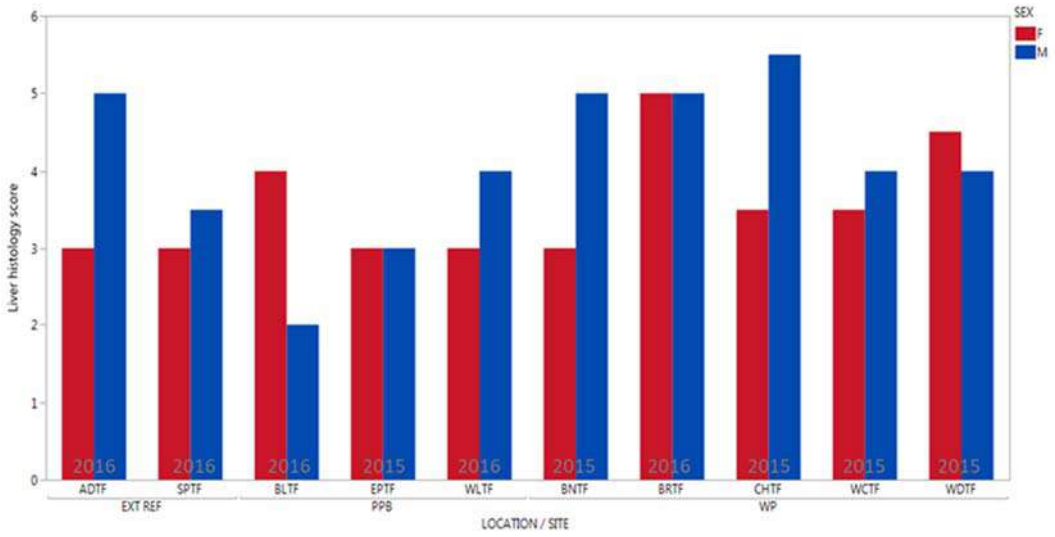

Figure 14. Median liver histology scores in female and male Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

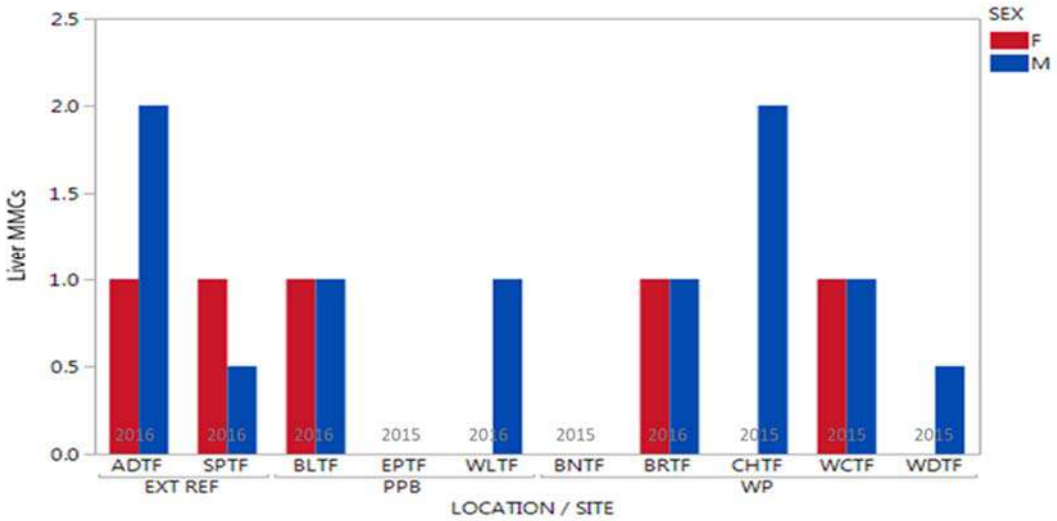

Figure 15. Median liver macrophage aggregate (MMCs) scores in female and male Smooth toadfish collected from two reference sites (EXT REF), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016.

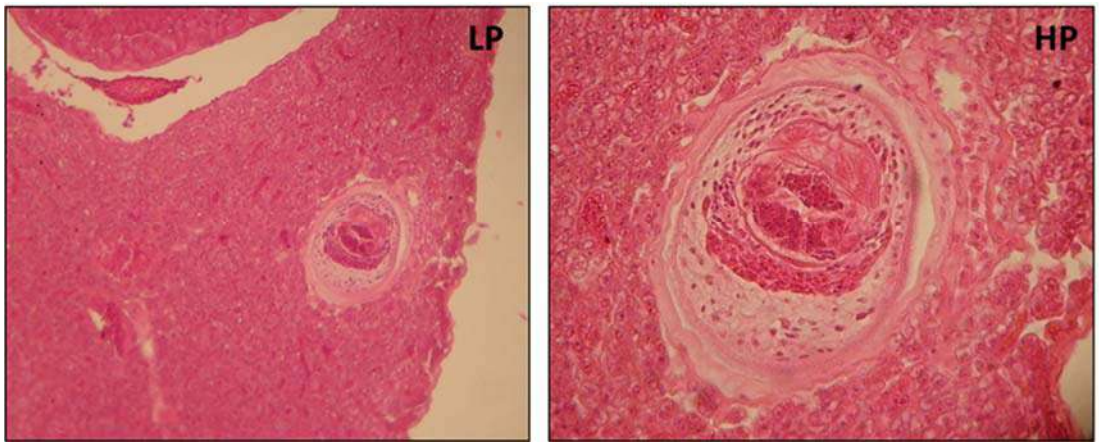

Figure 16. Low power (LP) and high power (HP) images of a smooth toadfish liver section, displaying a parasite embedded within tissue associated with a blood vessel.

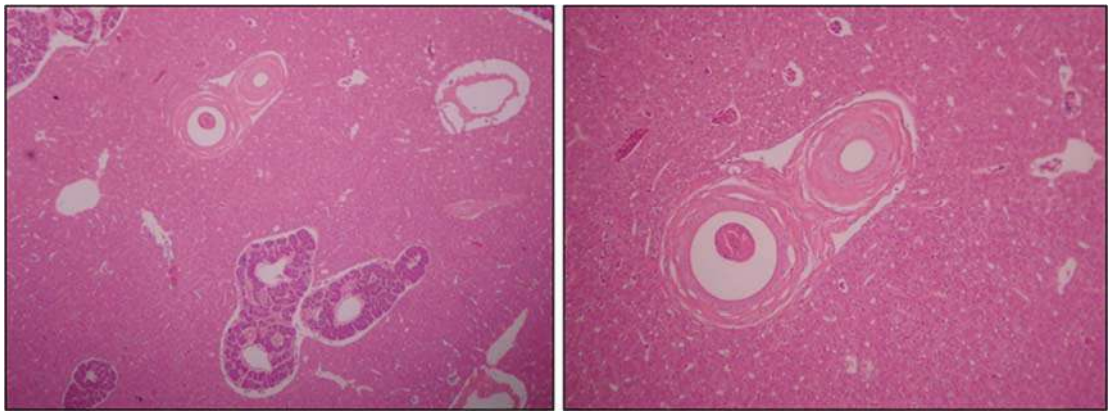

Figure 17. Low power (LP) and high power (HP) images of a smooth toadfish liver section, displaying a parasite within a bile duct.

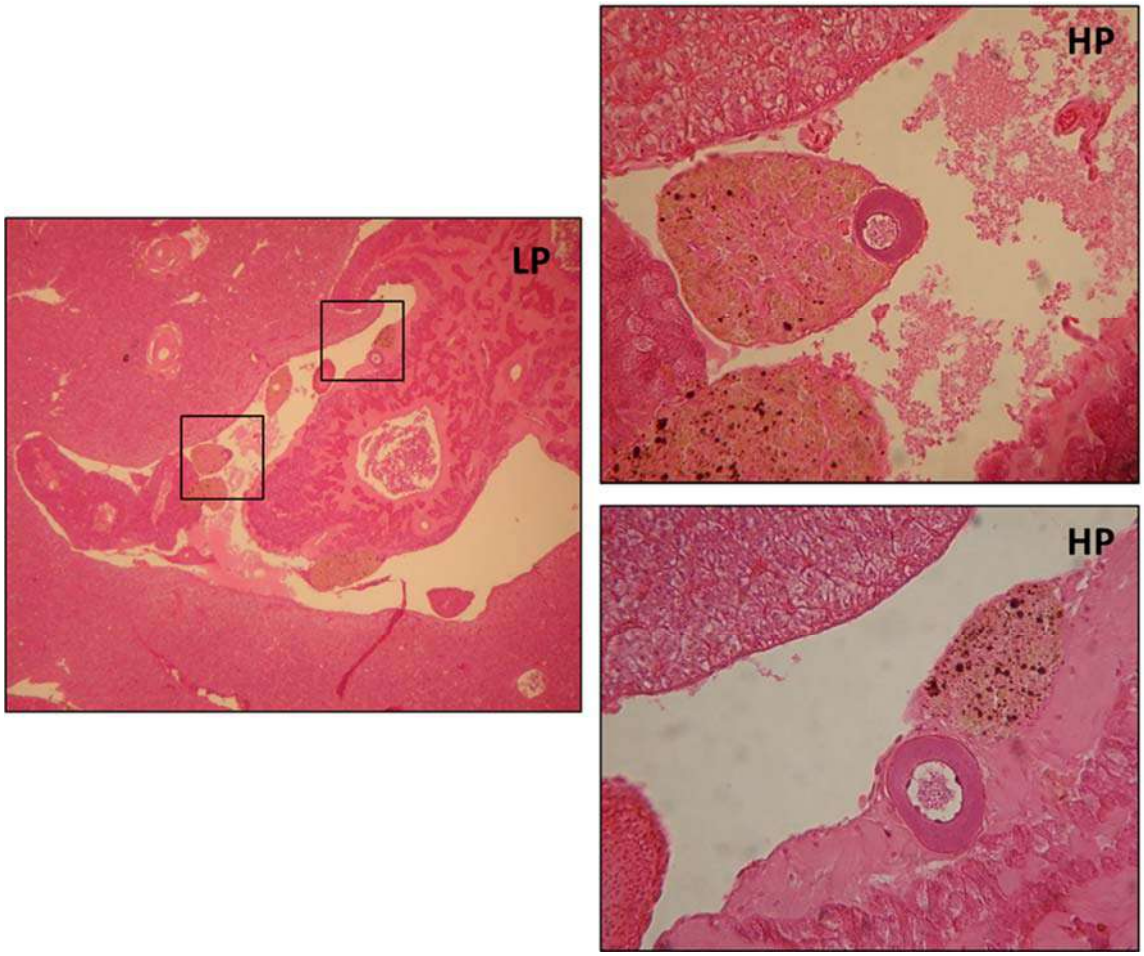

Figure 18. Low power (LP) and high power (HP) images of a smooth toadfish liver section, displaying ectopic oocytes associated with macrophage aggregates and pancreatic tissue.

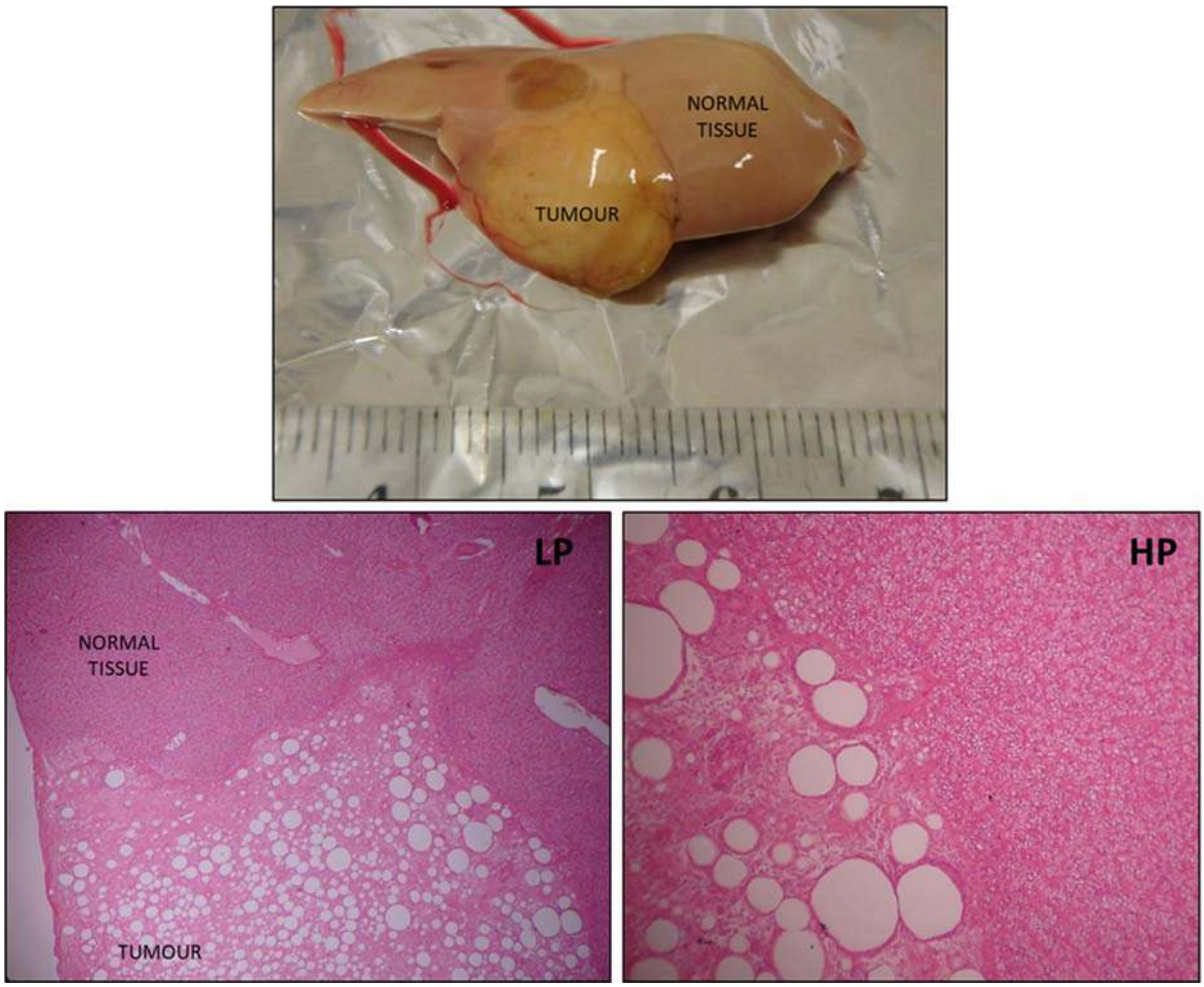

Figure 19. Smooth toadfish liver with a large hepatocellular carcinoma (malignant tumour). Low power (LP) and high power (HP) images of the liver section show that affected tissue contains adipocytes, necrotic tissue and pleomorphic cells.

Table 4. Histology scores and incidence of parasites and tissue changes in liver samples of smooth toadfish (*Tetractenos glaber*) sampled five locations within Western Port, three locations within Port Phillip and two external reference sites (EXT REF) during spring (November/December) 2015-2016. A semi-quantitative scoring system was used to categorise different histological features, where 0 = no incidence, 1 = low incidence, 2 = moderate incidence, 3 = high incidence. The overall liver score comprised 4 different markers, with the highest possible score being 12.

Site	Catchment	Sex	n	Liver Score*	Macrophage aggregates*	Parasites		Granuloma		FCA		Tumour (benign)		Tumour (malignant)	
SPRING 2015						n	%	n	%	n	%	n	%	n	%
Watsons Creek	WP	M	20	4 (1-9)	1 (0-3)	0	0	0	0	0	0	0	0	0	0
		F	10	4 (2-6)	1 (0-2)	0	0	2	20	0	0	0	0	0	0
Western Contour Drain	WP	M	24	4 (2-9)	1 (0-3)	0	0	3	12.5	0	0	0	0	0	0
		F	6	5 (3-8)	0 (0-3)	0	0	0	0	0	0	0	0	0	0
Churchill Island	WP	M	6	6 (2-8)	2 (0-3)	0	0	0	0	0	0	0	0	0	0
		F	24	4 (1-9)	0 (0-3)	3	12.5	2	8.3	3	12.5	0	0	0	0
Bunyip River	WP	M	14	5 (2-7)	0 (0-3)	0	0	2	14.3	0	0	0	0	0	0
		F	16	3 (1-6)	0 (0-1)	0	0	1	6.3	0	0	0	0	0	0
Edwards Point	PPB	M	7	3 (2-5)	0 (0-0)	0	0	3	42.9	0	0	0	0	0	0
		F	23	3 (1-6)	0 (0-2)	0	0	0	0	0	0	0	0	1	4.3
SPRING 2016															
Bass River	WP	M	9	5 (2-12)	1 (0-3)	1	11.1	2	22.2	3	33.3	0	0	0	0
		F	21	5 (1-11)	1 (0-3)	1	4.76	2	9.5	0	0	0	0	0	0
Blairgowrie	PPB	M	3	2 (2-5)	1 (0-1)	0	0	0	0	1	33.3	0	0	0	0
		F	27	4 (2-7)	1 (0-3)	0	0	1	3.7	8	29.6	0	0	1	3.7
Williamstown (Greenwich reserve)	PPB	M	3	4 (4-8)	1 (0-1)	0	0	0	0	1	33.3	1	33.3	0	0
		F	27	3 (1-6)	0 (0-1)	1	3.7	2	7.4	0	0	0	0	0	0
Shallow Inlet (Sandy Point)	EXT REF	M	2	3.5 (2-5)	0.5 (0-1)	0	0	0	0	1	50	0	0	0	0
		F	28	3 (0-7)	1 (0-3)	3	10.7	3	10.7	1	3.6	0	0	0	0
Anderson Inlet (Mahers Landing)	EXT REF	M	18	5 (3-9)	2 (1-3)	1	5.56	1	5.6	5	27.8	0	0	0	0
		F	12	3 (1-6)	1 (0-2)	1	8.33	2	8.3	0	0	0	0	0	0
*median (range)															

4.4 Summary of Biological Indicators

For each of the different indicators measured in smooth toadfish in this study, a rank was given to each site from lowest (1) to highest (10), then the ranks were grouped by location. The resultant table shows how each location (External Reference, EXT; Port Philip, PPB; Western Port, WP) ranked (Table 5). For many indicators, WP fish (male and female) showed the lowest ranks, indicating lower scores and therefore, for example lower levels of liver enzyme induction, and lower histological changes in the gonads and liver. However, there was a lot of variability in these ranks indicating no clear and consistent patterns between sites or locations. When all indicators are combined, the overall ranks for sites was EXT=PPB>WP for female toadfish and EXT>WP>PPB for male toadfish (Table 5).

Table 5. Summary of all physiological and histological endpoints measured in smooth toadfish sampled from two reference sites (EXT), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016. Values for each indicator were ranked from highest to lowest score for each site, then grouped by location. Indicators with (*) sampled only in 2016.

INDICATOR	FEMALE	MALE
Total Length (mm)	EXT>PPB>WP	EXT>PPB>WP
Weight (g)	EXT>PPB>WP	EXT>PPB>WP
GSI (%)	EXT=PPB>WP	EXT=PPB=WP
LSI (%)	EXT=PPB>WP	PPB>WP>EXT
CF (%)	EXT>PPB>WP	PPB>EXT>WP
External Parasites	PPB>EXT>WP	WP>EXT>PPB
Skin Lesions and Scarring	WP>PPB>EXT	WP>EXT>PPB
Liver Glycogen*	EXT>PPB>WP	EXT>WP>PPB
Liver Lipid*	EXT=PPB>WP	PPB>EXT>WP
Liver EROD	PPB>EXT>WP	PPB>EXT>WP
Liver LPO-MDA*	PPB>EXT>WP	EXT>PPB>WP
Liver Catalase*	PPB>EXT>WP	PPB>EXT>WP
Macrophage Aggregates	WP>EXT>PPB	EXT>WP>PPB
Testis-Ova		EXT=PPB=WP
Male Gonadal Changes		EXT=PPB=WP
Atresia	EXT=PPB=WP	
Female Gonadal Changes	EXT=PPB>WP	
Liver Score	WP>PPB>EXT	WP>EXT>PPB
Macrophage Aggregates	EXT>WP>PPB	EXT>WP>PPB
Parasites	EXT=WP>PPB	EXT=WP>PPB
Granuloma	WP>EXT>PPB	WP>PPB>EXT
Foci of Cellular Alteration (FCA)	PPB>WP>EXT	EXT>PPB>WP
Tumour (benign)	EXT=PPB=WP	PPB>EXT=WP
Tumour (malignant)	PPB>EXT=WP	EXT=PPB=WP
OVERALL	EXT=PPB>WP	EXT>WP>PPB

5 Population Genetics

5.1 Patterns of genetic diversity and population structure

Levels and patterns of genetic diversity (allelic richness, the percentage polymorphic loci and expected heterozygosity) did not vary significantly among sites (Table 6). Observed levels of heterozygosity were generally lower than expected resulting in small heterozygous deficits, however, none of the inbreeding co-efficients were significantly different from zero (Table 6).

Overall estimates of genetic differentiation based on F_{ST} were small and not significantly different from zero (Global F_{ST} = 0.0007, 95% CI = -0.003 to 0.0047). Estimates of pairwise F_{ST} between all population pairs revealed no significant differentiation between any of the sampled populations in this study. This lack of any significant structure was further confirmed from the DAPC analysis which showed significant overlap of most samples from the different populations, although samples from Churchill Island did appear to cluster separately from the rest (Figure 20).

These results indicate low levels of genetic structuring between populations, and therefore indicates strong mixing of individuals and gene flow between populations. This finding may have implications for pollution assessments with toadfish, because if the fish are moving around between sites, their 'pollution signatures' would not be distinct at different sites. However, if the dispersal and genetic mixing is occurring during the early life stages (which would seem more likely for this species), then site-specific pollution impacts may still be occurring. Assessment of contaminant levels within fish tissue would be the most appropriate way to determine this.

Table 6. Samples sizes and estimates of genetic diversity of toadfish (*Tetractenos glaber*) sampled from two reference sites (EXT), three sites within Port Phillip (PPB) and five sites within Western Port (WP) in spring 2015 and spring 2016. *N* = number of samples, AR = Allelic richness, % PI = percentage polymorphic loci, H_E = expected heterozygosity, H_O = Observed heterozygosity, F_{IS} = inbreeding coefficient.

Population	N	AR	% PI	H_E	H_O	F_{IS}
Anderson's Inlet	30	1.901	0.992	0.296	0.276	0.018
Blairgowrie	29	1.853	0.991	0.310	0.274	0.014
Bunyip River	20	1.913	0.978	0.286	0.276	0.010
Bass River	30	1.940	0.994	0.285	0.277	0.020
Churchill Island	21	1.921	0.980	0.286	0.279	0.011
Edward's Point	19	1.893	0.974	0.292	0.277	0.007
Sandy Point	30	1.777	0.991	0.340	0.275	0.015
Watsons Creek	23	1.894	0.983	0.294	0.276	0.011
Western Contour Drain	21	1.736	0.977	0.347	0.275	0.006
Williamstown	29	1.913	0.993	0.294	0.276	0.021
Average	***25.2***	***1.874***	***0.985***	***0.303***	***0.276***	***0.013***

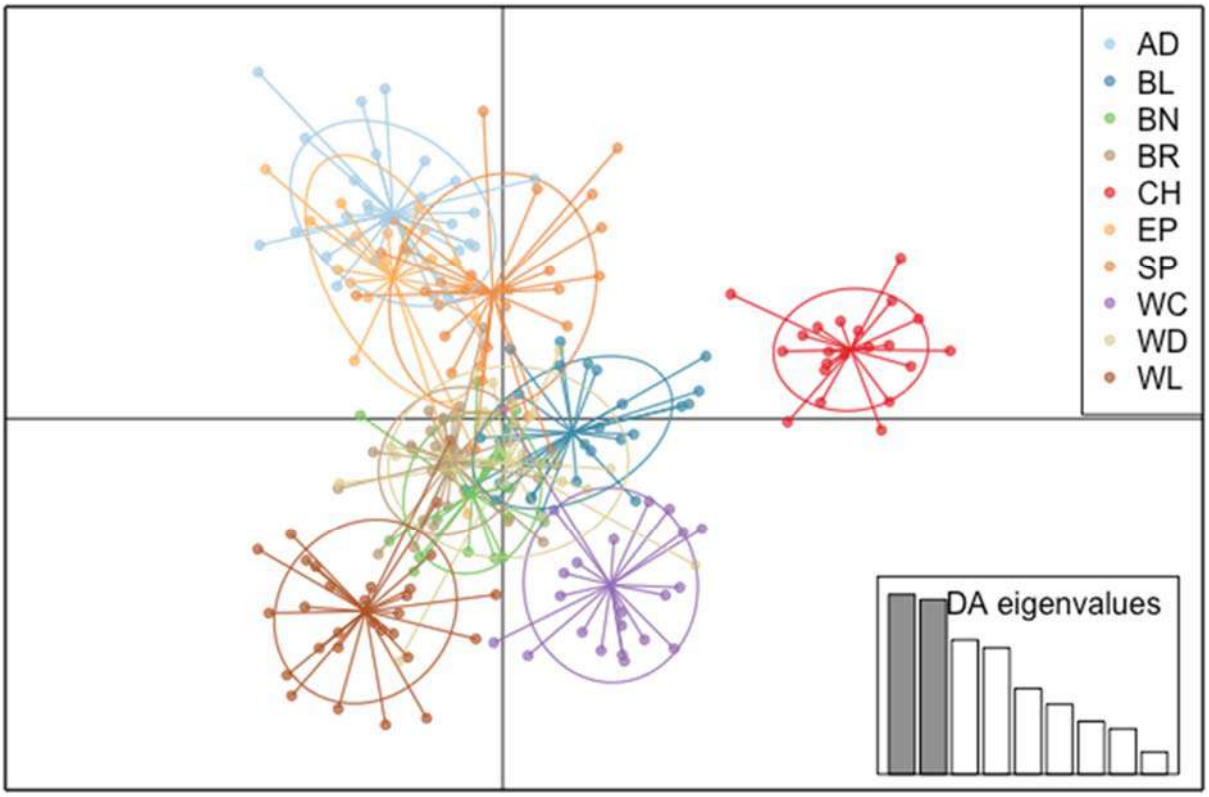

Figure 20. A discriminant analysis of principal components (PC's = 150) to identify genetic clusters. AD - Anderson's Inlet; BL – Blairgowrie; BN - Bunyip River; BR- Bass River; CH - Churchill Island; EP - Edward's Point; SP - Sandy Point; WC - Watsons Creek; WD - Western Contour Drain; WL – Williamstown.

6 Discussion

Across all indicators that were measured in this study, there was no one site that consistently showed the highest level of impact. In general, fish from the external reference sites tended to be ranked most highly (most impacted), but it was variable between indicators. The sex ratios for fish collected in 2015 were similar (52.7% female), whilst in 2016 there was a strong bias towards females, with an overall sex ratio of 76.7% female. The values varied substantially between sites, without any obvious patterns except that for toadfish collected from PPB sites, there was a consistent bias towards more females than males, regardless of site or year of collection. In 2016, mean fish sizes were larger than 2015, and there were differences between sites. This difference is a challenge, and not one that could be easily managed, since we had to take what fish we could catch. Sex ratios cannot be easily controlled for in smooth toadfish, since the species is not sexually dimorphic and therefore gender cannot be determined until dissection.

6.1 External parasites and condition indices

In some locations, greater than 80% of fish were infected with external parasites (such as anchorworm, *Lernaea* sp.), but overall the incidence, across all sites was relatively low (17.3%). Incidence rates were higher in fish collected in 2016 than 2015, whilst in contrast, skin lesions and scarring was more prevalent in 2015, with rates at some locations being up to 57.6%, but again, the overall incidence across all sites was low 14.0%. Anchorworm is a copepod parasite that infects freshwater and estuarine fishes, and infections are more commonly observed in warmer months (i.e. spring, summer) in stagnant or slow-moving water bodies (Noga, 2010). Little is known about the specific anchorworm species that occur in Australian waterways, or specific triggers for why outbreaks occur, but the results of infection are usually the same: chronic infection can lead to poor growth and reduced body condition, as well as increased likelihood of secondary infections (bacterial and fungal) developing. There was no correlation between lesion and parasite rates and body condition in the toadfish in this study.

There were significant differences in tissue condition indices: GSI, HSI and CF, indicating differences in energy allocation of fish sampled from each field site. These are general indicators that are influenced by factors such as food availability, nutrient enrichment, reproductive state and disease status, in addition to toxicant exposure. For example, fish populations impacted by nutrient enrichment showed elevated levels of these general indices relative to reference fish populations in Canadian rivers (Barrett and Munkittrick, 2010), whilst Mat Piah and Bucher (2014) observed a strong inverse relationship between HSI and GSI in toadfish sampled from the Richmond Estuary, NSW, which was attributed to seasonal changes in liver lipid content related to gonad maturation and spawning.

To understand the importance of diet in determining energy status in toadfish, we need a better understanding of food sources at the different field sites – this is likely to be quite variable, given how different the waterways are. For example, some collection sites were sandy, beach sites with no mangroves and low levels of suspended solids (BLTF, EPTF, ADTF, SPTF), whilst others were muddy, very tidal estuarine sites with mangroves and high levels of suspended solids (WLTF, CHTF, WCTF, WDTF, BRTF, BNTF). These differences are likely to influence what type of prey items the toadfish would be feeding on, as well as the types of contaminants they are exposed to (i.e. fish from sandy, beach sites might be more exposed to waterborne contaminants, whilst fish from muddy, tidal estuaries might be more exposed to sediment-bound contaminants). Measuring contaminant concentrations in the remaining tissue and carcasses of all of the toadfish used in the study would help establish site-based differences in contaminant exposure.

6.2 Energy allocation and biomarkers

Energy allocation was assessed through measuring differences in liver glycogen and lipid content between sites, and the profiles were variable both across, and within sites, without any clear patterns. No correlations were observed between fish size or GSI with energy markers, whilst a

weak correlation was seen between condition factor and lipid content (r^2 = 0.193) and a stronger correlation was observed between HSI and lipid content (r^2=0.381).

Total lipid and glycogen concentrations in female fish from BRTF were lower than levels in fish from all the other sites, as was EROD activity. These fish also displayed correspondingly low HSI values. Together these results suggest that fish from BRTF have a reduced energy capacity, which may explain why EROD activity was lower at this site compared to the other sites as chemical detoxification and/or metabolism is energy demanding and as these fish had low energy resources, there was no additional energy available for detoxification. Catalase activity was also lower at BRTF compared to the reference sites, but the difference wasn't statistically significant. Organisms respond to contaminants by increasing their capacity to metabolise these compounds so that they can be excreted (Van der Oost et al., 2003). Changes in EROD activity demonstrates that organisms are responding to contaminants by either inducing (increasing) or inhibiting (reducing) cytochrome P450 (which is a family of enzymes that can detoxify contaminants). Interestingly, in this study we also observed that female fish seemed to be more sensitive than males in the biological responses between sites. This has also been observed in previous studies (Edge et al., 2013 and Vu et al., 2016) where females were more sensitive than males to exposure to pollutants in some biological responses. Similarly, Vu et al. (2016) also found that reduced energy reserves in female amphipods following fungicide exposure was strongly correlated to growth with negative effects on both responses compared to controls in a laboratory exposure to boscalid, a fungicide found in estuarine environments around Victoria.

Our study also showed that EROD activity, glycogen and lipid content were more sensitive indicators in toadfish compared to catalase and lipid peroxidation (LPO). Therefore, we would recommend measuring these responses in future studies assessing fish health as they are cost effective and sensitive biomarkers.

In a similar study of toadfish health done in Sydney Harbour, Edge et al. (2013) reported site specific differences in tissue biomarker levels (EROD, GST and lipid peroxidation), as well as differences in concentrations of PAH metabolites in fish bile. They did not assess livers for the presence of tumours, however they did assess gonads, and reported distinct reproductive effects in female ovaries due to exposure to a dioxin contamination gradient. In that study, the contaminant source was well defined and showed a distinct gradient of reduced severity with increasing distance from the source. In the present study, there is no such defined contamination gradient. Rather, several toxicants have been detected in water and sediment samples collected as part of the Western Port Toxicants Study (Sharp et al., 2013; Myers et al., 2014; Melbourne Water, 2018). This includes herbicides (simazine, prometryn, linuron and metolachlor), fungicides (boscalid, oxadixyl, azoxystrobin, cyprodinil, tebuconazole, iprodione) and insecticides (pirimicarb, fenamiphos, malathion, bifenthrin, DDT) which were detected at trace levels. Contaminants were found to be most concentrated in the upper estuary and freshwater reaches, rather than the marine areas. The lack of any strong site-specific impacts in toadfish from this study may indicate that this species is pollution tolerant or that we require more sensitive bioindicators, or that the pollution within the sampled (marine) areas of Western Port and the external locations is low and fairly homogenous and evenly distributed. Contaminant analysis of all remaining toadfish tissues would be useful to determine the extent of contamination with legacy, bioaccumulative toxicants such as DDT and dieldrin, which have been detected in sediments or water samples from different locations around Western Port. This knowledge will help us interpret the findings of the present study (i.e. carcinogens) and will allow us to determine the levels of bioaccumulative substances in some of these fish which are up to 20 years old (and those collected from 2016 potentially even older), to potentially reconstruct exposure histories.

Genetics analysis shows low heterozygosity and good mixing between toadfish populations, with little genetic structuring. This may help explain why no strong site differences were observed in any indicators. However, this finding is in contrast to what was expected. Other studies have used acoustic tags to monitor movement in two estuarine pufferfish in New South Wales and concluded

that both species examined showed site fidelity and limited movement within a series of tidal channels (Mat Piah, 2011). An alternative explanation for the lack of genetic structuring between toadfish populations may be that dispersal and genetic mixing is occurring during the early life stages (which would seem more likely for this species), in which case site-specific pollution impacts may still be occurring. Assessment of contaminant levels within fish tissue would be the most appropriate way to determine this.

6.3 Gonad Histology

Fish from all locations were sexually mature and showed indications of recent spawning activity. All fish were sampled in the same season (Nov 2015 or Nov/Dec 2016) to minimise differences in seasonal reproductive maturity, and as much as possible fish of similar size were collected. Histological analysis of female gonads showed varying rates of oocyte atresia and female gonad scores between sites, but overall the gonad scores were low (highest score 5, of a possible 12). Histological scores are based on cumulative adverse changes, so low scores indicate 'healthier' fish tissues than high scores. Fish from Western Port showed higher numbers of MMCs/mm ovarian area compared to other sites, whilst the number of atretic follicles and post-ovulatory follicles was variable between sites. Male fish showed higher levels of gonad changes than females, with individual scores of up to 8 (of a possible 12) observed in some fish from Western Port. Median male gonad scores across all sites were highest in BLTF (PPB) and SPTF (EXT REF) fish. No male fish displayed any testis-ova and MMCs/mm testis area varied between sites. Macrophage aggregates (MMCs) are often associated with ageing, and gonad MMCs was correlated with total length in males (r^2=0.384), and to a less extend in females (r^2=0.206). Overall, some gonadal changes were observed in both female and male toadfish, however no fish showed any evidence of reproductive dysfunction or abnormal cell growth.

6.4 Liver Histology

The size, colour and general appearance of smooth toadfish livers was quite variable between fish from all sites. The overall presence of liver parasites was low, at just 3.67% across the entire population of smooth toadfish sampled, and similarly, the presence of granuloma was only 8.67%. Higher median liver scores were observed in larger fish, as expected, since the liver score incorporates the macrophage aggregate score, which is known to increase with size/age. However, fish size (total length) was not correlated with any other indicators.

A small number of fish were observed to have pre-cancerous (pre-neoplastic), or cancerous lesions in their livers, with overall presence of pre-cancerous changes in livers of 7.67% and just 1.0% for cancerous changes, including both benign and malignant tumours. In both sampling rounds the rates of pre-cancerous foci of cellular alteration (FCA) were low at each site (<35%, or 1-8 individuals), although if separated by sex, the incidence was as high as 50% (1 of 2 fish, due to low numbers of male fish at that location). Liver tumours, in flatfish such as flounder have been classified as direct indicators of chemical exposure, and as such histological assessment of livers has been widely used in marine environmental monitoring programmes worldwide (Cefas, 2007; Stentiford et al., 2009). FCAs have been described as 'transitional lesions', or pre-cancerous changes which are early indicators of hepatic neoplasia (tumours) (Stentiford et al., 2003). In that study, the authors reported incidence rates of up to 43.3% in fishes from four different British estuaries. In different toadfish populations, FCA incidence rates of up to 35% were reported, with the highest levels occurring in fish from BLTF (both sexes) and ADTF (males only). Across the entire toadfish population sampled during this study, FCA were only observed in 23 out of 300 fish (7.67%). Of those fish, 73.9% were female and 26.1% were male, and there was no correlation with size. Of greater concern however, was that some fish had benign or malignant liver tumours, and all of them were from PPB sites (EPTF, BLTF, WLTF). These fish were not the largest (or oldest) fish collected during the study. Whilst the overall levels of pre-cancerous and cancerous liver changes are low, they were observed in toadfish from multiple sites, especially PPB. Further investigation is required to determine if these levels represent 'natural' baseline levels or if they are the result of exposure to carcinogenic contaminants at some point during their lifetime.

Fish from the Bass River (WP) were the most distinct population of fish collected during the study. sites. BRTF fish were generally small, but not the smallest fish of all sites sampled. They had the lowest HSI values and the lowest EROD values, especially females. However, BRTF fish were also the highest ranked (most affected) for external lesions and parasites, and liver histology scores. The liver scores at this site were the highest for the study, due to the presence of parasites, granuloma and foci of cellular alteration. Yet no fish from BRTF displayed benign or malignant liver tumours. In addition to the high liver histology scores, some BRTF fish also showed high gonad histology scores. There appeared to be a sex-specific difference in gonad scores, with males showing low scores whilst females had high scores, mostly due to the presence of lots of atretic oocytes. Overall these findings indicate that environmental conditions within the Bass River are impacting resident fish (relative to fish from other sites), and a more detailed assessment of this particular river catchment is advised. In particular, further sampling to capture any seasonal differences would be worthwhile. Furthermore, gaining a better understanding of land use in this catchment (i.e. dairying and other agriculture) would be valuable for identifying the types of contaminants that are likely to be driving these changes in the resident fish. Toadfish from Western Contour Drain (WP) displayed a similar pattern of changes as BRTF, with WDTF fish also having low CF, HSI and EROD values. Unfortunately, the livers of fish from this site were not measured for lipid and glycogen content.

6.5 Genetics Analysis

Overall there is little genetic structure among the sampled locations based on SNPs. This suggests these sites are connected by high levels of gene flow for this species. Levels of genetic diversity appear high and consistent between sites, indicating that none of the sample sites are suffering from reduced population sizes or diversity. This is confirmed by the inbreeding co-efficient estimates that indicate no evidence of inbreeding in any of the locations sampled. As mentioned previously, this finding may help explain why no strong site differences were observed in any indicators, yet it is in contrast to what was expected. An alternative explanation for the lack of genetic structuring between toadfish populations may be that dispersal and genetic mixing is occurring during the early life stages and that site-specific pollution impacts may still be occurring. Assessment of contaminant levels within fish tissue would be the most appropriate way to determine this.

6.6 Major Findings

Smooth toadfish from all locations showed some indications of environmental stress. Fish collected from different sites within Western Port displayed some differences in physiological and histological endpoints compared to toadfish collected from Port Phillip and two external reference sites (Andersons and Shallow Inlets). For many indicators, WP fish (male and female) showed the lowest ranks, indicating lower scores and therefore lower levels of liver enzyme induction, and lower histological changes in the gonads and liver. However, there was much variability indicating no clear and consistent patterns between sites or locations.
Furthermore, between sites the order (rank) from most impacted to least impacted was not consistent, indicating that no one particular site was most affected, but rather fish from all 10 collection sites displayed some changes indicative of environmental stress.

- In general, fish from the external reference sites tended to be ranked most highly (most impacted), but it was variable between indicators, suggesting that fish from all sites displayed some changes indicative of environmental stress.

- When all indicators are combined, the overall ranks for sites (most impacted to least impacted) were EXT=PPB>WP for female toadfish and EXT>WP>PPB for male toadfish.

- In some locations, more than 80% of fish were infected with external parasites (such as anchorworm, *Lernaea sp*.), but overall the incidence, across all sites was relatively low (22.0%;66/300). Incidence rates were higher in fish collected in 2016 than 2015, whilst in contrast, skin lesions and scarring was more prevalent in 2015. There was no correlation between lesion and parasite rates and body condition in the toadfish in this study.

- There were significant differences in tissue condition indices (GSI, HSI and CF) between fish from different field sites. These are general indicators that are influenced by factors such as food availability, nutrient enrichment, reproductive state, disease status and toxicant exposure, and all of these factors may influence energy allocation.

- Energy allocation was assessed through measuring differences in liver glycogen and lipid content between sites, and the profiles were variable both across and within sites. No correlations were observed between fish size or GSI with energy markers, whilst a weak correlation was seen between condition factor and lipid content (r^2 = 0.193) and a stronger correlation was observed between HSI and lipid content (r^2 = 0.381).

- Total lipid and glycogen concentrations in female fish from Bass River were lower than levels in fish from all the other sites, indicating a reduced energy capacity. These fish also displayed correspondingly low HSI values, as well as low EROD and catalase activity levels.

- In this study EROD activity, glycogen and lipid content were more sensitive indicators compared to catalase activity and lipid peroxidation (LPO). Therefore, we would recommend measuring these responses in future studies assessing toadfish health as they are cost effective and sensitive biomarkers.

- Fish from the Bass River were the highest ranked (most affected) for external lesions and parasites, and liver histology scores. The liver scores at this site were the highest for the study, due to the presence of parasites, granuloma and foci of cellular alteration. Yet no fish from Bass River displayed benign or malignant liver tumours.

- There was no evidence of endocrine disruption-related gonadal changes (testis-ova, oocyte atresia) in fish from any sites, whilst there were some differences in female-specific and male-specific gonadal changes between sites.

- Female fish from Western Port showed higher numbers of gonad macrophage aggregates (MMCs) compared to other sites, whilst the number of atretic follicles and post-ovulatory follicles was variable between sites. MMCs are often associated with ageing, and gonad MMCs numbers were correlated with total length in males (r^2=0.384), and to a lesser extent in females (r^2=0.206).

- Male fish showed higher levels of gonad changes than females, with individual scores of up to 8 (of a possible 12) observed in some fish from Western Port. Median male gonad scores were highest (6) for fish from Blairgowrie (PPB) and Shallow Inlet (EXT).

- The size, colour and general appearance of smooth toadfish livers was quite variable between fish from all sites. The overall presence of liver parasites was low, at just 3.67% (11/300) across the entire population of smooth toadfish sampled, and similarly, the presence of granuloma was only 8.67% (26/300).

- One interesting observation was the presence of two ectopic oocytes associated with pancreatic tissue in the liver of a female fish from Williamstown (PPB). The significance of this rare finding is unknown.

- Across all toadfish sampled, a small number of fish were observed to have pre-cancerous (pre-neoplastic), or cancerous lesions in their livers. The overall presence of pre-cancerous changes of 7.67% (23/300) and just 1.0% (3/300) for cancerous changes, including both benign and malignant tumours.

- Three fish displayed benign or malignant liver tumours, and all of them were from PPB sites (Edwards Point, Blairgowrie and Williamstown). These fish were not the largest (or oldest) fish collected during the study.

- Whilst the overall levels of pre-cancerous and cancerous liver changes are low, they were observed in toadfish from multiple sites, especially PPB. This is concerning, since it indicates the fish have been exposed to carcinogenic contaminants at some point during their lifetime.

- Low levels of genetic structuring were observed between populations (indicating good mixing and high gene flow).

6.7 Recommendations

Based on the findings of this study there are several recommendations for further investigations to better understand the potential impacts of toxicants on fish health (in Western Port and other bays and estuaries):

1. Contaminant analysis of remaining carcass and liver samples to see if there has been any bioaccumulation of toxicants;
2. Have all remaining otoliths aged – this will assist interpretation of other findings, and may be useful for determining how long the fish may have been exposed to persistent bioaccumulative toxicants;
3. Further investigation into liver tumours in fish from Port Phillip sites;
4. Further investigation into the Bass River catchment to better understand why fish from this location showed higher levels of impact for some indicators than toadfish from other collection sites;
5. Focus future biomarker work with toadfish on EROD activity, glycogen and lipid content, which were more sensitive indicators compared to catalase and lipid peroxidation (LPO).
6. Further investigation into the importance of diet in determining energy status in toadfish, through a better understanding of food sources at the different field sites.
7. Thorough site characterisation to identify differences in substrate type (muddy or sandy), types of aquatic vegetation (i.e. seagrass and mangroves), tidal influence and levels of suspended solids, which would assist in understanding differences in food sources at different sites.
8. Catchment mapping and more detailed chemical analysis at each site to better characterise the differences in land use between these sites, which would drive differences in the types of contaminants present.
9. Examine the health of fish collected from upper estuary sites, since it is in these areas that toxicant concentrations have been found to be highest (Melbourne Water, 2018).

Priority 1 and 2: Contaminant analysis of remaining carcass and liver samples; have all remaining otoliths aged:

Measuring contaminant concentrations in the remaining tissue and carcasses of all of the toadfish used in the study will help establish if there are any site-based differences in contaminant exposure. This will also help clarify if the high levels of genetic mixing are due to dispersal in the early life stages, or dispersal as adults (i.e. strong site-based differences would be expected if dispersal was during early life stages only; weak site-based differences would be expected if dispersal occurs in adults). Other studies have observed accumulation of environmental contaminants in toadfish tissues, including metals (Alquezar et al., 2006) and dieldrin (Mat Piah, 2011). The otoliths for all fish collected in 2016 have been preserved and are available for age estimation. Contaminant

analysis and age estimation could be valuable to determine the extent of contamination with legacy, bioaccumulative toxicants such as DDT and dieldrin, which have been detected in sediments or water samples from different locations around Western Port. This knowledge will help us interpret the findings of the present study (i.e. carcinogens) and will allow us to determine the levels of bioaccumulative substances in some of these fish which are up to 20 years old (and those collected from 2016 potentially even older), to potentially reconstruct exposure histories.

Priority 3: Further investigation into liver tumours in fish from Port Phillip sites:
Whilst the overall levels of pre-cancerous and cancerous liver changes are low, further investigation is required to determine if these levels represent 'natural' baseline levels or if they are the result of exposure to carcinogenic contaminants at some point during their lifetime. Assessment of other long-lived fish species from Port Phillip would be valuable to determine if the prevalence of pre-cancerous and cancerous changes is restricted to smooth toadfish or is present in other species as well.

Priority 4: Further investigation into the Bass River catchment:
Smooth toadfish from the Bass River displayed low HSI and EROD values, low energy stores but high liver scores and a high presence of lesions and parasites. This indicates the fish are affected by stressors that may be compromising health and immunity. Toxicant screening and potentially further ecological assessment (algae, invertebrates and fish) within the Bass River catchment may assist in identifying what is driving this. In particular, further sampling to capture any seasonal differences would be worthwhile.

Priority 5: Focus future biomarker work with toadfish on EROD activity, glycogen and lipid content, which were more sensitive indicators compared to catalase and lipid peroxidation (LPO).
For any future projects that utilise smooth toadfish, we recommend using EROD activity and energy allocation markers in fish health assessments.

Priority 6 and 7: Further investigation into the importance of diet in determining energy status in toadfish.
Given how different some of the waterways were that were used in this study, the available food sources across sites was likely quite variable. For example, some collection sites were sandy, beach sites with no mangroves and low levels of suspended solids (BLTF, EPTF, ADTF, SPTF), whilst others were muddy, very tidal estuarine sites with mangroves and high levels of suspended solids (WLTF, CHTF, WCTF, WDTF, BRTF, BNTF). Future work should incorporate assessments of food web interactions, since some toxicants may not have serious direct effects on fish, but may have strong indirect effects on their food sources. For example, some herbicides are considered relatively non-toxic to fish, however if they affect phytoplankton, then zooplankton and other invertebrate prey may be affected, leading to poor food availability for the fish (and reduced resilience to other environmental stressors).

Priority 8: Catchment mapping and more detailed chemical analysis at each site to better characterise the differences in land use between these sites, which would drive differences in the types of contaminants present.
Toadfish from all 10 collection sites showed some indications of environmental stress, yet no particular sites showed strong patterns of impact across all indicators. Since toadfish are a long-lived species (>20 years), it is possible that their health may be influenced by toxicant exposure that occurred a long time ago. A better understanding of current (and legacy) issues within specific catchments may assist in identifying specific contaminants that might be contributing to the impacts that were observed.

Priority 9: Examine the health of fish collected from upper estuary sites, since it is in these areas that toxicant concentrations have been found to be highest (Melbourne Water, 2018). Other studies conducted by AQUEST (formerly CAPIM), have shown that toxicant concentrations, in particular some herbicides and fungicides are highest in the upper catchments, as opposed to the marine bay areas of Western Port. Therefore, fish inhabiting the upper catchments may be at higher risk of exposure to toxicants than fish in the lower estuary/bay areas, so a study to examine the health of fish inhabiting those sites would be valuable to determine the extent of any impacts caused by toxicants.

7 References

Barrett, T. J. & Munkittrick, K. R. (2010). Seasonal reproductive patterns and recommended sampling times for sentinel fish species used in environmental effects monitoring programs in Canada. Environ Rev 18: 115-135.

Booth, D. J. & Schultz, D. (1999). Seasonal ecology, condition and reproductive patterns of the smooth toadfish *Tetractenos glaber* (Freminville) in the Hawkesbury estuarine system, Australia. Proceedings of the Linnean Society of New South Wales 121: 71-84.

Cefas (2007). Monitoring the quality of the marine environment, 2004–2005. Science series aquatic environment and monitoring report. Cefas Lowestoft 59, available at www.cefas.co.uk

Dietrich, D.R. and Krieger, H.O. (2009). Histological Analysis of Endocrine Disruptive Effects in Small Laboratory Fish. Vol. 1. New Jersey: John Wiley & Sons Inc, 19- 133.

Edge, K. J., Chapman, J. C., Larsson, M. E., Hassell, K. L. and Roach, A. C. (2013). Rapid assessment of cumulative stressors on fish populations. Final report for environmental research project no: 2007/RD/RD017. Environmental Protection Science Branch, NSW Office of Environment and Heritage, December 2013.

Goede, R. W.; Barton, B. A., Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. American Fisheries Society Symposium 1990, 8, 93-108.

Hassell, K., French, J., Boyle, R. and Pettigrove, V. (2016), WESTERN PORT TOXICANT STUDY Stage 3 – Assessment of Fish Health in Western Port. Centre for Aquatic Pollution Identification and Management, Technical Report No. 63B, University of Melbourne, Victoria, Australia.

Johnson, R., Wolf, J. and Braunbeck, T. (2009). OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads, Organisation for Economic Cooperation and Development. 96 pp.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randal (1951). "Protein measurement with the Folin phenol reagent." *Journal of Biological Chemistry* 193: 265-275.

Mat Piah, R. (2011). 'Aspects of the ecology of small estuarine pufferfish relevant to their value as biomonitors of pollution', PhD thesis, Southern Cross University, Lismore, NSW. Copyright R Mat Piah 2011.

Mat Piah, R. and Bucher, D.J. (2014). Reproductive biology of estuarine pufferfish, *Marilyna pleurosticta* and *Tetractenos hamiltoni* (Teleostei: Tetraodontidae) in northern New South Wales: implications for biomonitoring. Proceedings of the Linnean Society of New South Wales 136, 219-229.

Melbourne Water (2018) Understanding the Western Port Environment: a summary of research findings from the Western Port Environment Research Program 2011-2017 and priorities for future research. Coleman R, Bathgate R and Keough MJK (eds). Melbourne Water, Victoria.

Myers, J. M., Sharp, S., Keough, M., Cinque, K., O'Niell, C., Coleman, R., Pettigrove, V. (2014). Western Port Toxicant Study Stage 2: Monitoring and evaluation of the risk of herbicides to key habitats in Western Port. CAPIM Technical Report Draft, September 2014.

Noga, E.J. 2010. Fish Disease: Diagnosis and Treatment, second ed. Wiley-Blackwell, Ames, IA.

Sharley, D., Hassell, K., O'Brien, A., Long, S. (2013). Assessment of invertebrate communities and fish health in the agriculturally influenced Watson Creek estuary, CAPIM Technical Report #28, August 2013.

Sharp, S., Myers, J.H., Pettigrove, V. (2013). An assessment of toxicants in Western Port and major tributaries. CAPIM Technical Report #27, 80 pp.

Stentiford, G. D., Longshaw, M., Lyons, B. P., Jones, G., Green, M., Feist, S. W. (2003) Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. *Mar Environ Res* **55,** 137–159.

Stentiford, G. D., Bignell, J. P., Lyons, B. P., Feist, S. W. (2009). Site-specific disease profiles in fish and their use in environmental monitoring. *Marine Ecology Progress Series*, **381**, 1–15.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental

risk assessment: A review. Environmental Toxicology and Pharmacology, **13**, 57-149.

Van Handel E. (1985a). Rapid determination of glycogen and sugars in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**:299–301.

Van Handel E. (1985b). Rapid determination of total lipids in mosquitoes. *J. Am. Mosq. Control Assoc.* **1**:302–304.

Vu, H, T, M. J. Keough, S. M. Long, and V. J. Pettigrove (2016). Effects of the boscalid fungicide Filan® on the marine amphipod *Allorchestes compressa* at environmentally relevant concentrations. *Environmental Toxicology and Chemistry*, **35**, (5), 1130–1137.

8 Appendices

Table A1: Summary of fish health indicators used for pollution assessment by AQUEST – Part A

Bioassay	Tissue	Description	Response observed	Pollutants/Stressor Identified
General observations	Whole fish, internal organs	Thorough observation of external and internal parts of each fish to quantify lesions, parasites, discolouration or deformity.	Expect to see an INCREASE in changes to general appearance and anatomy in association with exposure to environmental stressors, trauma, injury or disease.	General biotic stress. Non-specific.
Condition factor (CF)	Whole fish	The **condition factor** is an index used to describe the relative size and weight of each fish. This information is useful for comparing different groups of fish and provides some indication of general health status. **[Total body weight (g) / Fork length (cm)3] x 100**	May see an INCREASE or DECREASE in CF in association with exposure to environmental stressors. Values need to be interpreted in context, as they will change seasonally and with nutritional status.	General biotic stress. Non-specific.
Gonadosomatic Index (GSI)	Gonads	The **GSI** is an index used to describe the gonad weight relative to the overall weight of each fish. This information is useful for comparing different groups of fish and provides some indication of reproductive status and general health condition. **[Gonad weight (g) / Total body weight (g)] x 100**	May see an INCREASE or DECREASE in GSI in association with exposure to environmental stressors, and in particular reproductive toxicants. Values need to be interpreted in context, as they will change seasonally and with age.	General biotic stress and **reproductive toxicants**.
Hepatosomatic index (HSI)	Liver (or hepatopancreas*)	The **HSI** is an index used to describe the liver weight relative to the overall weight of each fish. This information is useful for comparing different groups of fish and provides some indication of nutritional and reproductive status, as well as general health condition. **[Liver weight (g) / Total body weight (g)] x 100**	May see an INCREASE or DECREASE in HSI in association with exposure to environmental stressors and specific hepatic toxicants. Values need to be interpreted in context, as they will change seasonally and with age.	General biotic stress and **hepatic toxicants**.
Gonad histology	*Gonads*	*Histology enables assessment of changes in cellular structure that might be related to pollutant exposure, general stress, infection and ageing.*	*See descriptions of specific responses observed below.*	*General biotic stress, **reproductive toxicants**, pathogens and parasites, **endocrine disrupting chemicals**.*
Histological changes - both sexes	Gonads	**General appearance, integrity and architecture of gonadal tissue.**	Expect to see an INCREASE in abnormal gonad appearance, loss of integrity and architecture, presence of infection, disease, inflammation and fibrous tissue in association with exposure to environmental stressors.	General biotic stress. Non-specific.
Histological changes - both sexes	Gonads	**Melanomacrophage centers (MMCs), or macrophage aggregates (MAs)** are cell aggregates that contain pigmented granules (hemosiderin, lipofuschin, ceroid, melanin).	Expect to see an INCREASE in MMCs in association with exposure to environmental stressors. Prevalence also increases with age. In female fish, MMC-like cell aggregates may be observed as late-stage atretic follicles are resorbed.	General biotic stress, ageing.
Histological changes - female specific	Ovaries	**Germ cell effects** (oocyte atresia, oocyte fragmentation, vacuoles, oocyte membrane folding, perifollicular cell thickening).	Expect to see an INCREASE in female germ cell effects in association with exposure to environmental estrogens, other endocrine disruptors and reproductive toxicants.	Indicative of exposure to **reproductive toxicants** including **environmental estrogens** and other **endocrine disruptors**.
Histological changes - female specific	Ovaries	**Post-ovulatory follicles (POF)** are what remain after ovulation (egg release) occurs and are indicative of recent spawning in fish.	Expect to see a DECREASE in POF in association with exposure to estrogens, other endocrine disruptors and reproductive	Indicative of exposure to **reproductive toxicants** including **environmental estrogens** and other **endocrine disruptors**.

			toxicants that can impair normal reproduction in fish.	
Histological changes - female specific	Ovaries	**Ovarian spermatogenesis** describes the presence of male testes tissue in female ovaries. Individual sperm cells or clusters of spermatocysts may be observed, with varying levels of severity and impacts on normal ovarian architecture.	Expect to see an INCREASE in ovarian spermatogenesis in association with exposure to certain endocrine disruptors and reproductive toxicants. Ovarian spermatogenesis is an uncommon histological change in female gonadal tissue.	Indicative of exposure to **reproductive toxicants** and certain **endocrine disruptors**.
Histological changes - male specific	Testes	**Germ cell effects** (apoptotic germ cells, syncytial cells, vacuoles).	Expect to see an INCREASE in male germ cell effects in association with exposure to environmental estrogens, other endocrine disruptors and reproductive toxicants.	Indicative of exposure to **reproductive toxicants** including **environmental estrogen**s and other **endocrine disruptors**.
Histological changes - male specific	Testes	**Testicular oocytes (testis-ova)** are female egg cells (oocytes) occurring in male testes tissue. Individual oocytes or clusters of oocytes may be observed, with varying levels of severity and impacts on normal testicular architecture.	Expect to see an INCREASE in testis-ova in association with exposure to environmental estrogens, other endocrine disruptors and reproductive toxicants. Presence of testis-ova is a well-established indicator of exposure to EDCs.	Indicative of exposure to **reproductive toxicants** including **environmental estrogen**s and other **endocrine disruptors**.
Histological changes - male specific	Testes	**Other cell effects** (hypertrophy/hyperplasia, eosinophilic granules, presence of other cell types).	Expect to see an INCREASE in other cell effects in association with exposure to environmental stressors.	General biotic stress. Non-specific.
Liver histology	*Liver*	*Histology enables assessment of changes in cellular structure that might be related to pollutant exposure, general stress, infection and ageing.*	*See descriptions of specific responses observed below.*	*General biotic stress, nutrition status, pathogens and parasites.*
Histological changes - both sexes	Liver	**Melanomacrophage centers (MMCs)** are cell aggregates that contain pigmented granules (hemosiderin, lipofuschin, ceroid, melanin).	Expect to see an INCREASE in MMCs in association with exposure to environmental stressors. Prevalence also increases with age.	General biotic stress. Non-specific.
Histological changes - both sexes	Liver	**Hepatocyte changes** include vacuolation, necrosis, apoptosis and nuclear pleomorphism.	Expect to see an INCREASE in hepatocyte changes in association with exposure to environmental stressors.	General biotic stress. Non-specific.
Histological changes - both sexes	Liver	**Bile and pancreatic* duct changes** include vacuolation, necrosis, fibrous tissue formation, cell hypertrophy and hyperplasia.	Expect to see an INCREASE in bile and pancreatic duct changes in association with exposure to environmental stressors.	General biotic stress. Non-specific.
Histological changes - both sexes	Liver	**Other cellular changes** include foci of cellular alteration and neoplasia.	Expect to see an INCREASE in other cellular changes, including formation of benign and malignant tumours in association with exposure to environmental stressors.	General biotic stress. Non-specific.

**liver morphology varies between species. Some fish have pancreatic tissue throughout liver (hepatopancreas), whilst in other species the two tissue types are separate.*

Table A1: Summary of fish health indicators used for pollution assessment by AQUEST – Part B

Bioassay	Tissue	Description	Response observed	Pollutants/Stressor Identified
Tissue Biomarkers	*Various tissues*	*Measurement of a response in specific tissues, using specific enzyme, mRNA, protein or metabolomic assays.*	*See descriptions of specific responses observed below.*	*Biomarkers may identify general biotic stress or evidence of direct exposure to specific contaminants (depending on which biomarker assay is used).*
Biomarker: Glutathione-S-transferase (GST) Activity	Gills and liver	**GST** is an antioxidant enzyme that is involved in Phase II (xenobiotic) metabolism and is a general stress biomarker.	Expect to see an INCREASE in GST activity in association with exposure to environmental stressors.	General biotic stress. Non-specific. General stress biomarkers provide an early warning marker of biological impairment.
Biomarker: Acetylcholinesterase (AChE) Activity	Gills, liver and brain	**AChE** is an enzyme that catalyzes the breakdown and metabolism of neurotransmitters.	Expect to see a DECREASE in AChE activity in association with exposure to specific pesticides.	Indicative of exposure to neuroactive pesticides, such as **organophosphates** and **carbamates**, as well as **organochlorines**.
Biomarker: Carboxylesterase (CBE) Activity	Gills, liver and brain	**CBE** is an enzyme that catalyzes the breakdown and metabolism of neurotransmitters.	Expect to see a DECREASE in CBE activity in association with exposure to specific pesticides.	Indicative of exposure to organic contaminants such as **organophosphate pesticides** and **synthetic pyrethroid insecticides**.
Biomarker: ethoxyresorufin-O-deethy-lase (EROD)	Liver	**EROD** is an assay that measures the catalytic activity of the CYP1A enzyme, which is involved in Phase I (xenobiotic) metabolism.	Expect to see an INCREASE in EROD activity in association with exposure to organic contaminants including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins.	Indicative of exposure to organic contaminants such as **polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs)** and **dioxins.**
Biomarker: bile metabolites	Bile (extracted from gall bladder)	During detoxification processes, metabolites get stored in bile prior to excretion. Measuring these **bile metabolite**s can provide an indication of recent exposure to compounds such as PAHs and petroleum hydrocarbons.	Expect to see an INCREASE in concentrations of contaminants in bile metabolites in association with exposure to environmental contaminants.	Indicative of recent exposure to organic contaminants such as **polycyclic aromatic hydrocarbons (PAHs)** and **petroleum hydrocarbons.**
Biomarker: Vitellogenin (VTG) induction	Blood plasma or skin mucus	**VTG** is a protein that should only be produced in maturing female fish. Production is stimulated by estrogen, so VTG induction in male fish has become a well-recognised biomarker of exposure to estrogenic compounds.	Expect to see an INCREASE in VTG levels in juvenile and male fish exposed to environmental estrogens. Expect to see a DECREASE in VTG levels in mature female fish exposed to reproductive toxicants (including estrogens and other EDCs).	Indicative of exposure to **environmental estrogens.**
Otolith ageing	Otoliths (ear bones)	**Otoliths** are calcified structures that lay down annual growth layers and can be counted to estimate age. Some fish are long lived (>5 years) so it is important to know how old the fish are to provide context for other biological findings.	Otolith analysis provides an estimation of fish age.	Nil. Age estimation will help with interpretation of other bioassay results.
Tissue contaminant analysis	Axial muscle (edible portion), liver	Concentrations of **contaminants in flesh and liver** assists in identifying which compounds are causing biological stress. Can infer bioaccumulation and biomagnification issues, which are important for long-lived fish and have both ecological and human health implications.	Expect to see an INCREASE in tissue contaminant levels in association with exposure to environmental contaminants.	**Bioaccumulative contaminants**. May enable identification of specific contaminants responsible for causing observed biological effects.

Table A2: Criteria for staging toadfish gonads (modified from Johnson et al. (2009) and Dietrich and Krieger (2009)).

Stage	Classification	Morphological Criteria
Male		
*	Juvenile	spermatogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
0	Undeveloped	exclusively immature phases (spermatogonia to spermatids); no spermatozoa.
1	Early spermatogenic	immature phases predominate, but spermatozoa may also be observed.
2	Mid-spermatogenic	spermatocytes, spermatids and spermatozoa present in roughly equal proportions.
3	Late spermatogenic	all stages may be observed, however, mature sperm predominate. Immature phases may be present throughout, or restricted to small nests of cells.
4	Spent	loose connective tissue with some remnant sperm.
Female		
*	Juvenile	oogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals.
0	Undeveloped	exclusively immature phases (oogonia to perinucleolar oocytes); no cortical alveoli.
1	Early development	vast majority (e.g., > 90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.
2	Mid-development	at least half of observed follicles are early and mid-vitellogenic.
3	Late development	majority of developing follicles are late vitellogenic.
4	Late devp./hydrated	majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.
5	Post-ovulatory	predominately spent follicles, remnants of theca externa and granulosa.