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Subcellular/tissue distribution and responses to oil exposure of the cytochrome P450-dependent monooxygenase system and glutathione S-transferase in freshwater prawns (*Macrobrachium malcolmsonii, M. lamarrei lamarrei*)

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Abstract Subcellular fractions (mitochondrial, cytosolic and microsomal) prepared from the tissues (hepatopancreas, muscle and gill) of freshwater prawns *Macrobrachium malcolmsonii* and *Macrobrachium lamarrei lamarrei* were scrutinized to investigate the presence of mixed function oxygenase (MFO) and conjugating enzymes (glutathione-S-transferase, GST). Cytochrome P450 (CYP) and other components (cytochrome b_5; NADPH-cytochrome *c* (CYP) reductase and NADH-cytochrome *c*-reductase activities) of the MFO system were predominantly present in the hepatic microsomal fraction of *M. malcolmsonii* and *M. lamarrei lamarrei*. The results are in agreement with the notion that monooxygenase system is mainly membrane bound in the endoplasmic reticulum, and that the hepatopancreas is the major metabolic tissue for production of biotransformation enzymes in crustaceans. Further, the prawns were exposed to two sublethal (0.9 ppt (parts per thousand) and 2.3 ppt) concentrations of oil effluent. At the end of 30th day, hydrocarbons and detoxifying enzymes were analysed in the hepatopancreas. The accumulations of hydrocarbon in the tissues gradually increased when exposed to sublethal concentrations of oil effluent and were associated with significantly enhanced levels of cytochrome P450 (180.6 ± 6.34 pmol mg^{-1} protein ($P < 0.05$ versus control, 136.5 ± 7.1 pmol mg^{-1} protein) for 2.3 ppt and 305.6 ± 8.5 pmol mg^{-1} protein ($P < 0.001$ versus control, 132.3 ± 6.8 pmol mg^{-1} protein] for 0.9 ppt of oil exposed *M. malcolmsonii*; 150 ± 6.5 pmol mg^{-1} protein ($P < 0.01$ versus control, 84.6 ± 5.2 pmol mg^{-1} protein) for 2.3 ppt and 175 ± 5.5 pmol mg^{-1} protein ($P < 0.01$ versus control, 87.6 ± 5.4 pmol mg^{-1} protein) for 0.9 ppt of oil exposed *M. lamarrei lamarrei*), NADPH cytochrome *c*-reductase activity (14.7 ± 0.6 nmol min^{-1} mg^{-1} protein ($P < 0.05$ versus control, 6.8 ± 0.55 nmol min^{-1} mg^{-1} protein) for 2.3 ppt and 12.1 ± 0.45 nmol min^{-1} mg^{-1} protein ($P < 0.01$ versus control, 6.9 ± 0.42 nmol min^{-1} mg^{-1} protein) for 0.9 ppt of oil exposed *M. malcolmsonii*; 12.5 ± 0.31 nmol min^{-1} mg^{-1} protein ($P < 0.001$ versus control, 4.6 ± 0.45 nmol min^{-1} mg^{-1} protein) for 2.3 ppt and 9.6 ± 0.32 nmol min^{-1} mg^{-1} protein ($P < 0.01$ versus control, 4.9 ± 0.41 nmol min^{-1} mg^{-1} protein) for 0.9 ppt of oil exposed *M. lamarrei lamarrei*) and cytochrome b_5 (124.8 ± 3.73 pmol mg^{-1} protein ($P < 0.01$ versus control, 76.8 ± 4.2 pmol mg^{-1} protein) for 2.3 ppt and 115.3 ± 3.86 pmol mg^{-1} protein ($P < 0.01$ versus control, 76.4 ± 4.25 pmol mg^{-1} protein) for 0.9 ppt of oil exposed *M. malcolmsonii* and 110 ± 3.11 pmol mg^{-1} protein ($P < 0.01$ versus control, 63.7 ± 3.24 pmol mg^{-1} protein) for 2.3 ppt and 95.3 ± 2.63 pmol mg^{-1} protein ($P < 0.01$ versus control, 61.4 ± 2.82 pmol mg^{-1} protein) for 0.9 ppt of oil exposed *M. lamarrei lamarrei*). The enhanced levels of biotransformation enzymes in oil-exposed prawns demonstrate a well-established detoxifying mechanism in crustaceans, and the response offers the possibility of use as a biomarker for the early detection of oil pollution.

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Introduction

Toxic bi-products of environmental chemicals have been studied at various subcellular levels in aquatic organisms (Peters et al. 1996). Accumulation of these metabolites can lead to toxic effects on organelle function of these organisms (Everaarts et al. 1998). Cytochrome P450-dependent monooxygenase systems are involved in the biotransformation of environmental chemicals and protection of aquatic organisms from the adverse effect of these hazardous substances (Livingstone 1991). In addition, the conjugating enzyme glutathione-S-transferase conjugates potentially harmful electrophilic reactive metabolites with endogenous glutathione and thus protects other nucleophilic centres such as protein and nucleic acids (George 1994). Even though much work has been carried out on cytochrome P450-dependent monooxygenase system in aquatic vertebrates, invertebrate species have received less attention (James and Boyle 1998). NADPH-cytochrome *c*-reductase activity is thought to principally measure CYP reductase activity, whereas NADH-cytochrome *c*-reductase activity reflects several components, including cytochrome b_5 and cytochrome b_5 reductase (Sato and Omura 1978; Lemaire and Livingstone 1994). In the present study, mitochondrial, cytosolic and microsomal fractions were prepared from the tissues (hepatopancreas, muscle and gill) of freshwater prawns *Macrobrachium malcolmsonii* and *Macrobrachium lamarrei lamarrei*. Cytochrome P450 and other components such as cytochrome b_5, NADH & NADPH-cytochrome *c*-reductase and GST activities were measured. Investigation on the existence of cytochrome P450-dependent monooxygenase system in freshwater crustaceans *M. malcolmsonii* and *M. lamarrei lamarrei* is of importance in the context of biomonitoring of aquatic pollution i.e., changes in the enzyme activities and MFO components of the system, in response to oil effluent could be used as a specific biomarker of oil pollution. Therefore changes of cytochrome P450 dependent monooxygenase components and enzyme activities were measured in the hepatopancreas of *M. malcolmsonii* and *M. lamarrei lamarrei* following exposure to 0.91 and 2.3 parts per thousand (ppt) of oil effluent (i.e., 10% and 25% of the 5-day-LC_{50}) for 30 days. Since the information on tissues and subcellular distribution of these enzymes and MFO components in crustaceans is limited, this study has been carried out on prawns. The main purposes of the study on the tissue distribution of selected biotransformation enzymes and MFO components in the subcellular fractions of prawn, are (i) to investigate the occurrence and possible mechanism of these enzymes at the cellular level and (ii) to find out whether these enzymes could serve as a biomarker to detect exposure to hydrocarbons in this species.

Materials and methods

Samples

The freshwater prawns (*M. malcolmsonii*; total length 6–6.5 cm, weight 2–2.5 g and *M. lamarrei lamarrei*; total length 4–4.5 cm, weight 1–1.5 g) were collected from river Cauvery (Tiruchirappalli, India) and were transported in aerated polythene bags to the laboratory. The prawns were held in tubs containing aerated and re-circulating freshwater. The prawns were acclimatized for 5 days before sacrifice of the animals for enzyme assay.

Tissue and subcellular fraction preparation

Prawns were decapitated and hepatopancreas, muscle and gill were dissected out for subcellular fractions. Pooled tissues were homnogenised in 20 mM Tris–HCl (pH 7.6) (1 g/4 ml) containing 0.25 M sucrose; 0.15 M KCl; 1 mM EDTA and 1 mM DTT and 100 μM PMSF. Subcellular fractions (mitochondrial, cytosol, microsomes) were prepared by the procedure of Livingstone and Farrar (1984). All the preparation procedures were carried out at 4°C. The homogenate samples were centrifuged at first on 600*g* for 10 min to sediment nuclei and cell fragments. Then, without transferring these fragments, samples were recentrifuged at 12,000*g* for 45 min. Consequently the supernatant was collected and used as a mitochondrial fraction. The resultant pellet was resuspended in homogenizing buffer and recentrifuged at 100,000*g* for 90 min, then, the supernatant was collected and used as a cytosolic fraction. Resuspension buffer consisting of 20 mM Tris pH 7.6, 1 mM Dithiothreitol, 1 mM EDTA and 20% v/v glycerol were added into the remaining pellet. This resuspended pellet was used as a microsomal fraction.

Exposure experiment

Based on a 5-day-LC_{50} value of oil effluent, sublethal concentration of 10% (0.9 ppt) and 25% (2.3 ppt) of LC_{50} were prepared and used for the exposure study. In this study, two sets of 10-l plastic tubs were used. In each tub prawns were exposed to 0.9 or 2.3 ppt of oil effluent. A control was also run simultaneously without the addition of oil effluent. After 30 days, the prawns were sacrificed for enzyme and hydrocarbon analysis.

Detection of oil derived hydrocarbons

Total hydrocarbons in oil and hepatopancreas were determined by the method of Law et al. (1988). Samples were extracted with hexane by using cylindrical glass separating funnel. The content of total hydrocarbon was measured with a fluorescence spectrophotometer (Ex. 310 nm; Em. 360 nm) using chrysene as a standard, since the pure aromatic compound chrysene strongly absorbs UV fluorescence intensity at 310 nm. Results are expressed as μg/g tissue in terms of chrysene equivalents.

Biochemical analysis

Total cytochrome P450 content was measured by the carbon monoxide difference spectrum of sodium dithionate reduced sample using an extinction coefficient of 91 mM^{-1} cm^{-1} (Livingstone and Farrar 1984). Cytochrome b_5 content was measured by reduction with NADH, using an extinction coefficient of 185 mM^{-1} cm^{-1}. The final NADH concentration was 30 μM (Estabrook and Werringloer 1978). NADH and NADPH-cytochrome *c*-reductase activities were measured by the method of Shimakata et al. (1972). The reaction mixture contained 1 mM KCN, 60 μm cytochrome *c* and 0.3 mM of NADPH or NADH in 10 mM Tris–HCl (pH 7.6). The increase in absorbance of cytochrome *c* was measured at 550 nm and the activity was calculated using an extinction coefficient of 19.6 mM^{-1} cm^{-1}. Glutathione-S-transferase was assayed according to Habig et al. (1974) with 1-chloro-2,4-dinitrobenzene as substrate. The standard assay mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM 1-chloro-2,4-dinitrobenzene and 50 μl of enzyme source. The complete assay mixture without enzyme was used as a control and the rate of the reaction was measured as the increase in absorbance at 340 nm. The chemical rate of the reaction was determined in the absence of the sample, which was subtracted from the total rate (1 μmol of GSH conjugated min^{-1} mg^{-1} protein was defined as one unit of GST). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Statistical analysis

Multiple values were presented as mean ± SEM (standard error of mean) for three determinations. Groups of values were analysed by Student's *t*-test and $P < 0.05$ was considered as statistically significant.

Results and discussion

In the present study, cytochrome P450 was predominantly found in the microsomal fraction of the hepatopancreas (Table 1). The maximum spectrum (CYP) of the carbon monoxide bound reduced hemoprotein was 450 nm. On the other hand, it was not detectable in mitochondrial and cytosolic fractions of gill and muscle. Cytochrome P40 content in the hepatic microsomal fraction was many folds higher than cytosolic fraction (no activity in mitochondrial fraction). Similar results were also obtained in mussel by Livingstone and Farrar (1984) who opined that during homogenization some materials from the endoplasmic reticulum get solubilised which accounts for the lesser amounts of cytochrome P450 and cytochrome b_5 in the cytosolic fraction of *Mytilus edulis*. An earlier study on cray fish (*Astacus astacus*) reported that the content of cytochrome P450 was found to be 1/100th of the rat cytochrome P450 (Lang et al. 1977). But in the case of *M. malcolmsoni*, the hepatic microsomal CYP 450 content was only seven-fold lower when compared to rat liver cytochrome P450. This variation may be partly due to the progress in methodology, in particular the use of enzyme stabilizers and protease in buffer e.g., protease inhibitor dithiothreitol, PMSF (phenymethylsulfonyl fluoride) (Lindstrom Seppa et al. 1983).

The cytochrome P450 and other components (NADH and NADPH-cytochrome *c*-reductase and cytochrome b_5) found in *M. malcolmsonii* and *M. lamarrei lamarrei* clearly indicate the existence of cytochrome P450-mediated mixed function-oxidase system in both these crustacean species. The present data of MFO components are comparable to those of other crustacean species (Table 2). Differences are also noted while comparing the MFO components in *M. malcolmsonii* and *M. lamarrei lamarrei* with other crustacean species. The level of cytochrome P450 for *M. malcolmsonii* was lower than that of blue crab (*Callinectes sapidus)* and higher than that of aeron barnacle (*Balanus eburnus*). On the other hand, in *M. lamarrei lamarrei*, the level of cytochrome P450 was lower than that of many other crustacean species (blue crab, cray fish, *M. malcolmsonii* and *Aeron barnacle*). In contrast the specific activity of NADPH-cytochrome *c*-reductase in *M. malcolmsonii* exhibits higher level than that of other crustacean species (cray fish, blue crab and spiny lobster). In the case of *M. lamarrei lamarrei* this activity was similar to those recorded for spiny lobster and less than those for blue crab and *Aeron barnacle*. Thus, variation of MFO components between different species was due to species variation as was described by Livingstone and Farrar (1984) in molluscan species. In *M. malcolmsonii* and *M. lamarrei lamarrei*, the specific activity of NADH and NADPH-cytochrome *c*-reductase in all the tissues (hepatopancreas, muscle and gill) were higher in the microsomal fraction than the mitochondrial and cytosolic fractions. Livingstone and Farrar (1984) also observed higher NADH and NADPH-cytochrome *c*-reductase activity in the

Table 1 Tissue/subcellular distribution of cytochrome P450-dependent monooxygenase and glutathione-S-transferase in *M. malcolmsonii* and *M. lamarrei lamarrei*

Enzymes	Hepatopancreas			Muscle			Gill		
	Mitochondrial	Cytosol	Microsomes	Mitochondrial	Cytosol	Microsomes	Mitochondrial	Cytosol	Microsomes
CYP[a]									
M. malcolmsonii	ND	6.8 ± 1.02	127 ± 3.01	ND	ND	5.5 ± 1.32	ND	ND	10.2 ± 1.52
M. lamarrei lamarrei	ND	5.6 ± 1.10	88.5 ± 4.44	ND	ND	4.8 ± 0.69	ND	ND	6.4 ± 0.66
NADPH-Cyt c-*red*[b]									
M. malcolmsonii	3.2 ± 0.37	2.3 ± 0.30	6.5 ± 0.40	0.5 ± 0.12	1.2 ± 0.27	2.1 ± 0.28	0.8 ± 0.11	2.6 ± 0.14	3.8 ± 0.26
M. lamarrei lamarrei	2.8 ± 0.23	2.1 ± 0.23	4.3 ± 0.20	0.2 ± 0.08	0.8 ± 0.11	1.7 ± 0.14	0.4 ± 0.12	1.6 ± 0.16	2.4 ± 0.23
NADH-Cyt c-*red*[b]									
M. malcolmsonii	5.2 ± 0.58	14.5 ± 1.09	27.4 ± 3.53	2.1 ± 0.32	6.32 ± 0.45	9.84 ± 0.54	3.0 ± 3.51	8.5 ± 0.49	13.45 ± 1.27
M. lamarrei lamarrei	4.3 ± 0.37	11.8 ± 0.56	18.7 ± 0.72	1.5 ± 0.23	4.2 ± 0.40	6.32 ± 0.65	2.15 ± 0.30	6.2 ± 0.52	9.83 ± 0.86
Cyt b5[a]									
M. malcolmsonii	ND	12.4 ± 1.65	77.5 ± 2.68	ND	8.5 ± 1.11	12.5 ± 1.08	ND	6.8 ± 0.69	38.5 ± 3.34
M. lamarrei lamarrei	ND	9.5 ± 1.30	62.4 ± 2.60	ND	7.4 ± 0.98	8.5 ± 1.12	ND	3.2 ± 0.23	21.5 ± 2.48
GST[c]									
M. malcolmsonii	0.587 ± 0.012	1.15 ± 0.042	1.04 ± 0.025	0.328 ± 0.007	0.671 ± 0.002	0.512 ± 0.003	0.125 ± 0.005	0.328 ± 0.007	0.283 ± 0.0035
M. lamarrei lamarrei	0.283 ± 0.004	1.06 ± 0.076	0.482 ± 0.005	0.185 ± 0.006	0.310 ± 0.005	0.232 ± 0.007	0.096 ± 0.002	0.217 ± 0.002	0.165 ± 0.0042

ND, Not detectable

[a] pmol mg^{-1} protein

[b] nmol min^{-1} mg^{-1} protein

[c] μmol min^{-1} mg^{-1} protein

Table 2 Cytochrome P450 (CYP) and NADPH-cytochrome *c*-reductase activities in crustacean species

Species	CYP (pmol mg^{-1}protein)	NADPH-cyt c red (nmol min^{-1} mg^{-1} protein)	Reference
Blue crab (*Callinectes sapidus*)	180	5.2	James et al. 1979
Aeron barnacle (*Balanus eburneus)*	110	28.6	Stegeman and Kaplan 1981
Cray fish (*Astacus astacus*)	127–310	2.23	Lindstrom-Seppa and Hanninen 1986
Spiny lobster (*Panulirus argus*)	910	4.3	James et al. 1979
Crab *(Carcinus aestuarii)*	–	~17	Fossi et al. 1998
Prawn (*M. malcolmsonii*)	126	6.5	Present study
M.lamarrei lamarrei	88	4.3	Present study

microsomal fraction of *Mytilus edulis*. Cytochrome b_5 content predominantly occurred in the microsomal fraction of all tissues followed by cytosolic fraction. But in the mitochondrial fraction, cytochrome b_5 was undetectable in both *M. malcolmsonii* and *M. lamarrei lamarrei*.

The phase II metabolizing (conjugating) GST activity was measured in various subcellular fractions of *M. malcolmsonii* and *M. lamarrei lamarrei*. Generally, the GST specific activity was two-fold higher in *M. malcolmsonii* than *M. lamarrei lamarrei* (Table 1). From this result it is presumed that the potential for glutathione conjugation is indicated to be higher in *M. malcolmsonii* than *M. lamarrei lamarrei*. In both species, the specific activity was higher in the cytosolic fraction of all tissues than other fractions. Nimmo et al. (1981) also observed a higher activity of GST in the cytosolic fraction of trout than microsomal, nuclear and mitochondrial fractions. Further, they concluded that the catalytic action for detoxification is mainly by cytosol rather than that of the microsomal fraction. In *M. malcolmsonii* and *M. lamarrei lamarrei*, it is possible that higher amount of GST present in the cytosolic fraction may play a major role in detoxification of xenobiotic compound. Thus, determination of activities of MFO and conjugating enzymes in the different subcellular fractions helps to gather knowledge of the metabolism of xenobiotics in the cellular compartment of freshwater prawns.

In Western countries, an increased number of studies have been carried out on biotransformation enzymes of aquatic organisms as possible biomarkers for detecting organic pollution in the aquatic medium (Livingstone 1993; Fossi et al. 1998). In contrast, relatively few studies have been conducted in the Asian region, particularly in

Table 3 Levels of total hydrocarbons and biotransformation enzymes in the hepatopancreas of *M. Malcolmsonii* and *M. lamarrei lamarrei* during oil effluent exposure

	M. malcolmsonii		*M. lamarrei lamarrei*	
	Control	30th day	Control	30th day
Hydrocarbon levels				
2.3 ppt	ND	32.5 ± 2.1	ND	25.4 ± 2.4
0.9 ppt	ND	20.4 ± 1.6	ND	19.3 ± 1.8
CYP				
2.3 ppt	136.5 ± 7.1	180.6 ± 6.34*	84.6 ± 5.2	150 ± 6.5**
0.9 ppt	132.3 ± 6.8	305.6 ± 8.5***	87.6 ± 5.4	175 ± 5.5**
NADPH cyt c-*reductase*				
2.3 ppt	6.8 ± 0.55	14.7 ± 0.6**	4.6 ± 0.45	12.5 ± 0.31***
0.9 ppt	6.9 ± 0.42	12.1 ± 0.45*	4.9 ± 0.41	9.6 ± 0.32**
NADH cyt c-*reductase*				
2.3 ppt	28.5 ± 2.82	20.6 ± 2.45	21.4 ± 1.4	14.3 ± 1.25
0.9 ppt	28.8 ± 2.53	25.6 ± 3.15	21.4 ± 1.86	22.4 ± 1.7
Cyt b_5				
2.3 ppt	76.8 ± 4.2	124.8 ± 3.73**	63.7 ± 3.24	110 ± 3.11**
0.9 ppt	76.4 ± 4.25	115.3 ± 3.86**	61.4 ± 2.82	95.3 ± 2.63**
GST				
2.3 ppt	1.18 ± 0.055	2.75 ± 0.082***	1.07 ± 0.083	2.83 ± 0.075***
0.9 ppt	1.2 ± 0.062	2.53 ± 0.075***	1.15 ± 0.076	2.12 ± 0.088***

Hydrocarbons, μg/g tissue; CYP, pmol mg^{-1} protein; NAD(P)H cyt *c*-red, nmol min^{-1} mg^{-1} protein; Cyt b_5, pmol mg^{-1} protein; GST, μmol min^{-1} mg^{-1} protein

Groups of values were analysed by Student's *t*-test and $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ were considered as statistically significant

India (Monirith et al. 2003; Pandey et al. 2003). Hence, we made an attempt to study whether biotransformation enzymes in the prawns were able to respond as a biomarker of oil pollution. Accumulation of hydrocarbons, in hepatopancreas of prawns, exposed to two sublethal concentration (0.9 and 2.3 ppt) of oil effluent, increased gradually (the levels were observed for every 7 days interval, results not shown), and many fold increased accumulation was observed at day 30 (Table 3). Similarly, the contents of cytochrome P450 and cytochrome b_5 and the activities of NADPH-cytochrome *c*-reductase and GST were increased significantly when compared to control prawns (Table 3). No difference was observed in the level of NADH-cytochrome *c*-reductase activity. From the above results, it is therefore possible that in freshwater prawns the enhanced level of microsomal electron transport components are involved in the metabolism of accumulated hydrocarbons. Further, the primary metabolites of the hydrocarbons may then be metabolized by enhanced GST.

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