

2007

EVALUATING THE IMPACT OF ORGANIC CONTAMINATION UPON THE PHYSIOLOGY OF THE SHORE CRAB *Carcinus maenas* (L.)

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<http://hdl.handle.net/10026.1/11113>

<http://dx.doi.org/10.24382/4708>

University of Plymouth

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EVALUATING THE IMPACT OF
ORGANIC CONTAMINATION UPON THE PHYSIOLOGY
OF THE SHORE CRAB *CARCINUS MAENAS* (L.)

AWANTHA DISSANAYAKE

PhD 2007

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**EVALUATING THE IMPACT OF ORGANIC CONTAMINATION
UPON THE PHYSIOLOGY OF THE SHORE CRAB *Carcinus
maenas* (L.)**

by

AWANTHA DISSANAYAKE

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences,
University of Plymouth

OCTOBER 2007

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Awantha Dissanayake

October 2007

Dedicated to Thathi

ACKNOWLEDGEMENTS

I would like to start by thanking my supervisors Prof. Tamara Galloway and Prof. Mal Jones for giving me this opportunity to fulfil a childhood dream of pursuing a PhD, trusting and allowing me to pursue my research ideas, for which I am eternally grateful.

To those there at the beginning. Muchas gracias para Ivan "El Puto" Ford y Darren "El Profe" Torres; por tu amistad durante todo los años, siempre estaras en mi corazon.

A big shout out to the Green Park Avenue Posse: Mark Browne and Ana Silva. Mark, for your many nuggets of Bristolian "wisdom". Ana, many thanks for your "An-eurisms" which have kept me amused for the past three years. To both of you, best of luck in your careers, I know you will be very successful!

A massive thank you to the Ecotox "family", both past and present members, for an enjoyable and supportive "working" environment. Vikki Cheung, Jo Hagger, Anne O'Neill, Giles Watson, Alan Scarlett, Martin "Weeman" Canty, Marie Hannam, Chris Pook and Ceri Lewis and Trevor Worsey. Specifically, to the members of "Da Ghetto" (Davy 415), Weeman, your "*you can never polish a turd*" quote, will remain with me forever. Marie, "Cheers then drive". To both of you, thanks for a working environment which was never quiet or dull! Chris "Dr Ninja" Pook, cheers for those Mount Gay evenings, I've never known a man who could get so enthusiastic about working with faeces. Many thanks also to Trev "T" Worsey, for his help with filming and video editing. Stan McMahon for his logistical help and friendship over the years. Noel Gary for aquarium tanks and fighting chamber construction. To Dave Lowe and Miké Kendall, my gratitude to both of you for your mentoring.

To all those too numerous to mention, but you know who you are, many thanks for making Plymouth my home. I would like to thank also those numerous watering holes (and those reprobates that frequent them) around North Hill, that made my social life interesting!

I worked at UOP, "lived" in Cuba, but my home is definitely Plymouth.

And the best for last, this thesis is dedicated to my father for his love, support and his belief in me.

So finally, to all those people whose paths have crossed with mine. To those, for inspiring me positively, my heartfelt gratitude and those negatively, this thesis is testament to my ambition in life and I have proved you wrong!

Evaluating the impact of organic contamination upon the physiology of the shore crab *Carcinus maenas* (L.)

Awantha Dissanayake

THESIS ABSTRACT

This thesis has focused on answering fundamental questions regarding the 'normal' physiological ranges of the male shore crab *Carcinus maenas*. Knowledge of the responses to environmental variables is needed before the effects of anthropogenic stress can be identified. Anthropogenic stress was imposed in the form of contamination by a ubiquitous priority pollutant of the aquatic environment (polyaromatic hydrocarbon, pyrene).

The first hypothesis focused on identifying the physiological differences between juvenile and adult male shore crabs. Differences at the cellular level (cell and immune function) determined the relative contaminant sensitivity between the two ontogenetic stages, with juveniles expressing increased physiological sensitivity to contaminant-imposed effects compared to adults.

Further questions relating to how the physiological condition of *Carcinus maenas* was altered by nutritional stress were examined in the adult stage. Physiological 'competency' or tolerance was shown to be dependent upon organism nutritional status. Shore crab physiological condition was robust to short-term starvation. This is interpreted to result from autophagy induction, whereby, when diet is restricted, energy is released via metabolism of protein, carbohydrate and lipid stores. The physiological implications of sublethal contaminant exposure under short-term induction included increased antioxidant status, signalling activation of compensatory mechanisms under contaminant-mediated challenge. The behavioural implications of nutritional status and contaminant exposure were investigated by staging intraspecific agonistic contests between pairs of shore crabs for a food resource.

Behavioural evidence revealed that the competitive ability (resource holding potential) of individuals was higher in pyrene-exposed compared to unexposed crabs, with higher proximate associated costs (energy expenditure) of entering agonistic contests in starved compared to fully-fed individuals. Shore crab competitive ability was concluded to be dependent upon the physiological condition of the contestant.

The final hypothesis investigated 'seasonal' differences in the physiology of *Carcinus maenas* to test whether there were any 'windows of sensitivity' to both environmental and contaminant-imposed challenges. The 'normal' pattern of seasonal variability was assessed from crabs collected from the Avon Estuary. Differences included higher immune function and lower antioxidant status between winter and spring compared to summer to autumn. These seasonal differences were shown to impact on the ability of shore crabs to respond to PAH exposure. Seasonal evaluation of shore crab physiological condition from estuaries of varying PAH input [Avon Estuary (low anthropogenic exposure) and Plym Estuary (relatively high anthropogenic exposure)], revealed significant seasonal differences between crabs as signalled by cellular endpoints (cellular integrity and viability) between January and June compared to July to December.

In summary, this study has revealed that shore crab physiology varies with intrinsic (age, nutritional status) and extrinsic (temperature) factors. Therefore, it is essential to establish the full extent of the 'normal' physiological ranges for *C. maenas* physiology to completely understand the impact of environmental and anthropogenic stress alike.

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee. This study was financed with the aid of a studentship from the University of Plymouth.

A programme of advanced study was undertaken, which included postgraduate courses on teaching/demonstrating skills. Relevant scientific seminars and conferences were regularly attended at which work was presented and several conference abstracts were prepared for publication.

PUBLICATIONS

- Dissanayake, A., Galloway, T. S. and Jones, M. B. (2005). Does size really matter? Ontogenetic differences in 'physiological competency' in *Carcinus maenas* (L.). *International Crustacean Congress: Symposium 4. The ecophysiology of crustaceans. Glasgow, Scotland*, pp.42.
- Dissanayake, A., Galloway, T. S. and Jones, M. B. (2006). Does size really matter? An evaluation of ontogenetic differences in the biomarker responses of *Carcinus maenas* (L.) to a priority pollutant. *Marine Environmental Research. Pollutant Responses in Marine Organisms (PRIMO 13)*. **62**, S400-S401.
- Dissanayake, A. (2006). *Crouching crab, Hidden cockle*. *Plymbiosis*. **2**, p5.
- Dissanayake, A., Worsey, T., Jones, M. B. and Galloway, T. S. (2007). A multidisciplinary approach to evaluating physiological condition and intra-specific behaviour in the shore crab *Carcinus maenas* (Crustacea: Decapoda). *Comparative Biochemistry & Physiology Part A*. **146**, S76.
- Piggott, C., Baldwin, C., Sloman, K. A. and Dissanayake, A. (2007). The effects of sublethal contaminant exposure on foraging behaviour in the shore crab *Carcinus maenas* (Crustacea: Decapoda). *Comparative Biochemistry & Physiology Part A*. **146**, S82.

PRESENTATIONS AND CONFERENCES ATTENDED

Sixth International Crustacean Congress, 18-22 July, Glasgow, Scotland, U.K.

Awantha Dissanayake, Tamara S. Galloway and Malcolm B. Jones.

Does size really matter? An evaluation of ontogenetic differences in the physiology of *Carcinus maenas* (L.). (Platform Presentation).

10th Annual meeting SETAC Europe, 7th-11th May 2006, The Hague, Netherlands.

Awantha Dissanayake, Trevor Worsey, Malcolm B. Jones and Tamara S. Galloway.

Bridging the gap: Does behaviour 'mirror' the effect at the cellular level? (Poster Presentation).

Society for Experimental Biology Annual Main Meeting 31st March – 4th April 2007, Glasgow, Scotland.

Awantha Dissanayake, Trevor Worsey, Malcolm B. Jones and Tamara S. Galloway.

A multidisciplinary approach to evaluating physiological condition and intraspecific behaviour in the shore crab *Carcinus maenas* (Crustacea: Decapoda). (Platform Presentation).

WORKSHOPS AND COURSES ATTENDED

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Design and Analysis of Ecological Experiments (2005). Prof. A.J. Underwood and Prof. M.G. Chapman.

Learning, Teaching and Assessment: Theory and Practice. (2005). Jon Yorke (University of Plymouth).

H.S.E. First Aid at Work. (2005). Frank Nott (University of Plymouth).

ASIA Pro Eco II (Eu Funded)

Sound Chemicals Management for a Healthier Environment .

Application and Validation of Biomarker Techniques (Chennai, India, October 2006).

Sri Ramanchandra Medical College, India. (Visiting lecturer).

ASIA Pro Eco II (Eu Funded)

Sound Chemicals Management for a Healthier Environment .

Rapid Assessment of Contaminant Effects using Biomarkers and Species Diversity (Hanoi, April 2007). Hanoi University of Science (University of Vietnam) (Visiting lecturer).

Coastal Biodiversity in Ranong project (Tsunami Impacts in Laem Son) TILS

(Kasetsart University, DMRC Thailand and NHM London). (Visiting lecturer).

Word count of main body of thesis: 33, 848.

Signed  _____
Date 6th DECEMBER 2007 _____

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CHAPTER 1:
Introduction

1. INTRODUCTION

1.1 Polyaromatic hydrocarbons and the marine environment

Coastal marine waters receive anthropogenic inputs containing many chemicals that are potentially toxic to aquatic organisms (Krahn et al. 1984). One group of chemicals of increasing concern is the highly lipophilic polycyclic aromatic hydrocarbons (PAHs) which are ubiquitous pollutants of the marine environment (Clarke et al. 2001). PAHs are a group of chemical compounds, containing two or more fused benzene rings, which are highly lipophilic ($\log K_{ow} = 3 - 8$) (OSPAR 2001; Readman et al. 2002). These compounds arrive in coastal waters from a diverse array of sources including petrochemical pollution, incomplete combustion processes (Clarke et al. 2001; Livingstone et al. 1992), metal smelting (Beyer et al. 1996; Naes et al. 1995) and electrolytic production of aluminium using anode technology (Aas et al. 2001; Beyer et al. 1998; MSC-E 2001). There are two broadly distinct PAH groups categorised dependent upon source. Petrogenic PAHs are derived from petroleum products, and are characterised by (relative) low molecular masses and have a 2 or 3-ringed structure; these include PAHs such as naphthalene and phenanthrene. Pyrolytic PAHs, however, are combustion derived and are formed as a result of high temperature combustion of organic matter and industrial processes (OSPAR 2001). Examples of pyrolytic PAHs include fluoranthene, benzo[a]pyrene and pyrene; the latter is a 4-ringed PAH that is included in the USEPA priority pollutant list (MSC-E 2001) (Figure 1.1). Natural sources of PAHs into marine systems are limited and include perylene, which is synthesised by bacteria and algae (Clarke et al. 2001). Concern over the fate and effect of PAHs in the marine environment is related to their persistence, bioaccumulation potential, and acute and chronic toxicity to marine organisms. The particular cause for concern regarding PAH toxicity was highlighted originally in vertebrates based on *in vivo* and *in vitro* experiments that revealed that various PAHs were potent mutagens and/or carcinogens (reviewed in Jacob 1996); around

200 years ago, scrotum cancer was found in chimney sweeps and attributed to coal and soot (Pott 1775). PAHs, with four or more benzene rings (e.g. pyrene), have been found to be mutagenic and/or carcinogenic and, in some cases, the resultant prevalence of neoplasia in fish has been associated with PAH levels in sediments (Aas et al. 2001; 2003; Ruddock et al. 2002; 2003; Sole et al. 1996; Van der Oost et al. 1994; 1997).

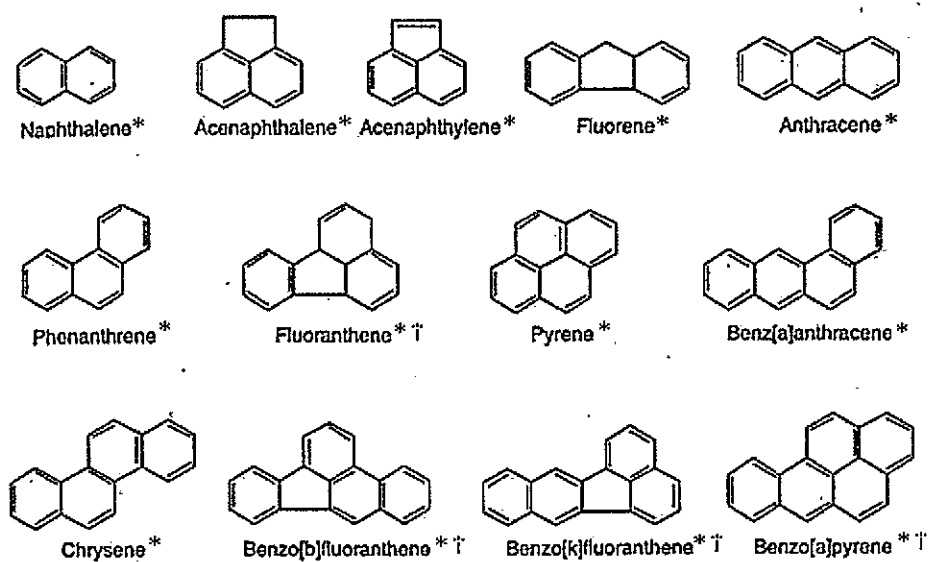


Fig. 1.1 Structures of selected polycyclic aromatic compounds, * United States Environmental Protection Agency priority pollutants, EPA₁₆, † European Union priority pollutants, EU₆. Taken from Nollet (2006).

1.2 Major pathways of PAH-induced damage

The possible molecular fate and effects of PAHs are dependent upon the biotransformation pathways involved in detoxification and excretion of organic compounds, and those pathways involved in the generation of molecular species (reviewed in Livingstone 1991). There are four potential mechanisms of PAH-induced damage; from the parent compound, from the production of primary and secondary metabolites, from free radical derivatives of the PAH, and from the enhanced production of oxyradicals.

1.2.1 PAH parent compound

Toxicity of the PAH parent compound may arise in several ways, such as interaction with enzymes, compartmentalisation within tissues or interference with gene expression, resulting in DNA adduct formation and/or strand breaks (Jha 2004).

1.2.2 PAH metabolites

Metabolism of PAH compounds results in their biotransformation and increased solubility, facilitating elimination from the body (Timbrell 1995). Metabolism is divided into two phases (Phase I: 'functionalisation' and Phase II: 'conjugation'), employing a wide range of enzymes; however, paradoxically, these reactions (mainly Phase I) result in toxic metabolites which can be more toxic than the parent compound. The biotransformation process results in bioactivation of the parent compound to secondary metabolites, which occurs due to the cytochrome P-450 enzymes and epoxide hydrolase function (Livingstone 1991, 1993, 1998).

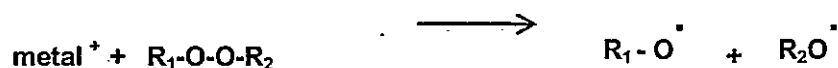
1.2.3 Free radicals

Free radicals of organic compounds are defined as compounds capable of existence that contain one or more unpaired electrons (Halliwell and Gutteridge 1989), and are produced from various single electron oxidations and other free radical reactions involving PAHs (Livingstone 1991) (Table 1.1).

Table 1.1 Free radical production involving organic compounds.

Possible sources of free radical production

1. Homolysis by redox coupling with metal ion

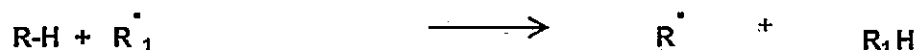


metal is a transitional metal capable of univalent redox change

R = alkyl or aryl group

R₂ = alkyl or aryl group

2. Transfer reaction with organic radical



3. Reaction with hydroxyl radical



4. Enzyme-mediated production where reactions proceed by sequential one electron steps and radicals can diffuse from enzyme surface before they are oxidised or reduced to an even electron species

5. Involvement in molecule assisted homolysis where, for example, decomposition of hydroperoxides is accelerated by the presence of an organic compound.
-

The protection of biological systems, such as membranes, occurs from the actions of free-radical scavengers, of which there are two types: water-soluble scavengers (e.g. ascorbate, glutathione, thiols and purine bases) and fat-soluble scavengers (e.g. carotene and retinol). An important feature of these scavengers is that they are oxidised during the reactions with free radicals (e.g. oxidised glutathione).

1.2.4 Oxyradicals

Oxyradicals are reactive oxygen derivatives [also known as Reactive Oxygen Species (ROS)] generated both endogenously as a by-product of normal oxygen metabolism (Winston and Di Giulio 1991), and also from the interaction between oxygen metabolism and organic compounds, such as PAHs. Reactive oxygen species include the superoxide ion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl ion (OH^-). Toxic effects of these ROSs include reaction with organic molecules such as lipids, thereby, yielding other ROSs responsible for lipid peroxidation and membrane damage, enzymatic inactivation/damage and reaction with DNA resulting in base modification or strand breakage (Livingstone 1991). Redox cycles are an important process as PAHs are reduced by an intracellular reductase enzyme, in a one-electron step to a reactive intermediate, which in turn reduces O_2 to O_2^- , thus forming a cycle of O_2 uptake and O_2^- generation. Protection against ROS is mediated by both enzymatic (e.g. superoxide dismutase) and non-enzymatic systems (e.g. α -tocopherol), known as antioxidants and either convert the oxygen species to less toxic or non-toxic products or by preventing their formation (Livingstone 1991). These protective systems are characterised by a high cellular activity, mainly cytosolic but also occurring in the mitochondria; the latter are proposed as the primary sites of (endogenous) aerobic cellular ROS production (Abele and Puntarulo 2004).

1.3 Pyrene as a model PAH

Pyrene, a 4-ringed PAH, has been found ubiquitously in coastal marine ecosystems as a result of use in combustion fuels such as diesel (King et al. 2004; Reeves et al. 2004). Exposure and subsequent metabolism of PAH compounds, such as pyrene, results in metabolites via the process of biotransformation. These metabolites are concentrated in body fluids, tissues and excreta. Analysis of such fluids can be used to detect exposure to bioavailable contaminants (Fillmann et al. 2002). Fluorescence detection methods of PAH metabolites were developed to signal PAH exposure in fish (Krahn et al. 1984), and have been used subsequently for detection of urinary PAH metabolites (e.g. 1-hydroxypyrene-type) in Crustacea (Dissanayake 2001; Dissanayake and Galloway 2004; Watson 2004; Watson et al. 2002; 2004b), thereby, facilitating the use of PAH metabolites as indicators of environmental PAH exposure. Accordingly, PAH metabolites have been used in this study to validate shore crab pyrene exposure. Figure 1.2 shows the metabolites identified and excreted in urine by *C. maenas* after both Phase I and II metabolism (Watson 2004; Watson et al. 2002; 2004b). Urinary PAH metabolites are used widely as indicators of PAH exposure in both vertebrates (Ruddock et al. 2002; 2003; Strickland et al. 1996; Strickland and Kang 1999) and invertebrates (Dissanayake 2001; Dissanayake and Galloway 2004; Watson 2004; 2004a; 2004b). Pyrene-exposed shore crabs produce phase I metabolites [e.g. 1-hydroxypyrene (1-OH pyrene)] and phase II metabolites (1-OH pyrene glucuronide and pyrene-1-sulphate) indicative of PAH exposure.

All PAHs absorb ultraviolet light followed by the emission of light of a longer wavelength. UV-fluorescence occurs due to the delocalised π -electrons within the benzene ring structure (Clarke et al. 2001). Each PAH compound has an optimal excitation and emission wavelength and signal intensity. The fluorescence properties vary between PAH compounds, as they are dependent upon size,

structure and corresponding constituents. A general trend is recognised in that the optimal excitation wavelength increases with increasing PAH molecule size. This variability between compounds has been used in PAH detection techniques (Aas et al. 1998; 2000b; 2001; Aas and Klungsoyr 1998; Krahn et al. 1984). Specific wavelength excitation and emission pairs have been used to differentiate between PAH compounds (naphthalene, 290/335 nm; pyrene, 341/383 nm and benzo[a]pyrene, 380/430 nm) from both laboratory- (Watson 2004; Watson et al. 2004b) and field-exposed shore crabs (Dissanayake 2001; Dissanayake and Galloway 2004; Watson et al. 2004a). The rationale for using urine collected from male shore crabs is based on the fact that urine from females may contain biogenic compounds, like cholesterol and progesterone, which also fluoresce and may lead to signal interference from these endogenous compounds (Hellou and Upshall 1995).

The toxic effect of PAHs originates from chemical processes at the molecular level when the contaminant impact exceeds the effects of compensatory physiological responses, the effect is expressed through the hierarchical levels of biological organisation i.e. biochemical, cellular and physiological levels (Fossi et al. 1994). Figure 1.3 provides a schematic representation of the metabolic fate and mechanisms of toxicity in *C. maenas*.

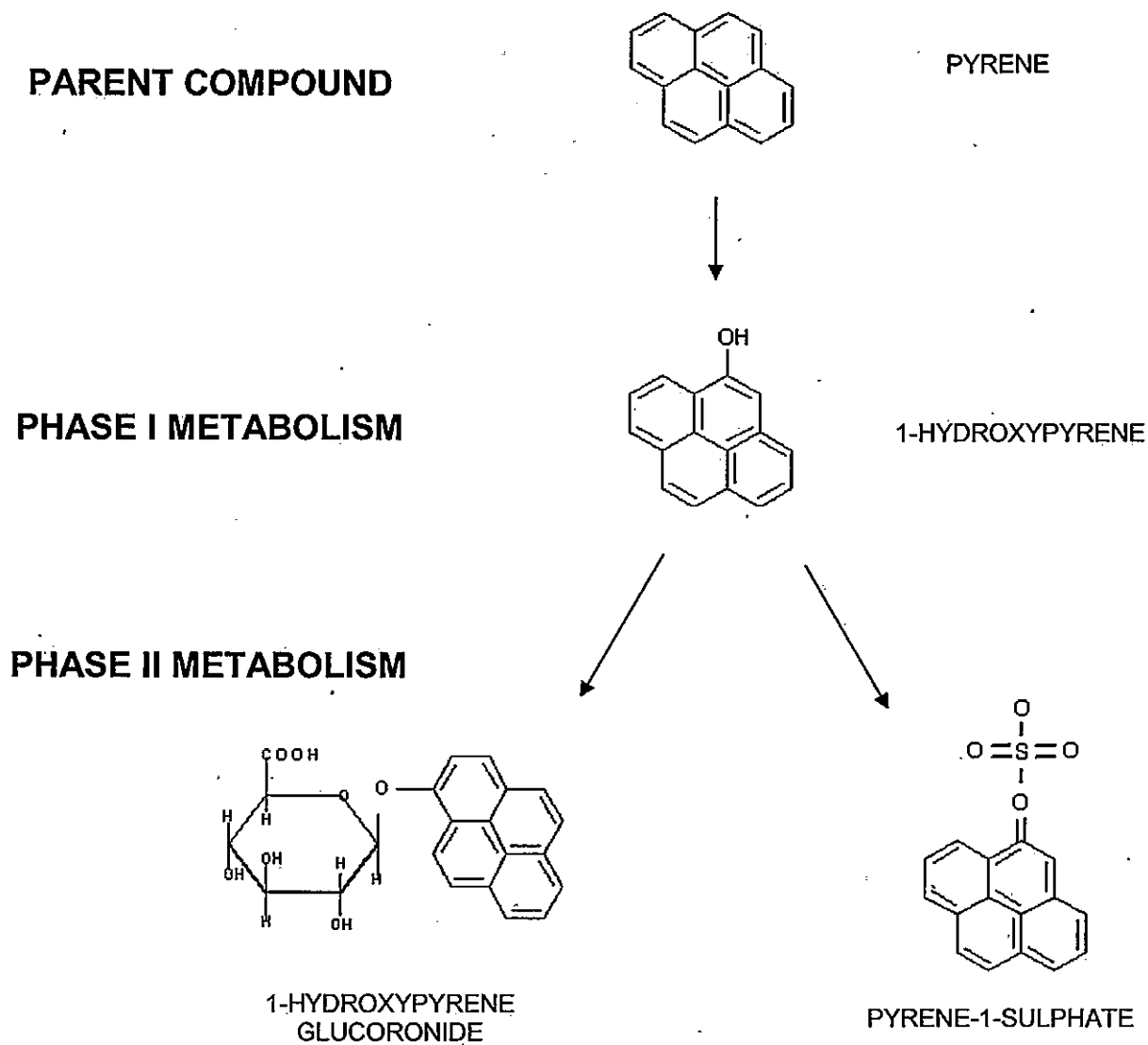


Fig. 1.2 Phase I and II metabolites tentatively identified in *Carcinus maenas* urine.

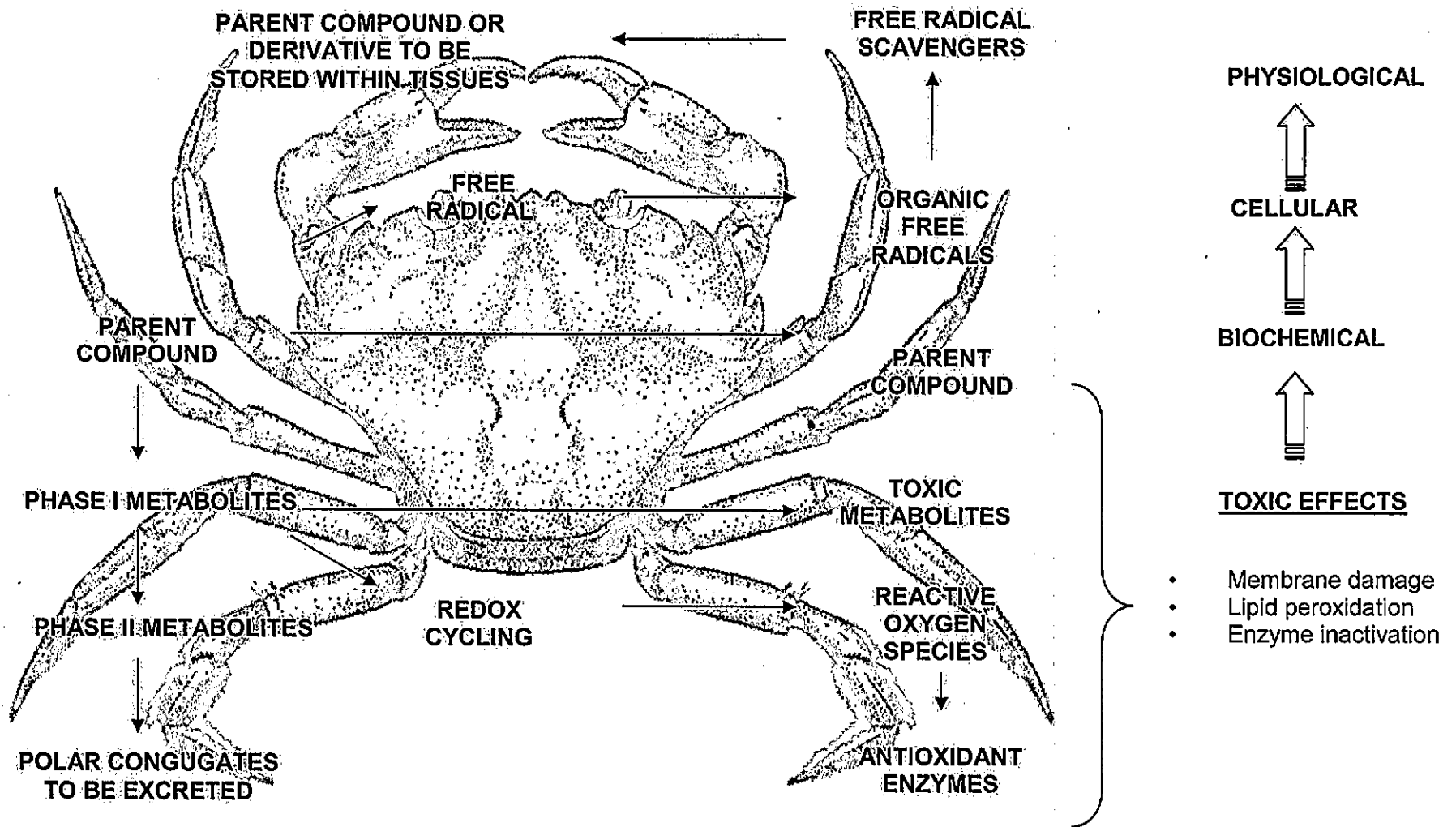


Fig. 1.3 Metabolic fate and mechanisms of toxicity in *Carcinus maenas*. Adapted from Livingstone (1991).

1.4 Evaluating the impact of PAHs

All individuals possess the ability to maintain key physiological functions in the face of environmental change, fluctuation or perturbation (homeostasis). An organism's capacity to adjust its physiology to operate within 'optimal' homeostatic efficiency in a variable environment is known as its adaptability (Bayne et al. 1985); however, any significant deviation from homeostasis will result in stress. Stress is defined as *"a state produced by an environmental or other factor (i.e. external constraints) which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced"* (Brett 1958). A stressor is therefore defined as a condition or situation that causes a system to mobilise its resources and increase energy expenditure (Lugo 1978). The response that arises from the effect of a stressor has been described as the General Adaptation Syndrome (Selye 1946), a general concept defined as the sum of all non-specific, systemic reactions of the body that ensue upon long-term exposure to stress (Selye 1946) (Fig. 1.4). Phase one (alarm reaction) relates to short-term responses whereby there are alterations in responses. During the second phase (resistance), adaptation is optimum, characterised by relatively longer-term responses such as enzyme activation. The third phase (exhaustion) is where, under prolonged stressful conditions, 'normal' physiological functioning of an organism is severely impaired (Fig. 1.4).

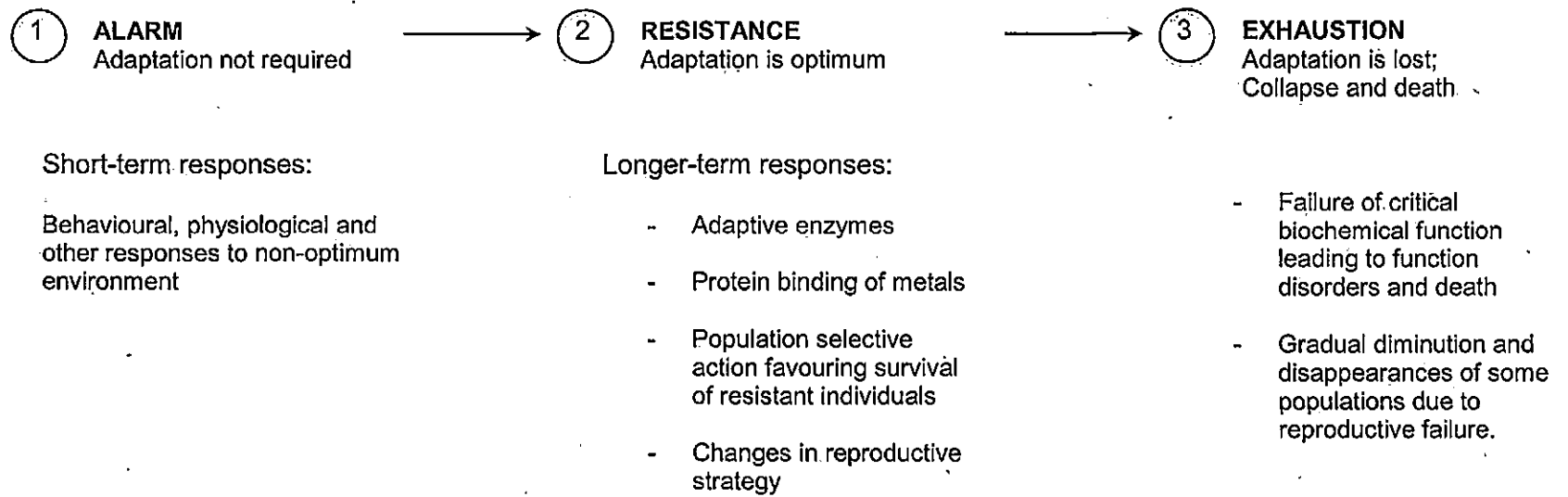


Fig. 1.4 The General Adaptation Syndrome of Selye (1976). Taken from Mayer (1992).

Physiological ecology (Ecophysiology) determines the basis of the physiological mechanisms involved in attaining constancy in an animal's *internal milieu* (proposed by Claude Bernard) (Selye 1956) and such physiological flexibility of an organism to be related to environmental demands (Bayne et al. 1985; Cannon 1935). In any attempt to measure the response of an organism to such stress, three general considerations should be borne in mind. Firstly, the effects of the stress will be an integrated response involving all levels of functional complexity within the organism (molecular, cellular and physiological). Secondly, the stress response is dynamic, and involves an alteration in functional properties over time. Thirdly, a potential stress may be neutralised by homeostatic physiological compensation. Although these processes may themselves be metabolically costly, it is when compensation for an environmental change is incomplete, or in the extreme, impossible, that lasting effects are measurable as a decline in the organism's fitness or, ultimately, as death (Koehn and Bayne 1989).

A more comprehensive definition of stress [extending Selye's (1946) definition] is "any environmental influence that impairs the structure and functioning of organisms such that their Darwinian fitness is reduced" (Calow 1989). This definition links responses at the level of the individual, such as organism survival probability, developmental rate and fecundity, as proposed by Koehn and Bayne (1989), to parameters that influence the density of their populations and their future contribution to the gene pool (Calow 1989). The immediate effect of an environmental stress can be referred to as the proximate response (Mayr 1961), whereby, an environmental variable must either increase or decrease from 'normal' and cause some change within the organism and population (Underwood 1989). Alternatively, the ultimate response, is the evolutionary

effect whereby direct adaptations in populations arise as a consequence of the evolution of stress tolerance (Mayr 1961).

The effects of stress, therefore, may be studied at the level of the individual and at the level of the population (Maltby 1999). However, there is no definitive level at which to study stress, as demands for scientific management of the biosphere involve prediction and analysis of stress responses at all levels from an individual organism to whole ecosystems (Grime 1989). Indeed, the different hierarchical levels of biological organisation provide insight, in combination, into stress effects, their mechanistic processes and their potential ecological consequences (Maltby 1999) (Fig. 1.5). Studies of populations can provide insight into the disruption of community structure (Warwick and Clarke 1993), but provide little information of the mechanisms of how such alterations occur. Conversely, studies at the organism level (molecular and biochemical), while contributing to our understanding of mechanistic alterations to physiological systems, fall short of providing information on higher-level consequences i.e. population and community level (Maltby 1999). Organisms are important operational units from both an ecological and evolutionary point of view (Underwood 1989) and, by studying effects of stress on individual organisms, elucidation of mechanisms of a) intra-individual variation, b) inter-individual variation and c) between population variation can be established. Physiological variation is a feature at each of the different hierarchical levels of biological organisation (Spicer and Gaston 1999). Organism-level responses can be used to monitor stress in natural environments, but this approach is limited currently by a lack of knowledge of the "normal" physiological ranges (Mehrlle and Mayer 1980).

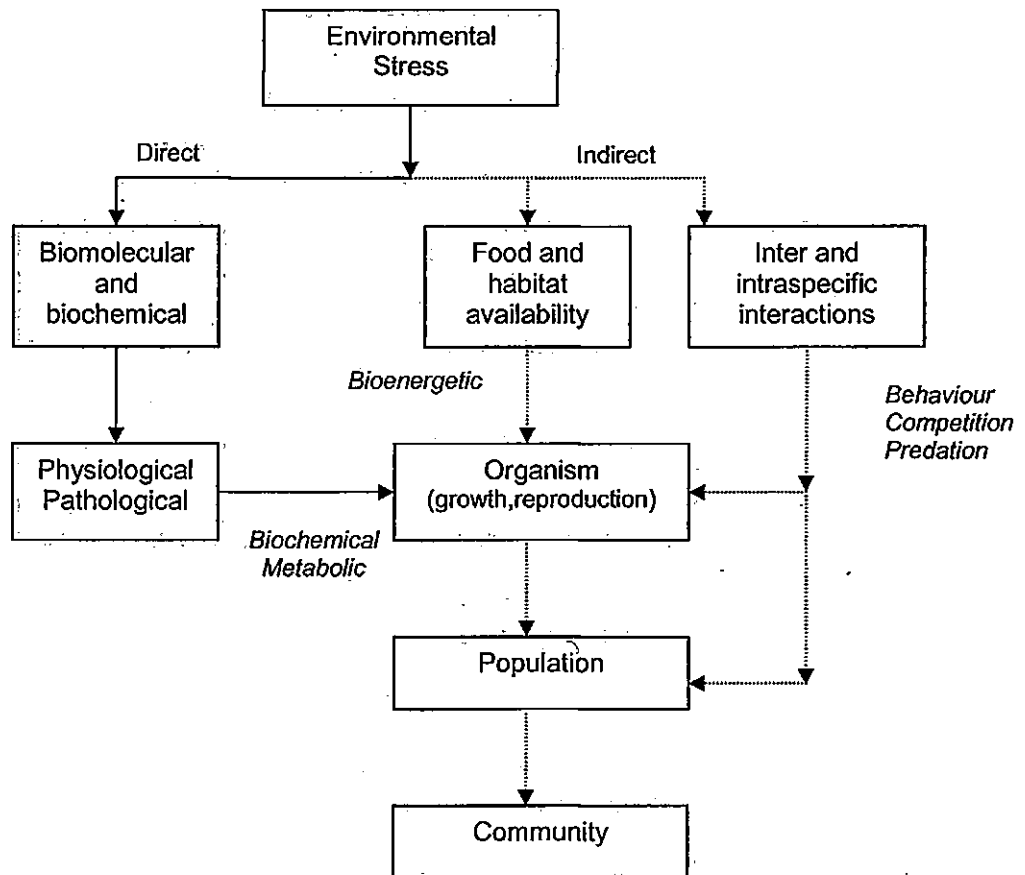


Fig. 1.5

Relationships between environmental stressors and direct and indirect effects on biological systems. Direct pathways affect organisms primarily through biochemical and metabolic processes and indirect pathways influence biota through effects on food and habitat availability and through intra- and interspecific interactions. Taken from Marshall Adams (2005).

1.5 *Carcinus maenas* as a model species

In this thesis, the common shore crab *Carcinus maenas* (L.) is taken as a model example of an eurytopic species as it is both euryhaline (Rainbow and Black 2001; 2002) and eurythermal (Cumberlidge 1977a; Taylor 1973). *Carcinus maenas* has a high fecundity, a long planktonic larval period and is an opportunistic predator (Crothers 1967; 1968). It is the most common crab species along European shores, extending from Tromsø (Norway) to Gibraltar (Clark et al. 2001; Hayward and Ryland 1990; Kuris et al. 2005). The discovery of introduced populations (Australia, Tasmania, South Africa, western North America and Japan) in the 1980s established this species as a global invader of temperate shores (Yamada et al. 2005).

Carcinus maenas responds quickly to environmental change, through changes in osmoregulation (Bjerregaard and Visle 1985; 1986), respiration rates and capability (Arudpragasam and Naylor. 1964a; 1964b; Dawirs 1983; Depledge 1985; Spicer and Weber 1991), and metabolic and cardiac activity (Cumberlidge 1977a; 1977b; Depledge 1984; Wallace 1972). The physiological ability of *C. maenas* is a major factor accounting for its widespread distribution (Rainbow 1997) and has contributed to its success as an invader (Roman and Palumbi 2004). Such an ability to respond to physiological challenges (defined as phenotypic plasticity) is likely to be a highly advantageous adaptive strategy (Brian et al. 2006) and may arise as a result of within-generation selective pressures. However, low genetic variability has been shown for shore crab populations around the U.K. coasts, indicating that patterns of phenotypic variability among shore crab populations are likely to reflect differences between local environments (Brian et al. 2006).

1.6 The biology of the shore crab

1.6.1 *Difference between colour morphs*

Crustacean growth is limited by the exoskeleton and only through the process of ecdysis (moulting) can an increase in body size be achieved (Crothers 1967; 1968). Active feeding occurs during the intermoult stage when energy reserves are built up prior to moulting (Crothers 1967). Two different colour forms of the shore crab *C. maenas* (red/green) are found in the adult benthic stage and occur due to differential duration of the intermoult stage. The colour forms are distinguished and classified according to carapace colour; green (green-yellow) or red (dark orange-red) (McGaw et al. 1992; Reid et al. 1997) and will be referred hereafter as green or red crabs. Physiological, behavioural and ecological distinctions have been documented between the two colour forms (McGaw et al. 1992; McGaw and Naylor 1992b; 1992c; Reid et al. 1997). Crothers (1968) noted that red crabs inhabited the subtidal and green crabs dominated the intertidal; Reid et al. (1997) related this zonation to differences in physiology between the colour morphs. Studies assessing the comparative physiology of the green and red forms have shown differences in respiration, osmoregulation and desiccation and starvation tolerances (Table 1.2). In summary, the green form appears to possess increased 'physiological tolerance' to natural abiotic and biotic factors (Table 1.2).

Table 1.2 Comparative differences between *Carcinus maenas* colour morphs. + signifies increased relative tolerance, - signifies decreased relative tolerance.

PARAMETER	MORPH		REFERENCE
	GREEN	RED	
Location	Intertidal	subtidal	(Reid et al. 1997)
Respiratory physiology	+	-	(Reid et al. 1997; Reid and Aldrich 1989)
Osmoregulatory physiology	+	-	(Reid et al. 1997)
Dessication capability	+	-	(Reid et al. 1997)
Low salinity tolerance	+	-	(McGaw and Naylor 1992b; 1992c)
Starvation tolerance	+	-	(Styrishave et al. 2000)

1.6.2 Integrated studies on the shore crab

Hebel et al. (1997) used a holistic approach to summarise the effects of copper upon the physiology of *C. maenas* (Fig. 1.6). Copper was selected as it is an essential trace metal in crustaceans (Hebel et al. 1997), enabling the respiratory pigment haemocyanin to transport oxygen (Mangum 1983) and represents 0.17 % of the functional haemocyanin molecule (Depledge and Bjerregaard 1989). Aquatic crustaceans, such as *C. maenas*, accumulate trace metals in their tissues from water-borne exposure or through dietary intake (Rainbow 1997). The transport route across membranes is as follows via carrier-mediated via membrane proteins, down concentration gradients through protein channels, passive diffusion (Lawson et al. 1995) leading to gill structural alterations, and finally endocytosis where the metal ion is detoxified via metal-binding proteins known as metallothioneins and stored, leading to accumulation within tissues and organs (Rainbow 1997). Physiological responses to copper include decreases in oxygen consumption, gill ventilation changes and alterations in ventilation/perfusion ratios (Hebel et al. 1997). Behavioural effects of copper exposure such as mating behaviour of male shore crabs, includes less directed searching, decreased display of mating behaviour, increased non-mating behaviour, such as pinching and the delayed establishment of the cradle carrying posture (Krang and Ekerholm 2006).

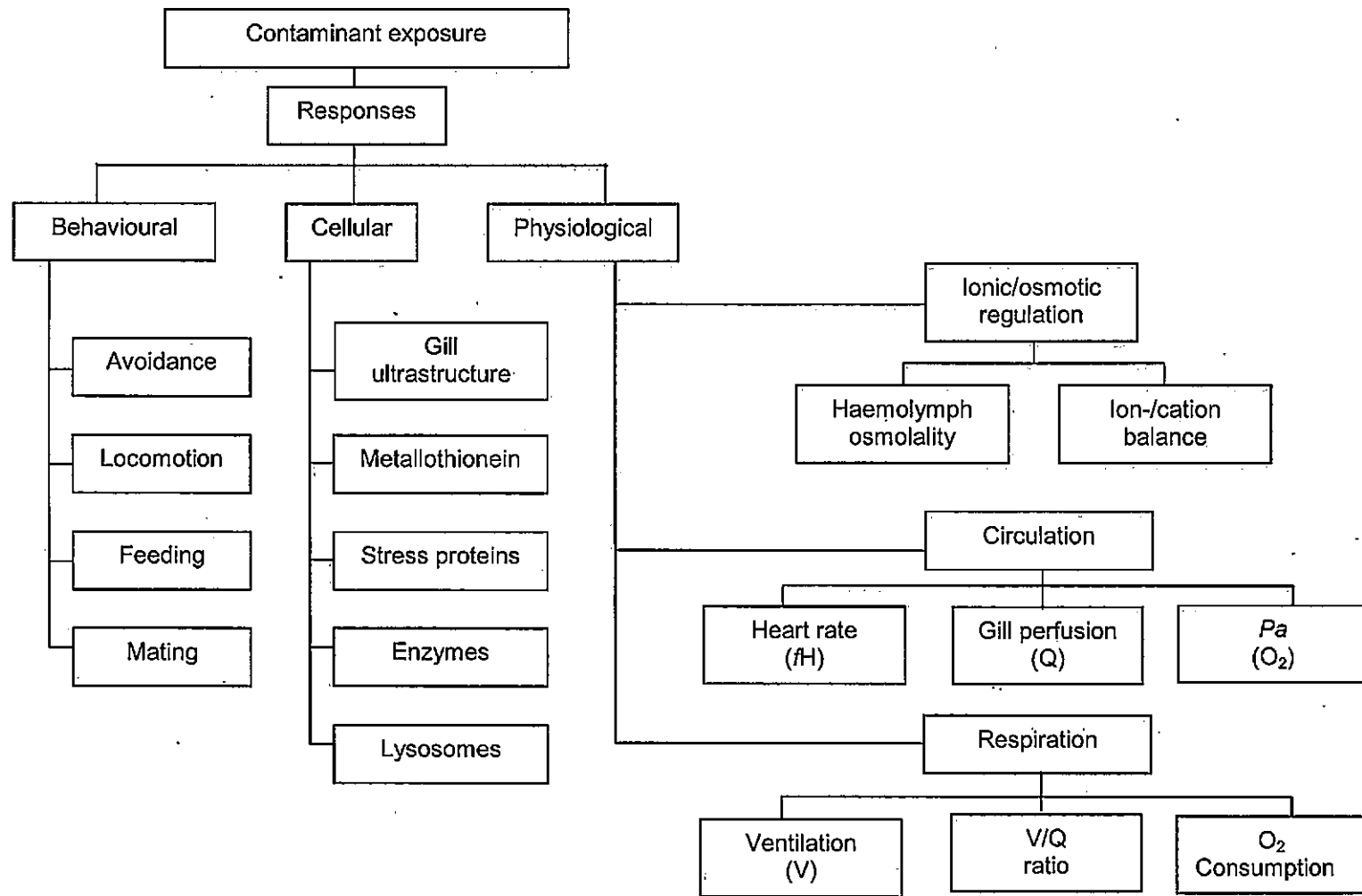


Fig. 1.6. Compensatory responses of *Carcinus maenas* following exposure to copper. Taken from Hebel et al. (1997).

The review by Hebel et al. (1997) highlighted the importance of an integrated approach to evaluating the physiological performance of an organism to either abiotic or biotic factors. These authors advocated the use of a combination of multiple measurements of variables (at the different levels of biological organisation) which signal the functional status of the organism, thereby, signalling the organism's integrated response (Hebel et al. 1997) (Fig.1.7); however, there is a currently a dearth of knowledge of studies that have utilised a holistic approach to evaluating effects at the level of the individual (Spicer and Gaston 1999). Studies that focus on linking the levels of biological organisation and consider the large-scale relevance of organism response are important in achieving the goal of determining ecosystem function (Attrill and Depledge 1997). This holistic, integrated approach is essential for identifying the full impact of chemical contamination on organisms (Galloway et al. 2004a; 2004b; 2004c).

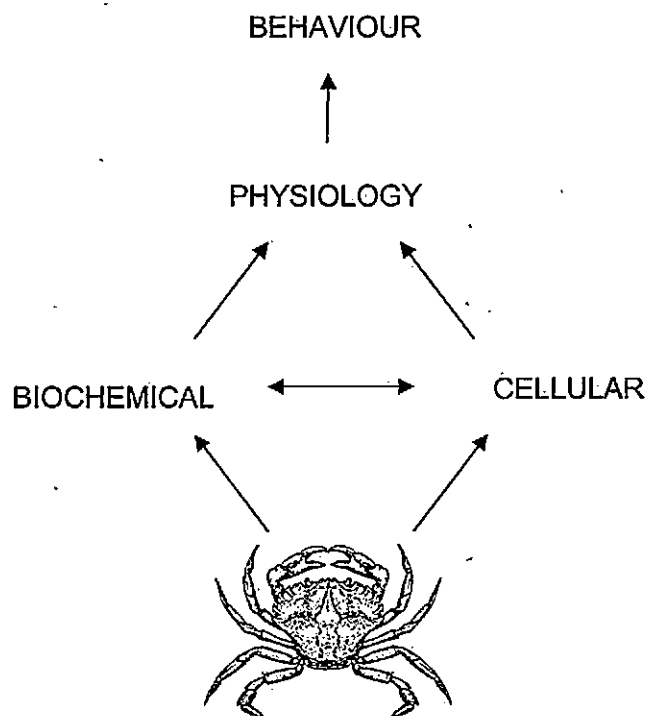


Fig. 1.7

Summary diagram representing the hierarchy of biological organisation, as a framework for assessing biological responses in *Carcinus maenas*.

The main emphasis of this thesis was on using a holistic ecophysiological approach to evaluating the impact of organic contamination upon *C. maenas* physiology.

1.7 Aims and objectives

The aim of this research programme was to elucidate the 'normal' physiological ranges of responses in *C. maenas*, by evaluating the effects of organic contamination (using pyrene as a model PAH) upon shore crab physiology. Specifically, the objectives were:

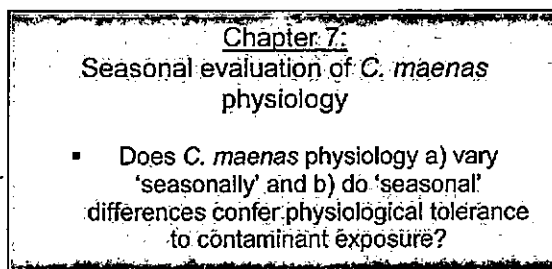
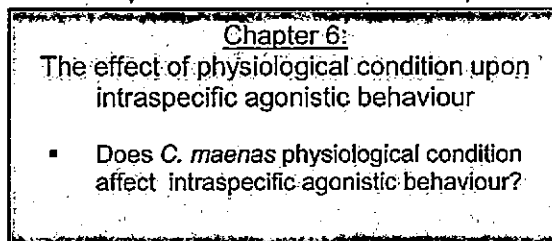
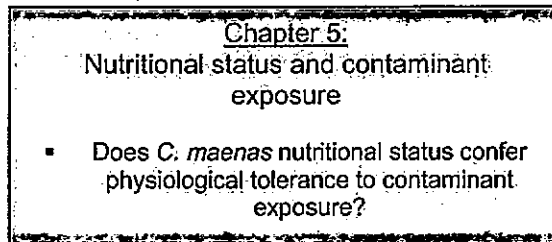
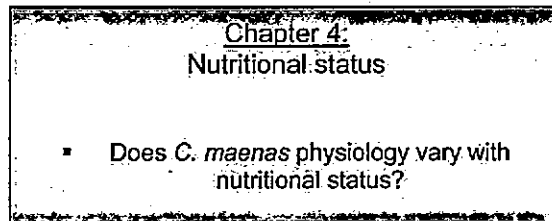
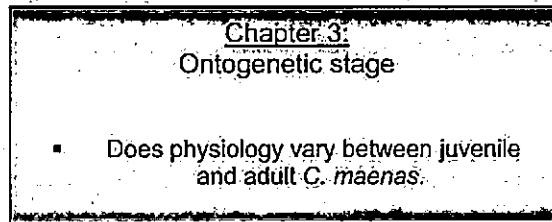
1. To investigate whether physiological differences exist between juvenile and adult *C. maenas* and whether any such differences confer increased susceptibility to pyrene exposure (Chapter 3).
2. To assess whether shore crab physiology varies with nutritional status (Chapter 4).
3. To investigate whether shore crab nutritional status is an important factor regarding pyrene exposure (Chapter 5).
4. To investigate the intraspecific behavioural implications (using agonistic behaviour) of pyrene exposure and nutritional status (Chapter 6).
5. To investigate whether a) shore crab physiology varies 'seasonally' within a year and b) whether 'seasonal' differences indicate *C. maenas* sensitivity to PAH exposure, evaluated in estuaries of varying PAH input (Chapter 7).

Outline of thesis

A schematic outline of the research objectives of the PhD thesis is provided in Figure 1.8.

Chapter 1: Introduction

Chapter 2: Materials & Method



Chapter 8: Final discussion and conclusion

Fig. 1.8

Outline of proposed research objectives for the PhD programme.

CHAPTER 2:
Material and Methods

2. MATERIAL AND METHODS

2.1 Shore crab collection

Male (green) intermoult shore crabs were collected from Jenkins Quay, Avon Estuary, Bantham, South Devon, U.K. (grid reference: SX 6623 4380) using mackerel-baited traps [Trappy tetra crayfish trap, Collins nets, U.K (dimensions: 40cm L x 30cm W x 22cm H, 1.5mm mesh)] (Fig. 2.1). Previous evidence (McGaw and Naylor 1992a), as well as a pilot study, revealed that two hours approaching high water was optimum time for shore crab collection. Shore crabs were transported back to the laboratory (45 min) in a cooler box with damp absorbent paper. Details of specific laboratory maintenance and exposure conditions are described within each experimental chapter.

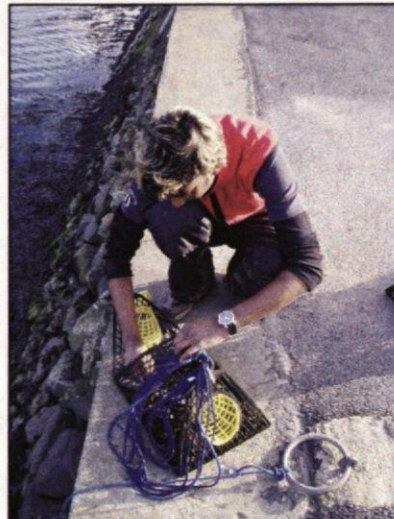
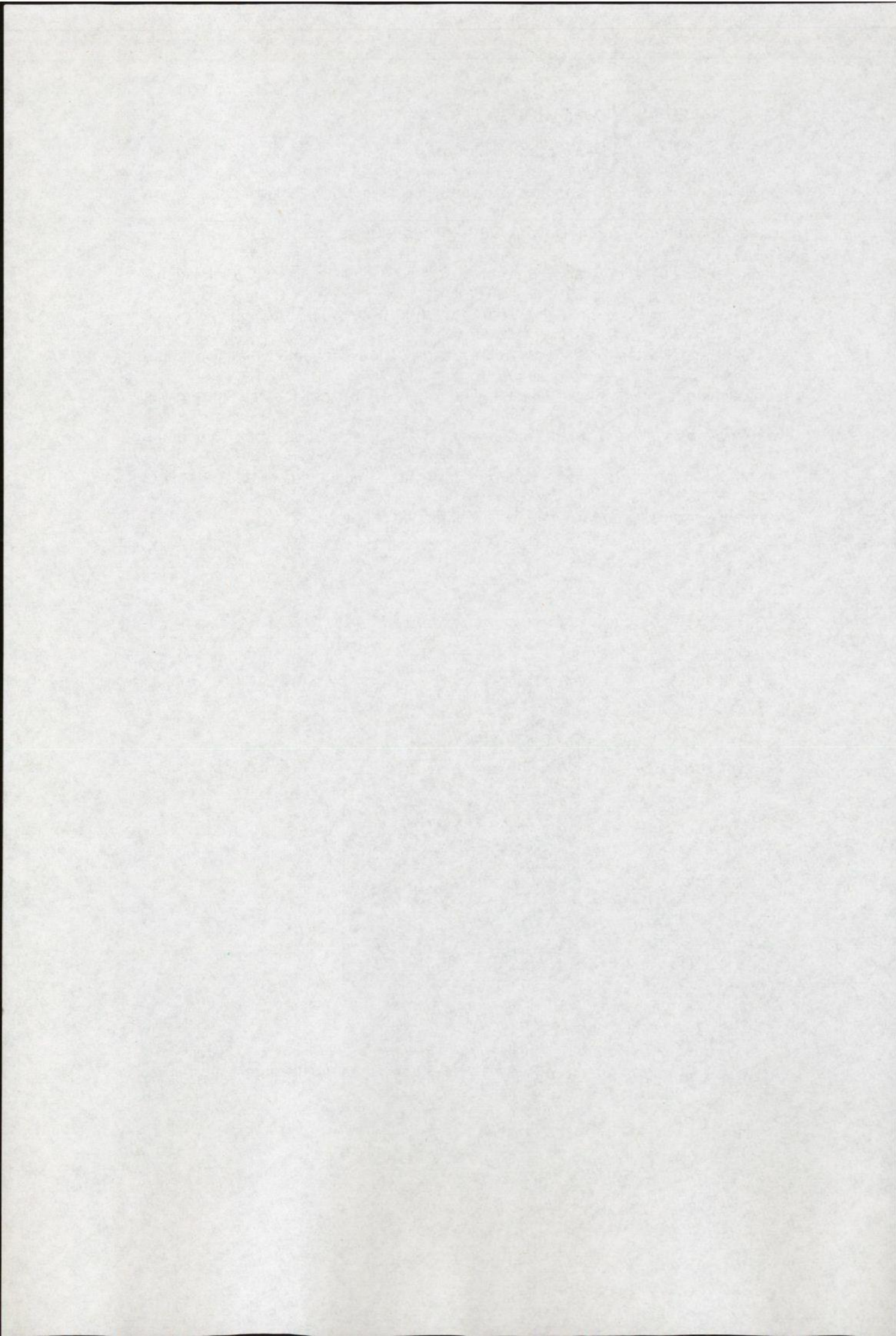


Fig. 2.1 Baited traps [dimensions: 40 cm L x 30 cm W x 22 cm H, (1.5 mm mesh)] used for shore crab collection.



2.2 Laboratory exposure to pyrene

Individual crabs were exposed to pyrene at a nominal concentration of $200 \mu\text{g L}^{-1}$ in 2 L tanks; this pyrene concentration is sublethal for *C. maenas* (Watson 2004; Watson et al. 2002; 2004b). Pyrene (98 %, cat no: 18, 551-5, Sigma-Aldrich, U.K) was added to seawater in an acetone carrier (ratio 1:1, w/v pyrene/acetone) to increase water solubility. Previous evidence has shown that the acetone carrier does not elicit any physiological effects on *C. maenas* (Dissanayake et al. 2006a; 2006b; Watson 2004; Watson et al. 2004b). Specific details of laboratory exposure duration are described within each experimental chapter (Chapters 3-7).

2.3 Validation of PAH exposure

2.3.1 Urine extraction

To extract urine, crabs were blotted dry using absorbent paper and restrained ventrally onto a backboard using elastic bands running across the chelipeds and pereopods (Fig. 2.2).

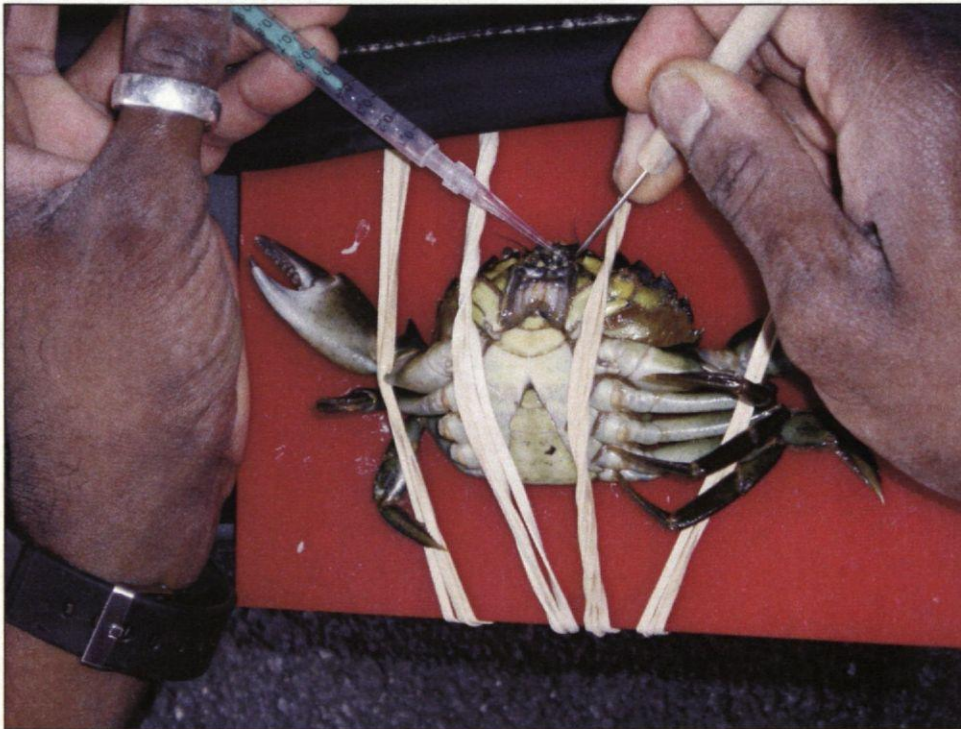
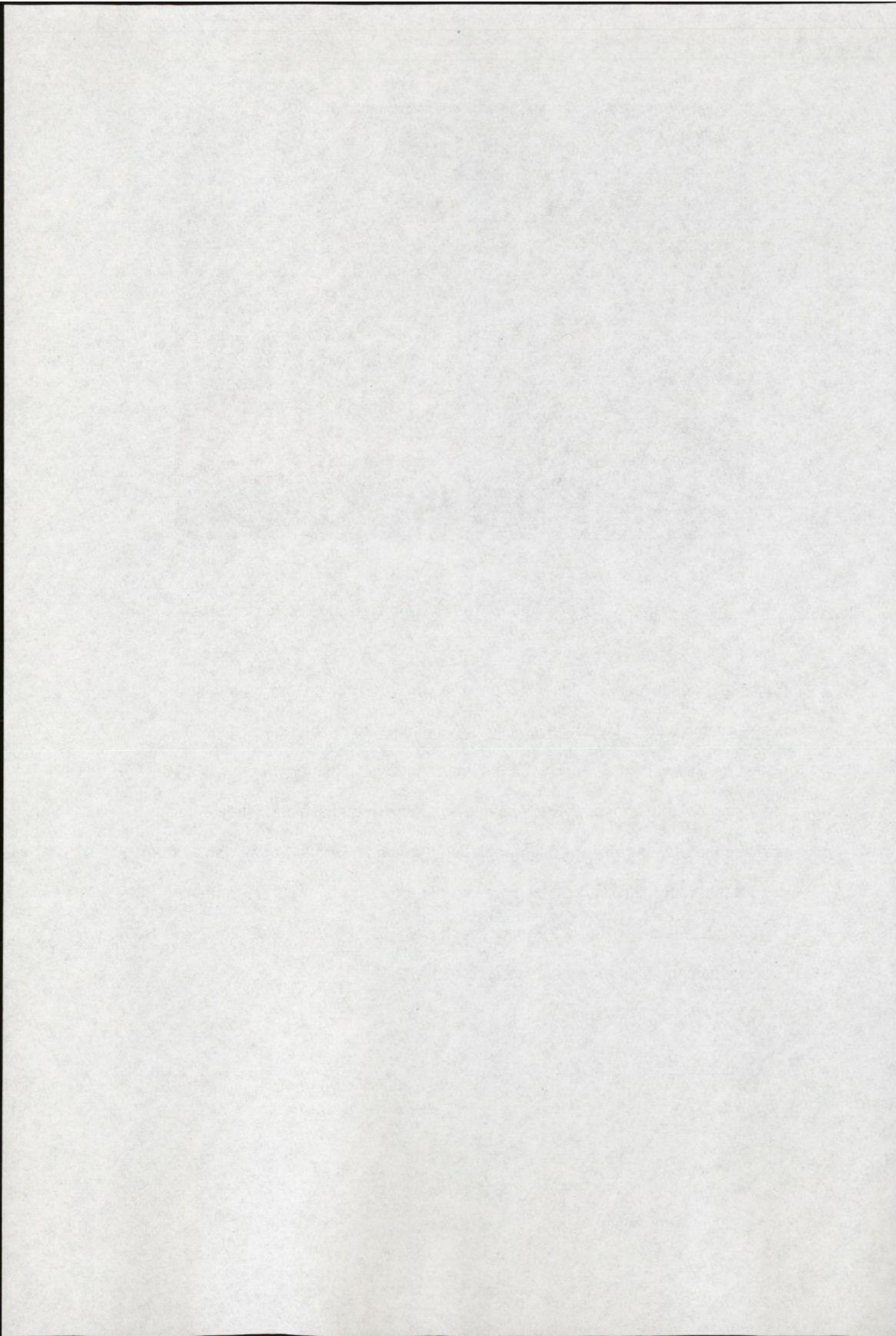


Fig. 2.2 Urine extraction and collection from *Carcinus maenas*.

Using a hooked seeker, the third maxillipeds were moved aside and secured using elastic bands, thereby exposing the epistome; to prevent urine dilution from water arising from the branchial exhalent chambers, residual water was blotted dry using absorbent paper. Each operculum of the antennal gland (situated below the second antennae) was prised open using a hooked seeker; urine flowed from bladders through the opercula and was collected using a 1 ml syringe with a 10 μ l pipette tip attached (Fig. 2.2). Urine samples (50-200 μ l) were transferred to siliconised centrifuge tubes and stored at -20 °C.



2.3.2 Urinary PAH metabolite analysis

Fluorescence analyses were performed using a microplate fluorescence spectrophotometer (BIOTEK FL-600) using specific wavelength filter pairs for either pyrene (PYR) metabolites (see Chapters 3, 4, 5, 6) or benzo[a]pyrene (B[a]P) metabolites (see Chapter 7). Prior to analysis, samples were thawed on ice (15 min). Fifty microlitre samples were diluted 1:20 with 50 % ethanol and screened for fluorescence using the wavelength pairs shown in Table 2.1; results are expressed as fluorescence units (arbitrary units).

Table 2.1 Wavelength pairs used to discern between PAH metabolite groups.

Filter set	Wavelength/ Bandwidth (nm)	
	PAH metabolite group	
	Pyrene	Benzo[a]pyrene
Excitation	340/11	360/20
Emission	420/50	420/50

2.3.3 1-hydroxypyrene standards

The primary metabolite, 1-hydroxypyrene (1-OH pyrene) (Sigma-Aldrich; 36, 151-8), was used as the standard to semi-quantitatively report the fluorescence from urine samples (not the parent compound, pyrene), as it takes into account the conjugation of a polar group. 1-hydroxypyrene also closely represents the peaks observed in urine samples from *C. maenas* (Watson et al. 2004b). Results are therefore reported as $\mu\text{g L}^{-1}$ of 1-OH pyrene equivalents. In Chapter 7, fluorescence from urinary benzo[a]pyrene metabolites are reported but are not in terms of 1-OH B[a]P equivalents, due to the unavailability of commercial standard solution of 1-OH B[a]P. The rationale for measuring metabolite fluorescence was to discern between sites where pyrene or B[a]P peaks may occur indicative of PAH exposure (Dissanayake and Galloway 2004).

2.4 Biochemical and cellular endpoints

2.4.1 Haemolymph extraction

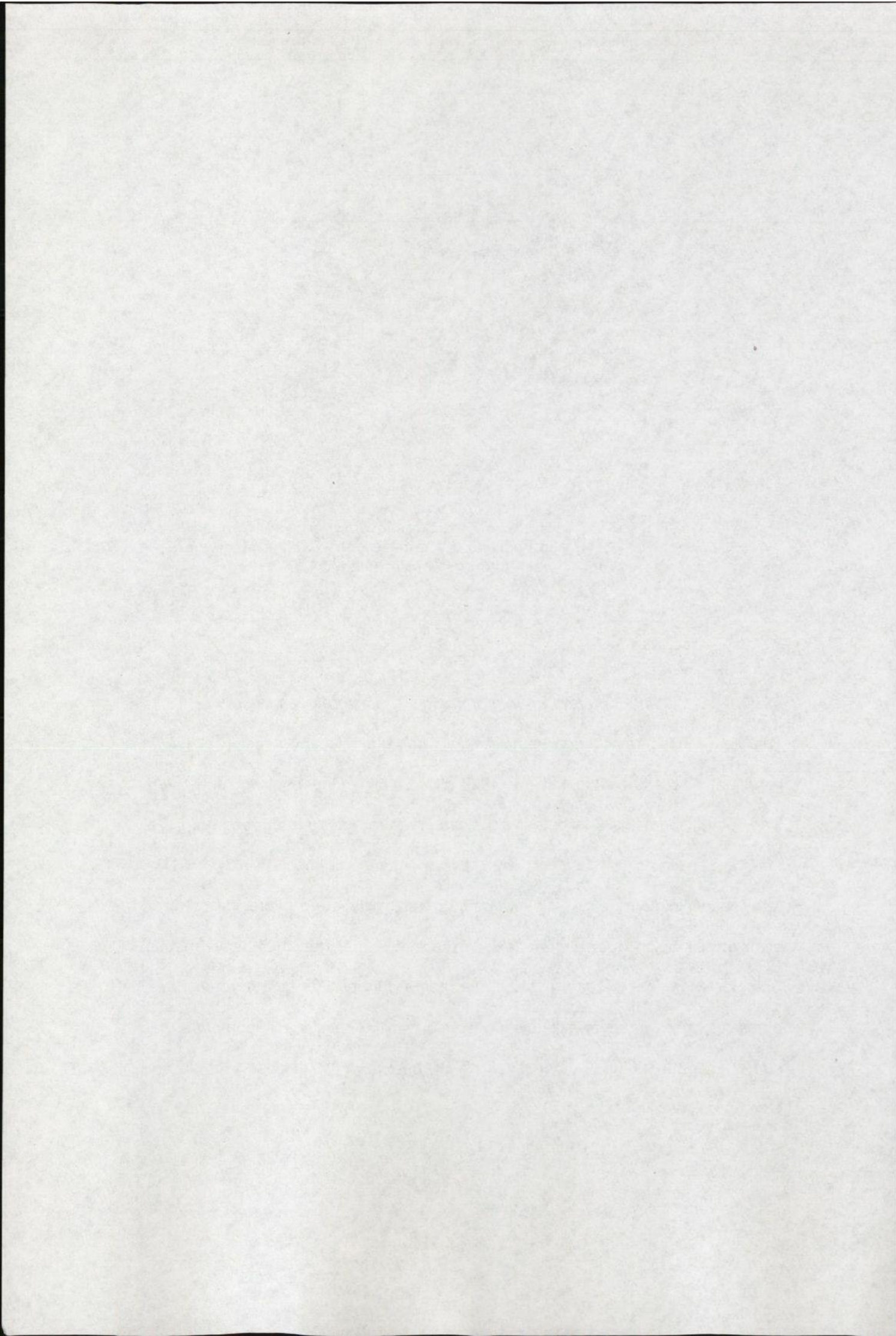
To extract haemolymph, crabs were blotted dry using absorbent paper and restrained as described previously. Haemolymph samples were taken from the arthrodistal membrane at the base of a walking leg (Fig. 2.3). Samples (500 μl) were withdrawn into a pre-chilled 1 ml syringe using a 21 gauge needle, transferred into siliconised centrifuge tubes, and stored on ice for analysis of 'live cell assays' (see Section 2.4.6) and stored at $-80\text{ }^{\circ}\text{C}$.



Fig. 2.3 The arrow indicates location of the arthroal membrane at the base of the walking leg where haemolymph was sampled.

2.4.2 Haemolymph preparation (Cold storage assays)

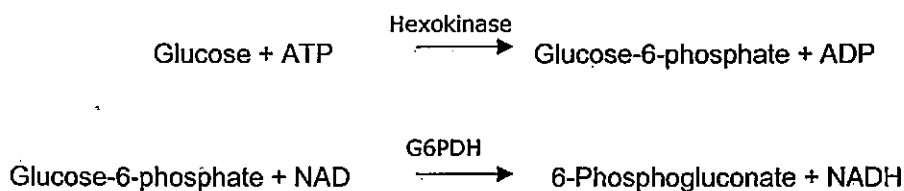
Thawed (4 °C) haemolymph samples were deproteinated by addition of 0.6 M perchloric acid and centrifuged at 10,000 g for 20 min. The resulting supernatant was neutralised using 2 M potassium bicarbonate to ensure a pH range of 6 - 8. Following the modification by Engel and Jones (1978), EDTA (ethylenedinitrilotetraacetic acid) was incorporated within the hydrazine buffer to eliminate inhibition of enzyme-catalysed reaction due to traces of heavy metals. Samples were centrifuged at 10,000 g for a further 20 min to remove the potassium perchlorate precipitate from solution; the resulting supernatant was stored at -80 °C for further analysis of haemolymph glucose and lactate concentrations.



2.4.3 Glucose analysis

Haemolymph glucose concentrations were determined using the method of Kunst et al. (1981). Glucose is phosphorylated by adenosine triphosphate (ATP) in the reaction catalysed by hexokinase. Glucose-6-phosphate (G6P) is then oxidized to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) in a reaction catalysed by glucose-6-phosphate dehydrogenase (G6PDH). During this oxidation, an equimolar amount of NAD is reduced to NADH, leading to a change in absorbance at 340 nm which can be measured spectrophotometrically. The consequent increase in absorbance is directly proportional to glucose concentration.

Principle:

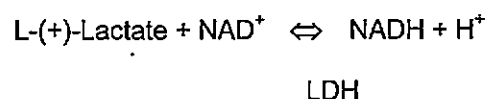


All reagents were supplied by Sigma-Aldrich Chemical Co. Ltd (Poole, U.K.).

2.4.4 Lactate analysis

Haemolymph L-lactate concentrations were determined using the method of Gutmann and Wahlefeld (1974) with the suggested modification by Engel and Jones (1978). The assay was carried out in a microplate format (Briffa and Elwood 2005). A mixture of glycine-hydrazine buffer, oxidized nicotinamide adenine dinucleotide (NAD) (40 mM) and lactate dehydrogenase (600 Units mg protein⁻¹) was added to 20µl of sample (ratio 20:1, v/v). Lactate concentrations were determined spectrophotometrically at 340 nm after 2 h at 37 °C. Lactate standards (0 – 2 mM) were used for lactate concentration determination.

Principle:



2.4.5 Total antioxidant status

Reactive oxygen species (ROS) are oxygen derivatives, produced both endogenously and as a result of PAH exposure, which may lead to molecular and cellular effects, such as DNA adducts and membrane damage (see Chapter 1, Section 1.2.4). The potentially harmful effects of ROS are negated by the enzymatic and non-enzymatic components of the antioxidant capability of the organism. A biological antioxidant is defined as any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge 1995). 'Antioxidant power' is referred to as the reducing ability of the substrate. A measure of antioxidant power is used here *in vitro* to determine the ferric reducing ability of plasma (FRAP) of *C. maenas* haemolymph. This method works using

reductants in a non-specific redox-linked colourmetric method employing an easily reduced oxidant (Fe^{III} -TPTZ complex) in stoichiometric excess, which is reduced to the ferrous form (Fe^{II} -TPTZ complex) to form an intense blue colour which strongly absorbs at 593nm (Benzie and Strain 1996).

Prior to analysis, samples were thawed on ice (15 min). Haemolymph samples were centrifuged at 4500 g and 10 μl of the sample supernatant (plasma) was added to 96-well microtitre plates for analysis. A working FRAP reagent was produced by mixing 300mM acetate buffer (pH 3.6), 10 mM 2, 4, 6 -tripyridyl-s-triazine (TPTZ) solution and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a 10:1:1 ratio. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate ($\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$) (Sigma, U.K) with 4 ml glacial acetic acid and brought to 1 L with distilled water. The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. Two hundred microlitre aliquots of the FRAP reagent mix was added to each sample and incubated at 37°C for 10 min. Standards of known Fe (II) concentrations ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were added between 0 and 1000 μM for FRAP determination. FRAP values were determined spectrophotometrically by measuring the absorbance at 593 nm, taken at both 0 and 10 min. Results were determined by subtraction of values at t10 from t0 and expressed as $\mu\text{M L}^{-1}$.

2.4.6 Haemolymph preparation ('Live cell' assays)

Haemolymph samples (30 μl) were pipetted into poly-L-lysine-coated 96-well microplates and analysed in duplicate. Plates were agitated gently in a plate shaker at 200 rpm for 60 sec and incubated at 10 °C for 50 min to promote adherence and formation of a cell monolayer.

2.4.7 Cellular integrity (Eosin Y dye exclusion assay)

Dye exclusion methods are based on the fact that, firstly, intact membranes are impermeable to large or charged molecules and, secondly, maintain cytoplasmic gradients thereby retaining intracellular concentrations of ions. Permeability of the cytoplasmic membrane indicates moribund or dead cells (Coder 1997). Eosin Y (tetrabromofluorescein) is a fluorescein-derivative stain with an absorption maxima between 515 and 518 nm (Constantino et al. 1995); the principle of this assay is that healthy cells with intact plasma membranes (full integrity) can exclude eosin Y, whereas, injured cells rapidly take up the dye (Lowe and Pipe 1994).

To measure membrane damage, haemolymph samples were incubated with Eosin Y dye at a 1:10 ratio at 10 °C for 10 min. Following incubation, cells were fixed using Baker's formal calcium (BFC) (2 % NaCl, 1 % Calcium Acetate, 4 % Formaldehyde), washed once with phosphate buffer (PBS; 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, Sigma-Aldrich, U.K), and centrifuged at 70 g for 5 min to remove residual dye. Cells were lysed using 1 % acetic acid-50 % ethanol solution (100 µl) to release any incorporated dye (Δ_{samples}). The optical density of each sample was determined spectrophotometrically at 518 nm. To quantify the amount of incorporated dye, and express percentage cellular integrity, 2 µl of Eosin Y and the acetic acid solution (98 µl) was used to solubilise the Eosin Y dye; this value was used as the absorbance of the dye (Δ_{dye}).

The lysed cell suspension was removed (90 μ l) following centrifuging at 70 g for 1 min and the remaining cell suspension (10 μ l) in the plate was analysed for haemocyte protein concentration (see Section 2.4.10). The following equation was used to calculate cellular integrity (see below). Cellular integrity is expressed as Eosin Y absorbance at 518 nm per mg protein.

$$\frac{\Delta_{\text{samples}}}{\Delta_{\text{dye}}} \times 100 = \% \text{ cellular integrity}$$

2.4.8 Lysosomes and oxidative stress

Lysosomes are highly conserved intracellular organelles within eukaryotic cells and are identified on the basis of two criteria: a surrounding membrane and the presence of enzymes (acid hydrolases) (de Duve 1983). Autophagy (self-eating) is a homeostatic intracellular process whereby intracellular degradation/recycling of proteins occur. It contributes to the turnover of cellular components by delivering portions of the cytoplasm and organelles to lysosomes, where they are digested (Yoshimori 2004). Autophagy takes part in cell survival and death, and has been implicated in development, aging, neurodegeneration and cancer (Deretic 2006). Lysosomes are integral to cellular functioning as they are the sites of macromolecular degradation and recycling via the system of digestive enzymes (de Duve 1983). This enzymatic action functions optimally at low pH and is maintained by an ATP-dependent proton pump located in the membrane (de Duve 1983).

The lysosomal system of cells has been the central focus of cellular-based toxicity studies, as these intracellular organelles are known for their ability to facilitate the sequestering of contaminants, such as metals and organic compounds (Babich

and Borenfreund 1991). On the basis of contaminant sequestering ability of lysosomes, mammalian cells have been used in regulatory toxicology studies, through use of a colourimetric dye method, as a tool for both detecting and quantifying the potencies of chemical compounds and in environmental monitoring studies (Babich and Borenfreund 1991; 1993). Consequently, lysosomes have been used as an indicator of cellular viability due to their sequestering properties, and have been evaluated in various freshwater and marine bioindicator species for environmental contamination (ICES 2004). For *C. maenas*, lysosomes are found within the hyaline cells which are important in terms of phagocytic activity (the innate immune function in crustaceans; see below) (Ratcliffe and Rowley 1979). The haemocytes of Crustacea have been reported to be involved in carbohydrate metabolism, to form a reservoir of free amino acids, to assist in the relocation of lipoproteins during moulting and to be actively involved in the host defence mechanisms (phagocytosis) (Ratcliffe and Rowley 1979).

Lysosomes play an important role in sequestration and detoxification of metals and organic compounds but previous studies have shown that overloading of the storage capacity may contribute to alterations in the integrity of lysosomal membrane (Lowe and Pipe 1994; Lowe et al. 1995b). Under severe 'stress', alteration of the lysosomal membrane may be so great as to result in loss of hydrolytic enzymes outside the organelles which may lead to autophagy. The latter forms the principle of using indicator dyes as tools for evaluating lysosomal function (Babich and Borenfreund 1991; 1993; Lowe et al. 1995a; Lowe et al. 1992; Lowe and Pipe 1994; 1995b). Winston and Di Giulio (1991) demonstrated in mussels that lysosomal destabilisation was affected by production of oxyradicals generated from contaminant exposure, both internally and externally to the lysosomal membrane.

2.4.9 Cellular viability (Neutral red retention assay)

The neutral red (NR) assay is based on the incorporation of the supravital dye NR within the lysosomes of cells. This weakly cationic dye penetrates cell membranes by non-ionic diffusion and binds intracellularly to sites of the lysosomal matrix. Contaminants that injure the plasma or lysosomal membrane decrease the uptake and subsequent retention of the dye. Dead or damaged cells cannot retain the dye after washing and fixation procedures (Babich and Borenfreund 1991).

Haemolymph samples were incubated with an excess (200 μ l) of neutral red solution (0.004 %) and incubated at 10 °C for 3 h to allow for uptake of the dye. Following incubation, the medium was removed and fixed using Baker's formal calcium (BFC). Any excess dye, which is not fixed intracellularly, is removed from solution by washing three times with phosphate buffered saline (PBS) and centrifuged at 70 g for 5 min. A 1 % acetic acid-50 % solution (200 μ l) was used to lyse the cells and extract the neutral red dye. The optical density of each sample was determined spectrophotometrically at 550 nm. An aliquot of the cell lysate suspension (190 μ l) was removed following centrifuging at 70 g for 1 min and the remaining cell suspension (10 μ l) in the plate was analysed for haemocyte protein concentration (see section 2.4.11). Cellular viability results are expressed as neutral red dye absorbance at 550 nm per mg protein (Galloway et al. 2004c).

2.4.10 Phagocytosis

All crustaceans have an open vascular system and hence the rationale for the blood to be termed haemolymph and the cells known as haemocytes (Bauchau 1981). Haemocyte functions include coagulation and wound repair, phagocytosis, transport and synthesis of carbohydrates, and indirectly, osmoregulation via contribution to the free amino acid pool (Bauchau 1981). The phagocytic capability of crustaceans serves to remove foreign particles, such as water-borne bacteria that may be incorporated into the open vascular system. Phagocytosis is performed by the alpha cells (Johnstone et al. 1973; Smith and Ratcliffe 1978) also known as the hyaline cells (Ratcliffe and Rowley 1979). There are ca. 2.5×10^8 circulating haemocytes in *C. maenas*, and around 80 % of these cells are capable of phagocytosis thereby underscoring the phagocytic ability of this species (Smith and Ratcliffe 1978).

A 50 μ l aliquot of neutral red (NR) stained, heat-stabilised zymosan (Sigma-Aldrich) particle suspension containing 1×10^8 particles ml^{-1} in PBS was added to the haemolymph. Microtitre plates were incubated at 10 °C for 30 min. Cells were fixed using BFC and the microtitre plates were centrifuged at 70 *g* for 5 min, and washed three times in PBS to remove excess zymosan particles. Suspensions of known zymosan concentrations were aliquoted (50 μ l) to duplicate wells just prior to the last centrifugation to provide a standard curve (0 – 5×10^7 zymosan particles). Acetic acid (1 %) in 50 % ethanol (100 μ l) was added to each well to solubilise the haemocytes and release the engulfed zymosan particles into suspension. An aliquot of the cell lysate suspension (190 μ l) was removed following centrifuging at 70 *g* for 1 min and the remaining cell suspension (10 μ l) in the plate was analysed for haemocyte protein concentration (see Section 2.4.11). The optical density of each sample was determined

spectrophotometrically at 550 nm. Phagocytic index was expressed as absorbance of NR-stained zymosan particles $\times 10^7$ per mg protein.

2.4.11 Haemolymph protein determination

Haemolymph protein concentration was quantified using a protein reagent kit (Pierce, U.K.). The Pierce bicinchoninic acid (BCA) protein reagent reacts with protein to form a purple reaction product and its absorbance can be determined spectrophotometrically at 550 nm. Two protein reagents (A and B) were mixed prior to protein determination (ratio of 1:50), the reagent mix was added (200 μ l) to haemolymph samples (10 μ l) and incubated at 37 °C for 30 min. Optical density was expressed as mg per ml of protein. Bovine serum albumin (Sigma, U.K) was used as a standard protein solution to produce a standard curve (0.2 - 2 mgml^{-1}).

2.4.12 Haemocyanin determination

Haemocyanin, the respiratory pigment of Crustacea, is metabolised under food-limiting situations (Taylor and Anstiss 1999), and used as an energy reserve (Uglow 1969). Concentrations of the respiratory pigment haemocyanin [Hc] were determined using the spectrophotometric method determined by Nickerson and van Holde (1983). Fifty microlitres of haemolymph diluted with 950 μ l of PBS (Sigma, U.K.) was used for [Hc] using 1.5 ml quartz cuvettes at an absorbance of 335 nm. Haemocyanin concentration was calculated using the extinction coefficient ($\epsilon = 14.2$) (see equation below) (Nickerson and van Holde 1983).

$$A_{335} = \epsilon c L$$

Where:

A = molar absorptivities

ϵ = molar extinction coefficient

c = molar concentration

L = Light path (1cm)

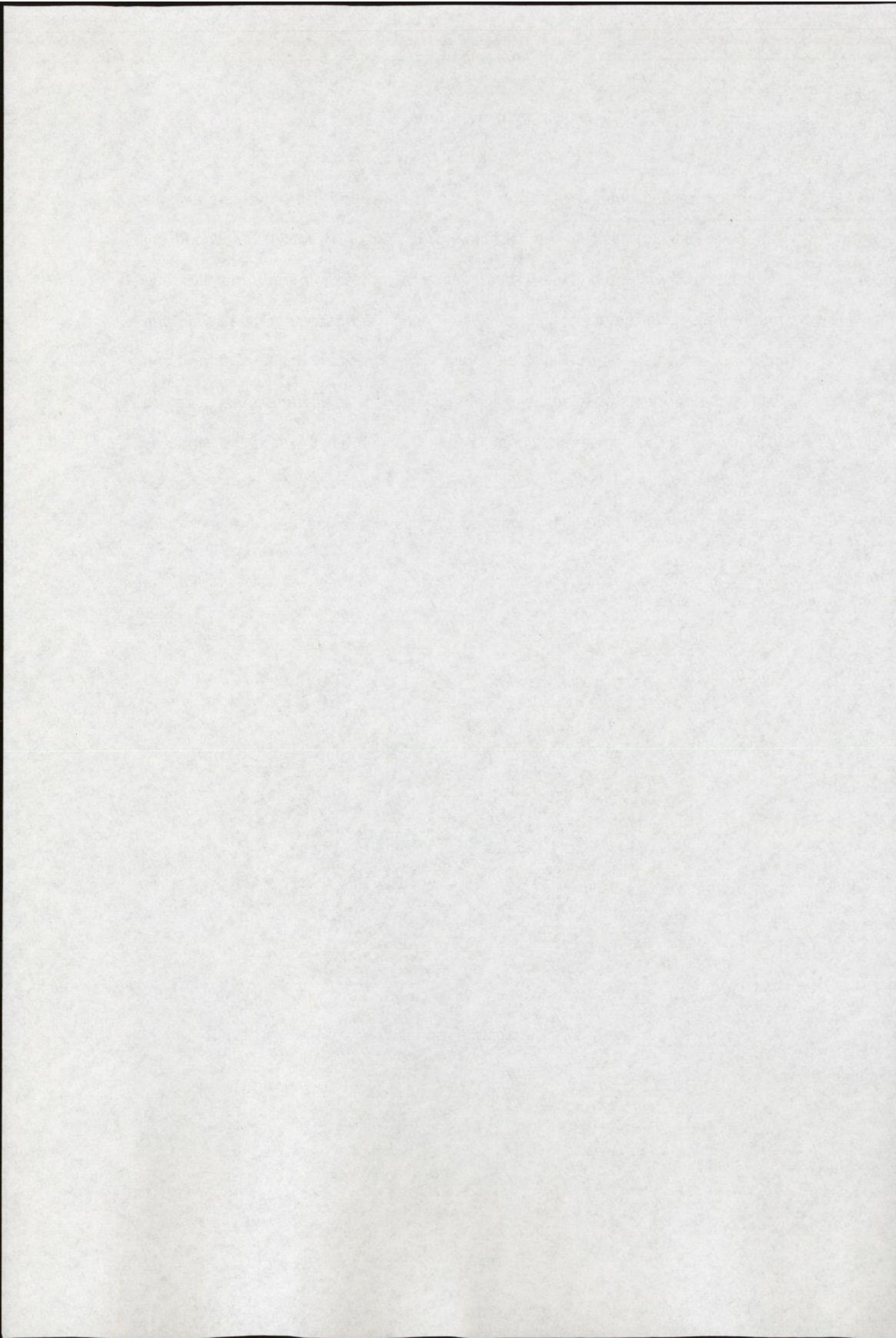
2.5 Physiological endpoints

2.5.1 Cardiac activity

The mean resting heart rate (f_H) of crabs was measured using the non-invasive Computer-Aided Physiological MONitoring system (CAPMON) described by Depledge and Andersen (1990). In summary, a coupled infrared transmitter and photo transducer detector unit is glued (using cyanoacrylate glue) onto the carapace above the heart (Fig. 2.3). Due to conformational changes of the heart with each heartbeat, the intensity of light reflected back to the detector is observed to fluctuate. The registered signal is fed to a computer where the signal is converted via an analogue digital converter. Heart rate can be determined continuously every minute for extended periods where the data are stored for analysis.



Fig. 2.4 The position on the crab carapace where the CAPMON sensor is placed to record cardiac activity.



2.5.2 *Scope for Growth*

Growth is a fundamental property of all living organisms and is imperative for the survival of a population. The amount of energy production represents the difference between an individual's energy intake and energy output (Bayne 1984). Changes in the amount of energy incorporated in growth or reproduction is described by the following equation:

$$P = A - (R + U)$$

Where:

P = energy incorporated into somatic growth and gamete production

A = energy absorbed from food

R = energy respired

U = energy excreted

This energy budget provides a means of integrating fundamental physiological processes (feeding, food absorption, respiration and excretion) into an energy index available for growth and reproduction, termed "Scope for Growth (SfG)" (Bayne 1984). Scope for growth is a useful index as it provides information about the organism's response to the environmental stimulus. Each component of the SfG equation was measured as outlined in the following sections and expressed as the integrated SfG index (Section 2.5.6).

2.5.3 *Measurement of oxygen consumption*

Oxygen measurements were performed using 1 L Perspex respiration chambers (with sealed o-rings) designed for oxygen consumption using a static recirculating system (Fig. 2.5). One crab was placed per chamber (30 min acclimation) with 'open' (i.e. recirculating water: open tap) water flow, and water samples were taken at the start (t_0) and following 30 min (t_{30}) during 'closed' (static water; closed tap) water flow to determine dissolved oxygen concentrations (mg L^{-1}). Concomitant heart rate measurements demonstrated that 30 min acclimation was adequate time for the heart rate levels to decrease from 'active' (i.e. handling stress) state to 'resting' levels. Oxygen concentrations were measured using a Cellox 325-3 oxygen probe (Multi 340i/SET, WTW, Germany). Oxygen consumption measurements (i.e. $t_{30} - t_0$) were performed in a temperature-controlled room (15 ± 1 °C) at 32 ppt. After each set of oxygen measurements, crabs were removed from test chambers, blotted dry using absorbent paper and wet-weighed (± 0.01 mg) using a balance (Sartorius B3100P) and converted to dry weight (Roast et al. 1999a) to allow for weight-specific expression of oxygen consumption rates and expressed as $\text{mg O}_2 \text{ L}^{-1} \text{ g dry weight}^{-1} \text{ hr}^{-1}$.

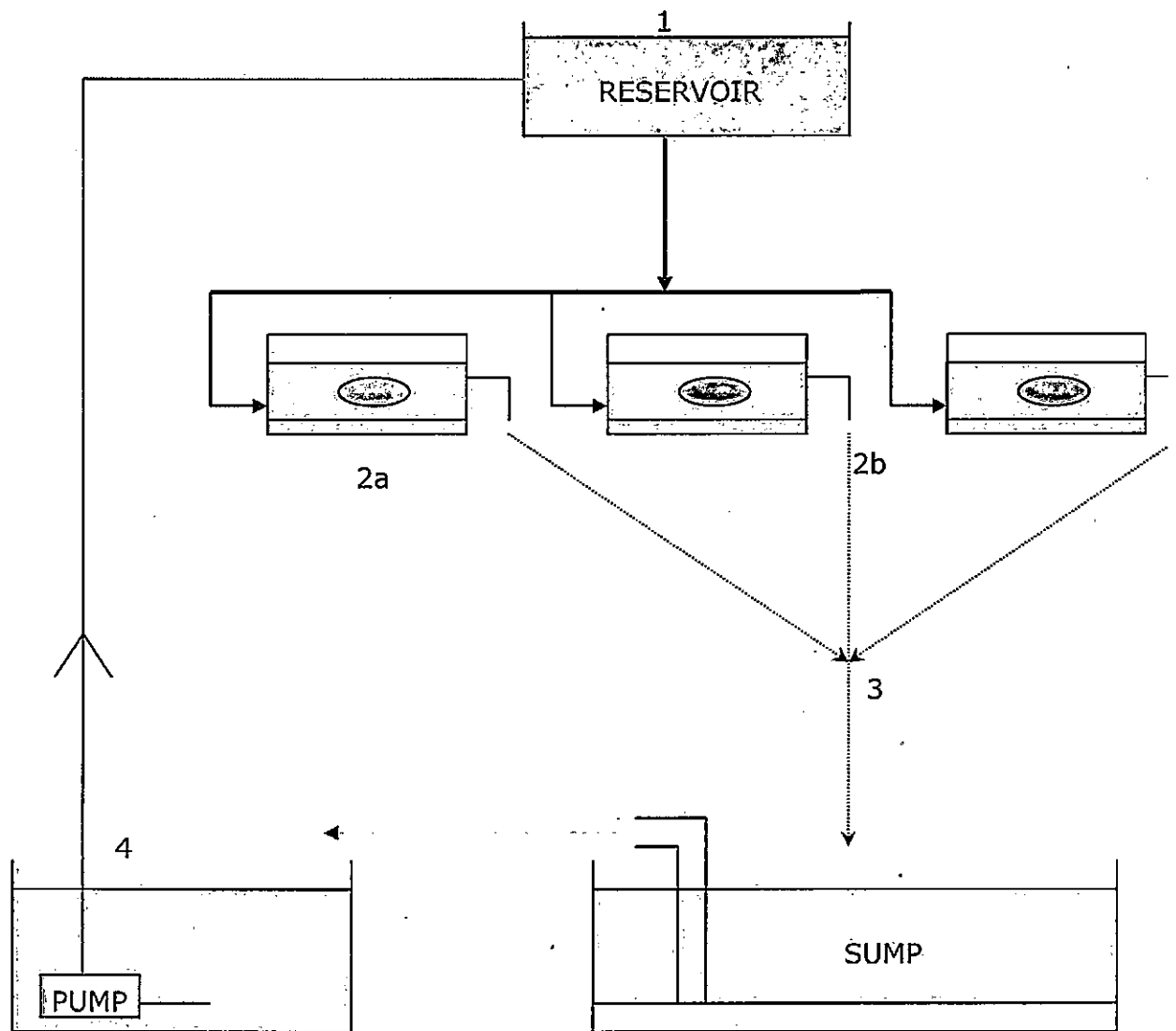


Fig. 2:5

Diagrammatic representation of a recirculating system used for oxygen consumption in *Carcinus maenas*. Water flow from reservoir (1) fed into individual chambers [represented by red lines (2a)], water taken for oxygen consumption measurements through chamber outflow valve containing decreased oxygen concentrations during 'closed' water flow (2b). During 'open' water flow, i.e. recirculating, water is collected into a sump (3) and transferred to a secondary sump (4) where it is filtered (10 μm filter) and pumped into the reservoir for recirculation.

2.5.4 Measurement of egestion rates

Egestion rates (faecal production) were measured as a surrogate for ingestion rates (as egestion correlates with ingestion) by adaptation of a method described by Roast et al. (1999b). Pre-weighed food [γ -irradiated cockle tissue (Gamma foods, TMC, Bristol, U.K) $1 \text{ g} \pm 0.01 \text{ mg}$] was introduced to individual crabs and faeces were collected using a plastic Pasteur pipette. Faeces were rinsed with distilled water and stored in pre-weighed centrifuge tubes at $-20 \text{ }^\circ\text{C}$ for analysis and expressed as mg per g dry weight per h.

2.5.5 Measurement of absorption efficiency

Crab absorption efficiency was estimated by the ratio method based on the proportion of the organic material (the ash-free material) in the food and the faeces (Conover 1966) and calculated using the following equation (1):

$$(1) \quad \text{Absorption efficiency} = (F - E) / [(1 - E) \times F]$$

Where:

F = ash free dry weight: dry weight ratio of food

E = ash free dry weight: dry weight ratio of faeces

Oven-dried ($60 \text{ }^\circ\text{C}$) material (food/faeces) ($\pm 0.01 \text{ mg}$) (dry weight) was placed into ashed, pre-weighed aluminium foil containers. The containers were ashed at $450 \text{ }^\circ\text{C}$ for 2 h to combust fully the organic material and re-weighed ($\pm 0.01 \text{ mg}$) to ascertain the ash content of the organic material (ash free dry weight).

2.5.6 - Calculation of Scope for Growth (SfG) Index.

Carcinus maenas SfG was calculated by converting oxygen consumption and ingestion rates into energy equivalents (J h^{-1}), and calculating the net energy gain/loss using the following equation (2):

$$(2) \quad P = A - (R + U)$$

Where:

$P = \text{SFG (J mg dry wt}^{-1} \text{ h}^{-1} \text{)}$

$A = \text{energy absorbed (J mg dry wt}^{-1} \text{ h}^{-1} \text{)}$

$R = \text{energy excreted (J mg dry wt}^{-1} \text{ h}^{-1} \text{)}$

Components of equation 2 (A and R) were calculated using equations 3 and 4 and transformed into energy equivalents:

Energy absorbed:

$$(3) \quad A = C \times \text{Absorption efficiency}$$

Where:

$A = \text{energy absorbed (J mg dry wt}^{-1} \text{ h}^{-1} \text{)}$

$C = \text{energy consumed (J mg dry wt}^{-1} \text{ h}^{-1} \text{)}$

Energy respired:

$$(4) \quad R = \text{oxygen consumption } (\mu\text{l O}_2 \text{ mg dry wt}^{-1} \text{ h}^{-1}) \times 0.02008.$$

$$R = \text{energy respired (J mg dry wt}^{-1} \text{ h}^{-1})$$

Oxygen consumption rates were converted to dry-weight specific rates (ca. 20 % for decapod crustaceans) (Roast et al. 1999b).

$$0.02008 \text{ J}(\mu\text{l O}_2^{-1}) = \text{heat equivalent of oxygen uptake (Gnaiger 1983)}.$$

The rate of ammonia excretion is usually correlated closely with respiration rate and contributes < 5 % of the metabolic energy expenditure and was therefore omitted from the SfG calculation (Widdows and Staff 1997). Ammonia excretion was not measured here, thus SfG was calculated from the equation $P = A - R$.

2.6 Statistical analyses

Two methods of statistical analyses are used throughout the thesis. To test for treatment differences in a single variable, univariate analyses are applied. To establish the integrated response of all physiological variables to signal physiological condition, multivariate analyses were used.

2.6.1 Univariate

Univariate analyses of variance (ANOVA) tests were performed using GMAV5 for Windows ® (Underwood 2005). ANOVA tests (otherwise stated) were performed using untransformed data. Significant differences between treatment groups are highlighted with rejection of the null hypothesis at the 5 % probability level ($P < 0.05$).

2.6.2 Multivariate

Multivariate analyses were performed using PRIMER © v6 (Plymouth Routines in Multivariate Ecological Research, PRIMER-E Ltd). Various analytical tests were used to identify the data. Tests are based upon triangular euclidean (geometric) distance resemblance matrices using normalised data (i.e. subtracted by the mean and divided by the standard deviation).

2.6.2.1 Analysis of similarity (ANOSIM)

ANalysis Of SIMilarity (ANOSIM) tests were used to test for differences between treatment groups. ANOSIM R values are expressed between 0 and 1, where large values indicate discrimination between pairwise interactions (Clarke and Warwick 2001). For analyses where there are more than two pairwise tests, a significant result is attained when the pairwise R value is greater than the Global R value.

2.6.2.2 *Index of multivariate dispersion (IMD)*

Pairwise comparisons between treatment groupings are expressed using IMD values (between a minimum of -1 and a maximum of +1) to denote the sample variability within a group compared to that of another group (Warwick and Clarke 1993). A value of +1 occurs when all similarities among impacted samples are lower than any similarities among control samples and the converse situation yields a value of -1. A value of 0 is indicative of no difference between treatment groups (Warwick and Clarke 1993).

2.6.2.3 *Similarity of percentages (SIMPER)*

SIMPER procedures are not formal statistical tests but exploratory analyses used to compare two groups at a time, identifying influential variables for each specific comparison (Clarke and Warwick 2001). SIMPER analyses are used here to indicate which physiological variables were responsible (i.e. percentage contribution) for the treatment differences (if any) as revealed by ANOSIM tests.

CHAPTER 3:

**Does physiology vary between juvenile
and adult *Carcinus maenas* (L.)?**

Does physiology vary between juvenile and adult *Carcinus maenas* (L.)?

Abstract

The aim of the present study was to test the hypothesis that adult and juvenile crabs are physiologically distinct and, consequently, will exhibit different sensitivities to contaminant exposure. Various aspects of the physiology of juvenile (< 35mm CW) and adult (> 60mm CW) crabs were measured immediately following collection; physiological assessment was based on a repertoire of biological responses at successive levels of biological organisation (biochemical to physiological). Univariate analysis of newly-collected crabs ($n = 18$) demonstrated that juveniles had lower immune capability (0.03 ± 0.02 vs. 0.07 ± 0.07 zymosan particles 10^7 mg protein⁻¹), lower metabolic energy (25.71 ± 14.42 vs. 36.88 ± 13.82 $\mu\text{g L}^{-1}$) and increased scope for growth (SfG) (24.62 ± 12.84 vs. 2.6 ± 1.18 J mg⁻¹ dry wt⁻¹ hr⁻¹) compared with adults. Following a seven-day sublethal exposure to pyrene (200 $\mu\text{g L}^{-1}$), exposed juveniles exhibited decreased immunocompetence (phagocytic index and cellular integrity) ($P < 0.05$), significantly elevated (19%) basal heart rate and significantly decreased (55 %) respiration rate (at rest) ($P < 0.05$) compared with unexposed juveniles. Although univariate analyses highlighted significant 'stress' responses in juvenile and adult crabs with exposure to pyrene, multivariate analysis revealed degradation in physiological condition in only juvenile shore crabs. Adult crabs were more tolerant than juveniles of pyrene exposure. Results confirm that juvenile and adult *C. maenas* vary physiologically and as such, juvenile shore crabs are more susceptible to the effects of pyrene exposure.

3.1 INTRODUCTION

The shore crab *Carcinus maenas* inhabits various coastal habitats including estuaries (Crothers 1968). Estuaries are highly productive habitats but are characterised by wide and acute fluctuations of abiotic conditions such as salinity and temperature (Rewitz et al. 2004). To cope with such environmental variability, shore crab physiology is highly robust and *C. maenas* is euryhaline (Abelló et al. 1997; McGaw and Naylor 1992c; Rainbow and Black 2001, 2002; Reid et al. 1997) and eurythermal (Aagaard 1996; Camus et al. 2004; Cumberlidge 1977a; Taylor 1973). In addition, adult crabs express a tidal migration from the subtidal to the intertidal and, by entering the estuary at high tide and leaving before low tide (Dare and Edwards 1981; Hunter and Naylor 1993; Rewitz et al. 2004; Warman et al. 1993), they exploit food resources and limit exposure to extreme environmental abiotic ranges (Kaiser et al. 1990; Reid et al. 1989). Juvenile shore crabs inhabit the intertidal (Crothers 1968), often within estuaries (Crothers 1968); the latter acting as a nursing ground, as reported for other crustacean species (Epifanio et al. 2003; Haywood et al. 1998; Morgan et al. 1996).

Many crustaceans move as adults from the intertidal to the subtidal (Rainbow and Black 2005) and it is believed that crustacean physiology needs to be 'physiologically competent' to cope with such ecological transitions (Morritt and Spicer 1999). It is well established that some aspects of crab physiology vary with developmental stage; for example, there is greater variation in heart rate of juvenile *C. maenas* compared to adults during the tidal cycle (Depledge 1992; Newell et al. 1972). The physiological tolerance at any life-cycle stage determines the survival potential of individuals (Hebel et al. 1997). Currently, there is a lack of information regarding the physiology of juvenile *C. maenas*. In this chapter, the hypothesis that the enhanced tolerance of adults compared with juvenile *C.*

maenas would be reflected in an increased ability of the adults to tolerate contaminant-induced stress (in the form of pyrene exposure) was tested.

Pyrene, a polyaromatic hydrocarbon (PAH), is one of a group of highly lipophilic organic compounds that are ubiquitous pollutants of the marine environment (Dissanayake and Galloway 2004). In the marine environment, PAHs originate from petrochemical pollution and from incomplete combustion processes (Clarke et al. 2001). There is concern over the fate and effect of PAHs in the marine environment due to their persistence, bioaccumulation potential, and acute and chronic toxicity to marine organisms (Clarke et al. 2001; Livingstone 1992, 1998). Pyrene toxicity can arise in the form of membrane damage, enzyme inactivation or damage by reactive oxygen species produced by pyrene metabolism (see Chapter 1, Section 1.2). A sublethal exposure was used here as means of imposing contaminant-induced injury, allowing assessment of the potential differential susceptibility of juvenile and adult stages of the shore crab to pyrene exposure. Exposure, and subsequent metabolism of PAH compounds by organisms, results in metabolites of the parent compound being concentrated in body fluids, tissues and excreta (Dissanayake and Galloway 2004). The presence of such metabolites in crab urine is used here as a surrogate measurement of detecting exposure to bioavailable contaminants (Fillmann et al. 2002).

The aim of this study was to establish how juvenile and adult *C. maenas* responded to sublethal exposure ($200\mu\text{gL}^{-1}$ for seven days) to the PAH, pyrene. To achieve this aim, the physiological condition of juvenile and adult crabs was measured (a) directly from the field (immediately upon collection within 24 h), and (b) after pyrene exposure (after seven days).

3.2 MATERIAL AND METHODS

3.2.1 Shore crab collection and maintenance

Juvenile (<35mm carapace width (CW)) and adult (>60mm CW) (Crother's 1967) male (green) intermoult *Carcinus maenas* ($n = 36$) were collected from the Avon Estuary, Bantham, South Devon, U.K. (grid reference: SX 6623 4380) on two occasions (23rd November and 26th November 2004) using mackerel-baited traps (Chapter 2, Section 2.1). In the laboratory, crabs were subjected to an assessment of their physiological condition within 48h of collection and maintained in static holding tanks containing filtered (10 μ m carbon filtered), aerated seawater (34ppt, $15 \pm 1^\circ\text{C}$) under a 12h light : 12h dark photoperiod for seven days. Crabs were fed twice weekly with γ -irradiated cockle (*Cerastoderma edule*) (Gamma foods, Tropical Marine Centre, Bristol, U.K.) and water was changed within 18h of each feeding.

3.2.2 Physiological assessment of newly-collected crabs

Each crab was numbered using a label attached (cyanoacrylate glue) to the carapace; carapace width (mm) and wet weight (g) were recorded, and crabs were grouped (based on CW) as juveniles (<35mm) and adults (>60mm CW) ($n = 18/\text{group}$). To establish physiological condition, the following biological responses were measured at successive levels of biological organisation: (1) biochemical (glucose determination, antioxidant potential), (2) cellular (cellular viability, cellular integrity and phagocytosis index) and (3) physiological [haemocyanin and scope for growth (SFG)]. Measurements of respiration and egestion rates (components of SFG) were taken prior to haemolymph sampling. Haemolymph samples (100 μ l) were extracted by puncture of the arthroal membrane at the base of the 4th walking leg using a pre-chilled 1ml syringe and 21 gauge needle (Sigma-Aldrich, U.K.). Cellular assays were conducted immediately post-haemolymph

sampling. Haemolymph samples were snap-frozen using liquid nitrogen and stored at -80°C for all subsequent analysis.

3.2.3 Biochemical techniques

Total antioxidant status: determined by measuring the combined reducing power of the electron-donating antioxidants present (i.e ferric reducing antioxidant potential) (Benzie and Strain 1996; Rickwood and Galloway 2004). A stoichiometric excess of the oxidant ferric tripyridyltriazine (Fe^{III} -TPTZ) was added to each 10 μl sample (300 μl of 10mM in 300 mM sodium acetate, pH 3.6) and its reduction to the ferrous form (Fe^{II}) measured spectrophotometrically after 10 mins at 593nm (see Chapter 2, Section 2.4.5 for details).

Glucose concentration in haemolymph: glucose concentrations were assayed according to the method of Kunst et al. (1981) (see Chapter 2, Section 2.4.3 for details).

3.2.4 Cellular techniques

Cellular viability: determined using a dye uptake method as described by Galloway et al. (2004c) (see Chapter 2, Section 2.4.9 for details).

Cellular integrity: cell functionality (i.e. dead or viable) was determined using a dye exclusion method whereby 2mg ml^{-1} Eosin Y was added to haemolymph samples (20 μl) at a ratio of 1:10. Following 10 min incubation and washing cycle, the absorbance of the residual dye was measured spectrometrically at 518nm. Viable cells stain light green (due to dye exclusion) whereas dead and moribund cells stain red (see Chapter 2, Section 2.4.7 for details).

Phagocytosis index: the phagocytic capability of haemocytes was evaluated by measuring the active uptake of neutral red-stained zymosan particles as described by Parry and Pipe (2004), and employed by Galloway et al. (2004c) (see Chapter 2, Section 2.4.10 for details).

3.2.5 Physiological techniques

Haemocyanin concentration [Hc]: was estimated for individuals using the established spectrophotometric method (Nickerson and van Holde 1983). Fifty microlitres of haemolymph were diluted in 950 μ l of physiological saline; the absorbance of the mixture was measured at 335nm using quartz cuvettes (Sigma, U.K) and expressed in mg ml⁻¹ using the 1% extinction coefficient given by Nickerson and van Holde (1983) (see Chapter 2, Section 2.4.12 for details).

Scope for growth (SFG): calculated measuring various physiological components (see Chapter 2, Section 2.5.2 for details) and the net energy gain/loss through these physiological processes using the following equation:

$$P = A - R$$

Where: $P = SFG$ (J mg dry wt⁻¹ h⁻¹), $A =$ energy absorbed (J mg dry wt⁻¹ h⁻¹) and $R =$ energy respired (J mg dry wt⁻¹ h⁻¹) (Widdows et al. 1995).

3.2.6 Experimental design and post-exposure assessment

Six groups, each of 6 crabs (a – c = juveniles, d – f = adults), were exposed individually (1 crab per tank; 36 in total) under the following treatments: controls (a and d); solvent controls (acetone, 0.005%) (b and e) and exposed 200µg l⁻¹ pyrene (nominal concentration) (c and f) (Table 3.1). Each group was exposed for 7 d; crabs were fed individually on 1g of γ-irradiated cockle every two days and water was changed within 18 h of feeding.

Table 3.1 Treatment groupings of exposed crabs. Subscript letters indicate treatment groups; a-e =juvenile treatments, d-f = adult treatments (*n* = 6).

Treatment	Juveniles	Adults
Control	6 ^a	6 ^d
Solvent control	6 ^b	6 ^e
PYR	6 ^c	6 ^f

Post-exposure assessment of physiological condition included the biological responses described previously (see Sections 3.2.3 – 3.2.5) with the additional physiological measurement of heart rate monitoring (30 min recordings) using the CAPMON system (Depledge and Andersen 1990). To semi-quantify pyrene exposure, fluorescence analysis for 1-hydroxypyrene-type metabolites (1-OH) was performed on diluted urine samples (10µl in 50% ethanol, ratio 1:20) sampled from the antennal gland (Dissanayake and Galloway 2004; Watson et al. 2004b) (see Chapter 2, Section 2.3.2 for details).

3.2.7 Statistical analysis

Two methods of statistical analyses were used to isolate significant differences in physiological condition of newly-collected juvenile and adult *C. maenas*, and to identify toxicant sensitivity via contaminant exposure. Univariate (*ANOVA* or Kruskal-Wallis) analyses were performed to test for differences for a single parameter between experimental groups. To attain an integrated summary of the contaminant impact, multivariate analyses tested for differences between the experimental groups via the complete repertoire of biological responses measured. Univariate statistical analyses were performed using GMAV5 for Windows ® or StatGraphics® Plus v5 (Statistical Graphics Corporation). Treatment differences were analysed using either the parametric *ANOVA* test or non-parametric Kruskal-Wallis test, dependent upon homoscedasticity of data (Kruskal and Wallis 1952) (see Chapter 2, Section 2.6.1 for details).



Multivariate analysis was carried out using PRIMER® v6 (Plymouth Routines in Multivariate Ecological Research, PRIMER-E Ltd). ANOSIM (ANalysis Of Similarities) tests were used to test for similarities between ontogenetic stages. ANOSIM *R* values are expressed between 0 and 1. Large values indicate discrimination between pairwise interactions (Clarke and Warwick 2001). A comparative index of multivariate dispersion (IMD) is also expressed and uses pairwise comparisons for denoting the sample variability within a group compared to that of another group (Warwick and Clarke 1993). IMD values are between a minimum of -1 and a maximum of +1. A value of -1 implies no difference between treatment groups and +1 occurs when all similarities among impacted samples are lower than any similarities among control samples (Warwick and Clarke 1993) (see Chapter 2, Section 2.6.2 for details).

3.3 RESULTS

3.3.1 Physiological assessment of newly-collected crabs

There were significant differences in several biological responses between juvenile and adult *C. maenas* (Table 3.2). Juveniles had significantly higher cell viability (ANOVA, $F_{1,34} = 16.90$, $P < 0.01$) (Fig. 3.1a), respiration rates (ANOVA, $F_{1,34} = 13$, $P < 0.01$) (Fig. 3.1b) and SFG (ANOVA, $F_{1,29} = 46.44$, $P < 0.001$) (Fig. 3.1c) but lower haemolymph glucose (ANOVA, $F_{1,34} = 5.62$, $P < 0.05$) (Fig. 3.1d) and phagocytic capability (ANOVA, $F_{1,34} = 7.07$, $P < 0.05$) (Fig. 3.1e) than adults. Pairwise ANOSIM tests confirmed that the physiological condition of the two ontogenetic stages was different, and that juveniles have different ranges for a number of their biological responses, whilst maintaining 'normal' function compared to adults [$R = 0.915$, $P (\%) = 0.001$, Table 3.3a].

Table 3.2 Physiological assessment of newly-collected juvenile and adult *Carcinus maenas*.

RESPONSES (mean ± SD)		JUVENILES (n = 18) 	ADULTS (n = 18) 	STATISTICAL SIGNIFICANCE
BIOCHEMICAL	GLUCOSE ($\mu\text{G L}^{-1}$)	25.71 ± 14.42	36.88 ± 13.82	ANOVA, $F_{1,34} = 5.62,$ $P < 0.05$
	ANTIOXIDANT POTENTIAL ($\mu\text{M L}^{-1}$)	331 ± 122	346 ± 147	NO DIFFERENCE
CELLULAR	CELLULAR INTEGRITY (%)	79.35 ± 11.35	75.69 ± 13.65	NO DIFFERENCE
	CELLULAR VIABILITY (O.D mg protein ⁻¹)	0.15 ± 0.07	0.08 ± 0.04	ANOVA, $F_{1,34} = 16.90,$ $P < 0.01$
	PHAGOCYTOSIS (zymosan 10 ⁷ mg protein ⁻¹)	0.03 ± 0.02	0.07 ± 0.07	ANOVA, $F_{1,34} = 7.07,$ $P < 0.05$
PHYSIOLOGICAL	HAEMOCYANIN (mg ml ⁻¹)	0.39 ± 0.14	0.32 ± 0.15	NO DIFFERENCE
	RESPIRATION (mg O ₂ L ⁻¹ g dry weight ⁻¹ hr ⁻¹)	5.06 ± 5.11	0.70 ± 0.52	ANOVA, $F_{1,34} = 13,$ $P < 0.01$
	SFG (J mg ⁻¹ dry wt ⁻¹ h ⁻¹)	24.62 ± 12.84	2.68 ± 1.18	ANOVA, $F_{1,29} = 46.44,$ $P < 0.001$

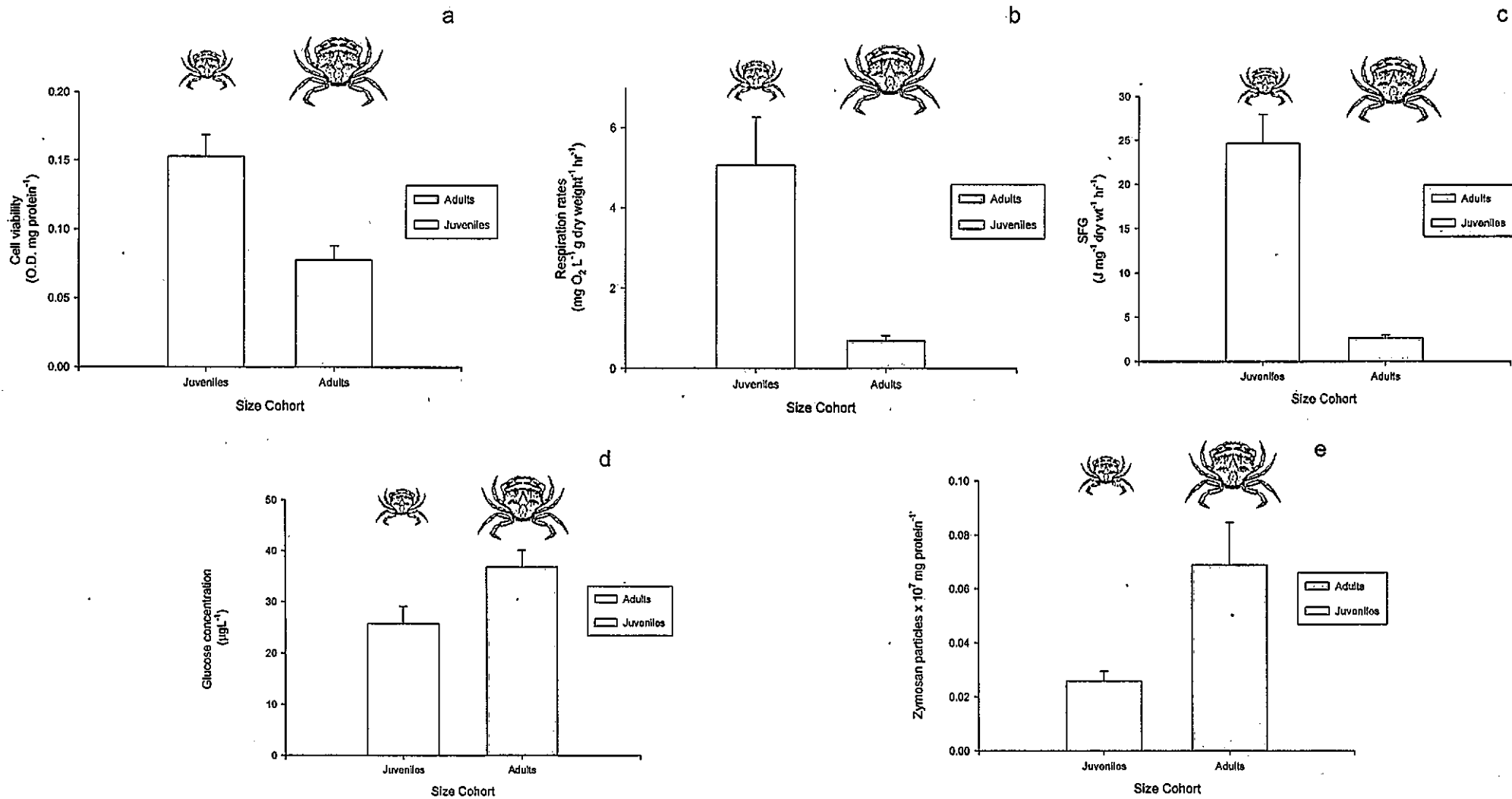


Fig. 3.1 a) Cell viability (OD mg protein⁻¹; mean ± 1 SE) (*n* = 18); b) Respiration rate (mg O₂ L⁻¹ g dry weight⁻¹ hr⁻¹); mean ± 1 SE) (*n* = 18); c) Scope for growth (J mg⁻¹ dry wt⁻¹ h⁻¹); mean ± 1 SE) (*n* = 18); d) Glucose concentrations (µg L⁻¹; mean ± 1 SE) (*n* = 18); e) Phagocytosis (zymosan particles 10⁷ mg protein⁻¹; mean ± 1 SE) (*n* = 18).

Table 3.3 Results from one-way ANOSIM tests, based on Euclidean distance similarities in biological responses from newly-collected crabs and post-exposure physiological assessments. *NS* = Not significant, *S* = Significant, CON = Control, PYR = Pyrene.

	Treatments	ANOSIM Global tests			ANOSIM pairwise tests			Significant difference	
		R	P (%)	P (value)	R	P (%)	P (value)		
(A) Newly-collected crabs	Juveniles vs. Adults	-	-	-	0.915	0.1	0.001	S	
(B) POST-exposure	Juveniles; CON vs. PYR	0.124	5.6	0.056	0.215	1.1	0.011	S	0.609
	Adults; CON vs. PYR	0.245	5.6	0.056	0.1	13.4	0.134	NS	0.04

3.3.2 Physiological assessment following pyrene exposure

Compared to controls, concentrations of 1-hydroxypyrene-type metabolites in the urine were significantly higher in both juvenile and adult pyrene-exposed individuals (*Kruskal-Wallis*, $TS = 13.86$, $P < 0.05$) (Fig. 3.2), validating exposure to the parent compound (pyrene). For juvenile crabs, six out of seven responses were significantly affected by pyrene exposure, whereas for adults, only two out of seven parameters were affected significantly (Table 3.4). Multivariate analysis (ANOSIM) identified significant differences in physiological state for juvenile crabs with exposure to pyrene, but not for adults; however, juveniles also showed greater variability in the overall response to pyrene exposure (0.609) compared to adults (0.04) (Table 3.3).

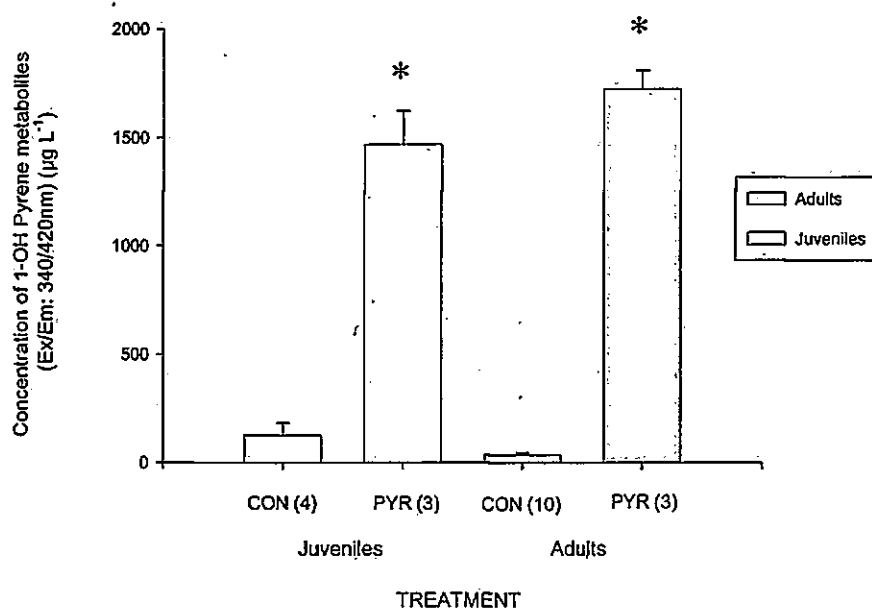




Fig. 3.2 Concentrations of 1-OH Pyrene-type metabolites (_{340/420}) (µg L⁻¹; mean ± SE) (CON = Control, PYR = pyrene exposed, * signify exposures are significantly different from controls $P < 0.05$) ($n = 6$ group⁻¹).

Table 3.4. Post-exposure (200 μ g L⁻¹ pyrene) assessment of juvenile and adult *Carcinus maenas* physiology ($n = 6$) based on changes in biological responses compared to controls.

		JUVENILES	ADULTS	
RESPONSES				STATISTICAL SIGNIFICANCE
CELLULAR	CELLULAR INTEGRITY (%)	Decreased cell integrity (25%)	No effect	ANOVA, $F_{5,30} = 2.54,$ $P < 0.05$
	CELLULAR VIABILITY (O.D mg protein ⁻¹)	Increased cellular viability (65%)	No effect	ANOVA, $F_{5,29} = 3.02,$ $P < 0.05$
	PHAGOCYTOSIS (zymosan 10 ⁷ mg protein ⁻¹)	Decreased phagocytic Capability (50%)	Increased phagocytic capability (30%)	ANOVA, $F_{5,30} = 4.74,$ $P < 0.01$
PHYSIOLOGICAL	HEART RATE (beats min ⁻¹)	Increased heart rate (20%)	No effect	ANOVA, $F_{5,30} = 4.74,$ $P < 0.01$
	RESPIRATION (mg O ₂ L ⁻¹ hr ⁻¹)	Decreased respiration rate (50%)	No effect	ANOVA, $F_{5,30} = 4.39,$ $P < 0.005$
	SFG (J mg ⁻¹ dry wt ⁻¹ h ⁻¹)	No effect	Decreased SFG (65%)	Kruskal-Wallis, TS = 17.86 $P < 0.005$
	FEEDING (% energy consumed)	Decreased energy consumption (10%)	No effect	Kruskal-Wallis, TS = 10.74 $P < 0.06$

3.4 DISCUSSION

Measurements taken directly from crabs from the field established significant physiological differences between juvenile and adult *C. maenas*. Juveniles showed higher weight-specific respiration rates and scope for growth, but lower immune function (phagocytic index) and metabolic energy (in the form of glucose) compared with adults. Respiration rates recorded here are consistent with those reported by Dawirs (1983) for *C. maenas* and confirm his finding that weight-specific respiration rates decrease with increasing biomass. Present findings also highlight that juvenile crabs have increased *scope for growth* compared with adults; these data are consistent with the hypothesis that they channel more energy towards somatic growth than adults (Styrishave et al. 2000). Differences between juveniles and adults were also identified at sub-organismal levels, with immunocompetence (cellular viability and phagocytosis) differing in juveniles compared with the 'adult pattern'. A higher cellular viability in juvenile crabs could be explained by varying ratios of cell types. Johnstone et al. (1973) provided evidence for two types of circulating haemocytes: alpha and beta cells. Alpha cells are the storage sites for glycogen and the presence of numerous large vacuoles in the peripheral cytoplasm is suggestive of phagocytic function. Beta cells are described as the 'explosive corpuscles' thought to be involved in coagulation. Alpha cells constitute ca. 80% and beta cells constitute ca. 20% of the circulating haemocytes found in adult crabs (Smith and Ratcliffe 1978) which is consistent with the present findings of ca. 80% cellular integrity in both size groupings. Variable ratios of alpha and beta haemocytes, throughout the developmental stage of the life cycle, would afford an explanation of the differential immunocompetence pattern seen here.

Energy available for metabolism, in the form of glucose, was higher in adult crabs than juveniles, and may result from differences in feeding behaviour. Adult male crabs are active foragers and undertake extensive migrations into estuaries to exploit food resources (Rewitz et al. 2004), whereas juveniles reside in estuaries, which serve as nursery areas (Morgan et al. 1996). The total energy pool available to an individual is a sum of the circulating glucose and stored glycogen (not measured here) (Briffa and Elwood 2004). High circulating glucose levels are necessary in energy-demanding activities such as exercise and fighting behaviour, as shown in *C. maenas*, *Necora puber* and *Pagurus bernhardus* (Briffa and Elwood 2004; Sneddon et al. 1999; Thorpe et al. 1995). The ontogenetic differences outlined here suggest a less developed immune capability in juveniles compared to adults, and differential physiological status between the two developmental stages.

The main aspect of this study was to assess if there were differential responses between the ontogenetic stages of *C. maenas* to sublethal toxicity to the priority pollutant pyrene. The global question was do juvenile and adult crabs have different 'physiological competencies' (defined as the tolerance afforded to the organism by the repertoire of inherent biological responses to stressors). Following pyrene exposure, significant biological responses were observed in each size grouping, with juveniles displaying significant deviations in seven cellular and physiological measurements. At the cellular level, pyrene exposure caused significant decreases in immunocompetence and cellular integrity for juveniles. The increased dye retention observed in the neutral red assay could have arisen due to an increase in the amount of lysosomes present as well as lysosomal volume. Findings of increased dye uptake in juveniles are in accord with other studies reporting increased lysosomal capacity following contaminant exposure (Grundy et al. 1996; Matozzo et al. 2002; Matozzo and Marin 2005; St-

Jean et al. 2002a, b). Cellular viability is based on the accumulation of the cationic neutral red dye into the lysosomes of viable cells. The apparent increase of dye retention could be due to the increase in lysosome number and volume induced by the low level of pyrene. This pattern of elevated dye retention in pyrene-exposed juveniles may be due to the intrinsic ability of lysosomes to accumulate a wide range of contaminants (St-Jean et al. 2002b). In the present study, lysosomes did not appear to reach their maximum retention capacity and probably preserved their integrity. According to St-Jean et al. (2002a) (and references therein), lysosomal membrane destabilisation is linked to oxidative stress. The current exposure conditions did not confer a persistent xenobiotic challenge, in either size cohort, to induce lysosomal membrane injury or oxidative stress, substantiating our results of no change in antioxidant potential. Increased heart rate, and concomitant decrease in respiration rates, were also observed here, which are in concordance with Spicer and Weber (1991) who reported a similar response in crustaceans exposed to heavy metals (copper and zinc at a sublethal concentration of 0.4 mgL^{-1}). Camus et al. (2004) report increased cardiac activity for *C. maenas* exposed to copper (0.5 mgL^{-1} for three days) and interpreted this as an increase in oxygen demand to compensate for physiological malfunctions. Impairment of gaseous exchange due to gill epithelium alterations would cause internal hypoxia, thereby, accounting for an increase in heart rate as a compensatory response (Hebel et al. 1997).

Present results show contaminant impacts for juveniles and adults are manifested at the cellular and physiological levels. To understand the implications of these responses an assessment of their effects upon Darwinian 'fitness' parameters needs to be made [i.e. the intra-population variation in physiological traits serves to increase the fitness of individuals (Spicer and

Gaston 1999)]. Results of the integrated measure of scope for growth (*SfG*) demonstrated that there was no significant effect of pyrene exposure on juveniles; however, a decrease in energy acquisition (due to decreased egestion rates) occurred. Suppression of crustacean feeding rates, following exposure to toxic contaminants, has been documented (Naylor 1989; Roast et al. 1999b) and help explain the present results. In adults, scope for growth was decreased significantly and this may be the result of re-channelling of energy from growth to compensatory mechanisms (e.g. energetic costs of immunocompetence) (St-Jean et al. 2002b). In general, *SfG* decreases with contaminant exposure; however, this conclusion is based solely on bivalve molluscs (Widdows et al. 1995; Widdows 1992; Widdows and Salkeld 1993). The results for *SfG* from this study highlight the implications of contaminant-exposure to energy budgets within crustaceans and demonstrate how they vary with developmental stage.

Although univariate analyses highlighted significant 'stress' responses in juvenile and adult crabs with exposure to pyrene, multivariate analysis revealed degradation in physiological-condition in only juvenile shore crabs. On the other hand, adult crabs were more tolerant than juveniles of pyrene exposure. These results highlight the robustness of adult shore crab physiology and the relative susceptibility of juveniles to contaminant exposure. Values for the index of multivariate dispersion (IMD) demonstrated greater variability in juvenile responses (0.609) compared with adults (0.04), suggesting increased plasticity of response for the former (Table 3.3). Newell (1972) stated that it was advantageous for juvenile crabs inhabiting the intertidal to be in an 'alert' state, thereby, possessing the ability to deal with unfavourable conditions. The variability of responses to contaminant exposure between crab stages can be used as an identifiable symptom of perturbation (suggestive of the phenotypic response to pyrene exposure) and has been used as such in environmental

impact studies (Warwick and Clarke 1993). This variability in response could be used also to identify toxicant sensitivity between the two developmental stages.

In the context of the present study, sublethal pyrene exposure resulted in alterations in single biological systems at various levels of biological organisation. Alterations to cellular and physiological parameters measured here suggest that juvenile shore crabs are more susceptible to the toxic effects of pyrene (after an acute sublethal exposure), possibly due to the higher metabolic capacity in the bio-activation of pyrene (indicated by higher respiration rates) (Table 3.4), although similar levels of 1-OH pyrene metabolite levels between pyrene-exposed juvenile and adults were observed (after 7 days). Multivariate analyses highlighted the robustness of the physiology of adult *C. maenas* compared with juveniles, and the susceptibility of juvenile shore crabs, via the inherent phenotypic responses to sublethal contaminant exposure.

In conclusion, differences in physiological condition existed between recently-collected adult and juvenile *C. maenas*, and these differences in turn determined the level of susceptibility of each ontogenetic stage to the effects of pyrene exposure. Results found here support the hypothesis that juveniles display increased sensitivity to contaminant-imposed toxicity compared to the fully-developed adult form. Having established the physiological capacity of adult and juvenile *C. maenas* differs, the next chapter examines whether nutritional status alters the physiological condition of adults.

CHAPTER 4:

**Does *Carcinus maenas* physiology vary
with nutritional status?**

Does *Carcinus maenas* physiology vary with nutritional status?

Abstract

Crustaceans are subjected to temporary periods of reduced food intake during moulting and at times of limited food availability. The aim of this study was to test if food limitation altered the physiological condition of *Carcinus maenas*, through the utilisation of energy reserves and the status of each physiological component of its system. To achieve this aim, short-term changes (7 and 14 days) in *C. maenas* physiological condition (biochemical and cellular) were evaluated.

Significant differences [as highlighted by univariate analysis, ($P < 0.05$)] in both biochemical and cellular [cellular viability and phagocytosis, (day 7); antioxidant status and phagocytosis, (day 14)] endpoints were observed in shore crabs between dietary treatments (starved, diet-restriction and fully fed) after both seven and fourteen days. An integrated evaluation of the physiological condition (via multivariate analysis) revealed that shore crab physiology is relatively robust to short-term changes (< 7 days) with significant changes to cellular functioning occurring after fourteen days. In conclusion, this study has shown that short-term starvation leads to a difference in cellular homeostasis only after fourteen days, thereby highlighting the robustness of shore crab physiology to tolerate periods of nutritional deprivation.

4.1 INTRODUCTION

The ability of *Carcinus maenas* to deal with periods of nutritional constraints, both intrinsic (i.e. moulting) and environmental (i.e. limited prey availability), is dependent on each physiological component of the organism's system, such as the energy available to the shore crab via mobilisation of energy stores, e.g. protein, carbohydrate and lipid. The repertoire of biological responses, from biochemical to physiological, to biotic and also abiotic stressors, ultimately, determines whether the organism can compete both intra- and interspecifically, i.e. to grow and finally reproduce (Hebel et al. 1997). In order to differentiate between the physiological effects of stressors and natural processes, a brief outline of cellular metabolism is needed.

In all eukaryotic cells, metabolism enables the cell to perform vital functions, including providing the energy to maintain the internal composition of the cell, as well as the synthesis (and recycling) of cell constituents (proteins, nucleic acids, lipids and carbohydrates) for the cell to fulfil its specialised function (Bronk 1999). The synthesis of cell constituents arises from one of three sources: firstly, absorption from metabolites external to the cell (i.e. dietary route), secondly, release from a metabolite from a source within the cell (i.e. storage), and/or finally, the metabolite may be formed by the metabolism of a precursor, which is either absorbed or derived from a source stored within the cell (Bronk 1999). In summary, metabolism enables cells to convert the energy found in nutrients (or from storage) into a form which will support both biosynthesis of molecules and maintenance of the internal environment. Cellular metabolism is driven by generation of energy (in the form of ATP) via the Krebs cycle (or TCA cycle). The energy is used to oxidise pyruvate formed during the glycolytic breakdown of glucose and also oxidises acetyl CoA arising from fatty acid degradation and proteolysis to form amino acids; the latter are then used to

synthesise new proteins required for survival or catabolised into Krebs cycle intermediates (e.g. citrate) to supply cells with energy (Hames and Hooper 2000). The intermediates in the cycle provide precursor molecules for various biosynthetic pathways; synthesis of fatty acids from citrate, amino acid synthesis, synthesis of purine and pyrimidine nucleotides and glucose conversion by gluconeogenesis. With regard to *C. maenas*, glycogen is stored in granules of the alpha cells, where glucose is subsequently released upon breakdown of this storage polysaccharide (Johnstone et al. 1973). Fatty acid (lipid) breakdown occurs within the mitochondria, whereas protein degradation occurs within the lysosomes of cells. Summary diagrams outline the intracellular energy pathways where energy is released via metabolism of the energy stores (Fig. 4.1) and the fate of these three cell constituents (under aerobic metabolism) via the Krebs cycle (Fig. 4.2).

Crustacean growth is limited by the exoskeleton and only through ecdysis (moulting) can an increase in body size be achieved (Crothers 1967; 1968). During the moulting cycle, feeding strategies are adopted which are dependent upon food availability (Sanchez-Paz et al. 2006). Active feeding occurs during the intermoult stage (C_4) when energy reserves are built up prior to moulting (Crothers 1967). Approaching ecdysis, feeding declines and ceases during the process of moulting. The feeding cycle resumes post-moult when the exoskeleton hardens (Sanchez-Paz et al. 2006). Starvation induction in crustaceans has been proposed as a tractable model to understand the molecular and enzymatic changes which determine the energetic and metabolic function of the organism (Sanchez-Paz et al. 2006). Short (< 4 weeks) and long-term (> 4 weeks) changes in biochemical, cellular and physiological parameters have been examined in various crustacean species, including *C. maenas*, and are the physiological changes during nutrition-deprived conditions.

summarised in Table 4.1. Physiological changes, resultant from starvation, range from metabolic alterations (oxygen consumption and cardiac activity) to depletion of energy stores, such as haemocyanin.

The aims of this study were to test whether short-term (7 and 14 days) food limitation would cause biochemical and cellular changes, thereby altering the physiological condition of *C. maenas*.

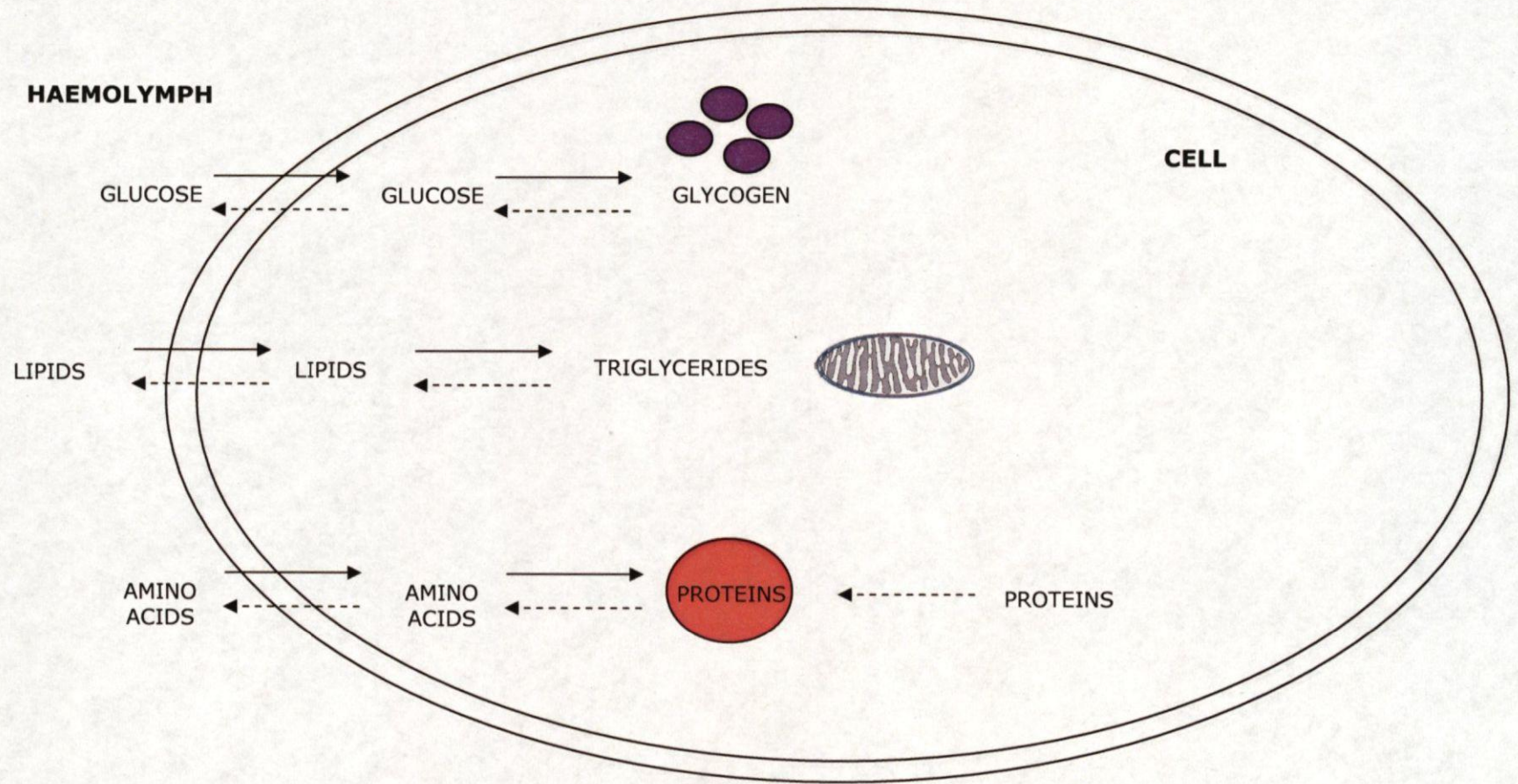
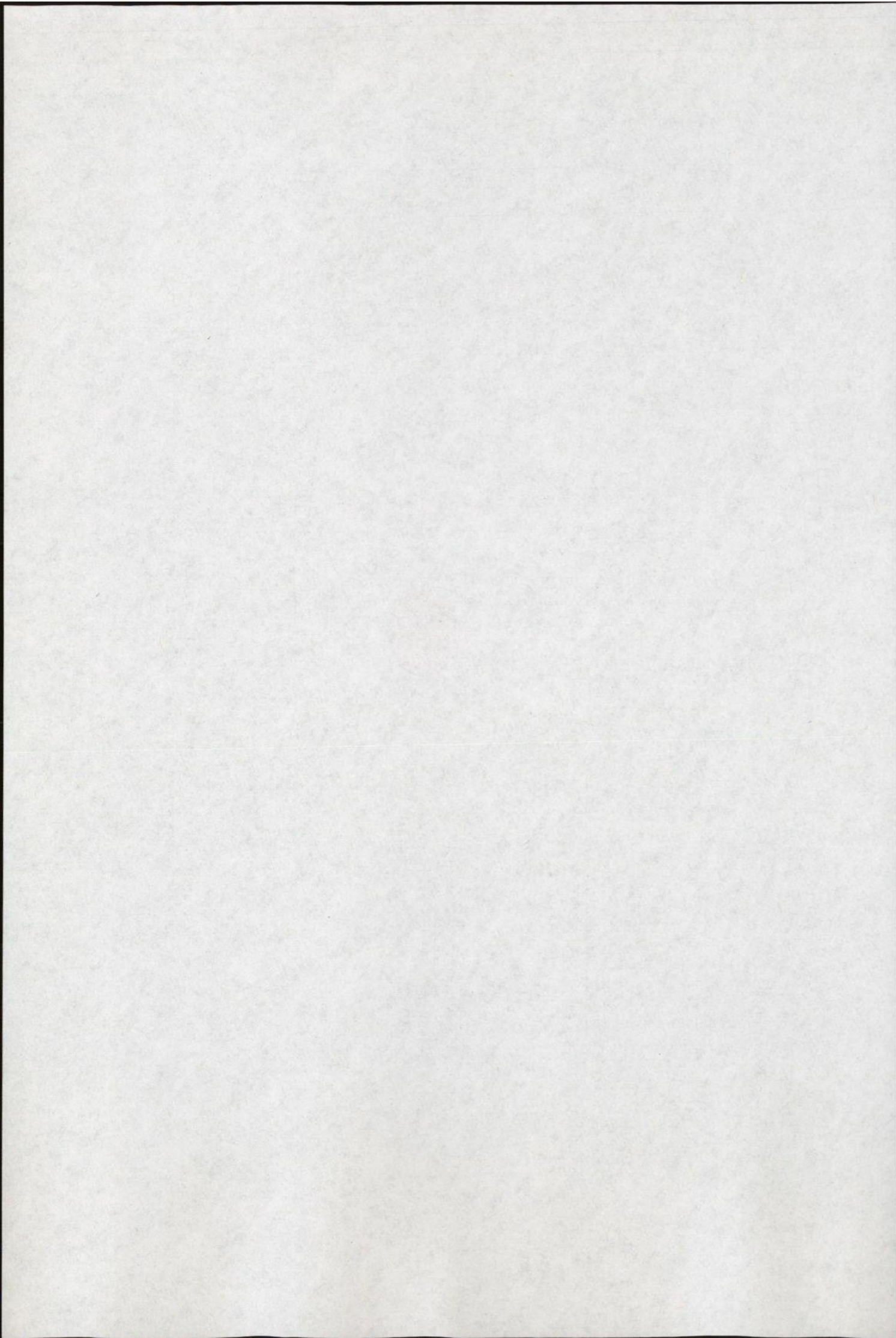


Fig. 4.1 Diagrammatic representation of pathways utilised for intra-and extracellularly energy release into the haemolymph in Crustacea. Adapted from Sanchez-Paz et al. (2006).



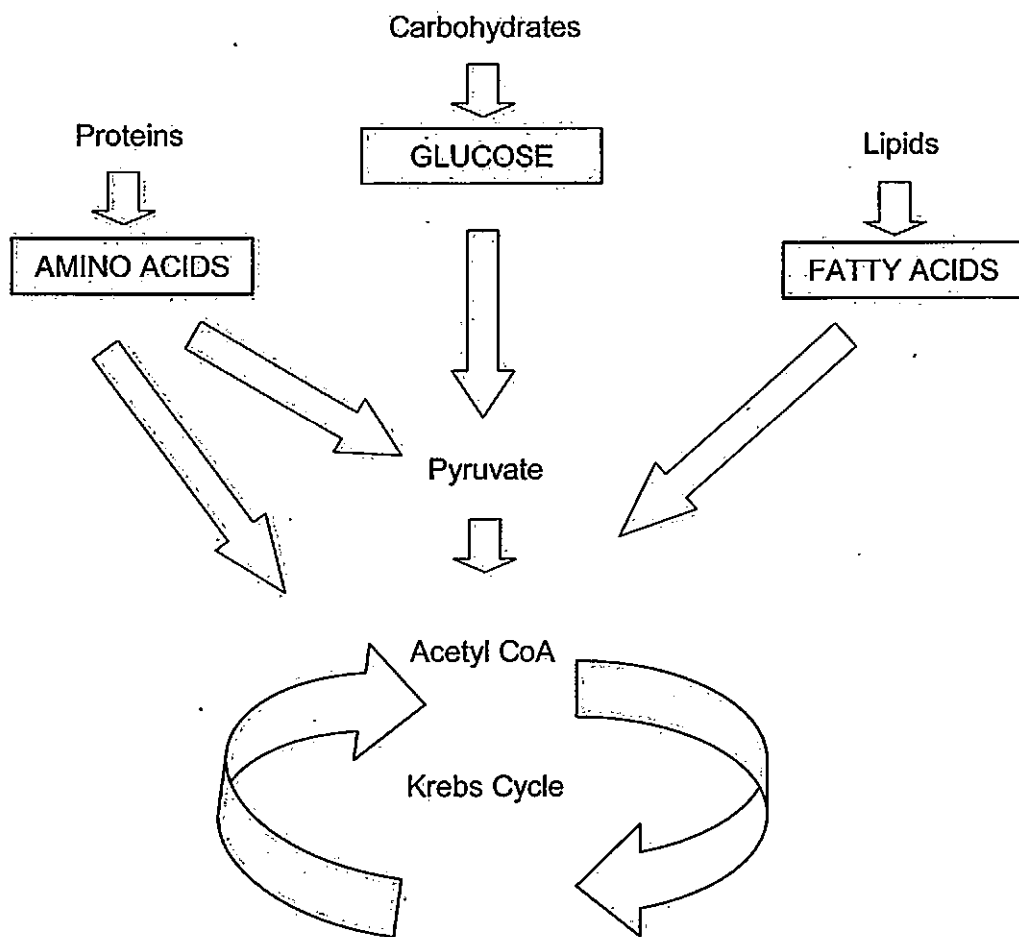


Fig. 4.2 Diagrammatic representation of the aerobic metabolism of three major cell constituents via the Krebs cycle (Boyer 2002).

Table 4.1 Short (< 4 weeks) and long (> 4weeks) term effects of starvation upon physiological parameters in *Carcinus maenas*.

TIME SCALE	PARAMETER	DESCRIPTION	REFERENCE
Short-term	Nutritional status	Diet restriction triggers autophagy and 'improved' lysosomal capacity	(Moore 2004)
Short-term	Glycogen and glucose	Glycogen depletion, especially in muscles was seen following exposure to hypoxia or starvation in <i>Nephrops norvegicus</i> Analysis of anthrone-responsive sugar concentration in the blood of <i>C. maenas</i> shows that the haemolymph sugars exhibit a cyclic pattern which is related to tidal state, with peak values associated with low tide period.	(Baden et al. 1994) (Williams 1985)
Short-term	Oxygen Consumption	Starvation in <i>C. maenas</i> in the laboratory at 15°C results in a progressive suppression of the metabolism compared with fully fed crabs. This effect becomes apparent after 2 weeks at 15°C but may occur sooner at higher acclimation temperatures. No significant differences in body weight over a 3 wk starvation period	(Marsden 1973)
Short-term	Heart rate	Heart rates were depressed following starvation and crabs were less active	(Depledge 1985)
Short and long-term	Haemocyanin	'slow' haemocyanin and apohaemocyanin may act as organic stores 'fast' haemocyanin suggests that this protein fulfils a basic respiratory need. [Hc] a reflection of feeding history Indicator of physiological stress	(Uglow 1969) (Spicer and Baden 2000; Spicer and Stromberg 2002) (Engel et al. 1993)
Short-term	Fatty acids	High lipid content in crabs of 'good' condition, as well as low water content in haemolymph. Storing energy in lipid form may be considered an advantageous strategy	(Styrishave et al. 2000)
Long-term	Midgut gland	May act as a temporary store of haemolymph copper when haemocyanin is catabolised during starvation or at the time of moulting (Rainbow 1988)	(Depledge 1989)
Long-term	Tissue free space	Shrinkage of tissue due to catabolism increases the tissue free space, compensated by uptake of water and expansion of haemolymph volume	(Scottfordsmand and Depledge 1993)
Long-term	Exoskeleton Structure	Thinning of the exoskeleton occurs (this is a long-term starvation cost)	(Scottfordsmand and Depledge 1993)

4.2 MATERIAL AND METHODS

4.2.1 *Shore crab collection and maintenance*

Adult (>60mm CW) (Crothers 1967) male (green) intermoult *Carcinus maenas* ($n = 54$) (mean CW 66.29 ± 5.07 mm) were collected from the Avon Estuary, Bantham, South Devon, U.K. (grid reference: SX 6623 4380) on two occasions (23rd and 26th May 2005) using mackerel-baited traps(see Chapter 2, Section 2.1). In the laboratory, crabs were maintained in static holding tanks containing filtered (10 μ m carbon filtered), aerated seawater (34ppt, $15 \pm 1^\circ\text{C}$) under a 12h light : 12h dark photoperiod for a maximum of 2 days and transferred into treatment tanks. Crabs were held in exposure tanks (2L) and fed according to the individual nutritional treatment conditions (see below) and starved or fed with γ -irradiated cockle (*Cerastoderma edule*) (Gamma foods, Tropical Marine Centre, Bristol, U.K). Water was changed every 48 h and within 18h of each feeding.

4.2.2 *Experimental design and post-exposure assessment*

Each crab was numbered using a label attached (cyanoacrylate glue) to the carapace. Carapace width (CW) (mm) and wet weight (g) of each crab was recorded. Crabs were assigned into one of three treatment groups: a) starved ($n = 18$); b) diet-restricted (DR) ($n = 18$) and c) fully fed ($n = 18$). Each group was 'exposed' for fourteen days with crabs being fed individually, either on 1g on alternate days [after an initial starvation period of 3 days (DR)] or 2g everyday (fully fed) of irradiated cockle (Fig. 4.3). Post-'exposure' assessment included the biological responses measured at: (1) biochemical (antioxidant potential) and (2) cellular levels (cellular integrity, viability and phagocytosis), after seven and fourteen days exposure. Haemolymph samples (500 μ l) were extracted by puncture of the arthroal membrane at the base of the 4th walking leg using a

pre-chilled 1ml syringe and 21 gauge needle (Sigma-Aldrich, U.K). Cellular assays were conducted immediately post-haemolymph sampling. Haemolymph samples were snap frozen using liquid nitrogen and stored at -80°C for subsequent biochemical analysis (see Chapter 2, Section 2.4 for details).

4.2.3 Post-exposure assessment

FRAP (Ferric reducing ability of plasma): antioxidant status was determined by measuring the combined reducing power of the electron-donating antioxidants present (i.e ferric reducing antioxidant potential) (Benzie and Strain 1996; Rickwood and Galloway 2004). A stoichiometric excess of the oxidant ferric tripyridyltriazine (Fe^{III} -TPTZ) was added to each 10 μl sample (300 μl of 10mM in 300 mM sodium acetate, pH 3.6) and its reduction to the ferrous form (Fe^{II}) monitored over 10 min at $A_{593\text{nm}}$ (see Chapter 2, Section 2.4.5 for details).

Cellular viability: stress-induced pathological change in the lysosomal compartments of haemocytes was determined using a dye uptake method as described by Galloway et al. (2004c) (see Chapter 2, Section 2.4.9 for details).

Cellular integrity: cell functionality (i.e. dead or viable) was determined using a dye exclusion method whereby 2mg ml⁻¹ Eosin Y was added to haemolymph samples (20 μl) at a ratio of 1:10. Following 10 min incubation and washing cycle, the absorbance of the residual dye was measured at 518nm. Viable cells stain light green (due to dye exclusion) whereas dead and moribund cells stain red (see Chapter 2, Section 2.4.7 for details).

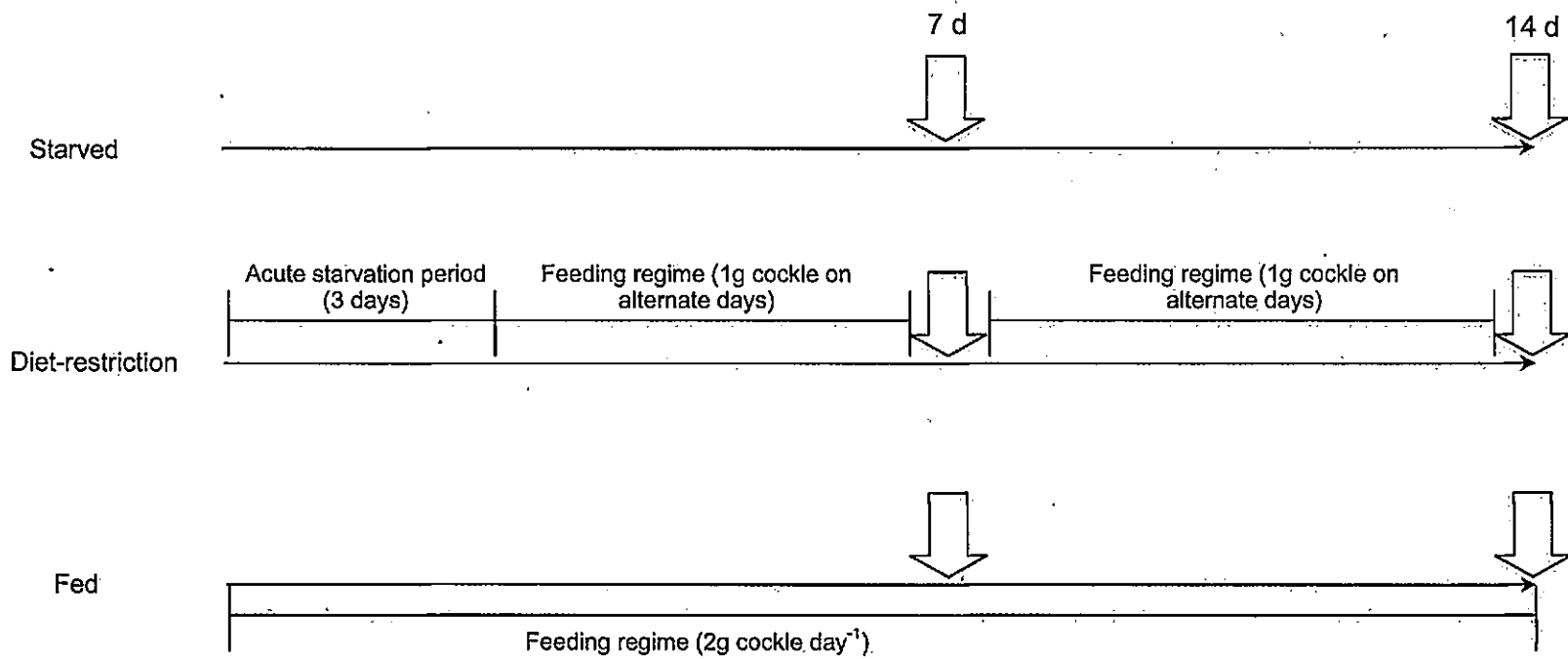


Fig. 4.3 Experimental feeding regime and exposure assessment of *Carcinus maenas* at 7 and 14 days.

Phagocytosis index: the phagocytic capability of haemocytes was evaluated by measuring the active uptake of neutral red-stained zymosan particles as described by Parry and Pipe (2004), and employed by Galloway et al. (2004c) (See Chapter 2, Section 2.4.10 for details).

4.2.4 Statistical analysis

Two methods of statistical analyses were used to isolate significant differences in physiological condition of adult *C. maenas*. Univariate analyses were performed to test for differences for a single parameter between experimental groups. To attain an integrated summary of the contaminant impact, multivariate analyses (ANOSIM, SIMPER) tested for differences between the experimental groups via the complete repertoire of biological responses measured (see Chapter 2, Section 2.6 for details).

4.3 RESULTS

4.3.1 Day 7

4.3.1.1 Univariate analyses

Significant differences in physiological condition (at the biochemical and cellular level) were revealed over time (following 7 and 14 days) under different feeding regimes (Tables 4.2 and 4.3 respectively).

Antioxidant status, as shown by the potential of the plasma to reduce ferric ions *in vitro*, showed no differences between dietary treatments. These results highlight the robustness of total antioxidant status in crab haemolymph. Cell integrity (%) did not differ between dietary treatments, supporting the conclusion that cellular integrity is maintained regardless of short-term and long-term starvation (i.e. 3 and 14 day in diet-restriction and starved treatments respectively). Significant differences between treatments were found for cellular viability, with increased neutral red dye retention in starved compared with fed individuals (ANOVA, $F_{2,51} = 3.66$, $P < 0.05$) (Table 4.2 and Fig. 4.4). Immune function, as shown by the phagocytic capability of haemocytes, was also found to be affected by dietary status, and significant differences were observed between starved and fed individuals; starved crabs expressed increased phagocytic capability (ANOVA, $F_{2,51} = 3.98$, $P < 0.05$) (Table 4.2 and Fig. 4.5).

Table 4.2 Physiological assessment of starved, diet-restricted (DR) and fully fed shore crabs (day 7) ($n = 18$).

Level	Parameters	Starved	Diet-restriction (DR)	Fed	Statistical significance
CELLULAR	Antioxidant status ($\mu\text{M L}^{-1}$)	161.27 \pm 67.16	159.13 \pm 76.40	169.72 \pm 84.38	ANOVA $F_{2,51} = 0.10$ $P = 0.91$
	Cellular integrity (%)	66.3 \pm 16	69.43 \pm 16	75.81 \pm 17	ANOVA $F_{2,51} = 0.48$ $P = 0.65$
	Cellular viability (O.D mg protein $^{-1}$)	1 \pm 0.71	0.68 \pm 0.42	0.56 \pm 0.40	ANOVA $F_{2,51} = 3.66$ $P < 0.05$
	Phagocytosis (zymosan particles 10^7 mg protein $^{-1}$)	2.56 \pm 1.44	2.33 \pm 0.84	1.82 \pm 0.77	ANOVA $F_{2,51} = 3.98$ $P < 0.05$

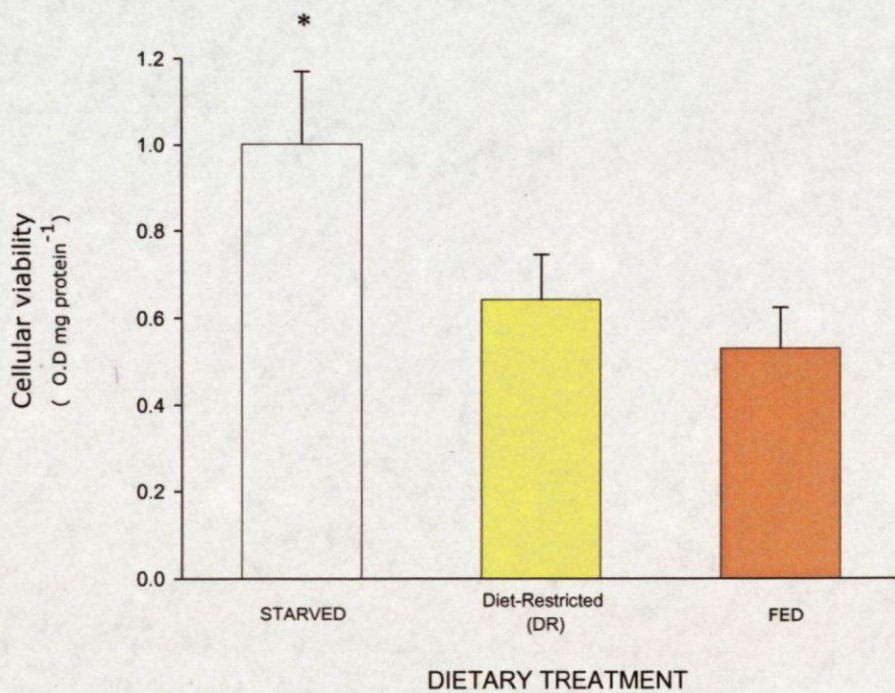


Fig. 4.4 Cellular viability (O.D mg protein⁻¹) assessed in starved, diet-restricted and fully fed *Carcinus maenas* ($n = 18$) (day 7). * signifies $P < 0.05$. Error bars signify ± 1 standard error.

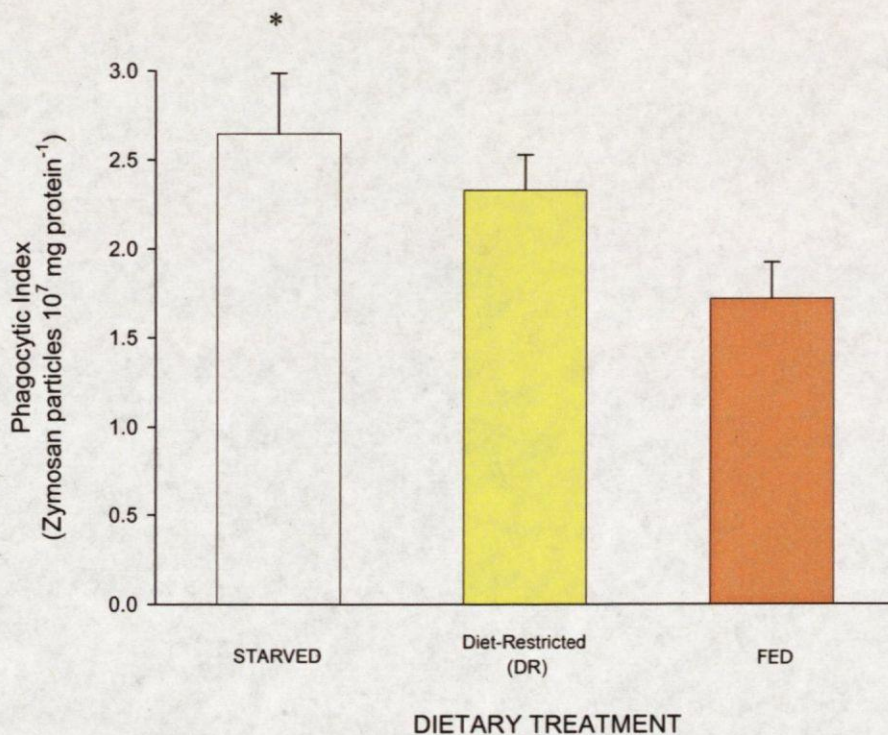
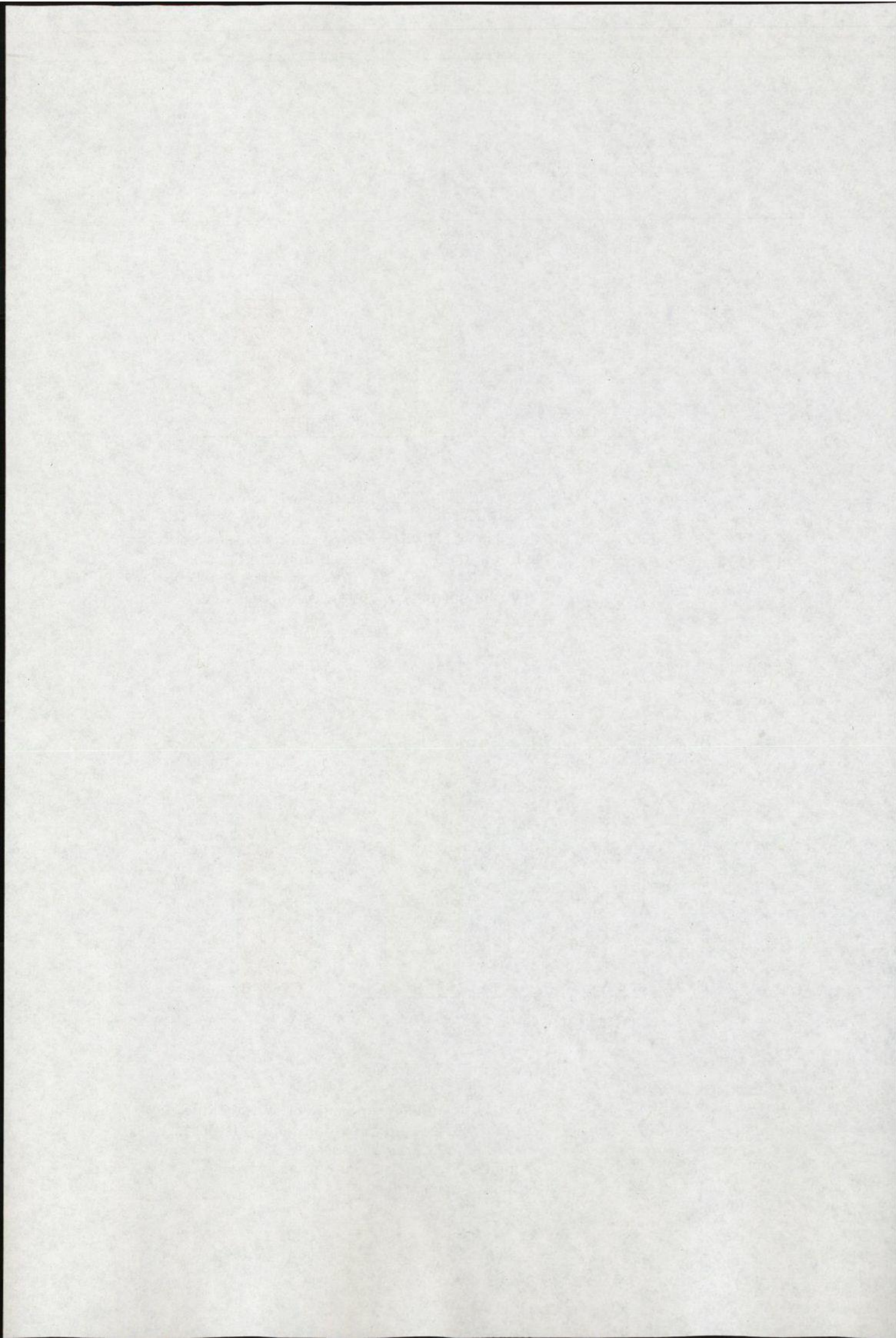


Fig. 4.5 Phagocytic index (zymosan particles 10⁷ mg protein⁻¹) assessed in starved, diet-restricted and fully fed *Carcinus maenas* (day 7). * signifies $P < 0.05$. Error bars signify ± 1 standard error.



4.3.1.2 *Multivariate analyses*

No significant differences in shore crab physiological condition were identified between dietary treatments (using ANOSIM) [*Global R* = 0.018, *P* (%) = 19.2]. These results suggest that shore crab physiological condition is robust and that during an acute starvation period (7 days), the biochemical and cellular functioning (i.e. total antioxidant status, cellular integrity, cellular viability and immune function) does not alter from that of fully-fed individuals.

4.3.2 *Day 14*

4.3.2.1 *Univariate analyses*

Following fourteen days under each dietary condition, diet-restriction (DR) crabs demonstrated lower antioxidant capability than the starved and fed groups (ANOVA, $F_{2,51} = 7.69$, $P < 0.05$) (Table 4.3 and Fig. 4.6). There were no significant differences at the cellular level between starved, fed and DR crabs (as demonstrated by cellular integrity and cellular viability), indicating no deterioration of cellular integrity or cellular functioning after 14 days of starvation. However, there was a significant difference with respect to starved and fed treatments, with starved crabs displaying an induction in phagocytic capability ($F_{2,51} = 3.98$, $P < 0.05$) (Fig. 4.7).

Table 4.3 Physiological assessment of starved, diet-restricted (DR) and fully fed shore crabs (day 14) ($n = 18$).

Level	Parameters	Starved	Diet-restriction (DR)	Fed	Statistical significance
BIOCHEMICAL	Antioxidant status ($\mu\text{M L}^{-1}$)	213.28 ± 52.24	170.56 ± 64.86	253.89 ± 72.36	ANOVA $F_{2,51} = 7.69$ $P < 0.05$
CELLULAR	Cellular integrity (%)	67.08 ± 12	75.44 ± 8	78.65 ± 15	ANOVA $F_{2,51} = 1.48$ $P = 0.24$
	Cellular viability (O.D mg protein $^{-1}$)	0.15 ± 0.04	0.18 ± 0.15	0.20 ± 0.14	ANOVA $F_{2,51} = 0.48$ $P = 0.64$
	Phagocytosis (zymosan particles 10^7 mg protein $^{-1}$)	2.65 ± 0.95	2.79 ± 2.59	1.69 ± 0.81	ANOVA $F_{2,51} = 7.98$ $P < 0.05$

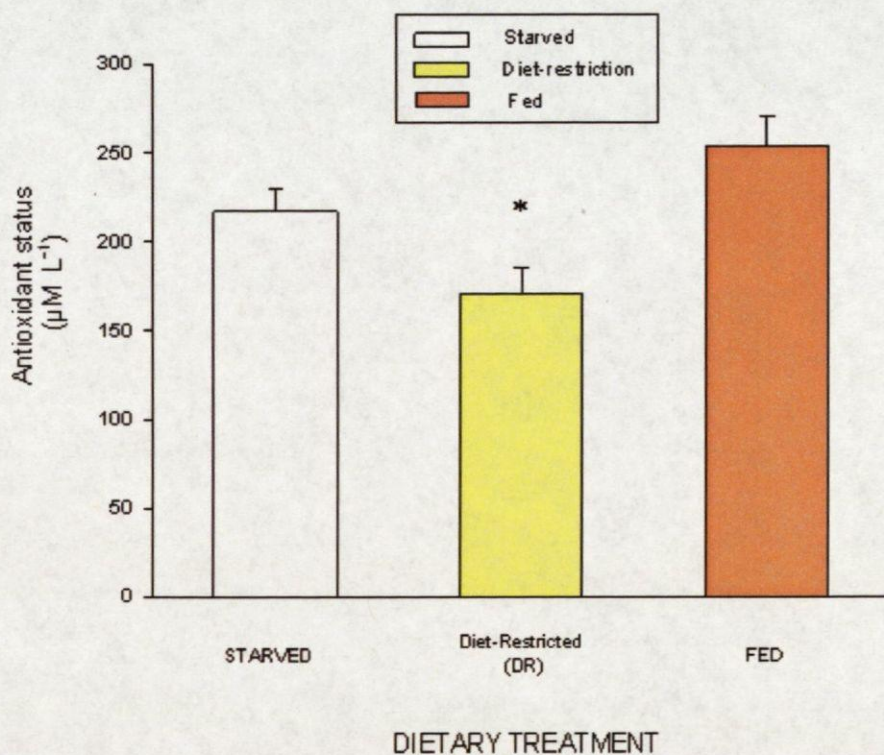


Fig. 4.6 Antioxidant status assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* (day 14). * signifies $P < 0.05$. Error bars signify ± 1 standard error.

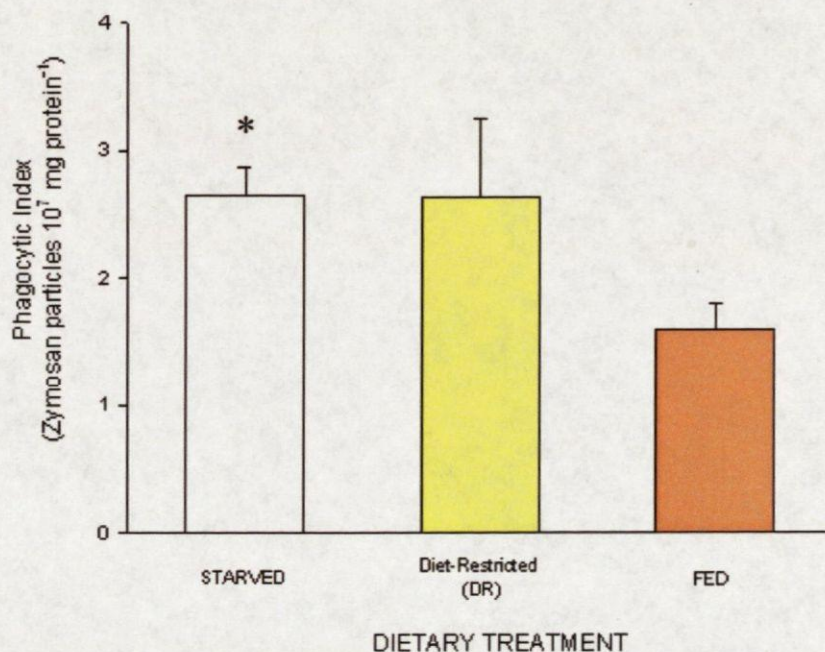
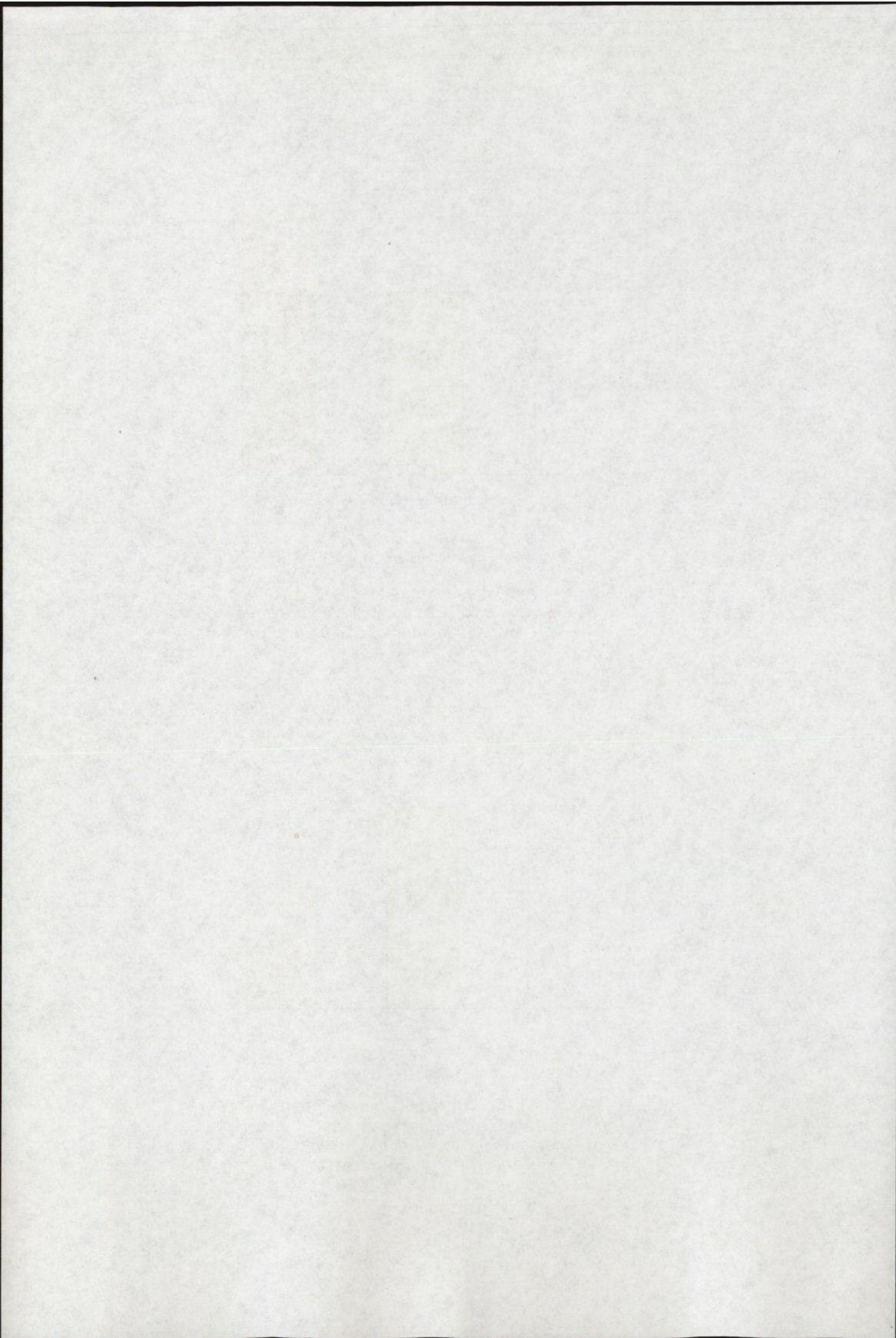


Fig. 4.7 Phagocytic index (zymosan particles 10⁷ mg protein⁻¹) assessed in starved, diet-restricted and fully fed *Carcinus maenas* (day 14). * signifies $P < 0.05$. Error bars signify ± 1 standard error.



4.3.2.2 *Multivariate analyses*

Significant differences in shore crab physiological condition were identified between treatment groups as revealed by the ANOSIM test [*Global R* = 0.134, *P* (%) = 0.1]. Differences were observed between starved and fed crabs [*R* = 0.209, *P* (%) = 0.2], and diet-restriction and fed treatments [*R* = 0.14, *P* (%) = 0.2] (Table 4.4a). Results from SIMPER analysis showed that cellular integrity (<42%) was the significant contributor to the overall separation of starved and fed treatments (Table 4.4b); however, antioxidant status was contributed < 31% to the overall difference between diet-restricted and fully-fed crabs (Table 4.4b). These results demonstrate the dichotomy in physiological status with dietary manipulation, with starvation (both 3 days and 7 days) and diet-restriction having a negative effect on cellular homeostasis, as indicated by cellular integrity and antioxidant status.

Table 4.4 Pairwise treatment comparisons of exposure assessment periods 7 and 14 days (a). *NS*= not significant, *S* = significant. (b) and (c) SIMPER analyses of variables contributing to the treatment differences observed in a). Significant differences and highest contributing variables are highlighted in bold.

a) Pairwise treatment comparisons			Exposure assessment	
			7	14
Starved	vs.	DR	<i>NS</i>	<i>NS</i>
Diet-restriction	vs.	Fed	<i>NS</i>	S
Starved	vs.	Fed	<i>NS</i>	S

Treatments: Starved vs. Fed crabs

b)	Contribution (%)	Cumulative (%)
Variable		
Cellular integrity	41.68	41.68
Antioxidant status	24.88	66.56
Cellular viability	22	88.56
Phagocytosis	11.44	100

Treatments: Diet-restriction vs. Fed crabs

c)	Contribution (%)	Cumulative (%)
Variable		
Antioxidant status	30.05	30.05
Cellular viability	26.91	56.96
Phagocytosis	26.51	83.47
Cellular integrity	16.53	100

4.4 DISCUSSION

Present results indicate that experimental manipulation of food intake affects shore crab physiology. In particular after fourteen days, there were significant differences at both the biochemical and cellular level, in starved compared to fully-fed shore crabs. The dietary conditions used were not intended to mimic seasonal nutritional conditions experienced by *C. maenas* but to test the hypothesis that differences in nutritional status give rise to different physiological conditions. However, these treatment groups give some insight into the potential differences in physiological state experienced by *C. maenas* during the course of a year at the adult stage of the life cycle.

Univariate analysis (ANOVA) revealed that two cellular variables were altered by food-deprived conditions after seven days. Phagocytic capability and cellular viability were shown to be induced by reduced nutrition (i.e. starvation), compared to fed conditions. However, physiological condition (multivariate analyses) by the interaction of all variables measured here, was unaltered by short-term starvation (seven days) comparing starved and fed crabs, highlighting physiological tolerance of this species to food-deprived conditions.

After fourteen days, however, significant differences in shore crab physiological condition were found between starved and fed crabs, and between diet-restriction and fed crabs. Although univariate analysis (ANOVA) revealed significant differences only in phagocytic capability (between starved and fed conditions) and decreased antioxidant status (between DR and fed crabs), multivariate analysis (SIMPER) signalled cellular integrity as the influential factor determining differences in physiological condition between starved and fed conditions (Table 4.4). This apparent contradiction in response may be related to depletion of the glycogen reserves within the alpha (hyaline) cells (Johnstone

et al. 1973) which are, subsequently, also the cells responsible for phagocytic function (Smith and Ratcliffe 1978). Phagocytosis is an energy-dependent process and is sensitive to intracellular energy levels (Galloway and Depledge 2001). For example, a decrease in cellular integrity may herald the breakdown of the hyaline cells for the utilisation of these energy reserves due to the imposed starvation conditions, resulting in subsequent induction of phagocytic function, compared with fed crabs.

The biochemical and cellular systems studied here, therefore, appear to be dependent upon nutritional status. For example, the total antioxidant capability (excluding glutathione) was stable over fourteen days of starvation signifying that the antioxidant systems (both enzymatic and non-enzymatic) in *C. maenas* are able to buffer oxidative stress. Under fully-fed conditions, antioxidant capabilities were higher than starved and diet-restricted individuals, indicating that, in this organism, the array of these enzymatic and non-enzymatic systems are dependent on the energy available through nutrition. It may postulated that in quiescent periods of the adult life stage of *C. maenas*, (December – April), when metabolic activity is low (Marsden 1973) and feeding is reduced due to reduced food availability (Depledge 1985), antioxidant capability of adult *C. maenas* is stable, but has the capability to increase with increase in nutritional intake. Furthermore, under periods of high activity (April – September), where adult males are foraging for food and channelling energy to somatic and gonadic growth (i.e. to reach reproductive maturity), the antioxidant capability of adult males would be augmented by the increase in food intake.

Differences in cellular viability were observed in starved individuals (day 7 compared to day 14) over the seven-day experimental period. It is concluded that induction of the lysosomal system [as shown by increased dye uptake, in

lysosomes of starved crabs compared with fed crabs (Fig. 4.4)] is triggered by acute starvation. These results are in accord with previous evidence of lysosomal up-regulation in mussels (Moore 2004; Moore et al. 2006) and signals autophagy. Autophagy is the process of 'self-eating' whereby degradation of cellular components occurs, such as defunct organelles and proteins (Moore et al. 2006). The concept of autophagy sustaining survival during nutrient-deprived conditions is not new (de Duve and Wattiaux 1966), and has led to the conclusion that autophagy is the major catabolic pathway for eukaryotic cells to generate intracellular nutrients (fatty acids and amino acids) for the purpose of maintaining energy production (ATP) and macromolecular synthesis (e.g. proteins) (Fig. 4.8). Autophagy has been proposed as a protective mechanism induced due to nutritional deprivation (Levine 2005).

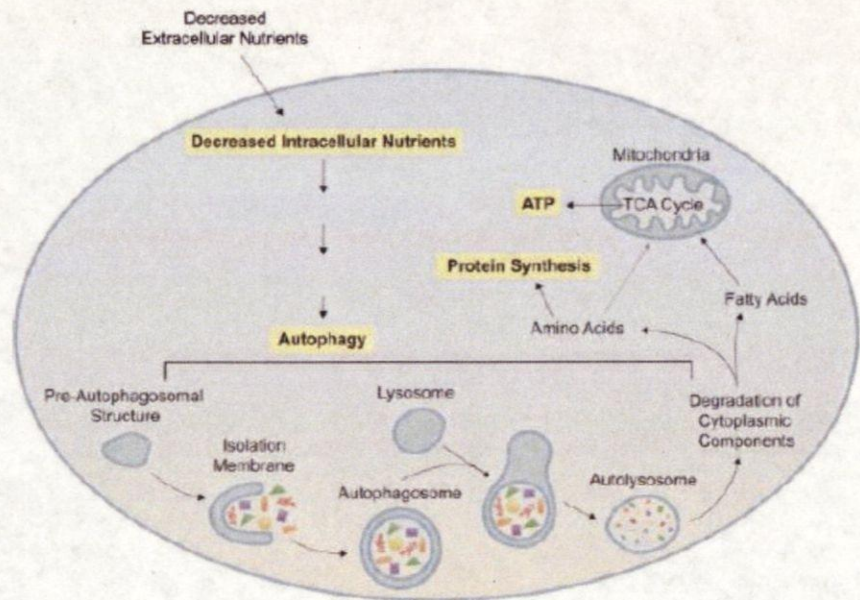
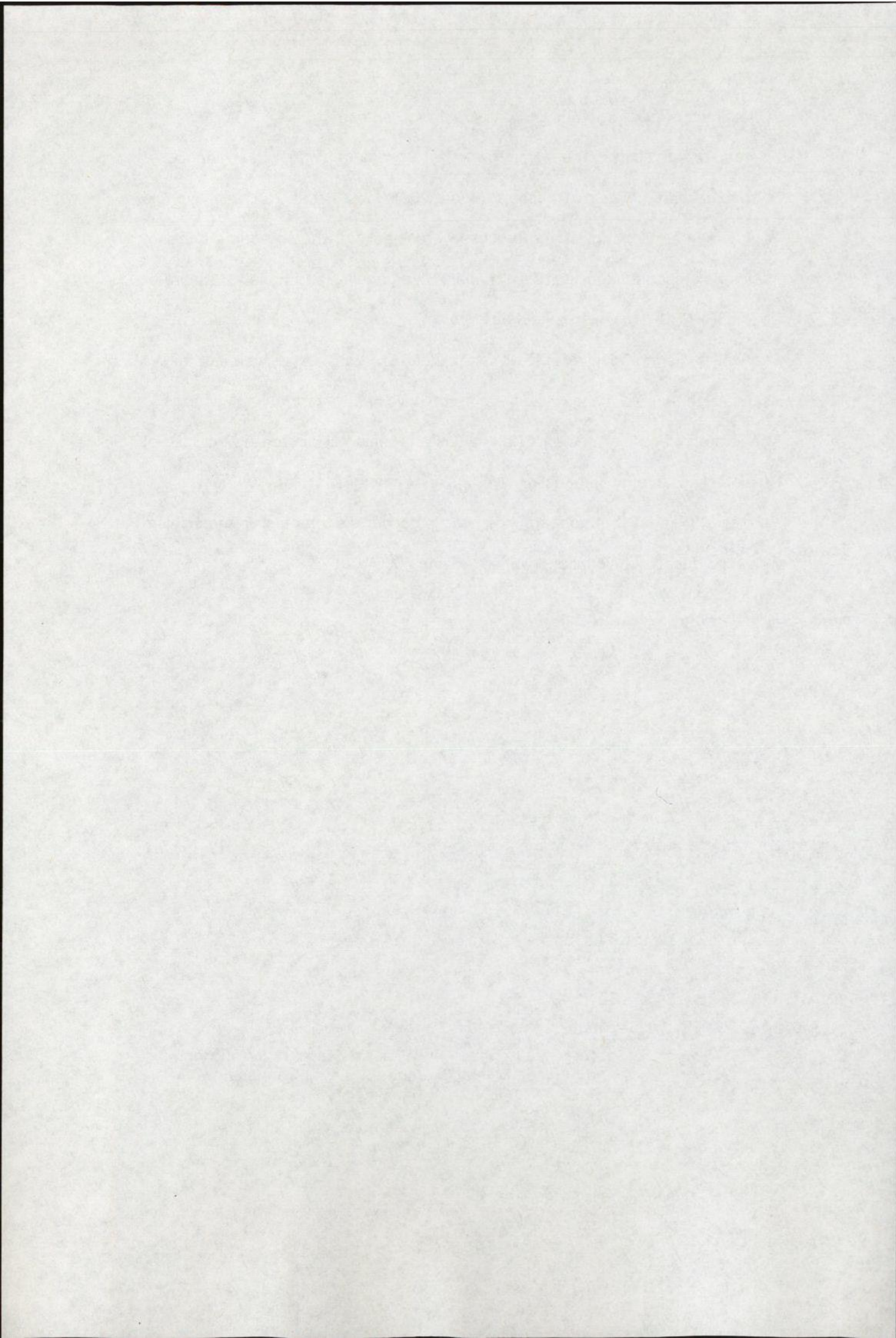


Fig. 4.8. Autophagy in cellular defence under nutrient-deprived condition. Adapted from Levine (2005).



Up-regulation of autophagic processes in starved shore crabs may function as a mechanism for breaking down protein and carbohydrate stores to release energy needed for survival. A fourteen-day starvation period has been shown to result in *C. maenas* utilising haemolymph protein to supply energy (Uglow 1969). Autophagy is proposed here as a mechanism of energy-production via the breakdown of proteins and cellular components as shown by the induction of the lysosomal system. Possible benefits of induced autophagy could be extrapolated to 'seasonal' differences in nutritional status (as outlined above). In dormant periods, such as winter, autophagy could be accountable as a process by which energy stores are metabolised, therefore, signalling an induction of the lysosomal system.

In summary, short-term starvation causes biochemical and cellular changes in shore crab physiology. This study has shown, however, that starvation leads to a significant difference in cellular homeostasis but only after fourteen days, highlighting the robustness of shore crab physiology to tolerate periods of nutritional deprivation.

CHAPTER 5:

Does *Carcinus maenas* nutritional status confer physiological tolerance to contaminant exposure?

Does *Carcinus maenas* nutritional status confer physiological tolerance to contaminant exposure?

Abstract

As shown previously (Chapter 4), cellular functioning was altered under food-deprived conditions, such as induction of haemocyte phagocytic ability. In this chapter, contaminant (pyrene) exposure is added to the experimental design to investigate whether shore crab nutritional status confers physiological tolerance to sublethal pyrene exposure.

Significant effects ($P < 0.05$) upon shore crab physiology (biochemical and cellular) were observed with both sublethal pyrene exposure ($200 \mu\text{gL}^{-1}$ for 14 days) and dietary manipulation (starved, diet-restriction and fully fed). Increased pyrene metabolite levels, both after seven and fourteen day's exposure, and increased protein levels (proteinuria) in the urine of starved crabs were observed in pyrene-exposed crabs. In summary, this study has shown that shore crab susceptibility to sublethal pyrene exposure is dependent upon nutritional status. In conclusion, susceptibility to the effects of contaminant exposure is dependent upon shore crab physiological condition, as shown by experimental manipulation of nutritional status.

5.1 INTRODUCTION

As shown previously in this thesis, cellular functioning was altered in starved compared with fed crabs (see Chapter 4). Shore crabs rely upon the readily available glycogen stores within haemocytes for energetically-costly cellular functioning, such as phagocytic activity of haemocytes. Induction of haemocytic phagocytic activity occurs under starvation conditions (assessed after both seven and fourteen days), resulting in changes to overall physiological condition (only after fourteen days) compared with fully fed crabs (see Chapter 4). As discussed previously, the nutritional status of an organism is paramount to maintaining its biochemical and cellular homeostasis. Functional components of the physiology of the organism can be up-regulated during nutrition-deprived conditions, leading to an 'improved cellular housekeeping' (Moore 2004) via the process of autophagy. Autophagy, or 'self-eating' (Finn and Dice 2006) is a non-selective, homeostatic process where cytosolic or intracellular organelles are sequestered by autophagosomes (a double-membrane structure) for transportation to lysosomes, where digestion occurs (Deretic 2006) (Fig. 5.1).

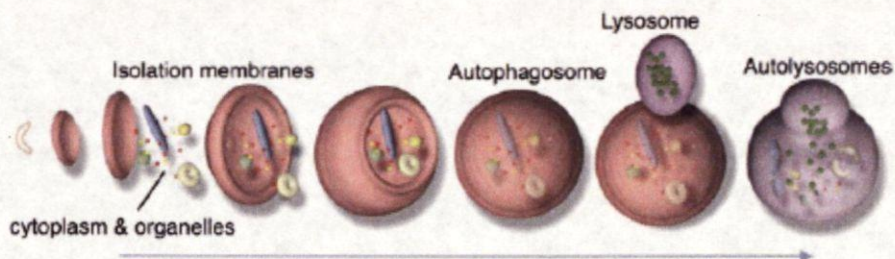
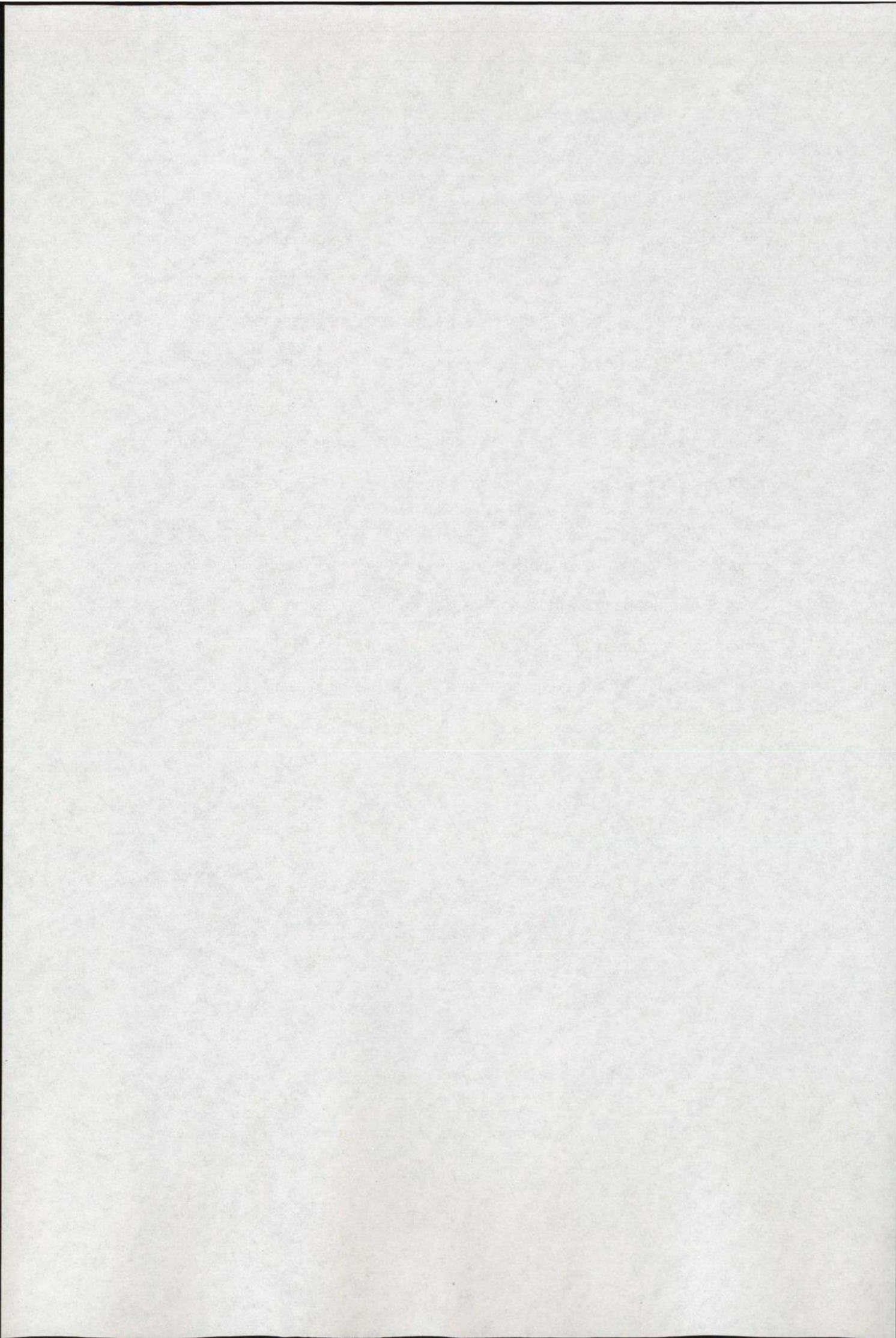


Fig. 5.1 Diagrammatic representation of the process of autophagy, mediated by the production of autophagosomes, which transport cellular components and organelles to the lysosomes for degradation. Taken from (Yoshimori 2004).



Autophagic processes are highly conserved in eukaryotic cells from yeast to mammals (Deretic 2006; Meijer and Codogno 2004). Autophagy plays an important role in intracellular degradation and recycling of long-lived proteins and organelles (i.e. cell survival and death) (Yoshimori 2004). A school of thought exists where this homeostatic process is implicated in cell development, ageing, neurodegeneration and cancer (Deretic 2006). Autophagy occurs in response to both internal (cellular 'housekeeping') and external (e.g. starvation) stimuli (Finn and Dice 2006).

The purpose of this chapter was to investigate the combined effects of contaminant-imposed perturbation and diet-restriction upon the biochemical, cellular and physiological components of *Carcinus maenas* physiology. The aims of this study, therefore, were to investigate whether nutritional status confers physiological tolerance to sublethal contaminant (pyrene) exposure.

5.2 MATERIAL AND METHODS

5.2.1 Shore crab collection and maintenance

Adult (>60mm CW) (Crothers 1967) male (green) intermoult *Carcinus maenas* ($n = 54$) (mean CW 66.29 ± 5.07 mm) were collected from the Avon Estuary, Bantham, South Devon, U.K. (grid reference: SX 6623 4380) on two occasions (23rd and 25th June 2005) using mackerel-baited traps (see Chapter 2 Section 2.1). In the laboratory, crabs were maintained in static holding tanks containing filtered (10 μ m carbon filtered), aerated seawater (34ppt, $15 \pm 1^\circ\text{C}$) under a 12h light : 12h dark photoperiod for a maximum of 2 days before being transferred to treatment tanks. Crabs were held in exposure tanks (2L) and fed according to the individual nutritional treatment conditions (see below) and starved or fed with γ -irradiated cockle (*Cerastoderma edule*) (Gamma foods, Tropical Marine Centre, Bristol, U.K.). Water was changed within 18h of each feeding.

5.2.2 Experimental design and post-exposure assessment

Each crab was numbered using a label attached (cyanoacrylate glue) to the carapace. Morphometric characteristics of each crab were recorded such as: carapace width (CW) (mm) and wet weight (g). Subsequently, crabs were assigned into one of six treatment groups: (1) starved unexposed ($n = 9$); (2) starved PYR-exposed ($n = 9$); (3) diet-restricted (DR) ($n = 9$); (4) DR PYR-exposed ($n = 9$); (5) fed unexposed ($n = 9$); and (6) fed PYR-exposed ($n = 9$). Each treatment group was held for fourteen days and fed crabs were fed individually, either 1g on alternate days (after an initial starvation period of 3 days) for diet-restricted crabs or 2g everyday in the fully-fed treatment. PYR-exposed crabs were subjected to a sub-lethal exposure regime of $200\mu\text{gL}^{-1}$ pyrene (nominal concentration) for 14 d. Post-exposure assessment included the biological responses measured at: (1) biochemical (antioxidant potential,

PYR-metabolites in urine, urinary protein concentration) and (2) cellular levels (cellular integrity, viability and phagocytosis), after 7 and 14 days exposure. Haemolymph samples (500µl) were extracted by puncture of the arthroal membrane at the base of the 4th walking leg using a pre-chilled 1ml syringe and 21 gauge needle (Sigma-Aldrich, U.K). Cellular assays were conducted immediately post-haemolymph sampling. Haemolymph samples were snap frozen using liquid nitrogen and stored at -80°C for subsequent biochemical analysis (See Chapter 2 for details).

5.2.3 Post-exposure assessment

FRAP (Ferric reducing ability of plasma): antioxidant status was determined by measuring the combined reducing power of the electron donating antioxidants present (i.e ferric reducing antioxidant potential) (Benzie and Strain 1996; Rickwood and Galloway 2004). A stoichiometric excess of the oxidant ferric tripyridyltriazine (Fe^{III} -TPTZ) was added to each 10µl sample (300µl of 10mM in 300 mM sodium acetate, pH 3.6) and its reduction to the ferrous form (Fe^{II}) monitored over 10 mins at 593nm (see Chapter 2, Section 2.4.5 for details).

Cellular viability: stress-induced pathological change in the lysosomal compartments of haemocytes was determined using a dye uptake method as described by Galloway et al. (2004c) (see Chapter 2, Section 2.4.9 for details).

Cellular integrity: cell functionality (i.e. dead or viable) was determined using a dye exclusion method whereby 2mg ml⁻¹ Eosin Y was added to haemolymph samples (20µl) at a ratio of 1:10. Following 10 min incubation and washing cycle, the absorbance of the residual dye was measured at 518nm. Viable cells stain light green (due to dye exclusion) whereas dead and moribund cells stain red (see Chapter 2, Section 2.4.7 for details).

Phagocytosis index: the phagocytic capability of haemocytes was evaluated by measuring the active uptake of neutral red-stained zymosan particles as described by Parry and Pipe (2004) and employed by Galloway et al. (2004c) (see Chapter 2, Section 2.4.10 for details).

5.2.4 Statistical analysis

Two methods of statistical analyses were used to isolate significant differences in physiological condition of adult *C. maenas*. Univariate analyses were performed to test for differences for a single parameter between experimental groups. To attain an integrated summary of the contaminant impact, multivariate analyses (ANOSIM and SIMPER) tested for differences between the experimental groups via the complete repertoire of biological responses measured. Univariate statistical analyses were performed using GMAV 5 for windows[®]. Treatment differences were analysed using the parametric analysis of variance test, due to homoscedasticity of data (Underwood 2005). Multivariate analysis was carried out using PRIMER[®] v6 (Plymouth Routines in Multivariate Ecological Research, PRIMER-E Ltd) (see Chapter 2, Section 2.6 for details).

5.3 RESULTS

Significant differences in shore crab physiological condition (at the biochemical and cellular level) was revealed over time (following 7 and 14 days exposure) (See Tables 5.1 and 5.4, respectively).

5.3.1 Day 7

5.3.1.1 Biochemical variables

In all three dietary treatments, urinary concentrations of 1-hydroxypyrene type metabolites were significantly higher in PYR-exposed individuals (ANOVA, $F_{2,50} = 10.19$, $P < 0.001$). A significant difference was observed also between the diet treatments, with increased metabolite concentrations found in the starved treatment compared to that of fed and DR crabs (ANOVA, $F_{2,48} = 11.76$, $P < 0.001$) (Fig. 5.2). Urinary protein concentrations were significantly higher in starved compared to the DR and fed crabs (ANOVA, $F_{2,48} = 23.25$, $P < 0.001$). Within the starved treatment; unexposed crabs had higher protein concentrations within the urine than PYR-exposed crabs ($F_{2,48} = 12.44$, $P < 0.001$) (Fig. 5.3). Antioxidant status, however, as shown by the potential of the plasma to reduce ferric ions *in vitro*, showed no significant differences between dietary treatments. Exposure to pyrene, in contrast (within dietary treatments), had a negative effect upon antioxidant status, with PYR-exposed crabs demonstrating a lower antioxidant capability than unexposed counterparts (ANOVA, $F_{1,50} = 21.40$, $P < 0.001$) (Fig. 5.4).

Table 5.1 Post-exposure physiological assessment of nutrition and contaminant-imposed physiological constraints in starved, diet-restricted (DR) and fully fed *Carcinus maenas* (mean \pm 1 SE) (day 7). *,**, *** signify $P < 0.05$, 0.01 and 0.001, respectively.

Significant changes to biological responses compared to treatment controls (DAY 7)	Parameters	Starved (Con vs. PYR)	Diet-restricted (DR) (Con vs. PYR)	Fed (Con vs. PYR)	Statistical significance	
					INTRA-treatment (Diet-Exposure Interaction)	INTER-treatment (Diet)
BIOCHEMICAL	Urinary PYR metabolites ($\mu\text{g L}^{-1}$)	315.22 \pm 22 vs. 724.95 \pm 94.17	34.14 \pm 34.34 vs. 806.15 \pm 236.30	70.65 \pm 19.49 vs. 570.68 \pm 68.28	***	(Unexposed) Starved > DR = Fed ***
	Urinary protein concentrations (mg ml^{-1})	0.39 \pm 0.07 vs. 0.18 \pm 0.02	0.07 \pm 0.02 vs. 0.08 \pm 0.01	0.10 \pm 0.01 vs. 0.14 \pm 0.01	***	***
	Antioxidant status ($\mu\text{M L}^{-1}$)	110.86 \pm 20.14 vs. 175.58 \pm 22.49	86.86 \pm 10.70 vs. 154.53 \pm 16.42	130.98 \pm 27.06 vs. 186.53 \pm 11.85	***	NO DIFFERENCE
CELLULAR	Cellular integrity (%)	84.73 \pm 0.41 vs. 80.92 \pm 0.99	86.73 \pm 0.03 vs. 80.8 \pm 2.13	84.24 \pm 0.46 vs. 80 \pm 0.67	***	NO DIFFERENCE
	Cellular viability (O.D mg protein $^{-1}$)	0.58 \pm 0.10 vs. 0.24 \pm 0.03	0.36 \pm 0.04 vs. 0.19 \pm 0.05	0.32 \pm 0.05 vs. 0.17 \pm 0.02	***	**
	Phagocytosis (zymosan particles 10^7 mg protein $^{-1}$)	2.93 \pm 0.68 vs. 0.75 \pm 0.26	2.37 \pm 0.36 vs. 0.69 \pm 0.17	1.62 \pm 0.41 vs. 0.53 \pm 0.15	***	NO DIFFERENCE

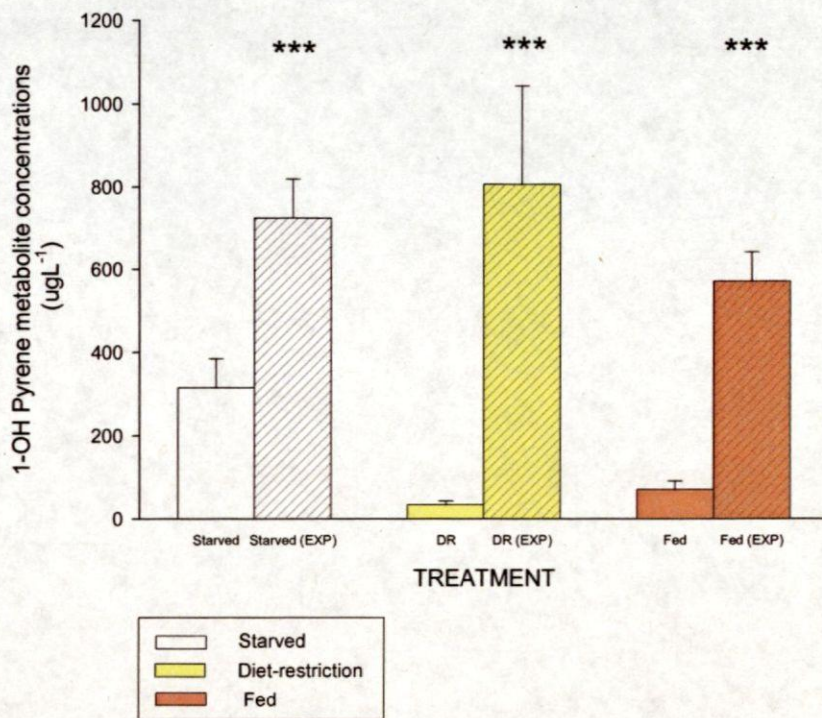


Fig. 5.2 Urinary PYR metabolite concentrations ($\mu\text{g L}^{-1}$) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. *** signifies $P < 0.001$. Error bars signify ± 1 standard error.

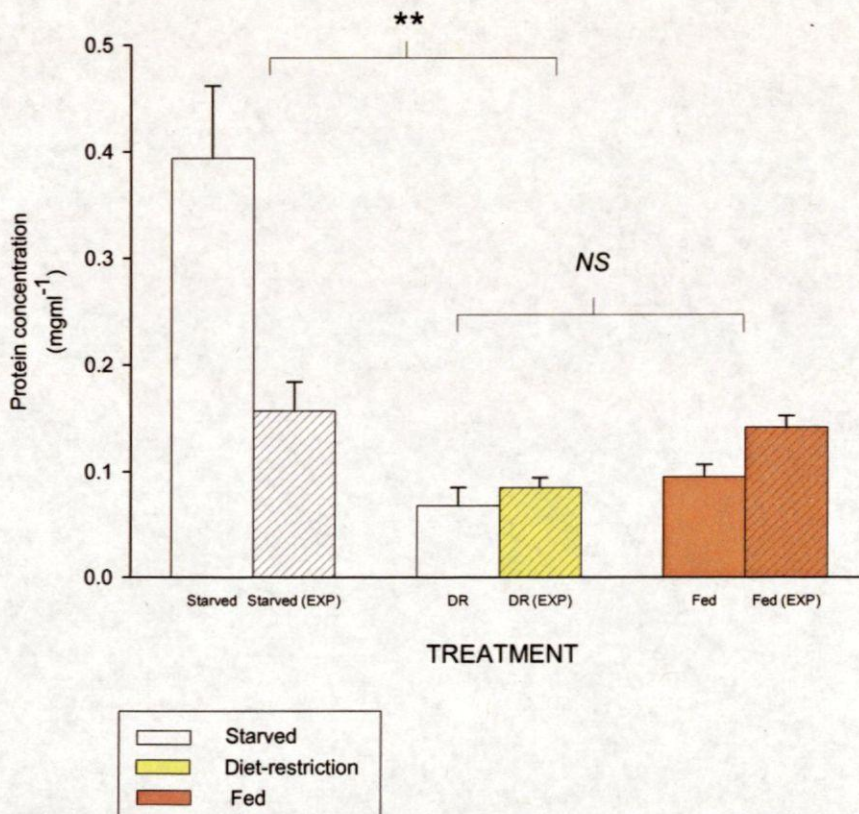
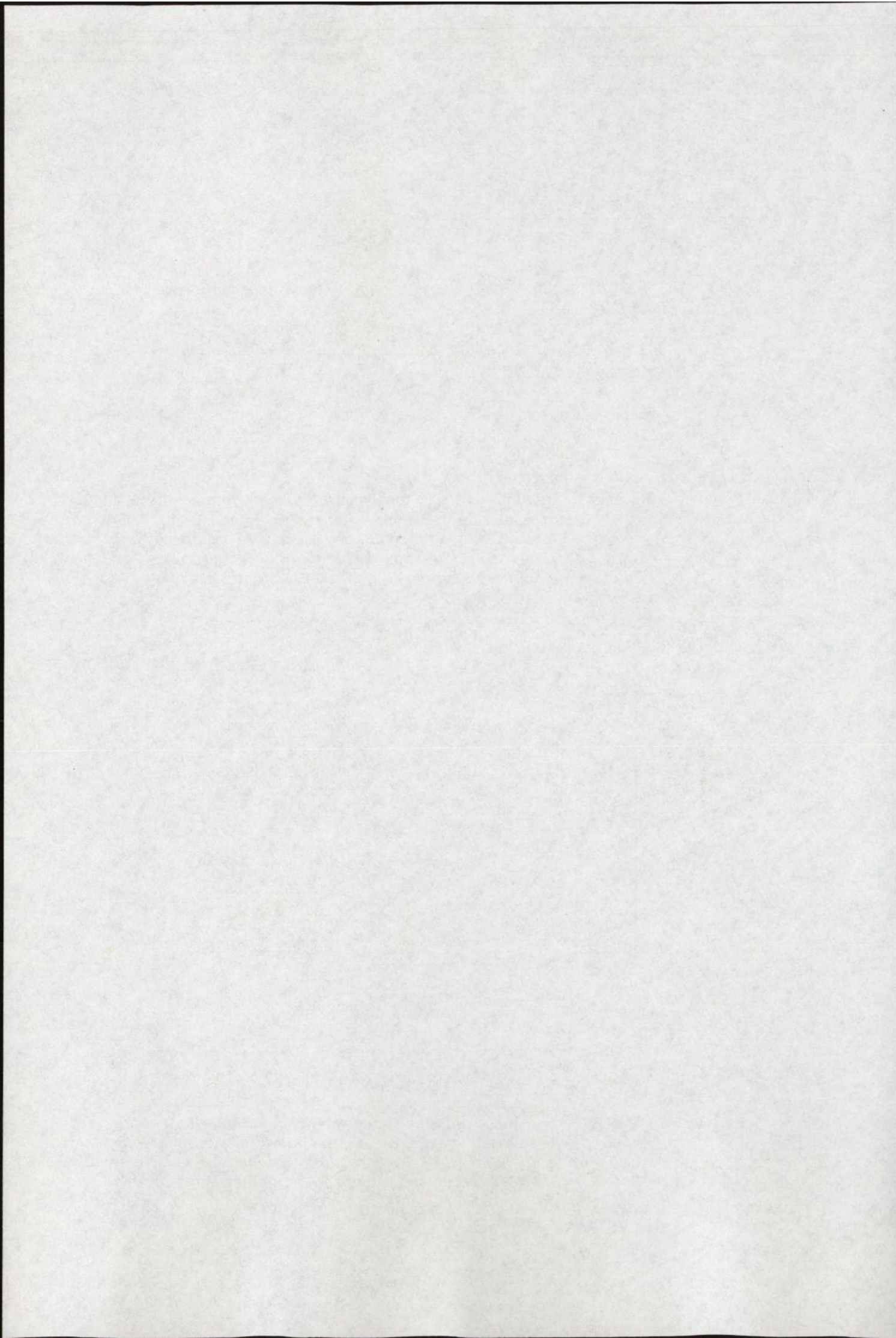


Fig. 5.3 Urinary protein concentration (mg ml^{-1}) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. ** signifies $P < 0.01$. NS = Not Significant. Error bars signify ± 1 standard error.



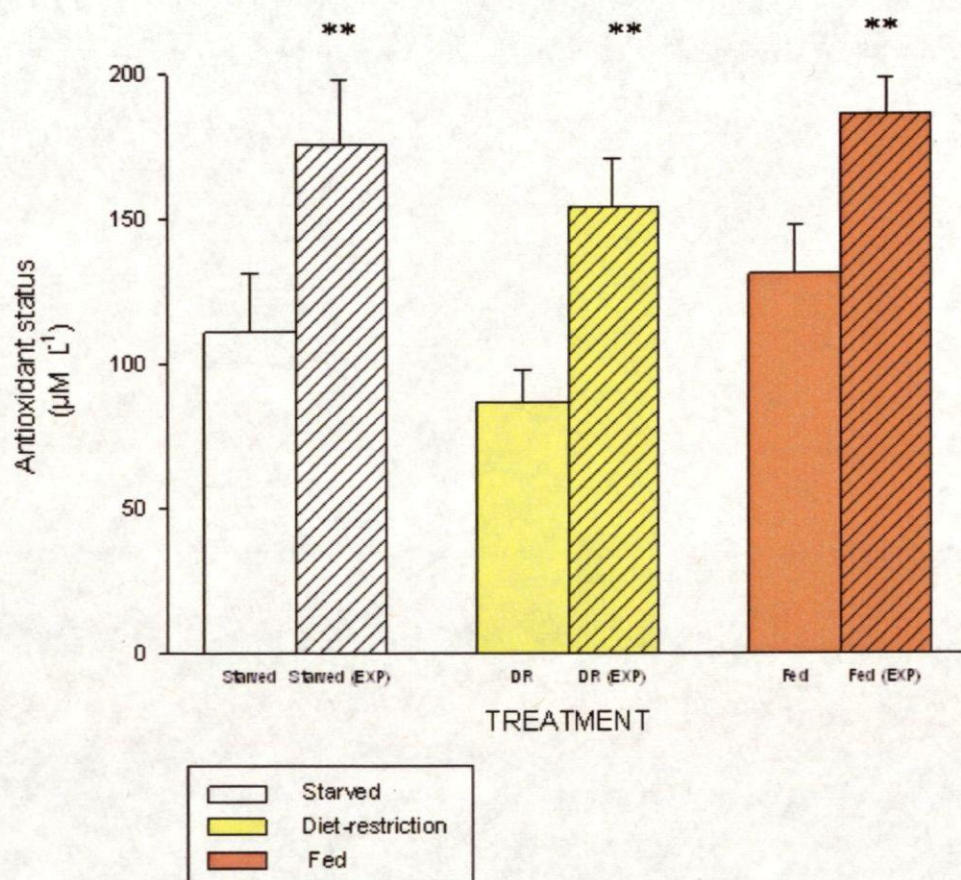
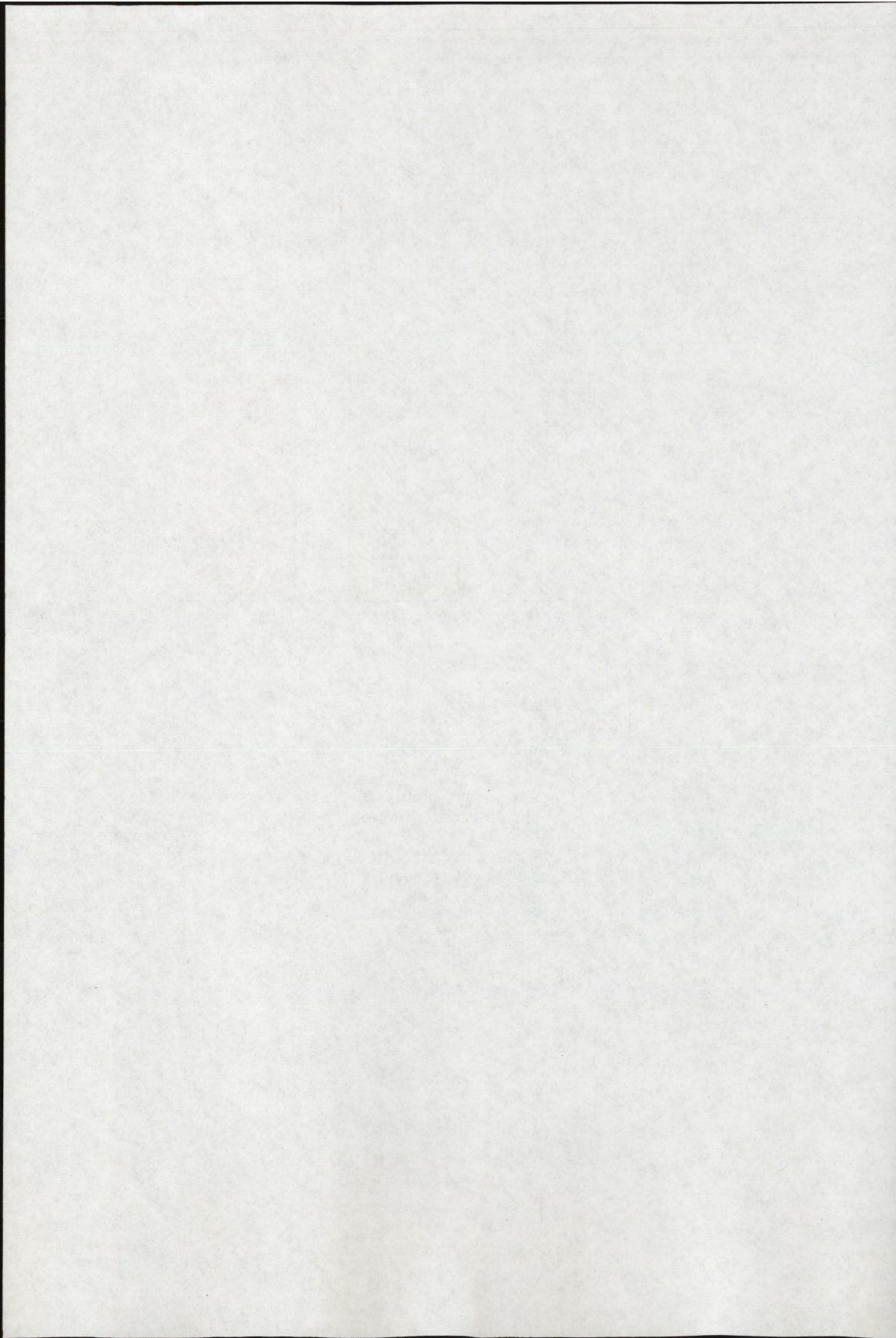


Fig. 5.4 Antioxidant status ($\mu\text{M L}^{-1}$) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. ** signifies $P < 0.01$. Error bars signify ± 1 standard error.



5.3.1.2 Cellular variables

Cell integrity (%) was higher in unexposed crabs compared to PYR-exposed crabs irrespective of dietary treatment (ANOVA, $F_{1,50} = 22.27$, $P < 0.001$). No significant differences, however, were observed between dietary treatments (Fig. 5.5).

Cellular viability was higher in unexposed compared to PYR-exposed crabs, irrespective of dietary treatment (ANOVA, $F_{1,50} = 25.27$, $P < 0.001$). Significant differences were also found between treatment groups, with increased cellular viability in starved compared with fed and DR crabs (ANOVA, $F_{2,50} = 5.06$, $P < 0.01$) (Fig. 5.6).

Immune function, as shown by the phagocytic capability of haemocytes, was affected by contaminant exposure and significant differences were observed between unexposed and PYR-exposed individuals, with contaminant-exposed crabs expressing a depressed phagocytic capability (ANOVA, $F_{1,48} = 29.13$, $P < 0.001$); however, no differences were observed, between dietary treatments (Fig. 5.7).

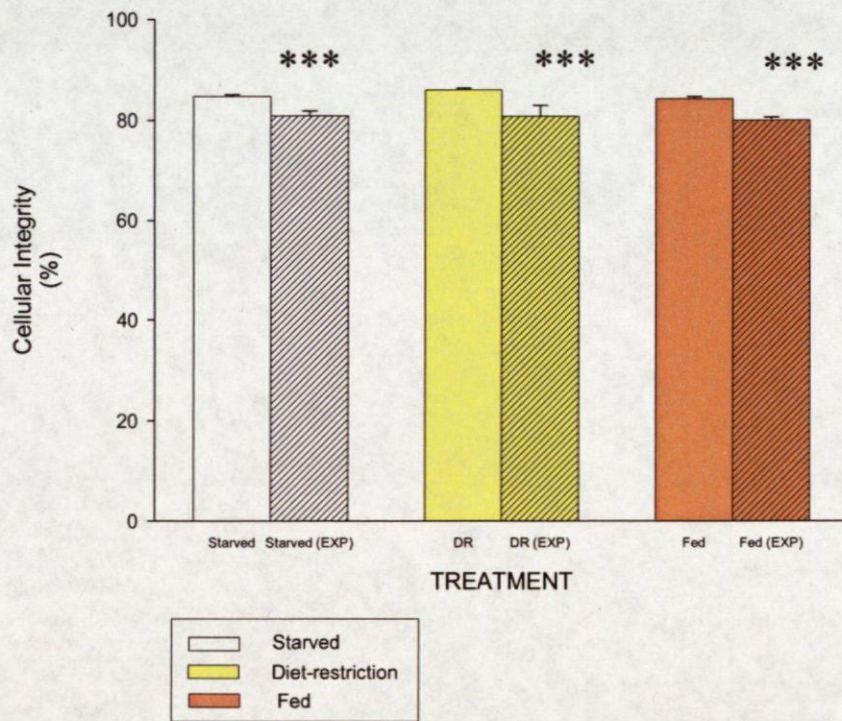


Fig. 5.5 Cellular integrity (%) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. *** signifies $P < 0.001$. Error bars signify ± 1 standard error.

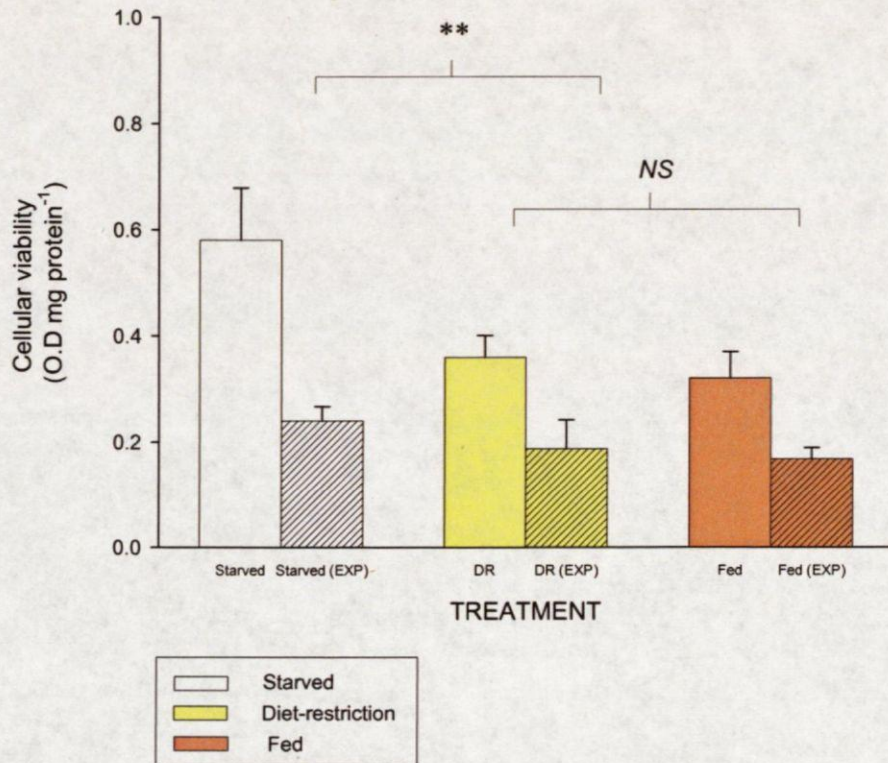
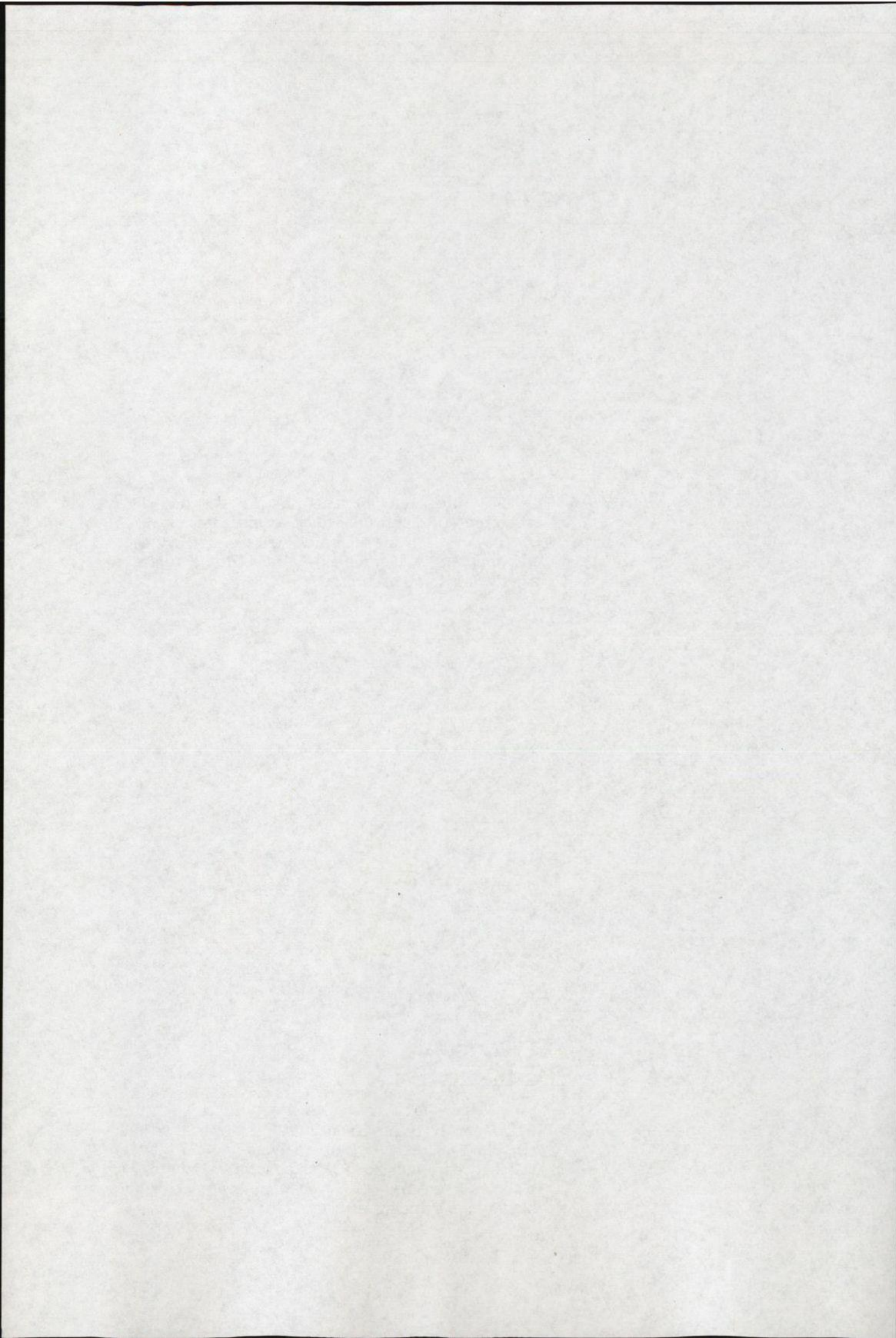


Fig. 5.6 Cellular viability assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. ** signifies $P < 0.01$. NS = Not Significant. Error bars signify ± 1 standard error.



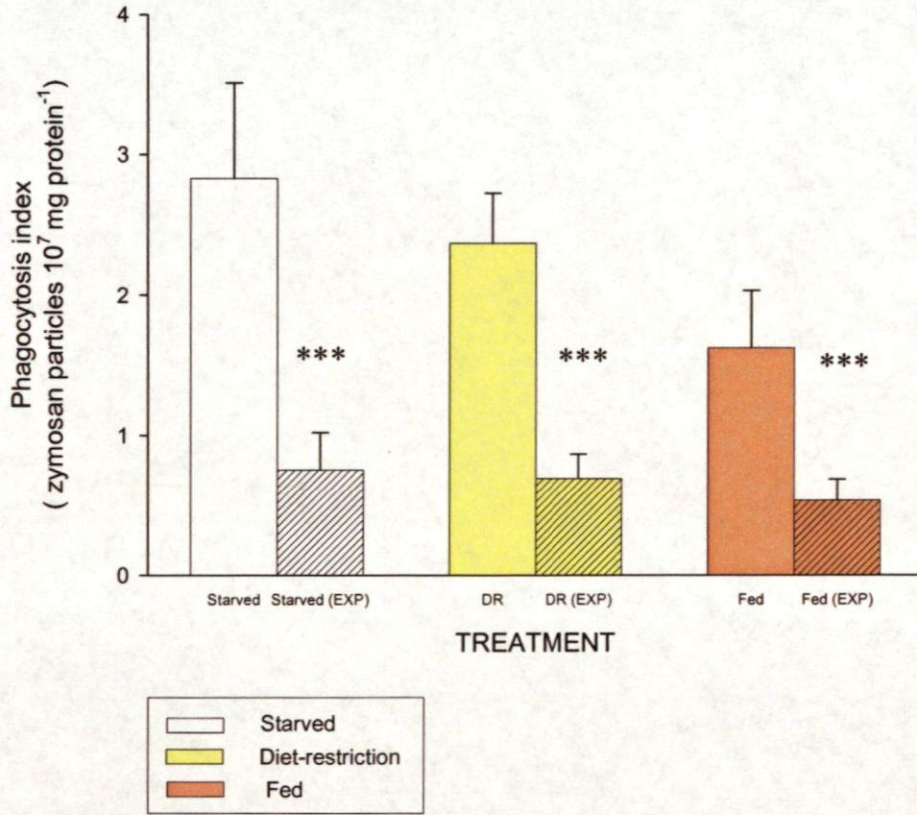
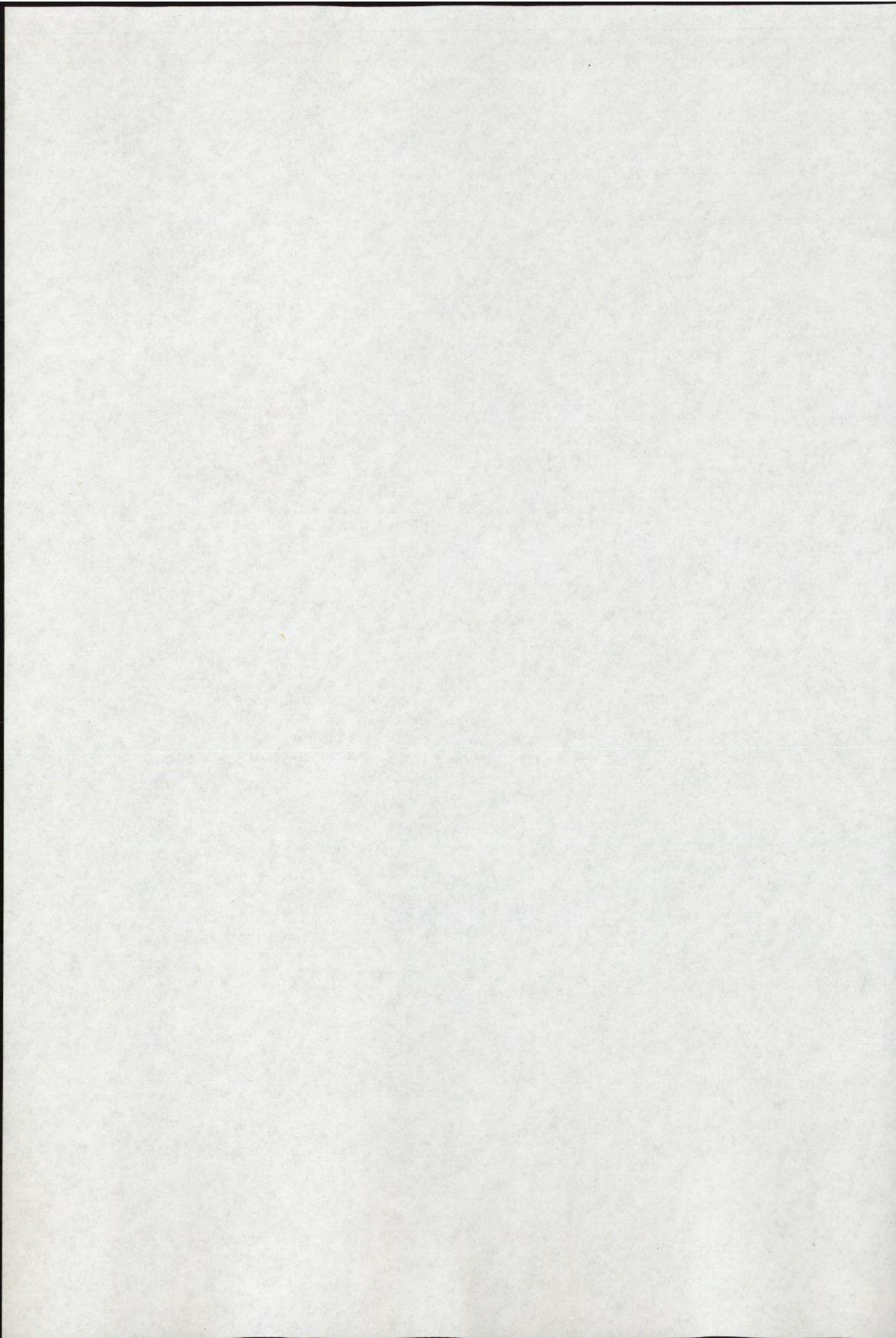


Fig. 5.7 Phagocytosis index (zymosan particles $10^7 \text{mg protein}^{-1}$) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 7). Hatched bars indicate PYR-exposed treatments. *** signifies $P < 0.001$. Error bars signify ± 1 standard error.



5.3.1.3 *Multivariate analyses*

Multivariate analysis (ANOSIM tests) identified significant differences between treatment groups [*Global R* = 0.393, *P* (%) = 0.1]. Significant differences were observed between pyrene-exposed and unexposed crabs, irrespective of dietary treatments: starved [*R* = 0.529, *P* (%) = 0.1]; diet-restriction [*R* = 0.564, *P* (%) = 0.1] and fully fed [*R* = 0.667, *P* (%) = 0.1] (Table 5.2). Results from SIMPER analysis revealed that, in each dietary treatment, a different variable was the significant contributor to the overall group separation (Table 5.3). Within the starved treatment, urinary protein concentrations contributed < 25% to the group differences (Table 5.3a). Antioxidant status (<35%) was the primary variable responsible for group differentiation for PYR-exposed and unexposed fed crabs (Table 5.3b). With regard to the diet-restriction treatment, however, urinary PAH metabolites (<25%) were the major contributing variable attributable for group separation (Table 5.3c).

Table 5.2 Post-exposure physiological assessment (day 7). Results from ANOSIM tests highlighting treatment differences between unexposed and pyrene-exposed (PYR) *Carcinus maenas*.

Treatment	Pair wise tests	ANOSIM GLOBAL tests		ANOSIM pairwise tests		Significant difference
		R	P%	R	P%	
STARVED	Unexposed vs. PYR-exposed	0.393	0.1	0.529	0.1	S
DIET- RESTRICTION	Unexposed vs. PYR-exposed	0.393	0.1	0.667	0.1	S
FED	Unexposed vs. PYR-exposed	0.393	0.1	0.564	0.1	S

Table 5.3 Pairwise treatment comparisons of percentage contribution of each physiological variable within each dietary treatment (day 7). Highest contributing variables are highlighted in bold.

a) Starved (unexposed) vs. Starved (PYR)

Variable	Contribution (%)	Cumulative (%)
Protein in urine	24.13	24.13
Cell viability	22.7	46.84
Phagocytosis	21.56	68.4
Antioxidant status	16.06	84.46
Cell Integrity	8.39	92.85
PAH metabolites	7.15	100

b) Fed (unexposed) vs. Fed (PYR)

Variable	Contribution (%)	Cumulative (%)
Antioxidant status	23.73	23.73
Cell Integrity	21.67	45.4
PAH metabolites	19.93	65.33
Phagocytosis	18.1	83.43
Cell viability	13.92	97.35
Protein in urine	2.65	100

c) DR (unexposed) vs. DR (PYR)

Variable	Contribution (%)	Cumulative (%)
PAH metabolites	33.91	33.91
Cell Integrity	29.01	62.92
Antioxidant status	13.52	76.44
Phagocytosis	12.94	89.38
Cell viability	9.61	99
Protein in urine	1	100

5.3.2 Day 14

Significant differences in shore crab physiological condition (at the biochemical and cellular level) were revealed following 14 days exposure (See Table 5.4).

5.3.2.1 *Biochemical variables*

Urinary concentrations of 1-hydroxypyrene-type metabolites were significantly elevated in PYR-exposed individuals in all three treatments, compared to that of unexposed crabs (ANOVA, $F_{2,50} = 51.79$, $P < 0.001$). A significant difference was observed also between the dietary treatments, with increased metabolite concentrations in the starved treatment compared to that of fed and DR crabs (ANOVA, $F_{2,48} = 24.47$, $P < 0.001$) (Fig. 5.8).

Urinary protein concentrations, were significantly higher in starved crabs compared to the DR and fed treatments (ANOVA, $F_{2,48} = 42.56$, $P < 0.001$). Within the starved treatment, unexposed crabs had higher protein concentrations within the urine than PYR-exposed crabs (ANOVA, $F_{2,48} = 28.36$, $P < 0.01$) (Fig. 5.9).

Antioxidant status demonstrated differences between dietary treatments, with the fed treatment demonstrating a greater antioxidant capability (in plasma) than both the starved and DR groups (ANOVA, $F_{2,48} = 5.35$, $P < 0.01$) (Fig. 5.10).

Table 5.4 . Post-exposure physiological assessment of nutrition and contaminant-imposed physiological constraints in starved, diet-restricted (DR) and fully fed *Carcinus maenas* (mean \pm 1 SE) (day 14). *,**, *** signify $P < 0.05$, 0.01 and 0.001, respectively.

Significant changes to biological responses compared to treatment controls (DAY 14)	Parameters	Starved	Diet-restricted (DR)	Fed	Statistical significance	
					INTRA-treatment (Diet-Exposure Interaction)	INTER-treatment (Diet)
BIOCHEMICAL	Urinary PYR metabolites ($\mu\text{g L}^{-1}$)	448.15 \pm 92.58 vs. 920.54 \pm 108.15	48.07 \pm 27.94 vs. 497.26 \pm 95.11	54.65 \pm 10.06 vs. 401.13 \pm 82.9	***	Starved > DR = Fed ***
	Urinary protein concentrations (mg ml^{-1})	0.42 \pm 0.06 vs. 0.17 \pm 0.01	0.11 \pm 0.02 vs. 0.08 \pm 0.02	0.13 \pm 0.02 vs. 0.10 \pm 0.03	**	***
	Antioxidant status ($\mu\text{M L}^{-1}$)	138.02 \pm 21.11 vs. 170.31 \pm 13.41	148.05 \pm 15.15 vs. 185.36 \pm 15.15	204.93 \pm 17.09 vs. 203.96 \pm 15.12	NO DIFFERENCE	Starved = DR < Fed **
	Cellular integrity (%)	86.28 \pm 0.45 vs. 87.09 \pm 0.13	84.80 \pm 1 vs. 86.30 \pm 1.36	86.92 \pm 0.63 vs. 86.13 \pm 0.46	NO DIFFERENCE	NO DIFFERENCE
CELLULAR	Cellular viability (O.D mg protein $^{-1}$)	0.27 \pm 0.02 vs. 0.56 \pm 0.12	0.19 \pm 0.04 vs. 0.45 \pm 0.05	0.19 \pm 0.03 vs. 0.34 \pm 0.04	***	*
	Phagocytosis (zymosan particles 10^7 mg protein $^{-1}$)	2.14 \pm 0.44 vs. 1.65 \pm 0.35	1.31 \pm 0.25 vs. 1.61 \pm 0.30	2.44 \pm 0.43 vs. 1.42 \pm 0.22	NO DIFFERENCE	NO DIFFERENCE

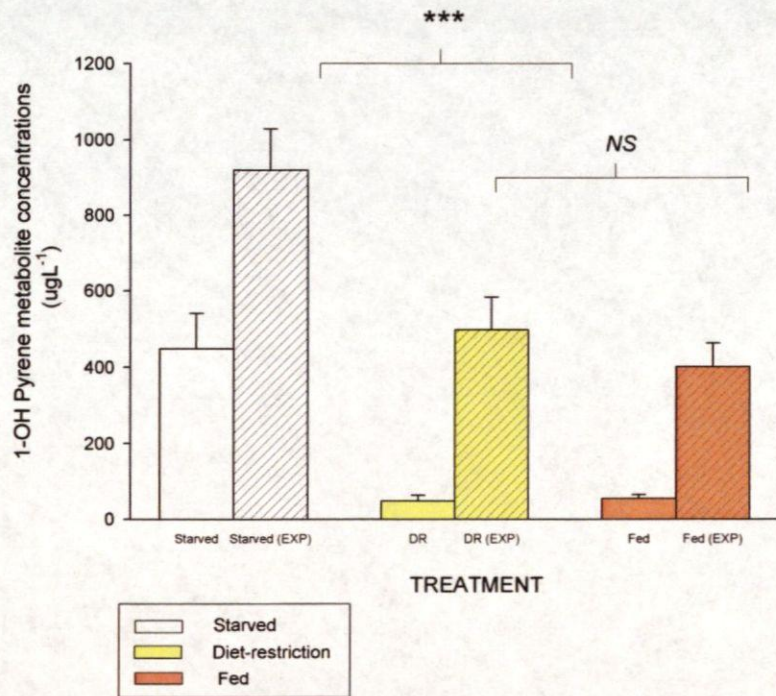


Fig. 5.8 Urinary PYR metabolite concentrations (μgL^{-1}) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 14). Hatched bars indicate PYR-exposed treatments. *** signifies $P < 0.001$, NS = Not Significant. Error bars signify ± 1 standard error.

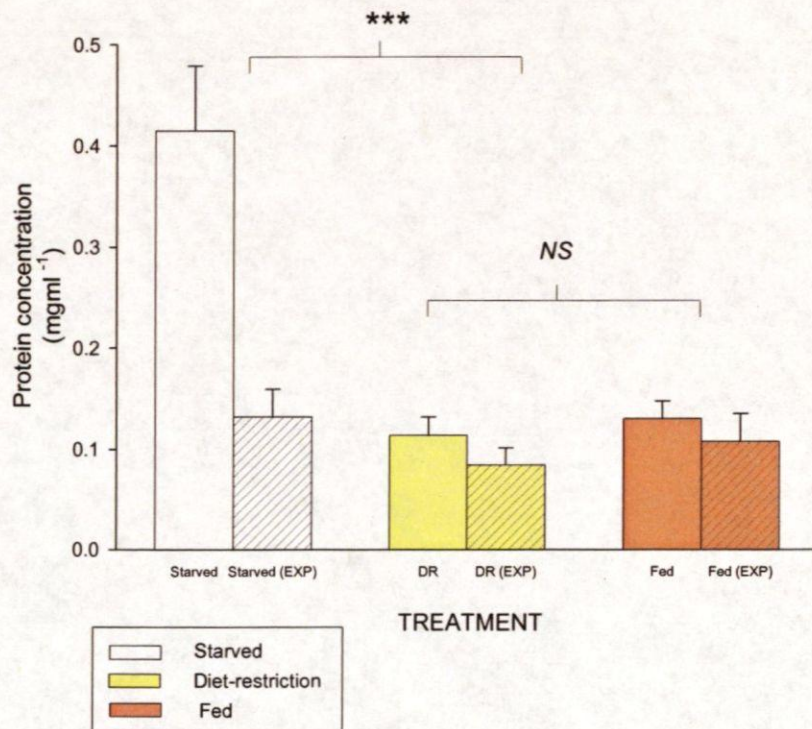
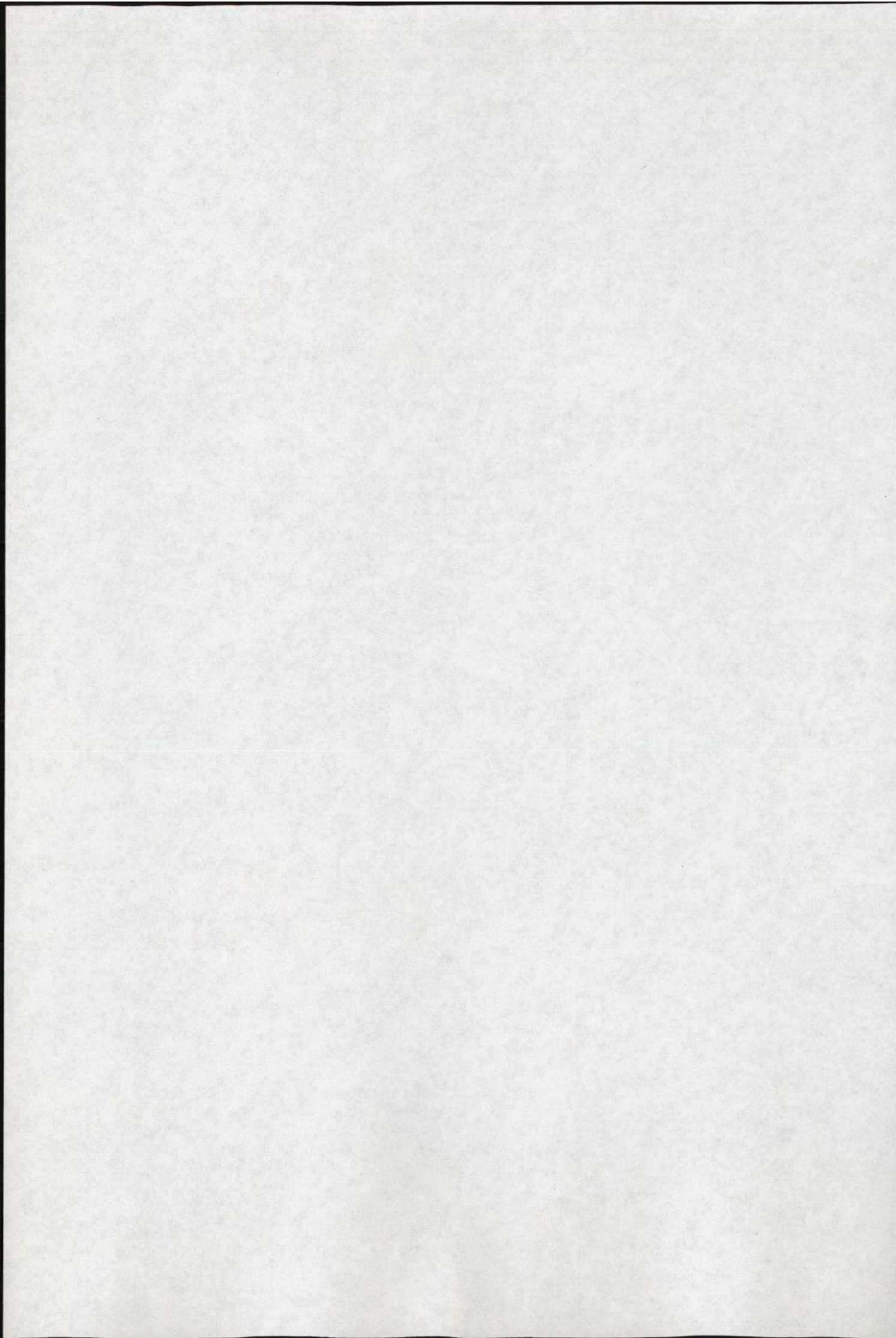


Fig. 5.9 Urinary protein concentration (mgml^{-1}) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 14). Hatched bars indicate PYR-exposed treatments. *** signifies $P < 0.001$. Error bars signify ± 1 standard error.



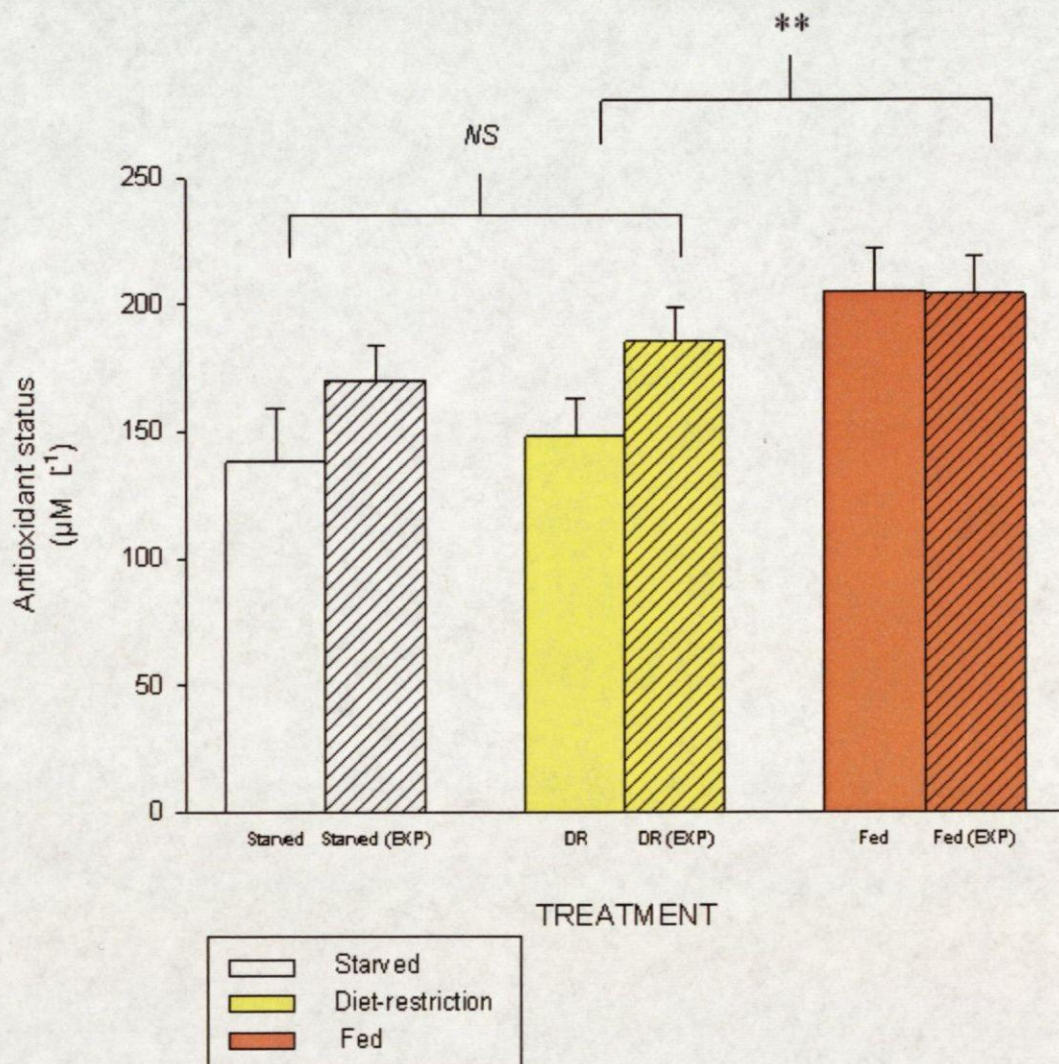
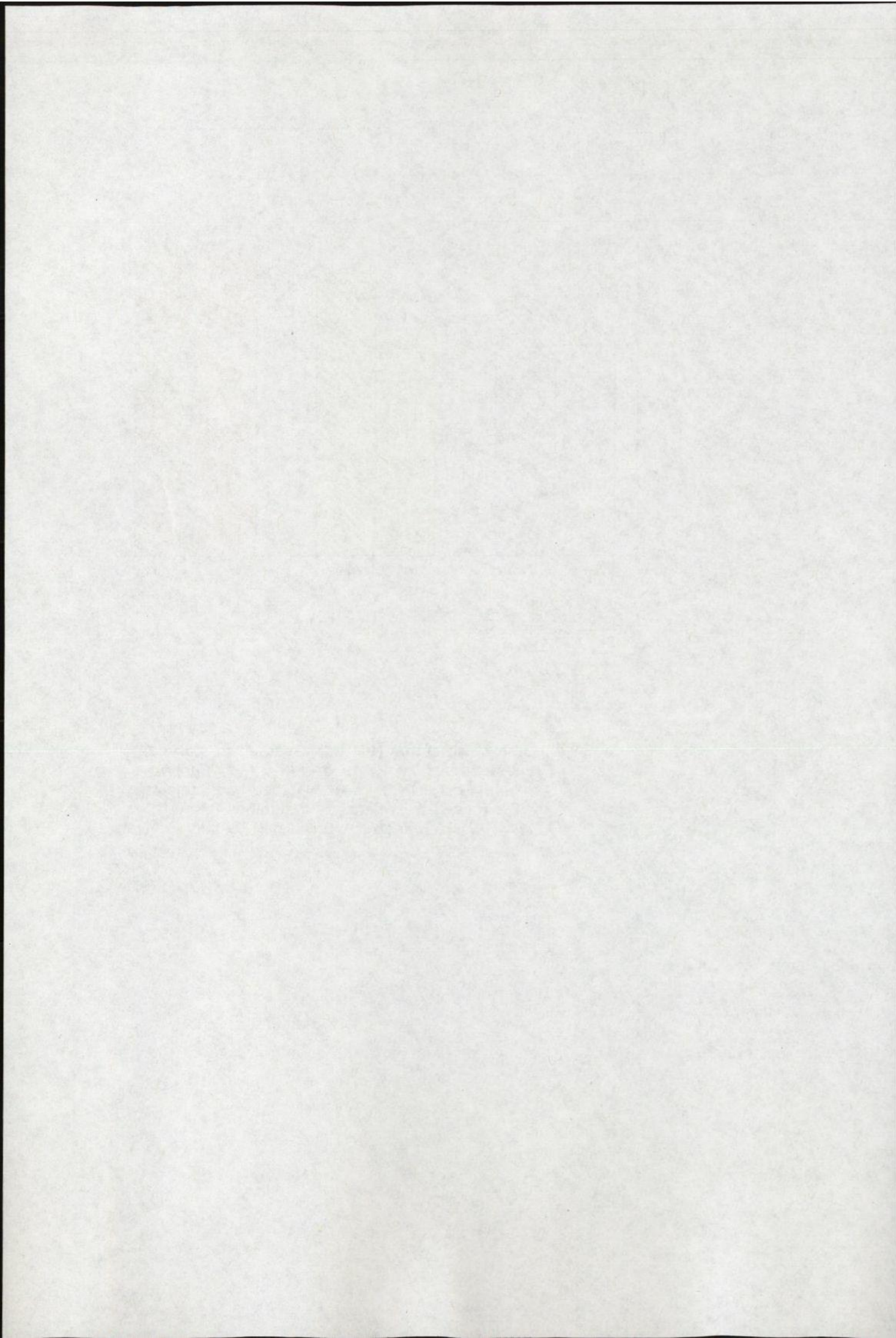


Fig. 5.10 Antioxidant status ($\mu\text{M L}^{-1}$) assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 14). Hatched bars indicate PYR-exposed treatments. ** signifies $P < 0.01$, NS = Not Significant. Error bars signify ± 1 standard error.



5.3.2.2 Cellular variables

Fourteen days pyrene-exposure had no apparent effect at the cellular level. PYR-exposed and unexposed crabs demonstrated similar cellular integrity and phagocytosis (Table 5.2). However, there was increased cellular viability in the PYR-exposed compared with unexposed crabs, regardless of dietary treatment (starved, DR and fed) (ANOVA, $F_{1,50} = 22.27$, $P < 0.001$) (Fig. 5.11).

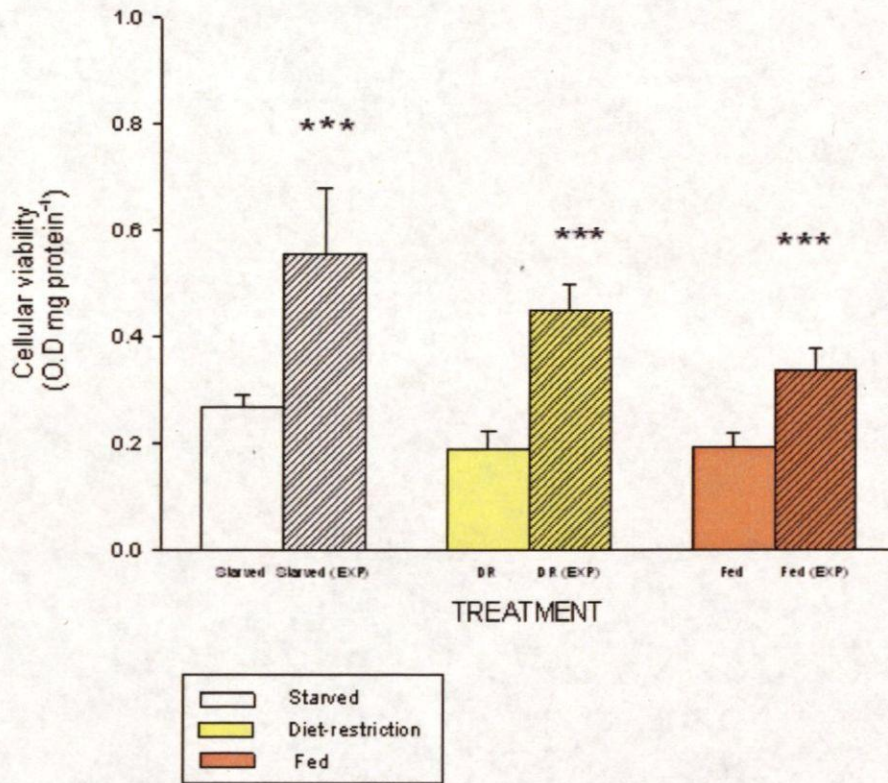
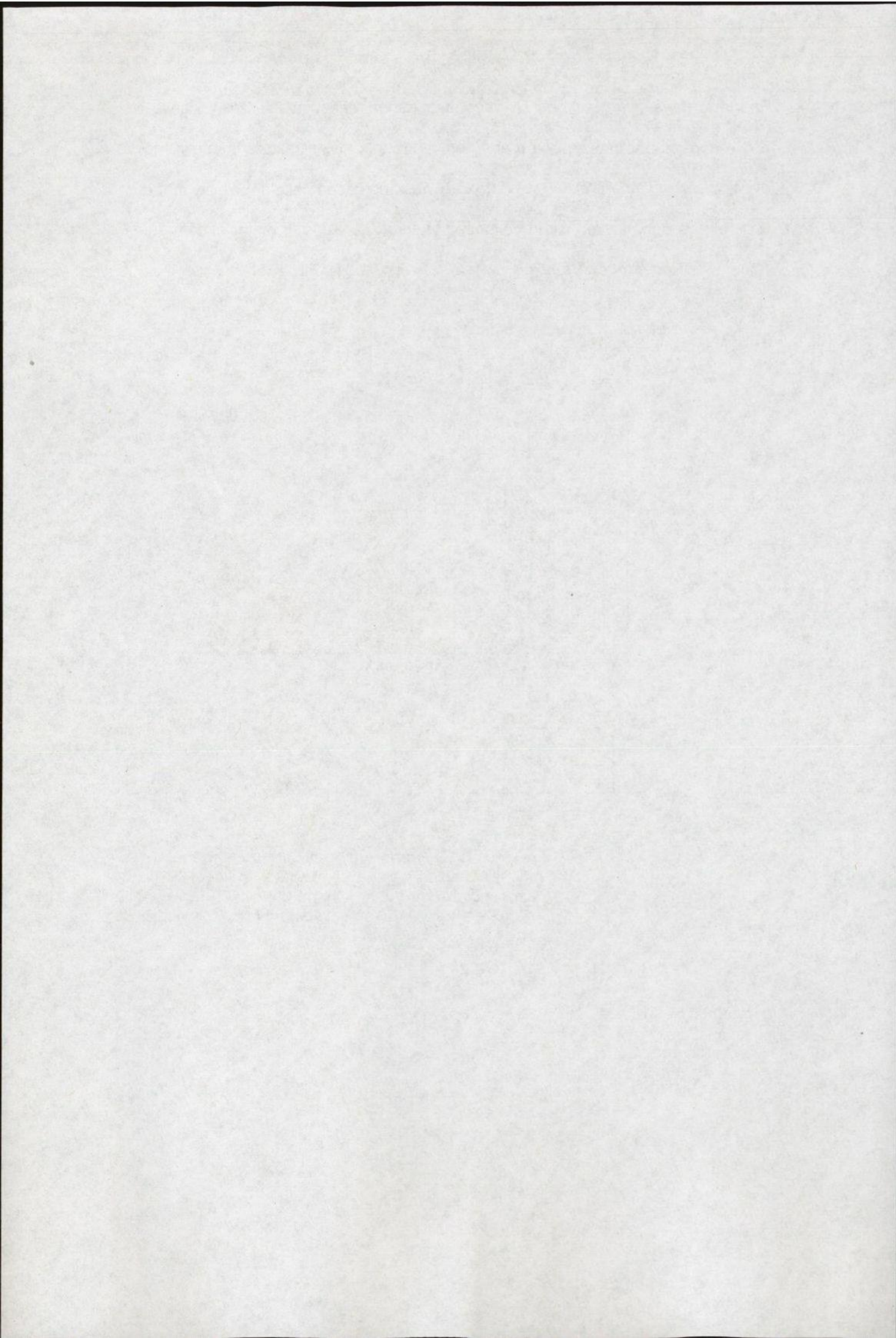


Fig. 5.11 Cellular viability assessed in starved, diet-restricted (DR) and fully fed *Carcinus maenas* ($n = 9$) (day 14). Hatched bars indicate PYR-exposed treatments. * signifies $P < 0.05$, NS = Not Significant. Error bars signify ± 1 standard error.



5.3.2.3 *Multivariate Analysis.*

Multivariate analysis (ANOSIM tests) revealed significant differences between treatment groups [*Global R* = 0.387, *P* (%) = 0.1] with significant differences only observed between starved pyrene-exposed and unexposed crabs, starved [*R* = 0.529, *P* (%) = 0.1] (Table 5.5). Results from SIMPER analysis revealed that with regard to the starved treatment, urinary protein concentrations were contributing < 36% to the group differences (See Table 5.6).

Table 5.5 Post-exposure physiological assessment (day 14). Results from ANOSIM tests highlighting treatment differences between unexposed and pyrene-exposed (PYR) *Carcinus maenas*.

TREATMENT	Pair wise tests	ANOSIM GLOBAL tests		ANOSIM pairwise tests		Significant difference
		R	P%	R	P%	
STARVED	Unexposed vs. PYR-exposed	0.387	0.1	0.648	0.1	S
DIET- RESTRICTION	Unexposed vs. PYR-exposed	0.387	0.1	0.285	0.1	NS
FED	Unexposed vs. PYR-exposed	0.378	0.1	0.158	0.1	NS

Table 5.6 Pairwise treatment comparisons of percentage contribution of each physiological variable within each dietary treatment (day 14). Highest contributing variables are highlighted in bold.

Starved (unexposed) vs. Starved (PYR)

Variable	Contribution (%)	Cumulative (%)
Protein in urine	35.91	35.91
PAH metabolites	18.4	54.31
Phagocytosis	15.61	69.91
Antioxidant status	13.81	83.72
Cellular Integrity	13.52	97.24
Cellular Viability	2.76	100

5.4 DISCUSSION

Experimental manipulation of the 'internal milieu' of the shore crab was accomplished via a combination of nutrition and contaminant exposure. Pyrene exposure had a significant effect upon cellular functioning after seven and fourteen days exposure.

Validation of pyrene exposure was achieved through evaluation of urinary pyrene metabolites. Irrespective of dietary treatment, after both 7 and 14 days exposure, pyrene-exposed crabs contained a higher concentration of urinary pyrene metabolites compared to unexposed crabs (Figs. 5.2 and 5.8, respectively). There were no differences in pyrene metabolite levels after 7 and 14 days exposure to pyrene for either starved (ANOVA, $F_{1,16} = 1.86$, $P = 0.19$), diet-restricted (ANOVA, $F_{1,16} = 1.51$, $P = 0.24$) or fed treatments (ANOVA, $F_{1,16} = 3.22$, $P = 0.092$), signifying that pyrene metabolism by shore crabs was constant throughout the study.

Antioxidant status, as shown by the ability of the plasma to sequester reactive oxygen species (ROS), is influenced by shore crab nutritional state (see Chapter 4). Fully-fed crabs have been shown in this study to possess a higher antioxidant capability than starved or diet-restricted (DR) crabs (irrespective of pyrene exposure). This increased dietary-influenced capability may arise due to the amount of energy channelled to produce compounds with antioxidant scavenging properties. Antioxidants have been studied in detail as possible indicators of oxidative stress in marine organisms (Ahmad et al. 2000; Burgeot et al. 1996; Cheung et al. 2001; Cossu et al. 1997; Fitzpatrick et al. 1997; Regoli et al. 1998; Winston and Di Giulio 1991). Evidence has shown that antioxidants (such as enzymes) are inducible with increasing contaminant concentrations and exposure (Ahmad et al. 2000; Burgeot et al. 1996; Cheung et al. 2001). Other studies have shown that antioxidant capabilities are diminished or saturated with contaminant exposure (Camus et al. 2002b; Cossu et al. 1997; Doyotte et al. 1997; Vijayavel et al. 2004). The mechanism by which antioxidant systems operate is dependent upon exposure concentration and duration of contaminant exposure. Acute exposure results in an induction of antioxidant activity; however, chronic or higher contaminant concentrations can result in a reduction in antioxidant activity/capability (Table 5.7).

Table 5.7 Summary table of effects of sublethal contaminant exposure on antioxidant systems in aquatic organisms.

Species	Contaminant	Effect	Reference
<i>Channa punctatus</i>	Paper mill effluent	Induction of antioxidant enzymes (e.g. glutathione peroxidase and catalase) over 15, 30, 60 and 90 days exposure	(Ahmad et al. 2000)
<i>Mullus barbatus</i>	PAHs	Induction of antioxidant enzymes (glutathione-s-transferase and catalase) in contaminated areas (37507.5 ngL ⁻¹) compared to 'clean' areas (47.2 ngL ⁻¹)	(Burgeot et al. 1996)
<i>Perna viridis</i>	PAHs	Induction of antioxidant enzymes with increasing PAH concentrations (8-307 ng g dry tissue ⁻¹)	(Cheung et al. 2001)
<i>Unio tumidus</i>	PAHs (42261 µg/kg dry sediment) metals (433.12 mg/kg dry wt)	After 15 and 30 days (caged) exposure to contaminants in the water column, antioxidants were inhibited by 60% and 80% in gills and digestive gland.	(Cossu et al. 1997)
<i>Mytilus galloprovincialis</i>	Trace metals (As, Cu, Fe, Mn, Pb, and Zn)	Lower levels of glutathione and higher enzymatic activities of glyoxalase compared to control mussels	(Regoli 1998)
<i>Hyas araneus</i>	14 day exposure to North sea oil: PAHs (234 195 µg kg ⁻¹)	Saturation of the total oxygen scavenging capacity in the midgut gland compared with controls	(Camus et al. 2002b)
<i>Macrobrachium malcolmsonii</i>	Hydrogen peroxide (in vitro)	Induction of glutathione peroxidase with increasing hydrogen peroxide exposure	(Arun et al. 1999)
<i>Scylla serrata</i>	Naphthalene (100 mg L ⁻¹) 96 hr exposure)	Reduction in antioxidant capability: in haemolymph, ovary and hepatopancreas for both: 1) Enzymes: catalase (CAT); glutathione peroxidase (GPX); superoxide dismutase (SOD) and 2) non-enzymatic antioxidants: vitamin C (ascorbic acid), vitamin E (tocopherol) and glutathione (GSH)	(Vijayavel et al. 2004)

The FRAP assay measures the total antioxidant level in a sample (Griffin and Bhagooli 2004). The drawback of this method is that glutathione is unable to reduce Fe^{III} and does not account for the thiol (SH) group of antioxidants (Prior and Cao 1999). Bell and Smith (1995) demonstrated the occurrence, distribution and activity of antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase) in the haemocytes and cell-free plasma of the shore crab *C. maenas*. Sub-lethal exposure to naphthalene (PAH) was investigated in *Scylla serrata* and revealed decreased antioxidant activities for both enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic antioxidants (vitamins C, E and glutathione) (Vijayavel et al. 2004), thereby, corroborating findings of antioxidants in crustaceans, and specifically allude to the antioxidants detected using the FRAP assay. The present results show that total antioxidant capability (excluding glutathione) in crab haemolymph is dependent on the individual's nutritional status.

Protein analysis of urine revealed that urinary protein concentrations were significantly higher in starved crabs than in fed or DR individuals, indicative of proteinuria (i.e. excess loss of protein in urine) (Kashif et al. 2003). There were no differences, however, in protein levels within the urine between days 7 and 14 exposure periods for either starved (ANOVA, $F_{1,16} = 0.11$, $P = 0.74$), diet-restricted (ANOVA, $F_{1,16} = 0$, $P = 0.99$) or fed treatments (ANOVA, $F_{1,16} = 1.68$, $P = 0.21$), signifying that protein excretion by shore crabs was constant throughout the study. There are four mechanisms of proteinuria (in humans): increased glomerular filtration, inadequate tubular reabsorption, overflow and increased tubular secretion and maybe indicative of kidney dysfunction (Kashif et al. 2003; Tonelli et al. 2006). The crustacean hepatopancreas, a multilobate sac or diverticulum of the midgut, is analogous to the vertebrate liver, pancreas and small intestine (Crothers 1967), and is a vital organ involved in excretion,

carbohydrate and lipid metabolism, enzyme secretion, digestion and food absorption, synthesis and secretion of plasma proteins and storage of energy reserves (Crothers 1967; Johnstone et al. 1973; Sanchez-Paz et al. 2007). In this present study, protein overflow may be postulated to arise when protein stores are broken down (in order to provide the body with energy during nutrition-deprived conditions) and subsequent increased protein levels overflow into the hepatopancreas, leading to excretion via the antennal glands. In *C. maenas*, starvation, has been induced experimentally (Meigh 2000; Uglow 1969). After fourteen days of starvation, haemolymph protein levels decreased by 20% (Uglow 1969). Meigh (2000) demonstrated that, due to a combination of starvation and exposure to mixed effluent of engine oil, significant decreases in haemolymph protein levels occurred and postulated that protein metabolism had been induced. In other crustacean species, a reduction in the hepatosomatic index (hepatopancreas weight: an indicator of energy reserves) (Woll et al. 2006) has been shown to occur through the use and mobilisation of energy reserves (Sanchez-Paz et al. 2007) (and references therein). Such evidence of protein metabolism for energy in times of nutritional deficiency may explain the excess protein excreted in urine, found here.

Present results indicate that contaminant exposure has not altered cellular function. The singular effect was increased cellular viability and this has been shown previously (Dissanayake et al. 2006b; Grundy et al. 1996; Matozzo et al. 2002; Matozzo and Marin 2005; St-Jean et al. 2002a, b). This elevated capability of lysosomes to accumulate contaminants (in this case pyrene) may be explained by the fact that maximal lysosomal capability has not been compromised and membrane destabilisation has not occurred (Dissanayake et al. 2006b). The significant differences of increased cellular viability between

starved and DR and fed crabs can be postulated to be linked with the process of autophagy. This process may confer pollutant-induced tolerance via up-regulation of the breakdown mechanisms of proteins through the use of intermediates for the end purpose of maintaining energy production (ATP) via the Krebs cycle (Moore et al. 2006). This process of augmented autophagy by short-term diet-restriction has been demonstrated also in mussels, using both model organic and heavy metal contaminants (Moore 2004).

The combined effect of dietary manipulation and contaminant-imposed perturbation was tested to investigate whether susceptibility to contaminant exposure was dependent upon shore crab nutritional status. Using multivariate methods it is possible to postulate the implications of both nutritional and contaminant-imposed constraints upon shore crab physiological condition, rather than measuring changes for one single variable. Changes to the shore crab physiological condition (using an integrated approach of multivariate analysis) signalled by a repertoire of biochemical and cellular responses are apparent with a seven-day and fourteen-day exposure period. Differences between pyrene-exposed individuals and unexposed crabs were observed for all dietary treatments (starved, fed and diet-restriction). For exposed crabs, increased pyrene metabolites and proteinuria levels were observed in starved crabs compared to fully-fed crabs assessed at seven and fourteen exposure periods (Figs 5.2, 5.3, 5.8 and 5.9, respectively). Also, antioxidant status was significantly different between fed and starved and diet-restricted crabs (Figs 5.4 and 5.10). Cellular viability, however, was lower in pyrene-exposed crabs (for all treatments) after seven days compared to unexposed crabs (Fig 5.6) after fourteen days exposure, however, induction of cellular viability was observed in pyrene-exposed crabs.

The mechanisms of biochemical and cellular processes observed here appear to be dependent upon shore crab nutritional status, thereby, affording contaminant tolerance. On the basis of these and previous results (Chapter 4), it is concluded that shore crab physiology is robust to short-term and sublethal contaminant-mediated challenges. In summary, this study has shown that the susceptibility of *C. maenas* to sublethal contaminant exposure is dependent upon its nutritional status (as shown by lower haemolymph total antioxidant capability in starved crabs compared to fully fed crabs). Short-term exposure to pyrene resulted in up-regulation of cellular processes, such as lysosomal capability (signalled by an increase in cellular viability).

CHAPTER 6:

**Does the physiological condition of
Carcinus maenas affect intraspecific
agonistic behaviour?**

Does the physiological condition of *Carcinus maenas* affect intraspecific agonistic behaviour?

ABSTRACT

In nature, intraspecific resource conflicts are prevalent, particularly male-male dyadic competitions. Ultimately, resource conflicts are resolved through contests and these depend on three distinct traits: resource holding potential (RHP; e.g. body size), resource value and aggressiveness. Male *Carcinus maenas* exhibit a characteristic and measurable series of agonistic behavioural patterns during inter-individual competition for resources (in this case, a whole cockle). This study tested the hypothesis that shore crab physiological condition is a key factor in determining the outcome of intraspecific agonistic resource contests in this species. For starved crabs, resource holding potential (RHP defined as the ability to win an all-out contest) evaluated using intraspecific agonistic behaviour was significantly higher in pyrene-exposed compared to unexposed crabs. Also for starved crabs, exposed individuals had increased resource possession (%) and decreased recuperation time compared to unexposed crabs ($P < 0.05$) irrespective of nutritional state. In conclusion, experimental manipulation of shore crab physiological condition (via a combination of dietary and sublethal contaminant exposure) has shown that under nutrient-deprived conditions (starved crabs), higher proximate metabolic costs are incurred [decreased lactate and glucose concentrations ($P < 0.05$)], compared to fully-fed individuals. Resource holding potential (RHP) was higher in pyrene-exposed compared to unexposed crabs, thereby, demonstrating how shore crab physiological condition determines the competitive ability of this species.

6.1 INTRODUCTION

In nature, intraspecific conflicts are prevalent for many species and arise over competition for limited resources, such as food, shelter (Briffa and Elwood 2001), territory (Brandt 2003; Taylor et al. 2001), and mates (Brandt 2003; Faber and Baylis 1993; Kosmala et al. 1998; Lailvaux et al. 2005) (reviewed in Huntingford and Turner 1987). Ultimately, resource conflicts are resolved through fighting (Hurd 1997) and may involve 'conventional' signals such as sexually-selected traits [(ornaments or badges of status; reviewed in Andersson (1994)] or 'costly' signals when performing an energy-demanding activity such as fighting (Briffa 2006). Both types of signals advertise fighting ability or resource holding potential (RHP). RHP is defined here as the ability to win an all-out contest (Maynard Smith 1982; Parker 1974). Resource holding potential has been assessed for several taxa using various measures including body size (Beaugrand et al. 1996), weight (Faber and Baylis 1993; Martin et al. 1997) and weapon size (e.g. claw size in *C. maenas*) (Sneddon et al. 1997b).

Game theory models of aggressive conflict behaviour are postulated to be dependent upon three distinct traits: resource holding potential, resource value, and aggressiveness. Experimental manipulation of these traits has helped clarify the degree they are influential in shaping intraspecific male-male dyadic contests: RHP (Faber and Baylis 1993; Gherardi 2006; Leiser et al. 2004; Neat et al. 1998; Petersen and Hardy 1996); resource value (Gherardi 2006; Sneddon et al. 1997a) and aggressiveness (Earley et al. 2000). In cases of asymmetrical contests, where one opponent is larger than the other, body size (RHP) is the important factor in determining the victor in pairwise contests (Beaugrand et al. 1996; Faber and Baylis 1993; Sneddon et al. 1997b). In symmetrical contests, however (where conspecifics are matched in terms of RHP, e.g. body size), resource value and aggression are important in settling conflicts (Hurd 2006).

During pairwise contests, quantitative estimates of the RHP difference between opponents are said to determine contest duration (Hurd 2006). Contest duration appears to persist until one opponent reaches a 'cost' threshold; this was proposed as 'the own RHP-dependent hypothesis' (Gammell and Hardy 2003). This latter hypothesis was developed from the work of Payne and Pagel (1997) who proposed that those opponents in such 'energetic wars of attrition' that are committed to expending the greatest amount of energy towards resolving the conflict will emerge as the contest victor. Phenotypic correlates (body size, claw size) (Briffa and Elwood 2004) of RHP are said to be fixed, as size of weapons will not vary during a fight (Briffa and Elwood 2004; Sneddon et al. 1997b). Intuitively, it may be proposed that shore crab physiological condition is an important factor in determining the individual RHP, outcome and associated costs of the contest, yet limited data exist to support this hypothesis.

The male shore crab *Carcinus maenas* exhibits a characteristic and measurable series of agonistic behavioural patterns during inter-individual competition for limited resources such as females and food (Sneddon et al. 1997a). Differences in physiological condition between opponents may lead to consequences in terms of competitive ability and, ultimately, contest outcomes. Exposure-concentration relationships have shed light upon the probable effects of acute-sublethal responses impacting upon an organism's physiology (Brown et al. 2004; Canty et al. 2007; Livingstone 1991; 1992; 1998; Rickwood and Galloway 2004; Scarlett et al. 2007). Behaviour links physiological function (internal biological processes) with ecological processes (e.g. growth, reproduction) (Scott and Sloman 2004). Unanswered questions remain about integrating the behavioural effects of toxicants with physiological processes. Instances where behaviour may be indicative/sensitive to contaminant-mediated injury can be considered to be potential indicators and have been characterised in detail in

fish (Scott and Sloman 2004). A paucity of information exists that link effects between the various hierarchical levels of biological organisation; biochemical through to behavioural. This study explored the hypothesis that experimental manipulation of shore crab physiological condition [via combined dietary manipulation and exposure to sublethal concentration of the aromatic hydrocarbon pyrene (see Chapter 5)], while keeping the phenotypic correlates fixed, (i.e. body size and weapon size), will be a key factor in shaping intraspecific agonistic contests in the shore crab *C. maenas* (Briffa and Elwood 2004).

6.2 MATERIAL AND METHODS

6.2.1 Shore crab collection and maintenance

Adult (>60mm CW) (Crothers 1967) male (green) intermoult *Carcinus maenas* ($n = 54$) (mean CW 66.29 ± 5.07 mm) were collected from the Avon Estuary, Bantham, South Devon, U.K. (grid reference: SX 6623 4380) on two occasions (23rd and 25th June 2005) using mackerel-baited traps (see Chapter 2, Section 2.1). In the laboratory, crabs were maintained in static holding tanks containing filtered (10µm carbon filtered), aerated seawater (34ppt, $15 \pm 1^\circ\text{C}$) under a 12h light : 12h dark photoperiod for a maximum of 2 days before being transferred into treatment tanks. Crabs were held in exposure tanks (2L) and starved or fed with γ -irradiated cockle (*Cerastoderma edule*) (Gamma foods, Tropical Marine Centre, Bristol, U.K.) according to the individual nutritional treatment conditions (see below). Water was changed within 18h of each feeding.

Each crab was numbered using a label attached (cyanoacrylate glue) to the carapace. Morphometric characteristics of each crab were recorded such as: carapace width (CW) (mm) and wet weight (g). The following treatment groups were used for agonistic encounters over a food resource (see Chapter 5, Section 5.2.2): (1) starved unexposed ($n = 9$); (2) starved PYR-exposed ($n = 9$); (3) diet-restricted (DR) ($n = 9$); (4) DR PYR-exposed ($n = 9$); (5) fed unexposed ($n = 9$); and (6) fed PYR-exposed ($n = 9$). Each PYR-exposed shore crab was paired with an unexposed conspecific from the respective dietary treatment (e.g. starved unexposed vs. starved PYR-exposed).

Table 6.1 Paired treatment groups used to evaluate agonistic behaviour ($n = 9$).

Dietary manipulation	Exposure treatment
Starved	Unexposed vs. PYR-exposed
Diet-restricted	Unexposed vs. PYR-exposed
Fully-fed	Unexposed vs. PYR-exposed

6.2.2 *Intraspecific contest behaviour*

To initiate agonistic encounters, two size-matched crabs (i.e. unexposed vs. PYR-exposed) were placed in a fighting chamber separated by vertically-sliding blackened glass dividers. The fighting chamber was blackened out from three sides to limit visual stimuli from altering contest behaviour. Contests commenced when a whole cockle was placed in the centre of the chamber and the dividers raised. Typically, the contest often began with a series of 'wrestles', where the crabs would grapple, often pushing and 'pinching' each other with their claws or trying to pin each other. In some cases, contests escalated to a fight, where the intensity of the aggression increased. Agonistic encounters were allowed to proceed for 15min, then crabs were separated and their physiological condition assessed based on haemolymph glucose and lactate analysis. Haemolymph samples (100µl) were extracted by puncture of the arthroal membrane at the base of the walking 4th walking leg using a pre-chilled 1ml syringe and 21 gauge needle (Sigma-Aldrich, U.K.) (see Chapter 2, Section 2.4.1 for details).

Behavioural patterns used here to describe contest behaviour in *C. maenas* were adapted from Sneddon et al. (1997a) (Table 6.2); with the addition of further categories (Table 6.2) based on intensity and duration of various postures adopted; and described here as wrestles and fights (Figs 6.1 and 6.2, respectively). Wrestles were identified as slow, low-intensity behaviour where opponents engaged in grappling by interlocking their chelae (Fig. 6.1). Wrestles were common and are interpreted as low aggression behaviour whereby injury is minimised (Brick 1998; deCarvalho et al. 2004; Warner 1970; Wells 1978). Fights, however, were more intense and accompanied a quicker series of movements where 'pinching' of opponents pereopods occurred (Fig. 6.2). Fighting is also reported commonly for crabs with consequent injury which often

decides the contest outcomes (deCarvalho et al. 2004; Huntingford and Turner 1987; Maynard Smith 1982; Parker 1974; Payne and Pagel 1997).

Agonistic encounters between contestants, recorded using a video camera (Sony DCR10 TRV), included various behaviours such as: resource possession (% time possession of the resource i.e. the cockle); recuperation time [period where opponent moves away from the aggressor (in sec)]; total number of recuperation times; fight and wrestle duration (in sec), contest intensity (no. of fights/no. of wrestles); and total contest duration [fight + wrestle duration (secs)] (Table 6.2). Crabs were held for a further two weeks after experiments to ensure they were not in proecdysis, which would potentially affect physiological condition and subsequent contest behaviour.

6.2.3 *Haemolymph analysis*

The concentration of L-lactate was determined using the method of Gutmann and Wahlefeld (1974) with the suggested modification by Engel and Jones (1978). The assay was optimised and carried out in a microplate format (Briffa and Elwood 2005) (see Chapter 2, Section 2.4.4 for details).

6.2.4 *Statistical analysis*

Two methods of statistical analyses were used to isolate significant differences between experimental treatments, using intraspecific agonistic contests and post-contest metabolite concentrations of lactate and glucose. Univariate analyses were performed to test for differences for a single parameter between experimental groups. To attain an integrated summary of the contaminant impact, multivariate analyses (ANOSIM, SIMPER) tested for differences between the experimental groups via the complete repertoire of biological

responses measured. Univariate statistical analyses were performed using GMAV 5 for windows[®]. Treatment differences were analysed using the parametric Analysis of variance test, due to homoscedasticity of data (Underwood 2005). Multivariate analysis was carried out using PRIMER[®] v6 (Plymouth Routines in Multivariate Ecological Research, PRIMER-E Ltd) (see Chapter 2, Section 2.6.2 for details).

Table 6.2. Types of behavioural patterns displayed in intraspecific contest behaviour in male *Carcinus maenas* adapted from Sneddon et al. (1997a).

TYPE	DESCRIPTION	QUANTIFIED
Wrestles	Crabs would engage each other in grapples, often interlocking chelae resulting in pushing and pinching to 'pin' the opponent down. This series of behaviours is slow and low in intensity. A wrestle can potentially escalate into a fight. (Duration between 30- 240 sec). (See Figure 1.)	Measured in sec
Fights	Crabs engage each other in the same postures as wrestles, however, aggression and intensity increases resulting in a more vigorous series of behaviours where pinching of pereopods occurs. This sequence of events is quicker, however, the duration is shorter, compared to wrestles. Fights can de-escalate to wrestles or both opponents seize aggressive activity. (Duration between 20- 40 sec). (See Figure 2.)	Measured in sec
Resource Possession (%)	% time in possession of the cockle.	Time in possession of the cockle / by contest duration (Expressed as %)
Recuperation time	Time taken to recuperate from either wrestles or fights, timed as soon as the crab breaks engagement and moves away from the opponent.	Measured in sec
N° of Recuperation times	Total number of recuperation times	Expressed as a frequency
Total contest duration	Total duration of wrestles and fights	Measured in sec
Contest intensity	Mean number of fights/ mean number of wrestles	Expressed as a ratio.

Fig. 6.1 Postures displayed in an example of a wrestle where opponents attempt to 'pin' each other.

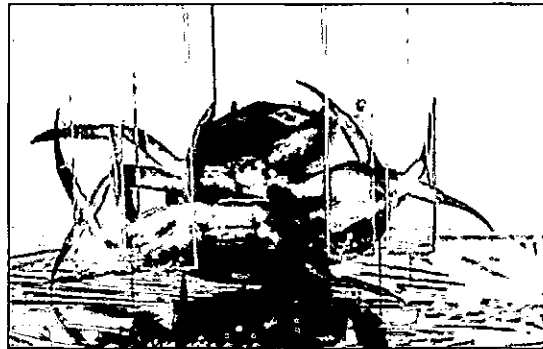
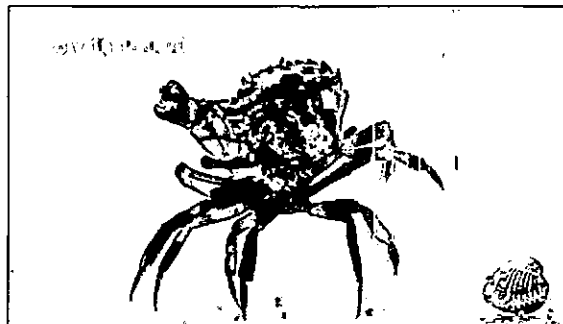


Fig. 6.2 Example of a fight where opponents use aggressive behaviours such as raising the body above the ground and use of the chelae for grasping and pinching.



6.3 RESULTS

6.3.1 Univariate

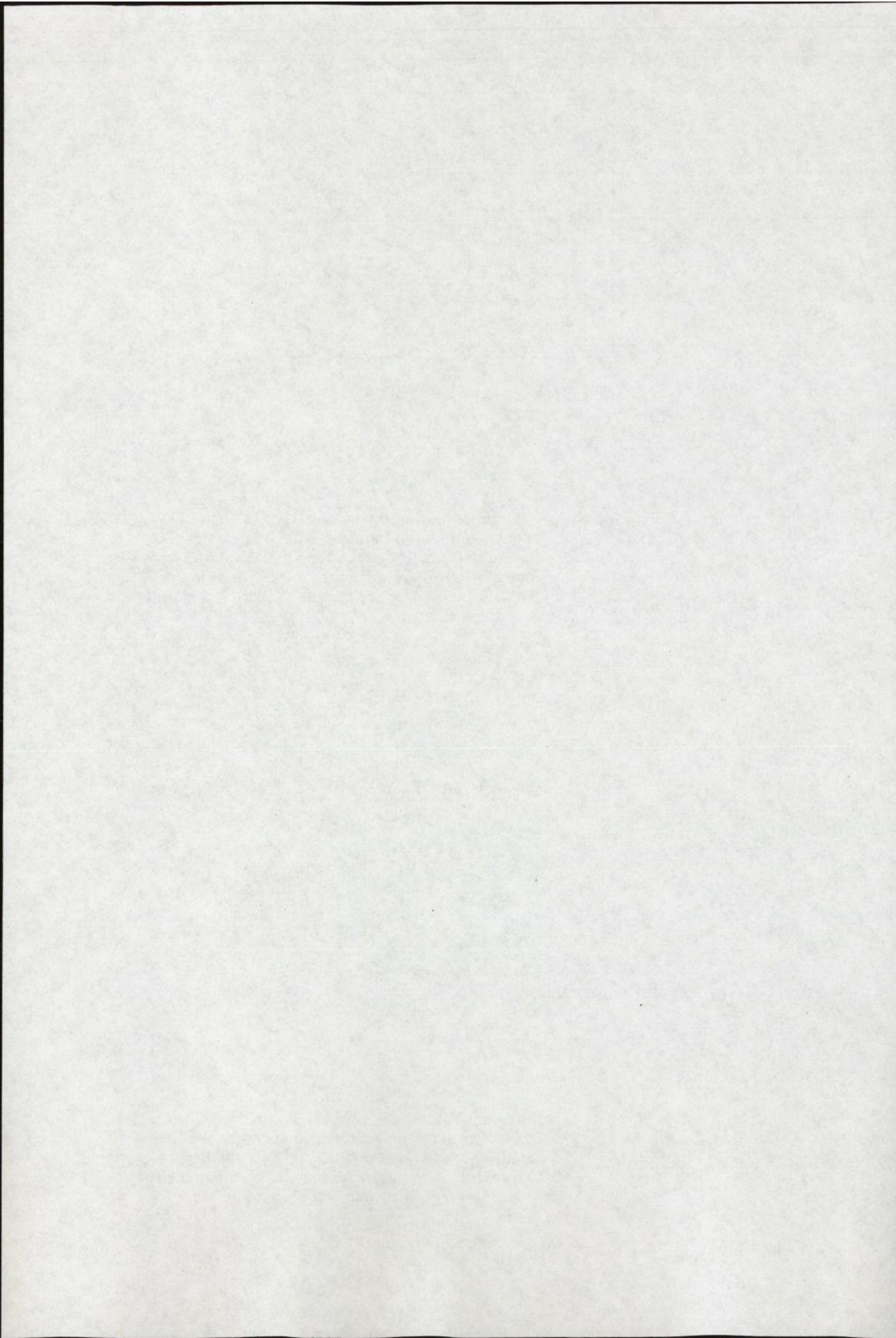
Results for biochemical (post-contest haemolymph lactate and glucose concentrations) and behavioural (intraspecific agonistic behaviours) endpoints are summarised in Tables 6.3 and 6.4, respectively.

6.3.1.1 Haemolymph analysis

Lactate concentrations did not differ significantly between unexposed and PYR-exposed crabs within the respective dietary treatments (starved, diet-restriction and fed), but there was a difference between the fed unexposed group and the starved and DR unexposed groups. For fully-fed crabs, unexposed individuals had significantly higher haemolymph lactate concentrations than PYR-exposed crabs ($F_{2,48} = 3.89$, $P < 0.05$) (Fig. 6.3). Haemolymph glucose concentrations did not differ significantly between the three dietary treatments but there was a significant difference in this measurement, due to the diet and exposure interaction, with the fed PYR-exposed group yielding lower glucose concentrations than their unexposed counterparts (22.49 ± 8.89 vs. 31.20 ± 9.42 μgL^{-1}) ($F_{2,48} = 4.23$, $P < 0.05$) (Fig. 6.4).

Table 6.3 Post-contest biochemical assessment of starved, diet-restricted (DR) and fully-fed shore crabs (mean ± 1 SE). * signifies $P < 0.05$. NS = Not Significant.

Level	Endpoints	Starved	Diet-restricted (DR)	Fed	Statistical significance	
					Intra-treatment (Diet-exposure interaction)	Inter-treatment (diet)
BIOCHEMICAL	Lactate (mmol L ⁻¹)	15.51 \pm 3.5	21.50 \pm 2.65	31.20 \pm 3.13	(Unexposed)	NS
		vs.	vs.	vs.	Starved > DR = Fed	
	23.60 \pm 3.06	27.52 \pm 3.03	22.49 \pm 2.96	*		
Glucose (μ g L ⁻¹)	29.09 \pm 1.7	28.59 \pm 3.10	34.51 \pm 4.89	*	NS	
	vs.	vs.	vs.			
	25.05 \pm 3.61	32.73 \pm 3.10	17.91 \pm 3.3			

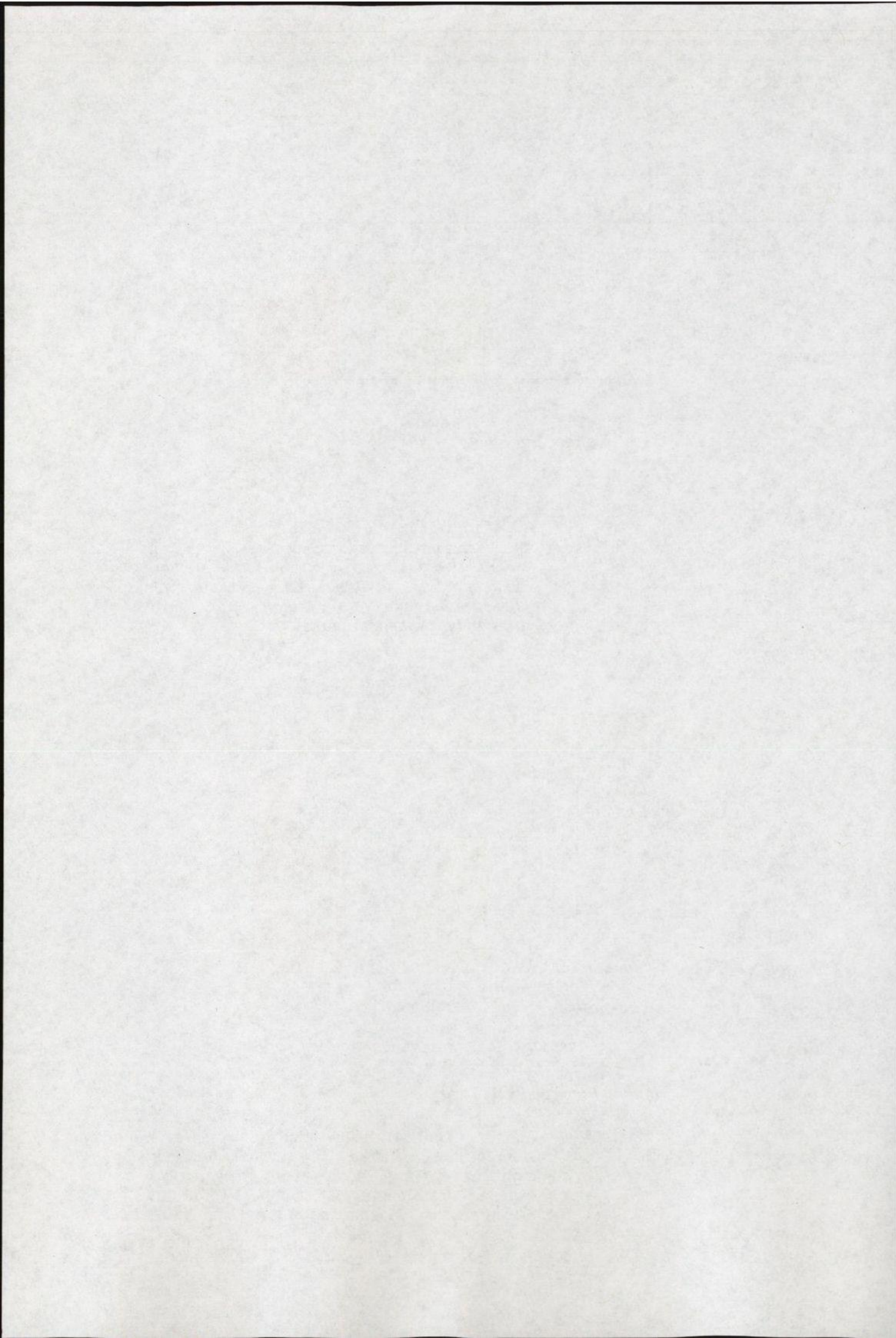


6.3.1.2 *Intraspecific contest behaviour*

Each pair of equally size-matched contestants engaged in agonistic encounters in the experimental fighting chamber. Agonistic encounters consisted of a series of behaviours (Table 6.2) ranging from cheliped displays to wrestles, subsequently escalating into fights. For all treatments, mean wrestle and fight durations ranged between 209 ± 37 s and 42 ± 13 s respectively. All results for behavioural variables are summarised in Table 6.4. Significant differences in resource possession were observed only within the starved treatment, with PYR-exposed individuals demonstrating a significantly higher resource possession than unexposed crabs (52 and 19% respectively) ($F_{1,16} = 4.53$, $P < 0.05$) (Fig. 6.5). Significant differences in recuperation time (sec) were observed only between unexposed and PYR-exposed crabs within the starved treatment. Unexposed, starved individuals took significantly longer to recuperate after fights or wrestles during agonistic encounters compared to PYR-exposed starved crabs (437.22 ± 28.23 vs. 166.44 ± 21.98 s) ($F_{2,48} = 3.76$, $P < 0.001$), (Fig. 6.6). No significant differences were observed in other agonistic behaviours including number of wrestles and fights, total contest duration and contest intensity.

Table 6.4 Intraspecific agonistic behavioural assessment of starved, diet-restricted (DR) and fully-fed shore crabs (mean \pm 1 SE). * and *** signify $P < 0.05$ and $P < 0.001$, respectively. NS = Not Significant.

Level	Endpoints	Starved	Diet-restricted (DR)	Fed	Statistical significance	
					Intra-treatment (Diet-exposure interaction)	Inter-treatment (diet)
BEHAVIOURAL	Resource possession (%)	19.44 \pm 1.19 vs. 51.89 \pm 1.20	32.78 \pm 1.20 vs. 37.67 \pm 1.53	33.89 \pm 1.04 vs. 25.56 \pm 1.17	*	NS
	Recuperation time (sec)	437.22 \pm 9.41 vs. 116.44 \pm 7.33	334.56 \pm 10.87 vs. 261.11 \pm 8.92	256.89 \pm 9.03 vs. 399.89 \pm 9.23	***	NS
	Number of recuperation times	4 \pm 0.10 vs. 1.78 \pm 0.08	3.11 \pm 0.09 vs. 3.67 \pm 0.14	4.56 \pm 0.18 vs. 3.89 \pm 0.08	NS	NS
	No. of wrestles	6 \pm 0.1	5.33 \pm 0.11	7 \pm 0.13	NS	NS
	No. of fights	2.44 \pm 0.06	2.22 \pm 0.04	1.78 \pm 0.06	NS	NS
	Total contest duration	246.67 \pm 2.3	229.32 \pm 0.9	277.25 \pm 4.8	NS	NS
	Contest intensity	0.43 \pm 0.01	0.56 \pm 0.02	0.36 \pm 0.01	NS	NS



6.3.2 *Multivariate*

Significant differences (ANOSIM pairwise tests) were identified only between contaminant (PYR) and unexposed shore crabs, with regard to the starved dietary treatment (Table 6.5). SIMPER analysis [evaluation of the percentage contribution of each endpoint (biochemical and behavioural) to the group differentiation], identified recuperation time (14.9%), resource possession (14.8%) and haemolymph lactate concentration (12%) as the three major contributing endpoints accounting for over 40% for the difference between treatment groups (Table 6.6).

Table 6.5 Results from one-way ANOSIM tests, based on euclidean distances in normalised variables from post-contest biochemical and intraspecific behavioural assessment. Significant treatment differences are highlighted in bold. *S* = Significant, *NS* = Not Significant.

TREATMENT	Pair wise tests	ANOSIM GLOBAL tests			ANOSIM pairwise tests			Significant difference
		R	P%	P value	R	P%	P value	
STARVED	Unexposed vs. PYR-exposed	0.052	6	0.06	0.21	1.1	0.011	S
DIET-RESTRICTION	Unexposed vs. PYR-exposed	0.052	6	0.06	-0.111	96	0.958	NS
FED	Unexposed vs. PYR-exposed	0.052	6	0.06	0.047	23	0.226	NS

Table 6.6 Pairwise treatment comparison of percentage contribution of each physiological variable within the starved treatment. Highest contributing variables are highlighted in bold.

Starved: Unexposed vs. PYR-exposed

Variable	Contribution (%)	Cumulative (%)
Recuperation time	14.93	14.93
Resource possession (%)	14.82	29.75
Lactate	12	41.75
Fight duration	11.59	53.33
Intensity	9.57	62.9
No. of recuperation times	9.2	72.1
No of Wrestles	8.88	80.99
Wrestle Duration	8.25	89.24
Glucose	5.99	95.23
No of Fights	4.77	100

6.4 DISCUSSION

Previous work has shown that fighting behaviour is metabolically costly for various crustacean species [*Necora puber* (Thorpe et al. 1995); *Carcinus maenas* (Sneddon et al. 1999) and *Pagurus bernhardus* (Briffa and Elwood 2004)]. For example, accumulation of the products of anaerobic respiration, such as lactate, result in reduced ability to persist in agonistic encounters and faster onset of exhaustion (Thorpe et al. 1995). In the current study, lactate concentrations were not measured from inactive crabs but previous studies have shown that lactate levels from crabs at rest range from negligible to 1.5 mmolL^{-1} . In the present study, post-contest lactate levels were high ($15\text{-}40 \text{ mmolL}^{-1}$), indicating that metabolic energy demands were met by anaerobic respiration, via the breakdown of pyruvate to lactate (Hames and Hooper 2000; Sneddon et al. 1999; Thorpe et al. 1995). Lactate is a harmful by-product of anaerobic respiration (arising under oxygen-limiting circumstances such as muscle contraction) (Hames and Hooper 2000) and has been used as an index of metabolic cost during aerobic activity of animals (particularly with a limited capacity for aerobic respiration) (Balogh et al. 2001; Matsumasa and Murai 2005). The costs of engaging in agonistic behaviour results in increased lactate levels and is proposed to impose a limit upon contest duration (Sneddon et al. 1998).

In the present study, accumulation of lactate was higher in unexposed fed crabs compared to unexposed starved and diet-restricted (DR) crabs. High lactate levels, coupled with increased concentration of free-circulating glucose concentrations, measured also in the haemolymph, implies an imposed proximate cost for energetic behaviour. Pyrene-exposed fed shore crabs showed no significant difference in levels of lactate post-contest, but unexposed

fed crabs had significantly higher glucose levels, suggesting that more energy was available to unexposed fed individuals as opposed to their PYR-exposed counterparts. It is known that for *N. puber*, and *C. maenas*, elevated glucose levels are observed in response to fighting (Sneddon et al. 1999; Thorpe et al. 1995). Also, it has been proposed that L-lactate levels influence behavioural decisions in contest behaviour such as decrease in shell-rapping behaviour and the decision to desist from contest behaviour, as seen in hermit crabs (Briffa and Elwood 2004).

The imposed 'physiological state' of the experimental crabs has an implication to the amount of energy reserves and, subsequently, the energy available for an energetically-demanding activity such as contest behaviour. Fed individuals can afford to 'cope' with higher L-lactate levels as well as mobilising glucose from energy reserves. Lactate is a metabolic substance that is produced at all times (even at rest), both due to oxygen availability and unavailability (Myers and Ashley 1997). In energy-demanding situations (e.g. agonistic contest behaviour), adenosine triphosphate (ATP) is catabolised to ADP and subsequently to AMP in the muscle fibres. This process is dependent upon glycolysis to ultimately generate ATP (Balogh et al. 2001). A metabolic change (within the cell) arises to enable full mitochondrial function to occur. Increase in the NADH/NAD⁺ (nicotinamide adenine dinucleotide) ratio (i.e. redox potential), which is transmitted to the cytosol whereby the lactate/pyruvate ratio is driven towards increased lactate production (Myers and Ashley 1997) in order to maintain the regeneration of NAD⁺ thereby allowing for the production of ATP via glycolysis (Hames and Hooper 2000).

The difference found here whereby fed crabs had higher concentrations of circulating glucose and lactate compared to starved or diet-restricted crabs, can be postulated to have arisen due to their higher energy reserves (in comparison to starved and diet-restricted individuals), thereby, increased glycolysis rate resulting in concomitant increased glucose and lactate production. This reasoning could explain why fed individuals were observed to 'cope' with higher L-lactate levels. In comparison, starved crabs, cannot cope with the high lactate levels, due to the lack of energy reserves and, hence have lower L-lactate levels, compared to fed and starved crabs. It has been suggested that there are few behavioural differences, and similar metabolite levels, between eventual contest winners and losers (Thorpe et al. 1995). The combination, however, of metabolite profiles may not be the sole reason influencing behavioural decisions to end encounters.

There are three traits that are inherently modelled in contest behaviour: resource holding potential (RHP); relative resource value (V) and aggressiveness (Hurd 2006). RHP is the ability to win a contest (Maynard Smith 1982) through inherent phenotypic (e.g. body size) and physiological attributes (e.g. energy reserves). Resource value or motivation is related to the value of the disputed resource (Sneddon et al. 1997a), such as food (foraging individuals) or females (reproductively active males). Aggressiveness is related to the individual's tendency to escalate a contest independently of both RHP and resource value (Hurd 2006). In symmetrical contests where RHP and resource value are matched, aggressiveness may be a trait that influences escalation decisions in agonistic encounters.

In this study, no differences were observed in contest duration, contest intensity and wrestle or fight duration, suggesting that crabs, irrespective of energy

reserves, engaged in intraspecific agonistic behaviour with comparable vigour or intensity. Sneddon et al. (1999) reported that shorter contests were observed in shore crabs that had been subjected to hypoxic conditions. De-escalation of fights to wrestles, (to a less energetically costly behaviour) is proposed by these authors to occur due to metabolic costs of agonistic encounters outweighing any possible benefits. Possible explanations for the discrepancy between earlier observations (Sneddon et al. 1999) and findings here may be explained by resource motivation. In the present study, resource motivation was imposed upon size-matched conspecifics by introduction of a food resource (whole cockle). Staged contests therefore presented contestants with motivation to engage in dyadic contests conferring a greater perceived benefit than cost. Contests often begin with wrestles escalating into fights (Sneddon et al. 1997a), contest de-escalation (from fights to wrestles) as witnessed by Sneddon et al. (1997a) can be explained by the lack of resource value in staged contests, thereby influencing an individual's motivation to pursue or evade agonistic encounters. In this current study, phenotypic correlates of RHP, such as body (carapace width) and weapon (cheliped) size were fixed; shore crab physiological condition was manipulated via a combination of dietary and contaminant exposure. Via autophagy (see Chapter 5), diet-restricted individuals are subjected to a survival strategy, whereby breakdown of long-lived proteins occurs allowing recycling of products into protein synthesis and energy-production pathways (Moore et al. 2006). Autophagy allows cells to be self-sustaining during nutrition-deprived conditions (Finn and Dice 2006; Yoshimori 2004). Under these conditions, resource value (and thus motivation) would be higher in starved crabs. This perceived increase in resource value could explain the significant difference in resource possession and recuperation time between the unexposed and PYR-exposed starved treatments. This interpretation corroborates predictions made by several game theory models that, as resource

value increases, behaviour during contests is related to the value of the disputed resource (in this case food) (Sneddon et al. 1997a). Corroborative evidence by Gherardi (2006) showed that resource value (i.e. gastropod shell quality) in the hermit crab *Pagurus longicarpus* was imperative to contest behaviour in this species. A hermit crab will initiate an attack upon a rival based upon information about its own shell quality (if its own shell is damaged or too small). Gherardi (2006) suggested that contest behaviour is dependent upon resource value.

Game theory models and experimental evidence suggest that strategies in agonistic encounters are determined by an individual's physiological condition. Manipulation of an individual's state, therefore, should elicit behavioural differences (Sneddon et al. 1999). The present study shows that the resource holding potential (RHP), and associated energetic consequences of agonistic encounters, was dependent on shore crab physiological condition. Physiological condition therefore determines the energy available to the shore crab for energetically-demanding activities (such as intraspecific agonistic behaviour) as shown here by higher lactate levels in fed crabs compared to starved crabs.

In conclusion, experimental manipulation of shore crab physiological condition (via a combination of dietary and sublethal contaminant exposure) has shown that, under nutrient-deprived conditions (starved crabs), resource holding potential (RHP) evaluated using the ecologically important intraspecific agonistic behaviour in this species was higher in pyrene-exposed crabs compared to unexposed crabs. Shore crab physiological condition, however, was not the sole reason accounting for the differences in behaviour, as resource value (evaluated here in the presence of a whole cockle) is concluded to influence an individual's motivation to enter agonistic encounters.

CHAPTER 7:

Does the physiology of *Carcinus maenas* vary 'seasonally' and do 'seasonal' physiological differences confer physiological tolerance to contaminant exposure?

Does the physiology of *Carcinus maenas* vary 'seasonally' and do 'seasonal' physiological differences confer physiological tolerance to contaminant exposure?

ABSTRACT

Environmental temperature has a direct influence upon poikilothermic organismal physiology. Knowing the full range of temperature-mediated metabolic and physiological abilities of an organism will contribute to our understanding of specific contaminant-imposed constraints upon organism physiological condition. The aims of this study were to a) outline the seasonal differences in shore crab physiology, and b) to evaluate the seasonal differences in physiological condition of crabs from estuaries [Avon and Yealm (low), Plym (high)] with relatively differential anthropogenic input (defined here as maritime activity and indicated by PAH metabolite levels). A seasonal difference in shore crab physiology (antioxidant status and immune function) was identified with physiological condition differing between winter-spring compared to summer-autumn periods in crabs from the Avon Estuary; these differences are taken to represent the 'normal' variability in shore crab physiology. Seasonal evaluation of shore crab physiological condition from estuaries of varying PAH input revealed significant seasonal differences between crabs as signalled by cellular endpoints [cellular integrity and viability ($P < 0.05$)]. The integrated response of shore crab physiological condition (multivariate) identified differences in physiological condition between Avon crabs (low anthropogenic exposure) and Plym crabs (relatively high anthropogenic exposure) between January and June compared to July to December. In conclusion, to aid understanding of the potential contaminant impacts on *C. maenas* physiology, it is imperative that, firstly, the natural temporal variability in organism physiological ranges is established by defining the 'normal' seasonal pattern in shore crab physiological condition and then contaminant effects upon physiology can be outlined.

7.1 INTRODUCTION

Environmental temperature is a major limiting factor in organism physiological function and is linked to climate (Davenport and Davenport 2005). For aquatic poikilothermic organisms, a change in environmental temperature results often in a direct change in the rate of all physiological and biochemical processes (Hochachka and Somero 2002); however, extremes are mitigated by temperature-mediated metabolic adjustments, leading to effects upon metabolic rates, locomotory and behavioural activity (Hochachka and Somero 2002; Sokolova and Portner 2003; Spicer and Gaston 1999).

Crustaceans have different physiological tolerances to abiotic factors such as hypoxia (Bernatis et al. 2007; Legeay and Massabuau 2000; Spicer and Baden 2001), salinity (Aagaard 1996; Jones 1981; Rainbow 1997; Rainbow and Black 2001, 2002; Roast et al. 1999a) and temperature (Hawkins et al. 1982; Stillman and Somero 1996; Styriehave et al. 1999; Truchot 1975). Many crustaceans, for example *Carcinus maenas*, are sensitive to abiotic factors (e.g. temperature) (Camus et al. 2004) but seasonal acclimatisation confers environmental modification of an individual's physiological processes (Spicer and Gaston 1999). The annual sea temperatures which shore crabs experience around British coastal waters ranges from 4°C (January to March) to 19°C (July to September) (Robertson et al. 2002). These temperature variations in temperate waters have important implications for shore crab physiology and behaviour, from cardiac activity to foraging behaviour (Table 7.1).

Table 7.1 Temperature-related effects upon aspects of shore crab *Carcinus maenas* physiology and ecology.

PARAMETER	WINTER - SPRING	SUMMER - AUTUMN	REFERENCE
Abundance in estuaries	Low	High	(Naylor 1962) (Crothers 1967) (Hunter and Naylor 1993) (Attrill and Thomas 1996)
Foraging activity	Low	High	(Aagaard et al. 1995) (Styrishave et al. 1999)
Cardiac activity	Low	High	(Aagaard 1996) (Camus et al. 2004) (Depledge 1985; 1992) (Styrishave et al. 1999)
Hepatopancreas fatty acid profile	Low	High	(Styrishave and Andersen 2000)

Although the differences in various aspects of shore crab physiology are primarily temperature related, they are also linked with the seasonal lifecycle of male and female shore crabs (Tables 7.2 and 7.3). Table 7.2 outlines the life cycle of a typical individual shore crab from copulation in summer to larval development over winter and spring. Adult male shore crabs, however, move offshore (>3m) during winter (December - February) and return to shallower waters as ambient temperature increases in spring (March - June) (Styrishave et al. 1999) (Table 7.3). Increases in water temperature leads to increased metabolic activity, resulting in concomitant increases in locomotory and foraging activity (Aagaard et al. 1995; Styrishave et al. 1999). Seasonal patterns are summarised as temporal variations *"usually responses to particular cues that tend to coincide with time of year. If such cues do not arise in any particular year, the response will not be elicited"* (Crowe 1999). Our lack of knowledge of the 'normal' seasonal physiological ranges has prevented the understanding of the associated effects of environmental change or fluctuation upon organism physiology. The aims of this present study were therefore to a) evaluate seasonality in shore crab physiology, and b) to evaluate seasonal differences in shore crab physiological condition in estuaries of differential anthropogenic input (defined here as maritime activity and assessed by screening for urinary PAH metabolites as an indicator of PAH exposure).

Table 7.2

General life cycle of *Carcinus maenas* [compiled from Crothers (1967)].

Step	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1							1a. Copulation			1b. Ovigerous females move offshore		
2	Berried females/ Eggs											
3				3a. Zoea								
4						4a. Megalopae			4b. Overwintering zoea			
5	4b. Overwintering zoea						5a. Young crab settlement			5b. Puberty		
6				Late settlers reach puberty								

Key

- 1a Copulation occurs during summer months; female is only receptive after moulting
 1b Ovigerous females move offshore
 2 Berried females move offshore; 'egg plug is cleaned and ventilated by the female, eggs turn bright orange to dull brown to grey
 3a Zoea appear in the plankton
 4a Megalopae settle onto the benthos
 4b Late developing larvae that do not metamorphose will settle in small number over the winter or overwinter to settle in the forthcoming spring
 5a Young crabs settlement onto the benthos
 5b Young crabs reach puberty; females quicker than males in autumn in the first year,
 6 Late settlers reach puberty in following spring.

Table 7.3 Seasonal lifecycle of adult *Carcinus maenas* [compiled from Crothers (1967) and Naylor (1962)].

Step	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1										Movement		
2	offshore'											
3	Anecdysis											
4	4a. Movement onshore								4b. Copulation			
5												

Key

- 1 Movement offshore due to environmental conditions; only specimens > 35 mm carapace width
- 2 Movement 'offshore' due to environmental conditions ('offshore' = > 3m depth)
- 3 Moulting cessation during the coldest months of the year
- 4a Increase in temperature initiates movements onshore
- 4b Breeding period begins with precopula pairing with female, ending with copulation in the late summer months

7.2 MATERIAL AND METHODS

7.2.1 Shore crab collection

Male (green) intermoult *C. maenas* were collected from three estuaries of varying anthropogenic input (Table 7.4, Fig. 7.1). Mackerel-baited cages were deployed two hours prior to high tide and collected at the predicted time of high tide. A maximum mean number of three adult male shore crabs ($\geq 60\text{mm CW}$) cage^{-1} (Fig. 7.2) were sampled for analysis of physiological condition. Crabs were transported to the laboratory (10 - 45 min) in cooler boxes with damp absorbent paper.

Table 7.4 Relative anthropogenic usage (defined as maritime activity) of three estuaries used to evaluate shore crab physiological condition (Avon, Yealm, and Plym), South Devon, U.K (grid references: SX 666 438, SX 540 480 and SX 483 538, respectively).

Location	Maritime activity (Boats/Ships year ⁻¹)	Reference
Avon Estuary	< 20	(QHM 2006c)
Yealm Estuary	< 250	(QHM 2006a)
Plym Estuary	> 1500	(QHM 2006b)



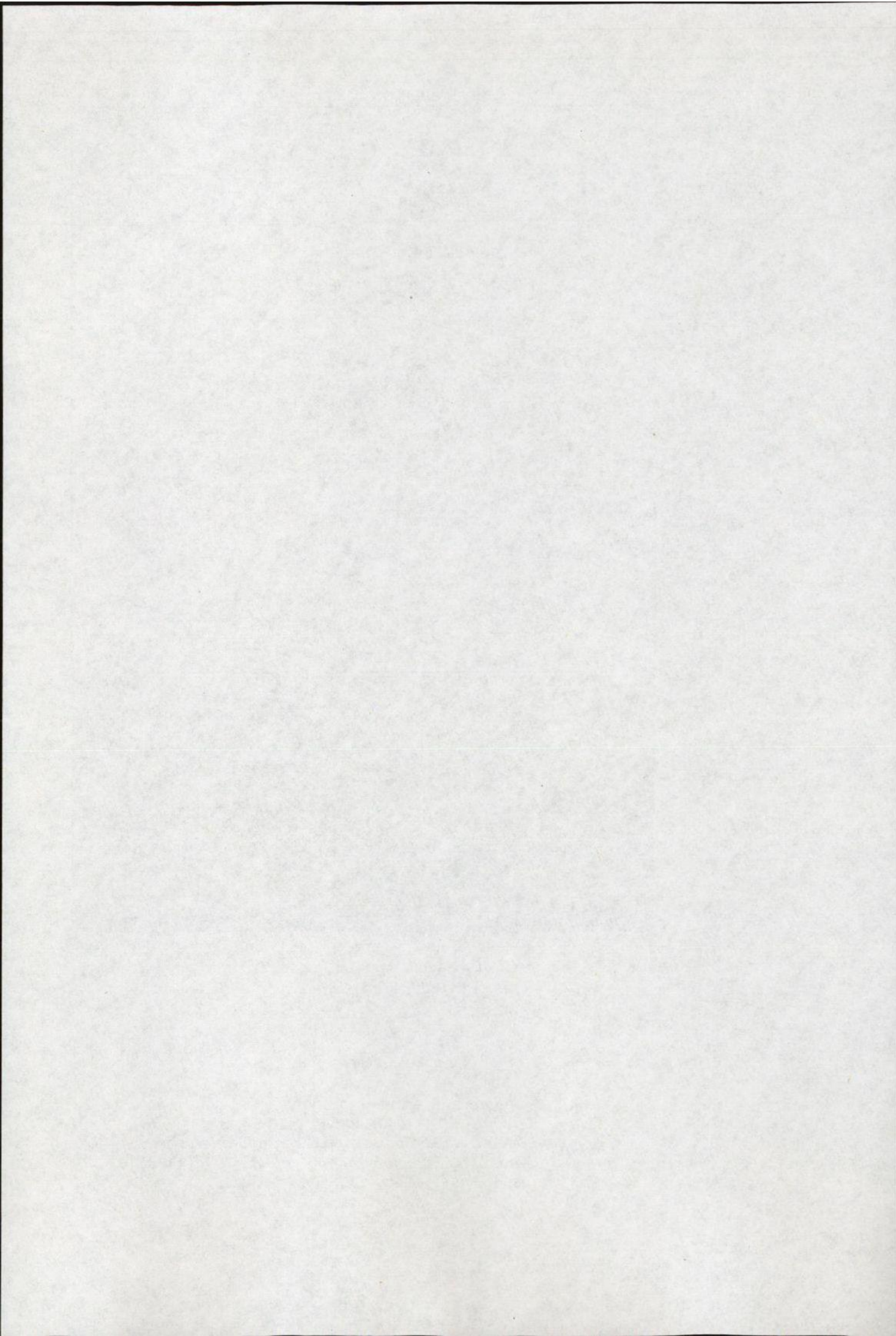
KEY

A = Plym Estuary

B = Yealm Estuary

C = Avon Estuary

Fig. 7.1 Map depicting estuary locations within southwest England (U.K) (Avon, Yealm and Plym Estuaries), South Devon, U.K (grid references: SX 666 438, SX 540 480 and SX 483 538, respectively) (Google 2007).



7.2.2 Seasonal sampling

Crabs were collected throughout the year (2006), as follows: season 1 ("winter" = January– March), season 2 ("spring" = April – June), season 3 ("summer" = July – September), season 4 ("autumn" = October – December). Sampling times were replicated temporally within each season with three sampling time points within each season (Fig. 7.2). Table 7.5 shows the mean seawater temperature and rainfall data common for all three estuaries sampled.

7.2.2.1 Physiological assessment

a) Sample collection

Within 24 h of collection, urine was extracted from each crab (as described in Chapter 2, Section 2.3.1) and stored for analysis. Within 24 - 48 h, haemolymph (500µl) was collected from each crab and analysed immediately using the live cell assays (see Section 7.2.2.1b). Remaining haemolymph was snap-frozen using liquid nitrogen and stored at -80°C for analysis of antioxidant status (as described in Chapter 2, Section 2.4.5). Following urine and haemolymph sampling, shore crabs from the three different estuaries were maintained in separate holding tanks containing filtered (10µm carbon-filtered) aerated seawater (34psu, 15 ± 1°C) under a 12h light : 12 dark photoperiod, until released back to the appropriate estuary. The feeding history of caught shore crabs was not known and, therefore, they were not fed at any time during collection or handling to not influence nutritional status.

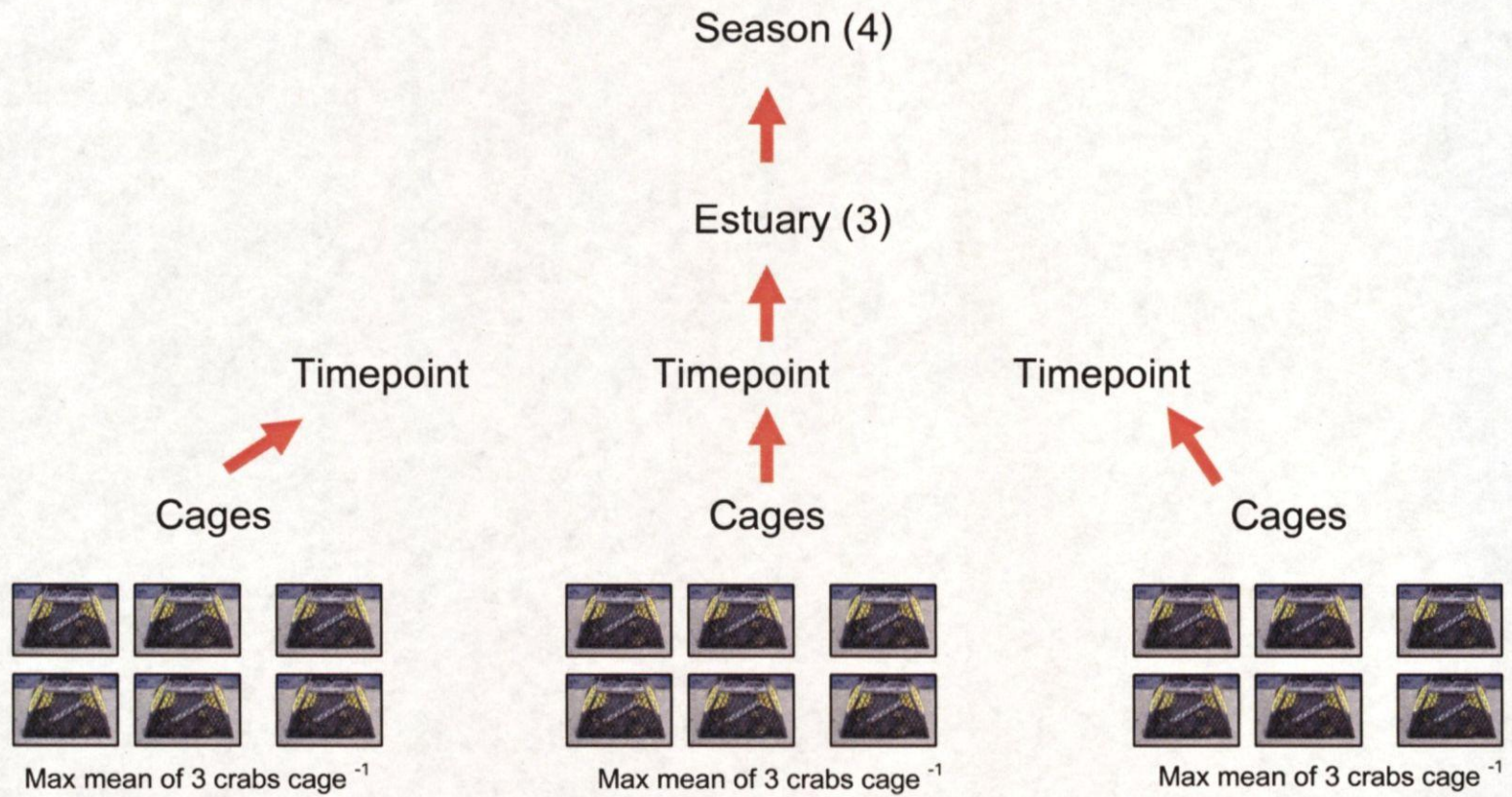


Fig. 7.2 Experimental procedure for seasonal collection of shore crabs. Sampling times were temporally replicated ($n=3$) at each estuary over the four seasons.

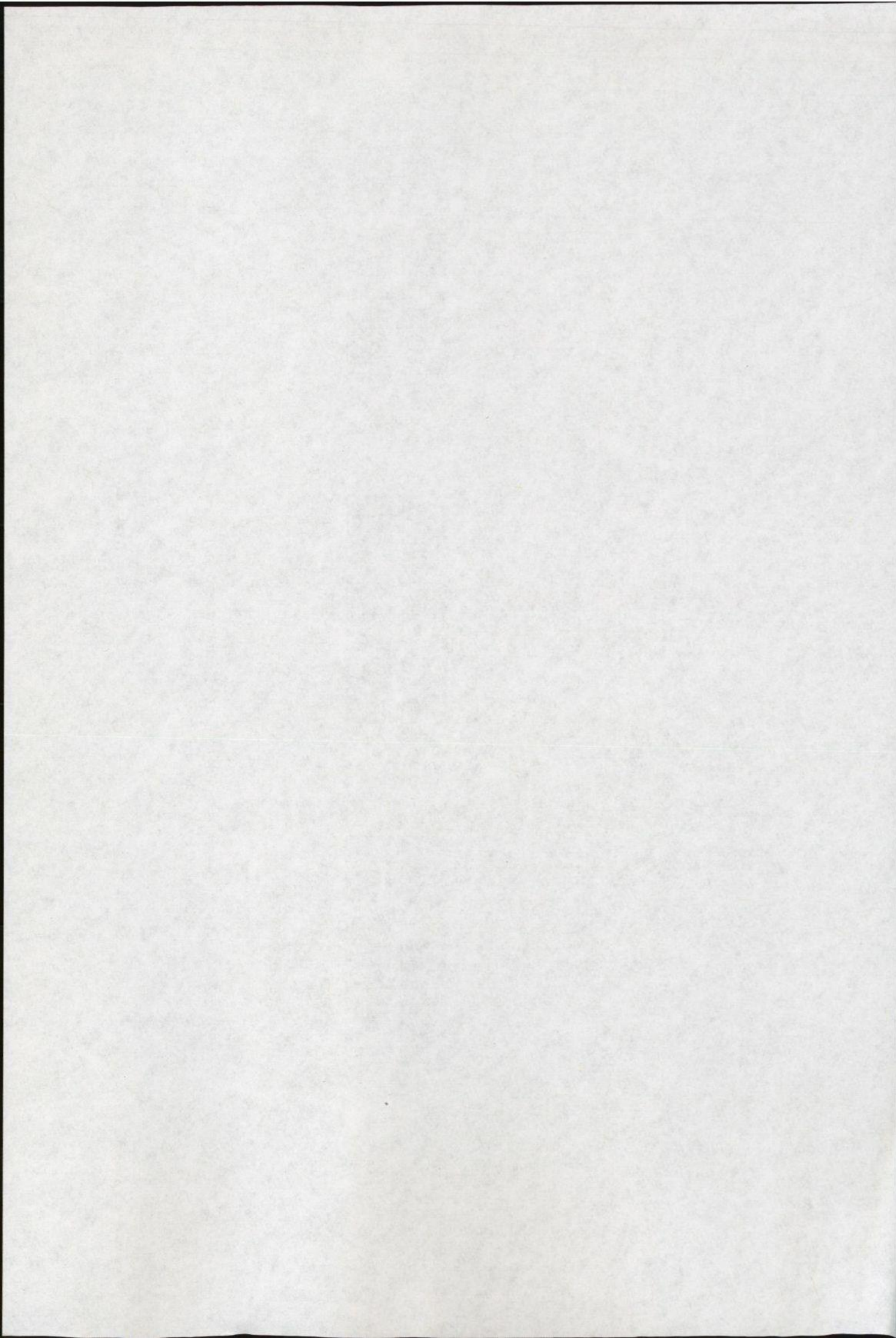


Table 7.5 Monthly precipitation and mean seawater temperatures in Southwest U.K. (2006). Precipitation data compiled from the Met Office (2007).

Season	Month	Mean seawater temperature ± 1 SD ($^{\circ}\text{C}$)	Precipitation (mm)	Mean seasonal precipitation ± 1 SD (mm)
1	JAN		32.7	60.23 ± 28.77
	FEB	7.5 ± 0.24	57.9	
	MAR		90.1	
2	APR		45.3	60.53 ± 48.58
	MAY	13.7 ± 0.37	114.9	
	JUN		21.4	
3	JUL		41.3	70.13 ± 25.01
	AUG	16.9 ± 0.33	85.9	
	SEP		83.2	
4	OCT		115.9	115.2 ± 0.96
	NOV	14.7 ± 1.66	115.6	
	DEC		114.1	

b) *Sample analysis*

Carcinus haemolymph samples collected within 24 – 48 h were analysed using both live cell (cellular integrity, cellular viability and phagocytosis) and cold storage techniques (total antioxidant status) (as described in Chapter 2, Sections 2.4.6 – 2.4.10). Urine samples were analysed for the presence of PAH metabolites with fluorescence spectrophotometry, using excitation and emission wavelength pairs for both pyrene (PYR) (345/382nm) and benzo[a]pyrene (BaP) (380/430nm) (see Chapter 2, Sections 2.3.2 -2.3.3).

7.2.3 *Statistical analysis*

Two methods of statistical analyses were used to differentiate between a) individual variables (univariate), and b) shore crab physiological condition (measured here by the integrated response of all variables) (multivariate). Univariate (ANOVA) and multivariate methodologies (ANOSIM and SIMPER) are described fully in Chapter 2, Section 2.6.

7.3 RESULTS

7.3.1 Seasonality of shore crab physiology (Avon Estuary)

7.3.1.1 Physiological endpoints

Significant seasonal differences ($P < 0.05$) in shore crab physiological variables were identified (Table 7.6). Increased immune function, signalled by a greater phagocytic index, was found in seasons 1 and 2 (winter and spring months), compared to seasons 3 and 4 (summer and autumn months) (ANOVA, $F_{3,8} = 12.71$, $P < 0.01$) (Fig. 7.3). Total antioxidant status was lower between January - March (season 1), compared to all other seasons (ANOVA, $F_{3,8} = 8.18$, $P < 0.01$) (Fig. 7.4). Presence of urinary PAH metabolites (PYR & BaP) was lower during seasons 1 and 2 (January - June) compared with seasons 3 and 4 (July - Dec) as observed with BaP metabolites (ANOVA, $F_{3,8} = 10.17$, $P < 0.01$) (Fig. 7.5). PYR metabolites, however, were higher only between October to December (season 4) compared to April - June (season 2) (ANOVA, $F_{3,8} = 4.57$, $P < 0.05$) (Fig. 7.5).

Table 7.6 Univariate summary of seasonal differences in physiological variables from shore crabs sampled from the Avon Estuary. Data are presented as means \pm 1 standard deviation. *NS* = Not Significant, *S* = Significant difference ($P < 0.05$).

<u>Variables</u>	Season 1 (January – March)	Season 2 (April - June)	Season 3 (July – September)	Season 4 (October – December)	Statistical differences	Seasonal differences
Carapace Width (mm)	53.97 \pm 12.77	66.15 \pm 3.53	60.74 \pm 4.34	56.01 \pm 2.94	<i>NS</i>	-
Wet weight (g)	47.75 \pm 12.28	68.36 \pm 11.65	53.97 \pm 9.79	41.71 \pm 6.85	<i>NS</i>	-
Cell Integrity (%)	81.08 \pm 18.98	85.08 \pm 5.14	89.64 \pm 1.26	86.39 \pm 2.46	<i>NS</i>	-
Cell Viability (O.D mg protein ⁻¹)	0.38 \pm 0.27	0.42 \pm 0.20	0.56 \pm 0.12	0.50 \pm 0.08	<i>NS</i>	-
Phagocytosis (zymosan particles 10 ⁷ mg protein ⁻¹)	1.86 \pm 0.78	1.73 \pm 0.41	0.93 \pm 0	0.90 \pm 0.05	<i>S</i>	1 & 2 > 3 & 4
Total antioxidant status ($\mu\text{M L}^{-1}$)	151.19 \pm 56.93	261.89 \pm 51.38	274.06 \pm 32.80	332.30 \pm 37.83	<i>S</i>	1 < 2 = 3 = 4
PYR metabolites (F.U.)	18.54 \pm 12.29	13.18 \pm 14.52	53.67 \pm 16.33	47.88 \pm 11.14	<i>S</i>	2 < 4
BaP metabolites (F.U.)	99.81 \pm 30.34	62.57 \pm 66.31	401.12 \pm 66.06	296.80 \pm 64.34	<i>S</i>	1 = 2 < 3 = 4

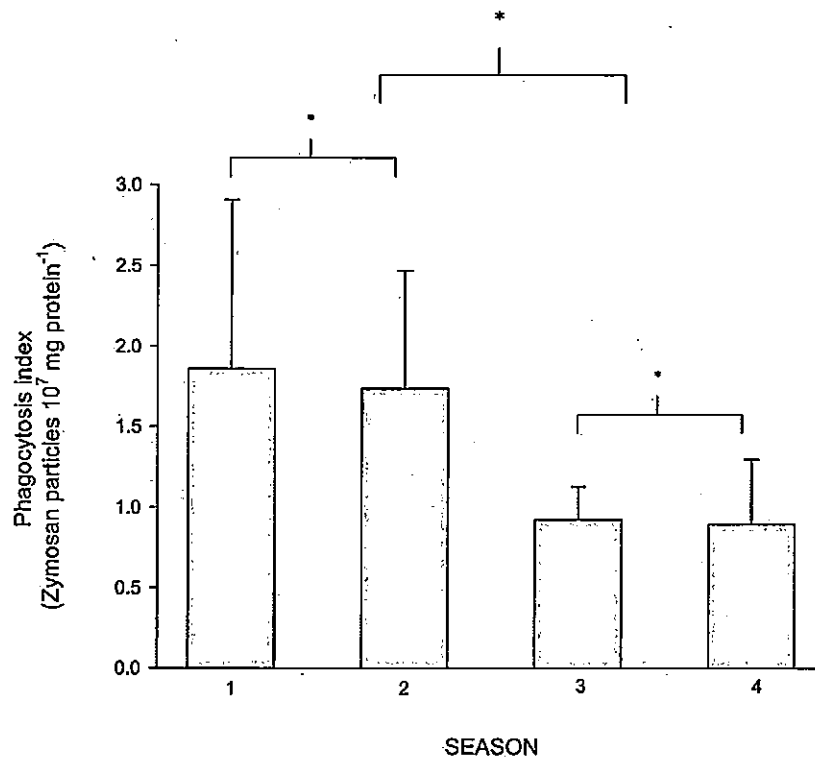


Fig. 7.3 Seasonal levels of phagocytosis index from assessed in shore crab haemocytes. ($n = 3$ timepoints). * signifies $P < 0.05$. Error bars signify ± 1 standard deviation.

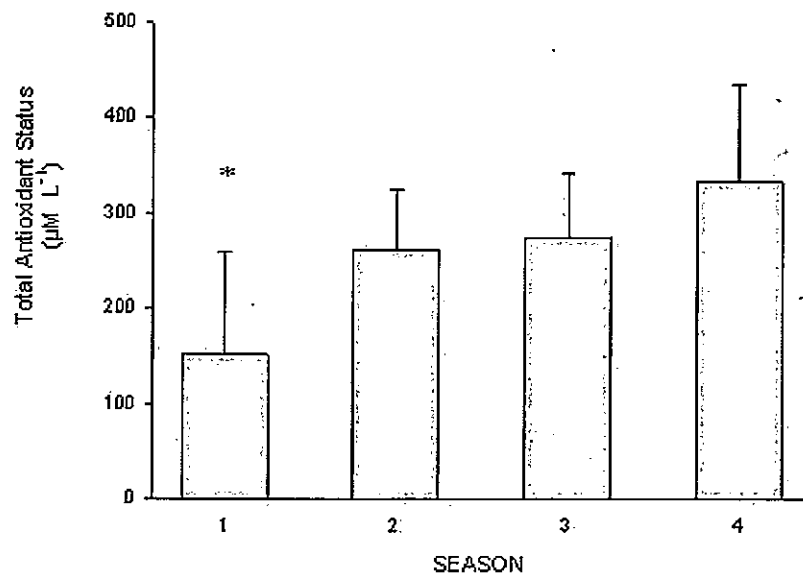


Fig. 7.4 Seasonal levels of total antioxidant status assessed in shore crab haemocytes. ($n = 3$ timepoints). * signifies $P < 0.05$. Error bars signify ± 1 standard deviation.

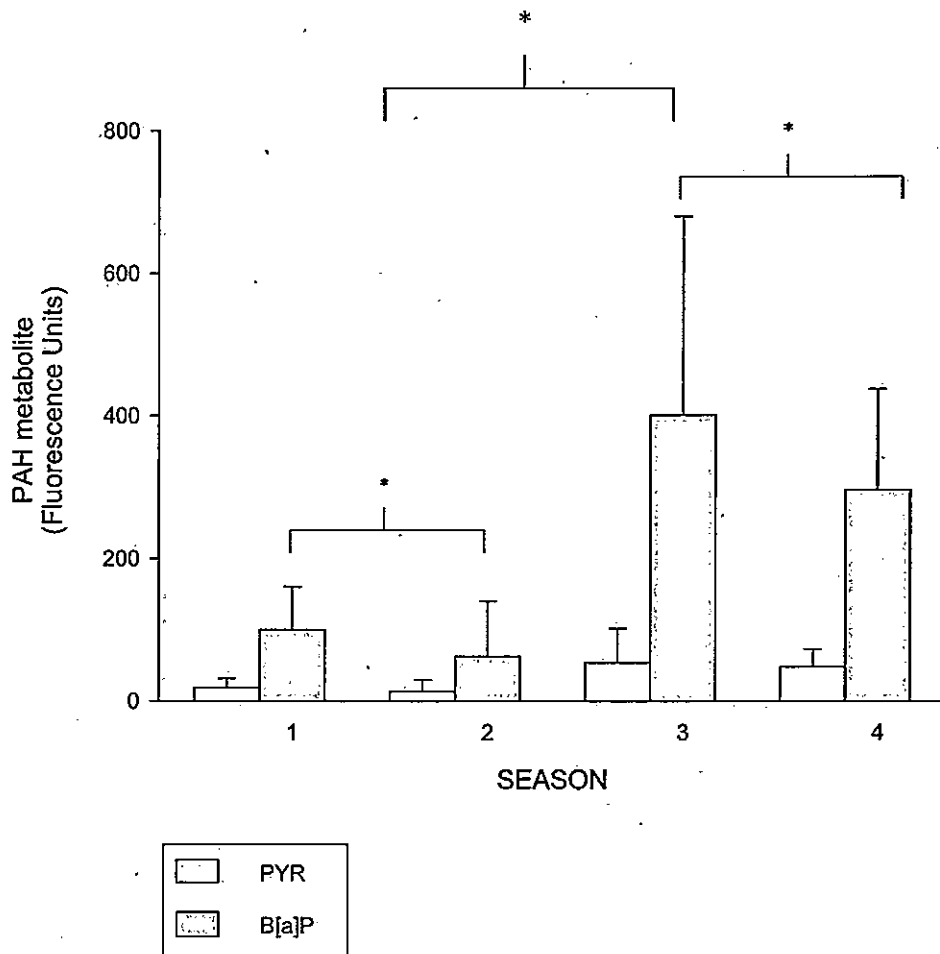


Fig. 7.5 Seasonal levels of PAH (PYR & B[a]P) urinary metabolites assessed in shore crabs. ($n = 3$ time points). * signifies $P < 0.05$. Error bars signify ± 1 standard deviation.

7.3.1.2 Shore crab physiological condition

Significant seasonal differences in *C. maenas* physiological condition (using the combination of several variables: shore crab wet weight, cellular integrity, cellular viability, phagocytic index and total antioxidant status) and environmental variables (PAH metabolites) were revealed by multivariate analyses (ANOSIM tests) (Table 7.7). Significant seasonal differences, with regard to all physiological variables, were observed early in the year compared with later in the year; 1 and 3 (winter-summer), 1 and 4 (winter-autumn), and 2 and 4 (spring-autumn) (Table 7.7). These results indicate that shore crab physiological condition varies seasonally with differences occurring between the months of January – June and July – December 2006.

Significant seasonal differences ($P < 0.05$) in PAH metabolites followed the same pattern as described for shore crab physiological condition, with higher concentrations of PAH metabolites in summer and autumn (seasons 3 and 4) compared with winter and spring (seasons 1 and 2) (Table 7.7). There were no significant correlations between seasonal shore crab physiological state and PAH exposure [season 1 (0.428), season 2 (0.027), season 3 (-0.064) and season 4 (0.146)].

SIMPER analysis identified the percentage contribution of each physiological variable to shore crab physiological condition within each season (Table 7.8). These results show that (of the variables measured here) each physiological endpoint was the significant driving variable to the observed differential shore crab physiological state within each season [s1: cell integrity (<38%); s2: phagocytosis (<32%); s3: cellular viability (<52%) and s4: antioxidant status (<38%)] (Table 7.8).

Table 7.7 Pairwise seasonal comparisons using physiological and environmental (PAH metabolites) variables. *NS* = Not Significant, *S* = Significant ($P < 1\%$).

Seasonal pairwise comparisons			Variables	
			Physiological (All variables)	Environmental (PAH metabolites)
s1	vs.	s2	<i>NS</i>	<i>NS</i>
s1	vs.	s3	<i>S</i>	<i>S</i>
s1	vs.	s4	<i>S</i>	<i>S</i>
s2	vs.	s3	<i>NS</i>	<i>S</i>
s2	vs.	s4	<i>S</i>	<i>S</i>
s3	vs.	s4	<i>NS</i>	<i>NS</i>

Table 7.8 Percentage contribution of each physiological variable within each season (assessed in Avon Estuary shore crabs): Highest contributing variables within each season are highlighted in bold.

Season 1 (January – March)		
Variable	Contribution (%)	Cumulative (%)
Antioxidant status	10.57	10.57
WW (g)	16.13	26.69
Phagocytosis	17.73	44.43
Cell Viability	17.8	62.23
Cell Integrity	37.77	100

Season 2 (April – June)		
Variable	Contribution (%)	Cumulative (%)
Cell Integrity	8.87	8.87
Antioxidant status	12.33	21.2
WW (g)	20.3	41.5
Cell Viability	26.98	68.48
Phagocytosis	31.52	100

Season 3 (July- September)		
Variable	Contribution (%)	Cumulative (%)
Cell Integrity	2.36	2.36
Phagocytosis	3.77	6.14
WW (g)	19.33	25.47
Antioxidant status	22.71	48.18
Cell Viability	51.82	100

Season 4 (October – December)		
Variable	Contribution (%)	Cumulative (%)
Cell Integrity	4.29	4.29
Phagocytosis	10.56	14.86
Cell Viability	23.05	37.91
WW (g)	24.49	62.39
Antioxidant status	37.61	100

7.3.2 Seasonal differences in physiological condition of shore crabs from estuaries of varying anthropogenic input

7.3.2.1 Physiological endpoints

Significant seasonal differences ($P < 0.05$) in shore crab physiology were observed in several physiological variables between estuaries of varying anthropogenic input (Table 7.9). Between January - March (season 1), increased benzo[a]pyrene metabolites were found in Plym and Yealm shore crabs compared to Avon crabs (ANOVA, $F_{2,6} = 8.52$, $P < 0.05$) (Table 7.9a and Fig. 7.9). No differences between Avon, Yealm or Plym crabs were observed between April and June (season 2) (Table 7.9b). Between July and August (season 3), however, Avon crabs displayed increased cellular viability compared to Plym and Yealm crabs (ANOVA, $F_{2,6} = 6.85$, $P < 0.05$) (Table 7.9c, Fig 7.10). During October to December (season 4), crabs sampled from the Yealm Estuary were significantly larger (ANOVA, $F_{2,6} = 6.45$, $P < 0.05$) and heavier (ANOVA, $F_{2,6} = 6.91$, $P < 0.05$) than those from the Avon and Plym (Table 7.9d); however, Plym crabs displayed higher cellular viability compared to crabs from the Avon and Yealm (Table 7.9d and Fig 7.9). Correlation analysis between cellular viability and PAH metabolites from Plym crabs (PYR and B[a]P metabolites) revealed modest correlations at only seasons 2 (0.43 and 0.54, for PYR and B[a]P metabolites, respectively) and season 4 (-0.34 and 0.59), for PYR and B[a]P metabolites, respectively), thereby, indicating that PAH exposure was not solely accountable for effects measured at the cellular level.

Table 7.9

Univariate summary of seasonal differences in physiological variables between estuaries of varying anthropogenic input. Data are presented as means \pm 1 standard deviation. *NS* = Not significant, *S* = Significant difference ($P < 0.05$).

a	Season 1: January – March			SIGNIFICANT DIFFERENCE	LOCATION DIFFERENCES
	Avon	Plym	Yealm		
Physiological variables					
Wet weight (g)	47.75 \pm 12.28	41.54 \pm 2.85	48.18 \pm 12.53	<i>NS</i>	-
Cell Integrity (%)	81.08 \pm 18.98	87.31 \pm 9.91	78 \pm 11.35	<i>NS</i>	-
Cell Viability (O.D mg protein ⁻¹)	0.38 \pm 0.27	0.20 \pm 0.05	0.33 \pm 0.17	<i>NS</i>	-
Phagocytosis (zymosan particles 10 ⁷ mg protein ⁻¹)	1.86 \pm 0.78	1.16 \pm 0.56	1.61 \pm 0.93	<i>NS</i>	-
Total antioxidant status ($\mu\text{M L}^{-1}$)	151.19 \pm 56.93	244.74 \pm 57.14	308.51 \pm 109.29	<i>NS</i>	-
PYR metabolites (F.U.)	18.54 \pm 12.29	30.11 \pm 12.75	19.63 \pm 10.41	<i>NS</i>	-
BaP metabolites (F.U.)	99.81 \pm 30.34	215.78 \pm 49.43	169.41 \pm 15.34	<i>S</i>	Avon < Plym = Yealm

b	Season 2: (April – June)			SIGNIFICANT DIFFERENCE	LOCATION DIFFERENCES
	Avon	Plym	Yealm		
Physiological variables					
Wet weight (g)	68.36 \pm 11.65	48.91 \pm 7.20	47.02 \pm 10.41	<i>NS</i>	-
Cell Integrity (%)	85.08 \pm 5.14	87.17 \pm 7.98	86.43 \pm 4.63	<i>NS</i>	-
Cell Viability (O.D mg protein ⁻¹)	0.42 \pm 0.20	0.29 \pm 0.05	0.32 \pm 0.15	<i>NS</i>	-
Phagocytosis (zymosan particles 10 ⁷ mg protein ⁻¹)	1.73 \pm 0.41	1.32 \pm 0.05	1.60 \pm 0.47	<i>NS</i>	-
Total antioxidant status ($\mu\text{M L}^{-1}$)	261.89 \pm 51.38	300.21 \pm 66.87	219.72 \pm 40.39	<i>NS</i>	-
PYR metabolites (F.U.)	13.18 \pm 14.52	43.85 \pm 22.25	12.93 \pm 22.39	<i>NS</i>	-
BaP metabolites (F.U.)	62.57 \pm 66.31	295.70 \pm 125.83	60.68 \pm 105.10	<i>NS</i>	-

Table 7.9 Univariate summary of seasonal differences in physiological variables between estuaries of varying anthropogenic input. Data are presented as means \pm 1 standard deviation. *NS* = Not significant, *S* = Significant difference ($P < 0.05$).

c

Physiological variables

Wet weight (g)
 Cell Integrity (%)
 Cell Viability (O.D mg protein⁻¹)
 Phagocytosis (zymosan particles 10⁷ mg protein⁻¹)
 Total antioxidant status ($\mu\text{M L}^{-1}$)
 PYR metabolites (F.U.)
 BaP metabolites (F.U.)

Season 3: July - September			SIGNIFICANT DIFFERENCE	LOCATION DIFFERENCES
Avon	Plym	Yealm		
53.97 \pm 9.79	54.75 \pm 9.98	50.04 \pm 20.59	<i>NS</i>	-
89.64 \pm 1.26	86.03 \pm 7.73	83.31 \pm 14.03	<i>NS</i>	-
0.56 \pm 0.12	0.32 \pm 0.04	0.28 \pm 0.13	<i>S</i>	Avon > Plym = Yealm
0.93 \pm 0	0.85 \pm 0.16	0.84 \pm 281.68	<i>NS</i>	-
274.06 \pm 32.80	275.33 \pm 85.13	281.68 \pm 46.44	<i>NS</i>	-
53.67 \pm 16.33	29.34 \pm 27.99	41.97 \pm 37.17	<i>NS</i>	-
401.12 \pm 66.06	237.01 \pm 225.08	319 \pm 246.71	<i>NS</i>	-

d

Physiological variables

Wet weight (g)
 Cell Integrity (%)
 Cell Viability (O.D mg protein⁻¹)
 Phagocytosis (zymosan particles 10⁷ mg protein⁻¹)
 Total antioxidant status ($\mu\text{M L}^{-1}$)
 PYR metabolites (F.U.)
 BaP metabolites (F.U.)

Season 4: October - December			SIGNIFICANT DIFFERENCE	LOCATION DIFFERENCES
Avon	Plym	Yealm		
41.71 \pm 6.85	44.90 \pm 11.17	68.05 \pm 4.74	<i>S</i>	Avon = Plym < Yealm
86.39 \pm 2.46	84.33 \pm 11.88	84.33 \pm 1.62	<i>NS</i>	-
0.50 \pm 0.08	0.91 \pm 0.21	0.55 \pm 0.09	<i>S</i>	Plym > Avon = Yealm
0.90 \pm 0.05	1.05 \pm 0.20	0.74 \pm 0.13	<i>NS</i>	-
332.30 \pm 37.83	217.13 \pm 170.10	398.29 \pm 82.78	<i>NS</i>	-
47.88 \pm 11.14	250.65 \pm 196.67	48.76 \pm 27.90	<i>NS</i>	-
296.80 \pm 64.34	1082.49 \pm 1170.67	339.65 \pm 181.07	<i>NS</i>	-

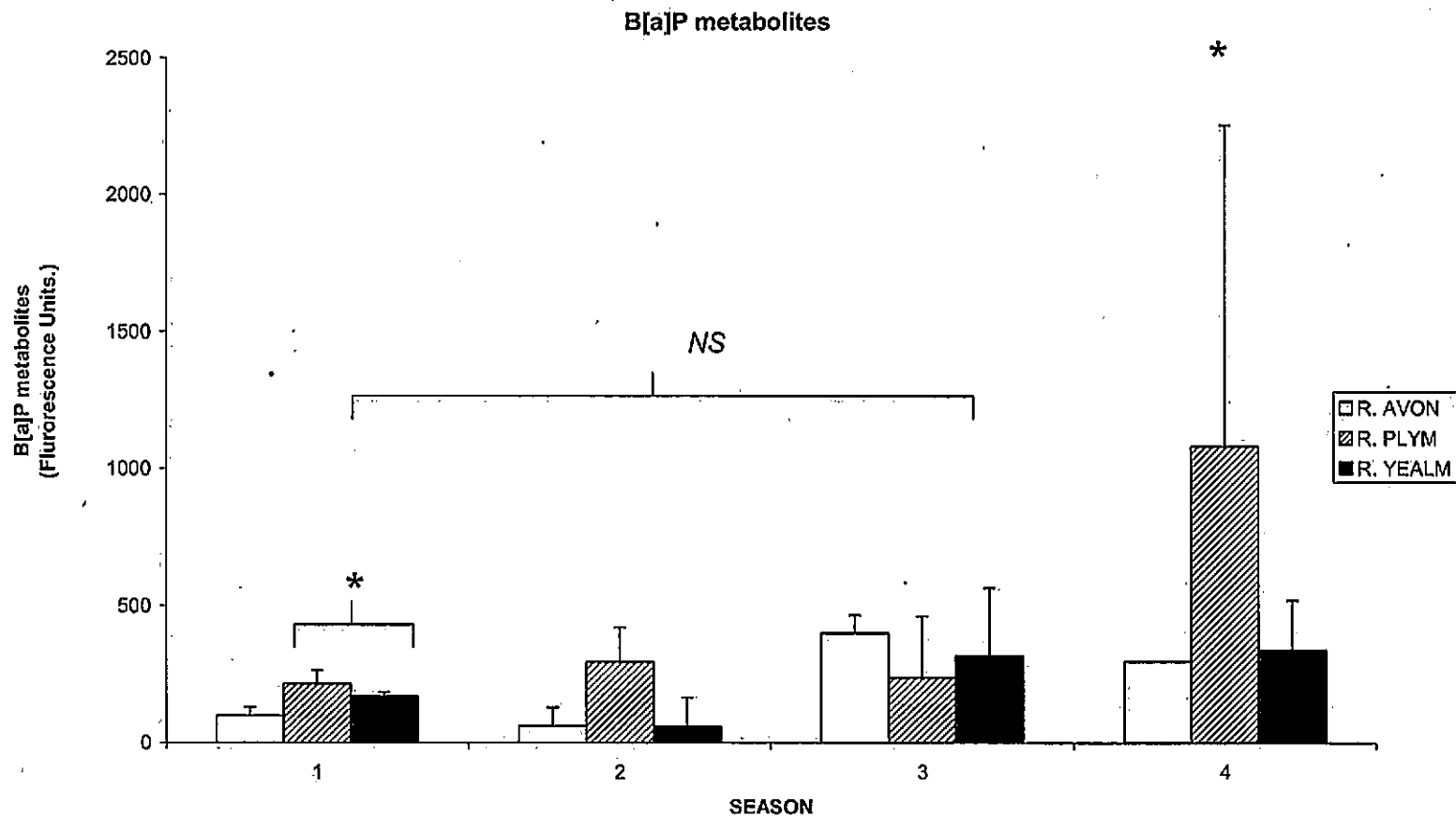


Fig. 7.9 Location and seasonal levels of B[a]P metabolites from three different estuaries. * signifies $P < 0.05$, NS = Not Significant. Error bars signify ± 1 standard deviation.

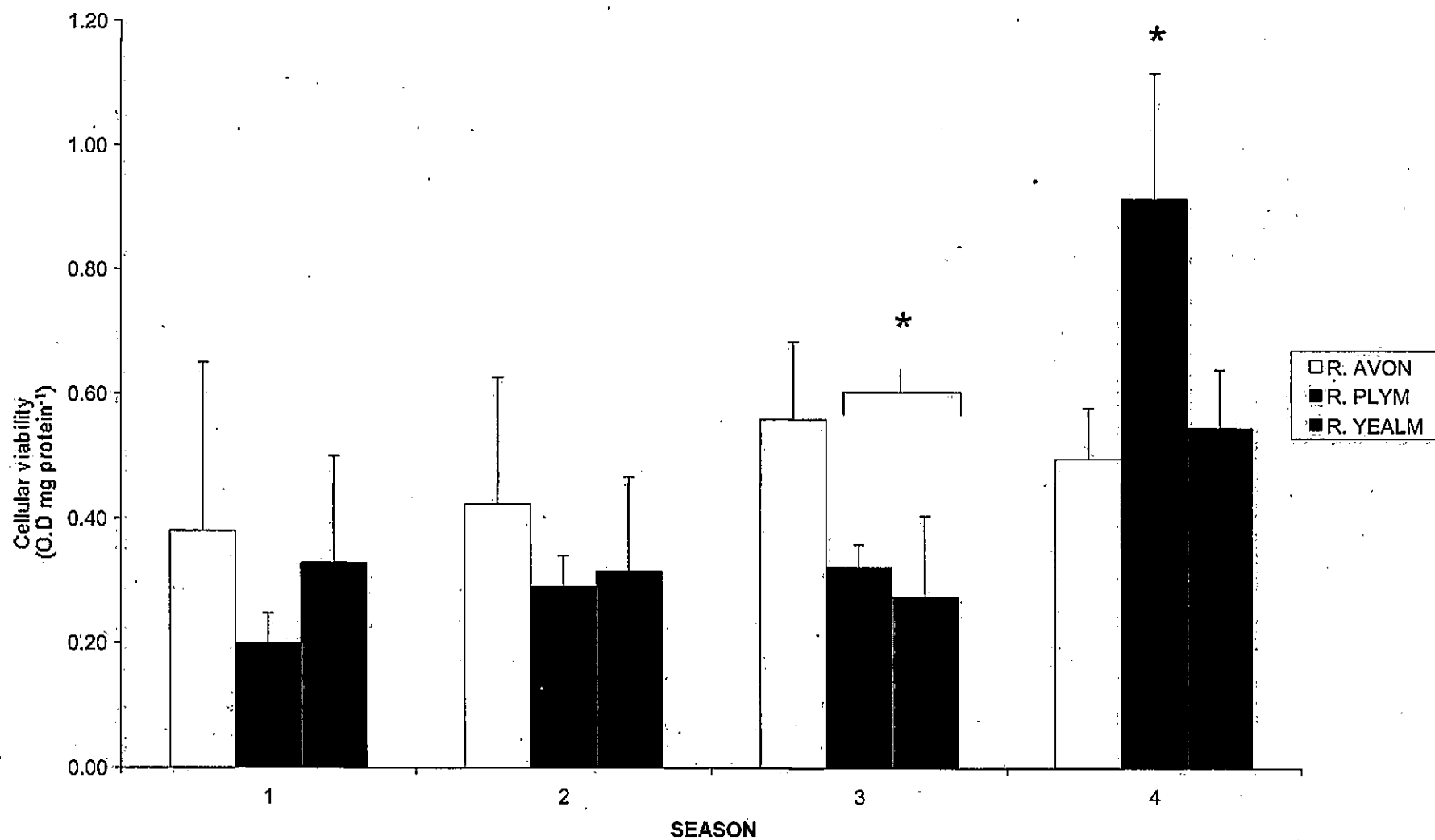


Fig. 7.10 Differences in cellular viability assessed in haemocytes from shore crabs from three different estuaries. * signifies $P < 0.05$. Error bars signify ± 1 standard deviation.

7.3.2.1 Shore crab physiological condition

Significant seasonal differences in physiological condition were found for *Carcinus maenas* sampled from the three estuaries with differences observed between Avon and Plym crabs in seasons 1 and 2 (Table 7.10a). No differences in shore crab physiological condition were found between Avon and Yealm shore crabs, and between Plym and Yealm crabs, in seasons 1 and 2 (Table 7.10a). Contrary to earlier in the year (seasons 1 and 2), no differences were identified between Avon and Plym crabs in seasons 3 and 4 (Table 7.10a). Significant differences, however, between Avon and Yealm *C. maenas* were observed in both seasons 3 and 4. The only seasonal difference found between Plym and Yealm crabs was observed later in the year (season 4). These results indicate differences in shore crab physiological condition between crabs from estuaries of varying PAH input at different times of the year.

Seasonal differences in PAH metabolites in urine between Avon and Plym crabs were observed throughout the year; however, no seasonal differences in crab physiological condition were observed between Avon and Yealm crabs (Table 7.10b).

Physiological condition of Plym and Yealm shore crabs, in comparison, differed throughout the year, except in season 1 (Table 7.10b). Correlation analysis revealed no significant correlations between shore crab physiological state and PAH exposure.

Physiological condition of Avon crabs was significantly different from Plym crabs, with cellular integrity (<26%) and cellular viability (<25%) accounting for the differences in physiological condition in season 1 and 2 (Table 7.11 and 7.12, respectively). These values indicate differential cellular functioning in

January – June in crabs from an estuary of low PAH input (Avon) compared to crabs from an estuary of (relatively) high PAH input (Plym). Physiological condition of Avon crabs was significantly different from crabs from the Yealm Estuary between July – December (seasons 3 and 4), with cellular viability (<29%) and shore crab wet weight (<35%) contributing to the overall difference in physiological condition, indicating differences in both cellular effects and weight of shore crabs sampled (Table 7.13 and 7.14). The only significant seasonal difference in shore crab physiological condition between Plym and Yealm crabs was observed between October and December (season 4), with total antioxidant status contributing <23% to the overall difference (Table 7.14), indicating effects manifested at the biochemical level. These results reveal that (of the variables measured here) different physiological variables (at a particular season) accounted for the significant differences in shore crab physiological condition between estuaries of varying anthropogenic input.

Table 7.10 Pairwise ANOSIM seasonal location comparisons using a) physiological and b) environmental (PAH metabolites) variables. *NS* = Not Significant, *S* = Significant ($P < 1\%$).

a

PAIRWISE COMPARISONS (LOCATION)	SEASON			
	1	2	3	4
Avon vs. Plym	S	S	<i>NS</i>	<i>NS</i>
Avon vs. Yealm	<i>NS</i>	<i>NS</i>	S	S
Plym vs. Yealm	<i>NS</i>	<i>NS</i>	<i>NS</i>	S

Physiological variables

Carapace Width (mm)

Wet weight (g)

Cell Integrity (%)

Cell Viability (O.D mg protein⁻¹)

Phagocytosis (zymosan particles 10⁷ mg protein⁻¹)

Total antioxidant status (μM L⁻¹)

PYR metabolites (F.U.)

BaP metabolites (F.U.)

b

PAIRWISE COMPARISONS (LOCATION)	SEASON			
	1	2	3	4
Avon vs. Plym	S	S	S	S
Avon vs. Yealm	<i>NS</i>	<i>NS</i>	<i>NS</i>	<i>NS</i>
Plym vs. Yealm	<i>NS</i>	S	S	S

Physiological variables

Carapace Width (mm)

Wet weight (g)

Cell Integrity (%)

Cell Viability (O.D mg protein⁻¹)

Phagocytosis (zymosan particles 10⁷ mg protein⁻¹)

Total antioxidant status (μM L⁻¹)

PYR metabolites (F.U.)

BaP metabolites (F.U.)

Table 7.11 Pairwise location comparisons of percentage contribution of each physiological variable within season 1. Significant location differences are highlighted in yellow, highest contributing variables are highlighted in bold.

Avon crabs vs. Plym crabs

Variable	Contribution (%)	Cumulative (%)
Cell Integrity	25.39	25.39
Cell Viability	22.03	47.42
Phagocytosis	20.59	68.01
WW (g)	19.58	87.59
Antioxidant status	12.41	100

Avon crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
Antioxidant status	22.72	22.72
Cell Viability	20.35	43.07
Cell Integrity	20.01	63.08
Phagocytosis	18.65	81.73
WW (g)	18.27	100

Plym crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
Antioxidant status	28.12	28.12
Phagocytosis	21.96	50.07
WW (g)	21.19	71.26
Cell Viability	17.25	88.51
Cell Integrity	11.49	100

Table 7.12 Pairwise location comparisons of percentage contribution of each physiological variable within season 2. Significant location differences are highlighted in yellow, highest contributing variables are highlighted in bold.

Avon crabs vs. Plym crabs		
Variable	Contribution (%)	Cumulative (%)
Cell Viability	24.69	24.69
Cell Integrity	20.46	45.16
WW (g)	20.42	65.58
Phagocytosis	18.15	83.73
Antioxidant status	16.27	100

Avon crabs vs. Yealm crabs		
Variable	Contribution (%)	Cumulative (%)
WW (g)	23.95	23.95
Cell Viability	22.57	46.52
Phagocytosis	20.1	66.61
Cell Integrity	16.71	83.32
Antioxidant status	16.68	100

Plym crabs vs. Yealm crabs		
Variable	Contribution (%)	Cumulative (%)
Antioxidant status	27.44	27.44
Cell Integrity	20.95	48.39
Phagocytosis	19.93	68.32
WW (g)	18.26	86.57
Cell Viability	13.43	100

Table 7.13 Pairwise location comparisons of percentage contribution of each physiological variable within season 3. Significant location differences are highlighted in yellow, highest contributing variables are highlighted in bold.

Avon crabs vs. Plym crabs

Variable	Contribution (%)	Cumulative (%)
Cell Viability	29.89	29.89
WW (g)	20.92	50.81
Cell Integrity	20.2	71
Antioxidant status	15.94	86.94
Phagocytosis	13.06	100

Avon crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
Cell Viability	28.47	28.47
Phagocytosis	20.24	48.71
Antioxidant status	19.69	68.4
WW (g)	16.54	84.94
Cell Integrity	15.06	100

Plym crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
Cell Integrity	23.44	23.44
Phagocytosis	22.41	45.85
WW (g)	21.52	67.37
Antioxidant status	21.15	88.52
Cell Viability	11.48	100

Table 7.14 Pairwise location comparisons of percentage contribution of each physiological variable within season 4. Significant location differences are highlighted in yellow, highest contributing variables are highlighted in bold.

Avon crabs vs. Plym crabs

Variable	Contribution (%)	Cumulative (%)
Cell Viability	25.13	25.13
Cell Integrity	20.6	45.73
Phagocytosis	20.51	66.25
Antioxidant status	17.87	84.12
WW (g)	15.88	100

Avon crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
WW (g)	34.73	34.73
Antioxidant status	24.35	59.08
Phagocytosis	20.82	79.9
Cell Integrity	10.94	90.83
Cell Viability	9.17	100

Plym crabs vs. Yealm crabs

Variable	Contribution (%)	Cumulative (%)
Antioxidant status	22.3	22.3
Cell Viability	20.44	42.74
Cell Integrity	19.92	62.66
WW (g)	19.74	82.39
Phagocytosis	17.61	100

7.4 DISCUSSION

7.4.1 Seasonality of shore crab physiology (Avon Estuary)

Significant seasonal differences in shore crab physiology were highlighted with univariate analysis; for example, winter and spring crabs had higher immune function compared to summer and autumn individuals (Fig. 7.3), and lower antioxidant status in winter compared to other seasons (Fig. 7.4). Multivariate analyses revealed seasonal differences in shore crab physiological state earlier in the year (winter-spring) compared to later in the year (summer-autumn); differences at the cellular and biochemical level were suggestive of differential levels of 'normal' physiological function.

Differences in immune function were observed with seasonal differences in water temperatures (Table 7.5). In crustaceans, phagocytic capability is the innate immune ability (Galloway and Depledge 2001) of the organism to combat foreign particles (e.g. bacteria) within the open circulatory vascular system (Bauchau 1981). As with all poikilothermic organisms, temperature dictates metabolic state, therefore, in *Carcinus maenas*, general activity (e.g. foraging) and food demand decreases in the colder winter months (Styrishave et al. 1999). Temperature has been suggested to cause an inhibitory effect upon phagocytosis (Bauchau 1981) and the phagocytic capability of *Mytilus edulis* was lower at 10°C than at 15°C (when exposed to copper) (Parry and Pipe 2004). Results found here, however, contradict previous evidence as the phagocytic capability of *C. maenas* was higher in the cold winter months compared to the summer. This same pattern is corroborated by findings for several fish species (Collazos et al. 1994; Dexiang and Ainsworth 1991; Le Morvan et al. 1997) and two bivalve mollusc species, *Crassostrea virginica* (Hegaret et al. 2003) and *Chamelea gallina* (Monari et al. 2007). Nikoskelainen et al. (2004), however, that higher environmental temperature had a negative

effect on the phagocytic ability of haemocytes in rainbow trout. At present, it appears that temperature effects on innate immunity are species-specific with no apparent clear summative pattern. Differences in immune function, found here in the shore crab, imply immune 'status' changes seasonally. Such seasonal changes may confer benefits to *C. maenas* at specific times of the year; for example, combating increases in bacterial communities in the water column (Sung et al. 1999). In this study, no attempt was made to quantify and categorise the haemocyte cell type (e.g. alpha or beta cells) present in the haemolymph at the different times of the year. Alpha cells are strongly basophilic and capable of phagocytosis and beta cells are thought to be the 'explosive corpuscles' responsible for coagulation (Johnstone et al. 1973). All alpha cells (i.e. hyaline, semigranular and granular cells) possess the capability to phagocytise, although hyaline and semi-granular cells appear more active than granulocytes (Bauchau 1981; Bell and Smith 1993). An investigation into whether differential ratios of the alpha cell types present throughout the yearly life cycle of the adult male shore crab could provide further insight into the seasonal differences in innate immune status.

A seasonal difference in total antioxidant status (excluding glutathione) was found in the shore crab yearly life cycle (Fig 7.4) and may occur as a result of metabolic state. In *C. maenas*, metabolic rate is a function of environmental temperature and varies at different times of the year (Styrishave et al. 1999). There is a positive relationship between heart rate and locomotory activity, and this relationship is significantly affected by temperature, which is highest during the summer months compared to autumn (Styrishave et al. 1999).

All forms of aerobic life experience production of potentially harmful partially reduced species of molecular oxygen, i.e. reactive oxygen species (ROS) that occur naturally as a by-product due to normal oxygen metabolism (Winston and Di Giulio 1991). Consequently, organisms possess a battery of protective defence mechanisms (antioxidants) against the potential damage of ROS, i.e. possible membrane, protein and DNA damage (Halliwell and Gutteridge 1989). Mitochondria are proposed as the primary sites of aerobic cellular ROS production, as these organelles are thought to utilise over 90 % of the cellular oxygen (Abele and Puntarulo 2004). In terms of cellular homeostasis, a balance in favour of ROS production is normal, as ROS perform roles that are integral to the organism (e.g. neurotransmitter function) (Abele and Puntarulo 2004). Oxidative stress, however, occurs due to increased ROS production whereby the ROS/antioxidant balance is altered (Evans and Halliwell 2001). Marine ectotherms, such as the shore crab, may face oxidative stress challenges through hypoxia (Abele and Puntarulo 2004) (and references therein), dietary and reproductive status (Regoli 1998), and contaminant-mediated injury (Camus et al. 2002a; 2002b; Frenzilli et al. 2001; Regoli and Principato 1995). Seasonal differences in antioxidant activity, corroborating findings here, have been reported in other marine organisms, including molluscs (Bocchetti and Regoli 2006; Borkovic et al. 2005; Lau et al. 2004; Lesser and Kruse 2004; Manduzio et al. 2004; Niyogi et al. 2001a; Ramos-Vasconcelos et al. 2005; Regoli et al. 2002; Wilhelm Filho et al. 2001), a polychaete worm (Geracitano et al. 2004), a barnacle (Niyogi et al. 2001b) and a fish species (Ronisz et al. 1999), and are suggestive of a seasonal trend of higher antioxidant enzyme activities during the summer months, compared to winter (Malanga et al. 2006). The seasonal variation in antioxidant status outlined here reflects a 'baseline' seasonal antioxidant profile in the adult male shore crab.

Present work identified seasonal alterations in lysosomal function for *Carcinus maenas*. Seasonal differences in lysosomal membrane destabilisation have been reported for the oyster *Crassostrea virginica* and the mussel *Mytilus sp.* with higher destabilisation rates in winter compared to summer (Harding et al. 2004; Ringwood et al. 2002). The general conclusion is that such seasonal differences are indicative of physiological differences or seasonal differences in bioavailability and bioaccumulation of contaminants (Ringwood et al. 2002). In this present study, PAH contaminant levels were relatively low and represent low anthropogenic input. Pyrene (PYR) metabolite levels (indicative of PAH contamination) within the Avon Estuary (low in maritime activity) were at environmentally lower levels ($<30 \mu\text{gL}^{-1}$) compared to PYR metabolite levels ($<350 - 7500 \mu\text{gL}^{-1}$) found in other studies around the U.K. (Severn and Tyne) (Law et al. 1997; 2002; Ruddock et al. 2002; 2003). B[a]P metabolite levels measured here could not be compared to levels found elsewhere due to the unavailability of benzo[a]pyrene standards needed to convert fluorescence units into B[a]P equivalents. The low PAH metabolite levels found here are regarded as indicative of low PAH input into the marine environment through low maritime activity. The low correlations [season 1 (0.428), season 2 (0.027), season 3 (-0.064) and season 4 (0.146)] found between the physiological multivariate 'pattern' and environmental (PAH metabolite) 'pattern' indicate that the seasonal differences outlined in shore crab physiology were not related to PAH exposure. For the present study, the conclusion is that seasonal differences in cellular viability (lysosomal function) are attributed directly to seasonal environmental conditions.

In summary for the Avon Estuary, there was a general trend that shore crab physiological condition differed in winter and spring from that observed in summer and autumn. Seasonal differences in shore crab physiology (Table 7.7) could confer seasonal 'windows' of increased physiological tolerance during the summer months compared to the winter (Hebel et al. 1997). The seasonal differences in shore crab physiology, observed here, may be regarded, therefore, as the 'normal' seasonal pattern in shore crab physiological condition.

7.4.2 *Seasonal differences in shore crab physiological condition between estuaries of varying anthropogenic input*

Comparison of shore crabs from the Avon (low anthropogenic activity) and Plym (high anthropogenic activity) estuaries showed differences in physiological condition between January and June (Table 7.10a). No differences in physiological condition between shore crabs were apparent, however, between July and December (Table 7.10a).

With regard to Plym and Yealm shore crabs, physiological differences were observed only between October and December. Comparison of crabs from the Avon and Yealm estuaries (both relatively low in maritime activity compared with the Plym Estuary) revealed physiological differences between July and December. Evaluation of the integrated shore crab physiological condition (SIMPER analysis) revealed that cellular integrity and/or cellular viability (between January - September) were the physiological parameters that most accounted for the differences. Previously, cellular viability has been shown to be impacted negatively by contaminants (Cheung et al. 1998; Dissanayake 2001; Galloway et al. 2004c; Lowe et al. 1995a; Lowe and Pipe 1994; Wedderburn et al. 2000). In this present study, although cellular integrity and

viability (when evaluated as single parameters) did not differ between estuaries, multivariate analyses indicated that cellular integrity was responsible, in conjunction with cellular viability, for seasonal differences between crabs from the Avon and Plym estuaries between January - June (Tables 7.11 and 7.12).

Geographical differences in shore crab physiological condition could be attributed to contaminant-mediated toxicity. For example, increased B[a]P metabolite levels [indicative of PAH exposure, (Fig 7.9)] could affect cell numbers (cellular integrity) and lysosomal function (cellular viability). Lysosomes have the capacity to bioaccumulate contaminants (Nicholson 2001; Regoli et al. 1998), with the negative effects, such as lysosomal membrane degradation, only becoming apparent after the maximal storage capacity is exceeded (Bayne et al. 1985; Regoli et al. 1998). Seasonal differences in lysosomal properties [e.g. membrane permeability (evaluated by measuring dye retention time), volume density, surface density, surface-to-volume ratio and numerical density] have been shown previously in *Mytilus edulis* (Etxeberria et al. 1995; Harding et al. 2004; Marigomez et al. 1996). Reduction in lysosome structure, morphology and membrane function during seasonally low environmental temperatures (e.g. in the winter and spring) has been observed, contrary to the summer and autumn seasonal pattern shown here. Coupled with this seasonal pattern, organic contamination, in the form of PAHs, has been causally linked with lysosomal alterations, such as size reduction (Marigomez and Baybay-Villacorta 2003; Marigomez et al. 1996). Evidence to show that lysosomal capability results from effects of contaminant-exposure include lysosomal size and density increase, under acute or sub-lethal exposure (Canty et al. 2007; Marigomez and Baybay-Villacorta 2003; Matozzo et al. 2002; Matozzo and Marin 2005; Rickwood and Galloway 2004; St-Jean et al. 2002a, b), lysosomal size reduction, decreased density and membrane impairment

caused by damage of the membrane-associated proton pump and leakage of hydrolytic enzymes to the cytosol, due to higher contaminant concentrations (Cajaraville et al. 2000; Cheung et al. 1998; Da Ros et al. 2002; Galloway et al. 2002; Lowe et al. 1995b; Nicholson 1999, 2001, 2003; Regoli et al. 1998).

PAH metabolites in crab urine indicated that crabs from the Avon and Yealm estuaries had significantly lower exposure to PAHs than those from the Plym in all seasons except winter (January – March). No significant seasonal differences in PAH metabolites were observed between Avon and Yealm crabs, confirming a low maritime anthropogenic input at these sites.

Results found here of increased PAH metabolites from high maritime activity are corroborated by findings by King et al. (2004) who reported seasonal differences in PAH concentrations from 16 PAH compounds (including pyrene and benzo[a]pyrene) in Brighton marina, U.K. These authors suggested that periods of rainfall would increase surface run-off from roads, thereby, increasing deposition of rainwater-dissolved PAHs directly to surface waters, as well as increased run-off (King et al. 2004). There is clear evidence by contaminant deposition (e.g. pesticides) within waterways being linked with precipitation events (Liess et al. 1999), supporting findings here of higher PAH metabolites between July - December [where there was higher rainfall (Table 7.5)] compared with January - June (ANOVA, $F_{2,6} = 4.45$, $P < 0.05$) (Fig 7.9).

In summary, the present study reported temporal (seasonal) variability in shore crab physiological condition at two reference locations and one location of (relatively) high anthropogenic impact (defined here as maritime activity). Although few seasonal differences in individual physiological parameters were identified (univariate), the integrated response of shore crab physiological

condition (multivariate) showed that seasonal differences in physiological condition were apparent between crabs at estuaries of varying anthropogenic input. However, physiological condition did not correlate with these differences in PAH contamination either between sites or seasons. While causality (to PAHs) cannot be attributed, multivariate analysis indicated that shore crab physiological condition differed between locations of varying anthropogenic activity.

In conclusion, to aid understanding of the potential contaminant impacts on *C. maenas* physiology, it is imperative that, firstly, the natural temporal variability in organism physiological ranges are outlined and, by defining the "normal" seasonal pattern in shore crab physiological condition, the contaminant effects upon physiology can be identified.

CHAPTER 8:
Final Discussion

FINAL DISCUSSION

Research conducted for this PhD focussed on answering fundamental questions regarding the physiology of the male shore crab *Carcinus maenas*. Individual organisms possess the ability to maintain key physiological functions in the face of environmental change or fluctuation (Spicer and Gaston 1999); however, if that ability is impaired, physiological dysfunction may arise. The main PhD research question centred on the effects of sublethal organic contaminant exposure upon shore crab physiology. The need for increasing understanding of the biochemical toxicology of aquatic organisms is impeded by the lack of knowledge of the basic biochemistry of the organisms, including 'normal' physiological ranges (Mehrlé and Mayer 1980). Determining the physiological mechanisms involved in maintaining homeostasis in an organism's *internal milieu* (Massabuau 2003; Spicer and Gaston 1999) is imperative in understanding its ability to function. Without this knowledge, responses (e.g. compensatory and/or 'stress' effects) to environmental factors, or anthropogenic stress, cannot be identified. Anthropogenic stress, in the form of contamination by a known ubiquitous priority pollutant of the aquatic environment (polyaromatic hydrocarbon, pyrene), was used here as a means of imposing physiological constraints and investigating the associated effects upon shore crab physiology. The physiological condition of the shore crab was evaluated throughout the thesis using a multidisciplinary approach, encompassing the biochemical, cellular, physiological and behavioural levels.

The first experimental chapter (Chapter 3) focussed on outlining physiological differences between the juvenile and adult benthic stages of the male shore crab. Differences in physiological condition at the biochemical (lower metabolic energy signalled by haemolymph glucose levels), cellular (lower immune function, as revealed by phagocytosis) and physiological (higher scope for

growth) levels were found between juveniles and adults. These 'normal' physiological differences determined the relative contaminant sensitivity between the ontogenetic stages, with juveniles expressing increased physiological sensitivity to contaminant-imposed effects (as revealed by decreased cell integrity, decreased phagocytic capability, increased heart rate, decreased respiration rates and decreased energy consumption) compared to the developed adult form (Dissanayake et al. 2006a, b).

Further questions relating to *Carcinus maenas* physiological condition subjected to nutritional stress (Chapters 4 and 5) and behavioural implications (Chapter 6) concentrated on the adult stage. Experimental manipulation of organismal physiological condition revealed that male shore crab physiology (in terms of the biochemical and cellular mechanisms) was dependent upon nutritional status. *Carcinus maenas* physiological condition was robust to short-term starvation (such as might occur during moulting or periods of decreased activity in winter months). It is postulated that this ability to withstand short periods of limited food supply occurs due to autophagy induction ('self-eating') (as revealed by an increase in cellular viability) (Deretic 2006), whereby energy is released via metabolism of protein, carbohydrate and lipid stores. The physiological implications of sublethal contaminant exposure under short-term nutritional duress included an increased antioxidant status, signalling activation of the compensatory mechanisms under contaminant-mediated challenge. Physiological 'competency' or tolerance is shown here to be dependent upon the organism's nutritional status and possible reasons for 'seasonal' differences in physiological condition were hypothesised. The behavioural implications of nutritional stress and contaminant exposure were investigated via intraspecific contest behaviour, i.e. staging contests between pairs of shore crabs for a food resource (Chapter 6). Behavioural evidence revealed that the energy

expenditure, stamina and associated costs of entering in agonistic contests were dependent on the physiological condition of the contestant, thereby, having an effect upon an individual's motivational state and hence competitive ability.

The final question of the research programme dealt with investigating possible 'seasonal' differences in *Carcinus* physiology, with the aim of identifying any 'windows of sensitivity' to both environmental and contaminant-imposed challenges. A seasonal field monitoring study was implemented and shore crab physiology was assessed throughout the year. Differences in 'seasonal' physiological condition were outlined such as a higher immune function and lower antioxidant status between November–April (winter and spring) compared to May–October (summer to autumn). These differences were related to shore crab ecology and regarded as the 'normal' seasonal pattern in shore crab physiological condition. These 'seasonal' differences were shown to have implications to shore crab physiological condition with regard to biochemical (antioxidant) and cellular (integrity, immune function and viability) mechanisms, in terms of organic contaminant-exposure, as revealed by the presence of urinary PAH metabolites. In summary, shore crab physiology was shown to be dependent on both intrinsic [age (Chapter 3), nutritional status (Chapter 4)] and extrinsic [temperature (Chapter 7)] factors. Both intrinsic and extrinsic factors are important in determining the physiological tolerance to contaminant-imposed conditions tested here (Chapters 3, 5 and 7). It could be argued that the combined effect of both intrinsic and extrinsic factors determine the 'plasticity' in responses observed here.

Phenotypic plasticity is defined as the production of multiple phenotypic responses from a single genotype and is dependent upon environmental conditions (Miner et al. 2005). Plasticity in responses include changes in

morphology (Cotton et al. 2004; Lee 1995; Price 2006), behaviour (Behrens Yamada et al. 1998; Cotton et al. 2004) and physiology (Russell 1998; Sinclair et al. 2006), and can be expressed within the period of a single individual's life span or across generations (Miner et al. 2005). Phenotypic plasticity; however, refers to ecological implications of variability in individual traits resultant from developmental processes as a consequence of external factors (e.g. environment, predators), and above all, refers specifically to irreversible traits (Piersma and Drent 2003). Confusion arises over the use of the term 'phenotypic plasticity' when it is applied in the physiological context, to reversible processes with changes in the external environment (Piersma and Drent 2003). Hence, the term 'phenotypic flexibility' was established and defined as the ability of organisms to show continuous but reversible transformations in behaviour, physiology and morphology (Piersma and Lindstrom 1997). The sea urchin *Diadema antillarum* was observed to respond to changes in food availability by adjusting body size and, consequently, optimising reproduction and survival (Levitan 1989), its feeding capacity, however, was unaltered as the Aristotle's lantern (mouth structure) remained unchanged (Levitan 1991). Similar effects have been shown with the green sea urchin *Strongylocentrotus droebachiensis*, whereby, low food availability resulted in utilisation of energy reserves from gonadal tissue (Russell 1998). Although such examples of phenotypic flexibility describe the variation in intra-individual traits as a function of external (environmental) factors, it falls short with regard to the degree of physiological compensation for the influence of the external factor.

Selye's (1973) General Adaptation Syndrome was proposed as a model of the response of an organism to a stressor. Gray's (1989) modification of the model showed that application of a stressor may lead to a measurable response within an organism (the 'alarm' reaction) and that if compensation to the stressor

occurs, 'normal' activity may resume. If continued application of the stressor (or at a greater concentration) occurs, the organism may not 'cope' and a decline in physiological condition occurs, ultimately resulting in death (Fig. 8.1). Example of 'alarm' reactions include induction of P450 enzyme expression and activity in response to PAH exposure (Aas et al. 2000a; Burgeot et al. 1994; Livingstone 1993). Gray's (1989) model illustrates possible alteration(s) in a biological response(s), but fails to depict the range in which compensation may occur. A schematic model originally developed in the context of human health (Hatch 1962), but also used to describe 'health status' and organism physiological condition (Depledge 1994), is adapted here and illustrates the extent to which the biochemical/physiological compensatory mechanisms can protect an organism before signs of damage are apparent (Depledge 1994; Spicer and Gaston 1999) (Fig. 8.2).

The solid curve defines the relationship between pathology (x axis) and physiological condition (y axis). An individual can occupy any point on the curve and can occupy different points at various times. The physiology of a 'healthy' organism can be observed to lie within the homeostatic range (Section A). Deviations from homeostasis will cause a change in the inherent biological responses, resulting in a decrease in physiological condition, thereby, a shift towards the compensatory zone (Section B). Any further exposure to a stressor(s) will result in a decline in physiological condition past the limit of physiological competency (i.e. the limit at which the physiological tolerance afforded the repertoire of inherent compensatory responses), resulting in physiological dysfunction and possible pathological damage (Section C). Physiological competency is no longer possible at this point and pathological damage may ensue, but any further decline and shift along the curve will surpass the point at which any pathological damage may be reversible (Section

D). The underlying importance of this diagram is ultimately dependent upon the repertoire of responses to stressor(s), which determines whether an organism can compete sufficiently well, both intra-specifically and inter-specifically, to grow and reproduce (Hebel et al. 1997).

Physiological competency is described as the physiological regulation that allows for any disturbances in physiological function, and can be defined as the predisposed physiological tolerance afforded to the organism by the repertoire of responses. Physiological competency can be measured within the individual at successive levels of biological organisation (biochemical to behavioural).

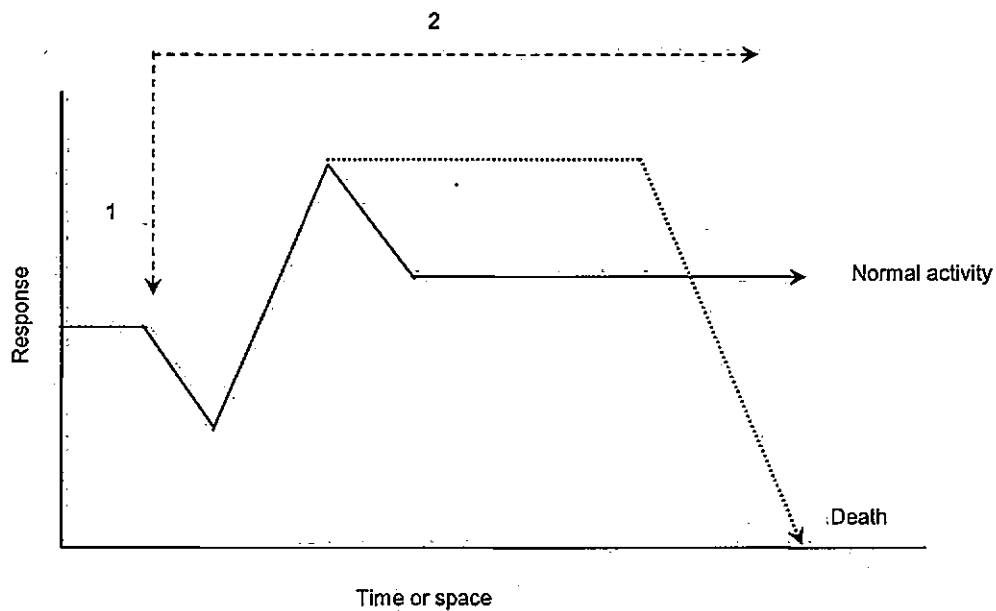


Fig. 8.1 Modification of Selye's General Adaptation Syndrome. Arrows show application of stressor. (1) alarm response and compensation to the stressor; (2) continuous line to single application (normal activity), broken line response to continuous application or increased level of stressor (leading to death). Taken from (Gray 1989).

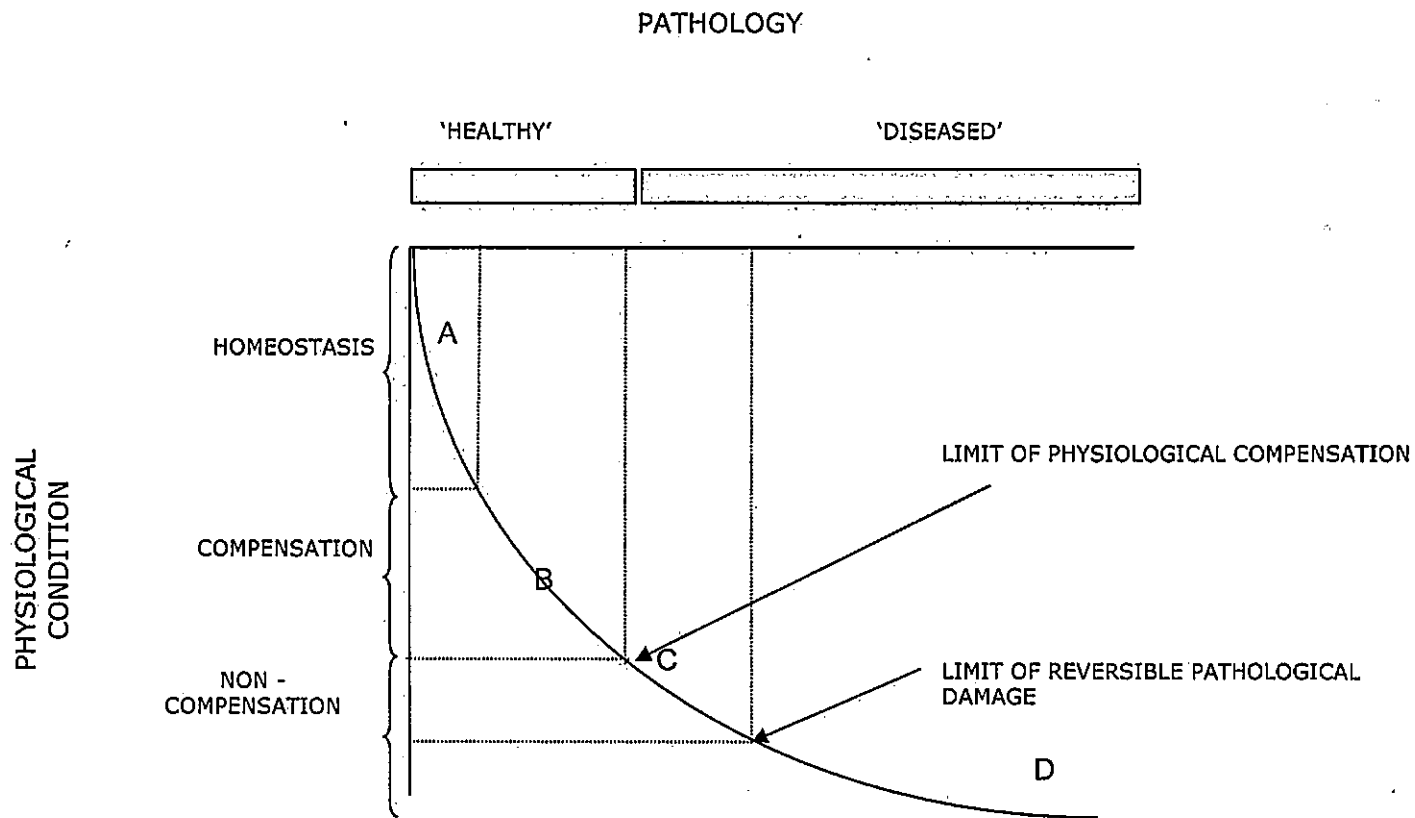


Fig. 8.2 Theoretical relationship between disease and dysfunction. (A) Individual function undisturbed; (B) function disturbed but physiological competency is possible; (C) limit of physiological compensation, until damage is repaired; (D) decline in physiological condition leading to death, due to irreversible pathological damage. Adapted from Hatch (1962) and Depledge (1994).

Using the evidence gathered from within this PhD, the degree of physiological competency may be outlined with regard to shore crab physiology. Outlining the difference between juvenile and adult male shore crabs allows for the elucidation of the 'normal' physiological function between the ontogenetic stages, through evaluation of biochemical (glucose); cellular (cellular viability and innate immune function); metabolic (basal respiration rates) and physiological (scope for growth) responses (Chapter 3). Super-imposition of a chemical stressor (pyrene) shows that the zone of physiological compensation (B) (Fig. 8.2) can be postulated to be lower in juveniles compared to adult shore crabs, due to increased sensitivity to contaminant-mediated effects (Chapter 3). Differences in physiological competency between ontogenetic stages have also been shown elsewhere with regard to salinity (Anger et al. 2000; Charmantier et al. 2001; Holdich et al. 1997; Morgan 1987) and osmoregulation (Charmantier et al. 2002; Morrill and Spicer 1999).

When focussing solely on the physiology of the male shore crab, it may be possible to outline the zones of homeostasis, i.e. normal function (A) and physiological competency (B) (Fig. 8.2). Chapter 4 described the effect of dietary manipulation (via nutritional intake) on the physiological condition of the adult male shore crab. Shore crab physiological condition was shown to be relatively robust to short-term (< 7 days) changes, postulated to occur via the utilisation of energy reserves (Chapter 4). Contaminant exposure, however, demonstrated that tolerance (physiological competency) afforded to the individual was significantly dependent upon the nutritional status of the organism (Chapter 5). Modulation in biochemical (increased antioxidant status) and cellular functions (immune function signalled by phagocytosis, cellular integrity and viability) were observed and can be postulated to be up-regulation of biochemical and cellular processes and indicative of physiological compensation by the repertoire of biological responses measured. Although short-term (14 days) effects of sublethal pyrene concentrations ($200\mu\text{gL}^{-1}$)

were evaluated here, they were not prolonged to evaluate the limit of reversible pathological damage (D) (Fig. 8.2) (i.e. by exposure to higher concentrations and/or longer exposure periods). It could be concluded that adult male shore crab physiology (during the intermoult stage) is robust to short-term changes in physiological condition. The degree of physiological competency, and the limit of physiological compensation to various stressors, has been shown previously in various species, with regard to nutritional state (Depledge 1985; Russell 1998), temperature (Cowling et al. 2003; Denny et al. 2006), osmotic stress (Calosi et al. 2005; Cowling et al. 2003), hypoxia (Bernatis et al. 2007; Brouwer et al. 2007; Davenport and Wong 1987) and contaminants (Sloman 2004), and serves to illustrate an organism's limit of physiological compensation.

Behaviour is an important component of physiological adjustment to the environment (Spicer and Gaston 1999) and is an energetically demanding activity. Intraspecific resource conflicts were shown here to be dependent upon crab physiological condition (Chapter 6). Shore crab physiological condition, manipulated by a combination of dietary and contaminant-imposed constraints, had repercussions for behaviour. Behavioural endpoints (resource possession and recuperation time) revealed that intraspecific agonistic contests were shaped by the physiological condition of the competitors and, consequently, had proximate biochemical (glucose and lactate concentrations) 'costs' resultant from energetically demanding behaviour. It may be concluded, therefore, that physiological compensation shown at the biochemical and cellular level translates to effects seen at higher levels (behaviour) within the individual. These have effects have ecologically-relevant implications to individual fitness, as evaluated here by the ability of shore crabs to compete for food.

Normal function or homeostasis in *C. maenas* physiology was shown to vary seasonally, with differences in biochemical (antioxidant status), cellular (phagocytosis) and shore crab physiology (multivariate) occurring between winter-spring and summer-autumn, accompanying changes in environmental (temperature) conditions (Chapter 7). This natural variation in physiology has implications, for the ability of crabs to tolerate contaminant exposure. The effects of contaminant exposure upon shore crab physiological condition (evaluated here in three estuaries of varying PAH input) revealed differences highlighting the importance of evaluating the natural physiological range of shore crab physiology.

To measure the response(s) of an organism to physiological stress, there are three important considerations. Firstly, the effects of the stress will be an integrated response involving all levels of functional complexity within the organism (molecular, cellular and physiological). Secondly, the stress response is dynamic and involves alteration in functional properties over time. Thirdly, a potential stress may be neutralised by homeostatic physiological compensation (Koehn and Bayne 1989). Therefore to study the effects of a stressor upon the physiology of organism, it is paramount that the normal physiological ranges be identified allowing understanding of the organism's ability to function. Without this knowledge, compensatory responses and consequently stress effects cannot be elucidated. In conclusion, this present study, has demonstrated that sublethal contaminant exposure (as a means of imposing physiological constraints) has resulted in alterations to single biological systems at various levels of biological organisation (biochemical to behavioural) (via univariate analysis). The integrated physiological condition (multivariate), allowed assessment of the normal physiological range and physiological competency, between ontogenetic stages (juvenile and adult), nutritional status and season.

The present study has attempted to elucidate the normal physiological ranges of the shore crab *C. maenas*. Therefore, it is essential to establish the full extent of the 'normal' physiological ranges for *C. maenas* physiology to fully understand the impact of environmental and anthropogenic stress alike.

REFERENCES

- Aagaard, A. (1996). In situ variation in heart rate of the shore crab *Carcinus maenas* in relation to environmental factors and physiological condition. *Marine Biology*. **125**, 765-772.
- Aagaard, A., Warman, C. G. and Depledge, M. H. (1995). Tidal and Seasonal-Changes in the Temporal and Spatial- Distribution of Foraging *Carcinus-Maenas* in the Weakly Tidal Littoral-Zone of Kerteminde Fjord, Denmark. *Marine Ecology-Progress Series*. **122**, 165-172.
- Aas, E., Baussant, T., Balk, L., Liewenborg, B. and Andersen, O. K. (2000a). PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. *Aquatic Toxicology*. **51**, 241-258.
- Aas, E., Beyer, J. and Goksoyr, A. (1998). PAH in fish bile detected by fixed wavelength fluorescence. *Marine Environmental Research*. **46**, 225-228.
- Aas, E., Beyer, J. and Goksoyr, A. (2000b). Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filter effect and signal interpretation. *Biomarkers*. **5**, 9-23.
- Aas, E., Beyer, J., Jonsson, G., Reichert, W. L. and Andersen, O. K. (2001). Evidence of uptake, biotransformation and DNA binding of polyaromatic hydrocarbons in Atlantic cod and corkwing wrasse caught in the vicinity of an aluminium works. *Marine Environmental Research*. **52**, 213-229.
- Aas, E. and Klungsoyr, J. (1998). PAH metabolites in bile and EROD activity in North Sea fish. *Marine Environmental Research*. **46**, 229-232.
- Aas, E., Liewenborg, B., Grosvik, B. E., Camus, L., Jonsson, G., Borseth, J. F. and Balk, L. (2003). DNA adduct levels in fish from pristine areas are not detectable or low when analysed using the nuclease P1 version of the P-32-postlabelling technique. *Biomarkers*. **8**, 445-460.
- Abele, D. and Puntarulo, S. (2004). Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*. **138**, 405-415.
- Abello, P., Aagaard, A., Warman, C. G. and Depledge, M. H. (1997). Spatial variability in the population structure of the shore crab *Carcinus maenas* (Crustacea: Brachyura) in a shallow-water, weakly tidal fjord. *Marine Ecology-Progress Series*. **147**, 97-103.
- Adams, S. M. (2005). Assessing cause and effect of multiple stressors on marine systems, *Marine Pollution Bulletin*, pp. 649-657.
- Ahmad, I., Hamid, T., Fatima, M., Chand, H. S., Jain, S. K., Athar, M. and Raisuddin, S. (2000). Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. *Biochimica Et Biophysica Acta-General Subjects*. **1523**, 37-48.
- Andersson, M. (1994). *Sexual Selection*. Princeton University Press.
- Anger, K., Riesebeck, K. and Puschel, C. (2000). Effects of salinity on larval and early juvenile growth of an extremely euryhaline crab species, *Armases miersii* (Decapoda : Grapsidae). *Hydrobiologia*. **426**, 161-168.
- Arudpragasam, K. D. and Naylor, E. (1964a). Gill ventilation and the role of reversed respiratory currents in *Carcinus maenas* (L.). *Journal of Experimental Biology*. **41**, 299-307.
- Arudpragasam, K. D. and Naylor, E. (1964b). Gill ventilation volumes, oxygen consumption and respiratory rhythms in *Carcinus maenas* (L.). *Journal of Experimental Biology*. **41**, 309-321.
- Arun, S., Krishnamoorthy, P. and Subramanian, P. (1999). Properties of glutathione peroxidase from the hepatopancreas of freshwater prawn *Macrobrachium*

- malcolmsonii. *The International Journal of Biochemistry & Cell Biology*. **31**, 725-732.
- Attrill, M. J. and Depledge, M. H. (1997). Community and population indicators of ecosystem health: Targeting links between levels of biological organisation. *Aquatic Toxicology*. **38**, 183-197.
- Attrill, M. J. and Thomas, R. M. (1996). Long-term distribution patterns of mobile estuarine invertebrates (Ctenophora, Cnidaria, Crustacea: Decapoda) in relation to hydrodynamical parameters. *Marine Ecology Progress Series*. **143**, 25-36.
- Babich, H. and Borenfreund, E. (1991). Cytotoxicity of T-2 toxin and its metabolites determined with the neutral red cell viability assay. *Applied and Environmental Microbiology*. **57**, 2101-2103.
- Babich, H. and Borenfreund, E. (1993). Applications of the neutral red cytotoxicity assay to risk assessment of aquatic contaminants: an overview. *ASTM STP*. **1179**, 215.
- Baden, S. P., Depledge, M. H. and Hagerman, L. (1994). Glycogen Depletion and Altered Copper and Manganese Handling in Nephrops-Norvegicus Following Starvation and Exposure to Hypoxia. *Marine Ecology-Progress Series*. **103**, 65-72.
- Balogh, N., Gaal, T., Ribiczeyne, P. S. and Petri, A. (2001). Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses before and after exercise. *Veterinary Clinical Pathology*. **30**, 214-218.
- Bauchau, A. G. (1981). Crustaceans. In: Ratcliffe, N. A. and Rowley, A. F. (Eds), *Invertebrate blood cells*, Academic Press, pp. 385-420.
- Bayne, B. L. (1984). Response to environmental stress: Tolerance, resistance and adaptation. *18th European Marine Biology Symposium*. Oslo.
- Bayne, B. L., Brown, D. A., Burns, K., Dixon, D. R., Ivanovici, A., Livingstone, D. R., Lowe, D. M., Moore, M. N., Stebbing, A. R. D. and Widdows, J. (1985). *The effects of stress and pollution on marine animals*. Praeger Publishers.
- Beaugrand, J. P., Payette, D. and Goulet, C. (1996). Conflict outcome in male green swordtail fish dyads (*Xiphophorus helleri*): Interaction of body size, prior dominance/subordination experience, and prior residency. *Behaviour*. **133**, 303-319.
- Behrens Yamada, S., Navarrete, S. A. and Needham, C. (1998). Predation induced changes in behavior and growth rate in three populations of the intertidal snail, *Littorina sitkana* (Philippi). *Journal of Experimental Marine Biology and Ecology*. **220**, 213-226.
- Bell, K. L. and Smith, V. J. (1993). In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.) *Developmental & Comparative Immunology*. **17**, 211-219.
- Bell, K. L. and Smith, V. J. (1995). Occurrence and distribution of antioxidant enzymes in the haemolymph of the shore crab *Carcinus maenas*. *Marine Biology*. **123**, 829-836.
- Benzie, I. F. F. and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*. **239**, 70-76.
- Bernatis, J. L., Gerstenberger, S. L. and McGaw, I. J. (2007). Behavioural responses of the Dungeness crab, *Cancer magister*, during feeding and digestion in hypoxic conditions. *Marine Biology*. **150**, 941-951.
- Beyer, J., Aas, E., Borgenvik, H. K. and Ravn, P. (1998). Bioavailability of PAH in effluent water from an aluminium works evaluated by transplant caging and biliary fluorescence measurements of Atlantic cod (*Gadus morhua* L.). *Marine Environmental Research*. **46**, 233-236.
- Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skare, J. U. and Goksoyr, A. (1996). Contaminant accumulation and biomarker responses in

- flounder (*Platichthys flesus* L) and Atlantic cod (*Gadus morhua* L) exposed by caging to polluted sediments in Sorfjorden, Norway. *Aquatic Toxicology*. **36**, 75-98.
- Bjerregaard, P. and Visle, T. (1985). Effects of mercury on ion and osmoregulation in the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology C*. **82**, 227-230.
- Bjerregaard, P. and Visle, T. (1986). Effect of copper on ion- and osmoregulation in the shore crab *Carcinus maenas*. *Marine Biology*. **91**, 69-76.
- Bocchetti, R. and Regoli, F. (2006). Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. *Chemosphere*. **65**, 913-921.
- Borkovic, S. S., Saponjic, J. S., Pavlovic, S. Z., Blagojevic, D. P., Milosevic, S. M., Kovacevic, T. B., Radojicic, R. M., Spasic, M. B., Zikic, R. V. and Saicic, Z. S. (2005). The activity of antioxidant defence enzymes in the mussel *Mytilus galloprovincialis* from the Adriatic Sea. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. **141**, 366-374.
- Boyer, R. F. (2002). *Concepts in Biochemistry*. John Wiley and Sons.
- Brandt, Y. (2003). Lizard threat display handicaps endurance. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **270**, 1061-1068.
- Brett, J. R. (1958). Implications and assessments of environmental stress *In*: Larkin, P. A. (Ed), *Investigations of fish-power problems*, British Columbia Press, pp. 69-83.
- Brian, J. V., Fernandes, T., Ladle, R. J. and Todd, P. A. (2006). Patterns of morphological and genetic variability in UK populations of the shore crab, *Carcinus maenas* Linnaeus, 1758 (Crustacea: Decapoda: Brachyura). *Journal of Experimental Marine Biology and Ecology*. **329**, 47-54.
- Brick, O. (1998). Fighting behaviour, vigilance and predation risk in the cichlid fish *Nannacara anomala*. *Animal Behaviour*. **56**, 309-317.
- Briffa, M. (2006). Signal residuals during shell fighting in hermit crabs: can costly signals be used deceptively? *Behav. Ecol.* **17**, 510-514.
- Briffa, M. and Elwood, R. W. (2001). Decision rules, energy metabolism and vigour of hermit-crab fights. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **268**, 1841-1848.
- Briffa, M. and Elwood, R. W. (2004). Use of energy reserves in fighting hermit crabs. *Proceedings of the Royal Society of London Series B-Biological Sciences*. **271**, 373-379.
- Briffa, M. and Elwood, R. W. (2005). Rapid change in energy status in fighting animals: causes and effects of strategic decisions. *Animal Behaviour*. **70**, 119-124.
- Bronk, R. (1999). *Human metabolism*. Addison Wesley Longman Ltd.
- Brouwer, M., Brown-Peterson, N. J. and Larkin, P. (2007). Molecular and whole animal responses of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia. *Journal of Experimental Marine Biology and Ecology*. **341**, 16-31.
- Brown, R. J., Galloway, T. S., Lowe, D., Browne, M. A., Dissanayake, A., Jones, M. B. and Depledge, M. H. (2004). Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology*. **66**, 267-278.
- Burgeot, T., Bocquene, G., Porte, C., Dimeet, J. and Santella, R. M. (1996). Bioindicators of pollutant exposure in the northwestern Mediterranean sea. *Marine Ecology-Progress Series*. **131**, 125-141.
- Burgeot, T., Bocquene, G., Truquet, P., Ledean, L. and Galgani, F. (1994). Induction of Erod Activity in Red Mullet (*Mullus-Barbatus*) Along the French Mediterranean Coasts. *Science of the Total Environment*. **142**, 213-220.

- Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Science of the Total Environment*. **247**, 295-311.
- Calosi, P., Ugolini, A. and Morritt, D. (2005). Physiological responses to hyposmotic stress in the supralittoral amphipod *Talitrus saltator* (Crustacea: Amphipoda). *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*. **142**, 267-275.
- Calow, P. (1989). Proximate and ultimate responses to stress in biological systems. *Biological Journal of the Linnean Society*. **37**, 173-181.
- Camus, L., Davies, P. E., Spicer, J. I. and Jones, M. B. (2004). Temperature-dependent physiological response of *Carcinus maenas* exposed to copper. *Marine Environmental Research*. **58**, 781-785.
- Camus, L., Jones, M. B., Borseth, J. F., Grosvik, B. E., Regoli, F. and Depledge, M. H. (2002a). Total oxyradical scavenging capacity and cell membrane stability of haemocytes of the Arctic scallop, *Chlamys islandicus*, following benzo(a)pyrene exposure. *Marine Environmental Research*. **54**, 425-430.
- Camus, L., Jones, M. B., Borseth, J. F., Regoli, F. and Depledge, M. H. (2002b). Heart rate, respiration and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to polycyclic aromatic compounds via sediment and injection. *Aquatic Toxicology*. **61**, 1-13.
- Cannon, W. B. (1935). Stresses and strains of homeostasis. *American Journal of Medical Science*. **189**, 1-14.
- Canty, M. N., Hagger, J. A., Moore, R. T. B., Cooper, L. and Galloway, T. S. (2007). Sublethal impact of short term exposure to the organophosphate pesticide azamethiphos in the marine mollusc *Mytilus edulis*. *Marine Pollution Bulletin*. **54**, 396-402.
- Charmantier, G., Gimenez, L., Charmantier-Daures, M. and Anger, K. (2002). Ontogeny of osmoregulation, physiological plasticity and larval export strategy in the grapsid crab *Chasmagnathus granulata* (Crustacea, Decapoda). *Marine Ecology-Progress Series*. **229**, 185-194.
- Charmantier, G., Haond, C., Lignot, J. H. and Charmantier-Daures, M. (2001). Ecophysiological adaptation to salinity throughout a life cycle: A review in homarid lobsters. *Journal of Experimental Biology*. **204**, 967-977.
- Cheung, C. C. C., Zheng, G. J., Li, A. M. Y., Richardson, B. J. and Lam, P. K. S. (2001). Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. *Aquatic Toxicology*. **52**, 189-203.
- Cheung, V. V., Wedderburn, R. J. and Depledge, M. H. (1998). Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong. *Marine Environmental Research*. **46**, 237-241.
- Clark, P. F., Neale, M. and Rainbow, P. S. (2001). A morphometric analysis of regional variation in *Carcinus* Leach, 1814 (Brachyura : Portunidae : Carcininae) with particular reference to the status of the two species *C. maenas* (Linnaeus, 1758) and *C. aestuarii* Nardo, 1847. *Journal of Crustacean Biology*. **21**, 288-303.
- Clarke, K. R. and Warwick, R. M. (2001). *Change in marine communities: An approach to statistical analysis and interpretation 2nd edition*. PRIMER-E.
- Clarke, R. B., Frid, C. and Attrill, M. J. (2001). *Marine Pollution*. Oxford University Press.
- Coder, D. M. (1997). *Current protocols in flow cytometry*. John Wiley & Sons Ltd.
- Collazos, M. E., Ortega, E. and Barriga, C. (1994). Effect of temperature on the immune system of a cyprinid fish (*Tinca tinca*, L). Blood phagocyte function at low temperature. *Fish & Shellfish Immunology*. **4**, 231-238.

- Conover, R. J. (1966). Assimilation of organic matter by zooplankton. *Limnology and Oceanography*. **11**, 338-345.
- Constantino, P. J., Budd, E. D. and Gare, N. F. (1995). Enumeration of viable *Candida albicans* blastophores using Tetrabromofluorescein (Eosin Y) and flow cytometry. *Cytometry*. **19**, 370-375.
- Cossu, C., Doyotte, A., Jacquin, M. C., Babut, M., Exinger, A. and Vasseur, P. (1997). Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels and lipid peroxidation in freshwater bivalves, *Unio tumidus*, as biomarkers of aquatic contamination in field studies. *Ecotoxicology and Environmental Safety*. **38**, 122-131.
- Cotton, P. A., Rundle, S. D. and Smith, K. A. (2004). Trait compensation in marine gastropods: shell shape, avoidance behaviour, and susceptibility to predation. *Ecology* **85**, 1581-1584.
- Cowling, J. E., Spicer, J. I., Weeks, J. M. and Gaston, K. J. (2003). Environmental tolerances of an invasive terrestrial amphipod, *Arcitalitrus dorrieni* (Hunt) in Britain *Comparative Biochemistry & Physiology Part A*. **136**, 735-747.
- Crothers, J. H. (1967). The biology of the shore crab *Carcinus maenas* (L). 1. The background -anatomy, growth and life history. *Field Studies*. **2**, 407-434.
- Crothers, J. H. (1968). The biology of the shore crab *Carcinus maenas* (L). 2. The life of the adult crab. *Field Studies*. **2**, 579-614.
- Crowe, T. P. (1999). Limits to generality: seasonal and temporal variation in dispersal of an intertidal gastropod. *Journal of Experimental Marine Biology and Ecology*. **232**, 177-196.
- Cumberlidge, M., Uglow, R.F (1977a). Size, temperature and scaphognathite frequency-dependent variations of ventilation volumes in *Carcinus maenas* (L.). *Journal of Experimental Marine Biology and Ecology*. **30**, 85-93.
- Cumberlidge, N., Uglow, R.F. (1977b). Heart and scaphognathite activity in the shore crab *Carcinus maenas* (L.). *Journal of Experimental Marine Biology and Ecology*. **28**, 87-107.
- Da Ros, L., Meneghetti, F. and Nasci, C. (2002). Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy). *Marine Environmental Research*. **54**, 817-822.
- Dare, P. and Edwards, D. B. (1981). Underwater television observations on the intertidal movements of shore crabs, *Carcinus maenas*, across a mudflat. *Journal of the Marine Biological Association of the United Kingdom*. **61**, 107-116.
- Davenport, J. and Davenport, J. L. (2005). Effects of shore height, wave exposure and geographical distance on thermal niche width of intertidal fauna. *Marine Ecology Progress Series*. **292**, 41-50.
- Davenport, J. and Wong, T. M. (1987). Responses of adult mud crabs (*Scylla serrata*) (Forsk.) to salinity and low oxygen tension. *Comparative Biochemistry and Physiology Part A: Physiology*. **86**, 43-47.
- Dawirs, R. (1983). Respiration, energy balance and development during growth and starvation of *Carcinus maenas* L. larvae (Decapoda: Portunidae). *Journal of Experimental Marine Biology & Ecology*. **69**, 105-128.
- de Duve, C. (1983). Lysosomes revisited. *European Journal of Biochemistry*. **137**, 391-397.
- de Duve, C. and Wattiaux, R. (1966). Functions of lysosomes. *Annual Review of Physiology*. **28**, 435-492.
- deCarvalho, T. N., Watson, P. J. and Field, S. A. (2004). Costs increase as ritualized fighting progresses within and between phases in the sierra dome spider, *Neriene litigiosa*. *Animal Behaviour*. **68**, 473-482.

- Denny, M. W., Miller, L. P. and Harley, C. D. G. (2006). Thermal stress on intertidal limpets: long-term hindcasts and lethal limits. *Journal of Experimental Biology*. **209**, 2420-2431.
- Depledge, M. H. (1984). Changes in Cardiac Activity, Oxygen-Uptake and Perfusion Indexes in *Carcinus-Maenas* (L) Exposed to Crude-Oil and Dispersant. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*. **78**, 461-466.
- Depledge, M. H. (1985). The Influence of Nutritional State on the Circulatory and Respiratory Physiology of the Shore Crab, *Carcinus-Maenas*. *Journal of the Marine Biological Association of the United Kingdom*. **65**, 69-78.
- Depledge, M. H. (1989). Studies on Copper and Iron Concentrations, Distributions and Uptake in the Brachyuran *Carcinus-Maenas* (L) Following Starvation. *Ophelia*. **30**, 187-197.
- Depledge, M. H. (1992). On the tidal, diurnal and seasonal modulation of the endogenous cardiac activity rhythms in the shore crab (*Carcinus maenas* L.). *Ophelia*. **XVIII**, 53-67.
- Depledge, M. H. (1994). The rationale basis for the use of biomarkers as ecotoxicological tools. In: Fossi, M. C. and Leonzio, C. (Eds), *Non-destructive biomarkers in vertebrates*, Lewis Publishers.
- Depledge, M. H. and Andersen, B. B. (1990). A Computer-Aided Physiological Monitoring-System for Continuous, Long-Term Recording of Cardiac Activity in Selected Invertebrates. *Comparative Biochemistry and Physiology a-Physiology*. **96**, 473-477.
- Depledge, M. H. and Bjerregaard, P. (1989). Haemolymph protein-composition and copper levels in decapod crustaceans. *Helgolander Meeresuntersuchungen*. **43**, 207-223.
- Deretic, V. (2006). Autophagy as an immune defense mechanism. *Current Opinion in Immunology*. **18**, 375-382.
- Dexiang, C. and Ainsworth, A. J. (1991). Effect of temperature on the immune system of channel catfish (*Ictalurus punctatus*)—II. Adaptation of anterior kidney phagocytes to 10[degree sign]C. *Comparative Biochemistry and Physiology Part A: Physiology*. **100**, 913-918.
- Dissanayake, A. (2001). The evaluation of a multibiomarker approach in assessing environmental contamination in the field. *MRes Thesis. University of Plymouth*.
- Dissanayake, A. and Galloway, T. S. (2004). Evaluation of fixed wavelength fluorescence and synchronous fluorescence spectrophotometry as a biomonitoring tool of environmental contamination. *Marine Environmental Research*. **58**, 281-285.
- Dissanayake, A., Galloway, T. S. and Jones, M. B. (2006a). Does size really matter? An evaluation of ontogenetic differences in the biomarker responses of *Carcinus maenas* (L.) to a priority pollutant. *Marine Environmental Research*. **62**, 400-401.
- Dissanayake, A., Galloway, T. S. and Jones, M. B. (2006b). Does size really matter? Ontogenetic differences in 'physiological competency' in *Carcinus maenas* (L.). *In Prep*.
- Doyotte, A., Cossu, C., Jacquin, M. C., Babut, M. and Vasseur, P. (1997). Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology*. **39**, 93-110.
- Earley, R. L., Hsu, Y. and Wolf, L. L. (2000). The Use of Standard Aggression Testing Methods to Predict Combat Behaviour and Contest Outcome in *Rivulus marmoratus* Dyads (Teleostei: Cyprinodontidae). *Ethology*. **106**, 743-761.

- Engel, D. W., Brouwer, M. and McKenna, S. (1993). Hemocyanin Concentrations in Marine Crustaceans as a Function of Environmental-Conditions. *Marine Ecology-Progress Series*. **93**, 235-244.
- Engel, P. and Jones, J. D. (1978). Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD⁺ in alkaline hydrazine buffers: improved conditions for the assay of l-glutamate, l-lactate and other metabolites. *Analytical Biochemistry*. **88**, 475-484.
- Epifanio, C. E., Dittel, A. I., Rodriguez, R. A. and Targett, T. E. (2003). The role of macroalgal beds as nursery habitat for juvenile blue crabs, *Callinectes sapidus*. *Journal of Shellfish Research*. **22**, 881-886.
- Etxeberria, M., Cajaraville, M. P. and Marigomez, I. (1995). Changes in Digestive Cell Lysosomal Structure in Mussels as Biomarkers of Environmental-Stress in the Urdaibai Estuary (Biscay Coast, Iberian Peninsula). *Marine Pollution Bulletin*. **30**, 599-603.
- Evans, P. and Halliwell, B. (2001). Micronutrients: oxidant/antioxidant status. *British Journal Of Nutrition*. **85**, 67-74.
- Faber, D. B. and Baylis, J. R. (1993). Effects of body size on agonistic encounters between male jumping spiders (Araneae: Saltidae). *Animal Behaviour*.
- Fillmann, G., Watson, G. M., Francioni, E., Readman, J. W. and Depledge, M. H. (2002). A non-destructive assessment of the exposure of crabs to PAH using ELISA analyses of their urine and haemolymph. *Marine Environmental Research*. **54**, 823-828.
- Finn, P. F. and Dice, F. J. (2006). Proteolytic and lipolytic responses to starvation. *Nutrition*. **22**, 830-844.
- Fitzpatrick, P. J., Ohalloran, J., Sheehan, D. and Walsh, A. R. (1997). Assessment of a glutathione S-transferase and related proteins in the gill and digestive gland of *Mytilus edulis* (L), as potential organic pollution biomarkers. *Biomarkers*. **2**, 51-56.
- Fossi, M. C., Leonzio, C. and Peakall, D. B. (1994). The use of Nondestructive biomarkers in the hazard assessments of vertebrate populations. In: Fossi, M. C. and Leonzio, C. (Eds), *Non-destructive Biomarkers in Vertebrates*, Lewis Publishers, pp. 3-34.
- Frenzilli, G., Nigro, M., Scarcelli, V., Gorbi, S. and Regoli, F. (2001). DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel, *Mytilus galloprovincialis*: a field study in a highly eutrophicated coastal lagoon. *Aquatic Toxicology*. **53**, 19-32.
- Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Cheung, V. V., Lowe, D., Depledge, M. H. and Jones, M. B. (2004a). The ECOMAN project - a novel approach to defining sustainable ecosystem function. (*In press*).
- Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Lowe, D., Jones, M. B. and Depledge, M. H. (2004b). Ecosystem management bioindicators: the ECOMAN project - a multi-biomarker approach to ecosystem management. *Marine Environmental Research*. **58**, 233-237.
- Galloway, T. S., Brown, R. J., Browne, M. A., Dissanayake, A., Lowe, D., Jones, M. B. and Depledge, M. H. (2004c). A multibiomarker approach to environmental assessment. *Environmental Science & Technology*. **38**, 1723-1731.
- Galloway, T. S. and Depledge, M. H. (2001). Immunotoxicity in invertebrates: Measurement and ecotoxicological relevance. *Ecotoxicology*. **10**, 5-23.
- Galloway, T. S., Sanger, R. C., Smith, K. L., Fillmann, G., Readman, J. W., Ford, T. E. and Depledge, M. H. (2002). Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. *Environmental Science & Technology*. **36**, 2219-2226.
- Gammell, M. P. and Hardy, I. C. W. (2003). Contest duration: sizing up the opposition? *Trends in Ecology & Evolution*. **18**, 491-493.

- Geracitano, L. A., Monserrat, J. M. and Bianchini, A. (2004). Oxidative stress in *Laeonereis acuta* (Polychaeta, Nereididae): environmental and seasonal effects. *Marine Environmental Research*. **58**, 625-630.
- Gherardi, F. (2006). Fighting behaviour in hermit crabs: the combined effect of resource-holding potential and resource value in *Pagurus longicarpus*. *Behavioral Ecology and Sociobiology*. **59**, 500-510.
- Gnaiger, E. (1983). Heat dissipation and energetic efficiency in animal aobixibiosis: economy contra power. *Journal of Experimental Zoology*. **228**, 471-490.
- Google (2007). Google Earth.
- Gray, J. S. (1989). Effects of environmental stress on species rich assemblages. *Biological Journal of the Linnean Society*. **37**, 19-32.
- Griffin, S. P. and Bhagooli, R. (2004). Measuring antioxidant potential in corals using the FRAP assay. *Journal of Experimental Marine Biology and Ecology*. **302**, 201-211.
- Grime, J. P. (1989). The stress debate: symptom of impending synthesis? *Biological Journal of the Linnean Society*. **37**, 3-17.
- Grundy, M. M., Moore, M. N., Howell, S. M. and Ratcliffe, N. A. (1996). Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology*. **34**, 273-290.
- Gutmann, I. and Wahlefeld, A. W. (1974). L-lactate determination with lactate dehydrogenase and NAD⁺. In: Bergmeyer, H. U. (Ed), *Methods of Enzymatic Analysis*, Academic Press, pp. 464-468.
- Halliwell, B. and Gutteridge, J. M. C. (1989). *Free radicals in biology and medicine*, Third edition edn. Oxford University Press.
- Halliwell, B. and Gutteridge, J. M. C. (1995). The definition and measurement of antioxidants in biological systems. *Free Radical Biology and Medicine*. **18**, 125-126.
- Hames, B. D. and Hooper, N. M. (2000). *Instant Notes: Biochemistry*, Second edition edn. BIOS Scientific Publishers Limited.
- Harding, J. M., Couturier, C., Parsons, G. J. and Ross, N. W. (2004). Evaluation of the neutral red assay as a stress response indicator in cultivated mussels (*Mytilus* spp.) in relation to post-harvest processing activities and storage conditions. *Aquaculture*. **231**, 315-326.
- Hatch, T. (1962). Changing objectives in occupational health. *Industrial Health Journal*. **62**, 1 - 7.
- Hawkins, A. J. S., Jones, M. B. and Marsden, I. D. (1982). Aerial and aquatic respiration in two mud crab crabs, *Helice crassa* Dana (Grapsidae) and *Macrophthalmus hirtipes* (Jacquinot) (Ocypodidae), in relation to habitat. *Comparative Biochemistry and Physiology*. **73A**, 341-347.
- Hayward, P. J. and Ryland, J. S. (1990). *The marine fauna of the British Isles & North-west Europe*.
- Haywood, M. D. E., Heales, D. S., Kenyon, R. A., Loneragan, N. R. and Vance, D. J. (1998). Predation of juvenile tiger prawns in a tropical Australian estuary. *Marine Ecology-Progress-Series*. **162**, 201-214.
- Hebel, D. K., Jones, M. B. and Depledge, M. H. (1997). Responses of crustaceans to contaminant exposure: A holistic approach. *Estuarine Coastal and Shelf Science*. **44**, 177-184.
- Hegaret, H., Wikfors, G. H. and Soudant, P. (2003). Flow cytometric analysis of haemocytes from eastern oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation: II. Haemocyte functions: aggregation, viability, phagocytosis, and respiratory burst. *Journal of Experimental Marine Biology and Ecology*. **293**, 249-265.

- Hellou, J. and Upshall, C. (1995). Monocyclic Aromatic Hydrocarbons in Bile of Flounder Exposed to a Petroleum Oil. *International Journal of Environmental Analytical Chemistry*. **60**, 101 - 111.
- Hochachka, P. W. and Somero, G. N. (2002). *Biochemical adaptation: Mechanism and process in physiological evolution*. Oxford University Press.
- Holdich, D. M., Harlioglu, M. M. and Firkins, I. (1997). Salinity Adaptations of Crayfish in British Waters with Particular Reference to *Austropotamobius pallipes*, *Astacus leptodactylus* and *Pacifastacus leniusculus*. *Estuarine, Coastal and Shelf Science*. **44**, 147-154.
- Hunter, E. and Naylor, E. (1993). Intertidal Migration by the Shore Crab *Carcinus Maenas*. *Marine Ecology-Progress Series*. **101**, 131-138.
- Huntingford, F. A. and Turner, A. K. (1987). *Animal conflict*. Chapman & Hall.
- Hurd, P. L. (1997). Is signalling of fighting ability costlier for weaker individuals? *Journal of Theoretical Biology*. **184**, 83-88.
- Hurd, P. L. (2006). Resource holding potential, subjective resource value, and game theoretical models of aggressiveness signalling. *Journal of Theoretical Biology*. **241**, 639-648.
- ICES, M. H. C. (2004). Report of the working group on biological effects of contaminants (WGBEC), ICES pp. 1-86.
- Jacob, J. (1996). The significance of polycyclic aromatic hydrocarbons as environmental carcinogens. *Pure and Applied Chemistry*. **62**, 301-308.
- Jha, A. N. (2004). Genotoxicological studies in aquatic organisms: an overview. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. **552**, 1-17.
- Johnstone, M. A., Elder, H. Y. and Spencer-Davies, P. (1973). Cytology of *Carcinus haemocytes* and their function in carbohydrate metabolism. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*. **46**, 569-581.
- Jones, M. B. (1981). Effect of temperature, season, and stage of life cycle on salinity tolerance of the estuarine crab *Helice crassa* Dana (Grapsidae). *Journal of Experimental Marine Biology and Ecology*. **52**, 271-282.
- Kaiser, M. J., Hughes, R. N. and Reid, D. G. (1990). Chelal morphometry, prey-size selection and aggressive competition in green and red forms of *Carcinus maenas* (L.). *Journal of Experimental Marine Biology & Ecology*. **140**, 121-134.
- Kashif, W., Siddiqi, N., Erhan Dincer, H., Dincer, A. P. and Hirsch, S. (2003). Proteinuria: How to evaluate an important finding. *Cleveland Clinic Journal of Medicine*. **70**, 535-547.
- King, A. J., Readman, J. W. and Zhou, J. L. (2004). Dynamic behaviour of polycyclic aromatic hydrocarbons in Brighton marina, UK. *Marine Pollution Bulletin*. **48**, 229-239.
- Koehn, R. K. and Bayne, B. L. (1989). Towards a physiological and genetic understanding of the energetics of the stress response. *Biological Journal of the Linnean Society*. **37**, 157-171.
- Kosmala, A., Migeon, B., Flammarion, P. and Garric, J. (1998). Impact assessment of a wastewater treatment plant effluent using the fish biomarker ethoxyresorufin-O-deethylase: Field and on-site experiments. *Ecotoxicology and Environmental Safety*. **41**, 19-28.
- Krahn, M. M., Myers, M. S., Burrows, D. G. and Malins, D. C. (1984). Determination of metabolites of xenobiotics in the bile of fish from polluted waterways. *Xenobiotica*. **14**, 633-646.
- Krang, A.-S. and Ekerholm, M. (2006). Copper reduced mating behaviour in male shore crabs (*Carcinus maenas* (L.)). *Aquatic Toxicology*. **80**, 60-69.
- Kruskal, W. H. and Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association*. **47**, 583-621.

- Kunst, A., Draeger, B. and Ziegerhorn, J. (1981). UV methods with hexokinase and glucose-6-phosphate dehydrogenase. *In: Bergmeyer, H. U. (Ed), Methods of Enzymatic Analysis*, Academic Press.
- Kuris, A. M., Lafferty, K. D. and Torchin, M. E. (2005). Biological control of the European green crab, *Carcinus maenas*: natural enemy evaluation and analysis of host specificity. *Second International Symposium on Biological Control of Arthropods*, pp. 102-115.
- Lailvaux, S. P., Hathway, J., Pomfret, J. and Knell, R. J. (2005). Horn size predicts physical performance in the beetle *Euoniticellus intermedius* (Coleoptera: Scarabaeidae). *Functional Ecology*. **19**, 632-639.
- Lau, P. S., Wong, H. L. and Garrigues, P. (2004). Seasonal variation in antioxidative responses and acetylcholinesterase activity in *Perna viridis* in eastern oceanic and western estuarine waters of Hong Kong. *Continental Shelf Research*. **24**, 1969-1987.
- Law, R. J., Dawes, V. J., Woodhead, R. J. and Matthiessen, P. (1997). Polycyclic aromatic hydrocarbons (PAH) in seawater around England and Wales. *Marine Pollution Bulletin*. **34**, 306-322.
- Law, R. J., Kelly, C. A., Baker, K. L., Langford, K. H. and Bartlett, T. (2002). Polycyclic aromatic hydrocarbons in sediments, mussels and crustacea around a former gasworks site in Shoreham-by-Sea, UK. *Marine Pollution Bulletin*. **44**, 903-911.
- Lawson, S. L., Jones, M. B. and Moate, R. M. (1995). Effect of copper on the ultrastructure of the gill epithelium of *Carcinus maenas* (decapoda: Brachyura). *Marine Pollution Bulletin*. **31**, 63-72.
- Le Morvan, C., Clerton, P., Deschaux, P. and Troutaud, D. (1997). Effects of environmental temperature on macrophage activities in carp. *Fish & Shellfish Immunology*. **7**, 209-212.
- Lee, S. Y. (1995). Cheliped size and structure: the evolution of a multi-functional decapod organ. *Journal of Experimental Marine Biology and Ecology*. **193**, 161-176.
- Legeay, A. and Massabuau, J. C. (2000). The ability to feed in hypoxia follows a seasonally dependent pattern in shore crab *Carcinus maenas*. *Journal of Experimental Marine Biology and Ecology*. **247**, 113-129.
- Leiser, J. K., Gagliardi, J. L. and Itzkowitz, M. (2004). Does size matter? Assessment and fighting in small and large size matched pairs of adult male convict cichlids. *Journal of Fish Biology*. **64**, 1339-1350.
- Lesser, M. P. and Kruse, V. A. (2004). Seasonal temperature compensation in the horse mussel, *Modiolus modiolus*: metabolic enzymes, oxidative stress and heat shock proteins. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*. **137**, 495-504.
- Levine, B. (2005). Eating Oneself and Uninvited Guests: Autophagy-Related Pathways in Cellular Defense. *Cell*. **120**, 159-162.
- Levitan, D. R. (1989). Density-dependent size regulation in *Diadema antillarum*: effects on fecundity and survivorship., *Ecology* pp. 1414-1424.
- Levitan, D. R. (1991). Skeletal changes in the test and jaws of the sea urchin *Diadema antillarum* in response to food limitation. *Marine Biology*. **111**, 431-435.
- Liess, M., Schulz, R., Liess, M. H. D., Rother, B. and Kreuzig, R. (1999). Determination of insecticide contamination in agricultural headwater streams. *Water Research*. **33**, 239-247.
- Livingstone, D. R. (1991). Organic xenobiotic metabolism in marine invertebrates. *In: Gilles, R. (Ed), Advances in Comparative and Environmental Physiology*, Springer-Verlag, pp. 46-185.

- Livingstone, D. R. (1992). Persistent Pollutants in Marine Invertebrates. *In: Walker, C. H. a. L., D.R. (Ed), Persistent Pollutants in Marine Ecosystems*, Pergamon Press.
- Livingstone, D. R. (1993). Biotechnology and Pollution Monitoring - Use of Molecular Biomarkers in the Aquatic Environment. *Journal of Chemical Technology and Biotechnology*. **57**, 195-211.
- Livingstone, D. R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology. Part A*. **120**, 43-49.
- Livingstone, D. R., Donkin, P. and Walker., C. H. (1992). Pollutants in marine ecosystems: an overview. *In: Walker, C. H. a. L., D.R. (Ed), Persistent Pollutants in Marine Ecosystems*, SETAC special publication. Pergamon Press, pp. 235-263.
- Lowe, D. M., Fossato, V. U. and Depledge, M. H. (1995a). Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: An in vitro study. *Marine Ecology-Progress Series*. **129**, 189-196.
- Lowe, D. M., Moore, M. N. and Evans, B. M. (1992). Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. *Aquatic Toxicology*. **91**, 135-140.
- Lowe, D. M. and Pipe, R. K. (1994). Contaminant induced lysosomal membrane damage in marine mussel digestive cells - an in-vitro study. *Aquatic Toxicology*. **30**, 357-365.
- Lowe, D. M., Soverchia, C. and Moore, M. N. (1995b). Lysosomal Membrane Responses in the Blood and Digestive Cells of Mussels Experimentally Exposed to Fluoranthene. *Aquatic Toxicology*. **33**, 105-112.
- Lugo, A. E. (1978). *Stress and Ecosystems*. U.S. Department of Energy Symposium Series CONF 77114. National Technical Information Services.
- Malanga, G., Estevez, M. S., Calvo, J., Abele, D. and Puntarulo, S. (2006). The effect of seasonality on oxidative metabolism in *Nacella (Patinigera) magellanica*. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*. **In Press, Corrected Proof**, 921.
- Maltby, L. (1999). Studying stress: The importance of organism-level responses. *Ecological Applications*. **9**, 431-440.
- Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F. and Rocher, B. (2004). Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a polluted area: major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione S-transferase. *Aquatic Toxicology*. **70**, 83-93.
- Mangum, C. P. (1983). Oxygen transport in the blood. *In: Mantel, L. H. (Ed), The Biology of Crustacea.*, Academic Press., pp. 373-419.
- Marigomez, I. and Baybay-Villacorta, L. (2003). Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. *Aquatic Toxicology*. **64**, 235-257.
- Marigomez, I., Orbea, A., Olabarrieta, I., Etxeberria, M. and Cajaraville, M. P. (1996). Structural changes in the digestive lysosomal system of sentinel mussels as biomarkers of environmental stress in mussel-watch programmes. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*. **113**, 291-297.
- Marsden, I. D., Newell, R.C., Ahsanullah, M. (1973). The effect of starvation on the metabolism of the shore crab, *Carcinus maenas*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*. **45**, 195-213.
- Martin, F., Beaugrand, J. P. and Laguë, P. C. (1997). The roles of hen's weight and recent experience on dyadic conflict outcome. *Behavioural Processes*. **41**.

- Massabuau, J. C. (2003). Primitive, and protective, our cellular oxygenation status? *Mechanisms of Ageing and Development*. **124**, 857-863.
- Matozzo, V., Ballarin, L. and Marin, M. G. (2002). In vitro effects of tributyltin on functional responses of haemocytes in the clam *Tapes philippinarum*. *Applied Organometallic Chemistry*. **16**, 169-174.
- Matozzo, V. and Marin, M. G. (2005). 4-Nonylphenol induces immunomodulation and apoptotic events in the clam *Tapes philippinarum*. *Marine Ecology-Progress Series*. **285**, 97-106.
- Matsumasa, M. and Murai, M. (2005). Changes in blood glucose and lactate levels of male fiddler crabs: effects of aggression and claw waving. *Animal Behaviour*. **69**, 569-577.
- Mayer, F. L., Vesteege, D. L., McKee, M. J., Folmar, L. C., Graney, R. L., McCume, D. C. and Rattner, B. A. (1992). Physiological and Nonspecific Biomarkers. In: Huggett, R. J., Kimerle, R.A., Mehrle, P.M., Bergman., H.L. (Ed), *Biomarkers; Biochemical, Physiological and Histological markers of anthropogenic stress.*, SETAC, Lewis Publishers., pp. 5-85.
- Maynard Smith, J. (1982). *Evolution and the theory of games*. Cambridge University Press.
- Mayr, E. (1961). Cause and effect in biology. *Science*. **134**, 1501-1506.
- McGaw, I. J., Kaiser, M. J., Naylor, E. and Hughes, R. N. (1992). Intraspecific morphological variation related to the moult-cycle in colour forms of the shore crab *Carcinus maenas*. *Journal of Zoological Society of London*. **228**, 351-359.
- McGaw, I. J. and Naylor, E. (1992a). Distribution and rhythmic locomotor patterns of estuarine and open-shore populations of *Carcinus maenas*. *Journal of the Marine Biological Association U.K.* **72**, 599-609.
- McGaw, I. J. and Naylor, E. (1992b). The effect of shelter on salinity preference behaviour of the shore crab *Carcinus maenas*. *Marine Behaviour and Physiology*. **21**, 145-152.
- McGaw, I. J. and Naylor, E. (1992c). Salinity preference of the shore crab *Carcinus maenas* in relation to colouration during intermoult and to prior acclimation. *Journal of Experimental Marine Biology and Ecology*. **155**, 145-159.
- Mehrle, P. M. and Mayer, F. L. (1980). Biochemistry/Physiology. In: Huggett, R. J., Kimerle, R. A., Mehrle, P. M. and Bergman., H. L. (Eds), *Biomarkers; Biochemical, Physiological and Histological markers of anthropogenic stress.*, SETAC, Lewis Publishers., pp. 5-85.
- Meigh, H. C. (2000). Models of oxidative stress induced by disease or pollution in invertebrates and vertebrates, *School of Biological Sciences*, University of Plymouth, pp. 282.
- Meijer, A. J. and Codogno, P. (2004). Regulation and role of autophagy in mammalian cells. *The International Journal of Biochemistry & Cell Biology Autophagy and Cell Fate*. **36**, 2445-2462.
- Miner, B. G., Sultan, S. E., Morgan, S. G., Padilla, D. K. and Relyea, R. A. (2005). Ecological consequences of phenotypic plasticity. *Trends in Ecology & Evolution*. **20**, 685-692.
- Monari, M., Matozzo, V., Foschi, J., Cattani, O., Serrazanetti, G. P. and Marin, M. G. (2007). Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*. *Fish & Shellfish Immunology*. **22**, 98-114.
- Moore, M. N. (2004). Diet restriction induced autophagy: A lysosomal. protective system against oxidative- and pollutant-stress and cell injury. *Marine Environmental Research*. **58**, 603-607.
- Moore, M. N., Allen, J. I. and Somerfield, P. J. (2006). Autophagy: Role in surviving environmental stress. *Marine Environmental Research Pollutant Responses in Marine Organisms (PRIMO 13)*. **62**, S420-S425.

- Morgan, S. G. (1987). Adaptive significance of hatching rhythms and dispersal patterns of estuarine crab larvae: avoidance of physiological stress by larval export? *Journal of Experimental Marine Biology and Ecology*. **113**, 71-78.
- Morgan, S. G., Zimmer Faust, R. K., Heck, K. L. and Coen, L. D. (1996). Population regulation of blue crabs *Callinectes sapidus* in the northern Gulf of Mexico: Postlarval supply. *Marine Ecology-Progress Series*. **133**, 73-88.
- Morritt, D. and Spicer, J. I. (1999). Developmental ecophysiology of the beachflea *Orchestia gammarellus* (Pallas) (Crustacea : Amphipoda : Talitridae) III. Physiological competency as a possible explanation for timing of hatchling release. *Journal of Experimental Marine Biology and Ecology*. **232**, 275-283.
- MSC-E (2001). Polycyclic aromatic hydrocarbon emission inventories and emission expert estimates, MSC-E,.
- Myers, J. and Ashley, E. (1997). Dangerous curves. A perspective on exercise, lactate, and the anaerobic threshold. *Chest*. **111**, 787-795.
- Naes, K., Knutzen, J. and Berglind, L. (1995). Occurrence of PAH in Marine Organisms and Sediments from Smelter Discharge in Norway. *Science of the Total Environment*. **163**, 93-106.
- Naylor, C., Maltby, L., Crane, P. (1989). Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. *Hydrobiologia*. **188/189**, 517-523.
- Naylor, E. (1962). Seasonal changes in a population of *Carcinus maenas* (L.) in the littoral zone. *Journal of Animal Ecology*. **31**, 601-609.
- Neat, F. C., Huntingford, F. A. and Beveridge, M. M. C. (1998). Fighting and assessment in male cichlid fish: the effects of asymmetries in gonadal state and body size. *Animal Behaviour*. **55**, 883-891.
- Newell, R. C., Ahsanullah, M. and Pye, V. I. (1972). Aerial and aquatic respiration in the shore crab, *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology*. **57A**, 239-252.
- Nicholson, S. (1999). Cytological and physiological biomarker responses from green mussels, *Perna viridis* (L.) transplanted to contaminated sites in Hong Kong coastal waters. *Marine Pollution Bulletin*. **39**, 261-268.
- Nicholson, S. (2001). Ecocytological and toxicological responses to copper in *Perna viridis* (L.) (Bivalvia : Mytilidae) haemocyte lysosomal membranes. *Chemosphere*. **45**, 399-407.
- Nicholson, S. (2003). Lysosomal membrane stability, phagocytosis and tolerance to emersion in the mussel *Perna viridis* (Bivalvia : Mytilidae) following exposure to acute, sublethal, copper. *Chemosphere*. **52**, 1147-1151.
- Nickerson, K. and van Holde, K. E. (1983). A comparison of molluscan and arthropod haemocyanin, I. Circular dichromism and absorption spectra. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*. **39**, 855-872.
- Nikoskelainen, S., Bylund, G. and Lilius, E.-M. (2004). Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. *Developmental & Comparative Immunology*. **28**, 581-592.
- Niyogi, S., Biswas, S., Sarker, S. and Datta, A. G. (2001a). Antioxidant enzymes in brackishwater oyster, *Saccostrea cucullata* as potential biomarkers of polyaromatic hydrocarbon pollution in Hooghly Estuary (India): seasonality and its consequences. *The Science of The Total Environment*. **281**, 237-246.
- Niyogi, S., Biswas, S., Sarker, S. and Datta, A. G. (2001b). Seasonal variation of antioxidant and biotransformation enzymes in barnacle, *Balanus balanoides*, and their relation with polyaromatic hydrocarbons. *Marine Environmental Research*. **52**, 13-26.
- Nollet, L. M. L. (2006). *Chromatographic analysis of the environment*. CRC Taylor & Francis.
- Office, M. (2007). England and Wales precipitation, Crown Copyright.

- OSPAR (2001). Polyaromatic hydrocarbons, *Hazardous substances series*, OSPAR Commission.
- Parker, G. A. (1974). Assessment strategy and the evolution of animal conflicts. *Journal of Theoretical Biology*. **47**, 223-243.
- Parry, H. E. and Pipe, R. K. (2004). Interactive effects of temperature and copper on immunocompetence and disease susceptibility in mussels (*Mytilus edulis*). *Aquatic Toxicology*. **69**, 311-325.
- Payne, R. J. H. and Pagel, M. (1997). Why do animals repeat displays? *Animal Behaviour*. **54**, 109-119.
- Petersen, G. and Hardy, I. C. W. (1996). The importance of being larger: parasitoid intruder-owner contests and their implications for clutch size. *Animal Behaviour*. **51**, 1363-1373.
- Piersma, T. and Drent, J. (2003). Phenotypic flexibility and the evolution of organismal design. *Trends in Ecology & Evolution*. **18**, 228-233.
- Piersma, T. and Lindstrom, A. (1997). Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends in Ecology & Evolution*. **12**, 134-138.
- Pott, P. (1775). *Chirurgical observations relative to the cataract, the polypus of the nose, the cancer of the scrotum, the different kinds of ruptures, and the mortification of the toes and feet*
- Price, T. D. (2006). Phenotypic plasticity, sexual selection and the evolution of colour patterns. *J Exp Biol*. **209**, 2368-2376.
- Prior, R. L. and Cao, G. (1999). In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology & Medicine*. **27**, 1173-1181.
- QHM (2006a). QHM (Newton Ferrers).
- QHM (2006b). QHM (Sutton Harbour).
- QHM (2006c). QHM Harbour Master (Bantham).
- Rainbow, P. S. (1997). Ecophysiology of trace metal uptake in crustaceans. *Estuarine Coastal and Shelf Science*. **44**, 169-175.
- Rainbow, P. S. and Black, W. H. (2001). Effects of changes in salinity on the apparent water permeability of three crab species: *Carcinus maenas*, *Eriocheir sinensis* and *Necora puber*. *Journal of Experimental Marine Biology and Ecology*. **264**, 1-13.
- Rainbow, P. S. and Black, W. H. (2002). Effects of changes in salinity and osmolality on the rate of uptake of zinc by three crabs of different ecologies. *Marine Ecology-Progress Series*. **244**, 205-217.
- Rainbow, P. S. and Black, W. H. (2005). Physicochemistry or physiology: cadmium uptake and effects of salinity and osmolality in three crabs of different ecologies. *Marine Ecology-Progress Series*. **286**, 217-229.
- Ramos-Vasconcelos, G. R., Cardoso, L. A. and Hermes-Lima, M. (2005). Seasonal modulation of free radical metabolism in estivating land snails *Helix aspersa*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. **140**, 165-174.
- Ratcliffe, N. A. and Rowley, A. F. (1979). A comparative synopsis of the structure and function of the blood cells of insects and invertebrates. *Developmental & Comparative Immunology*. **3**, 189-243.
- Readman, J. W., Fillmann, G., Tolosa, I., Bartocci, J., Villeneuve, J. P., Catinni, C. and Mee, L. D. (2002). Petroleum and PAH contamination of the Black Sea. *Marine Pollution Bulletin*. **44**, 48-62.
- Reeves, W. R., McDonald, T. J., Cizmas, L. and Donnelly, K. C. (2004). Partitioning and desorption behavior of polycyclic aromatic hydrocarbons from disparate sources. *Science of The Total Environment*. **332**, 183-192.

- Regoli, F. (1998). Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination and Toxicology*. **34**, 48-63.
- Regoli, F., Nigro, M., Chiantore, M. and Winston, G. W. (2002). Seasonal variations of susceptibility to oxidative stress in *Adamussium colbecki*, a key bioindicator species for the Antarctic marine environment. *Science of the Total Environment*. **289**, 205-211.
- Regoli, F., Nigro, M. and Orlando, E. (1998). Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology*. **40**, 375-392.
- Regoli, F. and Principato, G. (1995). Glutathione, Glutathione-Dependent and Antioxidant Enzymes in Mussel, *Mytilus-Galloprovincialis*, Exposed to Metals under Field and Laboratory Conditions - Implications for the Use of Biochemical Biomarkers. *Aquatic Toxicology*. **31**, 143-164.
- Reid, D. G., Abello, P., Kaiser, M. J. and Warman, C. G. (1997). Carapace colour, inter-moult duration and the behavioural and physiological ecology of the shore crab *Carcinus maenas*. *Estuarine Coastal and Shelf Science*. **44**, 203-211.
- Reid, D. G., Abello, P., McGaw, I. J. and Naylor, E. (1989). Phenotypic variation in sympatric crab populations. *Phenotypic responses and individuality in aquatic ectotherms*,. Dublin, pp. 40-60.
- Reid, D. G. and Aldrich, J. C. (1989). Variations in Response to Environmental Hypoxia of Different Color Forms of the Shore Crab, *Carcinus-Maenas*. *Comparative Biochemistry and Physiology a-Physiology*. **92**, 535-539.
- Rewitz, K., Styriahave, B., Depledge, M. H. and Andersen, O. (2004). Spatial and temporal distribution of shore crabs *Carcinus maenas* in a small tidal estuary (Looe Estuary, Cornwall, England). *Journal of Crustacean Biology*. **24**, 178-187.
- Rickwood, C. J. and Galloway, T. S. (2004). Acetylcholinesterase inhibition as a biomarker of adverse effect - A study of *Mytilus edulis* exposed to the priority pollutant chlorfenvinphos. *Aquatic Toxicology*. **67**, 45-56.
- Ringwood, A. H., Hoguet, J. and Keppler, C. J. (2002). Seasonal variation in lysosomal destabilization in oysters, *Crassostrea virginica*. *Marine Environmental Research*. **54**, 793-797.
- Roast, S. D., Widdows, J. and Jones, M. B. (1999a). Respiratory responses of the estuarine mysid *Neomysis integer* (Peracarida : Mysidacea) in relation to a variable environment. *Marine Biology*. **133**, 643-649.
- Roast, S. D., Widdows, J. and Jones, M. B. (1999b). Scope for growth of the estuarine mysid *Neomysis integer* (Peracarida : Mysidacea): effects of the organophosphate pesticide chlorpyrifos. *Marine Ecology-Progress Series*. **191**, 233-241.
- Robertson, R. F., Meagor, J. and Taylor, E. W. (2002). Specific dynamic action in the shore crab, *Carcinus maenas* (L.), in relation to acclimation temperature and to the onset of the emersion response. *Physiological and Biochemical Zoology*. **75**, 350-359.
- Roman, J. and Palumbi, S. (2004). A global invader at home: population structure of the green crab, *Carcinus maenas*, in Europe. *Molecular Ecology*. **13**, 2891-2898.
- Ronisz, D., Larsson, D. G. J. and Forlin, L. (1999). Seasonal variations in the activities of selected hepatic biotransformation and antioxidant enzymes in eelpout (*Zoarces viviparus*). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*. **124**, 271-279.
- Ruddock, P. J., Bird, D. J. and McCalley, D. V. (2002). Bile Metabolites of Polycyclic Aromatic Hydrocarbons in Three Species of Fish from the Severn Estuary. *Ecotoxicology and Environmental Safety*. **51**, 97-105.

- Ruddock, P. J., Bird, D. J., McEvoy, J. and Peters, L. D. (2003). Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in European eels *Anguilla anguilla* from United Kingdom estuaries. *Science of the Total Environment*. **301**, 105-117.
- Russell, M. P. (1998). Resource allocation plasticity in sea urchins: rapid, diet induced, phenotypic changes in the green sea urchin, *Strongylocentrotus droebachiensis* (Muller). *Journal of Experimental Marine Biology and Ecology*. **220**, 1-14.
- Sanchez-Paz, A., Garcia-Carreño, F., Hernandez-Lopez, J., Muhlia-Almazan, A. and Yepiz-Plascencia, G. (2007). Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). *Journal of Experimental Marine Biology and Ecology*. **340**, 184-193.
- Sanchez-Paz, A., Garcia-Carreño, F., Muhlia-Almazan, A., Peregrino-Uriarte, A. B., Hernandez-Lopez, J. and Yepiz-Plascencia, G. (2006). Usage of energy reserves in crustaceans during starvation: Status and future directions. *Insect Biochemistry and Molecular Biology Special Issue in Honour of John Law*. **36**, 241-249.
- Scarlett, A., Rowland, S. J., Canty, M., Smith, E. L. and Galloway, T. S. (2007). Method for assessing the chronic toxicity of marine and estuarine sediment-associated contaminants using the amphipod *Corophium volutator*. *Marine Environmental Research*. **63**, 457-470.
- Scott, G. R. and Sloman, K. A. (2004). The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology*. **68**, 369-392.
- Scottfordsmand, J. J. and Depledge, M. H. (1993). The Influence of Starvation and Copper Exposure on the Composition of the Dorsal Carapace and Distribution of Trace- Metals in the Shore Crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*. **106**, 537-543.
- Selye, H. (1946). The general adaptation syndrome and the diseases of adaptation. *Journal of Clinical Endocrinology*. **6**, 117-231.
- Selye, H. (1956). *The stress of life*. McGraw-Hill.
- Selye, H. (1973). The evolution of the stress concept. *American Science*. **61**, 692-699.
- Selye, H. (1976). *Stress in health and disease*. Butterworth Publishers.
- Sinclair, E. L. E., Thompson, M. B. and Seebacher, F. (2006). Phenotypic flexibility in the metabolic response of the limpet *Cellana tramoserica* to thermally different microhabitats. *Journal of Experimental Marine Biology and Ecology*. **335**, 131-141.
- Sloman, K. A. (2004). Tough toadfish: Surviving copper exposure. *J Exp Biol*. **207**, vii-
- Smith, V. J. and Ratcliffe, N. A. (1978). Host defence reactions of the shore crab, *Carcinus maenas* (L.), *in vitro*. *Journal of the Marine Biological Association of the United Kingdom*. **58**.
- Sneddon, L. U., Huntingford, F. A. and Taylor, A. C. (1997a). The influence of resource value on the agonistic behaviour of the shore crab, *Carcinus maenas* (L.). *Marine and Freshwater Behaviour and Physiology*. **30**, 225-237.
- Sneddon, L. U., Huntingford, F. A. and Taylor, A. C. (1997b). Weapon size versus body size as a predictor of winning in fights between shore crabs, *Carcinus maenas* (L.). *Behavioral Ecology and Sociobiology*. **41**, 237-242.
- Sneddon, L. U., Huntingford, F. A. and Taylor, A. C. (1998). Impact of an ecological factor on the costs of resource acquisition: fighting and metabolic physiology of crabs. *Functional Ecology*. **12**, 808-815.

- Sneddon, L. U., Taylor, A. C. and Huntingford, F. A. (1999). Metabolic consequences of agonistic behaviour: Crab fights in declining oxygen tensions. *Animal Behaviour*. **57**, 353-363.
- Sokolova, I. M. and Portner, H.-O. (2003). Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*, Gastropoda: Littorinidae) from different latitudes. *J Exp Biol*. **206**, 195-207.
- Sole, M., Porte, C., Biosca, X., Mitchelmore, C. L., Chipman, J. K., Livingstone, D. R. and Albaiges, J. (1996). Effects of the "Aegean Sea" oil spill on biotransformation enzymes, oxidative stress and DNA-Adducts in digestive gland of the mussel (*Mytilus edulis* L). *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*. **113**, 257-265.
- Spicer, J. I. and Baden, S. P. (2000). Natural variation in the concentrations of haemocyanin from three decapod crustaceans, *Nephrops norvegicus*, *Liocarcinus depurator* and *Hyas aranaeus*. *Marine Biology*. **136**, 55-61.
- Spicer, J. I. and Baden, S. P. (2001). Environmental hypoxia and haemocyanin between-individual variability in Norway lobsters *Nephrops norvegicus* (L.). *Marine Biology*. **139**, 727-734.
- Spicer, J. I. and Gaston, K. J. (1999). *Physiological diversity and its ecological implications*, 1 edn. Blackwell Science.
- Spicer, J. I. and Stromberg, J. O. (2002). Diel vertical migration and the haemocyanin of krill *Meganyctiphanes norvegica*. *Marine Ecology-Progress Series*. **238**, 153-162.
- Spicer, J. I. and Weber, R. E. (1991). Respiratory impairment in crustaceans and mollusks due to exposure to heavy-metals. *Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology*. **100**, 339-342.
- St-Jean, S. D., Pelletier, E. and Courtenay, S. C. (2002a). Hemocyte functions and bacterial clearance affected in vivo by TBT and DBT in the blue mussel *Mytilus edulis*. *Marine Ecology-Progress Series*. **236**, 163-178.
- St-Jean, S. D., Pelletier, E. and Courtenay, S. C. (2002b). Very low levels of waterborne butyltins modulate hemocyte function in the blue mussel *Mytilus edulis*. *Marine Ecology-Progress Series*. **236**, 155-161.
- Stillman, J. and Somero, G. (1996). Adaptation to temperature stress and aerial exposure in congeneric species of intertidal porcelain crabs (genus *Petrolisthes*): correlation of physiology, biochemistry and morphology with vertical distribution. *J Exp Biol*. **199**, 1845-1855.
- Strickland, P., Kang, D. and Sithisarankul, P. (1996). Polycyclic aromatic hydrocarbon metabolites in urine as biomarkers of exposure and effect. *Environmental Health Perspectives*. **104**, 927-932.
- Strickland, P. and Kang, D. H. (1999). Urinary 1-hydroxypyrene and other PAH metabolites as biomarkers of exposure to environmental PAH in air particulate matter. *Toxicology Letters*. **108**, 191-199.
- Styrishave, B., Aagaard, A. and Andersen, O. (1999). In situ studies on physiology and behaviour in two colour forms of the shore crab *Carcinus maenas* in relation to season. *Marine Ecology-Progress Series*. **189**, 221-231.
- Styrishave, B. and Andersen, O. (2000). Seasonal variations in hepatopancreas fatty acid profiles of two colour forms of shore crabs, *Carcinus maenas*. *Marine Biology*. **137**, 415-422.
- Styrishave, B., Petersen, M. F. and Andersen, O. (2000). Influence of cadmium accumulation and dietary status on fatty acid composition in two colour forms of shore crabs, *Carcinus maenas*. *Marine Biology*. **137**, 423-433.
- Sung, H.-H., Li, H.-C., Tsai, F.-M., Ting, Y.-Y. and Chao, W.-L. (1999). Changes in the composition of *Vibrio* communities in pond water during tiger shrimp (*Penaeus monodon*) cultivation and in the hepatopancreas of healthy and

- diseased shrimp. *Journal of Experimental Marine Biology and Ecology*. **236**, 261-271.
- Taylor, E. W., Butler, P. J., Sherlock, P. J. (1973). The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at different temperatures. *Journal of Comparative Physiology*. **86**, 95-115.
- Taylor, H. H. and Anstiss, J. M. (1999). Copper and haemocyanin dynamics in aquatic invertebrates. *Marine and Freshwater Research*. **50**, 907-931.
- Taylor, P. W., Hasson, O. and Clark, D. L. (2001). Initiation and resolution of jumping spider contests: roles for size, proximity, and early detection of rivals. *Behavioral Ecology and Sociobiology*. **50**, 403-413.
- Thorpe, K. E., Taylor, A. C. and Huntingford, F. A. (1995). How costly is fighting? Physiological effects of sustained exercise and fighting in swimming crabs, *Necora puber* (L) (Brachyura, Portunidae). *Animal Behaviour*. **50**, 1657-1666.
- Timbrell, J. A. (1995). *Introduction to Toxicology*. Taylor & Francis.
- Tonelli, M., Jose, P., Curhan, G., Sacks, F., Braunwald, E., Pfeffer, M. and Cholesterol and Recurrent Events (CARE) Trial Investigators (2006). Proteinuria, impaired kidney function, and adverse outcomes in people with coronary disease: analysis of a previously conducted randomised trial. *BMJ*. **332**, 1426-.
- Truchot, J. P. (1975). Factors controlling the *in vivo* and *in vitro* oxygen affinity of the hemocyanin in the crab *Carcinus maenas* (L.). *Respiration Physiology*. **24**, 173-189.
- Uglow, R. F. (1969). Haemolymph protein concentrations in portunid crabs - II. The effects of imposed fasting on *Carcinus maenas*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*. **31**, 959-967.
- Underwood, A. J. (1989). The Analysis of Stress in Natural Populations. *Biological Journal of the Linnean Society*. **37**, 51-78.
- Underwood, A. J. (2005). *Experiments in Ecology: Their logical design and interpretation using analysis of variance*. Cambridge University Press.
- Van der Oost, R., Van Schooten, F. J., Ariese, F., Heida, H., Satumalay, K. and Vermeulen, N. P. E. (1994). Bioaccumulation, biotransformation and DNA-binding of PAHs in feral eel (*Anguilla anguilla*) exposed to polluted sediments - a field survey. *Environmental Toxicology and Chemistry*. **13**, 859-870.
- Van der Oost, R., Vindimian, E., Van den Brink, P. J., Satumalay, K., Heida, H. and Vermeulen, N. P. E. (1997). Biomonitoring aquatic pollution with feral eel (*Anguilla anguilla*). 3. Statistical analyses of relationships between contaminant exposure and biomarkers. *Aquatic Toxicology*. **39**, 45-75.
- Vijayavel, K., Gomathi, R. D., Durgabhavani, K. and Balasubramanian, M. P. (2004). Sublethal effect of naphthalene on lipid peroxidation and antioxidant status in the edible marine crab *Scylla serrata*. *Marine Pollution Bulletin*. **48**, 429-433.
- Wallace, J. C. (1972). Activity and metabolic rate in the shore crab, *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology*. **41**, 523-533.
- Warman, C. G., Reid, D. G. and Naylor, E. (1993). Variation in the Tidal Migratory Behavior and Rhythmic Light-Responsiveness in the Shore Crab, *Carcinus-Maenas*. *Journal of the Marine Biological Association of the United Kingdom*. **73**, 355-364.
- Warner, G. F. (1970). Behaviour of two species of grapsid crab during intraspecific encounters. *Behaviour*. **36**, 9-19.
- Warwick, R. M. and Clarke, K. R. (1993). Increased Variability as a Symptom of Stress in Marine Communities. *Journal of Experimental Marine Biology and Ecology*. **172**, 215-226.

- Watson, G. M. (2004). Responses of the decapod crustacean, *Carcinus maenas*, following exposure to environmental contaminants. Development and application of multiple biomarkers., *School of Biological Sciences, University of Plymouth*, pp. 358.
- Watson, G. M., Andersen, O. K. and Depledge, M. H. (2002). A direct fluorimetric technique for the detection of PAH equivalents in crustacean urine and haemolymph: potential for use as an in situ biomarker of PAH exposure. *Marine Environmental Research*. **54**, 829-851.
- Watson, G. M., Andersen, O. K., Depledge, M. H. and Galloway, T. S. (2004a). Detecting a field gradient of PAH exposure in decapod crustacea using a novel urinary biomarker. *Marine Environmental Research*. **58**, 257-261.
- Watson, G. M., Andersen, O. K., Galloway, T. S. and Depledge, M. H. (2004b). Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph. *Aquatic Toxicology*. **67**, 127-142.
- Wedderburn, J., McFadzen, I., Sanger, R. C., Beesley, A., Heath, C., Hornsby, M. and Lowe, D. (2000). The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. *Marine Pollution Bulletin*. **40**, 257-267.
- Wells, K. D. (1978). Territoriality in the green frog (*Rana clamitans*): Vocalizations and agonistic behaviour. *Animal Behaviour*. **26**, 1051-1063.
- Widdows, J., Donkin, P., Brinsley, M. D., Evans, S. V., Salkeld, P. N., Franklin, A., Law, R. J. and Waldock, M. J. (1995). Scope for Growth and Contaminant Levels in North-Sea Mussels *Mytilus-Edulis*. *Marine Ecology-Progress Series*. **127**, 131-148.
- Widdows, J., Donkin, P. (1992). Mussels and environmental contaminants: bioaccumulation, and physiological aspects. In Gosling, E.M. (1992). *The mussel Mytilus: ecology, physiology, genetics and culture*. Elsevier Science. Amsterdam. pp383-398.
- Widdows, J. and Salkeld, P. N. (1993). Practical procedures for the measurement of scope for growth. *MAP Tech Rep Ser*. **71**, 147-172.
- Widdows, J. and Staff, F. J. (1997). Practical procedures for the measurement of scope for growth., Plymouth Marine Laboratory, pp. 1-48.
- Wilhelm Filho, D., Tribess, T., Gaspari, C., Claudio, F. D., Torres, M. A. and Magalhaes, A. R. M. (2001). Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture*. **203**, 149-158.
- Williams, J. A. (1985). An endogenous tidal cycle of blood-sugar concentrations in the shore crab *Carcinus maenas* (L.). *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*. **81**, 627-631.
- Winston, G. and Di Guilio, R. T. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*. **19**, 137-161.
- Woll, A. K., van der Meeren, G. I. and Tuene, S. (2006). Quality improvement by feeding wild-caught edible crab (*Cancer pagurus* L.): a pilot study. *Aquaculture Research*. **37**, 1487-1496.
- Yamada, S. B., Dumbauld, B. R., Kalin, A., Hunt, C. E., Figlar-Barnes, R. and Randall, A. (2005). Growth and persistence of a recent invader *Carcinus maenas* in estuaries of the northeastern Pacific. *Biological Invasions*. **7**, 309-321.
- Yoshimori, T. (2004). Autophagy: a regulated bulk degradation process inside cells. *Biochemical and Biophysical Research Communications*. **313**, 453-458.

