

BIOMARKER RESPONSES IN CORAL TROUT (*PLECTROPOMUS LEOPARDUS*) AS AN INDICATOR OF EXPOSURE TO CONTAMINANTS IN A CORAL REEF ENVIRONMENT.

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ABSTRACT

The Great Barrier Reef (GBR) is a resource of immense economic and social benefit to Australia and one that is highly valued by the international community. A number of reefs of the GBR have moored pontoons and frequent boat traffic to service the tourist industry. The aim of the present study was to test whether these human activities are having a measurable impact on selected reef environments as caused by anthropogenic contaminants. A recognised method for assessing changes in levels of exposure in biota to chemical contaminants released by boating activity is by assessing biological markers. Biomarker responses measured included: fluorescent aromatic compounds (FACs) in bile, changes in enzymatic responses (ethoxyresorufin *O*-deethylase: EROD and cholinesterase: ChE activities) and two general indices of condition (K and HSI) in a resident tropical reef species, coral trout (*Plectropomus leopardus*). EROD activity in liver was similar for both pontoon and non-pontoon (reference) sites suggesting no evidence of exposure to contaminants in coral trout. Similarly, the condition factor (K) and hepatosomatic index (HSI) failed to detect any evidence of exposure. However, results of FACs in bile suggest low-level exposure to naphthalene and its metabolites at both pontoon and non-pontoon reefs, and ChE activity was significantly inhibited in coral trout collected from reefs with pontoons as compared to trout from reefs without pontoons. These results raise concern that there may be other contaminants impacting these fish caused by exposure to chemicals such as antifoulants and further study is warranted to investigate these findings. This study is one of few to undertake assessment of biomarkers in fish from coral reefs of the GBR, and thus, provides a useful baseline reference for assessment of changes in environmental water quality in this part of the GBR.

Key words: coral trout; Great Barrier Reef; coral reef; biomarkers; contaminants; EROD; FACs; ChE.

INTRODUCTION

The Great Barrier Reef (GBR) annually attracts approximately 6.4 million visitors who contribute more than AUS\$5 billion to the Australian economy, employing more than 63 000 people (Access Economics 2005). Parts of the GBR are open to growing environmental pressure from tourism along the Queensland coast. The majority of tourists who visit the reef do so by means of large day trip boats travelling to specific reef sites (Moscardo and Ormsby 2004). Pontoons have been moored at a number of reefs to service these day boats, reducing impacts due to anchor damage and providing economically sustainable use of the reef. There are about 19 pontoons located on the GBR, mainly in the Cairns section of the GBR Marine Park (GBRMP) (Nelson and Mapstone 1998). Traffic by tourist boats to some reefs averages around 1000 trips per year, with each trip bringing 300-600 passengers. Some reefs receive much lower intensity of traffic, but on a daily basis these boats bring several thousand visitors to the reef pontoon system.

Many of these pontoons are moored at sensitive reef areas within the GBRMP and strict controls are placed on tourist activities in order to limit human impact. The Great Barrier Reef Marine Park Authority (GBRMPA), which manages the GBRMP, has implemented various monitoring programs on selected reefs with pontoons in order to assess the ecological impact of this type of tourist development. These studies have shown that fish aggregations are enhanced around the pontoons because of controlled feeding, and that benthic

community structure can be affected by the presence of pontoons (Sweetman 1996). Previous water quality studies around reef pontoons were concerned only with nutrients and based on these limited data, there was no evidence of an impact associated with pontoons (Nelson and Mapstone 1998). At present, there is no information available on environmental levels of anthropogenic contaminants that come from boat traffic around these pontoons or on the effects these activities may have on reef biota.

An established method for assessing changes in levels of exposure and potential effects, in biota, from anthropogenic contaminants is through using biological markers (e.g. biomarkers). Few studies have used biomarkers to assess the impact of contaminant exposure in the GBR region. Cavanagh et al. (2000) and Codi et al. (2004) investigated EROD in fish from river systems and estuaries in northern Queensland. Klumpp and von Westernhagen (1995) evaluated malformations and chromosome aberrations in developing fish eggs from reefs and coastal waters of the GBR. In both of these studies biomarkers were useful in identifying trends of increased environmental stress in areas close to human impact.

The focus of this study was to assess the potential use of fish biomarkers as indicators of exposure to bioavailable contaminants in the coral reef environment. Biomarkers were used to determine if there was a measurable impact of visits by tourist boats on the environmental quality of selected reefs as caused by anthropogenic contaminants. Petroleum

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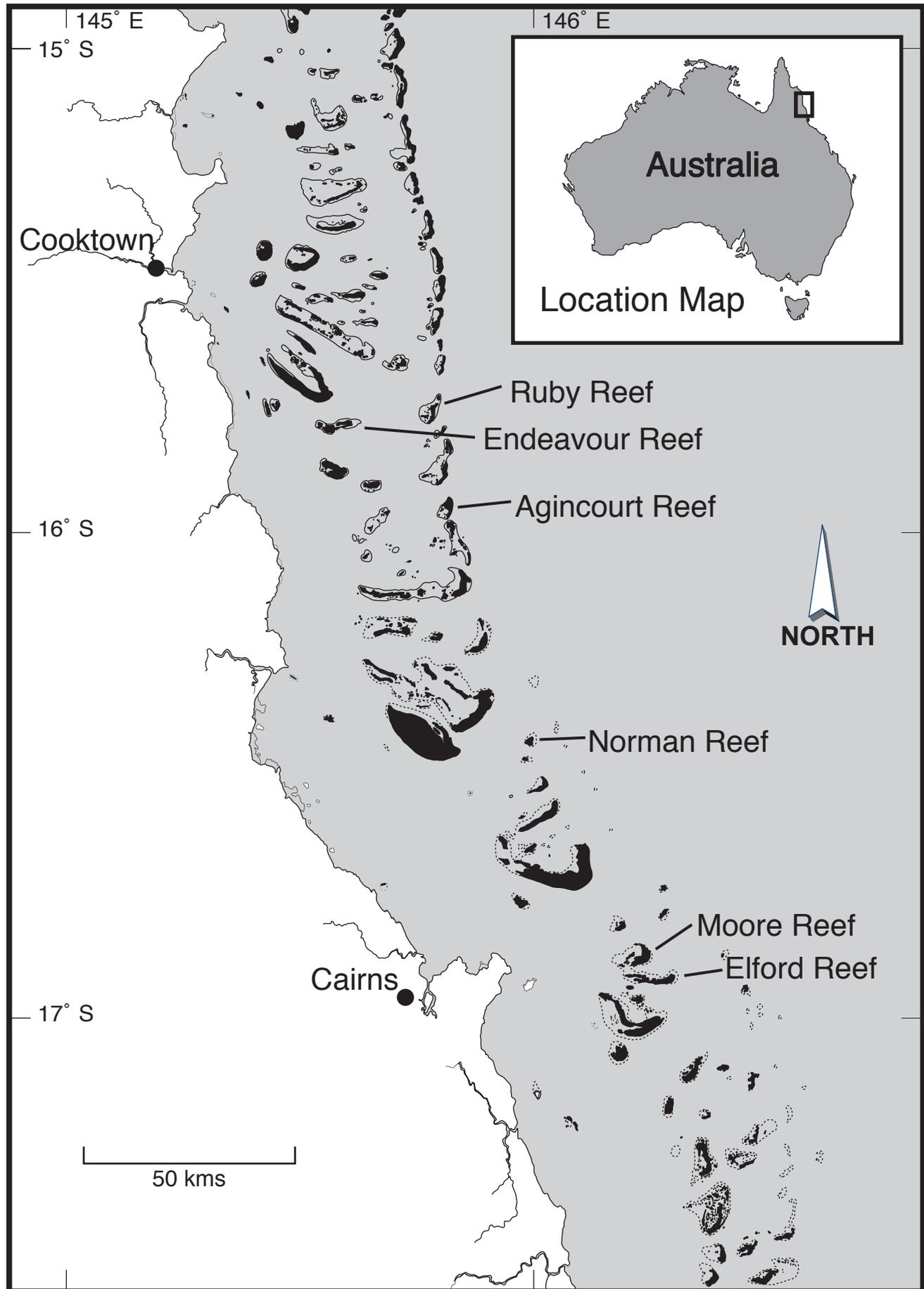


Figure 1. Map of sampling locations on the Great Barrier Reef.

hydrocarbons released from boat engines (e.g. n-alkanes, aliphatic and aromatic hydrocarbons), and antifouling agents derived from boat hulls (e.g. biocides and heavy metals), were expected to be potential chemical contaminants in this environment. The study compared three pontoon-reef systems that were known to get annual visits of 1000 tourist vessels or more with three reefs zoned "green" by GBRMPA (i.e. where all human activity is excluded) that have little or no boating activity. We measured five biomarkers of contaminant exposure or stress in an important commercial and recreational reef species, coral trout (*Plectropomus leopardus* (Lacepède, 1802)). These included: (1) analysis of fluorescent aromatic compounds (FACs) in bile; (2) induction response of the mixed function oxidation enzyme system by measuring the enzymatic activity of ethoxyresorufin *O*-deethylase (EROD); (3) cholinesterase (ChE), a neuro-transmission enzyme that is inhibited by certain toxins such as antifouling chemicals and metals; (4) condition factor (K); and (5) hepatosomatic index (HSI). This one of the few studies to undertake assessment of biomarkers in fish from coral reefs of the GBR, so these data provide a useful baseline reference for assessment of changes in environmental water quality in this part of the GBR.

MATERIALS AND METHODS

Coral trout were selected as the study organism for this study as they are a relatively common, large predatory reef fish and represent an important commercial and recreational fishery on the Great Barrier Reef (Kailola et al. 1993). They have been shown to be relatively sedentary with the majority of fish remaining on the same reef over a twelve-month period, therefore they would be representative of the study area (Kuhl 1994; Samoily 1997). Previous work on a closely related serranid species, *Plectropomus maculatus*, has demonstrated the suitability of this fish for use as a bioindicator organism in tropical waters of Australia (Codi King et al. 2005).

Fifteen coral trout were collected in May 2000 from each of three pontoon reefs (Moore [16° 52' S, 146° 12' E], Agincourt [15° 58' S, 145° 49' E] and Norman Reefs [16° 25' S, 145° 59' E]) and three reference or "green zone" reefs (Ruby [15° 44' S, 145° 47' E], Elford [16° 55' S, 146° 15' E] and Endeavour [23° 10' S, 150° 56' E] Reefs). Locations of these reefs are shown in Figure 1. Fish were sacrificed as soon as possible (on average 100 min after capture) by cervical dislocation, and liver and muscle tissue (from just anterior to and below the dorsal fin) were excised immediately and snap-frozen in liquid nitrogen for later analysis of EROD induction and ChE inhibition, respectively. Bile was also collected from each individual fish for analysis of fluorescent aromatic compounds (FACs). Fork length, fish weight, gut and liver weight were recorded for each fish.

Bile samples were analysed for two polycyclic aromatic hydrocarbons, naphthalene and phenanthrene, and their metabolites according to the method outlined in Krahn et al. (1984), with some modifications for use with tropical reef species (Codi King et al. 2005). Separations were performed on a GBC liquid chromatograph 1440 system with a 150 X 4.6 mm Platinum EPS C₁₈ column (100 Å, 5 µm) (Alltech™), protected by a 2 cm guard column pre-packed with LC₁₈

packing (Alltech™). Solvent composition changed from 100% water to 100% acetonitrile linearly over 20 min, at a flow rate of 1 mL min⁻¹, and then was held for 15 min before returning to starting conditions. Bile samples (20 µL) were analysed by direct injection into the HPLC and monitored using a Varian 9070 fluorescence detector set at excitation and emission wavelength pairs specific for naphthalene and its metabolites (290 nm/335 nm) and phenanthrene and its metabolites (340 nm/380 nm). The detection limit for both naphthalene and phenanthrene and their metabolites was 1 µg g⁻¹.

Microsomes for EROD analysis were prepared from the frozen liver. During homogenisation the liver tissue was always kept at 4°C. Livers were weighed individually and homogenised 1:4 w/v in 0.1 M phosphate buffer (pH 7.4). Homogenates were then centrifuged at 20 000 × g for 20 min at 4°C. The resulting pellet was discarded and the supernatant was centrifuged at 100 000 × g for 60 min at 4°C. The supernatant was discarded and the mitochondrial pellet was resuspended in 2 mL of 0.1 M phosphate buffer (pH 7.4), containing 20% glycerol. Resuspended microsomes were stored in liquid nitrogen for subsequent analysis.

EROD activity was determined fluorospectrophotometrically based on the methods of Burke and Mayer (1974) and as further described in Krüner and von Westernhagen (1999). Reactions were carried out in a 1 cm quartz cuvette in which 1865 µL of 0.1 M phosphate buffer (pH 7.4), 75 µL of 50 mM 7-ethoxyresorufin, 50 µL of sample and 10 µL of 1.0 mM NADPH were added. The enzyme kinetics was monitored on a Hitachi F-4000 fluorescence spectrophotometer at excitation/emission wavelengths of 530/585 nm respectively for a period of one minute. The measured fluorescence corresponded to the amount of resorufin produced by the enzymatic reaction. The activity of the sample measured was reported as production of resorufin per minute per mg protein (pmole min⁻¹ mg protein⁻¹).

Protein was determined by the method of Lowry et al. (1951).

Acetylcholinesterase (AChE, E.C.3.1.1.7) and non-specific butyrylcholinesterase (BChE, E.C.3.1.1.8) are the two forms of cholinesterases likely to be present in marine fish muscle (Sturm et al. 2000). Since we have not distinguished between these forms we have used the term "cholinesterase" (ChE) throughout this paper.

Frozen muscle tissue (1.5 to 2.5 g) was homogenised in 0.02 M phosphate buffer (pH 7.0) containing 0.1% Triton X-100. The tissue was homogenised 1:5 w/v for 1 min using a Heidolph Diax 900 homogeniser set on 4. The homogenate was then centrifuged at 10 000 × g for 20 min at 4°C and an aliquot of the supernatant used in the ChE and protein assays.

ChE activity was determined spectrophotometrically by the method of Ellman et al. (1961) as modified for microplate reading by Bocquené and Galgani (1998). To each well of the microplate 30 µL of 0.02 M phosphate buffer (pH 7.0), 20 µL of dithiobisnitrobenzoic acid (DTNB 0.01 M) and 10 µL of

sample were added successively. After 5 min incubations, 10 μL of acetylthiocholine iodide (ACTC 0.1 M) was added to start the reaction. The enzyme kinetics was monitored on a microplate reader (Wallac Spectra II) at 405 nm for two min, in which time the response was linear. Two types of blanks were analysed: 1) blanks in which the sample was omitted, and 2) blanks in which the substrate was omitted. Any activity due to these blanks was subtracted from the sample activity. Enzyme activity is given as the amount of enzyme which catalyses the hydrolysis of 1 μmole of acetylcholine per minute per mg protein ($\mu\text{mole ACTC min}^{-1} \text{mg protein}^{-1}$).

Condition factor (K) was calculated according to Bolger and Connolly (1989):

$$K = S_w / L^3$$

where S_w was the somatic weight in grams (total weight of the fish, less gonad and intestines in grams) and L was fish length in millimetres. Hepatosomatic index (HSI) was calculated according to Slooff et al. (1983):

$$\text{HSI} = (L_w / S_w) \times 100$$

where L_w represents the total liver weight in grams and S_w is the somatic weight as given above.

Data reported are untransformed means \pm standard error. Data were checked for homogeneity of variances by means of Bartlett's test, and normality by normal-probability plots. If required, data were Log-transformed to meet the assumption of homogeneity of variances and normality implicit in analysis of variance (Zar 1996). Significant effects due to the presence of pontoons on EROD, FACs and ChE activity were examined by analysis of covariance (ANCOVA), with sex as the covariable. Results were considered to be significantly different if $p < 0.05$. When a significant effect due to the presence of pontoons was found, individual comparisons were made between the pooled reference sites (there was no significant difference between the reference sites) and each reef using Dunnett's test, $p < 0.05$. Linear regression analyses were carried out to test for the effects of handling, fish size and liver weight on EROD activity and ChE inhibition.

RESULTS AND DISCUSSION

The mean bile metabolite data for naphthalene (expressed as naphthalene equivalents) for coral trout collected from the non-pontoon (Endeavour, Ruby and Elford) and pontoon (Agincourt, Norman, Moore) sites are presented in Figure 2a.

The mean naphthalene value for the three pontoon reefs of $5.81 \pm 4.02 \mu\text{g g}^{-1}$ was not significantly higher than the mean value of the three non-pontoon reefs of $5.67 \pm 3.2 \mu\text{g g}^{-1}$. The data clearly demonstrate that there is no significant difference in exposure between coral trout inhabiting reefs with and without pontoons. The mean phenanthrene bile metabolite data determined in coral trout from the non-pontoon reefs and the pontoon reefs were below detection ($< 1 \mu\text{g g}^{-1}$). Previous studies with *Plectropomus maculatus* showed differences in bile metabolites in response to differences in environmental PAH levels (Codi King et al. 2005). In the present study there appeared to be low level exposure of coral trout from pontoon

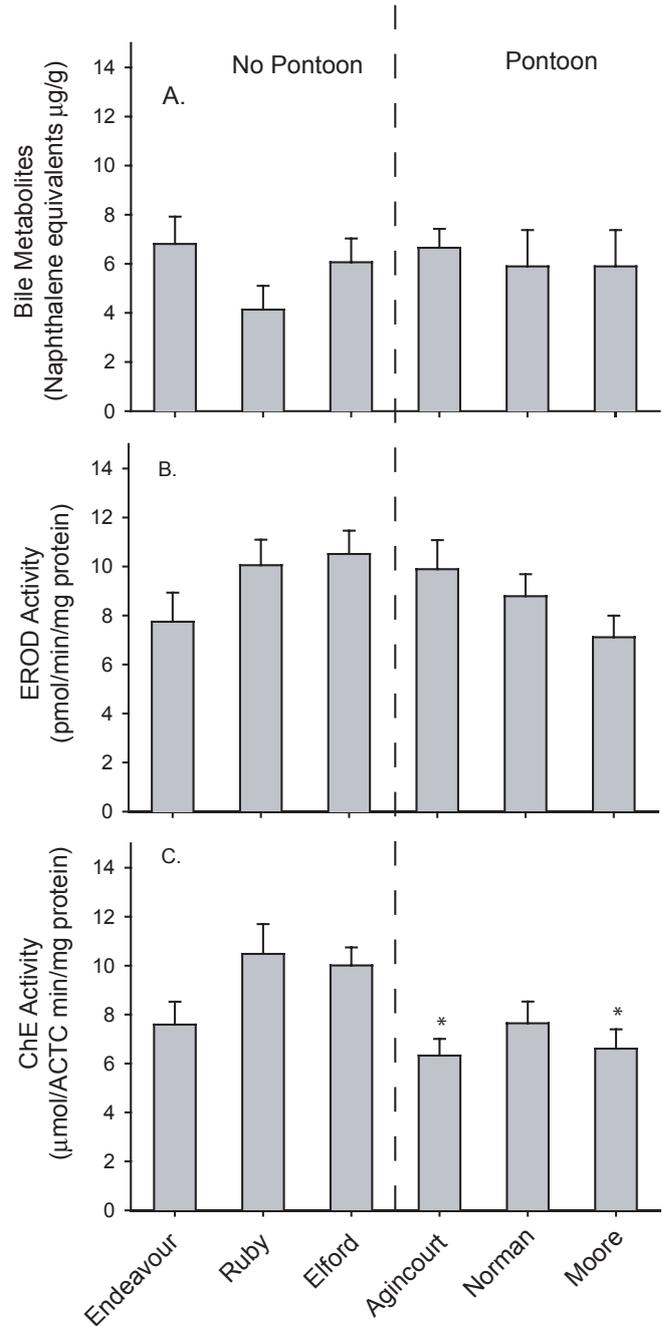


Figure 2. Biomarkers in *Plectropomus leopardus* from pontoon and non-pontoon reefs (mean \pm s.e.).

(a) FACs, (b) EROD activity and (c) ChE activity

* represents significantly different from the pooled reference sites. (n = 90).

and non-pontoon reefs to naphthalene and its metabolites at the time of sampling, which may be indicative of a more general source of PAH contamination.

Presence or absence of pontoons on reefs did not have a significant effect on the EROD activity in livers of coral trout (ANCOVA, $F_{(5,83)} = 1.702, p > 0.05$; Figure 2b). There was also no significant effect due to sex on EROD activity (ANCOVA, $F_{(1,83)} = 0.1677, p > 0.05$). Thus from these results there is no evidence to suggest that coral trout inhabiting reefs

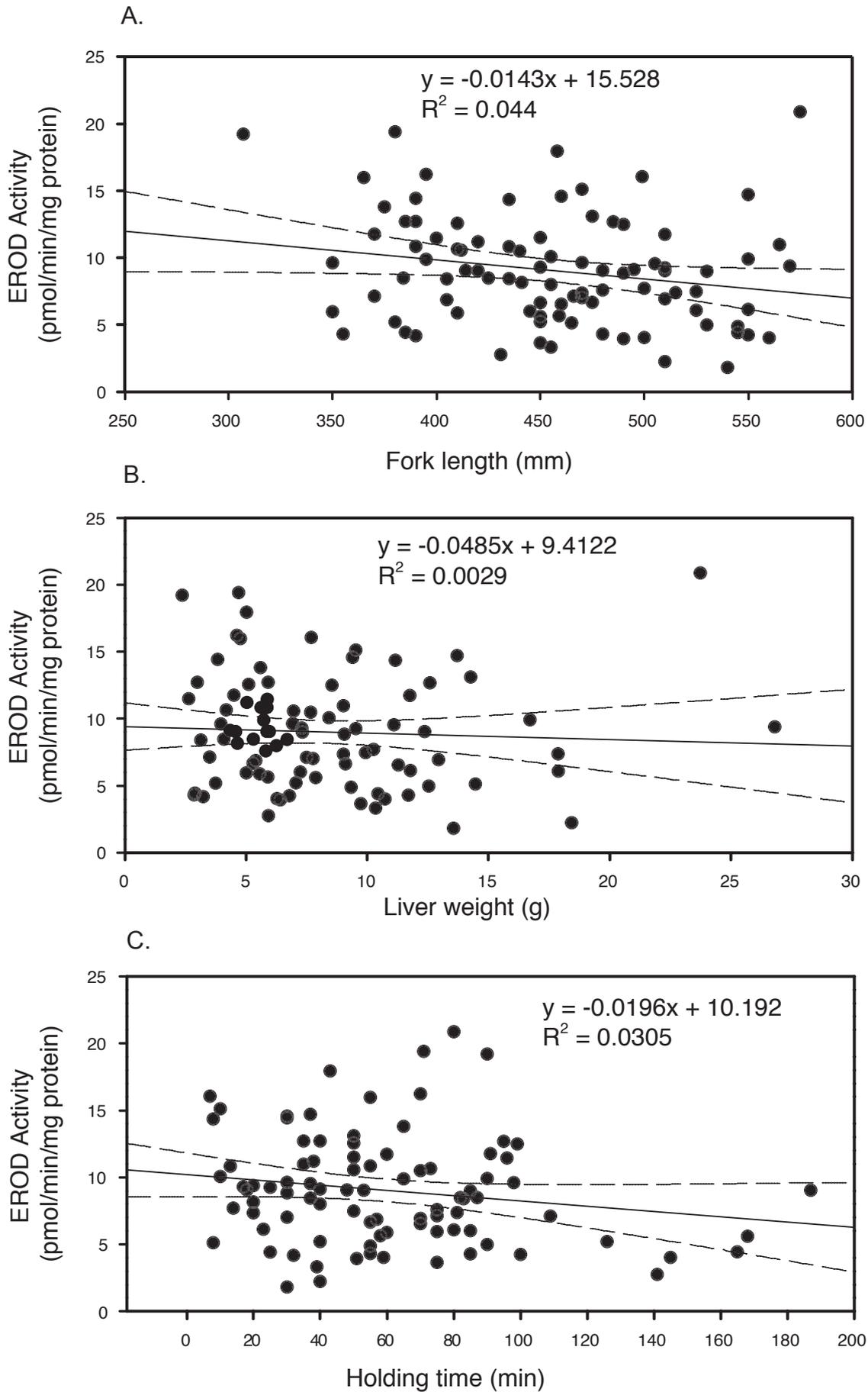


Figure 3. Variation in EROD activity in liver of *Plectropomus leopardus* in relation to (a) fish size, (b) liver size and (c) holding time.

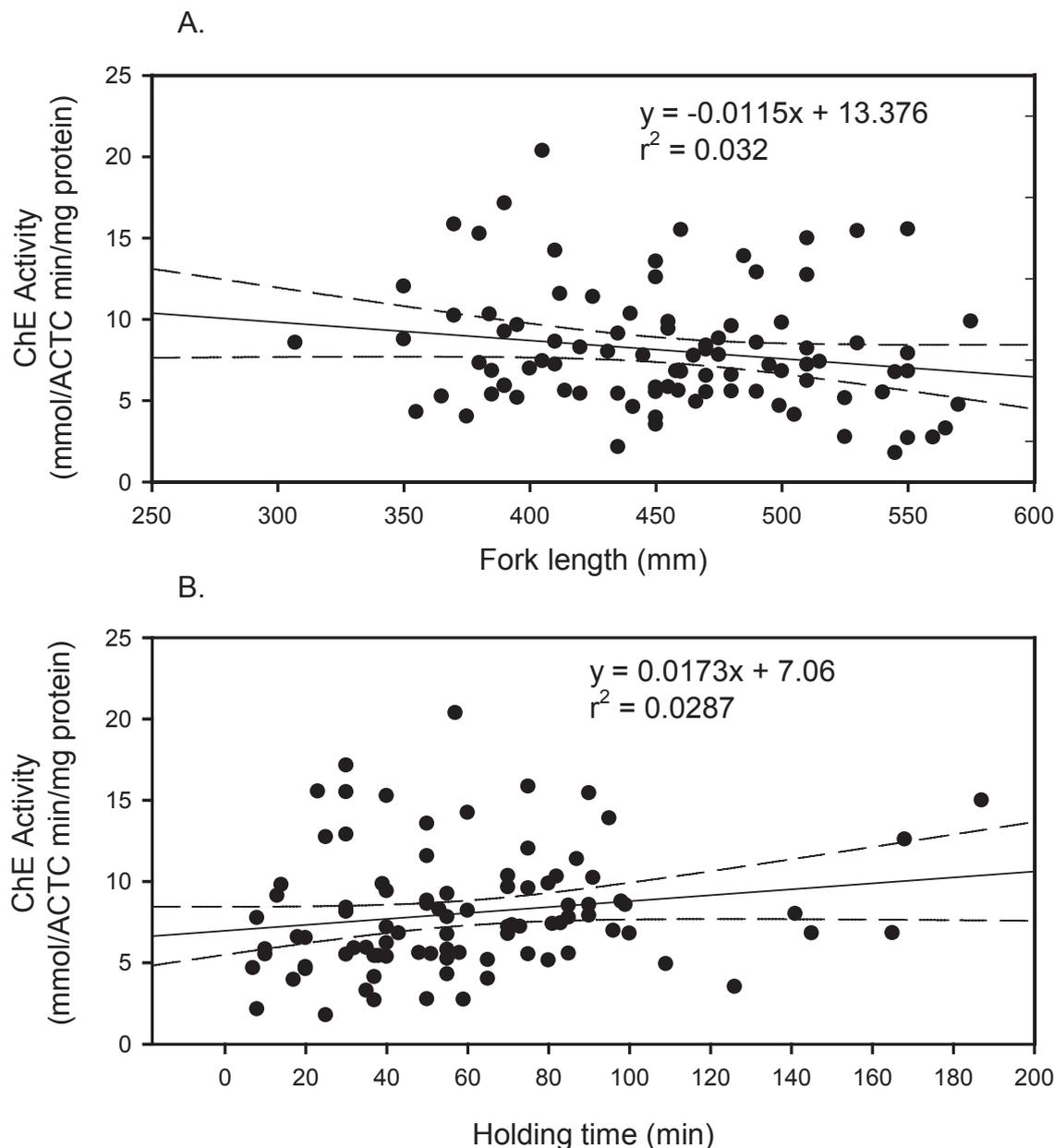


Figure 4. Variation in ChE activity in *Plectropomus leopardus* in relation to (a) fish size and (b) holding time.

where there is boat traffic and pontoons are more exposed to known contaminant inducers such as petroleum hydrocarbons than fish from other reefs. This is consistent with the above results on PAH metabolites in bile.

The presence of pontoons, and the associated high levels of boat traffic on reefs had a highly significant effect on the ChE activity in coral trout muscle (ANCOVA, $F_{(5,83)} = 14.61, p < 0.002$). There was no significant effect of sex of the fish on ChE activity (ANCOVA, $F_{(1,83)} = 0.0096, p > 0.05$). In general those fish sampled from reefs with pontoons tended to have lower enzyme reaction rates (average $6.86 \mu\text{mol ACTC min}^{-1} \text{mg protein}^{-1}$) than those from non-pontoon reefs (average $9.36 \mu\text{mol ACTC min}^{-1} \text{mg protein}^{-1}$) (Figure 2c). There was no significant difference between the three reference reefs, so these were pooled and a Dunnett's test carried out where each of the reefs with pontoons was compared

to the reference reefs. The Dunnett's test showed that both Agincourt and Moore Reefs were significantly different to the reference reefs. There was no significant difference between Norman Reef and the reference sites. ChE is a sensitive and specific indicator of toxic effects caused by exposure to chemicals such as pesticides and other compounds (Davies et al. 1994; Payne et al. 1996), some of which are commonly used in antifoulants such as copper (Garcia et al. 2000) and diuron (Bretaud et al. 2000). These results raise concern that activities around the reef pontoons may have resulted in contamination of the environment with cholinesterase-inhibiting chemicals.

Variables such as fish size and sex and handling of samples can be a source of error in EROD determinations (Krüner and von Westernhagen 1999). In this study there was a significant relationship between fork length and EROD activity ($R^2 =$

Table 1. Effect of the presence of pontoon, with sex as a co-variable, on bile metabolites, EROD activity and ChE activity in *Plectropomus leopardus*.

	No Pontoons			Pontoons		
	Endeavour Reef (n = 15)	Ruby Reef (n = 15)	Elford Reef (n = 15)	Agincourt Reef (n = 15)	Norman Reef (n = 15)	Moore Reef (n = 15)
Fork length (mm)	481±14	436±16	430±11	462±12	464±14	469±19
Weight (g)	1747±178	1302±144	1111±109	1488±119	1446±118	1614±181
Liver weight (g)	10.92±3.05	7.33±1.09	6.29±.061	7.90±0.87	7.78±0.77	9.07±1.26
HSI	0.64±0.05	0.60±0.04	0.64±0.04	0.57±0.05	0.58±0.04	0.61±0.05
K (x 10 ⁵)	1.34±0.02	1.37±0.03	1.31±0.03	1.37±0.04	1.30±0.02	1.35±0.02

Table 2. Length, weight, liver weight, condition factor (K) and hepatosomatic index (HSI) of *Plectropomus leopardus* from the six reefs.

	df	MS	F	p	Dunnett's test
(a) Bile metabolites					
Reef	5,49	11.977	0.9999	0.4278	
Sex	1,49	39.547	3.3013	0.0753	
(b) EROD activity					
Reef	5,83	27.994	1.7267	0.1432	
Sex	1,83	0.6680	0.0407	0.8405	
(c) ChE activity					
Reef	5,83	0.0428	4.2924	0.0016	Reference = Norman > Agincourt = Moore
Sex	1,83	0.0001	0.0096	0.9222	

0.033, $F_{(1,88)} = 4.08, p < 0.05$; Fig 3a) and between fish length and ChE activity ($R^2 = 0.047, F_{(1,88)} = 5.36, p < 0.02$; Fig 4a). However, the results were not biologically significant as fish size contributed to only 4 to 5% of the variability in EROD and ChE activity. Despite this small effect of fish size on both ChE and EROD activity, there was no significant difference in fish size between the six reefs sampled (ANOVA, $F_{(5,84)} = 1.821, p > 0.05$; Table 1). Fish sex had no effects on any of the biomarker activities (ANCOVA, Table 2). The period the fish were held alive between the time of capture and sacrifice (holding time) did not significantly affect EROD activity ($R^2 = 0.02, F_{(1,88)} = 2.90, p > 0.1$; Fig 3c) or ChE activity ($R^2 = 0.032, F_{(1,88)} = 3.95, p > 0.05$; Fig 4b). Liver weight ($R^2 = 0.003, F_{(1,88)} = 0.2726, p > 0.6$) had no significant effect on EROD activity (Fig 3b).

Two indices of general condition were investigated in coral trout from the six reefs: condition factor (K) and hepatosomatic index (HSI) (Table 1). The condition factor is based on the length-weight relationship of a fish and has often been used as an indication of general fitness of a fish (Bagenal and Tesch 1978; Bolger and Connolly 1989) and has also been used to investigate the effects of contaminants (e.g. Laroche et al. 2002; Bervoets and Blust 2003; Pyle et al. 2005). In this study no significant difference was found in the K of fish sampled from the six reefs (ANOVA $F_{(5,84)} = 1.2155, p > 0.1$). Similarly there was no significant difference in the HSI of the fish from the six reefs (ANOVA $F_{(5,84)} = 0.4232, p > 0.7$). The HSI is a measure of energetic reserves in the liver and metabolic activity and has been shown to increase in response to chemical contamination (Slooff et al. 1983).

This enlargement of the liver is due to either an increase in cell size (hypertrophy) or an increase in cell number (hyperplasia) (van der Oost et al. 2003).

CONCLUSIONS

The aim of the study was to test the hypothesis that tourist boat traffic associated with reef pontoons on the GBR was having a measurable impact on the reef environment by using biological indicators of exposure in coral trout, *Plectropomus leopardus*, a common and economically important reef fish. Petroleum hydrocarbons released from boat engines (e.g. n-alkanes, aliphatic and aromatic hydrocarbons), and antifouling agents derived from boat hulls (e.g. biocides and heavy metals), were expected to be potential chemical contaminants in this environment.

For the first time sensitive biomarkers of contaminant exposure (FACs, EROD, ChE, K and HSI) have been applied on coral reefs in GBR waters to evaluate coral trout for effects of exposure to hydrocarbons and other chemical contaminants derived from tourist boat traffic and pontoons. Analysis of FACs in bile suggests evidence of low-level exposure to naphthalene and its metabolites across all reefs, both those with pontoons and those without pontoons. EROD activity in livers of fish shows no evidence that fish living near to these pontoons are affected by contaminants such as petroleum hydrocarbons. Cholinesterase measurements did demonstrate that pontoon reef fish had significantly lower ChE activity as compared with fish from non-pontoon reefs and it is unclear at this stage the reason for this inhibition. Further study is warranted to confirm and investigate these findings.

Finally, the data reported in this study represent important ecotoxicological information for the Great Barrier Reef, and will provide a useful baseline reference for assessment of changes in environmental exposure in this highly valued and sensitive ecosystem.

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