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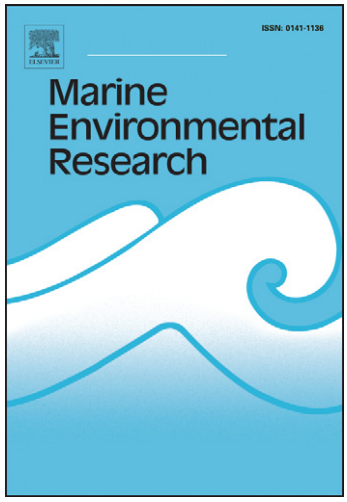

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Marine Environmental Research

Biomarker induction in tropical fish species on the Northwest Shelf of Australia by produced formation water

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ABSTRACT

Normal operation of oil well platforms results in the discharge of produced formation water (PFW). The expression of CYP1A, CYP2M1– and 2K1–like proteins was examined for use as possible biomarkers of PFW exposure. A pilot study on the Northwest Shelf of Australia had indicated that PFW contamination possibly contributes to induction of CYP1A-like proteins in Gold–Spotted Trevally (*Carangoides fulvoguttatus*). The pilot study samples were re-examined for CYP1A, and, in addition, CYP2K1/2M1-like proteins. In a subsequent caged-fish study in the same location a second species, Stripey seaperch (*Lutjanus carponotatus*), caught at a clean site, were distributed to three caging sites in a PFW gradient from the Harriet A production platform: A (near-field), B (far-field) and C (a non-impacted reference site). Fish were sampled at time (T) T=0, T=3 and T=10 days. Significant increases of CYP1A, one CYP2K1- and two CYP2M1-like proteins were noted at Site A at T=10 d. For another CYP2K1-like protein, a significant increase was observed at Site A only at T=3 d. These results support a previous study indicating that CYP1A protein is sensitive to PFW exposure. Importantly, statistically significant environmental induction of both CYP2M1- and CYP2K1-like proteins in tropical fish due to PFW exposure had not previously been described and induction of enzymes in the CYP2 family suggest new biomarkers for PFW. In addition, the novel response of one CYP2K–like protein requires further verification, but offers promise for improved monitoring of sub-lethal responses in marine organisms.

Keywords: CYP1A, CYP2K1, CYP2M1, produced formation water, tropical fish, Australia, biomarkers

1. Introduction

Produced formation water (PFW), the oily water usually discharged from a platform after separation from the oil, is made up from formation water (water associated with the oil in the reservoir) and potentially includes water which was injected into the reservoir to maintain pressure for oil production (Holdway, 2002). As oil fields age, the amount of PFW increases as the reservoir fills with injected water (Henderson et al., 1999).

When discharged, the PFW contains a large variety of naturally occurring materials from the formation, which include residual volatile and non-volatile hydrocarbons not removed by the separation regime, and also contains chemicals added to the production stream (Burns et al., 1999). Therefore, the composition of the discharged PFW is very complex. Almost all offshore oilfields produce formation water that can have significant environmental effects if not handled properly. Australia's oil and gas production activity is predominantly on the Northwest Shelf of Western Australia, off

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the continental margins between the North West Cape and Dampier (Swan et al., 1994). There has been excellent work on the toxicology of PFW (Holdway, 2002; Neff, 2002; Neff et al., 2006). Unfortunately, little is known of the effects of PFW on the tropical marine environment, especially for tropical reef fish. One study demonstrated minor toxicity of PFW from Harriet A oil platform to corals and their symbiotic algae (Jones and Heyward, 2003). Another study illustrated a preliminary toxicological assessment of PFW including analytical detection and acute toxicity bioassay using European sea bass larvae (Mariani et al., 2004). An independent scientific review commissioned by the Australian Petroleum Production and Exploration Association (APPEA) and the Energy Resource Development Corporation (ERDC) highlighted the need to conduct environmental assessments of industry practices in shallow coastal ecosystems around Australia.

In 1998, the Australian Institute of Marine Science (AIMS) conducted a pilot study to evaluate the use of sub-lethal stress indicators in fish for assessing exposure to organic contaminants associated with PFW discharged from an oil and gas production platform on the Northwest Shelf of Australia. The pilot study results confirmed that within 1000 m from the Harriet A platform there was potential for biological effects in fish populations exposed to PFW effluent and that further investigation was warranted at this site (Codi King et al., 2005a). For this study, these samples were re-examined for CYP1A using more universally repeatable conditions, and, in addition, the response of CYP2K1/2M1-like proteins was determined. In May 2003, AIMS conducted a comprehensive biological and chemical assessment of PFW effluent at the Harriet A platform. This more comprehensive study utilized a controlled system in which fish from a reference site were collected and placed in cages, and the cages were located in a gradient line moving away from the point source of PFW being discharged at Harriet A. The work described here is a subset of the suite of biomarkers conducted for the 2003 study and presents in detail the cytochrome protein biomarkers in an ecologically relevant reef species, Stripey seapearch (*Lutjanus carponotatus*) for the evaluation of the potential effects of PFW discharge at the Harriet A Platform on the Northwest Shelf of Australia.

2. Materials and Methods

2.1 Pilot study: study site and fish collection

Harriet A, an oil and gas production platform located 135 km west of Karratha on the Northwest Shelf of Australia, was the main focus of this study (Figure 1). The hydrocarbon concentration of PFW effluent in the sea-surface micro-layer, water column, sediments and biota, at this location, have been well documented (Burns and Codi, 1999) The site selections for the pilot study have previously been described in Codi King et al. (2005a). Gold-spotted Trevally was chosen for the pilot study because of its importance both commercially and recreationally to the NW Shelf of Australia. A total of 41 Gold-spotted Trevally were collected from each of three sites: Harriet A (n=23, Mean $\pm$ SD; 3817 $\pm$ 1532 g total weight, 654 $\pm$ 103 mm total length, age 1-5 yr); Harriet C (n=6, Mean $\pm$ SD; 3608 $\pm$ 1503 g total weight, 654 $\pm$ 103 mm

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total length, age 2-6 yr); and reference site off the Montebello Islands (n=12, Mean ± SD; 3992 ± 1122 g total weight, 660 ± 75 mm total length, age 2-5 yr). The data demonstrated there were no significant differences in total weight, total length and age of fish between the three study sites ($P < 0.05$). All fish were processed as previously described in Codi King et al. (2005a). Briefly, livers were removed, frozen in liquid nitrogen and transported to AIMS via a liquid nitrogen dry shipper remaining stored in liquid nitrogen until thawed for microsomal preparation (see Section 2.3).

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2.2 Caged fish study: Study site and fish collection

The PFW plume from Harriet A forms a fine surface slick that moves in a NNW direction on the ebb tides and SSE direction on the flood tide. The PFW hydrocarbons in the sea-surface microlayer were detectable to a distance of 1 km. The lighter MW components (naphthalene and homologs) were in the dissolved phase while the heavier MW hydrocarbons (phenanthrene and homologs) were associated with the particulate phase. The particulate phase was removed from the water column within a distance of 0.9 – 2 km of Harriet A (Burns and Codi, 1999; Holdway and Heggie, 1998). Therefore for the caged fish study, the cages were situated along the gradient of PFW formed as the plume moves away from the discharge site. Site A (within 200 m NNW of Harriet A) was the near-field site; Site B (~ 1000 m NNW of Harriet A) was the far-field site and Site C (~20 km NNW of Harriet A; in the Montebello Islands) was chosen as the reference site due to the lack of known contamination (Figure 2). The field component of this project was conducted aboard the AIMS *RV* Cape Ferguson from May 16-31, 2003. Initially, the target species was Red Emperor (*Lutjanus sebae)*, since it could be caged successfully (Mike Cappo, pers. comm.). On the first day of collection; however, only 5 juvenile *L. sebae* were caught and another species, Stripey seaperch (*Lutjanus carponotatus)* were being trapped at a much higher proportion (10:1). *L. carponotatus* is an important recreational fishery in Dampier Harbor and preliminary biomarker research with this species from the Harriet A pilot study (Codi et al., 2001) suggested that it would suffice as a substitute species for *L. sebae*. From May 16-19 2003, an estimated 130 *L. carponotatus* were collected by fish traps along the Dampier Harbor LNG pipeline and maintained in a 1000-L holding tank with running seawater and air. On May 21, all fish were placed in cages with 2 cages per station, 15 fish per cage at each of 3 sites (Figure 2). Fish were fed individually quick frozen (IQF) pilchards of the highest quality (Western Australia Bait Supply, Fremantle), collected from clean local waters off of Albany, WA. Food canisters were replenished every two days during the field exposure. Fish were sampled at T=0, 3 and 10 d.

A total of 50 Stripey seaperch were analyzed for CYP1A, CYP2M- and CYP2K-like proteins from the holding tank (T=0), and T=3 and T=10 for cage Sites A, B, and C. There were no significant differences for total weight, total length and age between the study sites ($P < 0.05$; Table 1). All fish were handled and processed in a similar manner as stated for the pilot study.

2.3. Microsomal preparation and Western blot

Hepatic microsomal samples were prepared at the Australian Institute of Marine Science, Townsville, Qld (AIMS) following methods previously described (Haasch, 2002) and shipped on dry ice to The University of Mississippi, University, MS. Hepatic microsomal samples were stored at –80˚C until analyzed. Protein content of the microsomal fraction was determined by Bio-Rad DC Protein Assay (Hercules, CA) using bovine serum albumin (BSA) as a standard. Microsomal proteins (10 µg

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173 per well) were separated using pre-cast Criterion SDS-PAGE gels (10%T, 2.7%C
174 resolving gel; 4%T, 2.7%C stacking gel; Bio-Rad) at 200V for 1 h with the Bio-Rad
175 Criterion apparatus. Proteins were then electrotransferred (100V for 0.75 h) to a
176 PVDF membrane (FluoroTrans W, 0.2 µm; VWR) together with one lane containing
177 MagicMark protein standards (contain proteins with IgG binding site; Invitrogen,
178 Carlsbad, CA) detectable by chemiluminescence, for molecular weight determination
179 and for evaluation of transfer efficiency. Sample loading buffer also contained
180 Pyronin Y which is visible on the membrane and marks the dye front. Detection of
181 proteins of interest was accomplished by using one of the primary polyclonal
182 antibodies; rabbit anti-fish CYP1A (two synthetic peptides used as immunogens;
183 BioSense, Bergen, NO distributed by Cayman Chemical, Ann Arbor, MI), rabbit
184 anti-trout CYP2K1, rabbit anti-trout CYP2M1 (both gifts from Donald R. Buhler,
185 PhD, Oregon State University, Corvallis, OR). Proteins of interest were visualized
186 with goat anti-rabbit alkaline phosphatase conjugate (Bio-Rad) and the Western
187 Breeze Novex Western Blot Immunodetection Kit (Invitrogen) according to the
188 manufacturer's directions using Western Lightening CDP-Star Chemiluminescent
189 reagent (Perkin Elmer Life Sciences, Boston, MA). Image analysis was accomplished
190 using the VersaDoc 3000 Imaging System and QuantityOne analysis software
191 (Bio-Rad). A summary of CYP1A results of the pilot study samples was previously
192 presented (Codi King et al., 2005a) in which the samples were analyzed with a
193 different primary antibody (anti-trout $CYP1A1_{277\text{-}294/KLH}$) under different SDS-PAGE
194 conditions (mini-Protean II, individually poured gels in the previous study) and with
195 different methods for quantification (film, scanning densitometry and NIH Image® in
196 the previous study). For this study, a commercially available antibody was used and
197 the number of samples that could be compared on one gel was increased improving
198 the statistical analyses.

199 **2.4 Densitometry and Statistical Analysis**

200 The optical density of the samples is given as a Trace Quantity (QTY; Bio-Rad
201 QuantityOne) or the quantity of a band as measured by the area under its intensity
202 profile curve. Trace QTY units are intensity ×area. For standardization, one sample in
203 triplicate was loaded on each gel. The mean Trace QTY of this sample was used as a
204 gel factor for standardization between gels. Trace QTY results are presented as an
205 Arbitrary Optical Density (without gray-scale calibration). Statistical analysis was
206 performed using GraphPad Prism. For the pilot study, a two-way ANOVA was
207 performed using both gender and site as covariates. The results indicated that for each
208 individual isozyme site was a significant factor, but not gender, and there were no
209 interactions between these two factors. Therefore, for the one-way ANOVA the males
210 and females were pooled, and the ANOVA results were analyzed by Turkey's multiple
211 comparison test ($P < 0.05$, 0.01 and 0.001). Similar analysis of covariates allowed the
212 use of the same statistical approach for the caged fish study.

213 **3. Results**

214 **3.1 Pilot study**

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The results of the immunodetection of the CYP1A-, CYP2M1-, and CYP2K1-like proteins indicated that proteins of the appropriate molecular weight range for each isozyme were detectable in Gold–Spotted Trevally. Most Gold-Spotted Trevally showed one immunodetectable band of CYP1A-, CYP2M1- and CYP2K1- like proteins; some samples had a second CYP2M1- like protein. Because this second protein was not present in each sample, only the first band of lower molecular weight was quantified (Figure 3). Similar to the previous study (Codi King et al., 2005a), CYP1A-like protein levels in Gold-Spotted Trevally from the near-field (Harriet A) site were significantly higher than those from the far-field (Harriet C) site and the reference site. Although not significant, Gold-Spotted Trevally also exhibited a trend of increased CYP2M1-like protein levels at Harriet A as compared to Harriet C and the reference site (Figure 3).

For CYP2K1-like protein, a significant increase was observed at the Harriet C site compared to the reference site (Figure 3). The induction of CYP2K1-like proteins at the Harriet C site in Trevally may be due to possible exposure to hydrocarbons from natural oil seepages found in this region, or due to the movement of fish between platforms. The elimination of these variables was one of the main reasons for undertaking the subsequent controlled field study using caged fish.

3.2. Caged fish study

Immunodetection of CYP1A-like proteins indicated that proteins of the appropriate molecular weight range were detectable in Stripey seaperch at all three sites and over the time of the exposure (Figure 4a). The results for the CYP1A-like proteins (Figure 4b) demonstrated that by Day 3 of exposure both Site A and Site B had higher mean values than Site C, albeit not significant. By Day 10, fish collected at Site A had significantly higher induction of CYP1A-like proteins as compared to the fish at Site B and Site C. Comparisons also demonstrated that Site A fish after 10 days of exposure had significantly induced levels of CYP1A-like proteins as compared to the holding tank fish (T=0).

Figure 5a presents the optical densities for the species-specific pattern of CYP2M1-like proteins determined in Stripey seaperch, with two distinct appropriate molecular weight proteins in most samples; CYP2M1-like Protein 1 (52.8 kDa) and CYP2M1-like Protein 2 (49.0 kDa). Immunodetection of CYP2M1-like proteins demonstrated a similar pattern of expression for both Protein 1 and Protein 2 in Stripey seaperch with significant induction occurring at Day 10 for both proteins (Figures 5b and 5c) and at Day 3 the same increasing but not significant trend. For Site A both Day 3 and Day 10 exposed Stripey seaperch had significantly higher levels of CYP2M1-like proteins than the holding tank fish. It is interesting that Site C Day 3 exposed fish also demonstrated site-specific differences from the holding tank fish.

Figure 6a presents the optical densities for the species-specific pattern of CYP2K1-like proteins determined in Stripey seaperch, again showing two distinct appropriate molecular weight proteins; CYP2K1-like Protein 1 (52.5 kDa) and

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CYP2K1-like Protein 2 (48.2 kDa). Unlike the two CYP2M1-like proteins, the induction patterns for the two CYP2K1-like proteins were dissimilar. For CYP2K1-like Protein 1, levels (Figure 4b) were significantly elevated for Site A Day 3 as compared to fish collected on Day 3 from Site B and Site C. At Day 10 there were no site-specific differences in expression, although Site A Day 10 and Site A Day 3 Stripey seaperch had significantly higher CYP2K1-like Protein 1 levels than the holding tank fish. For CYP2K1-like Protein 2 levels (Figure 4c) the induction trend was similar to the pattern observed for CYP1A- and both CYP2M1-like Protein 1 and Protein 2 in that by Day 10, Site A fish demonstrated the highest mean level of induction. The same site-specific differences were also observed with Site A Day 10 fish and Day 3 fish having significantly higher CYP2K1-like Protein 2 induction levels than the holding tank fish.

4. Discussion

Fish are important organisms for monitoring environmental contaminants because of their position in the food web, their lifestyle, their relative abundance and their adaptable physiology. Fish CYP1A induction is an extremely useful biomarker of chemical exposure in the environment (Flammarion et al., 2002), particularly for persistent pesticides (Levine et al., 1999), polychlorinated biphenyls (Burton et al., 2002; Haasch et al., 1993), dioxins (Zodrow et al., 2004; Smith, 2003), pulp mill effluents (Oikari et al., 2002), xenoestrogens (Katchamart et al., 2002), spilled crude oil (Jewett et al., 2002), and polycyclic aromatic hydrocarbons (PAHs) (Stagg et al., 2000). The induction of CYP1A has implications for human fish consumption, as well as for the health status of aquatic organisms (Arinc et al., 2000).

The induction of CYP2 family proteins in bluegill and catfish due to exposure to peroxisome proliferating agents (PPAs; Haasch, 1996) was the first description of an inducible P450 in fish other than CYP1A. The CYP2M1- and 2K1-like proteins in bluegill and catfish were later shown to be lauric acid hydroxylases (Haasch et al., 1998). Bivalves exposed to petroleum water-accommodated fraction (WAFs) produce peroxisome proliferation (Cajaraville et al., 1997). Therefore, petroleum hydrocarbon, as the major contaminant in PFW, may be considered a PPA, even though the mechanism is unknown. These findings provided an indication that a petroleum hydrocarbon-initiated peroxisome proliferation response may occur in lower vertebrates similar to the model PPA-mediated induction of CYP2M1/2K1 (Ackers et al., 2000; Johnson et al., 1996).

These two CYP2 family P450 isozymes have been most thoroughly characterized in rainbow trout, (*Oncorhynchus mykiss*; Buhler and Wang-Buhler, 1998). The molecular weight of CYP2K1 in rainbow trout is about 54 kDa, and has been shown to catalyze the (ω-1) and (ω-2) hydroxylation of lauric acid and some longer chain fatty acids; the molecular weight of CYP2M1 in rainbow trout is about 50 kDa, and this isozyme is the only known P450 responsible for laurate hydroxylation at the (ω-6) position. Both isozymes also catalyze the hydroxylation of estradiol, testosterone and progesterone (Buhler and Wang-Buhler, 1998). It follows that induction of CYP2K1- and

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CYP2M1-like proteins in fish may have effects on fatty acid and steroid homeostasis, with possible links to developmental and reproductive toxicity as well as carcinogenesis (Biegel et al., 1995; Vanden Heuvel, 1996; Vanden Heuvel et al., 1991). A variety of structurally diverse xenobiotics and pharmaceuticals are known to produce peroxisome proliferation and concordant fatty acid metabolizing enzyme induction in mammals, therefore, the monitoring of CYP2 family isozymes in fish may have implications for human health and has the potential to become an important indicator of environmental health.

The immunodetection results indicated one highly immunoreactive protein for CYP1A and multiple immunoreactive proteins for the CYP2-like proteins. Some cross-reactivity between CYP2-like proteins is expected with the polyclonal anti-CYP2K1 and anti-CYP2M1 antibodies (some affinity for the same protein epitopes) as these antibodies have cross-reactivity to both rainbow trout CYP2K1 and CYP2M1 (Buhler and Wang-Buhler, 1998). Further evidence for cross-reactivity was evident in samples that also included one or more minor proteins of intermediate size that were specific to individuals and not consistently represented across all individuals. The CYP2 family is known to be fairly complex in number of isozymes and expression levels (Buhler and Wang-Buhler, 1998). Multiple cross-reactivity was not unexpected and did not detract from the relatively clear induction of two proteins using the anti-CYP2 antibodies. The proteins were designated CYP2M1- and CYP2K1-like proteins because appropriate standards for the tropical fish species do not yet exist and were beyond the scope of this study. Nevertheless, it is evident that the CYP2 family antibodies recognize constitutively present and inducible proteins that could potentially be used as biomarkers. Furthermore, it is possible, with appropriate sequence information, for commercially-available anti-CYP2 family antibodies to be developed.

The pilot study CYP1A results supported the findings of an earlier analysis of those samples using a different methodology indicating induction of CYP1A (Codi King et al., 2005a). Unlike the previous analysis, the current study did not indicate gender-specific responses. Multiple differences between the two analyses could contribute to the discrepancy including the use of a different primary antibody preparation. Similar to the previous analysis, site-specific responses were detected. Analysis of the pilot study samples for CYP2M1- and CYP2K1-like proteins indicated possible site-specific induction and prompted the further examination of these proteins in the caged fish study.

Studies of environmental contaminant-mediated induction of the cytochromes P450 in tropical fish are lacking (Codi King et al., 2005a; Vrolijk et al., 1994). Both the pilot and caged fish field studies indicated significant results pertaining to the induction of cytochromes P450 in tropical fish species by PFW. First, the induction of CYP1A in Gold-Spotted Trevally and Stripey seaperch at the near-field site indicated that even though PFW discharge limits are being met (Codi King et al., 2005b), exposure to CYP1A inducing chemicals within the diluted PFW produced a significant induction

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effect. Numerous studies have demonstrated that chronic low level exposure of fish and especially larvae to PAHs can have a significant impact and can produce long-term, adverse health and reproductive effects (Incardona et al., 2004; Stegeman et al., 2001). These adverse effects are most likely to be more serious to those organisms attached to the platform or living under or near the platform in a restricted range but could also be detrimental to organisms cyclically exposed to pools of PFW. Second, the significant induction of the CYP2K1/2M1–like proteins in both the Gold-Spotted Trevally and the Stripey seaperch is the first documented significant environmental induction in fish of any cytochrome P450 isozyme other than CYP1A1. The similar induction pattern of CYP1A1 and CYP2K1-like Protein 2 and CYP2M1-like Protein 1 and 2 may indicate that all of these isozymes are induced by the same chemical class contained within the PFW. While this finding alone is a reason for further study, it is perhaps even more interesting that there is an apparent response difference for induction of particular CYP2K1/2M1-like proteins. Only the CYP2K1-like Protein 1 was induced at the near-field site (Site A) on Day 3, but not on Day 10. The apparently different induction of the CYP2K1 Protein 1 compared to the other CYP isozymes potentially represents a unique chance to develop another bioindicator of PFW exposure. Although the metabolic activities of the CYP2M1 and CYP2K1 isozymes in Stripey seaperch are not known, it is reasonable to assume that the fatty acid and reproductive steroid hormone substrates would differ among the different proteins. Induction of different isozymes may therefore have different short- and long-term effects on the exposed organisms.

In support of the previous findings, the results indicate that CYP1A protein is sensitive to PFW exposure. Importantly, statistically significant environmental induction of both CYP2M1- and CYP2K1-like proteins in tropical fish due to PFW exposure had not previously been described and CYP2 family induction may represent possible new biomarkers (other than CYP1A) associated with PFW. In addition, the novel response of one CYP2K-like protein requires further verification but offers promise for improved monitoring of sub-lethal responses in marine organisms.

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Table 1: Characteristics of Stripey seaperch used in the caged fish study. All of the fish were of intermediate reproductive status (sex-stage) and had plasma vitellogenin levels that were below the detection limit.

Holding Tank or Cage Site	Time (days)	Sex Ratio (Female/Male)	Body Weight* (g)	Body Length* (mm)	Age* (yr)
Holding Tank	**0**	(4/7)	271 ± 85	261 ± 22	5 ± 2
Reference Site C	**3**	(4/4)	292 ± 83	265 ± 30	6 ± 4
	10	(4/4)	284 ± 52	257± 19	4 ± 2
Far-Field Site B	**3**	(0/5)	273 ± 123	260 ± 34	5 ± 3
	10	(0/5)	324 ± 92	269 ± 21	3 ± 2
Near-Field Site A	**3**	(3/5)	371 ± 88	285 ± 20	5 ± 3
	10	(1/4)	293 ± 79	262 ± 28	5 ± 2

*Values are mean ± SD for male and female combined.

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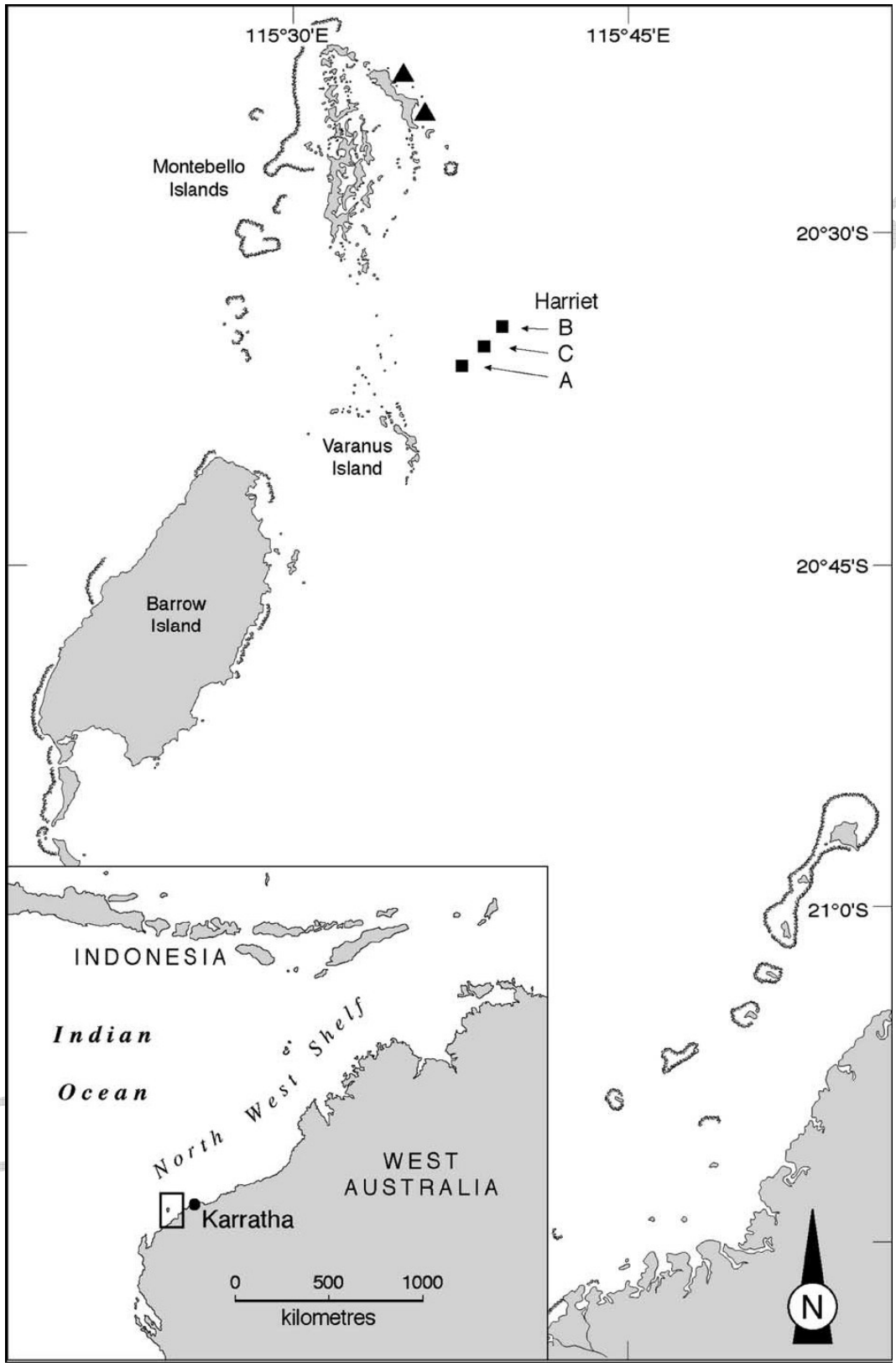

Figure 1: Pilot study site locations: Harriet A, Harriet B, Harriet C, and the reference sites, indicated by triangles, off the Montebello Islands.

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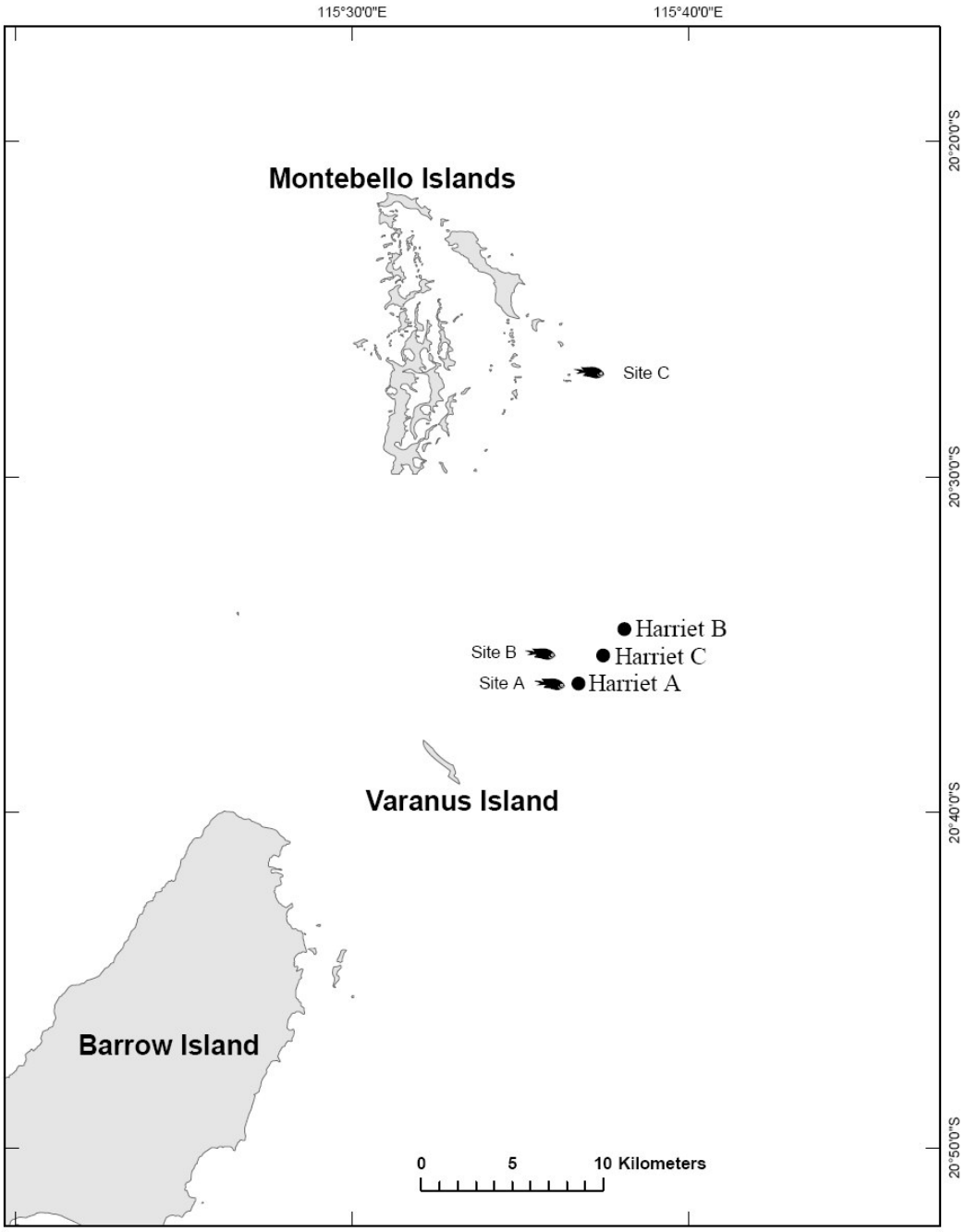

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393 Figure 2. Site locations of the caged fish study: (Site A ~200 m, Site B ~1000 m and Site C ~20 km)
394 relative to the Harriet A PFW discharge.

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396 Figure 3. Pilot study: the species specific pattern of CYP1A-, CYP2M1-, and CYP2K1- like proteins
397 determined in Gold-Spotted Trevally (shown for comparison of isozyme expression not for illustration
398 of site effects), and graphical representation of the optical densities (Mean ± SD) determined for each
399 site. The molecular weight range of the proteins is typical of cytochromes P450 and is between 45 and
400 60 kDa for all three isozymes. Numbers above the bars indicate the number of female and male fish
401 (e.g., 6/2, female/male) represented in the average densitometry values. No statistical difference was
402 observed between genders.

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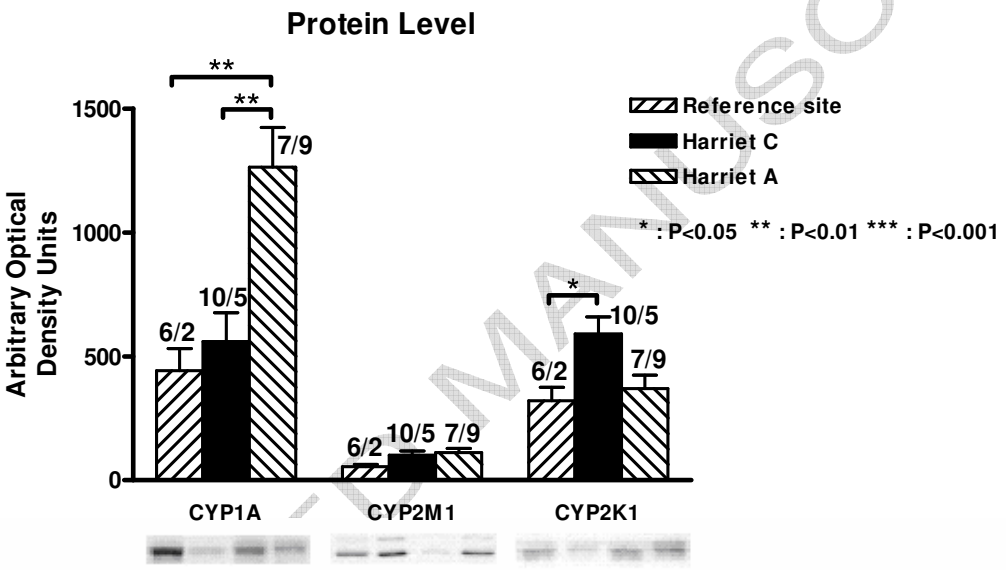

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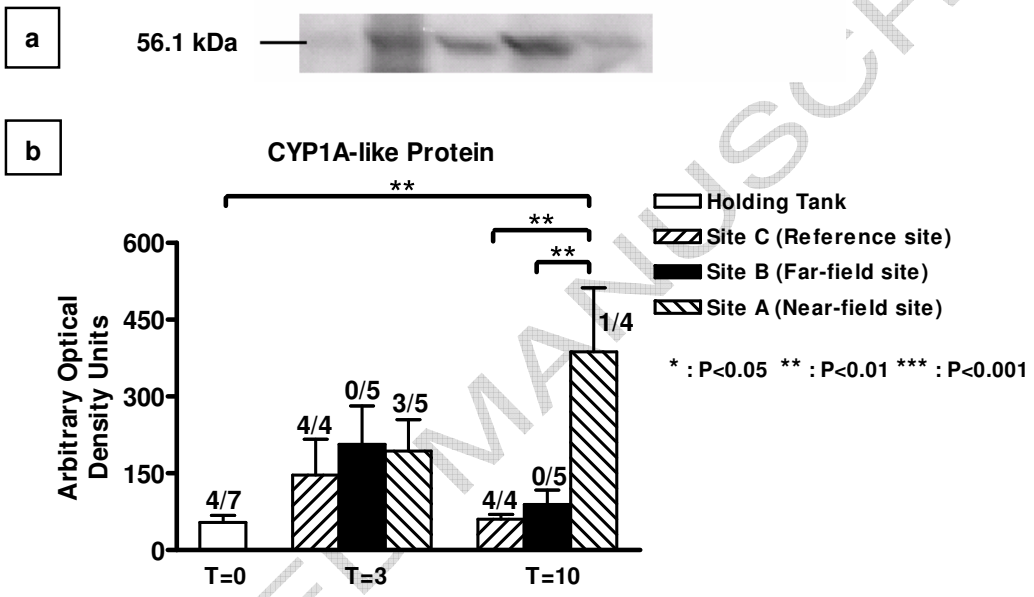
Figure 4 a-b. Caged fish study: the species-specific pattern of CYP1A-like proteins determined in Stripey seaperch. (a) Image of the chemiluminescence detection of CYP1A-like proteins with a single distinct band (56.1 kDa) in the appropriate molecular weight range (shown for comparison of isozyme expression not for illustration of site effects), and (b) graphical representation of the optical densities (Mean ± SD) determined in Stripey seaperch at each site over time. Numbers above the bars indicate the number of female and male fish (e.g., 4/7, female/male) represented in the average densitometry values. No statistical difference was observed between genders.



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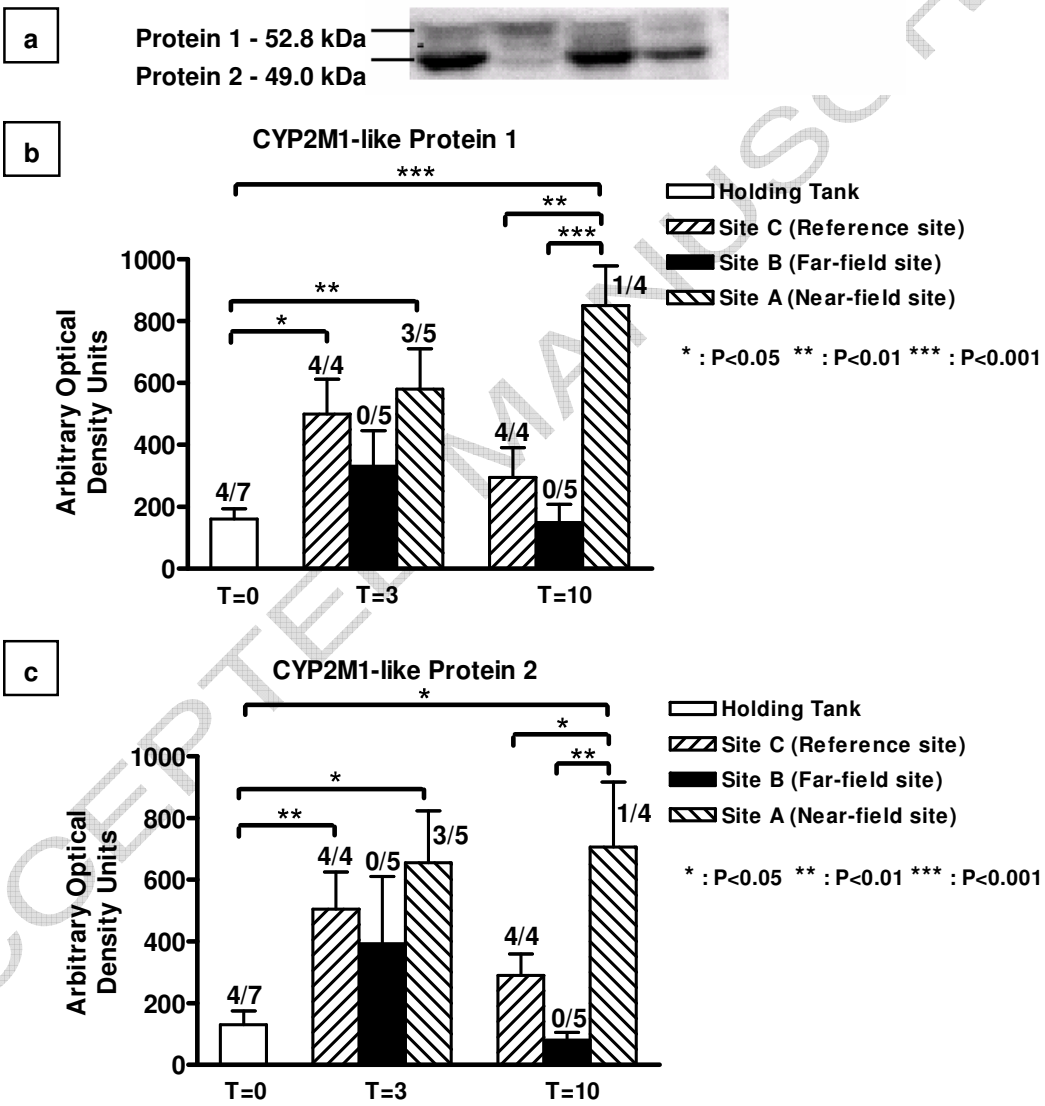
413 Figure 5 a-c. Caged fish study: the species specific pattern of CYP2M1-like proteins determined in
414 Stripey seaperch. (a) Image of the chemiluminescence detection of CYP2M1-like proteins with two
415 distinct bands of protein (shown for comparison of isozyme expression not for illustration of site
416 effects), (b) the graphical representation of optical densities (Mean ± SD) determined for Protein 1 and
417 (c) for Protein 2. Numbers above the bars indicate the number of female and male fish (e.g., 4/7,
418 female/male) represented in the average densitometry values. No statistical difference was observed
419 between genders.



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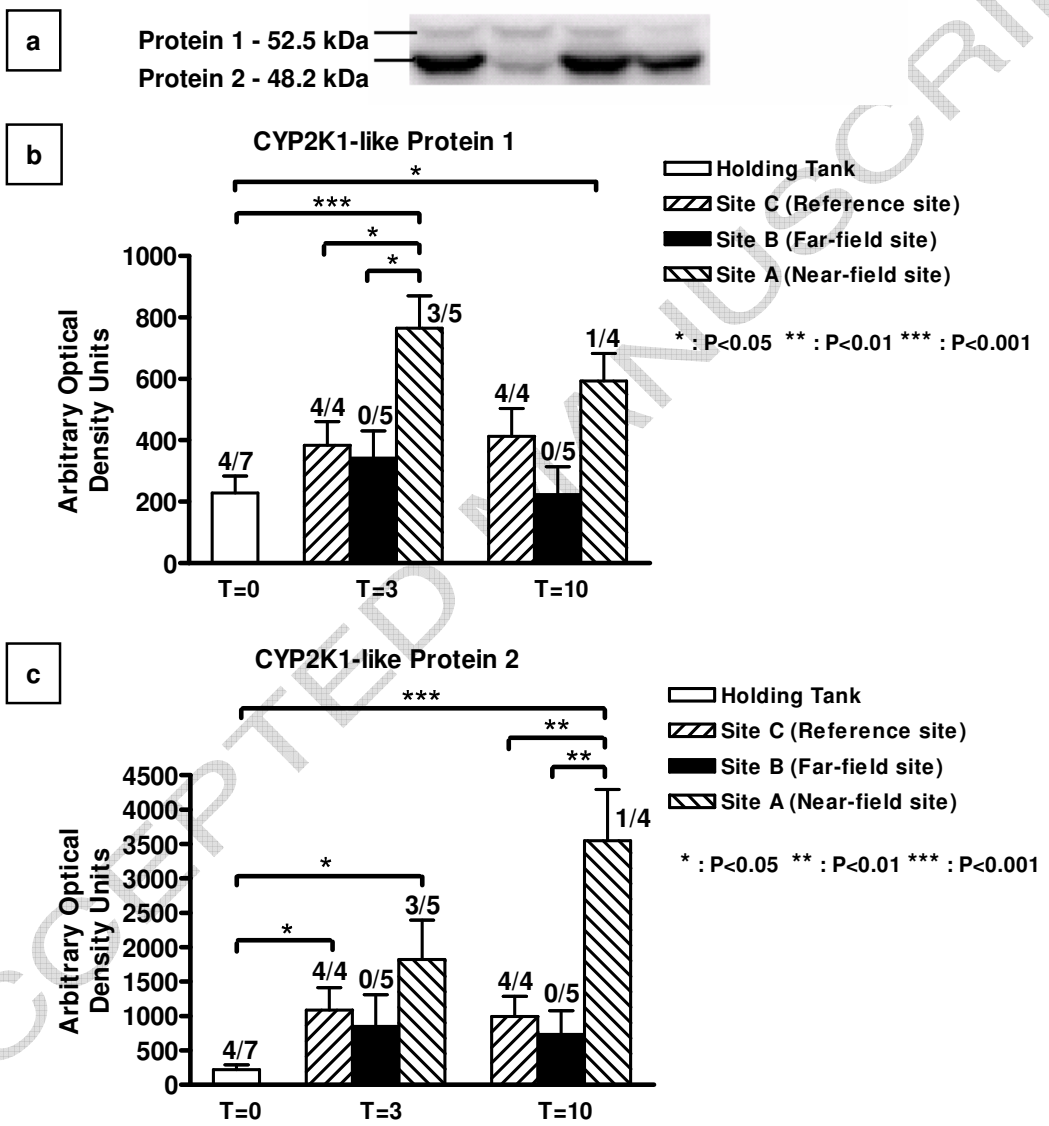
421 Figure 6 a-c. Caged fish study: the species specific pattern of CYP2K1-like proteins determined in
422 Stripey seaperch. (a) Image of the chemiluminescence detection of CYP2K1-like proteins with two
423 distinct bands of protein (shown for comparison of isozyme expression not for illustration of site
424 effects), (b) the graphical representation of optical densities (Mean ± SD) determined for Protein 1 and
425 (c) for Protein 2. Numbers above the bars indicate the number of female and male fish (e.g., 4/7,
426 female/male) represented in the average densitometry values. No statistical difference was observed
427 between genders.