Depurated fish as an alternative reference for field-based biomarker monitoring

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ABSTRACT

The entire Swan-Canning Estuary, south-western Australia, is impacted by human activity, and the selection of a reference site to assess the impact of contamination on the health of biota is not possible. To determine whether the use of fish depurated under laboratory conditions is a suitable substitute, adult *Acanthopagrus butcheri* were collected from the estuary and maintained in clean water (24 ppk) for 3 months. A suite of biomarkers were assessed, namely; mixed-function oxygenase enzymes [ethoxycoumarin-*O*-deethylase (ECOD) and ethoxyresorufin-*O*-deethylase (EROD)] activities, serum sorbitol dehydrogenase (s-SDH), naphthalene-, pyrene-, and benzo[*a*]pyrene-type biliary metabolites, DNA strand breaks, and heat shock protein (HSP70) levels. The results were compared to field captured black bream from three sites within the estuary (Ascot, Claisebrook, and Riverton), and to hatchery-bred juvenile fish. Biomarker levels were lower (up to 3.8 times) in depurated fish compared to field captured fish, while DNA integrity was higher. EROD activity was marginally lower in the hatchery-bred black bream than the depurated fish while s-SDH levels were 2 times higher in the hatchery fish, comparable to levels measured in the field. From the results obtained, field captured fish depurated for 3 months are suitable to determine reference/baseline levels for biomarker of health studies in estuarine environments.

Keywords: *Acanthopagrus butcheri*, alkaline unwinding assay, bile metabolites biomarkers, biomonitoring, DNA strand breaks, s-SDH, HSP70

INTRODUCTION

Defining the reference condition is considered to be an essential feature of ecosystem risk assessment to determine a baseline against which all experimental evidence is compared (Baird and Burton Jr 2001; Suter *et al.* 2007; Schmidt *et al.* 2009). However, this assumes that a reference baseline exists in each ecosystem that is constant, except when influenced by human activity (Kapustka 2008). Suter *et al.* (2007) defines different types of reference conditions, such as historical, self-reference, local, regional natural, regional acceptable, or no reference. None of these conditions can be applied to biochemical marker of fish health studies in estuaries such as the Swan-Canning Estuary (SCE) in south-western Australia, where anthropogenic inputs affect the whole system, with the exception of the no reference definition. This is in accord with Foran and Ferenc (1999) who state that a priori definition of a reference sites is not always possible especially where all sites within a study area are considered impaired.

The SCE is a highly modified, wave-dominated salt wedge estuary (NLWRA 2002) with an open (via training walls and dredging) connection to the ocean at Fremantle (Fig. 1). Like all estuarine environments this estuary is a complex ecosystem undergoing high levels of natural variability (Elliott and Quintino 2007; Schulte 2007). Physico-chemical conditions are extremely variable with daily and seasonal movements of the salt wedge within the estuary playing an important role in the distribution of soluble hydrocarbons at the sediment/surface water interface (Thomson *et al.* 2001; Twomey and John 2001; Westbrook *et al.* 2005).

The SCE is the focal point around which the city of Perth, with a population approaching 1.6 million people, has developed. There are no areas within its catchment that have not

been impacted by some form of human activity, either through urban, light industrial or agricultural development. Direct discharge, surface runoff and groundwater all have the potential to contribute nutrients, pesticides, fertilisers, heavy metals, and polycyclic aromatic hydrocarbons (PAHs) in the form of fuel and oil into the estuary (Foulsham *et al.* 2009; Nice 2009; Nice *et al.* 2009).

Fish biomarker of health studies in the Swan-Canning Estuary commenced in 2000 at a limited number of sites within the system (Webb and Gagnon 2002) after 170 years of expanding human development throughout the catchment. A historical baseline for biomarker studies in fish from this estuary is therefore not available. Estuaries similar to the SCE in south-western Australia are all influenced by anthropogenic inputs from agriculture, urbanisation and other human activity. Foran and Ferenc (1999) suggest assessing site results based on a gradient of biochemical response from low to extremely stressed. However studies to date show the proximity of major roads and stormwater drains, former landfill sites and industrial precincts, significant weather events, are the most significant factors affecting biomarker levels in biota. There is no evidence of gradients of biomarker responses up- or downstream in the SCE (Webb and Gagnon 2002; Webb *et al.* 2005a; b; c).

Depuration is a process designed to cleanse or remove contaminants from an animals gut or tissue. This process is often used in purging shellfish of pathogens (Barile *et al.* 2009), or to remove detritus from the gut of crustaceans (McClain 2000), to improve hygiene or palatability for human consumption. Other studies use depuration techniques to determine the bioaccumulation potential of lipophilic compounds in aquatic organisms (Ellgehausen *et al.* 1980).

In this study black bream (*Acanthopagrus butcheri*), a fish species endemic to estuaries in south-western Australia, were depurated in order to determine a baseline (reference) condition for the assessment of health of fish in the estuary using biochemical markers. Black bream were captured from the estuary and maintained under laboratory conditions using clean water replicating the physico-chemical conditions within the estuary for a period of three months. At the end of 3 months the depurated fish were sacrificed and a selection of biomarkers were compared with fish, of a similar size and age, collected directly from the estuary at the same time the laboratory depuration was terminated. Further comparison was undertaken for several biomarkers measured in juvenile fish sourced from a fish hatchery. It was hypothesised that laboratory depurated fish would be suitable to use to determine a reference state for fish health biomarker studies in estuarine environments.

METHODS

Depuration of Fish

Ten (10) black bream (*Acanthopagrus butcheri*) were collected from the Swan River in January 2006 by a commercial fisherman using a 120 m, 100 mm monofilament haul net then transferred to a 1000 litre tank at the Curtin Aquatic Research Laboratory with UV sterilised recirculating water filtration. The fish were maintained at 24ppk and 20^{o}C for 92 days, and fed human food quality mussels (*Mytilus* sp.). On the 92nd day the fish were sacrificed and tissue and fluid biopsies taken

Field collection

In April 2006, 56 black bream were collected from two sites in the Swan River (Claisebrook and Ascot; $N = 20$ respectively**)** and one site in the Canning River (Riverton; $N = 16$; Fig 1). The fish were sacrificed within 2 hour of capture and biopsies taken.

Hatchery-bred Fish

Twenty (20) juvenile black bream, purchased from the Challenger Institute of Technology hatchery Fremantle, Western Australia, had been maintained as control fish for an unrelated laboratory experiment. These fish were kept in 4 x 100 litre aquariums under similar conditions to the hatchery (37ppk, 20^{o}C) in semi-static water conditions with 50% daily water changes. These fish were sacrificed on day 21.

Sample Collection

Total weight, gutted weight and standard lengths were recorded for each fish. An external examination was conducted for abnormalities and any sign of parasite infestation. A blood sample was taken from the caudal vein using a vaccutainer, which was allowed to clot on ice for 15 minutes then centrifuged for 10 minutes at 3000 x g. Each fish was killed by the method of ike jime, bile collected from the gall bladder, and gill and liver biopsies taken. All samples were immediately placed in liquid nitrogen then later transferred to a freezer and held at –80 ^{o}C until analysis. Total protein content in the bile, and liver and gill supernatants, was determined using the method of Lowry *et al.* (1951).

Biomarker Analyses

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver supernatant using the methods of Hodson *et al.* (1991) adapted to black bream as detailed in Webb (2005). The fluorescence of the supernatant was read on a Perkin-Elmer LS-45 Luminescence

Spectrometer at excitation/emission wavelengths of 535/585 nm (slit 10 ex/10 em). EROD activity was expressed as picomoles of resorufin produced, per mg of total protein, per minute (pmol R mg Pr^{-1} min^{-1}).

Bile Metabolites - Three biliary metabolite-types were measured, naphthalene, pyrene and benzo(*a*)pyrene (B[*a*]P) by fixed wavelength fluorescence (FF) using the methods of Krahn *et al.* (1986) for pyrene-type metabolites and Lin *et al.* (1996) for both naphthalene-type and B(*a*)P-type metabolites. Biliary PAHs are standardised to biliary protein (metabolite mg $protein^{-1}$).

Serum sorbitol dehydrogenase (s-SDH) activity - The s-SDH assay was modified from the Sigma Diagnostics (St Louis, USA) Sorbitol Dehydrogenase Procedure No. 50-UV as described by Webb and Gagnon (2007). The linear decrease in the rate of absorbance (ΔA) over one minute was read on a Pharmacia UV-Visible Spectrophotometer at 340 nm. The s-SDH activity is expressed as milli-International Units (mU) mL^{-1} serum.

The following biomarkers were measured in the depurated and field captured fish only. Ethoxycoumarin-O-deethylase (ECOD) activity was measured in liver supernatant using methods previously detailed by Webb *et al.* (2005a). The fluorescence of the supernatant was read on a Perkin-Elmer LS-45 Luminescence Spectrometer at excitation/emission wavelengths of 380/452 nm. ECOD activity was expressed as picomoles of 7-hydroxycoumarin produced, per mg of total protein, per minute (pmol H mg Pr^{-1} min^{-1}).

DNA strand breakage - DNA strand breaks in liver supernatant was determined using the alkaline unwinding assay method of Shugart (1996). Incubation times and temperatures to

obtain partially unwound DNA (DSS) and single stranded DNA (SS) in each sample were optimized for black bream. Hoechst dye 33258 is used to bind with the isolated DNA in solution. When DNA is in the single-stranded form, the intensity of fluorescence of the bound dye is reduced to approximately one half of that observed for double-stranded DNA. This development constitutes the basis for determining the amount of double- and single-stranded DNA present in a sample of DNA during the alkaline unwinding assay (Shugart 1996). The fluorescence of the double stranded DNA (DS), DSS (incubated at 35^{o}C for 5 mins), and SS (incubated 85^{o}C, 30 mins) present in each sample was read on a Perkin-Elmer LS-5 Luminescence Spectrometer at excitation/emission wavelengths of 350ex/453em nm. The ratio of double-stranded DNA in the sample (F value) was calculated using the equation, F = (DSS - SS)/(DS - SS). The F value is a measure of DNA integrity with a high value corresponding to high DNA integrity.

Stress protein (HSP70) - Stress protein response was measured in gill supernatant using the methods of Martin et al. (1996) optimized for black bream as outlined in Webb and Gagnon (2009). HSP70 levels in the black bream are expressed as pixel density per μg of total protein (pixels μg pr^{-1}).

Statistical analysis

Data are presented as mean ± standard error (SE) and analysis was done using the SPSS statistical package (Version 17; SPSS GmbH, Germany). Where necessary, Log_{10} transformations were done for each biomarker to achieve normality and homoscedasticity. Student *t*-tests were carried out to determine whether any sexual differences were present for each biomarker ($\alpha = 0.05$). As no interactions were found in the data sets main effects were analysed using one-way ANOVAs. Where significant differences were found ($p <$

196 0.05), a Dunnett's (2 sided) test was run to compare the field and hatchery-bred fish with
197 the depurated fish group.
198
199 **RESULTS**
200 No significant differences were detected between male and female black bream for any
201 morphological measure, physiological indices, or biomarker analysed in this study ($p \geq$
202 0.05) so the results for both sex were pooled for each variable.
203
204 The length and weights of the black bream purchased from the hatchery were significantly
205 smaller than either the depurated fish or the freshly captured fish from the estuary (field
206 captured fish; $p \leq 0.001$; Table 1). The liver somatic index (LSI) of the hatchery-bred fish
207 was also much smaller compared to the other fish in the study ($p \leq 0.001$). However, the
208 hatchery-bred fish had a significantly higher condition factor (CF) compared to both the
209 depurated fish and the field captured fish ($p \leq 0.001$; Table 1). The depurated fish and the
210 field captured fish had similar morphology and physiological indices ($p \geq 0.05$; Table 1).
211
212 No significant differences were found in EROD activity ($p = 0.21$; Fig 2a), naphthalene-
213 type biliary metabolites ($p = 0.43$; Fig 3a), pyrene-type biliary metabolites ($p = 0.52$; Fig
214 3b) and B[*a*]p-type biliary metabolites (p=0.99; Fig 3c) measured in the hatchery-bred
215 black bream when compared with the depurated fish. Compared to the depurated fish, the
216 hatchery-bred black bream measured significantly higher s-SDH activity (p = 0.006; Fig 4)
217 which was at similar levels to the field collected fish.
218
219 *Field-captured vs. Depurated black bream*

EROD activity in the black bream was lower in the depurated fish than the field captured fish ($p \leq 0.001$). This difference was significant between the depurated fish and fish captured from Ascot ($p = 0.01$) and Claisebrook ($p \leq 0.001$) but not Riverton ($p = 0.81$; Fig 2a).

All biliary metabolites measured in the depurated black bream were lower than the field captured fish in this study. This difference was significant at Claisebrook for all metabolites (naphthalene and pyrene-type $p \leq 0.001$; B[*a*] P-type $p = 0.01$; Figs 3a, b, c). The fish collected from Ascot had significantly higher pyrene-type metabolites ($p = 0.016$; Fig 3b) while the fish from Riverton had higher B[*a*] P-type ($p = 0.02$; Figs 3c) when compared to the depurated fish.

The activity of s-SDH was lower in the depurated black bream compared to all field captured fish in this study but this difference was only significant when compared to the fish from Ascot ($p = 0.003$) and Claisebrook ($p = 0.004$; Fig 4).

ECOD activity was significantly lower in the depurated fish ($p = 0.003$) than the field captured black bream. This difference was statistically significant between the depurated fish and fish captured from Claisebrook ($p = 0.04$) but not Ascot ($p = 0.99$) nor Riverton ($p = 0.30$; Fig 2b).

The depurated black bream displayed higher DNA integrity than the field captured fish ($p = 0.01$), which was only significant compared to the fish from Riverton ($p = 0.004$). Ascot ($p = 0.06$) and Claisebrook ($p = 0.07$) also had lower DNA integrity but this was not statistically lower (Figure 5a).

HSP70 levels were lower in the depurated black bream compared to the field captured fish ($p = 0.01$) but this difference was only significant compared to Riverton ($p = 0.02$) and not Claisebrook ($p = 0.06$) or Ascot ($p = 0.93$; Fig 5b).

DISCUSSION

This study has clearly validated the use of depurated fish to establish baseline levels for a suite of biochemical markers of fish health in the black bream from the Swan-Canning Estuary.

The biomarkers measured in the depurated black bream are all significantly lower that those measured in the fish collected directly from the field. There was an almost 50% reduction in the Cytochrome (CYP) detoxification enzymes as measured by both EROD and ECOD activity induction levels. Serum SDH levels in the depurated were also up to 50% below the levels measured in the field fish demonstrating that the lower levels of EROD and ECOD levels were not due to liver damage. There was a 100 to 275% improvement in the levels of biliary metabolites measured. DNA integrity was higher in the depurated fish although this was only significant when compared to the fish captured from the Riverton site. Finally, HSP70 expression was between 119% lower that the black bream measured from Claisebrook and 155% lower than the fish analysed from Ascot indicating a reduction in oxidative stress levels. These results clearly show that the biomarker levels had come close to, or attained, baseline levels in the black bream which compares well to the results of Ferreira *et al.* 2007 using sea mullet (*Mugil cephalus*).

Ferreira *et al.* 2007 studied long term depuration on the levels of oxidative stress biomarkers in *M. cephalus*). The fish, chronically exposed to contaminants in the Douro estuary, were transferred to and maintained in unpolluted seawater for periods of 1, 4, and 8 months. The researchers found that the mullets had the capacity to recover oxidative damage following long term depuration. Mixed results were observed after 1 month depuration whereas the activities of antioxidant enzymes, as well as stress induced oxidative damaged proteins, had returned to normal values at 4 months.

Depurated fish also provided an improved benchmark when compared to hatchery-bred fish. The fish collected for depuration were adults of comparable size/age and with similar life histories as the fish sampled directly from the field whereas as the hatchery-bred fish were appreciably smaller juvenile fish (Table 1) that had been bred and reared under artificial conditions. Although EROD activity induction was lower in hatchery-bred fish, measured s-SDH levels were comparable to the highest readings measured in the field captured fish. This result suggests the possibility that some hepatocellular injury had occurred in the juvenile black bream impacting the CYP1A detoxification enzymes.

3. Implications for ERM

- According to Baird and Burton Jr (2001) the establishment of an appropriate benchmark or reference condition for comparison is required to assess biochemical responses of fish to contaminant exposure under field conditions.
- Preston 2002 – Reference sites in ecological risk assessment used to compare an impacted to a ‘pristine’ or non-impacted site. Often located in close proximity. Reference may be compromised by indirect results. E.g. periodic foraging of a fish in a relatively small contaminated patch may have adverse effects over a much larger

geographic area with adjacent areas. Adjacent non-contaminated ecosystems may still be affected at a distance by contamination and therefore not suitable as reference.

- Elliott and Quintino 2007 – Estuarine Quality Paradox – the difficulty in separating natural and anthropogenic stress in estuaries – repercussions for implementation of all environmental management systems reliant on ability to detect changes based on a defined reference condition.
- Lobry *et al.* 2006 - particularly difficult in estuarine situations to define a reference condition as estuaries are complex ecosystems and fluctuate with time (seasonally).
- This is the case in many estuarine environments, such as the Swan-Canning Estuary (SCE), where anthropogenic inputs affect the whole system.

4. Conclusions

From the results obtained, field captured fish depurated for 3 months are suitable to determine baseline levels and therefore the reference state for biomarker of health studies in estuarine environments.

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420 TABLE 1: Mean (± SEM) measurements and physiological indices of black bream
421 collected during autumn in the Swan-Canning Estuary. Within columns, values marked
422 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

Site	N	Standard length (cm)	Gutted weight (g)	Condition Factor[1]	Liver Somatic Index[2]
Depurated	7	26.1 ± 1.2	534 ± 59	2.98 ± 0.14	1.62 ± 0.27
Ascot	20	25.3 ± 0.4	460 ± 25	2.81 ± 0.03	1.24 ± 0.08
Claisebrook	10	24.8 ± 0.3	439 ± 12	2.87 ± 0.03	1.17 ± 0.04
Riverton	16	25.8 ± 0.8	517 ± 58	2.89 ± 0.03	1.56 ± 0.09
Hatchery bred	17	13.8* ± 0.03	91* ± 6	3.37* ± 0.07	1.02* ± 0.07
p-value		≤0.001	≤0.001	≤0.001	0.008

423 [1] Condition Factor = (gutted weight/standard lenght^3) x 100. [2] Liver Somatic Index = (Liver
424 weight/gutted weight) x 100.
425

426 **List of Figures**

427 **Figure 1.** Field collection sites within the Swan-Canning Estuary (adapted from Swan
428 River Trust, 1999).

429

430 **Figure 2**. Mixed function oxygenase activities (mean ± SEM) in black bream (A) EROD
431 activity (pmol R mg Pr^{-1} min^{-1}); (B) ECOD activity (pmol H mg Pr^{-1} min^{-1}). Bars marked
432 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

433

434 **Figure 3**. Biliary metabolites (mean ± SEM) in black bream; (A) naphthalene-type (mg
435 metabolite mg $protein^{-1}$); (B) pyrene-type (μg metabolite mg $protein^{-1}$); (C) B[*a*]p-type (μg
436 metabolite mg $protein^{-1}$). Bars marked with an asterisk are significantly different to the
437 depurated fish ($p \geq 0.05$).

438

439 **Figure 4**. s-SDH activity (mU; mean ± SEM) in the serum of black bream. Bars marked
440 with an asterisk are significantly different to the depurated fish ($p \geq 0.05$).

441

442 **Figure 5**. Biomarkers of effect (mean ± SEM) in black bream. (A) DNA integrity (F
443 value); (B) HSP70 levels (pixels μg pr-1). Bars marked with an asterisk are significantly
444 different to the depurated fish ($p \geq 0.05$).

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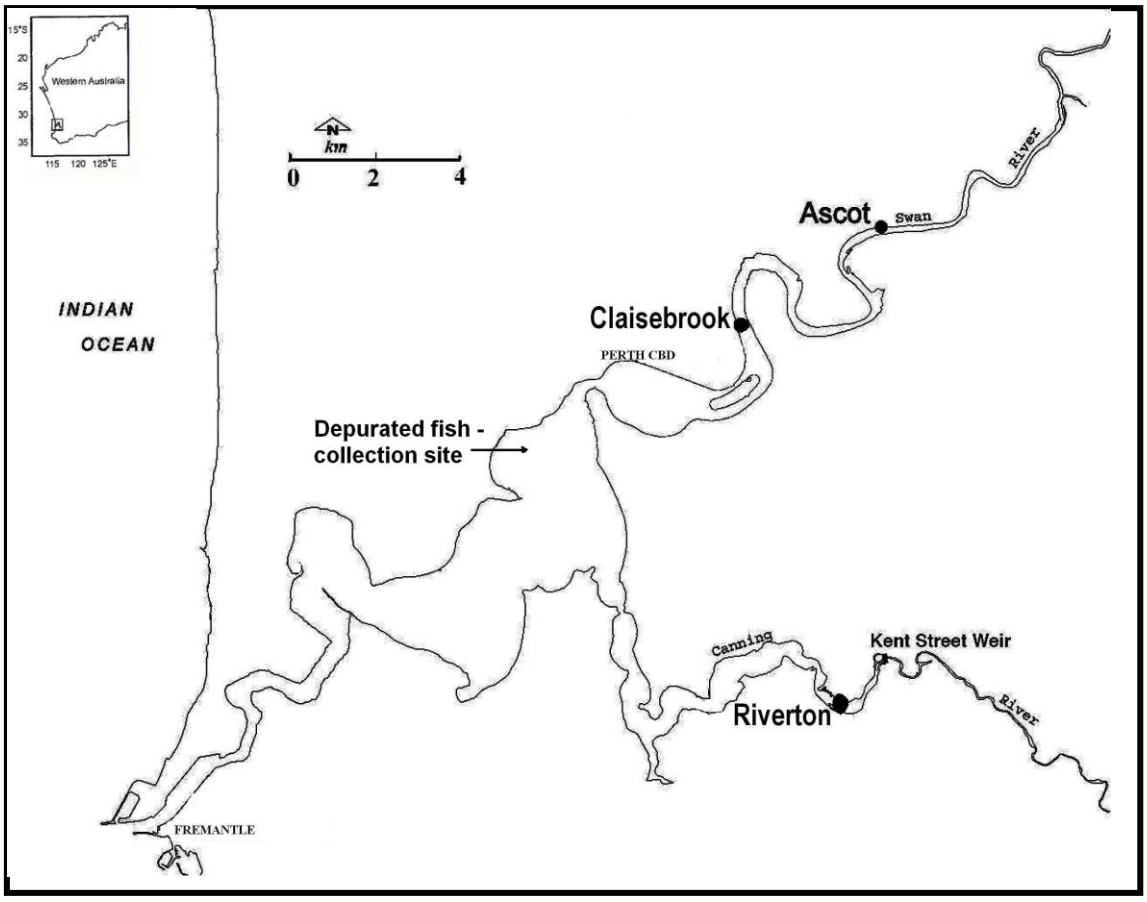
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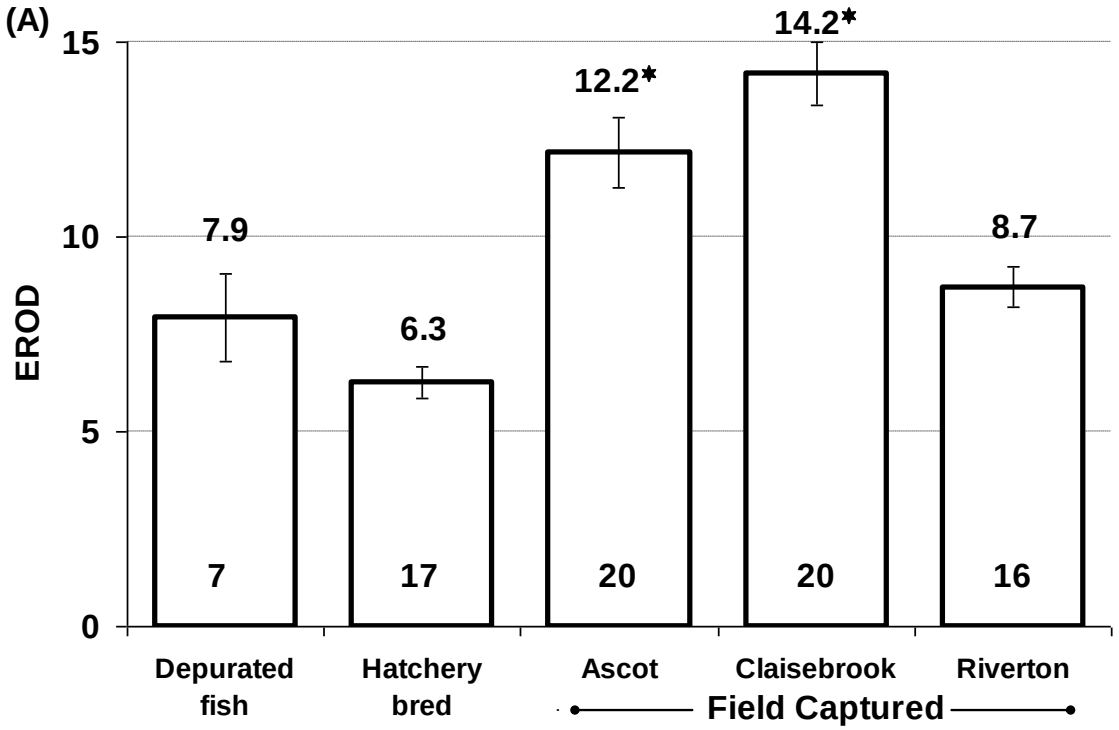

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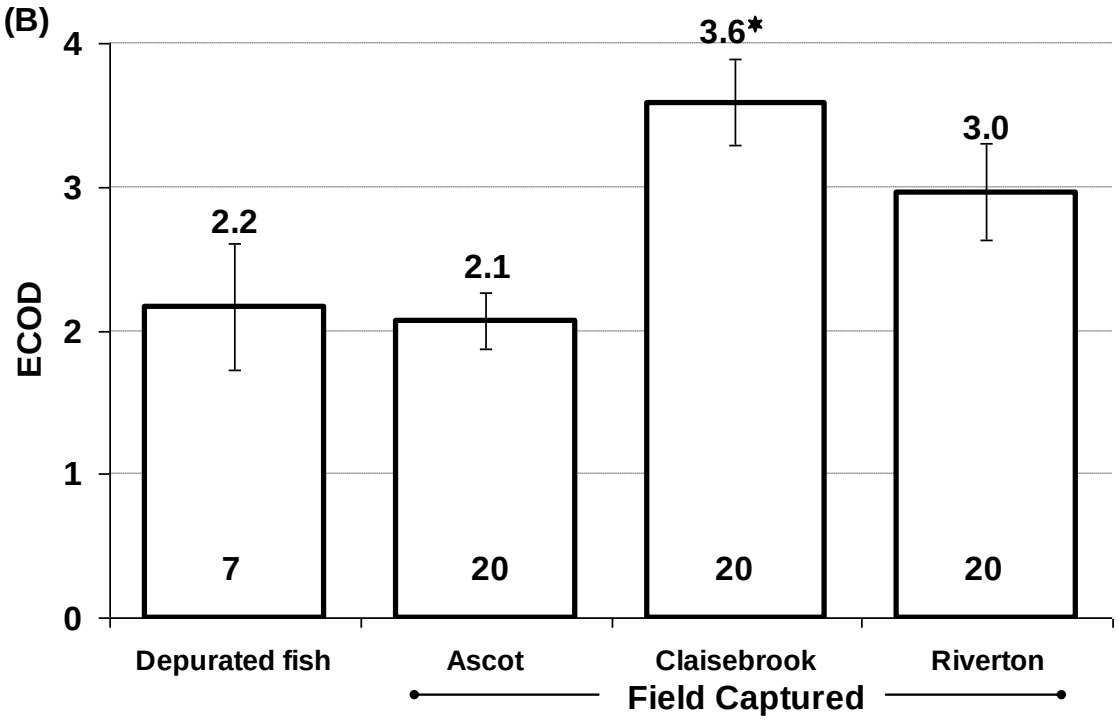
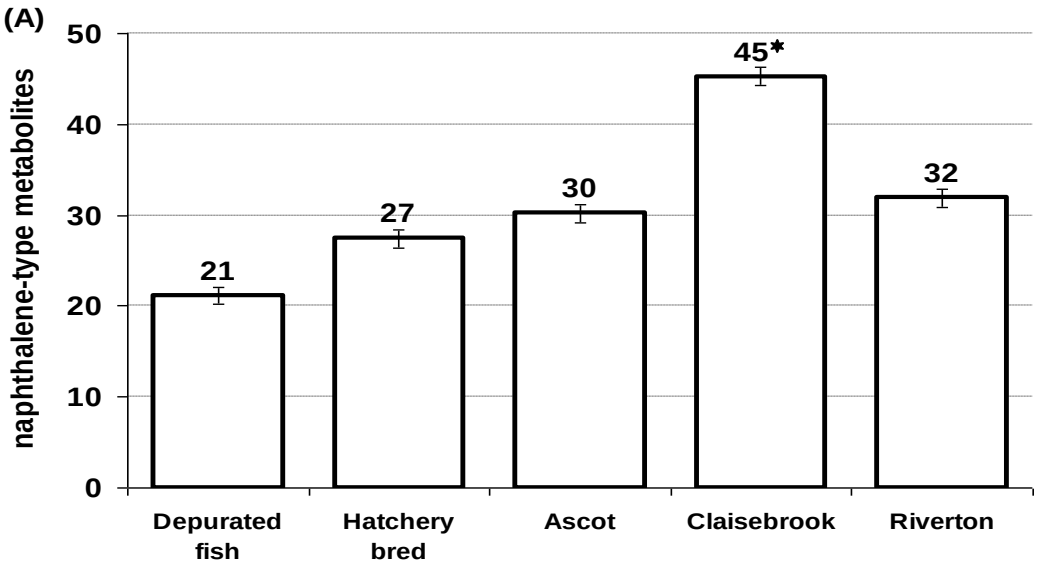
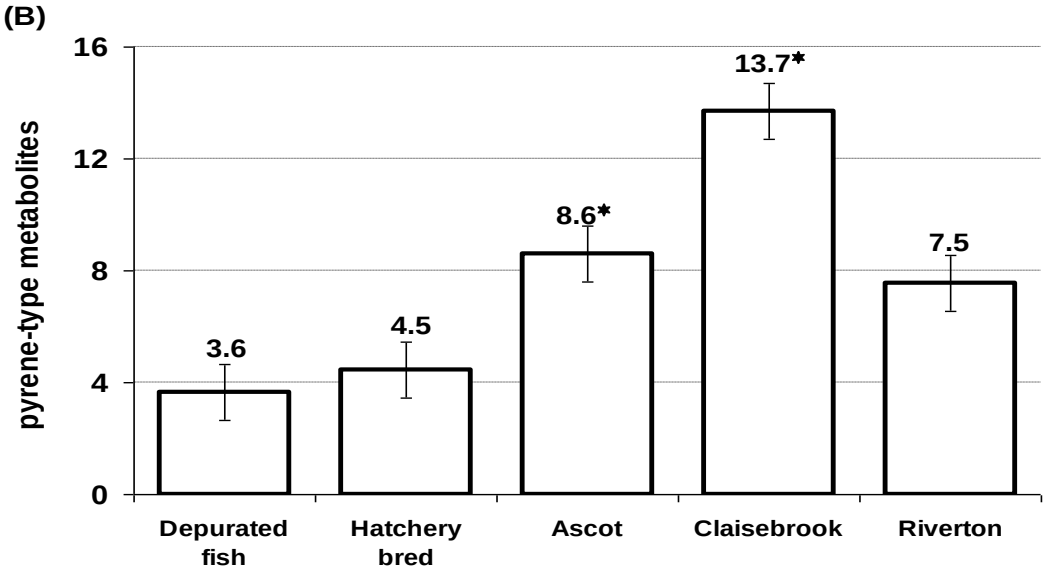


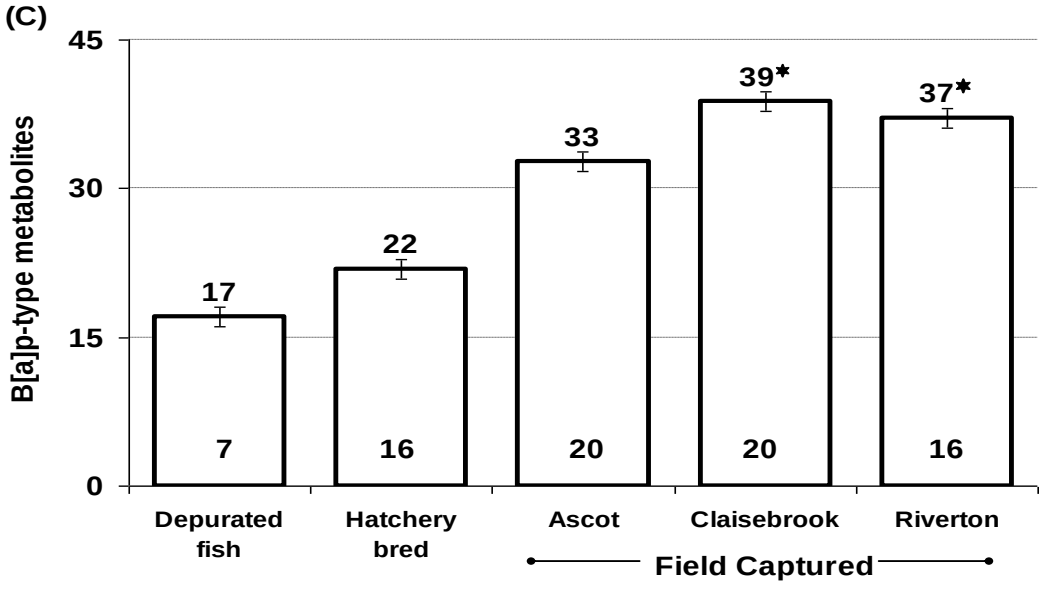
450
451



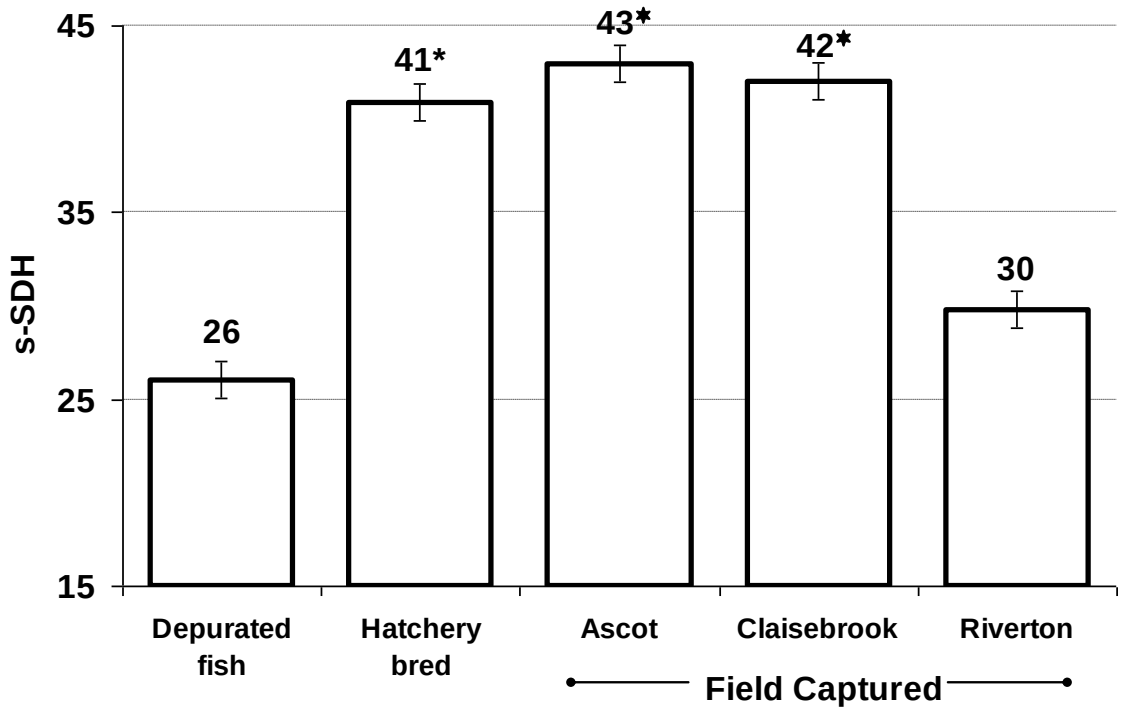
452



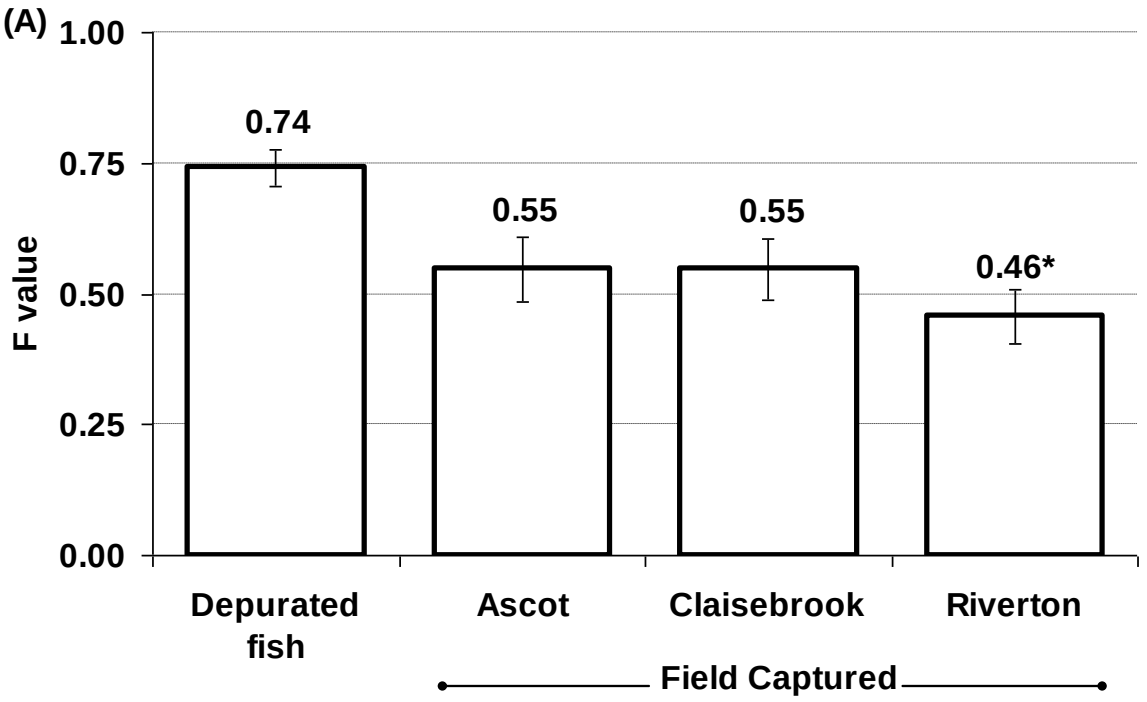
453



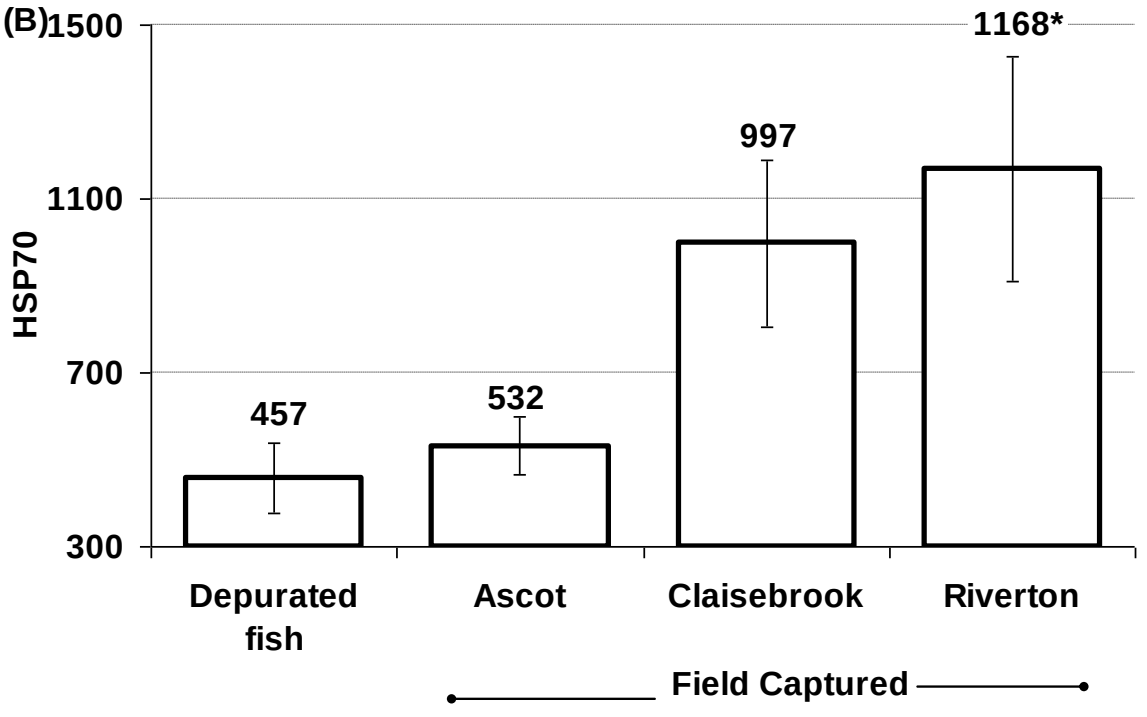
454
455


45
35
25
15
s-SDH
26
41*
43*
42*
30
Depurated fish
Hatchery bred
Ascot
Claisebrook
Riverton
Field Captured

456
457


(A)
1.00
0.75
0.50
0.25
0.00
F value
0.74
0.55
0.55
0.46*
Depurated fish
Ascot
Claisebrook
Riverton
Field Captured

458


(B)
1500
1100
700
300
HSP70
457
532
997
1168*
Depurated fish
Ascot
Claisebrook
Riverton
Field Captured

459