THE UNIVERSITY OF HULL

Aspects of the physiology of decapod crustaceans with particular reference to the live marketing of *Cancer pagurus* (L) and *Necora puber* (L)

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ABSTRACT

The crabs *Cancer pagurus* (L) and *Necora puber* (L) are exported live, in bulk, from the UK to various continental countries. The success of this relatively new trade is marred by the incidence of mortalities and impaired quality of the delivered product. These studies addressed various causes - procedural and biological - of these events.

Descriptions are given of detailed examinations of handling and other marketing protocols for both species from point of capture to arrival at continental dealer's premises. Such examinations were made with the help of a number of major dealers in the UK, Spain and France and included studies of handling, packing, holding and transportation methods, physical damage assessments before and after consignment, and chemical and biochemical analyses of seawater and blood samples.

Dissolved ammonia levels were found to increase greatly in the fixed volume water of vivier tanks and this was found to be matched by correspondingly high blood ammonia values of the contained animals. The measurement of both free ammonia and ionic ammonia efflux rates of juvenile and adult *C.pagurus* and *N.puber* in media with high dissolved ammonia levels was investigated and was found to be related to concentration gradients between the internal and external media. The fluxes could be explained on the basis of diffusion down concentration gradients. When animals were transferred to media with higher ammonia levels than those in blood, a cessation of efflux, or even a net influx of ammonia (NH_4^+) occurred.

During emersion, blood ammonia concentration rose. Such accumulated ammonia was very rapidly off loaded when the animals were re-immersed.

The data produced has been discussed in the context of crustacean physiology and of improving the expectations of delivering a live, quality product after journeys of several days.

TECHNICAL NOTE

The term NH_4 has been used in this text to indicate total ammonia *ie* [NH_3] and [NH_4^+] combined.

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Tables and Figures are included with the relevant Chapters.

Chapter 1

General Introduction

A comprehensive review of the fishery for the edible (brown) crab in British waters reported that *Cancer pagurus* was the "only species of crab regularly fished for human consumption in European waters" (Edwards, 1979). Since this time, three other species of crab have become regularly fished in Britain for culinary markets, almost entirely for European countries, and these are the spider crab, *Maja squinado*; the velvet swimming crab, *Necora puber* (MacMullen, 1983a); and the green or shore crab, *Carcinus maenas*. While these species were fished prior to 1978 in Spain and France an apparent decline in the fisheries, especially for *Necora* and other portunid species in Spain after this time (FAO statistics, MacMullen, 1983a), must have encouraged the fisheries which developed in the UK.

At present, the markets for the crab species mentioned above are primarily for live product in Europe, but a proportion of freshly cooked, vacuum packed product is also required (MacMullen & Uglow, *pers comm*). In Britain only a very small market for live crab is present (normally for the restaurant trade) - the main requirement being for 'dressed crab' in season or processed product. The species consumed is mainly *Cancer* with a small amount of *Maja* (generally where it is fished in SW England).

Edwards (1979) suggested that many of the stocks of brown crab were underexploited in the UK and the future of the brown crab fishery in Britain was dependant upon improved marketing of this species to the UK population. Failing this, an increase in demand might come about by marketing to our European neighbours. The latter situation has transpired over the past 15 years with the development of fisheries in other species. A trade in live transport of *Maja squinado* and a small amount of

Cancer pagurus to Spain was reported around 1976 (Edwards, 1979) and was proving to have larger financial returns than the local markets. The future development of British crab was not forseen to be in the live marketing of species due to "problems of limited storage life, seasonal limitations and the problems of transporting such a perishable product", (Edwards, 1979). While the latter problems still arise frequently, the live trade of crab species has escalated and, due to European demand, has increased crab fisheries in the UK since the 1970s.

1.1. *Cancer pagurus* fishery & *Necora puber* fishery and their live markets

1.1.1. *Cancer pagurus* - the brown, or edible crab

The fishery for *C.pagurus* is an historic one and has been recorded as early as 1800 with references prior to this, probably as far back as Roman times (Edwards, 1979). *C.pagurus*, at present is fished around most parts of the UK coast. Fishing is largely on rocky substrata using a baited trap or pot, the design and construction of which alters depending on the history and development of the fishery in any particular area (Edwards, 1979). The type of boat used and the hauling gear alters also, depending on the number of pots being used and the distance of the fishery from port.

The brown crab fishery areas of UK importance have altered during its recorded history. In England and Wales the proportion of the brown crab catch landed in the three main fishing areas (Table 1.1) was highest on the east coast (at 81 - 90%) until the 1950s when the proportion started to decrease and declined to 31% of the catch during 1970-75 and 25% during 1976-80. The west coast fishery represented only a small proportion over this time and the south coast fishery a varying proportion (9 - 16% from 1904) up to the 1950s and thence an increase, when in 1970 this region contributed the largest proportion to the overall landings (68% during 1970-75 and

72% over the period 1976-80). The areas where these landing were made are represented by the top ten crab ports in England and Wales, and Scotland in 1975 and 1982 (Table 1.2). The information from 1982 reflects the development of the Scottish crab fishery northwards on the Scottish east coast to Shetland and Orkney and also greater catches from the west coast, especially the Western Isles.

Edwards (1979) indicated that the main British fishing grounds for *C.pagurus* were along the east coast of Scotland and England, the Devon coast, around Cornwall and off Orkney and Shetland. Smaller fisheries were prosecuted in Wales and Ireland. He suggested that increased exploitation of Welsh and eastern English Channel stocks could be sustained as well as those of the west coast of Scotland. The Scottish west coast fishery has developed in line with demand form France and Spain, and to a lesser extent in Wales. Developments in existing fisheries have also occurred. These have involved exploitation of brown crab stocks further offshore on the east coast in the Yorkshire (recent years) and Norfolk (previous 7 years) fisheries; and a move from a seasonal to a year-round fishery. The former of these developments has been due to reduced catches inshore to maintain the normal crab landings (Edwards & Uglow, *pers comm*), most of these are transported live for the export market.

The value of crab landings is documented and quoted as part of the landing statistics recorded by the Ministry of Agriculture, Fisheries and Food in the UK. Edwards (1979) quoted average landings of about 6,500 tonnes of crab during 1972-76 worth £1,500,000. In 1982 the average annual UK catch over 5 years was 9,500 tonnes worth £4 million at first sale (Table 1.3). The documented landings from 1982 for crabs increased to 13,000 tonnes in 1988 (Key Indicators, 1989), with an estimated value of £19 million at first sale. Crab landed in England and Wales were worth more than in Scotland (£1088 and £986 per tonne respectively; N.B. the latter value is for brown crab only: Key Indicators, 1989). Landing figures for the early 1990s are quoted as being around 20,000 tonnes annual average for the UK, 40% of which are

used in processing and 60% for live transport/export (Jacklin, *pers comm*).

1.1.2. Value of product along marketing chain

Values for shellfish at different stages of the marketing chain are difficult to obtain, since official documentation does not normally occur. Final sale, restaurant prices in Spain in the early 1980s were recorded as being £4-£4.50 and £7.50 per kilo for brown crab at a 'medium' and 'luxury' restaurant respectively (Table 1.4; 800-900 ptas and 1,500 ptas respectively - using 1989 exchange rates of about 200 ptas per £1 sterling). Values for first sale of different crab species of landed live product in the UK and Spain are also given in Table 1.4, the value for brown crab varies considerably from £0.40 to £1.50 per kilo depending on season.

Table 1.5 gives information from a single importing dealer in Spain regarding his total UK intake of different shellfish species. This information is taken from his own records - not official data. The information for 1987 is taken from January to December. Over 45 tonnes of brown crab were imported in 1988 from one UK dealer. A Spanish dealer such as this often owns part of the marketing chain including retaurants and other retail outlets (MacMullen *pers comm*) - the possibility of large returns (considering the restuarant prices given - Table 1.4) is therefore apparent.

1.1.3. *Necora puber*, the velvet swimming crab

Necora puber (L) is known to occur on all coasts of France and Spain and is common on west coast areas of the UK, including Eire, and is present as far north as southern Norway. Lower densities of this species occur on the UK east coast. The Spanish stocks have been depleted by overfishing and this situation has led to the development of the fishery for the velvet swimming crab in the UK and especially Scotland, where it was previously considered as a pest (MacMullen, 1983a). Small fisheries in Wales and

SW England have also developed. The Scottish stocks are considered to be reasonably unexploited - although there are areas of localised over exploitation; this is not of great concern since larger individuals compete successfully with smaller ones for the traps and re-population from surrounding areas is fairly rapid (MacMullen, 1983a). Extensive biological study of these populations and the impact of the fishery has not been undertaken, although some investigations of the stocks and breeding cycles have begun.

Necora is fished using a baited trap in the Spanish fishery and in Scotland. Not all Scottish catchers fish full-time for *Necora* - many are part time for the main season (Autumn to Winter) or this species comprises part of a by-catch with other species MacMullen, 1983a). Where velvets are fished full-time, other species, (lobsters and Norway lobsters) are often taken as a by-catch (MacMullen, 1983b). Although some traps are specifically designed for velvet fishing, traps available for fishing other species are used often - especially when fishing for velvets is a part-time practice. The most common alternative used is a trap designed for *Nephrops norvegicus*, the prawn or Norway lobster. The 'D'-type design (as opposed to the ink-well or creel design) is used acceptably well by many catchers - although some gear damage occurs. Traps are placed at a depth of 4-5 fathoms (8-10m) and left for up to 2 days, but usually overnight or up to 4 hours for a second haul during the day (MacMullen, 1983b). White fish, herring or mackerel are the baits used and the freshness influences catches (MacMullen, 1983b). Many Scottish areas are currently operating a voluntary ban of velvet fishing during the late summer months - covering the moult and post-moult periods

Studies of many trap designs and types of bait undertaken in Spain demonstrated that a traditional wood and net design was overall better for management purposes and that sardine, bogue and horse mackerel were the most effective baits (Gonzalez-Escalente

& Gonzalez-Gurriaran, 1985). Since damage occurs to the prawn traps (designed for soft substrata) when used for *Necora* on hard areas in the UK, some investigation of creel design was undertaken by the Sea Fish Industry Authority to reduce damage caused by hard ground, to effect some size selction of the animals, to maintain by-catches or to increase the *Necora* catch and reduce escapement of this highly mobile (swimming) species (MacMullen, 1983b). The use of escape holes for size selection of this species was considered to be useful only with trap soak times of greater than 2-4 hours (Gonzalez-Escalente & Gonzalez-Gurriaran, 1985).

UK catch statistics for *Necora* are difficult to obtain since, if reported, they are included with other crab species landing figures. Estimates, based on approximate known landings are around 20 tonnes per week during November and December (time of peak demand in Europe). Lower landings occur throughout the rest of the year - giving overall, 500 tonnes annually (MacMullen, 1983a). Table 1.5 gives import figures for one Spanish dealer which indicates that 97.5 tonnes of *Necora* were supplied from the UK fishery in 1988 - from one UK dealer. This indicates that the above estimate is likely to be low.

The value of landed *Necora* at first sale, in 1983 was £1 per kilo (MacMullen, 1983a & b). Table 1.6 gives a breakdown of the estimated cost of live transport for *Necora*, depending on the mortality rates and the volume of crab transported. The 'at-cost' value of the crab at sale to the import dealer could vary between £1.70 and £3.50. Considering the value of Spanish landed crab (Table 1.6), the potential for high returns on transport of this species are good. The live market trade from the UK, however, has a generally low confidence in Europe. This has been affected by the occasional high mortalities. Confidence is gradually increasing with time (as evidenced from SFIA internal reports, 1980-84).

1.2. Ammonia excretion

NB Where ammonia excretion is mentioned, it is considered to be the same as ammonia efflux rate.

1.2.1. Site of Excretion

The two main sites of excretory ammonia in ammonotelic animals are *via* the excretory organ (kidney, in teleosts or antennal gland in crustaceans) and across the gill epithelium. The proportions produced from each site have been measured in teleosts and crustaceans and by far the greatest part of the ammonia is excreted across the gills.

In *Callinectes sapidus* ammonia produced in the urine was found to be 1-2% of the total ammonia excreted (Cameron and Batterton, 1978) and urinary ammonia contribution to total efflux was found to be negligible in *Carcinus maenas* (Harris and Andrews, 1985). *Jasus edwardsii* was found to excrete less than 2% ammonia via the antennal gland (Binns and Petersen, 1969).

Ammonia excreted across the gills may not comprise totally all of that produced in the tissues and then transferred from the blood across the gills. Deamination of amino acids in the blood may occur in the gill epithelium itself and, therefore, contributes another source of ammonia to the total net flux from the animal. Most investigations concerning deamination at the gill have been carried out with teleosts, where amounts of 20 - 40% of total ammonia production are estimated to originate in the gill tissue (see Kormanik and Cameron, 1981a). Evidence for ammonia production in *Eriocheir sinensis* gills was put forward by Vincent-Marique & Gilles (1970), since they found proline oxidase activity in a gill preparation of this crab. The activity was greater in the three posterior gill pairs than the anterior gill pairs.

The three posterior gill pairs in *Eriocheir sinensis* have been identified as having an osmoregulatory function (Gilles and Pequeux, 1985) due to changes in structure identified with the onset of more dilute conditions. Such changes do not occur in marine osmoconformers, even those which are slightly euryhaline. It can be suggested, therefore, that animals with an osmoregulatory capacity - shown by morphological changes in the gill - may produce more ammonia than osmoconformers due to deamination across the gill membrane. When considered along with the volume regulation mechanisms found in animals exposed to osmotic stress and which involves the release of proteins and amino acids from the tissues (Gilles and Pequeux, 1983), the elevated excretion rates produced during osmotic stress may be due to deamination at the gill as well as tissue deamination during adaptation. Normal excretion rates are found after acclimatisation.

Therefore, the site of nearly all ammonia excretion in Crustacea is the gills. Most of the ammonia movement across the gills derives from ammonia carried in the blood and smaller, more variable amounts derive from deamination in the gill tissue (Harris & Andrews, 1985).

1.2.2. Ammonia excretion in Crustacea

Ammonia excretion comprises over half of the nitrogenous waste of aquatic Crustacea (Regnault, 1987). Quantities from 55% to 68% are reported in the literature (Prosser & Brown, 1961). Urea, uric acid, amino acids, purine and trimethylamine oxide (TMAO) are also excreted as nitrogenous waste of Crustacea. Ammonia excretion is continuous in aquatic crustaceans, the molecular or un-ionised form (NH_3) being converted to the ionised form (NH_4^+) on release into the surrounding medium in a normal situation (Regnault, 1987). Excretion of ammonia is considered to be the most primitive system for nitrogen release and least expensive energetically (Forster & Goldstein, 1969). In the present investigations, ammonia excretion was measured with

no attempt to quantify other nitrogen waste products excreted (except in one experimental series - see Chapter 6). Many of these studies were undertaken with an emphasis on problems associated with live transport of Crustacea and the high ammonia production measured during these activities. Therefore, the focus was on ammonia 'dynamics' and not on overall nitrogen waste excretion in these organisms. It is realised that ammonia production from the gill can also occur by deamination of amino acids at the gill surface (Harris & Andrews, 1985).

Table 1.7 gives excretion rates taken from the literature and recently measured for a number of crustacean species. Where possible, all values have been converted to μmol g^{-1} l^{-1} for fresh or dry weight, for comparison with the measurements derived for the present work. Some of the conditions prevailing when the rate measurements were made are also detailed. The ammonia excretion rates vary considerably both inter- and intra-taxonomic grouping. This can be attributed to differences in experimental methodologies used and different analytical techniques. More accurate methods of ammonia analysis have developed as well as a deeper understanding of how experimental conditions interfere with ammonia measurements in recent years (Regnault, 1987). Overall, it can be deduced that ammonia excretion in Crustacea is highly variable.

The latter comment is borne out by consideration of Q_{10} determinations of excretion rate values calculated from the literature (Table 1.8); these vary between 15.24 and negative values. Differences are observed with different developmental stage and with environmental situation, such as salinity changes, although alterations of Q_{10} over different temperature ranges are known to occur (Wilson, 1979). Q_{10} values for most biological rate processes normally fall into the range 1.1 - 4, with values of 2 or above indicating chemical reactions ; values close to 1 indicate physical processes such as diffusion (Wilson, 1979). Reasons for the high variability in Q_{10} are probably similar

to those for differences in the ammonia excretion rates.

1.3. Ammonia toxicity

The toxicity of ammonia to marine shellfish is of particular interest for the present work due to the high ammonia levels which are measured in vivier tanks during live transport (>6mmol l^{-1}; see Table 3.6) and the suggestion that high ammonia levels may effect mortality of species transported live (Quinlan, 1984; MacMullen, 1983a) or contribute to a combination of stressors leading to mortality or a considerable reduction in quality (Uglow *et al*, 1986; Whyman *et al*, 1985). Toxicity measures are usually considered on an acute (acting in a short time-period) or chronic (acting over a long time-period, *eg* a series of developmental life-stages) basis. The normal measurement of acute effects of a substance is by determining a dose which is lethal to 50% (LD_{50} or LC_{50}) of a sample of organsims over a quoted time period (often 96 hours). LC_{50} values have been found for various marine invertebrates for given ammonia concentrations but the component of total ammonia which is considered to be toxic is the un-ionised proportion (NH_3; see section 1.5.1.) (Warren, 1962; and Alabaster & Lloyd, 1982). Any toxicity of the ionised form of ammonia is considerably less than the un-ionised form (Willingham *et al*, 1979, cited in Seager *et al*, 1988).

In the determination of environmental water quality standards - a process which is at present being undertaken in the UK, co-ordinated by the National Rivers Authority, (1993) - the most useful assessments for ammonia toxicity are made by analysing LC_{50} data in terms of the un-ionised component (Seager *et al*, 1988). Ammonia is a List II substance (a category given to substances which may have a deleterious effect on the marine environment (and are considered to be less dangerous than List I substances)) under the 'Dangerous Substances Directive' (76/464/EEC - CEC, 1976) and is also

included in the 'Shellfish Waters Directive' (79/923/EEC - CEC, 1979) for coastal and estuarine areas. The derived LC_{50} values are used to determine suitable standards for water quality.

Acute LC_{50} values calculated for marine invertebrates range from 0.31 to 30.5 mg NH_3-N l^{-1} (equivalent to 22.1 to 2,178.6 μmol l^{-1}). Larval stages of invertebrates are more susceptible than adults to acute ammonia exposure (Seager *et al*, 1988). This is also demonstrated by comparison of some more recent figures for acute 96h LC_{50} concentrations for *Homarus americanus* stage II larvae (1.7 mg NH_3-N l^{-1}) and adults (3.25 mg NH_3-N l^{-1}) (Young-Lai *et al*, 1991). Some investigations of the chronic toxicity of ammonia to marine invertebrates have also taken place - mainly with respect to aquaculture species (Wickins, 1976).

A suggested water quality standard for ammonia for the protection of saltwater fish and shellfish given for the Department of the Environment by the Water Research Centre (WRc) in the UK is 0.021 mg NH_3-N l^{-1} (equivalent to 1.5 μmol l^{-1}) annual average concentration; this is based on experimental data, since field data obtained were not reliable (Seager *et al*, 1988). This may give a suitable measure for assessing conditions during vivier transport.

LC_{50} determinations for ammonia may often be unreliable due to differences in experimental conditions and species used, and the ability of certain species to detoxify high ammonia levels present in the blood, thus affecting the effective toxicity, and not being quantified (Seager *et al*, 1988). Reduced salinity reduces acute toxicity of ammonia, and other environmental factors may affect toxicity (*eg* a reduction in dissolved oxygen resulted in increased toxicity effects in *Salmo salar*, (Alabaster *et al*, 1979 & 1983). Acclimation of these animals to sub-lethal levels of ammonia resulted in increases in LC_{50} 24h levels. Other environmental effects must also be taken into

account, these include the pH of the external medium, the temperature and the salinity - all of which are known to affect the proportions of ionised and un-ionised ammonia present (Bower & Bidwell 1987; Seager *et al*, 1988). Acute LC_{50} concentrations for ammonia toxicity were not determined for the present study as other factors probably contributed to mortality during live transport. Haemolymph measurements were made, however, which allowed calculation of the un-ionised component of the total ammonia present in the animal - a measure which may be a more useful indication of the toxicity of ammonia to organisms studied for this work.

1.4. Blood ammonia

Table 1.9 gives values for blood ammonia determinations for Crustacea taken from the literature and converted to μmol l^{-1} for comparative purposes. The values measured range from over 1000 μmol l^{-1} (1 mmol l^{-1}) to 105 μmol l^{-1}. The values given pertain to those in normal, low ammonia situations, and immersed. As with ammonia excretion rate measurements, the methodology involved in analysis has developed since some of the first measurements were made (Myers, 1920; Delauny, 1927). Despite this, some of the early measurements of blood ammonia are similar to present day determinations (*eg* 176.5 μmol l^{-1} (Souterbicq, 1935) and 129.6 μmol l^{-1} (present studies) for *Cancer pagurus*. The values presented in Table 1.9 are mean levels, sometimes measurements were taken of pooled haemolymph samples (Myers, 1920). The individual variability of blood ammonia can, however, vary within a given study group (*eg Nephrops norvegicus* 115 - 170 μmol l^{-1} and *Carcinus maenas* 235 - 647 μmol l^{-1}, Table 1.9). The level of blood ammonia and variability of measurements will also be affected by treatment of the animals before analysis within experimental treatments and between species.

The ammonia concentrations in the haemolymph of crustaceans are much greater than

ambient concentrations in the surrounding water. This will favour transfer across the gills along a concentration gradient. Release of ammonia from the tissues to the haemolyph occurs since ammonia build-up will eventually be toxic to the organism. The rates of movement between tissues and haemolymph are difficult to quantify. Efflux across the gill surface is continuous (Regnault, 1987) and, therefore, it may be considered that movement from tissue to haemolyph is also continuous.

In assessing the toxicity of ammonia to the organism in terms of blood ammonia, the pH of the haemolymph is important, as with water levels of ammonia. The blood pH is also affected by other factors, which in turn may affect the toxicity of ammonia. Where this is relevant in the following accounts, the method by which the relative components of ammonia are calculated is described.

1.5. Small Review: Ammonia Excretion in Marine Organisms

1.5.1. Ammonia and its different Forms in Water

When ammonia comes into contact with water it dissolves, according to Henry's Law. The following equation shows the chemistry of ammonia in water:-

$$NH_3 + H_2O \rightleftharpoons NH_4OH \rightleftharpoons NH_4^+ + OH^-$$

The equilibrium constant or pK of this equation varies with the pH of the medium. At normal physiological pH (6.5 - 8) and the pH of seawater, most of the ammonia will exist in the NH_4^+ form. The ammount of NH_4^+ relative to NH_3 is important when investigating ammonia fluxes across the gills of aquatic animals but information on pK values with variations in temperature and concentration of medium are scarce in the literature. Such values are logarithmic - small inaccuracies creating large errors in calculations.

Free ammonia (NH_3) is a small molecule and is regarded as a lipophilic molecule diffusing readily across lipid membranes (Kormanik and Cameron, 1981a). The ease with which a molecule can permeate a membrane is determined by its partition coefficient, (or solubility) in a lipid (*eg* olive oil) and water, and also its diffusional coefficient. The high solubility of free ammonia in human plasma is thought to be due to the lipoproteins present (Jaques *et al*,1959) but clear values for free ammonia solubility are uncommon in the literature. Recent determinations of NH_3 solubility or partition coefficient give low values in the range 0.04 - 0.08, in a range of lipids (including chloroform and olive oil) (see Evans and Cameron, 1986). These values are lower than expected for a mobile highly lipid-soluble molecule and throw into question the classically regarded process of free ammonia membrane permeation. Since both water and free ammonia are non-electrolytes in aqueous solution, the possibility exists that NH_3 can also follow water diffusional pathways in gill permation.

The ammonium ion, NH_4^+ is a much larger molecule than the free ammonia (NH_3) molecule and is charged. The NH_4^+ion would not disslove in the lipid component of membranes because of its high charge and movement along water diffusional pathways may not be possible because of its size.

The diffusional net flux of non-electrolyte movement through a membrane can be described as follows:

$$Jnet = -A.D.\frac{(C^{in} - C^{out})}{X}$$ (see Taylor, 1989).

(negativity demonstrating net flux from the animal).

A = exchange area (m^2);

D = barrier diffusion constant ($m^2\ sec^{-1}$);

C^{in} - C^{out} = internal and external chemical concentrations;

X = barrier thickness (m).

D/X represents the permeability coefficient (P; msec-1) and C^{in} - C^{out}/X, the gradient of potential energy across the barrier. D cannot be measured in practice and X may vary (*eg* the difference between respiratory and regulatory epithlia in the gills) so P is determined experimentally.

For a homogeneous, symmetrical membrane:

$$P = D^m K \,/\, X$$

D^m is the molecular diffusion coefficient within the membrane;

K is the dimensionless local lipid water partition coefficient.

Circulation of blood through the gill and ventilation of the gill may change so that the exchange area for diffusion may vary. The 'transfer factor' used in respiratory physiology is useful here as a 'practical permeability' (Taylor, 1989):

$$A\,D \,/\, X$$

a function of the ratio effective area/effective thickness.

Taking into account ammonia flux across the gill epithelium, molecular ammonia diffusion will take place due to differences in the partial pressure gradient. The solubility of the molecular form of ammonia must also be taken into account so the net flux will be:

$$Jnet = - A. D. \frac{\alpha.\Delta Pp}{X}$$ (Kormanik and Cameron 1981a)

α = Bunsen solubility coefficient (ml l^{-1} $torr^{-1}$);

ΔPp = partial pressure gradient (torr). Ammonia excretion may vary due to different conditions (natural or experimental). Considering different conditions - the ratio of ammonia flux is useful in comparing the effect of these conditions. Using the above equation:

$$\frac{J^1_{net}}{J^2_{net}} = \frac{\alpha^1.\Delta Pp^1}{\alpha^2.\Delta Pp^2}$$

1.5.2. Gill Permeability and Diffusive Mechanisms of Excretion

Gill permeability is concerned with the passive movement of molecules across the gill surface in the direction of a chemical or electrical gradient. Whole-animal permeability has been much studied; Nagel (1934) showed that iodine penetrated more rapidly across the body surface of stenohaline marine crustaceans than estuarine species and these animals were, in turn, more permeable than freshwater species. This principle also holds for teleosts (Evans 1985) and applies generally to euryhaline animals acclimated to different salinities.

The permeation of free ammonia or ammonium ions across the gill surface will depend upon the size of the molecule involved. Therfore, whether the 'aqueous' solution (be it haemolymph or external medium) is an electrolyte or non-electrolyte is important. In

the following discussion these circumstances will be dealt with separately.

1.5.2.1. Permeability of Non-electrolytes

A non-electrolyte is a solute the molecules of which stay intact in aqueous solution. Water is a non-elecrolyte since the molecule does not dissociate but hydrogen bonds with its neighbours in the aqueous phase. The free ammonia (NH_3) form of ammonia in aqueous solution is also a non-electrolyte.

Water permeability of aquatic animals has been studied in order to better understand body fluid regulation with respect to the external environment. In fish, more than 90% of diffusional water exchange occurs at the gills (Motais *et al*, 1969), primarily through the respiratory cells of trout (Isaia, 1982) which cover about 96% of the gill surface. In decapod Crustacea, the gills are identified as the principal site of osmotic water movements. Water flux studies of euryhaline dacapods, *Rithropanopeus* sp. (Capen, 1972) and *Uca pugilator* (Hannan and Evans 1973) revealed that 86-90% of movement was across the gills. Two types of gill epithelia are found in decapods depending on whether the animal has the ability to osmoregulate (see Gilles and Pequeux, 1985). In the strong osmoregulator, *Eriocheir sinensis*, the three anterior gill pairs are respiratory in function, with cuticle 1 μm and the epithelium thickness 2-4 μm. The three posterior gill pairs consist of a 'salt transporting' epithelium which is 10 μm or more thick and with a cuticle 0.3 μm thick. In marine, stenohaline forms the gills are comprised of respiratory epithelium only.

Permeability to water varies with the environment inhabited, as shown by water flux studies using heavy water experiments. A stenohaline marine crab, *Macropipus depurator*, a euryhaline crab *Callinectes sapidus* and the freshwater crayfish, *Astacus fluviatus* were studied and a decrease in the water influx or permeability constant

ocurred with change from a marine to more dilute environment (Rudy 1967). Crustacea inhabiting environments of fluctuating salinity are able to adjust their permeability to water - becoming less permeable after acclimation to dilute media (Campbell and Jones, 1990). This follows the adaptive requirements of inhabiting fluctuating environments, *ie* a decrease in permeability to reduce water loss and an uptake of salts to balance salt loss. Such animals also alter their salt balance and this ability to regulate may confer additional or different means of ammonia excretion compared with animals with less ability to regulate.

In euryhaline teleosts, a change in gill membrane permeability is also found with environmental salinity changes. Morphological changes in gill structure in animals adapted to salt water almost triple the amount of chloride cells in the eel, *Anguilla japonica* (Shirai and Utida, 1970) and *Anguilla anguilla* (Sargent *et al*, 1977). A "leaky" membrane is formed by immature chloride cells extending into mature chloride cells during acclimation (Sardet *et al*, 1979). Gill permeability is, therefore, increased in the non-respiratory epithelium or chloride cells. In the respiratory epithelium, after acclimation to salt water, the percentage of the sphingomyelin content of the gill cells doubles. The permeability of molecules decreases with increasing sphingomyelin content (Barenholz and Thompson, 1980). Compared with other epithelia, the gill is relatively impermeable to non-electrolytes (Isaia 1984). This is reflected in the water permeability of the apical respiratory epithelium which is eight times greater than an inner epithelial barrier - probably the basal membrane (Isaia *et al*,1978). Whether the sphingomyelin component of the gills plays any part in this remains unclear but the relative impermeability of gills with 'leaky junctions' compared to other epithelia (*eg* toad bladder, which has tight junctional complexes) demonstrates the complexity of gill permeability in teleosts.

The 'salt transporting' epithelium of euryhaline crustaceans undergoes functional,

morphological changes when the animal experiences a salinity change. These changes are functional. How strongly the animal is able to regulate under such environmental fluctuations is also reflected by the extent of the morphological changes (*eg* in *Carcinus maenas* the changes are not as extensive as those in the extremely euryhaline *Eroicheir sinensis*). The salt-transporting epithelium in *Eriocheir sinensis* acclimated to dilute media has an infolding system of the plasma membrane at both the apical and basal borders. The basal folds are packed with mitochondria. The ATPase activity is also greater in the posterior gill pairs than the anterior gill pairs. Although these changes in morphology are linked to ionic, probably active mechnisms of transport, there is also evidence of an effect on the diffusive ionic permeability of these epithelia.

The ammonia permeability in the crustacean *Callinectes sapidus*, as determined by Kormanik and Cameron (1981b), is 0.023 cm sec^{-1}. Olert *et al* (1968) give an ammonia permeability value of >0.01 cm sec^{-1} for the mammalian cortical nephron, the value for *C.sapidus*, therefore is comparable.

1.5.2.2. Permeability of electrolytes

An electrolyte is a solute the molecules of which dissociate into constituent ions in aqueous solution. Ionic movements across the gills of aquatic animals are classically considered in exchange mechanisms. However, diffusional movement of ions along electrochemical gradients also occurs. Evidence for NH_4^+ permeability of membranes does exist (Kormanik and Cameron, 1981a & b) and measurements of permeability have been made in bullfrog and rabbit gallbladder (perm NH_4^+/K^+ = 1.41, NH_4^+/Na^+ = 2.48 and NH_4^+/K^+ = 1.05, NH_4^+/Na^+ = 3.23 respectively, Diamond, 1975). Here, NH_4^+ permeability is higher than Na^+ and K^+. Gall bladder is a relatively leaky tissue with intercellular or junctional complexes, which is similar to teleost non-respiratory epithelium (see earlier).

Na^+ fluxes were studied in freshwater adapted *Eroichier sinensis* by Gilles and Pequeux (1985). In the anterior gill pairs (epithelium respiratory), Na^+ movements were found to be passive. These movements were also dependant on the external level of Na^+ in the media. From isolated gill studies a decrease in permeability of gills ocurred on introduction of dilute media *ie* an 'immediate adaptability' of Na^+ permeability to reduce salt loss from the animal. Osmotic stress, for this animal is reduced therefore and a 'long term' decrease in permeability also occurs. In the posterior gill pairs, where the salt-transporting epithelium is found, Na^+ transport is esentially active since Na^+ influx was demonstrated but no efflux detected so uptake by means of Na^+ exchange takes place in this epithelium. In marine forms, virtually no change in gill permeability to ions was found on exposure to dilute media (Mantel and Farmer, 1983). The ecological significance of gill permeability in such weak or non-regulating decapods is relevant. Animals not exposed to dilute media in their habitat require no means of regulation for it. Since the epithelium of essentially marine forms is respiratory, passive movement of ionic forms through the epithelium, especially Na^+ or ions of similar size, seems probable, based on data for *E.sinensis*.

Evidence exists for the incidence of NH_4^+ diffusion in ammonia excretion of Crustacea (Evans and Cameron, 1986; Cameron 1986) and fish (Evans, 1985). The proportion of the total ammonia excreted as NH_4^+ varies, but may be related to the 'leakiness' of gill epithelia in marine forms. In euryhaline and freshwater species NH_4^+ is involved in exchange mechanisms for salt balance and excretion.

This thesis outlines the methods involved in live transport, reports on studies which have been undertaken to investigate the stresses imposed on live-marketed species as part of this work, gives experimental evidence for improving protocols associated with this trade and gives recommendations for further scientific work in this field. An additional element which has resulted from these investigations is the study of the processes involved in nitrogenous excretion in these *Cancer pagurus* and *Necora puber*.

Table 1.1. Proportion of the crab catch landed in three different fishing areas of England & Wales, 1904 - 1980.

Time period	East Coast (Berwick - Deal)	South Coast (Dover - Isles of Scilly)	West Coast (Sennen Cove - Silloth)
1904 - 13	81%	16%	3%
1914 - 23	81%	16%	3%
1924 - 33	85%	12%	3%
1934 - 43	89%	9%	2%
1944 - 53	90%	9%	1%
1954 - 59	78%	21%	1%
1960 - 69	66%	33%	1%
1970 - 75	31%	68%	1%
1976 - 80	25%	72%	3%

Data from SFIA reports and Edwards (1979).

Table 1.2. Top ten ports for crab landings in 1975 and 1982.

1975	
England & Wales	Scotland
R Dart (Kingswear)	Fraserburgh
Salcombe	Gairloch
Plymouth	St Abbs
Weymouth	Hoy
Selsey	Burnmouth
Bridlington	Westray
Cromer	Stromness
Sheringham	Dubar
Filey	Rousay
Littlehampton	Gourdon

1982			
England & Wales	Wt landed (tonnes)	Scotland	Wt landed (tonnes)
Dartmouth	1233	Western Isles (Stornoway/N Uist)	796
Salcombe	602	Orkney	556
Cromer	332	Wick	353
Bridlington	267	Leith	280
Newlyn	240	Anstruther	192
Sheringham	229	Fraserburgh	160
Plymouth	155	Arbroath	156
Weymouth	143	Eyemouth	125
Helford	116	Shetland	106
Whitby	112	Gairloch	101

Data from SFIA reports and Edwards (1979).

Table 1.3. Crab landings from different parts of the UK, and their value 1973-1984.

Year	England & Wales (tonnes)	Scotland (tonnes)	Northern Ireland (tonnes)	Total United Kingdom (tonnes)	Value (£1000)
1984	9070	4952	4	14,026	8422
1983	7310	4004	4	11,318	6373
1982	5404	3161	2	8,567	4246
1981	7104	2637	4	9,745	4258
1980	7147	2530	6	9,683	3968
1979	6658	2380	-	10,425	3906
1978	6166	2638	-	9,579	3242
1977	6081	2394	-	8,628	2682
1976	5672	1996	-	7,717	1918
1975	4897	1686	-	6,586	1332
1974	4267	2300	-	5,850	1199
1973	4772	2250	-	6,950	1041

NB Figures for England & Wales exclude spider crabs and species other than the edible crab.

Table reproduced from figures taken from SFIA reports and Edwards (1985).

Table 1.4. Restaurant prices (final sale) for crustacean shellfish in Spain. Figures are typical of a holiday season.

Species	Price in a 'medium' restaurant (Ptas per kilo)	Price in a 'luxury' restaurant (Ptas per kilo)
Lobster	2600	4000
Brown/Edible crab	800/900	1500
Spider crab	3500	4000
Norwegian lobster	2000	4000
Crawfish	3300	5000
Velvet crabs	2500	3200

Information from SFIA draft report from early 1980's.

As a guide, 1989 exchange rate values were about 200 Ptas per £1 Sterling.

Table 1.5. Total weights of different crustacean shellfish imported from the UK by a Spanish dealer in northern Spain in one year, 1987.

Species	Total import (kg)	Proportion of shellfish (%)
Brown/Edible crab	45,660	23
Velvet crab	97,515	49
Green crab	40,672	20.5
Lobster	12,120	6
Crawfish	2,908	1.5

Information compiled from figures recorded by Spanish dealer himself.

1 metric tonne = 1000 kg.

Table 1.6. Breakdown of estimated cost of live transport for the velvet swimming crab (*Necora puber*) and the edible crab (*Cancer pagurus*).

Necora puber, price range £1 - £1.50 per kilo. *

Cancer pagurus, price range £0.40 - £1.50 per kilo. *

Average first sale price £1/kg.

Cost of sending a vivier, return to Spain from Scotland is £3000.

Normal loading capacity 4 - 9 tonnes.

Mortality 20% - 50%.

Costing description	4 tonnes shellfish transported		9 tonnes shellfish transported	
	20% mortality	50% mortality	20% mortality	50% mortality
Effective first sale price, allowing for mortality	£1.25/kg	£2.00/kg	£1.25/kg	£2.00/kg
Transport cost	£0.94/kg	£1.50/kg	£0.42/kg	£0.67/kg
Delivered price, excluding margins	£2.19/kg	£3.50/kg	£1.67/kg	£2.67/kg

For comparison of price:

Spanish first sale price £5 per kilo *Necora puber*. *

Spanish first sale price £3 - £6 per kilo *Cancer pagurus*. *

* Source SFIA Internal Report No 1170, August 1984.

Table 1.7. Ammonia excretion rates cited in the literature for different species of Crustacea. (Reference source given in parenthesis).

Species	Ammonia excretion rate (μmol g^{-1} h^{-1} - or other specified)	Comments
Artemia salina (Moffett & Fisher, 1978)	167-798 per day	15-25^{o}C, 34‰
Ceriodaphnia reticulata (Gophen, 1970)	5.4-13 per day	15-27^{o}C
Trigriopus brevicornis (Harris, 1973)	3.5-75 dry wt h^{-1}	5-25^{o}C
Penaeus chinensis (Chen & Nan, 1993)	5.7-9	
Penaeus esculentus (juveniles) (Hewitt & Irving, 1989)	0.5-6 dry wt h^{-1}	Post feeding rates, protein diet 30-50%
Penaeus japonicus (Spaargaren *et al*, 1982)	23-51 (NH_4^+-N)	25^{o}C 22-35‰
	2-8 (NH_4^+-N)	10-14^{o}C 22-35‰
Penaeus indicus (Gerhardt, 1980)	0.06 per day	28^{o}C 30‰
Pandalus platyceros (Quarmby, 1985)	0.6	2.5-9^{o}C 26‰
Crangon crangon (Hunter, 1991)	0.78-1.95	Seawater
Crangon crangon (Regnault, 1981)	0.31	12-14^{o}C Seawater
Crangon franciscorum (Nelson *et al*, 1979)	2-9 per g dry weight	
Palaemonetes varians (Hunter, 1991)	2.05-2.47	

Table 1.7. continued

Species	Ammonia excretion rate (μmol g^{-1} h^{-1} - or other specified)	Comments
Macrobrachium rosenbergii (Armstrong *et al*, 1978)	0.95-1.4 per g dry weight	
Macrobrachium rosenbergii (Nelson *et al*, 1977)	2.4-2.7	
Macrobrachium lar (Nelson & Kropp, 1985)	1.1-5	Freshwater
Nephrops norvegicus (Hosie *et al*, 1991)	0.05	6°C 32‰
	0.15	12°C 32‰
Nephrops norvegicus (Hagerman *et al*, 1990)	0.16	10°C 32‰
Jasus edwardsii (Binns & Peterson, 1969)	0.11 per g dry weight	
Jasus lalandii (Zoutendyk, 1987)	0.2-0.7 per g dry weight	
Cancer pagurus (present studies)	0.02	12°C 30-32‰
Cancer irroratus (Kormanik & Evans, 1984)	0.007	
Callinectes sapidus (Cameron, 1986)	0.15	
Carcinus maenas (Spaargaren, 1982)	0.11-0.72 (Nitrate)	4-20°C 5-42‰
Carcinus maenas (Haberfield *et al*, 1975)	0.26-0.54	
Necora puber (present studies)	0.03	12°C 30-32‰

Table 1.8. Q_{10} values given in the literature and calculated for various Crustacea. (Citations in parenthesis indicate sources for excretion rate data or actual Q_{10} quotations).

Species	Q_{10} value	Temperature range (^{o}C)
Artemia salina (Moffett & Fisher, 1978)	3.1	15-20
	2.9	20-25
Ceriodaphnia reticulata (Gophen, 1970)	2.69	15-22
	1.47	22-27
Trigriopus brevicornis (Harris, 1973)	1.4	5-15
	1.47	5-25
Penaeus japonicus (Spaargaren *et al*, 1982)	8.54	10-25, 100% Seawater
	5.11	10-25, 50% Seawater
Penaeus indicus (Gerhardt, 1980)	2.16	16-28, large animals
	1.9	16-28, small animals
Pandalus platyceros (Quarmby, 1985)	15.24	2.5-9
Nephrops norvegicus (Hosie *et al*, 1991)	6.5	6-12

Table 1.9. Blood ammonia values for Crustacea in the literature under normal (aquarium) conditions, unless specified. (Values have been converted to μmol l^{-1} for ease of comparison, where possible).

Species	Blood ammonia concentration (μmol l^{-1}, units are specified, if different)
Crangon crangon (Hunter, 1991)	189.5-265.9
Crangon crangon (Hunter, 1991)	305.3-436.8 (post-captured animals)
Palaemonetes varians (Hunter, 1991)	167.2
Nephrops norvegicus (Hosie *et al*, 1991)	115.4-170.6
Nephrops norvegicus (Hagerman *et al*, 1990)	130
Nephrops norvegicus (Robertson, 1961)	280
Homarus vulgaris (Florkin & Frappez, 1940)	611-706
Homarus americanus (Young-Lai, 1991)	114
Astacus fluviatus (Florkin & Frappez, 1940)	752
Astacus fluviatus (Delauny, 1927)	1035

Table 1.9. continued

Species	Blood ammonia concentration (μmol l^{-1}, units are specified, if different)
Panulirus argus (Vermeer, 1987)	339.8
Panulirus argus (Florkin, 1960)	105
Panulirus vulgaris (Nicol, 1967)	376.5
Panulirus elephas (Delauny, 1927)	1177
Maja squinado (Nicol, 1967)	850
Maja squinado (Souterbicq, 1935)	114-588
Maja squinado (Delauny, 1927)	914
Cancer pagurus (present studies)	129.6
Cancer pagurus (Souterbicq, 1935)	176.5
Cancer pagurus (Delauny, 1927)	470.6
Cancer irroratus (Kormanik & Evans, 1976)	335
Cancer productus & antennarius (Myers, 1920)	152.7
Callinectes sapidus (Kormanik & Cameron, 1981b)	785 (as NH_4^+)
Callinectes sapidus (Mangum *et al*, 1976)	330

Table 1.9. continued

Species	Blood ammonia concentration (μmol l^{-1}, units are specified, if different)
Carcinus maenas (Harris & Andrews, 1985)	250
Carcinus maenas (Binns, 1969)	234
Carcinus maenas (Souterbicq, 1935)	235-647
Carcinus maenas (Delauny, 1927)	1235.5
Necora puber (present studies)	269.4
Eriocheir sinensis (Mollitor, 1937)	342

Chapter 2

General Materials And Methods

2.1 Animal Supply

Adult specimens of *Cancer pagurus* (L) were obtained variously from a number of suppliers in Britain (see Table 2.1). Supply was dependent on the time of year and the practical logistics of obtaining the animals reasonably cheaply. If a visit was planned to a supplier within a few hours driving distance of Hull, then animals were obtained directly from that supplier. These animals were transported dry, in polystyrene boxes, which also contained bags of ice to minimise temperature fluctuations. During summer months, adult *Cancer* could be obtained from the local, Yorkshire seasonal fishery; animals were obtained from a shellfish processor, Hartley Ltd., Hull, or from Bridlington Trawlers Ltd., Yorkshire. At other times of year, especially during the winter months, a supply of animals was obtained directly from intercepted vivier lorries travelling from Scotland to ports in southern Britain. This interruption minimised the period of aerial exposure for these animals, since the Scottish fishery was the closest ongoing fishery at this time and thus the only source of supply.

Juvenile *Cancer pagurus* were obtained intertidally from the rocky shore at Filey, Yorkshire during the months May or June to September. They were collected at low water during spring tides. After collection, the animals were placed in a domestic ice box along with damp seaweed and bags of ice, to help minimise dehydration and temperature fluctuations during journey to the aquarium at Hull.

Adult *Necora puber* (L), were also obtained from intercepted vivier lorries, along with adult *Cancer pagurus* (as mentioned above). They were brought to the Hull aquarium

dry, in polystyrene boxes with bags of ice, to minimise aerial and thermal stresses for these animals.

2.2 Animal Husbandry

Experimental animals were kept in large (25 - 40 l) aquaria supplied with recirculating seawater (30.0-32.0‰, S) at 10 - 12°C. This aquarium system was used for preliminary physiological experiments, but, it became unsuitable for some experiments, as large fluctuations (5°C) in temperature occurred in the summer months. For this reason a separate, independant aquarium system was built. This recirculating system contained basic filter beds which removed particulate material and maintained water ammonia levels at low concentrations. Marble (calcium) chips were also included in the filters to aid the seawater buffering capacity. An independant cooling system was used to maintain water temperatures unless large variations of weather temperature were experienced. In these instances, temperature varied by 2°C (maximum) per day which was not considered to be too stressful to the animals concerned. Experiments during such times of temperature change were avoided. Summer water temperatures were 15°C and winter temperatures were 8-10°C.

Animals were kept for at least a week, normally two, before they were used for experiments. In the case of juveniles, this ensured the elimination of any tidal rythm which may have affected metabolism and, in all cases, allowed recovery from stresses of transportation. As far as possible, the same batch of animals was used for an experiment to ensure any population differences were minimised.

The animals were fed twice-weekly with thawed, cooked *Mytilus* flesh. On occasions when this food was not available, partly-cooked fish was used. At least 24 hours were allowed to elapse after feeding before ammonia efflux measurement experiments were

made or before blood samples were taken (because of predigestive effects on excretion measurements).

Animals were kept under ambient day:night periods. As it was impractical to attempt to eliminate photoperiod rhythms, experiments were made at the same period during the day, as far as possible, during a set of experiments.

2.3 Haemolymph Samples

Haemolymph samples were obtained from the sinus at the base of the fourth or fifth peraeopod by the insertion of a Pasteur pipette through the arthrodial membrane and allowing the haemolymph to drain into an Eppendorf tube (Treff, Scotlab). Blood sample volumes of 1.0 - 1.5 ml were taken from juvenile and adult *Cancer pagurus*, but, often no more than 0.7 ml were obtained from *Necora puber* specimens. In the latter species, the haemolymph was more viscous and had a tendancy to coagulate rapidly. This necessitated keeping the samples chilled and centrifuging them very soon after their collection.

Laboratory readings of the blood pH were made immediately after the haemolymph sample was taken. It was not possible to make pH readings of blood samples in the field. For ammonia analyses, blood samples were kept on ice to minimise any enzymatic activity which might affect the ammonia levels. Haemolymph samples taken in the field were immediately frozen in a deep freezer (on-site at the dealer's premises), or they were kept on ice for later analysis or were assayed immediately on a machine set up in the field for dissolved ammonia determination. Haemolymph samples taken in the laboratory or field samples, after thawing, were centrifuged (3000g for 5 mins) before further biochemical tests were made.

2.4 Water Samples

Samples of water were rarely retained for later analysis in the laboratory, since they were assayed at the time of sampling. Field water samples were kept cool or were frozen until analysed for ammonia. The ammonia levels of frozen water samples were known not to alter significantly for over a month of freezing at -10°C (Koroleff, 1983).

2.5 Ammonia Measurement

Ammonia measurement was by means of a flow injection/gas diffusion technique (Clinch et al, 1988; Hunter & Uglow, 1993). The principle involves using a carrier stream of NaOH (0.4g l^{-1}) into which a sample is injected (Rheodyne valve, Anachem Ltd). The strong base converts all ammonia in the sample to the free or gaseous form (NH_3) which moves across a PTFE, gas-permeable membrane. On the opposite side of the membrane Bromothymol Blue (0.5g l^{-1}, pH 6.5 - 7.0) flows through. This is a pH-sensitive indicator solution, and changes colour, due to the addition of NH_3 (Fig 2.1). The colour change is detected colorimetrically and the optical density change (measured as peak height on a potentiometric chart recorder (Kipp & Zonen BD4004)) is linearly-related to ammonia concentration. The sample volume varied (from 200 µl - 1.0 ml) to optimise the sensitivity of the machine, the sample volume remaining the same for a series of samples and their standards.

Standards were made from dilutions of a stock solution of ammonium sulphate (1000µmol l^{-1} in ultrapure water (Fistreem R060, Reverse Osmosis, Fisons; Nanopure II, Barnstead)) in the appropriate media for the experiment. Excretion experiment standard solutions were made from the same stock of seawater (low ammonia or enriched with ammonia) that was used in the experimental aquaria. Standard solutions

for blood ammonia measurements were made from the same stock of saline (NaCl 9g l^{-1}) used for dilution of blood samples. In all cases (including the stock chemicals for the ammonia detector) solutions were prepared in acid-washed (HCl, 10%) glassware, which was rinsed in ultrapure water and allowed to dry.

A fresh set of standard solutions (eg 10 - 50 μmol) was made up at the beginning of an experiment and used to produce a linear calibration curve (r=0.9 or better). Following this, a single standard concentration (often 40 μmol total ammonia or NH_4 l^{-1}) was used frequently, and usually between sample replicates throughout the experiment or series of measurements. This allowed correction for any change in sensitivity of the machine, which ocurred especially with seawater analyses, due to precipitation of salts on the PTFE membrane. Standard solutions were made at the relevant concentration ranges of the experiments.

The limit of the detection was down to 0.2 μmol NH_4 l^{-1} and the precision of detection was (0.9-3.3%) for seawater and diluted haemolymph samples (Hunter & Uglow, 1993).

2.6 Measurement of Ammonia Efflux Rate

Animals were placed in individual glass aquaria (14 or 15cm x 14.5cm x 30cm for juvenile *Cancer* or *Necora puber*; 29 or 29.5cm x 19cm x 20cm for adult *Cancer pagurus*) which had been acid washed (10% HCl), rinsed in ultrapure water (Fistreem R060, Reverse Osmosis, Fisons; Nanopure II, Barnstead) and allowed to dry to remove any microorganisms which may affect ammonia water levels. The experimental aquaria were set up in a recirculating water bath to minimise temperature fluctuations. Aeration was provided during all normoxic ammonia excretion measurements since preliminary investigations demonstrated no decrease in medium

ammonia concentration, even with high ammonia levels in the medium. For normal excretion measurements, low ammonia concentration seawater (S=30.0-32.0 ‰) was used (5-6 l for adult *Cancer pagurus*; 2-4 l for juvenile *Cancer* and *Necora puber*) and the animals were allowed to settle for at least one hour before hourly water samples were taken. Efflux measurement experiments were not continued for more than six hours to ensure that any natural microorganism increase in the medium did not affect ammonia levels. In some cases it was possible for the animals to settle in the experimental aquaria overnight with stock recirculating, low ammonia concentration seawater flowing through the aquaria. Before the experiment started the flow was stopped and the water siphoned to the required volume for the experiment.

2.7 Experimental Ecophysiological Methods

Hypoxic water conditions were created by bubbling N_2 gas through the aquarium water and monitoring oxygen saturation with an oxygen electrode (Radiometer E5046, Strathkelvin Instruments) with oxygen meter (Model 781, Strathkelvin Instruments). The oxygen monitor was previously calibrated with a mixture of borax and sodium sulphite solution (0% saturation) and fully oxygenated seawater (100% saturation). On occasions 'natural hypoxia' was investigated, in these instances the animals were left for some time without aeration so that the oxygen was removed by the animal itself.

The effects of 'ammonia enriched' conditions, as sometimes occur (eg. during eutrophication), were investigated by artificially increasing the water ammonia levels using ammonium sulphate solution. Standard solutions for these experiments were made at about the same concentration as the 'enriched concentrations'.

When the combined effects of hypoxia and 'ammonia enrichment' were investigated, the aquarium water was first enriched with ammonia and then the oxygen was purged

with nitrogen, as described above.

Animals were emersed either by removing aquarium water with a siphon or by physically removing the animals from the aquarium. Once the animals were emersed, the container they were in was always covered with with black polythene sheeting to reduce any visual disturbances and to aid the maintenance of a high ambient humidity (*circa* 80% RH). During emersion/re-immersion experiments, on introduction of the animals to the prepared aquaria, a water sample was taken immediately to ensure measurement of any subsequent ammonia unloading by the animals.

Haemolymph samples were taken at the end of most of the ecophysiological experiments. Animals used for efflux measurements in the case of emersion/re-immersion experiments were not bled as this would have created an additional unquantifiable stress; in such instances a separate set of animals were used to obtain haemolymph samples.

2.8 Haemolymph Analyses

2.8.1 Total Protein

The Sigma Kit 540 (Sigma Chemical Co) was used for total protein assays. The priciple is based on the Biuret method of protein measurement and is quantitative as follows:

$$\text{Copper + Serum proteins} \xrightarrow{\text{NaOH}} \underset{\text{(purple)}}{\text{Copper protein compexes}}$$

The Biuret reagent with copper (stabilised with tartrate) reacts with the peptide bonds of the serum proteins to give a blue/purple colour which has an absorbance at 540-

545nm. The colour development is proportional to the total protein concentration in the serum.

10 µl of sample haemolymph was added to 1.0 ml of Biuret reagent. A blank and standard sample were made using 10 µl distilled water and 10 µl protein standard (80 mg ml^{-1} human albumin or bovine serum albumin) respectively added to 1.0 ml Biuret reagent. All test solutions were incubated at 20°C for 15 mins. Sample absorbances were read against the blank at 540 nm using a CE 303 Grating Spectrophotometer (Cecil Instruments). With reference to the standard absorbance the total protein content was calculated:

$$\text{Haemolymph Protein Concentration (mg ml}^{-1}\text{)} = \frac{\text{Sample Absorbance x Standard Concentration (mg ml}^{-1}\text{)}}{\text{Standard Absorbance}}$$

A simple programme was written for the BBC computer so large numbers of sample protein concentrations could be calculated, since large numbers of blood samples were often assayed.

2.8.2 Glucose Analysis

Sigma Kit 510 (Sigma Chemical Co.) was used for glucose analysis of haemolymph samples. It is a quantitative procedure using the enzymes glucose oxidase and peroxidase. The procedure is based on the following reactions:

i) Glucose + $2H_2O$ + O_2 $\xrightarrow{\text{Glucose oxidase}}$ Gluconic acid + $2H_2O_2$

ii) H_2O_2 + O-Dianisidine $\xrightarrow{\text{Peroxidase}}$ Oxidised O-Dianisidine

(Colourless) (Brown)

The intensity of the brown colour at 425-475nm is proportional to the original glucose concentration.

100µl of haemolymph was added to 200 µl of 0.33N perchloric acid, it was mixed and centrifuged at 3000g for 5 mins. 100 µl of the supernatant was added to the Combined Enzyme Colour Reagent (containing glucose oxidase, peroxidase and O-Dianisidine). Standards (2.5, 5.0, 10.0, and 20.0 mg $100ml^{-1}$) and a blank of distilled water were prepared by addition of 100 µl of each to 1.0 Combined Enzyme Colour Reagent. All test solutions were incubated at 37°C for 30 mins. Absorbances of the samples were read against the blank at 450 nm using CE 303 Grating Spectrophotometer (Cecil Instruments) or an Ultrospec 4050 (LKB(Biochrom)). Glucose concentrations of the haemolymph samples were read from the standard curve produced by plotting the standard concentrations against the absorbances measured.

2.8.3 Lactate Analyses

Lactic acid contents of haemplymph samples were measured using Boehringer

Mannheim Kit 139084 (Boehringer Mannheim). The principle of the method is that L-Lactic acid (L-Lactate) is oxidised to pyruvate by nicotinamide-adenine dinucleotide (NAD) in presence of L-Lactae dehydrogenase (L-LDH):

$$\text{L-Lactate} + \text{NAD+} \xrightarrow{\text{L-LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The equilibrium of this reaction lies almost completely on the side of L-Lactate. By trapping the pyruvate in a subsequent reaction (catalysed by the enzyme glutamate-pyruvate transaminase GPT in the presence of L-glutamate), the equlibrium can be displaced in the favour of pyruvate and NADH:

$$\text{Pyruvate} + \text{L-glutamate} \xrightarrow{\text{GPT}} \text{L-alnine} + \text{2-oxyglutarate}$$

The amount of NADH formed is stochiometric with the amount of L-lactic acid. The increase in NADH is determined by means of its absorbance at 334, 340 or 365 nm.

100 µl of haemolymph was added to 200 µl of 0.6N perchloric acid, mixed and cetrifuged at 3000g for 5 mins. A blank for each experimental series and the samples were prepared directly in 1.3 ml cuvettes (1cm path) and the assay performed as follows:

Solutions to be pipetted	Blank	Sample
Glycylglycine buffer (pH 10.0); L-glutamic acid (440 mg) andstabilizers	0.50 ml	0.50 ml
NAD (200 mg), lyophilisate	0.10 ml	0.10 ml
Double distilled water	0.50 ml	0.45 ml
Glutamate-pyruvate transaminase suspension (1100 U)	0.01 ml	0.01 ml
Sample	----	0.05ml
L-lactate dehydrogenase solution (3800 U)	0.01ml	0.01ml

Solutions were mixed and absorbances read against the blank at 340 nm (A1) after 5 minutes. Then 0.01 ml of L-lactate dehydragenase (3800 U) solution was added to the blank and sample cuvettes and mixed. After 20 minutes the absorbances of the samples were read against the blank at 340 nm (A2) and the actual absorbances (ΔA)

of the samples calculated (A2-A1).

The lactate concentrations of the haemolymph samples were determined from the known absorbance coeffecient of NADH at 340 nm (6.3 l $mmol^{-1}$ cm^{-1}):

$$\text{Lactate concentration (g } l^{-1}) = \frac{V \times MW}{e \times d \times v \times 1000} \times \Delta A \times F$$

Where:

V = Final volume in cuvette (ml);

MW = Molecular weight of L-lactic acid (g mol^{-1});

e = Absorbtion coeffecient of NADH at 340 nm;

d = Light path (cm);

v = Sample volume (ml);

F = Dilution factor of the blood due to the fluid content and after deproteinisation.

In this assay Lactate concentration (g l^{-1})

$$= \frac{1.12 \times 90.1}{6.3 \times 1 \times 0.05 \times 1000} \times \Delta A \times F$$

The blood dilution factor, F is obtained from the sample volume added to the perchloric acid (0.1 ml), the volume of perchloric acid (0.2 ml), the specific gravity of the sample (1.06 g ml^{-1}), the fluid content of the blood (0.8), the sample volume of the

supernatant (0.05 ml) and divided by the volumes of sample and perchloric acid used:

$$F = \frac{(0.1 \times 1.06 \times 0.8 \times 0.2) \times 0.05}{0.1 \times 0.2}$$

$$= 0.0424.$$

2.8.4. pH Measurements

Immediately after a haemolymph sample was obtained, a pH probe (combination micro-electrode; 4 x 120mm (Whatman 6200 0160)) connected to a pH meter (Jencon PHM2) was used to determine pH. This particular probe had a small diameter rod which was able to reach to the base of Eppendorf tubes (Treff, Scotlab). The probe was calibrated to pH 4 and 7 (Whatman Labsales buffer) before each sample series and was calibrated to room temperature each day.

2.8.5 Urea measurements

Urea measurements were made using a colorimetric method provided by Sigma Chemicals Co., Kit 640. The principle of the method is to convert all the urea present into NH_2 in the presence of urease, the NH_2 is then measured colorimetrically:

i) $\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_2 + CO_2$

ii) $NH_2 + \text{Phenol-Hypochlorite} \xrightarrow{\text{Nitroprusside}} \text{Indophenol (Blue)}$

Urease solution 0.5ml was added to 10µl of distilled water (blank), serum or plasma

(test)' or 10µl of urea standard solution (30 mg dl^{-1}). The mixture was then incubated in a 37°C water bath for 5-10 mins or at room temperature (*circa* 20°C) for 15-20 mins. This allowed for conversion of the urea to ammonia. Then 1.0 ml of nitroprusside, 1.0ml of alkaline hypochlorite and 5.0ml of distilled water was added to the enzyme solution, mixing after the addition of each solution. The colour of the reaction was allowed to develop for 20-30 mins and the absorbance measured against a blank at 570nm using an Ultrospec 4050 (LKB (Biochrom)). With reference to the standard absorbance the urea concentration was calculated:

Urea concentration (mg dl^{-1})

$$= \frac{\text{Sample absorbance x Standard concentration}}{\text{Standard absorbance}} \text{ (mg } dl^{-1}\text{)}$$

Table 2.1. Dealers used to supply animals and their locations of supply.

Dealer	Location of supply
Wilsons of Holyhead	West Scotland (Oban) and Western Isles (Benbecula and Islay)
Holyhead Fish Processors	West Scotland - Islay and Western Isles
Shin Game Ltd.	North and Eastern Scotland
Pat Jenkins	Southwest Wales
The Lobster Farm	North Wales
Monteum Ltd.	South England
Hartley	East England - off Spurn Point
Bridlington Trawlers Ltd.	East Yorkshire coast

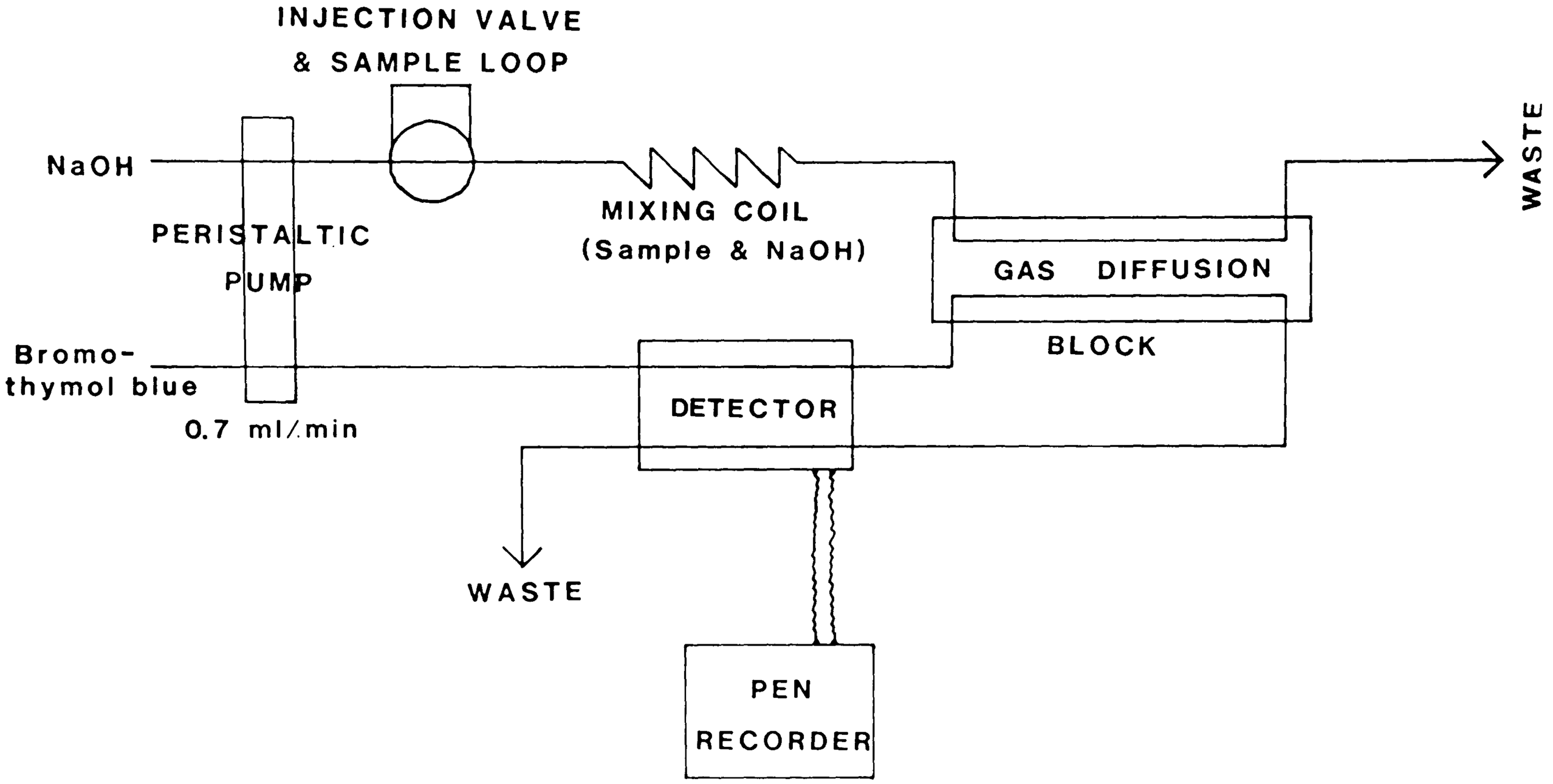


Figure 2.1. Diagram of the flow injection/gas diffusion system for ammonia analysis.

Chapter 3

Overview of Protocols Observed in the Live Transport of Crustacea

These descriptions are mainly derived from personal observations and measurements made during the course of these studies. In many cases they are the first documented accounts of these practices, except where reference is made to cited work. This account attempts to summarise those practices which are pertinent to the field and laboratory work described in this thesis and, therefore, is a summary and not an exhaustive account.

The UK trade in live crustaceans is almost exclusively dependant upon product (= animals) caught in UK waters and landed at UK ports. Although some Welsh dealers may purchase and handle product caught by Irish fleets, some of this product is used in the processing industry as well as the live trade. The principal species involved in live marketing are:-

- *Cancer pagurus* (L)*, the brown crab, which is landed in most of the fishing ports of the UK;

- *Necora puber* (L)*, the velvet swimming crab, principally a West Coast species which is landed in south west and western ports in the UK, as far north as the Scottish Hebridean islands;

- *Maja squinado* (L), the spider crab, which is landed in South Wales and the Channel ports;

- *Homarus gammarus* (L), the lobster, which is landed in most fishing ports in the UK;

- *Palaemon serratus* (L), the shrimp, landed on the west Wales coast during the summer and autumn months only;

- *Carcinus maenas* (L), the green or common shore crab, which is fished and landed principally on the west coast of Scotland;

- *Nephrops norvegicus* (L)*, scampi, prawn or Norway lobster, which is landed in northern east and west coast ports;

(* - Species actually studied in this work).

These species have their principal 'seasons' of maximum landings and these timings vary geographically. This, coupled with the wide geographical separation between the most northerly and most southerly ports produces a complex pattern of seasonal and spatial availability of product. This has underlined the need for the development of appropriate handling and transport techniques to cope with this situation. Many of these developments are recent and have occurred with the rapid and large expansion of the export trade in live crustaceans to the near continent over the past 15 years. Developments occurring within the time that these studies were made, have enabled problems to be identified which affect all aspects of the trade - problems associated with the principal aim of maintaining the intrinsic quality (the quality of the animal at the time of first capture) of the product along the whole marketing chain and which is represented diagrammatically in Figure 3.1. The brown crab and the velvet crabs are species that, previously, were not landed at all by Scottish fishermen. Therefore, the development of the Scottish trade in these species has been heavily dependant on the

import of necessary expertise from other fishing areas - an exercise which, as with many new or growth projects, has not been always accomplished smoothly.

3.1. Post Harvest Treatment

Figure 3.1 shows schematically the principal events which may occur in a simplified marketing chain. These events can be segregated into those that occur at sea (shipboard), those that occur when landed (first sale, temporary storage *etc*) and the peculiar situation when they are 'in transit' from one place to another. A final phase - after delivery to the continental dealer also occurs but has not been extensively studied here because of financial constraints.

Taking the phases in order:-

3.1.1. Shipboard

Edwards (1979) has described the principal means of capturing crabs using baited pots. Lobsters, velvet crabs and prawns are all caught also using baited trap methods. Although some prawn and crab are caught by trawling methods also, the incidence of physical damage to the product with this fishing method usually precludes such animals from consideration for the 'live' trade.

Figure 3.2 shows schematically the crab handling practices used currently by the fishermen in the SW UK fishery - probably the most sophisticated methods employed in the UK and which have developed from a long established trade to satisfy the French market demand (which is for a very high quality of delivered product). This success is based on meticulous monitoring of the crabs, by 'nicking' them (cutting articulating membranes of the chelipeds, see section 3.1.1.3 to prevent damage to other animals during storage, by removing badly damaged or

irreparable individuals for processing and 'repairing' any broken legs. Leg repairs are done by inducing the animal to autotomise the damaged limbs which leaves a membrane at the point of autotomy and thereby prevents continuous and excessive blood loss. Usually the animals are held for a few days before being sold-on. This period allows the removal of weak individuals and gives the consignment an improved chance of arriving at the destination in a good condition. "Weak" individuals may not survive the journey and their death may foul the water and thus exacerbate the problems of water quality which are caused normally by the holding of large numbers of animals in a set volume of water by fouling the water further.

Scottish practices (Fig 3.3) have developed more recently and vary widely. Until recently there was a common practice of killing all brown crabs caught as they were considered to be a pest which competed with lobster for the bait in pots. Nicking is now standard practice, but was not always widely used as in the SW UK and selection of the animals is still (1993) not as thorough as is routine in the Channel fisheries. On occasions, even where selection has been undertaken, the load is sometimes later supplemented by freshly landed or unselected animals. This unwise practice is due to possible financial pressures, with the need to maximise loads consigned and also to compete effectively for product with other dealers.

The fishery operating in South Wales (Fig 3.4) is quite small and only involves local dealers, who have stipulated required practices to catchers to land product suitable for vivier transport. The animals are generally well-selected and treatment of the animals during handling, is satisfactory - mainly due to the immigration of some catchers from the Channel fishery. These fishermen have come to the Welsh fishing grounds because of increased competition for crab fishing in the Channel fishery. However, the practice of putting newly caught animals onto a vivier lorry may bring about problems, since these animals are not held in keep pots (section

3.2.1) and weak individuals are not selected out at all. The problem is lessened. however, because the total journey time to the Brittany dealers is less than 24 hours from South Wales. Consignments for Scotland, however, normally have to endure a further 24 hours in vivier tanks before delivery. Consequently Welsh dealers prefer to have a fast 'turn round' of product to avoid having to hold product for any length of time.

A very recent development is the live trade in crab from the Yorkshire and East Coast stocks. Currently, the East coast is providing weekly consignments of live brown crabs and lobsters to French dealers and an outline of the marketing chain is given in Figure 3.5. The 'season' of this fishery has become 'year round' and the traditional fisheries of 'inshore' regions are now greatly augmented by 'offshore' catches. This has coincided with a recent (1993) shortage of crabs - the reasons for which may be environmental or due to possible overfishing.

3.1.2. *Necora puber*

The velvet swimming crab, *Necora puber* is a more valuable product than *Cancer pagurus*. This is a new species for commercial exploitation in the UK and the trade has arisen because of Spanish (to a lesser extent, French) demand for this species. Consequently, new fishing, handling, holding and transportation methods have been developed in the past few years. This has been an expensive process as continental dealers usually refuse to pay for any dead or poor quality product - no matter what species. In the early 1980's, mortality rates of *Necora puber* during consignment sometimes reached 80-100% (Whyman *et al*, 1985).

Necora is not common in the North Sea and the principal fisheries occur in SW England, west Wales and the west coast of Scotland. At first it was prosecuted

mainly as a by-catch of other species (principally lobsters), but now there are specialised fisheries for this species (MacMullen, 1983a, b). The species can tolerate short periods (3-4 hours) in air, but any longer than this is a commercial risk, the animals being required to be returned to water as their intrinsic quality deteriorates rapidly in air. Consequently, Scottish practices have developed to cater for the long distance journey times involved in the live marketing of this species (Whyman *et al*, 1985).

The crabs are placed in plastic mesh containers the size and shape of crates that are used to market Spanish oranges. Weights of 8kg of the *Necora* can be packed/kept in this way (MacMullen, 1983a; Quinlan, 1984). The animals are usually put into these containers as soon as they are caught and are then held tethered in mid-water or over rocky ground until onward sale to a dealer. Such dealers then either hold the animals in holding ponds or place them in crates again for onward transport. Often the first buyer is a Spanish dealer working from a vivier lorry on behalf of a dealership in Spain and the purchased animals are packed directly into lorry-based vivier tanks.

3.1.3. 'Nicking' and 'Repairing' *Cancer pagurus*

In this context 'nicking' refers to the severing of the tendons associated with opening/closure movements of the chelipeds and 'repairing' refers to the process whereby crabs are induced to autotomise any legs which are physically damaged and leaking blood.

Nicking greatly slows the opening/closing of the chelipeds and thus reduces the incidence of chela-related damage incurred by husbanded animals. It also minimises the incidence of injury to any persons which may have need to handle the

product.

Figure 3.6 shows diagrammatically the two principal means by which crabs are nicked. The traditional English method of nicking (Fig.3.6a) is to sever the dorsal tendon at the point of articulation with the cheliped. Market pressure (and a generally accepted view that the technique is easier and more effective) has resulted in the French nicking method (Fig.3.6b) being the predominant, if not only, method in current use. Nicking is usually done with a blunt edged knife and involves no actual cutting or sawing. The tendon is put under pressure by means of a special implement (Fig.3.7) and very light pressure on the tendon is sufficient to snap it with minimal or no blood loss. The method, properly done is rapid and does not result in excessive subsequent necrosis of the cheliped muscle.

When a crab has had both claws nicked, the customary practice is to examine it quickly to see if any other legs (walking legs and chelae) are damaged or whether any physical damage has been sustained. Any damage distal to the plane of autotomy on one leg (Fig.3.8)- the most common damage is that to the dactylopodite - results in the operator inducing that limb to be autotomised. This is done by holding one segment of the limb firmly and twisting the segment distal to it. The autotomised limb wound is automatically sealed with little or no blood loss. Crabs with damage proximal to the plane of autotomy are rejected from those selected for the live trade and are usually swiftly processed. So too are animals which may have excessive limb loss or which have lost both chelae. Such product does not command a premium and, therefore, would only serve to reduce the overall value of the consignment.

Experienced operators advocate that freshly nicked/repaired product are rapidly returned to water holding conditions and, ideally, allowed at least 24 hours to

recover before being consigned.

Nicking and repair operations are applied to *Cancer pagurus* only. The much smaller size - hence numbers per kilo - precludes such labour intensive practices being applied to *Necora puber*.

3.2. Systems used for holding product before selling on

3.2.1. Shipboard to first sale

When brown crabs are first captured they are removed from the trap and then may either be placed in plastic boxes or in large plastic drums, popularly known as 'bongos' (Figs.3.2-3.5). In some of the larger vessels, special 'vivier' wells are constructed (Figs.3.2-3.4). These allow the free passage of seawater through them *via* stopcocks. Small vessels, which make short (< 1 day) trips usually keep the crabs dry until landed. Many of the smaller Channel crabbing boats will use the boat hose-pipe to constantly irrigate the bongos full of crabs. These fishermen will nick their crabs either at sea or after landing - usually dependant on the particular instructions of the dealer they supply. Larger boats may operate several hundred traps in a series of 'fleets' (a group of 10-20 pots attached, in line and at intervals to an anchored rope and which are hauled in using a powered block, Edwards, 1979). Such vessels may be used for trips lasting a number of days and the crabs are usually nicked at sea.

The same procedures are used for the other crustacean species fished. In the majority of cases the animals are initially placed in plastic boxes which have sea water hosed to them. A certain amount of selection is carried out at sea to comply with existing legislation on minimum sizes. Some, newly moulted 'soft' animals have no commercial worth and are returned to the sea. The only other restriction

against the landing of product with low intrinsic quality is set by the demands of the purchasing dealer.

In several of the more remote parts of the UK - west Wales, and around the Scottish coast - the crab are sold-on to dealers who arrive with large vivier lorries each week. In such cases, the catch is held in keep pots until the time of first sale. Keep pots are usually made of wooden planks which have several holes perforating them. They are moored so that they float just below or at the surface of the water and the held product is inspected daily. Scottish fishermen in particular have to take care that the keep pot is not affected by silt at low tide times or by freshwater runoff which serves to form a cold surface layer over sea lochs during winter months. Some of the English Channel port dealers have elaborate floating pontoons moored inshore. These can accommodate several tonnes of product and contracted catching vessels land directly to these.

Catchers will normally empty their keep pots in advance of the arrival of the weekly dealer. This may involve a journey and a wait of several hours - all of which time the animals are in the air. They may or may not be covered with damp sacking during this period. Such 'dry' periods apply to any other species for sale (*eg* *Necora, Homarus, Maja, Carcinus* or *Nephrops*). The dealer may then transport the purchased product 'dry' to his establishment where holding ponds are available. Some dealers - especially continental ones - will have vivier tanks of aerated, chilled seawater on-board their lorries.

3.2.2. Dealer Holding Systems

A variety of holding systems are used by UK and continental dealers. Many are now some years old and of traditional design whilst others are newer and have

incorporated some novel features.

3.2.2.1. The Traditional Pond

In the UK these are usually indoor facilities. The enclosures are of concrete and usually close to a supply of good quality seawater which is piped/pumped to the ponds periodically each day. Some dealers may use a simple gravel or shell filter and recirculate the water whilst others use no form of filtering and rely on an appropriate throughput of water. Usually very little attention is paid to monitoring the temperature or the salinity of the water used. Some ponds do not have means of aerating the water, but all are designed to be drained when the product is to be packed and sold-on. The ponds may suffer from inadequate aeration and fouling from dead animals or poorly maintained filter systems.

3.2.2.2. Modern Holding Systems

These usually are of a similar construction to those described above, but with a covering of 'resinous' paint to line the tank. Aeration of the ponds is standard and an efficient biofilter is usually built into the system. Some of the larger installations incorporate complete water quality maintaining systems, which include protein skimmers and ozonation treatments. Such systems can utilise recirculating water safely.

3.2.2.3. Cascading Systems

These systems utilise a stacked array of perforated holding tanks which are supplied by an overhead supply of seawater which then penetrates successively through all the tanks. Such systems are effective for holding product for some days or weeks.

are relatively cheap and can be produced and dismantled in step with the supply of product. Usually, the product is checked twice a day, when this happens the top box becomes the bottom of the new stack.

In such systems the supply of seawater has to be sufficient to cater for the numbers of stacked boxes used. The top box is used to break the impact of the water and the box at the base of the stack collects particulate debris. A sump may or may not be used with such systems.

In Canada such cascading facilities have reached an optimum development. Here they are used for the bulk holding of lobsters and are known as 'champagne' systems. Some of the installations may have over a million lobsters held this way at any given time. The holding trays may be sub-divided into 8X4 compartments and such trays are palletised in stacks which may reach 20 high.

3.2.2.4. Vivier Tanks

These are usually associated with road-based transport systems and comprise large chests of aluminium or fibre glass which line the walls of dedicated vivier lorries. Although supplied with aeration, there are no methods used to improve or even maintain water quality whilst the product is being held. The normal size of the vivier is such that 1 tonne of *Cancer*, (brown crab) is placed in 1 tonne of seawater and 1 tonne *Homarus* or *Necora*, (lobster/velvet swimming crab) are placed in 2 tonnes of seawater. Normally, the interior of the lorry is chilled to 6^{o}C - though this rarely reduces the temperature of the water by any substantial amount. Many of the lorries in current use can carry 8-10 tonnes of product (plus seawater) and total journey times of $>$48 hours are reasonably common.

Cancer, Homarus, and *Maja* are normally packed 'loose' into the vivier tanks. *Carcinus* are packed into mesh sacks and *Necora* are usually packed into small plastic crates the same size and dimensions as wooden crates used for marketing oranges. *Necora* is a high priced commodity and warrants the extra handling procedure (though the packing procedure often has an adverse effect on the product). The crates usually contain 8 kilo of crabs which are very tightly packed together to prevent them damaging each other. The crates themselves are stacked closely together which reduces the chances of aerated water reaching some of the animals (Whyman *et al*, 1985).

3.3. Vivier conditions

Oxygen supply to the animals often becomes limiting, despite vigorous aeration of the vivier water during transport (Whyman *et al*, 1985). This is especially so for the velvet crabs, packed closely in boxes with conditions in the centre becoming hypoxic for the animals. Lactate is also produced by the animals under these conditions, but at a slower rate at low vivier temperatures compared with normal ambient temperatures for the crabs (Whyman *et al*, 1985). Crowding of the animals in the vivier tanks is evident, and can produce some damage to the animals (Chapter 4). High ammonia is also produced in the viver tanks due to excretion from the animals, and decomposition of the dead animals (Whyman *et al*, 1985).

3.3.1. 'In transit' Water Temperature

In the majority of cases, vivier lorries are supplied with air chilling equipment. However, the water used in their vivier tanks is frequently considerably higher than 6^{o}C commonly used as the in-lorry air temperature. Few operators seem to appreciate that air-chilling only very slowly lowers water temperature and, in many cases, such air chilling in completely ineffectual in producing a significant alteration

of water temperature. At least one successful dealer transports his product at ambient water temperature. The objective of chilling is to lower the general metabolic rate of the product so that is less mobile (hence less prone to inflict damage on neighbouring animals) and will consume less oxygen and excrete less ammonia per unit of time. Chilling to 2°C too quickly can have the effect of inducing autotomy which very much reduces the commercial value of the product and, in the case of lobsters results in a serious loss of value of the animals (Whiteley & Taylor, 1986).

3.4. Handling of Product

It is generally advocated that all live crustaceans are 'placed' not 'thrown'. This minimises aggressive interaction between animals and minimises the risk of physical damage to them being incurred. Particular care is usually taken when animals are removed from the catching pots or keep pots. Animals often project one or more limbs through perforations or meshes and rough handling at such times can result in limb damage to the animals.

3.5. Alternatives to Vivier Systems

Some attention is currently being paid to devising effective methods of holding and, particularly transporting product with the minimum of water to enable larger loads of animals to be carried and thereby give a better financial return on a consignment. During the present studies, two novel systems were observed in use - one utilising a sump and spray system with one large butyl container filling the floor area of the lorry (a cascade system) and the other with the interior of the lorry fitted with especially designed perforated trays and used with a mist spray system (a mist system). Neither system worked particularly well because of the lack of any provision to remove excreted metabolites from the water or to cater for debris such

as cast limbs. Commercial dealers tend to be very secretive about such systems which they hope will confer to them a commercial advantage over their competitors. However, it is understood that at least two UK dealers are using forms of cascading systems in especially designed vivier lorries.

3.6. CASE STUDIES

3.6.1. Vivier Transport Studies

The following case studies demonstrate the types of stresses which are imposed upon crustaceans when they are transported alive in bulk. The data given include those of water and blood samples analysed using techniques current at the time of sampling.

The principal stresses imposed comprise those of aerial exposure, physical handling, temperature fluctuations and prolonged exposure to poor quality water (low oxygen saturation and high dissolved ammonia content). Different stresses are imposed at different stages of the marketing chain and this is summarised in a Table which accompanies each case study described. These descriptions serve to show how varied and non-standard the various marketing protocols are and what the possible cumulative effects on the animals may be.

3.6.2. CASE STUDY 1: Monitoring of a consignment of *Cancer pagurus* from Benbecula (Outer Hebrides, Scotland) to Santander (N Spain) - (Journey undertaken during August).

Table 3.1 summarises the principal events at various places during the trip and

points out what the suspected stresses imposed were. The consignment was made up of a series of small batches of animals purchased locally and successively added to the dealer's ponds. This supply was then augmented by product bought in from the Isle of Barra. Animals were sent by road to Holyhead in two lorries, one with animals packed 'dry' in plastic boxes and the remaining animals in conventional vivier tanks. At Holyhead, all the animals were transferred to a Spanish dealer's vivier lorry for transport to Santander.

The data relating to the analyses of the haemolymph samples are given in Table 3.2. The total protein and haemocyanin concentrations showed no clear trend of change whilst the animals were in the UK, but the mean concentrations of both were much lower on arrival at Santander. When the relative concentration of haemocyanin (% of total blood protein) is considered then there is a trend of haemocyanin loss from an original mean of 96% in Benbecula to a final mean of 73.8% in Santander.

The high blood lactate values reflect the periods in air. Thus levels were at 40.2 mg/100ml blood after >6 hours aerial exposure in Benbecula, but this dropped subsequently to a mean of 17.1 mg/100ml blood after 30 hours in the holding ponds. Animals transported 'dry' to Holyhead (*ca* 8 hours) had blood lactate values of 63.6 mg/100ml blood (*cf* those sent in vivier tanks, 20.3 mg/100ml). The animals at Santander had relatively low blood lactate levels (11.7 mg/100ml blood) which probably reflects a good level of water aeration in this Spanish lorry.

A Kruskal-Wallis one way analysis of variance of the data was undertaken using a PC based program, SPSS. This nonparametric method was used because the data collection was not easily predetermined and the source of the data was not always from the same sampling groups. The results are shown in Table 3.3. Since significant differences were found between the data values of the different groups

for all the blood determinations a multiple comparison procedure was undertaken to determine which groups of data were causing the significance in the differences. The results of these comparisons are also shown in Table 3.3, the underlining demarking the mean rank values which are similar; groups which are underlined can be considered to be different from one another.

The results for both the haemocyanin and total protein contents of the blood were not clearly defined, since two groups of values for the sample groups were found, but within the comparisons four sample groups overlapped. The sample groups which did not overlap are those responsible for the differences detected by the ANOVA. In the case of haemocyanin, the values found for the groups from Santander and in the holding ponds at Benbecula were significantly different from those determined for the group from Barra. This may be explained by the fact that the mean of the values obtained from the animals arriving by vivier from Barra was the highest obtained during the investigation of this journey. The sample groups which showed clear differences of total protein levels by the multiple comparison procedure were the animals which were freshly bled at Holyhead and those sampled from the holding ponds at Benbecula which were significantly different from the group which was sampled from the vivier consignment at Holyhead. The heamocyanin values of the group which arrived in Santander by vivier and the group sampled in the ponds at Benbecula were significantly lower (<0.05 in both cases) than that of the group which arrived at Benbecula from Barra.

The multiple comparison of the lactate values demonstrated that there was an overlap of some sample sets. However, two easily-defined sets were determined by the procedure (Table 3.3). One of these two groups included the sample groups from the vivier lorry at Santander, a group from the animals which arrived to Benbecula from Barra by vivier boat and the group from the holding ponds, at

Benbecula. The other set includes the animals which had been held 'dry' at Benbecula, a group which were sampled from the animals transported dry to Holyhead and a group of the same animals which were freshly bled at Holyhead. The clear difference between these two sets is that one relates to groups held in water (either holding ponds or vivier lorries) and the second relates to animals which were being held in air.

3.6.3. CASE STUDY 2: Vivier consignment from S Wales to Audierne, Brittany. (Journey undertaken in February).

Both *Cancer pagurus* and *Necora puber* were monitored on this journey, although the consignment included *Homarus gammarus* and *Maja squinado*. Table 3.4 summarises the main events which occurred during the trip and includes haemolymph data obtained. This journey was the first during which blood ammonia measurements were made relating to various times during the trip.

The Table 3.5 shows the data obtained from two different ports, Solva and Fishguard (South Wales) and the recent history of the product sold to the dealer. Mean total protein levels of brown crabs were not significantly changed throughout the journey, but blood lactate levels were found to be elevated on arrival at Audierne. Lactate levels showed signs of dropping back towards original levels after a night in the French dealer's ponds. The picture for *Necora puber* was slightly different as blood total protein levels were lowered after the overnight stay in the French ponds. Blood lactate and blood glucose values of *Necora* were lower than those of *Cancer* and *Necora* did not show the large increase in blood lactate on arrival at Audierne (*cf Cancer*). The *Necora* blood lactate levels were low after recovering overnight in the French holding ponds.

Both species shared a pattern of showing a greatly increased blood ammonia

concentration on arrival at Audierne. *Necora* had a mean value of nearly 5 mmoles l^{-1} and *Cancer* >3.9 mmoles l^{-1}. These values had dropped to >1.3 and >1.5 mmoles l^{-1} respectively after the overnight recovery in the ponds. Table 3.6 summarises the data obtained on the seawater ammonia concentrations measured at various points in the journey. These data demonstrate the very high ammonia levels that develop during the course of a typical vivier journey.

Statistical analyses were again performed on the data. A Kruskal-Wallis one-way ANOVA was performed - a nonparametric method since the data source could not be predefined before data collection commenced. A multiple comparison analysis was carried out where the results were found to be significant. The results of these analyses are given in Table 3.7.

The statistical analysis of lactate and ammonia for *Cancer pagurus* (Table 3.7) revealed some significance ($P<0.05$) differences between the groups. Two sets were found within the lactate data which overlapped in terms of the sample groups. The groups which were clearly different were the animals sampled at Solva *cf* those sampled on arrival at Audierne by vivier lorry. Two clearly separate sets of ammonia data were defined and these related to animals sampled before loading in South Wales and animals sampled in Audierne. The high blood ammonia concentrations found at Audierne, even after overnight recovery of the animals in holding ponds were probably the cause of the differences found. For *Necora puber* the significant difference ($P=0.000$) found for blood ammonia derivations, again was due to the separation of two sets of sample groups. These corresponded to those samples taken prior to vivier transport, and after overnight settling in holding ponds in Audierne.

The particular French dealer who was involved in this particular investigation used a system whereby the seawater was replaced at each high tide and was recirculated

between such times. This accounts for the relatively high seawater ammonia value of 57.1 μmoles NH_4 l^{-1}. The water in the French dealer's vivier tanks was also high (25.1 μmoles l^{-1}) as some animals had been loaded previous to the commencement of monitoring.

The temperature changes experienced by the animals were considerable. In South Wales the vivier water was $<10^{o}C$ and this was allowed to increase to an arrival temperature in France of $12^{o}C$. The French ponds used ambient seawater temperature which was $18^{o}C$ and during recirculation some tanks reached $21^{o}C$. The temperature advocated by Seafish (Seafish Industry Authority) for the transport of live crustaceans is $6^{o}C$. Clearly, transporting animals at ambient temperature results in acceptably low mortality rates, but reception temperatures used here may represent a risk if animals happened to be delayed in transit or were received in a highly-stressed condition. As it is, the blood ammonia levels of *Cancer* and *Necora* were still extremely high compared with normal animals- even after several hours in low ammonia seawater following unloading.

3.6.4. Other Transport Studies

Alternative methods of transport compared to vivier transport were developing at the time that these studies were undertaken. The opportunities for study of these alternatives were limited but data were collected during a journey which was undertaken using a novel cascade method and, less comprehensively, during a trip using an especially fitted lorry for a mist system of transport.

3.6.5. CASE STUDY 3: Journey from Kyle of Lochalsh (W Scotland) to Roscoff (Brittany). (Journey undertaken in May).

This journey involved the use of a cascade method of transport, the data collected are

given in Table 3.9 and a summary of the events at the dealer's premises before, during and after the trip are given in Table 3.8. A cascade method of holding the animals before onward-transport was used at the dealer's premises in Kyle of Lochalsh (Scotland) and data related to samples taken during the use of this method are given with the journey data in Tables. It was during the data collection for this particular trip that blood ammonia determinations were first made and relate to haemolymph samples taken at Roscoff only.

Data obtained from animals in the holding facilities at the Kyle of Lochalsh are shown in Table 3.9. Two systems of recirculating water were in use. The holding facilities were as described previously in section 3.2.2.3. using stacks of plastic boxes. The seawater supplied was pumped directly via the quay from the sound of Kyle, which was adjacent to the holding facilities. Water drained through the stack of tanks and was allowed to drain directly back to the quay side, the water supply being used only once for a stack of boxes. A recirculating system was also set up in tanks which were originally of the conventional holding type and which had the same source of water supply as the other stacks of boxes, but which was in fact recirculated by means of a pump for a number of hours before renewal. Blood total protein appears to show a negative relationship to holding-time in the system with an approximate loss of 20-25% protein (41.75-34.24 mg ml^{-1}), a >45% increase in lactate (29.3-58.22 mg $100ml^{-1}$) also occurred over a 2 week holding period (Table 3.9). The animals in the recirculating system, however, had a higher mean lactate concentration (58.2 mg $100ml^{-1}$) than even the freshly landed animals which may indicate high levels of stress are associated with this holding system. The blood glucose concentrations showed little variation while in the holding system which may be argued as evidence of little imposed stress.

The data obtained during the journey (Table 3.9) can be compared with those relating

to conditions where animals are held. Blood glucose determinations made of samples taken in France were high (*cf* Kyle samples) - even for animals sampled after an overnight recovery in ponds. The mean final lactate level (7.1 mg ml^{-1}) is comparable to values measured for animals held in the recirculating water system before the journey. The higher blood protein and glucose levels found in Roscoff may be attributed to conditions prevailing during transport with this novel cascade method. Certainly, detectable nitrite levels were found in the water used for the cascade journey, the production of nitrite is likely to be increased at times of hypoxia (and such conditions also induce an increase in haemocyanin synthesis in Crustacea (Hagerman *et al*, 1990; Hagerman & Uglow, 1985).

A nonparametric statistical analysis was performed on the data, using Kruskal-Wallis one way ANOVA. The results are summarised in Table 3.10 and also show the multiple comparison analyses undertaken for the incidence where significance was found. No significant segregation of the groups was found in the case of haemolymph total protein levels. Separation of sets of sample groups comparing other haemolymph constituents of groups of animals studied at the holding premises and after the journey at Roscoff was possible, although in all cases some groups were not clearly segregated. Glucose and lactate levels were clearly different (for relevant comparisons) in the samples taken from animals arriving after cascade transport at Roscoff from those sampled in the cascade holding systems or those freshly landed at Kyle of Lochalsh. The sampling which led to a detectable difference in haemolymph lactate concentrations was again that made of the animals on first arrival at Roscoff and compared with animals in the flow-through cascade systems for both long term and short term holding in Scotland.

3.6. CASE STUDY 4: Mist System (Journey undertaken in March).

Investigations during this study were confined to those made in France. The mist

system has been described earlier (section 3.5), but essentially has been designed to allow the animals being held or carried to be in very humid conditions. The principle being that the conditions allow the normal gas and ion exchanges across the gills to occur so that the animals can still respire and excrete. The data collected for this journey are given in Table 3.11. The journey took place between Stornoway in the Scottish Outer Hebrides and Cherbourg in France totalling a journey time of about 46 hours. Analyses of the haemolymph of crabs which arrived in Cherbourg revealed that very high blood and water ammonia levels (1629.9 μmol l^{-1}) had accumulated in these animals. This particular trip also resulted in a high "in-transit" mortality of crab. An analysis of the recovery of animals after the journey was not possible. Nitrite was detected in a water sample which was taken from the sump of the mist system. This nitrite, accompanied by the high levels of ammonia found in the haemolymph, may have caused the mortalities The mist system itself, although used successfully elsewhere is a much better alternative to holding the animals in dry air but does not allow complete efficiency of gaseous ammonia exchanges across the gill surface to occur and so some accumulation of metabolites in the animal is probably inevitable. A combination of the design behind this system and that used for cascade-holding of animals has been shown to work effectively for the long distance road transport of the Dungeness crab, *Cancer magister*, in the United States. In that instance, low volumes of water were pumped and cascaded through trays of animals which were kept alive for journeys of up to 4 days (Barnett *et al*, 1973).

Table 3.1. Detail of possible consequent stresses which crabs may sustain during stages of the live marketing from Benbecula to Santander.

Place	Action	Possible consequent stress
BENBECULA and nearby islands	Keep-pots raised. Crabs placed in plastic boxes covered with damp sacking.	Mechanical through handling, physical damage to the animals and Physiological through aerial exposure
BENBECULA at various landing ports	Dealer buys animals from fishermen who transfer them to dealer's boxes	Additional Mechanical stress and Physiological stress animals held >6hours before reimmersion
BENBECULA Holding ponds	Animals transferred to holding ponds *	Mechanical stress and Thermal stress. Water quality vital
BENBECULA Holding ponds	Animals arrive from Barra by vivier, transferred to ponds *	Mechanical and Thermal stresses
BENBECULA Holding ponds 30hours since arrival of Barra animals	Animals transferred to vivier - some selection *	Mechanical and Thermal stresses
BENBECULA TO HOLYHEAD	Transport by vivier *	Mechanical due to lorry motion and competition between animals in tanks. Physiological due to high ammonia levels and other deleterious water quality elements
BENBECULA TO HOLYHEAD	Transport dry *	Mechanical due to transport motion. Physiological, aerial exposure 12-14hours
HOLYHEAD	Animals transferred to Spanish vivier lorry	Mechanical and Thermal stresses
HOLYHEAD TO SANTANDER	Transport by vivier	Mechanical due to lorry motion and competition between animals in tanks. Physiological due to high ammonia levels and other deleterious water quality elements
SANTANDER	Animals inspected by customs *	Mechanical due to handling. Thermal due to temperature increase on opening of refrigerated container

NB *denotes when haemolymph samples are taken

Table 3.2. Haemolymph data relating to samples taken during a consignment from Benbecula to Santander. Values are given as means ± standard errors. Numbers in parenthesis refer to numbers of animals sampled.

Cancer pagurus.

Sample	Total protein (mg ml^{-1})		Haemocyanin (mg ml^{-1})		% Haemocyanin of total protein	Lactate (mg $100ml^{-1}$)	
BENBECULA > 6hours aerial exposure	42.28 ± 4.63	(20)	43.71 ± 5.43	(20)	100.00	40.25 ± 3.04	(19)
*BENBECULA Animals as above after 30h in holding ponds	29.32 ± 5.53	(10)	26.37 ± 3.90	(10)	89.94	17.08 ± 2.85	(9)
BENBECULA Animals arrive by vivier from Barra	42.75 ± 4.05	(20)	53.76 ± 4.86	(20)	100.00	31.95 ± 2.88	(20)
HOLYHEAD Animals arrive 'dry' from Benbecula and subsample taken of Benbecula sample group *	40.84 ± 4.31	(18)	41.80 ± 4.70	(18)	100.00	65.64 ± 3.13	(18)
HOLYHEAD Animals arrive 'dry' from Benbecula and freshly bled	27.78 ± 5.91	(6)	35.70 ± 8.48	(6)	100.00	57.45 ± 8.96	(6)
HOLYHEAD Animals arrive by vivier from Benbecula	66.34 ± 6.74	(10)	46.95 ± 5.84	(10)	70.77	–	
SANTANDER Arrival by vivier	40.19 ± 6.42	(8)	23.85 ± 4.20	(8)	59.34	11.75 ± 1.96	(8)

Necora puber

Sample	Total protein (mg ml^{-1})	Haemocyanin (mg ml^{-1})	% Haemocyanin of total protein	Lactate (mg $100ml^{-1}$)
HOLYHEAD Animals arrive by vivier from Benbecula	36.65 ± 4.09 (10)	32.80 ± 6.33 (10)	89.49	20.42 ± 2.29
SANTANDER Animals arrive by vivier from Benbecula	55.86 ± 24.20 (10)	26.03 ± 4.01 (10)	46.60	–

Table 3.3. Results of nonparametric oneway analysis of variance (Kruskal-Wallis) tests and consequent multiple comparison analysis (denoted by underlining) performed on the data derived for the vivier journey from Benbecula to Santander.

Groups compared	Group number
BENBECULA > 6hours aerial exposure	1
*BENBECULA Animals as above after 30h in holding ponds	2
BENBECULA Animals arrive by vivier from Barra	3
HOLYHEAD Animals arrive 'dry' from Benbecula and subsample taken of Benbecula sample group *	4
HOLYHEAD Animals arrive 'dry' from Benbecula and freshly bled	5
HOLYHEAD Animals arrive by vivier from Benbecula	6
SANTANDER Arrival by vivier	7

Summary of statistics including multiple comparisons

Cancer pagurus

Blood constituent	Sample groups compared	Significance (P)	Level
TOTAL PROTEIN (mg ml^{-1})	5 2 7 4 1 3 6	0.0071	**
HAEMOCYANIN (mg ml^{-1})	7 2 5 4 1 6 3	0.0039	**
LACTATE (mg $100ml^{-1}$)	7 2 3 1 5 4	0.0000	***

Necora puber

Blood constituent	Significance (P)
TOTAL PROTEIN (mg ml^{-1})	Not significant
HAEMOCYANIN (mg ml^{-1})	Not significant
LACTATE (mg $100ml^{-1}$)	Not significant

Table 3.4. Detail of possible consequent stresses which crabs may sustain during stages of the live marketing from South Wales to Audierne.

Place	Action	Possible consequent stress
Fishguard & Solva S WALES	Animals raised from keep pots, placed in plastic boxes and covered with damp sacking or animals freshly caught	Mechanical stress due to handling and Physiological stress due to aerial exposure (up to 4hours)
Fishguard & Solva S WALES	Animals bought by dealer after inspection and transferred to vivier, *Necora* were first placed in string bags *	Additional Mechanical and Physiological stress
S WALES TO AUDIERNE	Transport by vivier	Mechanical due to lorry motion and competition between animals in tanks. Physiological due to high ammonia levels and other deleterious water quality elements
Roscoff, FRANCE	Animals inspected by customs	Thermal stress possibly when refrigerated container is opened
AUDIERNE	Transfer to holding ponds *	Physiological stress briefly on aerial exposure, Mechanical and Physiological stress due to handling and water temperatures
AUDIERNE	Animals settled overnight in recirculating ponds with tidal replenishment of water *	Physiological stress associated with settling

NB *denotes when haemolymph samples are take

Table 3.5. Haemolymph data relating to samples taken during a consignment from South Wales to Audierne. Values are given as means ± standard errors. Numbers in parenthesis refer to numbers of animals sampled.

Cancer pagurus.

Sample	Total protein (mg ml^{-1})	Lactate (mg $100ml^{-1}$)	Glucose (mg $100ml^{-1}$)	Ammonia (μmol l^{-1})
FISHGUARD 30 mins since keep pots raised or freshly fished	–	27.59 ± 6.53 (12)	5.00 ± 0.49 (13)	431.21 ± 43.07 (12)
- 1 hour after keep pots raised	57.28 ± 2.83 (19)	32.53 ± 2.83 (18)	5.98 ± 0.71 (16)	276.61 ± 21.28 (18)
- freshly caught or raised from pots	–	–	6.76 ± 1.18 (10)	330.95 ± 68.31 (10)
SOLVA 2-4hours after raising keep pots	49.90 ± 3.43 (14)	28.12 ± 4.59 (9)	7.30 ± 1.16 (14)	412.34 ± 38.77 (14)
AUDIERNE after vivier transfer from S Wales.Max 48 hours in vivier	49.33 ± 4.98 (10)	53.20 ± 6.59 (10)	–	4185.45 ± 415.33 (10)
AUDIERNE overnight In Holding Tanks	47.69 ± 5.53 (10)	36.61 ± 12.89 (10)	–	1549.70 ± 562.05 (10)

Necora puber

Sample	Total protein (mg ml^{-1})	Lactate (mg $100ml^{-1}$)	Glucose (mg $100ml^{-1}$)	Ammonia ($\mu mol\ l^{-1}$)
FISHGUARD - freshly caught or raised from pots 1 hour	_	20.20 ± 4.67 (9)	3.05 ± 1.01 (8)	189.76 ± 27.78 (11)
SOLVA 2-4hours after raising keep pots	41.05 ± 8.46 (6)	11.67 ± 2.86 (5)	3.76 ± 2.60 (6)	575.63 ± 106.81 (6)
AUDIERNE after vivier transfer from S Wales.Max 48 hours in vivier	41.57 ± 43.79 (10)	12.12 ± 2.85 (6)	_	4565.57 ± 284.57 (10)
AUDIERNE overnight In Holding Tanks	32.25 ± 3.91 (10)	6.25 ± 2.75 (8)	_	1315.61 ± 329.26 (9)

Table 3.6. Seawater ammonia levels measured at various times during the journey from South Wales to Audierne, France.

Water sample details	Water ammonia concentration (μmol l^{-1})	Comment
IN WELSH DEALER'S VIVIER TANKS		
WALES One vivier chest filled with fresh local seawater	25.1	Temperature 9.5 ^{o}C
Over 6hours of loading	25.1 - 1010.4	Brown crab loaded
Three vivier chests with seawater already on board (Water 2-3 weeks in tanks)	57.1	Temperature 9.8 ^{o}C
Over 9hours of loading:- Vivier chest 1	757.8 - 1302.4	Brown crab partially loaded
Vivier chest 2	59.1 - 422.4	Lobster partially loaded
Vivier chest 3	147.1 - 809.6	*Necora* (boxed) & *Maja* partially loaded
FRANCE Arrival at Audierne (Journey time 36hours)	Readings taken from Vivier chests 1-3	Temperatures 19-21 ^{o}C
Vivier chest 1	5421.0 - 6107.0	Ratio of animal to water in the load 55:45
Vivier chest 2	1542.0 - 1910.0	Ratio of animal to water in the load 45:55
Vivier chest 3	1941.0 - 2204.0	Ratio of animal to water in the load 45:55
AT HOLDING FACILITIES IN FRANCE		
Audierne pond holding water	System filled with fresh seawater with each tide and recirculated	Temperature 18 ^{o}C

Table 3.7. Results of nonparametric oneway analysis of variance (Kruskal-Wallis) tests and consequent multiple comparison analysis (denoted by underlining) performed on the data derived for the vivier journey from South Wales to Audierne.

Groups compared	Group number
FISHGUARD 30 mins since keep pots raised or freshly fished	1
FISHGUARD - 1 hour after keep pots raised	2
FISHGUARD - freshly caught or raised from pots	3
SOLVA 2-4hours after raising keep pots	4
AUDIERNE after vivier transfer from S Wales.Max 48 hours in vivier	5
AUDIERNE overnight in holding tanks	6

Continued.....

Summary of statistics including multiple comparisons

Cancer pagurus

Blood constituent	Sample groups compared	Significance (P)	Level
TOTAL PROTEIN ($mg\ ml^{-1}$)		Not significant	-
LACTATE ($mg\ 100ml^{-1}$)	4 1 6 2 5	0.0483	*
GLUCOSE ($mg\ 100ml^{-1}$)		Not significant	-
AMMONIA ($\mu mol\ l^{-1}$)	2 3 4 1 6 5	0.0000	***

Necora puber

Blood constituent	Sample groups compared	Significance (P)	Level
TOTAL PROTEIN ($mg\ ml^{-1}$)		Not significant	-
LACTATE ($mg\ 100ml^{-1}$)		Not significant	-
GLUCOSE ($mg\ 100ml^{-1}$)		Not significant	-
AMMONIA ($\mu mol\ l^{-1}$)	1 4 5 6	0.0000	***

Table 3.8. Detail of possible consequent stresses which crabs may sustain during stages of the live marketing from Kyle of Lochalsh to Roscoff.

Place	Action	Possible consequent stress
KYLE OF LOCHALSH	Animals raised from keep pots, placed in plastic boxes and covered with damp sacking or animals freshly caught	Mechanical stress due to handling and Physiological stress due to aerial exposure
KYLE OF LOCHALSH Holding cascade system	Animals bought by dealer after inspection and transferred to holding cascade system *	Additional Mechanical and Physiological stress
KYLE OF LOCHALSH Holding cascade system	Animals selected and unacceptable animals rejected from sytem during checking process. Animals held for different lenths of time in system as load builds up *	Mechanical due to handling and competition between animals in tanks
KYLE OF LOCHALSH	Animals transferred to cascade (container) lorry	Mechanical, Physiological and Thermal stresses due to handling, some aerial exposure and different seawater held for some time in refrigerated container.
KYLE OF LOCHALSH TO ROSCOFF	Transport by cascade vivier	Mechanical due to lorry motion and competititon between animals. Physiological due to deterioration in water quality with time of journey
ROSCOFF	Animals inspected by customs	Thermal stress possibly when refrigerated container is opened
ROSCOFF	Transfer to traditional type holding ponds *	Physiological stress briefly on aerial exposure, Mechanical and Physiological stress due to handling and water temperatures
ROSCOFF	Animals settled overnight in holding ponds *	Physiological stress associated with settling

NB *denotes when haemolymph samples are taken

Table 3.9. Haemolymph data relating to samples taken during a consignment from Kyle of Lochalsh to Roscoff. Values are given as means ± standard errors. Numbers in parenthesis refer to numbers of animals sampled.

Cancer pagurus

Sample	Total protein (mg ml^{-1})	Lactate (mg $100ml^{-1}$)	Glucose (mg $100ml^{-1}$)	Ammonia (μmol l^{-1})
KYLE OF LOCHALSH Freshly caught or raised from keep pots	46.44 ± 3.77 (12)	37.93 ± 5.58 (11)	2.15 ± 0.18 (11)	_
KYLE OF LOCHALSH Short term cascade holding conditions	41.75 ± 2.74 (14)	29.30 ± 7.25 (12)	2.11 ± 0.14 (14)	_
KYLE OF LOCHALSH Long term cascade holding conditions	34.24 ± 3.41 (9)	19.87 ± 5.85 (11)	2.16 ± 0.15 (10)	_
KYLE OF LOCHALSH Recirculating cascade holding conditions	42.16 ± 3.42 (34)	58.22 ± 12.73 (34)	2.40 ± 0.05 (34)	_
KYLE OF LOCHALSH Rejects from recirculating cascade	40.89 ± 3.84 (21)	45.14 ± 9.86 (20)	3.21 ± 0.10 (21)	_
ROSCOFF After cascade vivier transfer from Kyle of Lochalsh	56.17 ± 3.86 (16)	124.81 ± 10.68 (16)	7.99 ± 0.75 (16)	511.44 ± 94.41 (18)
ROSCOFF Overnight in holding tanks	53.36 ± 4.14 (12)	47.17 ± 13.65 (11)	6.13 ± 0.38 (12)	81.18 ± 10.09 (13)

Table 3.10. Results of nonparametric oneway analysis of variance (Kruskal-Wallis) tests and consequent multiple comparison analysis (denoted by underlining) performed on the data derived for the vivier journey from Kyle of Lochalsh to Roscoff.

Groups compared	Group number
KYLE OF LOCHALSH Freshly caught or raised from keep pots	1
KYLE OF LOCHALSH Short term cascade holding conditions	2
KYLE OF LOCHALSH Long term cascade holding conditions	3
KYLE OF LOCHALSH Recirculating cascade holding conditions	4
KYLE OF LOCHALSH Rejects from recirculating cascade	5
ROSCOFF After cascade vivier transfer from Kyle of Lochalsh	6
ROSCOFF Overnight in holding tanks	7

Summary of statistics including multiple comparisons

Cancer pagurus

Blood constituent	Sample groups compared	Significance (P)	Level
TOTAL PROTEIN ($mg\ ml^{-1}$)	3 5 4 2 1 7 6	0.0063	*
LACTATE ($mg\ 100ml^{-1}$)	3 2 7 5 4 1 6	0.0000	***
GLUCOSE ($mg\ 100ml^{-1}$)	3 1 2 4 5 7 6	0.0000	***
AMMONIA ($\mu mol\ l^{-1}$)	Two groups only	0.0000	***

Table 3.11. Haemolymph and water data relating to a consignment using a novel 'mist system' from Stornoway to Cherbourg, sampled in France only. Journey time about 46 hours. Crab:water ratio, 5:2 tonnes. Values are given as means ± standard errors. Numbers in parenthesis refer to number of animals sampled.

Haemolymph analysis	Concentration
Total protein (mg ml^{-1})	34.61±3.63 (18)
Lactate (mg $100ml^{-1}$)	83.96±9.11 (20)
Glucose (mg $100ml^{-1}$)	5.93±1.24 (14)
Ammonia (µmol l^{-1})	1629.9±2732.9 (20)

Water analysis	Concentration
Ammonia (µmol l^{-1})	3680.77

Figure 3.1 Typical Handling Practices of Crab during Live Transport

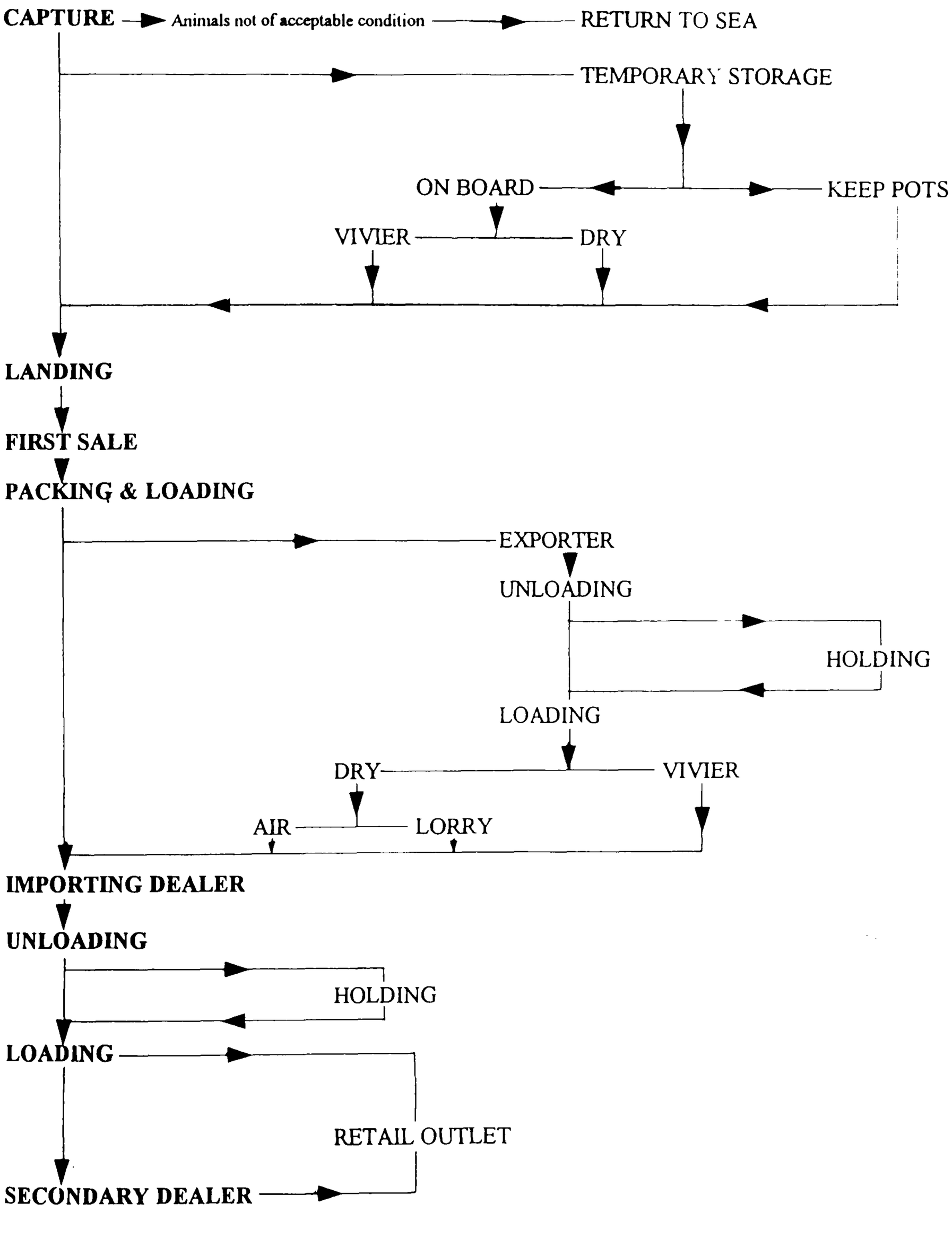


Figure 3.2 South Western Fishery - Typical Handling Practices of Crab during Live Transport

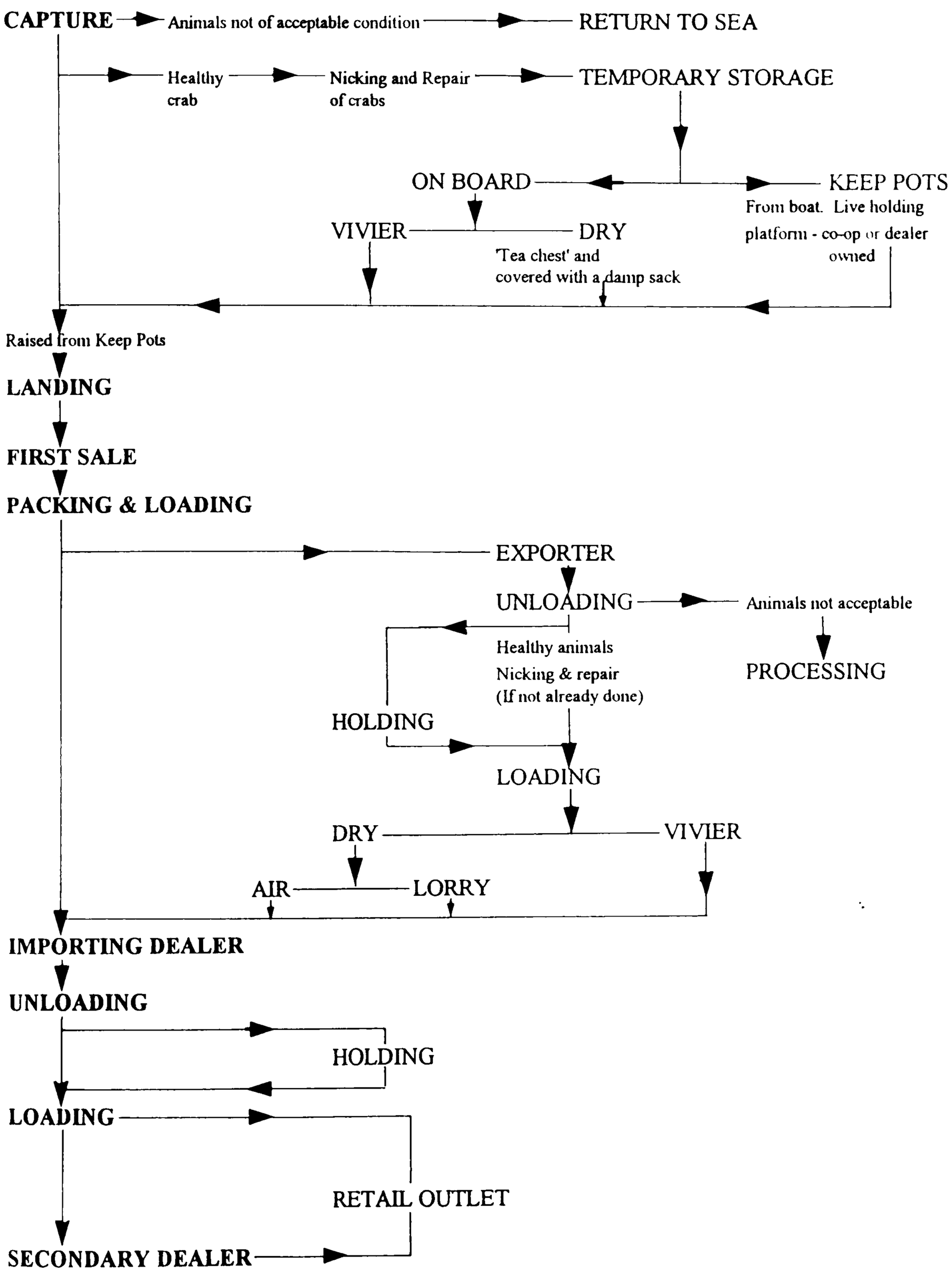


Figure 3.3 Scottish Fishery - Typical Handling Practices of Crab during Live Transport

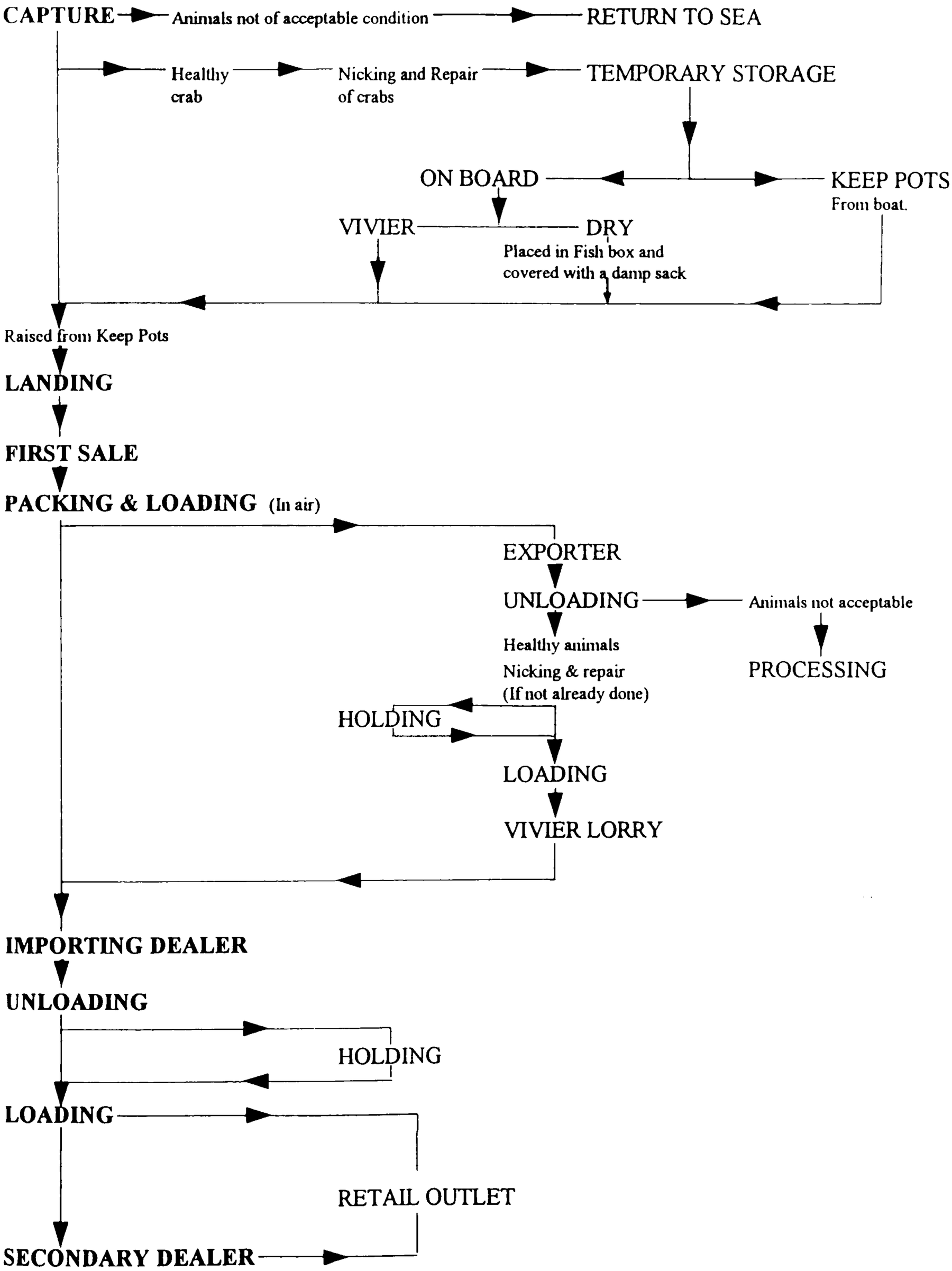


Figure 3.4 South Wales Fishery - Typical Handling Practices of Crab during **Live Transport**

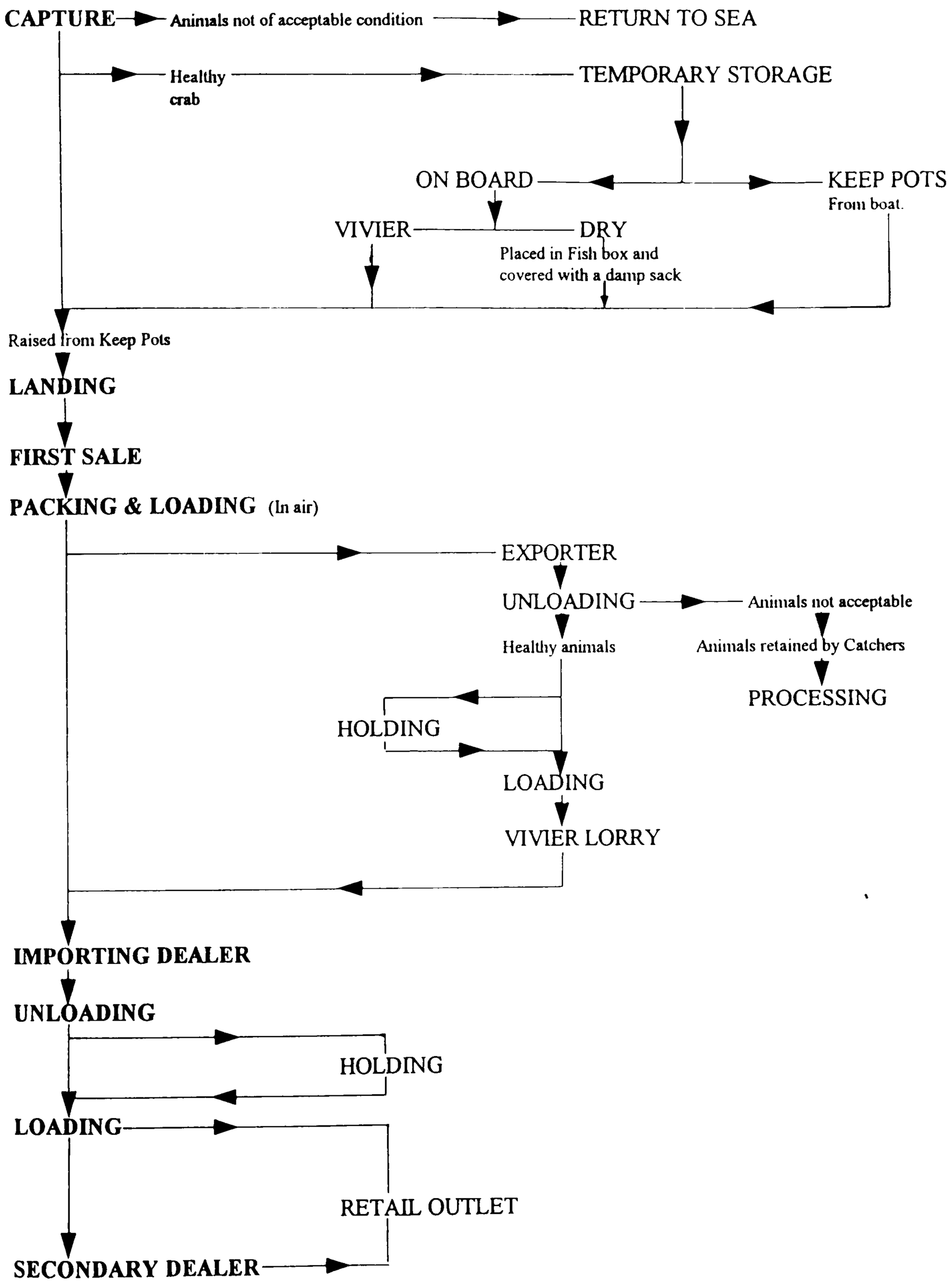
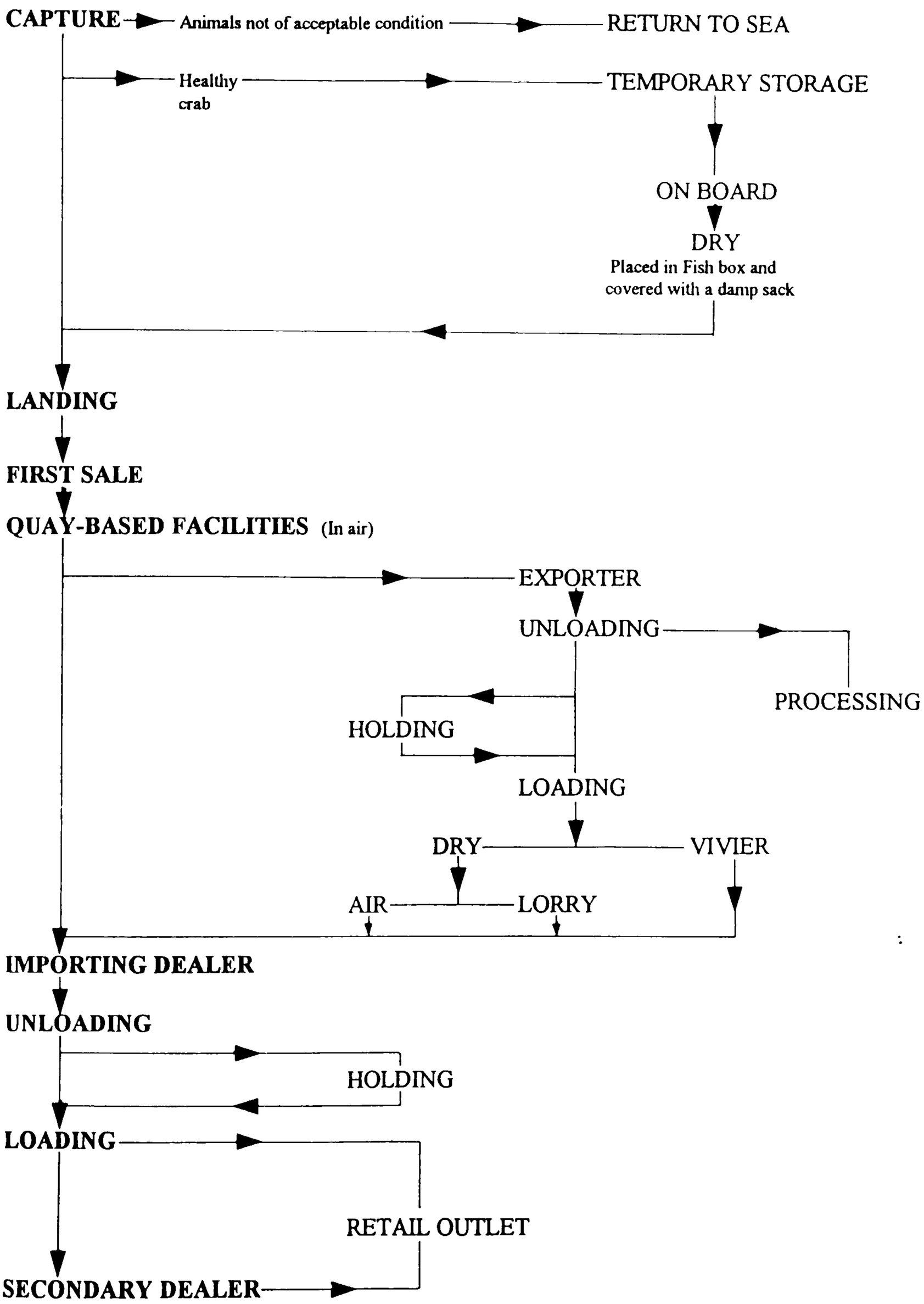


Figure 3.5 Yorkshire Fishery - Typical Handling Practices of Crab during Live Transport



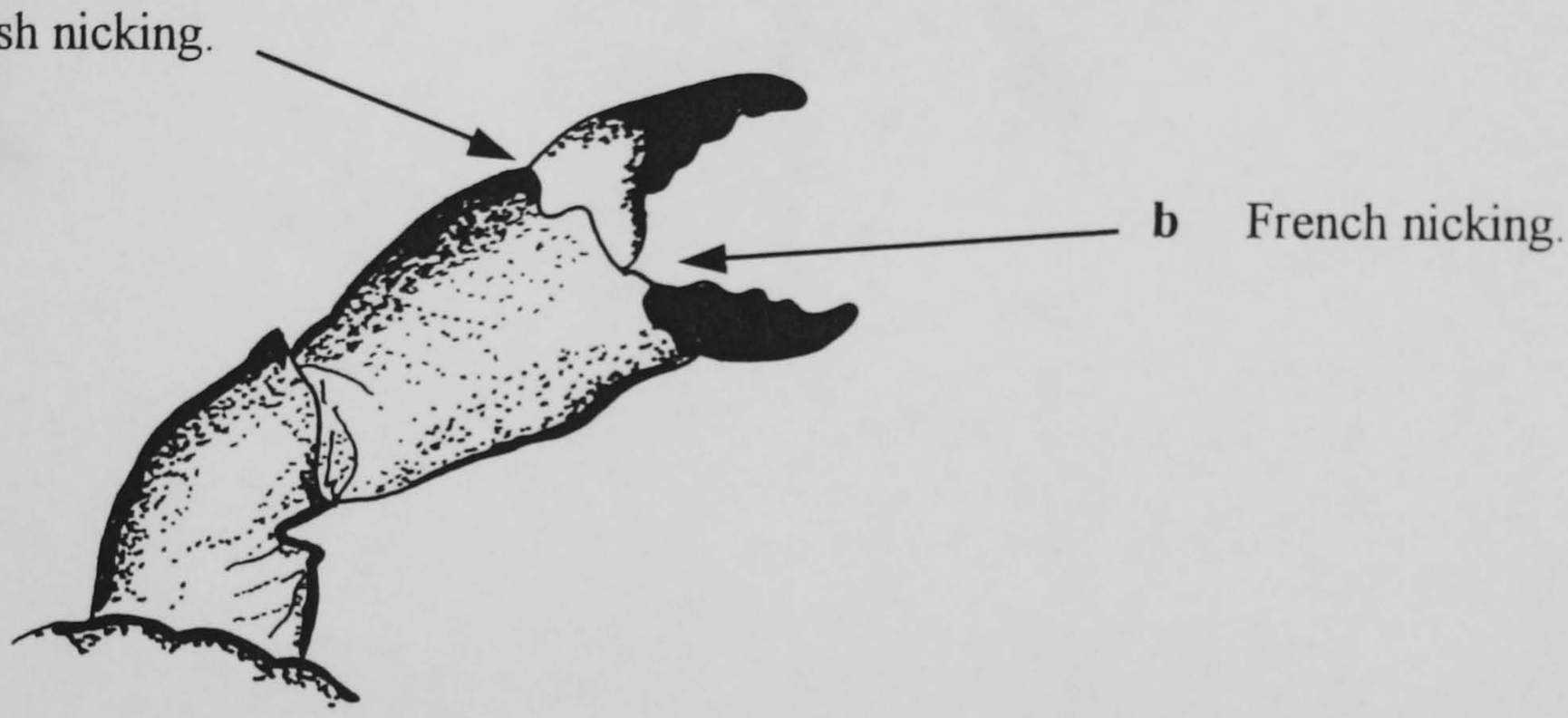


Figure 3.6. *Cancer pagurus*.

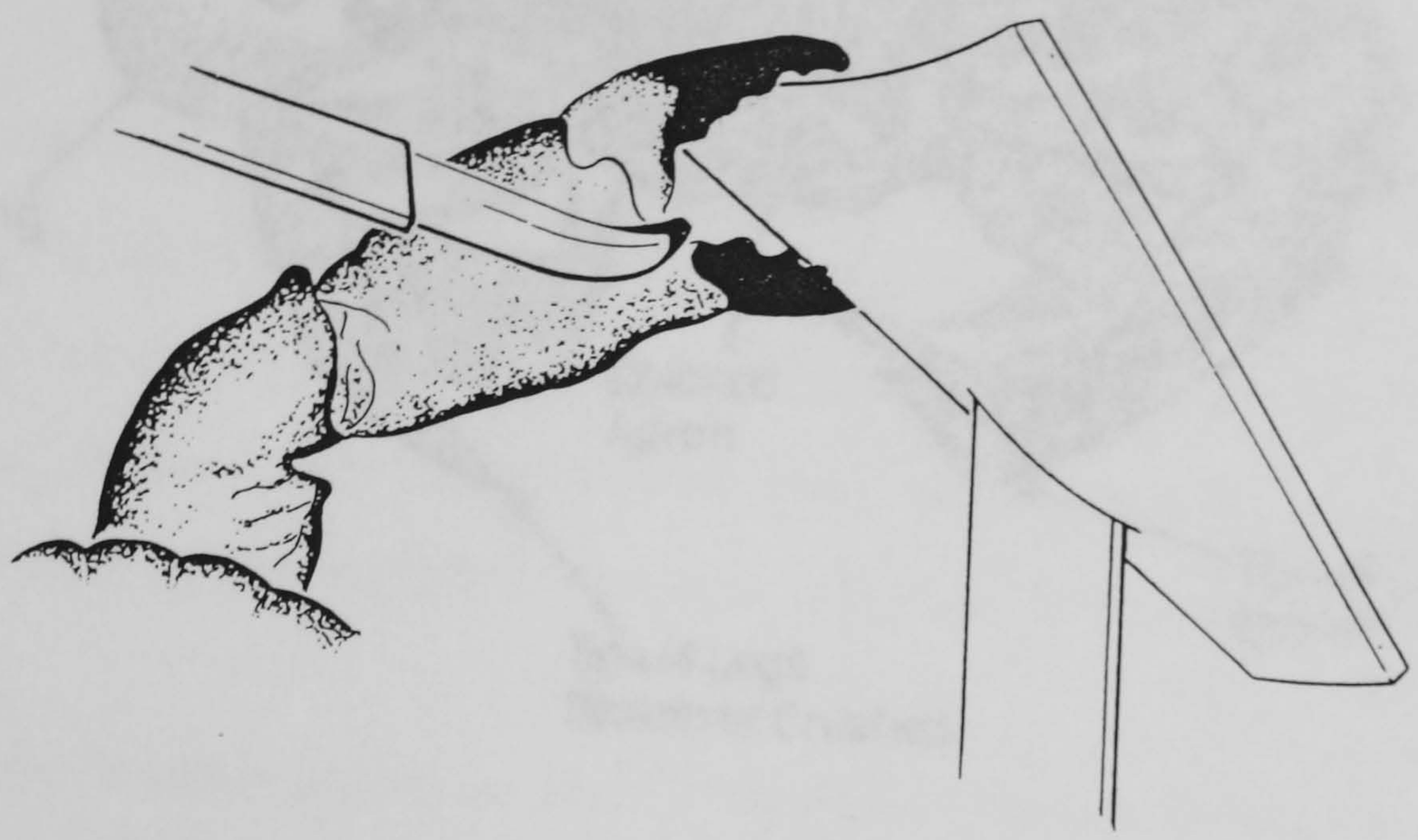
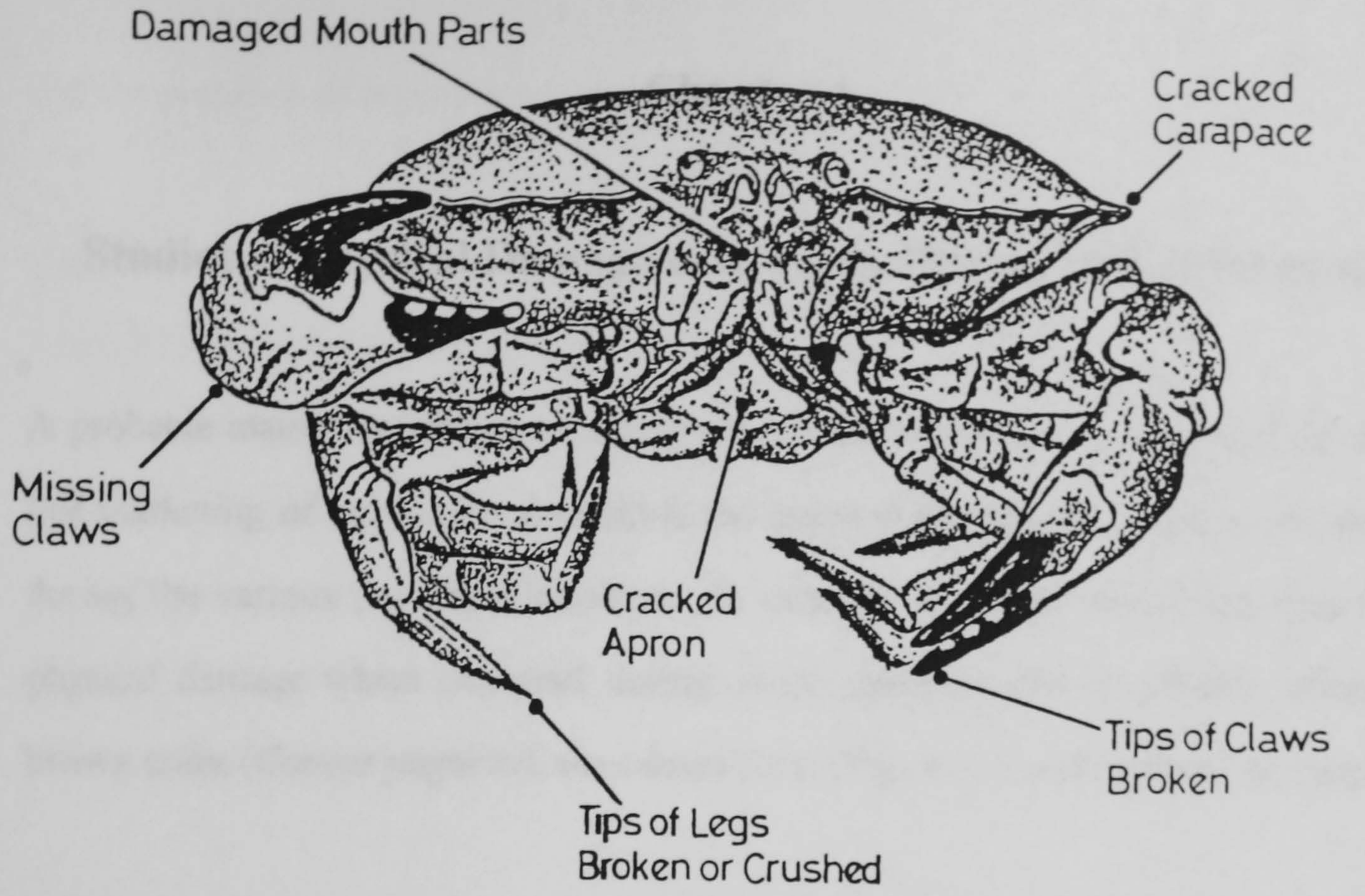
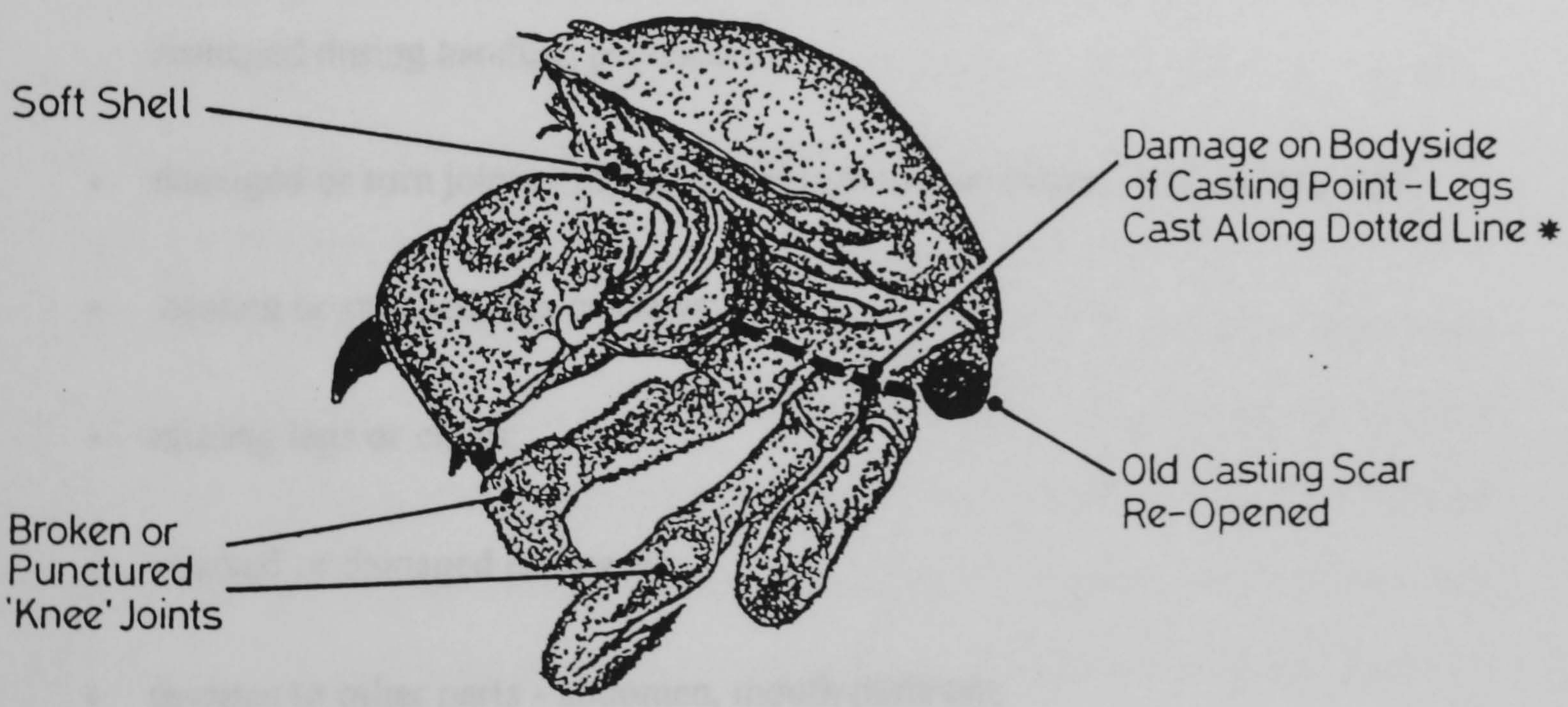


Figure 3.7. Use of nicking implement with a nicking bar.





* Damage to Legs Can Be 'Repaired' by Casting; Damage Inside the Casting Point Cannot Be Made Good

Figure 3.8. *Cancer pagurus*. Examples of common types of damage sustained by crabs during live transport.

Chapter 4

Studies of Physical Damage to Animals During Vivier Transport

A probable major contributor to decreased intrinsic quality, or even mortality, during live marketing of crustacean shellfish is the physical damage sustained by the animals during the various processes involved. In order to study the extent and type of the physical damage which occurred during vivier transport and its possible effects on brown crabs (*Cancer pagurus*), the census form (Fig. 4.1) was developed and used.

The types of data recorded are shown in Figure 4.1. These include physical damage of all types, whether the animal was dead or alive and the sex and relative size of the crab (Fig. 3.8). The principal types of damage which were experienced included:

- broken dactyls - the last segment of the walking legs - these were frequently damaged during handling procedures;
- damaged or torn joints - including damage to scar tissues over missing legs;
- broken or snapped legs or claws;
- missing legs or claws;
- cracked or damaged carapace;
- damage to other parts - abdomen, mouth parts *etc*;
- intact animals - no physical damage seen.

The damage was identified as recent or old; old having black scar tissue, and adjudged to be at least several days old. The condition of the animal concerned was also noted and this included whether the animal was hard or soft (whiteface = recently moulted)

and the presence of regenerated legs.

The data collected are shown in Appendix 1. Some of the data correspond with journeys on which haemolymph data were also collected from the animals (see Chapter 3). The two data types are different and not always from the same animals, therefore only inferred information can be gained from the comparison of these data. However, the damage data do give useful information concerning the vivier transport of these animals and served to formulate a set of recommendations for the catchers and dealers in this market (MacMullen *et al*, 1986b).

The data given in Appendix 1 are derived from a number of sources. Studies have been made of animals which were freshly-landed or samples which comprised animals which had been recently caught and then held in keep pots for a number of days. Some information on the status of animals held in holding and cascade ponds has also been collected, and provides a source of information of the physical effects of such holding conditions. Data have also been collected at stages along a journey of a consignment of brown crabs carried by vivier from Scotland to Santander in Spain. The bulk of the animals studied were at dealers' premises in Spain; these data have been collected from loads which originated variously from different parts of Britain and give information relating to differences in trade practices which may have a bearing on the ultimate survival and condition of the delivered load. Finally, some damage data were collected from the load involved in a novel cascade method used during transport to France thus allowing a comparison with data from the conventional methods of vivier transport.

4.1. The 'Quality' Product

A freshly-caught crab has the highest intrinsic quality of an animal in the live marketing chain though selection for commercial worth is also practised so that only those that satisfy selection criteria are (or should be) marketed. The maintenance of this quality is important for final live sale and hence the transport of these animals in seawater has developed as an attempt to fulfil this objective. However, the attributes which a purchaser regards as providing a 'quality' product may not be satisfied by just presenting a living animal at the time of sale. Other issues of importance to quality may be based less on the animals themselves, but on the reputation of the fishery. It is pertinent, therefore, to outline the main characteristics which Spanish and French dealers consider determine the quality of the product (*ie* those that should be selected for). The following details refer to the brown crab, *Cancer pagurus*:

- An animal which is a prime quality product for final live sale is intact and alive, in the case of a male crab it has more worth if it is large with large chelipeds. The animals should be 'full';

- An animal which is selected as for prime quality for final live sale is intact and demonstrably alive;

- Male or 'cock' crabs are usually more valuable than female 'hen' crabs because of their relatively large chelae. Females with large ovaries (coral) are also of high worth;

- The delivered consignment should have a large proportion of very high quality animals which are still actively mobile. Animals which are virtually immobile on delivery and/or which include many with missing or damaged limbs can reduce the

value of the load or affect the negotiated price for a repeat order;

- Crabs with missing chelae are known as 'cripples' and, along with those missing 3 or more legs, are of value to the processed trade only.

4.1.1. Other Factors

Other factors which were recorded and may have a bearing on the quality of the product on the Continental market include the presence of regenerated legs. During one of the investigations into the damage and mortality of crab during vivier transport, it was found that regenerated appendages were prone to damage during the normal interactions of the animals in the vivier tanks (MacMullen *et al*, 1986). This, therefore, might be considered as a detrimental characteristic and was included in the summary information for discussion in the following sections. It was also found that scars from previously cast legs also became damaged and were a source of blood loss which, over an extended journey, seriously weakened the crabs.

The presence of 'soft' or light-weight (relative to normal) animals in the consignment, while demonstrating a lack of appropriate selection of the animals, also presents an increased risk of mortality and fouling of the vivier water. Such animals are of no worth to the dealer since 'soft' animals cannot be cooked as the 'shell' disintegrates and the light animals would contain very little meat. This category was also included in the summary Tables 4.1-4.5 along with other aspects which might be considered beneficial in terms of the live transport definition of 'quality' identified above.

4.2. Analysis of data collected from newly-landed animals

A summary of the pertinent results of damage data taken from newly-landed animals is given in Table 4.1. The information was collected at different times of year and from

different locations - the first two sets of data were from the west coast of Scotland; the Isle of Islay and Kyle of Lochalsh, and the data were collected in October and May respectively. The remaining data were collected from three landings in South Wales in January. It is important to note that the data presented are not always a comprehensive representation of the landings at this time, but was often taken from a sample of the animals in whatever time was available within the particular dealer's timetable.

The information presented here provides a baseline on which to consider the later data collected on live transport journeys. The treatment of animals at first catch, or their survival in keep pots may have a significant bearing on their condition on arrival at the dealer's premises and also their quality at final sale to the exporting dealer.

The proportion of intact and alive individuals on landing was low in the sample of animals analysed on the Isle of Islay compared with the other groups. More animals had old and recent leg damage (in the less than 3 legs category) in the Scottish groups than the remaining sets analysed. However, the proportion of male crabs in the catch and large individuals was higher in the Islay group than in the other groups analysed. The ratios of male to female crabs varied in the landings sampled here, which demonstrates the sex-ratio variability with season and location. Many more data would be required for a full analysis of the seasonality of the crab populations considered here.

Of the data which may be considered to give 'low quality' characteristics to the landed crabs, the information given in Table 4.1 shows that the Islay crab had the highest statistics of negative attributes. The landings of the Welsh catches had least damage in this category. Overall, the Scottish landings had a higher level of damage than the Welsh landings, which may reflect the short length of time that the fishery has been ongoing and the experience of the catchers in this region. The Islay group studied was

also the smallest (Appendix 1.1) and it is known that the catchers in this area commonly use the fishery as a supplement to their income rather than pursue it as a full time concern.

4.3. Assessment of physical damage and mortality at stages during the live transport chain

The data presented in Table 4.2 are a summary of information given in Appendix 1.2 which is relevant to quality criteria determined by French and Spanish dealers. All the data refer to *Cancer pagurus* and were collected during a vivier journey from Scotland (including animals from the Outer and Inner Hebrides and the West coast). The UK dealer was responsible for buying the crab from Scotland and transporting them to Holyhead in Wales where a transfer of live animals to a vivier lorry consigned by a Spanish dealer took place. It was at this transfer point that the first set of data was collected. The animals were then taken to Spain via France and sampled again in Santander in northern Spain. Here, animals were off loaded, sorted, repacked and then transported to Madrid where they were delivered to a number of wholesalers. At one of these premises in Madrid a consignment from the Channel Isles and Falmouth in the UK was studied for comparison with the data collected for the Scottish crab. Each stage of the journey took approximately 36-40 hours and therefore the 'sampling times' were consistent. The analyses of these vivier loads took place during the month of October.

In respect of these characteristics which are considered to be beneficial for the load on arrival, a higher proportion of the Falmouth and Channel Isle load was recorded as intact and alive and a higher proportion also comprised large crabs. A low proportion, comparable with the first investigation of the Scottish load, had suffered recent damage to up to two legs and this had had less recent blood loss so a higher ratio of these

animals would be suitable for holding in the Spanish dealer's ponds. The proportions of male and female animals recorded from the different areas is due only to the seasonality of the populations in these regions, although some selection of the crabs may have occurred prior to transport from the Falmouth and Channel Isles fisheries. Some continental dealers may stipulate a preponderance of one or other gender in their consignment. The analysis made of the Scottish consignment at Santander demonstrates that quality loss had occurred since departure at Holyhead. Dead animals were removed from the original load at the Holyhead transfer point, these results therefore are derived from the live animals which were transferred at that time.

The above observations are demonstrated more clearly when considered with the detrimental aspects of the loads which are given in Table 4.2. Here, it is seen that the consignment from Falmouth and the Channel Isles were delivered with a larger proportion of the load in better condition than in the Scottish consignment. None of the animals from the English load were dead on arrival at Madrid and only a small proportion of the load showed recent damage which would lower their commercial value. The proportion of Scottish load which was dead on arrival at Santander was greater than that found in the load at Holyhead. In all other aspects the two loads were comparable except for the proportion of soft animals found, since this analysis is of the same animals that were sampled at Holyhead, these details were omitted in the first sampling.

Analyses of the dead animals was carried out in an attempt to identify the possible causes of death due to physical damage. It is important to note that the results which are given in Table 4.2 for the analysis of dead crabs is based on percentages of the whole consignment under consideration. Large numbers of animals were examined; 618 at Holyhead and 574 at Santander, the total number of animals which were found to be dead or moribund on arrival from the two loads was 118 individuals. This is a

large proportion of the original load of 618 animals (19.1%). The proportions which are presented in Table 4.2 for the analysis of dead animals appear to be quite low, based on the whole number transported, but in terms of the number of dead individuals, nearly 20% of those that died or became moribund during the journey were intact on arrival. A large proportion of the load was found to have sustained recent damage to the legs or carapace (calculated as 44.9% of animals recorded dead or moribund).

That a high proportion of the dead crabs were intact is a problem which has been identified by UK dealers, commonly with the Scottish consignments to Spain. It implies that some of the mortalities may be due to poor intrinsic condition of the animals (rather than sustained damage), which may be due to a number of factors relating to the population seasonality. The animals which had recent physical damage will have suffered blood loss and this provides some evidence for the possible if not probable cause of mortality. Of those animals analysed at Santander, 30% had sustained old damage to two or less legs and 15% had sustained similar recent damage. During off loading from the vivier lorry at Holyhead, many animals may have suffered such damage upon removal from the vivier tank. The base of each tank consisted of a mesh screen sited above the aeration pipework and lively, strong crabs were observed to move to the bottom of the tank where the water conditions were probably preferable. However, such crabs would wrap their legs through the mesh and thus suffer damage when they were removed, as was recorded at Santander. Such damage is also known to occur between individual crabs which clutch each other.

4.4. Results of further loads analysed in Spain

A summary (Table 4.3) of relevant information was derived from Appendix 1.3. These data are taken from a series of consignments which journeyed to Santander from

Scotland. The UK dealers who were responsible for the load concerned were different from those used for other loads analysed, so the data is not directly comparable with the results discussed formerly in section 4.3. The examinations were made of these consignments over a four week period in July and August. On one occasion, a sample of a load which arrived from France was analysed, for comparison with the other results - although this load originated in France the source of the crab could have been from the UK as well as France. Such details are difficult to obtain, once the animals are mixed in holding tanks. Also, a sample of a consignment taken to a wholesaler's premises in Madrid was examined, this was a part of one of the loads which arrived and was analysed at Santander (Santander 5). The data, again refer to the brown crab *Cancer pagurus* and were collected from vivier consignments.

The proportions of the loads which arrived alive and intact increased over the sampling period from July to the end of August, with high numbers, 78% and 80% for the Santander 4 and Santander 5 loads (compared with the loads sampled from France (35%) and in Madrid (3%)). The incidence of recent damage to two or less legs (3 - 8%) was reasonably consistent over the sampling period at Santander; (the load delivered to Madrid contrasting markedly at 15%), and was lower than that recorded previously for a consignment at Santander (section 4.3). Some of these loads may not have been loaded into the vivier lorry at a transfer point and may have been brought directly from Scotland. Even with this level of physical damage, a high proportion of the load would still have been suitable for holding at the Spanish holding ponds before onward sale. On one of the loads, a fairly high proportion of old damage was found associated with two limbs or less (15% on Santander 2 load) compared with the other loads (between 1% and 2%) - while this is fairly minor damage it would be detrimental to the load since these animals would have incurred blood loss at some point and this could have been alleviated by removal of the leg concerned (section 3.1.3). One interesting feature of the information in Table 4.3 is the large proportions of female

crabs in the loads, the seasonality of these females in the Scottish fishery may have been responsible for the large numbers of intact/alive individuals arriving in Spain.

While study of the beneficial attributes of the loads arriving into Santander implies the loads were successful, the proportions of animals which were arriving dead or moribund are quite high, compared with the other load data (section previously and French and Madrid loads compared in Table 4.3). A high proportion (29%) of the complete load was dead or moribund on the Santander 1 consignment and in the following load it was also quite high (19%). Even the Santander 5 load, which arrived with 80% alive and intact, carried 11% dead and moribund crabs. Considering the number of crabs which were sampled in these loads (Appendix 1.3) this is a high number of individuals (143, 97 and 47 individuals for Santander loads 1, 2, and 5 respectively). The proportions of these crabs which were intact appear small since they are expressed in terms of the full load in Table 4.3. However, based on the number of dead animals only (20 - 13.7% of the dead animals, 20 - 21.1% and 13 - 27.3% for Santander loads 1, 2, and 5 respectively), the actual numbers are high considering the probable good condition of the animals in general (*cf* previous paragraph). Damage may also have brought about some of the mortalities, the incidence of recent damage to more than three legs was expressed as 3% of the load for the Santander 4 analysis; old damage to three legs or more was fairly consistent on all the loads (Table 4.3) and it is possible that such damage was incurred early in the journey of these consignments. The high temperature experienced at this time of year may be responsible for some of the mortalities, the temperature difference between Scotland and Spain being greater than during winter months and, despite the vivier trucks being refrigerated, the increased ambient temperatures will cause the cooling of water to be increasingly difficult and actual water temperatures during transport may be several degrees centigrade higher in the summer than during the winter months.

The load delivered to Madrid from Santander, although suffering no mortalities, was not of good quality. An additional journey of up to 40 hours using animals which recently arrived in Santander would appear to be very stressful (temperatures were not recorded) and the recovery of these animals in holding ponds would have provided a better product in Madrid. Analysis of the second load which was delivered to Santander is worth mentioning separately since a large number of crippled (both chelipeds missing) animals were included (52%, Table 4.3). This is an uncommonly large proportion and despite 41% of the load arriving intact and alive, this situation demonstrates a lack of proper sorting of animals prior to live transport from this fishery. Overall the consignments considered in this section and previously (section 4.3) demonstrate that, despite the problems associated especially with live transport from Scotland, commercially successful loads are received in Spain.

4.5. Results of physical damage sustained during 'cascade' holding of crab

A summary of the advantages and disadvantages in terms of live transport quality is given in Table 4.4, derived from Appendix 1.4 which provides a convenient means of comparison. The study in this case was undertaken during an investigation of the cascade method of holding and transport. The examination was of dead or moribund crabs which were rejected from the holding conditions in the UK, or by the French dealer on arrival at his premises and the data were compared with that relating to freshly-landed product in order to determine the possible case of death. The information was collected for *Cancer pagurus* and during the month of May.

The information given here for the newly-landed crabs has been compared previously with other, similar data (section 4.2) and shows that the animals concerned were of reasonable commercial quality. The data given for the animals that were rejected

(Table 4.4) reveals that these had few of the attributes that confer quality to the product. The ratios of male to females animals have been included for completeness but most of the animals were dead or moribund (Appendix 1.4). Considering that these animals were rejects, the numbers of them which are intact animals is quite high, especially those sorted at Roscoff and the proportion included in the 'intact dead' category (Table 4.4). The data relating to damage are noteworthy in that many of the animals rejected from the cascade holding were cripples (26%). The main reason for this was poor quality of nicking and these animals showed the highest figures for nicking damage, of all the analyses undertaken in these studies. During the time when examinations were being made at the holding premises at Kyle of Lochalsh (section 3.6.5), the newly-landed animals were being nicked. This was done badly and resulted in many damaged chelipeds (which were later cast) and also resulted in much bleeding from forced wounds. The proportions of 'soft' and 'light' animals recor ed amongst the dead, rejected crabs were also high and surprising, since the animals were handled and checked several times during their holding and again, finally, when they were loaded onto the cascade lorry. These are opportune times for the proper selection of animals to ensure suitable condition for live transport.

The results of this particular method for the live transport and holding of crustacean shellfish were very promising and indicated a potentially useful and novel way of reducing water quantities for such transport. A load of 3 tonnes of animals was transported using 600 l of seawater in three separate changes of 200 l for the journey from Kyle of Lochalsh to Roscoff. After overnight in the French holding ponds, a total of 7.7% mortality occurred (see also section 3.6.5).

4.6. Examination of animals after vivier transport and recovery in holding ponds

A summary of a small amount of information collected (Appendix 1.5) from animals transported live to Audierne in Brittany, France from south Wales is presented in Table 4.5. The results pertain to the brown crab, *Cancer pagurus* and the velvet swimming crab *Necora puber*, on arrival at a dealer's premises after vivier transport and again after overnight recovery in holding ponds.

The proportions of the load which were found to be intact and alive were the same on examination after an overnight recovery in the holding ponds as upon arrival at the end of the vivier journey. The sex ratio was unaltered and the numbers of male and female crabs which were sampled did not change and the damage incidence recorded on arrival at the holding ponds was unchanged after overnight recovery. The damage which was recorded was mainly minor and did not affect the value of the load (Table 4.5).

The condition of the crabs on landing, before loading into the vivier lorry was generally good (Table 4.1 and section 3.6.3). Only small amounts of old and recent damage were recorded when the animals considered here were landed. This provided a good quality load for transport and little further, detectable physical damage was sustained during the journey. The UK dealer who was involved with these investigations was unusual in that he made no attempt to reduce the temperature of the vivier water to below ambient temperature. This and the stringent requirements he made to his suppliers who landed to him provided a successful means of live transport.

Table 4.1. *Cancer pagurus*: Summary of damage data collected from freshly-landed animals

Sampling group	Isle of Islay	Kyle of Lochalsh	Wales-Hobbs Point	Wales-Fishguard 1	Wales-Fishguard 2
Beneficial attributes					
Intact/Alive	27	61	83	60	80
<3 legs recent damage	57	11	0	4	4
Large animals	29	9	0	0	0
<3 legs old damage	18	0	0	14	0
Male	86	32	44	52	20
Female	14	68	56	48	80
Detrimental aspects					
Dead/Moribund	0	0	0	0	0
3+ legs recently damaged	0	0	0	0	0
Recent carapace damage	4	1	0	0	0
3+ legs old damage	0	0	0	0	0
Old and recent cripple	15	12	0	10	0
Leg regeneration	7	9	0	4	0
Nicking damage	0	0	0	0	0
Soft animal	32	0	0	0	0
Light animal	29	9	0	0	0

Table 4.2. *Cancer pagurus*: Summary of damage data collected at stages in a typical vivier journey from Scotland to Santander, Spain.

Sampling group	Holyhead from Scotland	Santander from Holyhead	Madrid from Falmouth
Beneficial attributes			
Intact/Alive	34	13	42
<3 legs recent damage	5	15	6
Large animals	8	8	17
<3 legs old damage	4.5	30	35
Male	17	25	28
Female	83	75	72
Detrimental aspects			
Dead/Moribund	4	15	0
3+ legs recently damaged	3	1	0
Recent carapace damage	1	0.5	1
3+ legs old damage	0	0.5	0
Old and recent cripple	4	5	2
Leg regeneration	6	2	8
Nicking damage	0	0	0
Soft animal	0	9	1
Light animal	0.5	0	0
Analysis of dead animals			
Intact animals	2	2	0
3+ legs recently damaged	2.5	5.5	0
Recent carapace damage	0.5	0.5	0
3+ legs old damage	0	0.5	0
Old and recent cripple	1.5	1	0
Leg regeneration	0.5	1	0
Nicking damage	0	0.5	0
Soft animal	2	1	0
Light animal	0	0.5	0

Table 4.3. *Cancer pagurus*: Summary of damage data collected from many consignments transported to Spain. All animals originally from Scotland.

Sampling group	Santander 1	Santander 2	Santander 3	Santander 4	Santander 5
Beneficial attributes					
Intact/Alive	44	41	54	78	80
<3 legs recent damage	3	7	4	5	8
Large animals	0	0	0	0	0
<3 legs old damage	1	15	2	1.5	1
Male	29	22	19	22	20
Female	71	78	81	78	80
Detrimental aspects					
Dead/Moribund	29	19	9	14	11
3+ legs recently damaged	1	0	0.5	1	1
Recent carapace damage	0	0.5	0	0.5	0.5
3+ legs old damage	0.5	1	1.5	0	0
Old and recent cripple	7	52	6	0	4
Leg regeneration	0	0	0	0	0
Nicking damage	0	0	0	0	0
Soft animal	0	0	0	0	0
Light animal	0	0.5	0	0	0
Analysis of dead animals					
Intact animals	9	4	4	4	3
3+ legs recently damaged	0	1	1	3	0
Recent carapace damage	0	0	0	1	1
3+ legs old damage	1.5	1.5	2	0.5	1.5
Old and recent cripple	0	0	0	0	0
Leg regeneration	0	0	0	0	0
Nicking damage	0	0	0	0	0
Soft animal	0	0	0	0	0
Light animal	0	2	0	0	0

Table 4.3 continued. Animals originating from places other than Scotland.

Sampling group	French Load	Madrid
Beneficial attributes		
Intact/Alive	35	3
<3 legs recent damage	16	15
Large animals	0	0
<3 legs old damage	3	3
Male	19	37
Female	81	63
Detrimental aspects		
	0	0
Dead/Moribund	0	0
3+ legs recently damaged	0	0
Recent carapace damage	0	0
3+ legs old damage	0	0
Old and recent cripple	0	0
Leg regeneration	0	0
Nicking damage	0	0
Soft animal	0	0
Light animal		
Analysis of dead animals		
Intact animals	0	0
3+ legs recently damaged	0	0
Recent carapace damage	0	0
3+ legs old damage	0	0
Old and recent cripple	0	0
Leg regeneration	0	0
Nicking damage	0	0
Soft animal	0	0
Light animal	0	0

Table 4.4. *Cancer pagurus*: Summary of damage data sustained by animals held in a "cascade" system and transported using a novel cascade method.

Sampling group	Kyle of Lochalsh	Cascade holding rejects	Roscoff rejected animals
Beneficial attributes			
Intact/Alive	61	5	3
<3 legs recent damage	11	0	0
Large animals	9	0	0
<3 legs old damage	0	0	0
Male	32	61	40
Female	68	39	60
Detrimental aspects			
Dead/Moribund	0	95	90
3+ legs recently damaged	0	0	3
Recent carapace damage	1	0	0
3+ legs old damage	0	0	0
Old and recent cripple	12	0	0
Leg regeneration	9	0	0
Nicking damage	0	0	0
Soft animal	0	0	0
Light animal	9	0	0
Analysis of dead animals			
Intact animals		2	13
3+ legs recently damaged		0	0
Recent carapace damage		10	0
3+ legs old damage		0	0
Old and recent cripple		26	0
Leg regeneration		0	0
Nicking damage		10	17
Soft animal		0	7
Light animal		0	17

Table 4.5. Summary of damage data sustained by two species of crab during a vivier journey from South Wales to Audierne, France.

Sampling group	Vivier at Audierne *Necora*	Audierne overnight in holding ponds *Necora*	Vivier at Audierne *Cancer*	Audierne overnight in holding ponds *Cancer*
Beneficial attributes				
Intact/Alive	20	20	40	40
<3 legs recent damage	5	6	1	1
Large animals	3	2	3	0
<3 legs old damage	0	0	0	0
Male	80	80	50	50
Female	20	20	50	50
Detrimental aspects				
Dead/Moribund	0	0	0	0
3+ legs recently damaged	0	1	0	0
Recent carapace damage	0	0	0	0
3+ legs old damage	0	0	0	0
Old and recent cripple	1	0	0	0
Leg regeneration	1	0	0	0
Nicking damage	0	0	0	0
Soft animal	0	0	0	2
Light animal	0	0	0	3

COLLECTION SAMPLE DETAILS Notation_______________

No.	SEX	DAMAGE: Recent: Missing	DAMAGE: Recent: Damaged	DAMAGE: Old: Missing	DAMAGE: Old: Damaged	COMMENTS
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	
		R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	R 12345 L 12345	

'Damage' in this scheme refers to legs. 'R' and 'L' refer to right and left sides and the numbers identify each leg, number 1 being the claw.

Figure 4.1. Sample data sheet.

Chapter 5

Some effects of Emersion and Hypoxia on Blood Chemistry and Ammonia Efflux Rates of *Cancer pagurus* (L)

5.1. INTRODUCTION

Cancer pagurus (L) is an economically important species of crab (Chapter 1, section 1.1.1.; Edwards, 1979) which is common intertidally as a juvenile in the U.K., but is subtidal as an adult. Procedures during live marketing of this species frequently include periods when the animals experience aerial exposure and hypoxia (see Tables 3.1, 3.4, 3.6 & 3.8 and Chapter 3) and thus in some respects resemble the intertidal events which adults would not normally experience, but which juveniles can cope with (Wanson *et al*, 1983). A comparison of the responses of *Cancer pagurus* adults and juveniles in a series of situations which mimic the marketing procedures serves to identify those factors which are particularly stressful for the animal (or which could impair intrinsic quality) and those for which the animal can compensate. Such information could contribute to a greater understanding of the likely problems which could occur during live marketing and identify possible means of avoiding them.

Ammonia is the principal end-product of protein metabolism in Crustacea. It is released from metabolising tissues into the haemolymph (production) and excreted from there to the external medium across the surface of the gills (>95%) and in the urine (<2% in studies undertaken by Binns & Peterson, 1969; Harris & Andrews, 1985; Cameron & Batterton, 1978) to the more dilute external medium (in normal situations).

Ammonia excretion rates given in the literature, vary considerably (Table 1.7) and have been shown to be temperature (Needham, 1957; Quarmby, 1985) and salinity

dependant (Spaargaren *et al*, 1982). During long-term experiments with *Nephrops norvegicus*, excretion was found to have a linear relationship with oxygen tension (Hagerman *et al* , 1990) and other studies have reported a decrease in efflux rate with short-term hypoxia Laxminarayana & Kutty, 1982; Hosie *et al*, 1991).

Haemolymph ammonia levels are much greater than ambient concentrations in the normal, natural environment, favouring a removal from the body along a concentration gradient. Measurements are reported in the literature since 1920 (Table 1.9) and can be very variable, even within a single experimental group (Spaargaren, 1982; Hosie *et al*, 1991). The effects of hypoxia are reported to lead to a reduction in blood ammonia in investigations undertaken with *Nephrops norvegicus* (Hagerman *et al* , 1990; Hosie *et al*, 1991). An increase in blood ammonia is reported for *Cancer productus* and *Panulirus argus* on emersion (DeFur & McMahon, 1984b; Vermeer, 1987), which increases with time of emersion (Regnault, 1992) in juvenile *Cancer pagurus*.

→ Elevations of blood ammonia have been observed during live transport of crustaceans (>4 mmol l^{-1}, Table 3.5) in commercial vivier tanks full of seawater, also containing high concentrations of ammonia (>6 mmol l^{-1}, Table 3.6). Blood ammonia levels also increase during aerial holding practiced within the normal marketing chain (Table 3.5). These studies were undertaken to investigate the effects of these practices, with an emphasis on ammonia levels within the animals and in the water.

5.2. MATERIALS AND METHODS

Specimens of juvenile *Cancer pagurus* (L) were collected intertidally at Filey, Yorkshire, U.K. and adult animals were obtained from the local inshore fisheries. The animals (62.2-103.0g and 235.7-539.6g) were kept in the aquarium in running, aerated seawater (T=9°C or 15°C, S=30-32‰) for at least 7 days before being used in experiments as described in Chapter 2 'General Materials and Methods'.

Ammonia efflux measurements were made at 9°C and 15°C using weighed animals in individual, acid washed, glass aquaria (14 or 15cm x 14.5cm x 30cm for juvenile *Cancer*; 29 or 29.5cm x 19cm x 20cm for adult *Cancer pagurus*) which were gently aerated while normoxic efflux rates were being sampled. When measurements from groups of individuals were required, the animals were placed in a large plastic tank with a known volume of seawater (25 - 40 l). Some sampling was made "*in situ*" on the shore from the caught juvenile *Cancer pagurus* and plastic aquaria (19cm x 15cm x 9.5cm), which were easy to transport to and from the sampling location were used at such times.

Ammonia (total ammonia = NH_4) determinations were made using a modified flow-injection/gas diffusion technique (Clinch *et al*, 1988; Hunter & Uglow, 1993) as described in Chapter 2 'General Materials and Methods'. During these experiments 0.25 ml sample injection volumes were used and the limit of detection was 0.5-1.0 μmol NH_4 l^{-1} for seawater and diluted blood samples. All solutions were prepared using fresh, ultrapure water (Fistreem R060, Reverse Osmosis, Fisons; Nanopure II, Barnstead). Water samples were taken at timed regular intervals and the efflux rates were calculated on a weight specific basis.

When the effects of emersion were studied, the animals were carefully removed from

water and placed in plastic tanks which were then covered with a polythene bag to prevent excessive air flow and maintain the relative humidity of the air (RH80%, 15°C). After the required period of emersion had elapsed, a haemolymph sample was taken from each animal by piercing the arthrodial membrane at the base of the fourth or fifth peraeopod with a glass pasteur pipette. The pH of the blood was measured immediately, if required, using the procedure described in Chapter 2 'General Materials and Methods'. The blood was then diluted with a variable, but measured, volume of saline (9g l^{-1} NaCl) for ammonia content measurement. The methodology described in Chapter 2, 'General Materials and Methods' was used for blood ammonia determinations and the standard solution for such measurements was made up with the same saline solution that was used to dilute the haemolymph. Ammonia efflux was measured after timed periods of emersion when the animals were re-immersed in fresh low ammonia seawater (<5 μmol NH_4 l^{-1}). Water samples for ammonia determinations were taken at regular timed intervals. For shore-based experiments, emersed crabs were covered with damp seaweed to maintain the R.H. and blood samples taken were kept on crushed ice in the field and then kept frozen prior to thawing, dilution and analysis in the laboratory following the recommendations of Hunter (1991).

The effects of hypoxia were measured under two situations, one with tanks containing groups of animals (n=6) and the other where individual animals were each placed in individual aquaria. The oxygen content of the holding water was lowered by the normal respiration of the animals (since this was directly similar to the situation in commercial live transport) and measurements of water or blood ammonia were made from samples taken at predetermined times and normally at PwO_2=10% (or 16 torr). Water oxygen levels were also lowered, if required, by purging with nitrogen gas until the required P_wO_2 level was obtained; water PwO_2 levels were subsequently monitored using a Strathkelvin oxygen meter (Model 781, Strathkelvin Instruments)

together with a Radiometer E5046 oxygen electrode.

The total ammonia (NH_4) present in a water or blood sample is comprised free ammonia (NH_3) and the ionised form (NH_4^+) in ratios that depend on the pH and the pK of the medium. As described by the Henderson-Hasselbalch equation:-

$$pH = pK + \log ([NH_3]/[NH_4^+]) \quad (1)$$

The $[NH_3]$ can be calculated as follows:-

$$[NH_3] = \frac{[NH_3] + [NH_4^+]}{(1 + 10)^{pK - pH}} \quad (2)$$

(Armstrong *et al*, 1978)

The pK values were taken from values given for seawater (Bower and Bidwell, 1987) and the blood osmotic concentrations were taken to be similar to seawater.

Lactate determinations, when required, were made as described in Chapter 2 'General Materials and Methods'.

Where appropriate, data on efflux rates were subjected to analysis of variance using the statistical package SPSS (Subprograms of the Statistical Package for the Social Sciences) on a PC (Norusis, 1986, manuals pA1-H11).

5.3. RESULTS

Mean haemolymph ammonia levels (± standard error of the mean) relating to samples taken during the emersion and hypoxia experiments (as specified at 9°C and 15°C) are given for adult and juvenile *Cancer pagurus* in Table 5.1. The values found are quite variable and appear to depend upon the duration of the experimental treatment. (The $[NH_3]$ and $[NH_4^+]$ were calculated from the mean blood ammonia concentrations (= total ammonia) and pH values for each group). A oneway ANOVA of all the blood total ammonia data for juvenile and adult animals resulted in a high significance (P=0.0000) in both cases. A Tukey multiple comparison test identified the 8h emersion treatment group of adults to have significantly different blood ammonia levels from the other adult groups (Appendix 2.1). A multiple comparison procedure (Tukey test) performed on the juvenile groups showed that the 12h emersion group was significantly (higher) different from all the others (Appendix 2.1).

The $[NH_3]$ and $[NH_4^+]$ values given in Table 5.1 for each group were calculated for the group mean values for ammonia concentration using a pK_a value of 9.65 (taken from tables given in Bower and Bidwell, 1987). The relative proportion (%) of the total blood ammonia present in the gaseous form (NH_3) decreased (*cf* the control, aquarium animals) whilst under hypoxia or during emersion (Table 5.1, col 6). Changes measured in blood pH values indicate that an acidosis occurred with emersion and, overall, with hypoxia.

Weight-specific net ammonia-efflux (excretion) rates are given for juvenile and adult *Cancer pagurus* in Table 5.2 at various levels of hypoxia. Statistical comparison of all experimental data presented in Table 5.2 was significant (oneway ANOVA; P=0.0000) due to differences between adult and juvenile efflux rates (Appendix 2.2). Comparison of the adult or juvenile data alone was not significant (oneway ANOVA; P=0.5608 and

P=0.9613 respectively). No differences due to temperature were therefore found with efflux rate in adult *Cancer*.

Table 5.3 shows the results for efflux rates and blood ammonia levels of juvenile and adult *Cancer pagurus* after 4h emersion. Comparison of the blood ammonia data and efflux rates in the two stages of *C.pagurus* gave significant differences (t-test; P=0.046 and P=0.041 for blood ammonia and efflux rate respectively; Appendix 2.3) due to the higher efflux rate and blood ammonia levels found in juvenile *Cancer*.

Table 5.4 presents the results of experiments carried out on the shore with juvenile *Cancer pagurus*. Such experiments were not possible with adult *Cancer pagurus*, as the opportunity of taking samples of such post-capture animals was not presented during these studies. A oneway Analysis of Variance of the blood ammonia data revealed no significant differences (oneway ANOVA; P=0.5255) due to extremely high associated variability. Lactate levels of haemolymph samples collected during the shore-based experiment on juvenile *Cancer* and are also given in Table 5.4. A oneway Analysis of Variance of these data revealed a highly significant (P=0.0000) increase in the emersed group following efflux rate measurement (Tukey's multiple comparison procedure; Appendix 2.4).

5.4. DISCUSSION

The blood ammonia levels found in these studies range from 84.08 - 643.79 μmoles NH_4 l^{-1} in adults and 50.40 - 3284.65 μmoles NH_4 l^{-1} in juvenile *Cancer*. The normal mean values found (174.24 ± 19.83, n=14) and (71.4 ± 8.01, n=14) for adults and juveniles respectively are at the lower range of values given in the literature for this and other crustacean species (Table 1.9). Recently, Regnault (1992) has provided a specific value of 245 μmoles NH_4 l^{-1} for juvenile *Cancer pagurus*- but the present results indicate that large deviations from this value may be expected

5.4.1. Emersion

Blood ammonia levels were found to be directly related to emersion duration in both adult and juvenile *Cancer pagurus* (Table 5.1). Regnault (1992) found blood ammonia concentrations of 151, 260 and 501 μmoles NH_4 l^{-1} for juvenile *Cancer pagurus* emersed for 1, 4 and 12 hours respectively. Her values are lower than those found in these studies, but the temperature regimes (16-18^{o}C) were similar for both studies. The relative humidities may have differed during the emersion periods in the two studies or the differences may merely reflect physiological differences between the two quite widely separated populations studied (North Brittany and North Yorkshire). Environmental factors are also known to influence the ammonia metabolism in Crustacea (Regnault, 1987; Hunter, 1991) and tidal level differences at the sites of collection may also have influenced the values found. Juvenile *Cancer pagurus* collected on the beach at Roscoff are commonly found associated with the areas that have considerable runoff water (Uglow, *pers comm*) whereas, at Filey, such animals are invariably found buried in sand or emersed under boulders. Thus the Yorkshire animals may not have been able to utilise the gill chamber reservoirs to the same extent that the Roscoff animals could.

The data given in Table 5.1 also show that blood ammonia values of adults emersed for 0.5 or 1.0 hours were lower than those of the control values relating to animals in the holding conditions. This was also found by Regnault (1992) with juveniles but was not found with the juveniles tested in the present studies.

The calculated NH_4 values relating to emersed groups ranged from 0.83 - 5.84 μmoles NH_4 l^{-1} with both adult and juvenile groups showing a direct relationship between blood NH_4 and emersion duration. Control animals had higher blood NH_4 values than adults emersed for 0.5 hours, whereas juveniles showed little alteration to their blood NH_4 during the first hour of emersion. As emersion proceeded there was a progressive increase in both blood [NH_3] and [NH_4^+] levels and a drop in blood pH levels (acidosis). At all corresponding stages of emersion and hypoxia, the blood pH of juveniles was lower than that of adults - but no explanation of this can be given yet.

5.4.2. Hypoxia

The changes to blood ammonia levels under hypoxia differed between the adult and juvenile groups. In the former, ammonia levels dropped throughout the hypoxic period whereas, in juveniles, the 4h hypoxia level was higher and the 8h hypoxia level was lower than the respective control groups. A negative relationship between hypoxia duration and blood ammonia levels has been found to occur in *Nephrops norvegicus* (Hagerman *et al*, 1990; Hosie *et al*, 1991) but without the initial increase that *Cancer pagurus* juveniles showed in these studies. Other experiments described elsewhere in this thesis (Chapter 6) describe events during 6 hours of hypoxia in *Cancer pagurus* and *Necora puber* adults which also included initial increases in blood ammonia levels which suggests that the phenomenon may not be normal.

Under hypoxia, the blood pH of juveniles and adults decreased from control, normoxic values, however, in the case of adults an increase was found with short term hypoxia

which dropped below normoxic levels with a longer time period of hypoxia.

5.4.3. pH

It has been suggested previously that blood total ammonia levels (of which NH_4^+ is by far the greater component) do not play a significant part in acid-base regulation (Hosie *et al*, 1991), but haemolymph HCO_3^- and CO_2 levels are important in this respect (Regnault, 1992). From the animal's welfare point of view, a low pH value co-incident with a high blood ammonia level is beneficial as this would lower the proportion of the higly toxic NH_3 present, but whether this is a factor in blood pH regulation is not known. Several other changes to blood acid-base chemistry occur during emersion or hypoxia and these could effect detoxification mechanisms which, at present, are little understood.

5.4.4. The relationships between temperature and hypoxia and ammonia efflux rates.

The normoxic weight-specific efflux rates for juveniles was found to be greater than those of adults at 15°C which suggests that efflux rates are size-dependant and such data plotted against weight demonstrates this (Fig. 5.1). Maximum efflux rates were found to be higher at 15°C than at 9°C, suggesting temperature dependence also, although no significance was found between the excretion rate at these two temperatures (oneway ANOVA; $P>0.05$; Appendix 2.2). The efflux rates found here accord well with those found for several other species given in the literature (see Hagerman *et al*, 1990; Hosie *et al*, 1991; Hunter, 1991; Regnault, 1992 and Table 1.7). Under hypoxia, the efflux rates decreased in both juveniles and adults and such hypoxia-induced decreases have been found also to occur with *Nephrops norvegicus* (Hagerman *et al*, 1990; Hosie *et al*, 1991).

A calculated Q_{10} (9-15°C) is 0.54 for ammonia efflux rate in adults under normoxia in these studies. A similar calculation made for *Crangon crangon* (Hunter, 1991) was found to be negative and for *Nephrops norvegicus* under normoxia (from the data of Hosie *et al*, 1991) a Q_{10} (6-12°C) of 6.5 was obtained. Thus the Q_{10} values of ammonia efflux rates found here for *Cancer pagurus* are rather low compared with values found for other species (see Table 1.8). Some of this variability will be due to the temperature range and to the relative weights of the animals used here and some will probably be due to the particular care taken to avoid stressing the animals and also the rejection of data relating to the immediate post-handling settling period for the animals.

5.4.5. Weight-specific ammonia efflux rates after emersion

Immediate post-emersion efflux rates were found to be higher (Table 5.3) than those measured with normal, immersed animals (Table 5.2). Such high efflux values suggest that ammoniogenesis proceeds during anareobiosis (given that lactate levels increase during emersion Uglow *et al*, 1986; Table 3.2) and that, somehow, the animals cope with the resulting ammonia accumulation and rapidly off-load such ammonia upon re-immersion. The data provided in Table 5.3 were used to calculate the weight-specific ammonia production rates during the 4.75 hours the animals spent emersed and re-immersed before sampling and yielded the values of 0.032 and 0.185 μmoles NH_4 g^{-1} h^{-1} for the adult and juvenile groups respectively (Table 5.5). Such values accord well with normoxic ammonia efflux rates given for such groups in Table 5.3 and indicate that, for the first 4 hours of emersion at least, ammonia production continues at normal immersed rates.

Blood ammonia levels also rise during emersion. At mean body weights of 396g (adults) and 76.6g (juveniles) and an average blood volume of 30% fresh body weight (*eg* Nicol, 1967; Spaargaren, 1972; Prosser, 1973 and Gleeson & Zubkoff, 1977), the

ammonia produced during 4 hours of emersion would have increased the blood concentration by 427.6 and 2465.2 μmoles NH_4 l^{-1} for adults and juveniles respectively (Table 5.5). In fact, when the 4 hour emersion blood ammonia levels for adults and jveniles are compared (Table 5.1), the values are very similar and less than those that could be predicted on the assumption that all ammonia is retained in the blood. The observed discrepancy between the calculated blood content and the measured release following re-immersion indicates that an alternative site is used to "store" the ammonia produced whilst in air. This "alternative" was not sought during this study because of time constraints but has been followed-up by others (see (Couper, 1993 and Kwee, 1993). Possible candidates to be tested include the reservoirs of water in the gill chamber, temporary storage in the bladders, conversion to some alternative form of nitrogeneous end-product (e.g. urea, Spaargaren, 1982 or, perhaps as as alanine as occurs in *Mytilus* during emersion (Widdows & Schick, 1991). Whichever strategy is employed it is such that a very substantial quantity of ammonia is released very quickly - an observation which would tend to favour the use of gill chamber reservoirs rather than the use of high efflux rates across the gill surfaces.

5.4.6. Blood ammonia and efflux rates of post-capture juvenile *Cancer pagurus*

On initial analysis (Table 5.4), it is clear that the blood ammonia levels and efflux rates are much greater for shore-based (*in-situ*) crabs than those held in the laboratory. The normal efflux rate of these shore-based individuals was equivalent to that found in the laboratory for juveniles following 4h emersion (Table 5.3). The 'immersed' *in-situ* individuals serve as the control groups throughout the experiment. Blood ammonia levels of the emersed group were found to be similar following efflux rate (and therefore emersed) measurement to those determined for a separate group immersed in experimental aquaria on the beach for 30 minutes. While the blood ammonia in the experimental group following emersion was greater than that of the immersed group

the statistical variability of the group was high since no statistical differences were found between these two groups (oneway ANOVA; P=0.5255; Appendix 2.4). The ammonia levels returned to a level similar to this control group following re-immersion.

The weight-specific blood ammonia levels were greater for the 30 minute emersed crabs (0.82 μmol g^{-1}), than for any of the other groups which all shared levels of around 0.4 μmol g^{-1}; after re-immersion of the experimentally emersed individuals, the weight-specific blood ammonia returned to that of immersed crabs.

Thus *Cancer pagurus* shows a reduction of blood ammonia during hypoxia and a progressive increase in blood ammonia during emersion. Adjustments to the blood acid-base chemistry are such that the animals are able to survive periods with supra-normal blood ammonia levels and this has clear advantages to a species which may become emersed during an intertidal period of its life. This also has clear advantages to those who would market the species alive as studies have shown that such marketing of crustaceans results in high ambient water ammonia concentrations and attendant high blood ammonia levels.

High levels of lactate accumulated during emersion in post-capture animals (*cf* other measurements taken from emersed animals - Chapter 3; Uglow *et al*, 1986). These levels decreased to concentrations comparable with the control groups upon re-immersion. A previous study where efflux rates and blood ammonia of post-capture animals were measured was undertaken by Hunter (1991). An inverse relationship was found between blood ammonia level and time elapsed since capture in both *Crangon crangon* and *Palaemonetes varians*. Levels in the aquarium 5d after post-capture field measurements were lower than found immediately post-capture and 10 minutes post-capture. The levels of post-capture efflux rate and blood ammonia found here for

juvenile *Cancer* are much greater than found in the laboratory. Overall metabolism in the field post-capture animals appears to be much greater than found in the laboratory. The high levels of lactate accumulated during 30 minutes emersion and the subsequent efflux rates reflect this high metabolic rate (*cf* aquarium animals).

These results have evident consequences for the live transport of adult *Cancer pagurus*, especially where freshly caught animals are loaded into vivier lorries. Considerations need to be made similarly for animals freshly landed and stored in holding ponds so that frequent water changes are effected.

Logical follow-up work will now focus on the effects of exceptionally high blood ammonia levels imposed for periods of time. The studies will address the survival after re-immersion and the impairment of the product.

Table 5.1. *Cancer pagurus*: Blood ammonia levels (± SEM) measured in crabs subject to different experimental conditions sustained during live transport (salinity=31‰, temperature=15°C).

Experimental condition	n	Blood ammonia μmol NH_4 l^{-1}	pH	$[NH_3]$	$\%NH_3$	$[NH_4^+]$	Survival
Adult							
Aquarium	14	174.00 ± 19.83	7.46 ± 0.03	1.12	0.64	173.12	-
0.5 hours emersion	4	84.00 ± 9.50	7.69 ± 0.02	0.91	1.08	83.17	4/4
1 hour emersion	4	148.00 ± 22.20	7.59 ± 0.02	1.28	0.86	146.75	4/4
4 hour emersion	4	376.00 ± 25.35	7.25 ± 0.06	1.49	0.40	374.98	4/4
8 hour emersion	4	644.00 ± 121.80	7.22 ± 0.08	2.38	0.37	641.41	4/4
Short-term hypoxia (2 hours; P_wO_2=16torr)	6	132.00 ± 16.77	7.54 ± 0.04	1.02	0.77	130.96	-
Long-term hypoxia (4 hours; P_wO_2=16torr))	6	97.00 ± 16.02	7.39 ± 0.08	0.53	0.55	96.44	-
Juvenile							
Aquarium	14	71.41 ± 8.01	7.72 ± 0.05	0.83	1.16	70.58	-
0.5 hours emersion	4	120.38 ± 19.96	7.49 ± 0.00	0.83	0.69	119.55	4/4
1 hour emersion	4	173.99 ± 16.89	7.34 ± 0.17	0.85	0.49	173.14	4/4
4 hour emersion	4	400.69 ± 114.83	7.49 ± 0.03	2.75	0.69	397.94	3/4
8 hour emersion	4	688.01 ± 137.63	7.17 ± 0.08	2.27	0.33	685.74	3/4
12 hour emersion	4	2185.45 ± 871.04	7.05 ± 0.19	5.48	0.25	2180.0	0/4
Short-term hypoxia (4 hours; P_wO_2=16torr)	6	120.43 ± 8.01	7.47 ± 0.07	0.79	0.66	119.64	-
Long-term hypoxia (7 hours; P_wO_2=16torr))	6	50.4 ± 3.08	7.06 ± 0.06	0.13	0.26	50.27	-

Table 5.2. *Cancer pagurus*: Ammonia excretion rates of adult and juveniles at various water oxygen saturations (P_WO_2) (Salinity=31‰). Values are means (±SEM) over 3 hours following a water change and 2 hour settling period.

Water Oxygen Saturation (P_WO_2)	Ammonia efflux rate ($\mu mol\ NH_4\ g^{-1}\ h^{-1}$)
Adult (T=9^{0}C)	
Normoxia (P_WO_2=155torr)	0.049 ± 0.0092 (n=13)
50% saturation (P_WO_2=78torr)	0.0395 ± 0.0059 (n=7)
10% saturation (P_WO_2=16torr)	0.0304 ± 0.0054 (n=6)
Adult (T=15^{0}C)	
Normoxia (P_WO_2=155torr)	0.034 ± 0.0072 (n=6)
10% saturation (P_WO_2=16torr)	0.029 ± 0.0066 (n=6)
Juvenile (T=15^{0}C)	
Normoxia (P_WO_2=155torr)	0.181 ± 0.029 (n=6)
35% saturation (P_WO_2=54torr)	0.178 ± 0.033 (n=6)
25% saturation (P_WO_2=39torr)	0.128 ± 0.016 (n=6)

Table 5.3. *Cancer pagurus*: Ammonia efflux rates (±SEM) after 4 hours experimental aerial emersion and blood ammonia levels following 45 minutes re-immersion for adults and juveniles (salinity=31‰, temperature=15°C)

Weight	Efflux rate (μmol NH_4 g^{-1} h^{-1})	Blood ammonia (μmol NH_4 l^{-1})	Weight-specific blood ammonia (μmol g^{-1})
Adult			
396.2 ± 76.04	0.20 ± 0.01	92.10 ± 8.91	0.028
Juvenile			
76.60 ± 90.10	1.17 ± 0.17	283.80 ± 72.32	0.085

Table 5.4. Juvenile *Cancer pagurus*: Results of experiments carried out on the shore (± SEM), measuring the blood ammonia levels and excretion rates found in natural conditions (n=6 in all cases).

Measurements	Immersed (for 0.5 hours)	Emersed (for 0.5 hours)
Blood ammonia after treatment (μmol l^{-1})	1582.52 ± 450.36	3284.65 ± 1471.55
Weight (g)	111.32 ± 23.95	54.01 ± 12.03
Weight-specific blood ammonia (μmol g^{-1})	0.40	0.82
Lactate (mg $100ml^{-1}$)	17.58 ± 2.69	30.72 ± 4.41
Efflux rate after treatment (μmol g^{-1} h^{-1})	0.46 ± 0.18	3.56 ± 0.64
Weight (g)	50.44 ± 10.70	45.46 ± 6.07
Lactate (mg $100ml^{-1}$)	30.00 ± 10.26	117.10 ± 14.65
Blood ammonia after efflux measurement (μmol l^{-1})	1638.17 ± 1016.17	1590.03 ± 532.00
Weight-specific blood ammonia (μmol g^{-1})	0.41	0.40

Table 5.5. *Cancer pagurus.* The estimation of ammonia production of animals during emersion for 4 hours, based on ammonia efflux rates on re-immersion.

Adult

Mean weight (g)	Blood volume (wt x 0.3)*	Efflux rate measured (μmol g^{-1} h^{-1})	Efflux rate (μmol g^{-1} h^{-1}) over 45 mins actual measurement time	Efflux rate/production rate therefore (over 4.75 hours of emersion and re-immersion *ie* 0.15/4.75) (μmol g^{-1} h^{-1})
396.6	118.8	0.2	0.15	0.032 (*cf* 0.034 normoxic rate)

*calculated according to references in Materials & Methods

Total ammonia produced over 4h emersion = 0.032x4x396.6

=50.8 μmol.

If ammonia production over this time is retained in the haemolymph - this equates to a predicted additional (measureable) concentration in the blood of

$$\frac{50.8 \times 1000}{118.8}$$

=427.6 μmol l^{-1} (above normal aquarium levels) over 4h emersion.

Juvenile

Mean weight (g)	Blood volume (wt x 0.3)*	Efflux rate measured (μmol g^{-1} h^{-1})	Efflux rate (μmol g^{-1} h^{-1}) over 45 mins actual measurement time	Efflux rate/production rate therefore (over 4.75 hours of emersion and re-immersion *ie* 0.88/4.75) (μmol g^{-1} h^{-1})
76.6	23.0	1.17	0.88	0.185 (*cf* 0.181 normoxic rate)

*calculated according to references in Materials & Methods

Total ammonia produced over 4h emersion = 0.185x4x76.6

=56.7 μmol.

If ammonia production over this time is retained in the haemolymph - this equates to a predicted additional concentration in the blood of

$$\frac{56.7 \times 1000}{23.0}$$

=2465.2 μmol l^{-1} (above normal aquarium levels) over 4h emersion.

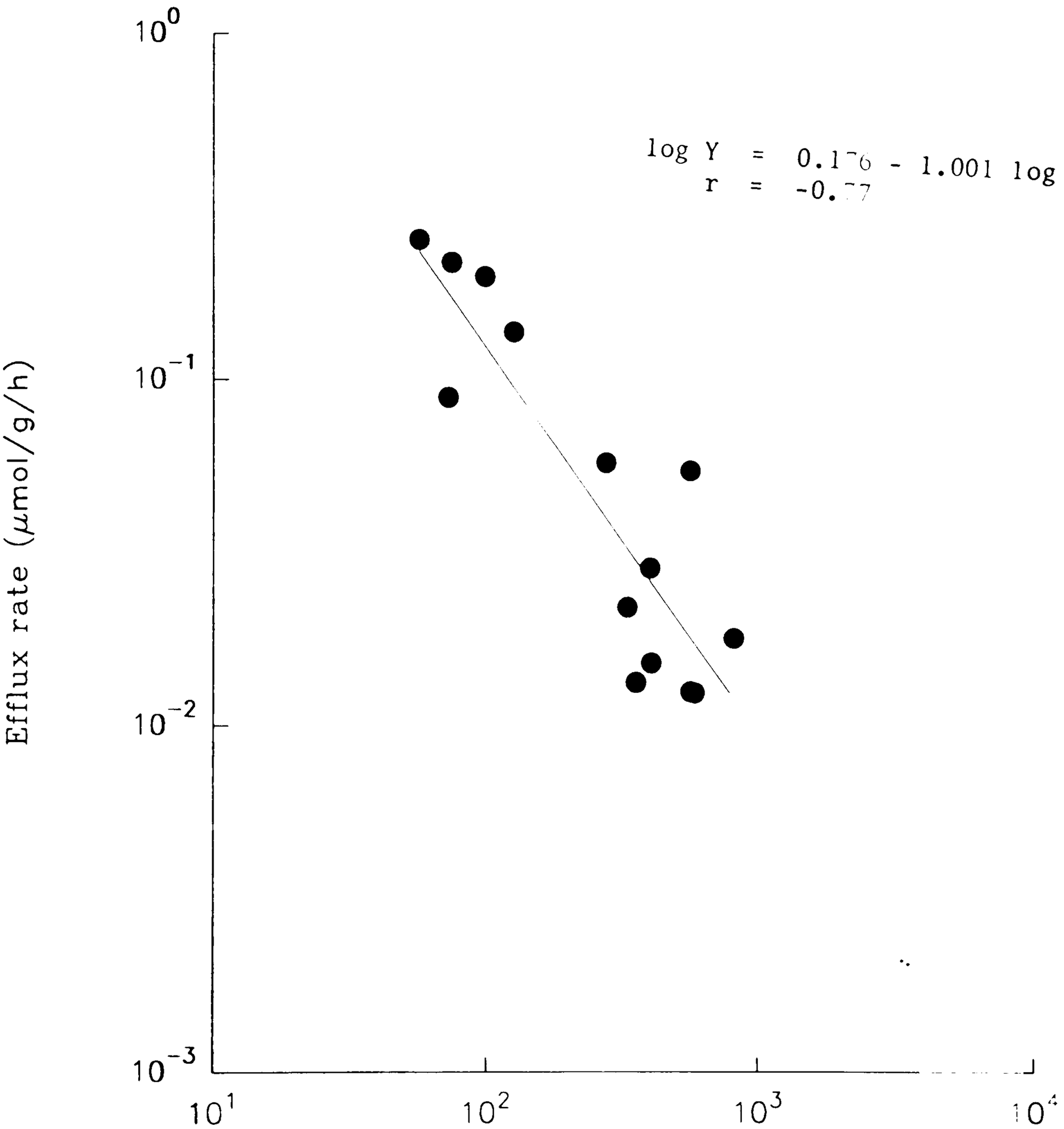


Figure 5.1. *Cancer pagurus.*

Plot of efflux rate (μmol g^{-1} h^{-1}) by fresh weight (g). All data collected at 15°C and 30-32‰.

Chapter 6

Effects of hypoxia and medium ammonia enrichment on efflux rates and circulating levels of ammonia in *Cancer pagurus* (L) and *Necora puber* (L)

6.1. INTRODUCTION

Ammonia, the principal end-product of protein metabolism in decapod crustaceans is transferred from the tissues to the haemolymph, from where it is excreted across the surface of the gills to the generally more dilute external medium. More than 95% of circulating ammonia is excreted at the gill and up to 2% in the urine. Efflux rates were found to be directly related to temperature in *Carcinus maenas* (Needham, 1957), in some penaeid species (Spaargaren *et al*, 1982) and in *Pandalus platyceros* (Quarmby, 1985). Spaargaren, (1982) found that 6-day acclimated blood ammonia levels of *Carcinus maenas* were strongly regulated by means of a variable flux of NH_4^+ and, in high external ammonia conditions, high NH_4^+ efflux was supplemented by urea formation. Regnault (1986) found that ammonia excretion of *Crangon crangon* was not influenced by external ammonia concentrations up to 87 μmol l^{-1}. In *Nephrops norvegicus* ammonia efflux (excretion) rates were variable and uptake occurred in enriched conditions up to 300 μmoles NH_4 l^{-1} (Hosie *et al*, 1991). This uptake was reflected by increased blood ammonia levels.

The proportion of ammonia excreted in the urine is very small; 1-2% of the total amount produced in *Callinectes sapidus* (Cameron and Batterton, 1978), less than 2% in *Jasus edwardsii* (Binns and Petersen, 1969) and a negligible amount in *Carcinus maenas* (Harris and Andrews, 1985). Because of its relatively small contribution to the total ammonia efflux (*cf* branchial excretion) a special study of urinary ammonia

was not included in these studies.

Hypoxia has been shown to cause a decrease in excretion rate in *Nephrops norvegicus* - a relationship which is linear and negative with respect to external oxygen tension (Hagerman *et al*, 1990). Haemolymph ammonia levels in this species also decrease under short term (Hosie *et al*, 1991) and long term hypoxia (Hagerman *et al*, 1990). An effect which is also seen under combined enrichment (300 μmoles NH_4 l^{-1}) and hypoxia (24 torr) compared with enriched conditions alone. *Cancer pagurus* (L) and *Necora puber* (L) are decapods of commercial importance in European waters. They are also species which are transported in bulk live consignments by road from the UK to markets principally in France and Spain. Such 'vivier' transport systems utilise high biomass to water ratios (1:1 in the case of *Cancer* and 1:2 for *Necora*) and journeys may take 2-3 days. Very high (1000-2000 μmol l^{-1}) external ammonia concentrations can develop along with moderate to severe hypoxia during this time (MacMullen *et al*, 1986).

Ammonia excretion has been the subject of investigations with regard to many crustaceans and fish involved in aquaculture. Many studies have been made on ammonia excretion (or efflux) rates, especially in freshwater and brackish species, but less on marine types. The form in which the ammonia is excreted is still a matter of debate. A common method of study has been to increase ambient medium ammonia levels, which causes excretion to cease (Cameron, 1986; Kormanik & Evans, 1984) and then to make subsequent experiments and measurements in an attempt to clarify the mechanisms taking place. The present studies, therefore, also attempt to study the mechanism of ammonia excretion in *Cancer pagurus* and *Necora puber*.

Eutrophication is becoming a well known problem in inshore areas around Europe and is mainly caused by agricultural runoff and other factors, especially in the North Sea

and in the Kattegat/Skaggerak region. Such environmental effects can mean that epibenthic-living organisms may be subjected to periods of strong hypoxia and high ammonia levels as tidal and other agencies alter the positions of these 'pockets' of water within an area. Despite the fact that some of these organisms are mobile and will move when conditions become unfavourable, they may still be subject to such poor conditions for short periods of time. Some of these 'pockets' of low oxygen water can be very extensive, however, and cause the organisms to suffer some physiological embarrassment (Hagerman & Phil-Baden, 1988). Under hypoxia, an ammonia efflux from the sediment of 100 mmol m^2 h^{-1} was found by Kristensen (1984). It is likely, therefore, that there are periods when high concentrations of ammonia combined with hypoxia may occur for these animals. Abnormally high ammonia concentrations and hypoxia also occur during the live marketing of *Cancer* and *Necora* - often with financial consequences (Chapter 3). The effects of such ecological and commercial conditions on ammonia effluxes in *Cancer* and *Necora* are considered in these studies which also provide information regarding the mechanism of ammonia efflux in these two species.

6.2. MATERIALS AND METHODS

Experiments were carried out over the months May to September 1990. A supply of animals was obtained, mainly from the Yorkshire fisheries (*C.pagurus*) or Scottish fisheries (*N.puber*) as described in Chapter 2 'General Materials and Methods'. The *C.pagurus* (329.24 g - 821.14 g fresh weight) and *N.puber* (67.01 g - 111.5 g fresh weight) were maintained as described in Chapter 2 'General Materials and Methods' for at least seven days before being used for experiments.

For each species, using groups of 6 animals, net ammonia fluxes were measured at a temperature of 12°C, at two oxygen concentration levels (normoxia, P_wO_2 = 100% or 155 Torr and P_wO_2= 15% or 24 Torr) and at four ammonia concentrations (normal seawater, <5 μmol NH_4 (total ammonia) l^{-1} and enriched seawater of 200 μmol NH_4 l^{-1}, 400 μmol NH_4 l^{-1}, and 1200 μmol NH_4 l^{-1}, a range of concentrations covering those commonly found during vivier transportation. Animals were allowed to acclimate for at least 7 days to the intended experimental temperature before being left to settle overnight in individual glass aquaria which were provided with a low flow-through of the normal aquarium seawater. At the start of the experiment, the flow of water was stopped and the water in each individual aquarium was carefully siphoned off until about 6 l were available to *C.pagurus* individual animals or 3 l in the case of *N.puber*. Oxygen levels were lowered at this time, if required, by purging with nitrogen gas as described in Chapter 2 'General Materials and Methods'. During normoxia experiments, the individual aquaria were aerated. The animals were left to settle for two hours before the first water sample (~1ml) was taken (T_0). Further water samples were taken every hour for five consecutive hours (ie a 4 hour excretion period, T_0-T_4). In the hypoxia experiments P_wO_2 values were checked regularly.

Thirty minutes after the last water sample was taken, a haemolymph sample was taken

from each animal as described in Chapter 2 'General Materials and Methods'. The pH of the haemolymph was measured immediately after taking the sample and the sample was diluted in saline (9g l^{-1} NaCl) for measurement of blood ammonia as described formerly (Chapter 2 'General Materials and Methods').

Ammonia concentrations were measured using a modified flow-injection/gas diffusion technique (Clinch et al, 1988, Hunter & Uglow, 1993), as described in Chapter 2 'General Materials and Methods'.

The total ammonia (NH_4) present is comprised free ammonia (NH_3) and the ionised form (NH_4^+) in ratios that depend on the pH and the pK of the medium. As described by the Henderson-Hasselbalch equation:-

$$pH = pK + \log ([NH_3]/[NH_4^+]) \qquad (1)$$

The $[NH_3]$ can be calculated as follows:-

$$[NH_3] = \frac{[NH_3] + [NH_4^+]}{(1 + 10)^{pK - pH}} \qquad (2)$$

(Armstrong *et al*, 1978)

The pK values were taken from values given for seawater (Bower and Bidwell 1987) and the blood osmotic concentrations were taken to be similar to seawater. From the calculated value of $[NH_3]$ the appropriate value of pNH_3 was obtained:-

$$pNH_3 = [NH_3] \times (22.09/\alpha) \qquad (3)$$

where α = Bunsen solubility coefficient = 1.46, given by Washburn (1928); cited in Kormanik & Cameron (1981a).

Urea analyses were made of the blood and water samples using Sigma Chemicals Kit No. 650 as described in Chapter 2 'General Materials and Methods'.

Where appropriate, data were subjected to Analysis of Variance using the statistical package SPSS (Subprograms of the Statistical Package for the Social Sciences) on a PC (Norusis, 1986, manual pA1-H11).

6.3. RESULTS

Notation of the results used in the text is as follows:-

Normoxia, P_wO_2 = 100% or 155 Torr and non-enriched conditions - (N);

Hypoxia, P_wO_2 = 15% or 24 Torr and non-enriched conditions - (H);

Normoxia and enriched conditions - NE200, NE400 and NE1200 for 200 μmol NH_4 l^{-1}, 400 μmol NH_4 l^{-1}, and 1200 μmol NH_4 l^{-1} enrichment respectively;

Hypoxia and enriched conditions - HE200, HE400 and HE1200 for 200 μmol NH_4 l^{-1}, 400 μmol NH_4 l^{-1}, and 1200 μmol NH_4 l^{-1} enrichment respectively.

Tables of results are presented with the same format in this Chapter as those given in Hosie *et al*, 1991, in order to facilitate comparisons; a copy of this paper is presented in Appendix 4.

The weight-specific net ammonia efflux rates of groups of animals (n=6) in all the experimental conditions are given in Table 6.1a and b for *C.pagurus* and *N.puber* respectively. These data show the rates to be very variable in each species, each group including some animals with no measured net efflux, or even an apparent net uptake, over one or more hours (Table 6.2a and 6.2b). Oneway Analyses of Variance of all the efflux data for each species (whole data and hypoxic or normoxic groups of data alone) revealed no significant differences ($P>0.05$; Appendix 3.2) demonstrating that the individual variability of efflux rate was high and did not enable significant differences to be determined between the experimental groups within a species.

Mean values of blood ammonia concentration at the end of the experiments are also included in Table 6.1a and 6.1b. In *C.pagurus* a comparison of all the hypoxic groups (H, HE200, HE400, and HE1200) was not significant (P>0.05; Appendix 3.4). comparison of all the normoxic groups (N, NE200, NE400, NE1200) did show significance (oneway ANOVA; P=0.0045; Appendix 3.3) revealing differences within this group for *C.pagurus*. Comparison of the whole data set for *N.puber* showed significant differences in all the experimental groups (oneway ANOVA; P=0.0131; Appendix 3.6). The variability in the non-enriched groups was high and prevented significant differences from being calculated in the normoxic experimental groups (N, NE200, NE400, and NE1200) (P>0.05; Appendix 3.7), comparisons of all the hypoxic groups (H, HE200, HE400, and HE1200) did show significance (P=0.0185; Appendix 3.8).

The rough estimate that blood volume = 0.30 ml g^{-1} fresh body weight - an approximation based on the blood volume estimates given for other decapods (eg Nicol, 1967; Spaargaren, 1972; Prosser, 1973; Gleeson & Zubkoff, 1977) was used to estimate the blood ammonia content data and the weight-specific blood ammonia content data given in Table 6.3a and b. Statistical comparisons of the blood ammonia content data for *C.pagurus* showed no significance for any comparisons (Appendix 3.2-3.4). However, significant differences were found for the *C.pagurus* weight specific blood ammonia content data for comparisons of all the groups (P=0.0072; Appendix 3.2) and for the data of the normoxic groups (P=0.0045; Appendix 3.3), no significant differences were found for comparisons of the hypoxic groups only (*ie* H, HE200, HE400, HE1200; Appendix 3.4). In *N.puber*, statistical comparisons of blood ammonia content data and weight-specific blood ammonia data showed significance for similar comparisons of the data; (oneway ANOVA; P=0.0239 and P=0.0200 for the whole blood ammonia content data set and the groups subject to hypoxia respectively and P=0.0131 and P=0.0185 for the same comparisons for weight specific blood

ammonia data; Appendix 3.6 & 3.8); no significant differences were found for the normoxic data only (Appendix 3.7).

The mean weight-normalised data for efflux rates (Table 6.1a and b) and blood ammonia contents (Table 6.3a and b) were used to produce estimates of the time required to effect a complete replacement (or turnover) of the blood ammonia (assuming a steady production and efflux rate) at each of the experimental conditions (Table 6.3a and b).

The Fick equation allows a calculation of NH_3 or NH_4^+ flux due to the partial pressure or pNH_3 gradient (data from Table 6.5) or the NH_4^+ concentration difference across the gills, as follows:-

$$J_{NH_3} = D_{NH_3} \cdot A \cdot \alpha \cdot \Delta P_{NH_3}/x \qquad (4)$$

where:

J_{NH_3}=total ammonia flux (mol s^{-1}); D_{NH_3}=diffusive coefficient of the membrane (m^2 s^{-1}); A = surface area of exchange (m^2); α = Bunsen solubility coefficient of ammonia (ml NH_3 l^{-1} $torr^{-1}$); ΔP_{NH_3}= partial pressure gradient of NH_3 (torr); x = barrier thickness (m)

or

$$J_{NH_4^+} = D_{NH_4^+} \cdot A \cdot (C^{in}_{NH_4^+} - C^{out}_{NH_4^+})/x \qquad (5)$$

where: $J_{NH_4^+}$= total ammonia flux (mol s^{-1}); and ($C^{in}_{NH_4^+} - C^{out}_{NH_4^+}$) = concentration gradient (mol m^{-3}).

If NH_3 diffusion is the dominant mode of excretion, the measured ammonia flux should be predicted by changes in the pNH_3 gradient; similarly if NH_4^+ diffusion is the dominant mode of excretion the measured ammonia flux should be predicted by the NH_4^+ concentration gradient. Kormanik & Cameron (1981a,b) used the ratio of two such estimates relating to different experimental conditions to predict changes in the net ammonia flux attributable to the changes in conditions. Using J_{NH_3} or $J_{NH_4^+}$ = J_{amm}:-

$$\frac{J^1_{amm}}{J^2_{amm}} = \frac{\Delta P^1_{NH_3}}{\Delta P^2_{NH_3}} \qquad (6)$$

or

$$\frac{J^1_{amm}}{J^2_{amm}} = \frac{(C^{1\,in}_{NH_4^+} - C^{1\,at}_{NH_4^+})}{(C^{2\,in}_{NH_4^+} - C^{2\,at}_{NH_4^+})} \qquad (7)$$

Normoxic (N) net efflux values were used as J^1_{amm} estimates in equations (6) and (7) to produce predictions of flux for the other experimental conditions. These estimates along with the actual measured values are given in Table 6.4a and 6.4b. For *C.pagurus*, in all cases, except that of NE200 the better estimate is that based on the pNH_3 concentration gradient. The better estimate for *N.puber* in most cases is that based on the pNH_3 concentration gradient but in the following situations of hypoxia only, normoxia enriched at 1200 μmol NH_4 l^{-1} (NE1200), and hypoxia enriched at 1200 μmol NH_4 l^{-1} (HE1200) the gradient was best decribed by the $[NH_4^+]$ gradient.

Under normal conditions seawater ammonia levels are very low compared with

haemolymph ammonia levels. This situation is shown by the high blood:medium ratios of $[NH_3]$ and $[NH_4^+]$ given in Table 6.5a and 6.5b for *C.pagurus* and *N.puber* respectively. These ratios become reduced in ammonia-enriched conditions and become <1:1 for $[NH_4^+]$ ratios in enrichment only. In *C.pagurus*, the $[NH_3]$ ratio generally decreased with an increase in enrichment in both normoxic and hypoxic conditions, except in the highest enrichment, normoxic group (NE1200), where a very high ratio blood:medium was found. The $[NH_4^+]$ ratio in *C.pagurus* was negatively related to enrichment (Table 6.5a) and the range of ratios was less in the hypoxic experimental groups compared with the normoxic groups. The $[NH_3]$ and $[NH_4^+]$ ratios calculated for *N.puber* were negatively related to enrichment under both normoxic and hypoxic conditions (Table 6.5b).

Gradients of the unionised and ionised ammonia concentrations are also given in Table 6.5a and b. The $[NH_3]$ gradient in *C.pagurus* was consistently low and varied slightly, showing a decrease in some enriched conditions except in the highest enrichment, normoxic (NE1200) group, where a gradient over two hundred times the normal normoxic gradient was found. The $[NH_4^+]$ gradients for this species became negative with enrichment, except in the least enriched hypoxic group (HE200). In *N.puber* the $[NH_3]$ gradient was quite consistent and often low in the normoxic groups compared with similar gradients under hypoxia. The *N.puber* $[NH_4^+]$ gradients became negative with enrichment, except in the normoxic group which was subjected to enrichment at 200 μmol NH_4 l^{-1} (NE200).

In *C.pagurus*, under hypoxia, the pNH_3 gradient was negatively related to enrichment, and a significant difference was found in the data (oneway ANOVA; P=0.0153) attributable to the differences between the non-enriched group (H) and the most highly enriched group (HE1200) being significant at the 5% level (Tukey multiple comparison testing - Appendix 3.4). The pNH_3 gradients (*C.pagurus*) in the normoxic groups

were variable and did not show a clear trend, the gradient in the NE1200 group was very high and caused significant differences to be found in the statistical testing (oneway ANOVA; P=0.0000, for both comparisons of all the experimental groups and normoxic groups only). Where effects of hypoxic conditions were studied in *N.puber* (H, HE200, HE400, HE1200), the pNH_3 gradient was negatively related to enrichment, and a significant difference was found in the data (oneway ANOVA; P=0.0050) due to the differences between the non-enriched group (H) and the most highly enriched group (HE1200) being significant at the 5% level (Tukey multiple comparison testing - Appendix 3.8). The pNH_3 gradients for the normoxic groups were consistent (*N.puber*), except where the enrichment level was 400 μmol NH_4 l^{-1} (NE400). All comparisons of these normoxic experimental groups were insignificant at the 5% level (Tukey multiple comparison testing - Appendix 3.7).

The blood and final water pH values obtained are given in Tables 6.1a and b. In Table 6.1a (for *C.pagurus*) the blood pH data for the hypoxic groups show an acidosis effect which was negatively related to enrichment. However, under normoxic conditions, enrichment causes an initial blood alkalosis but then revealed a negative relationship with enrichment and showed original pH conditions in NE1200. Statistical comparisons of the *C.pagurus* blood pH data revealed no significant differences between the experimental groups (P>0.05 in all cases, Appendix 3.2). However, comparisons of all the final water pH data for all experimental groups for *C.pagurus* showed a high significance (oneway ANOVA; P=0.0000; Appendix 3.2), which similarly was found within the hypoxic data only (P=0.0000; Appendix 3.4), but no significance was demonstrated by comparing the normoxic groups only (P=0.2237; Appendix 3.3). A trend of external media becoming more acidic with experimental enrichment was found in the normoxic experiments, which also ocurred under hypoxic conditions in the *Cancer* experiments (except at the onset of enrichment, HE200), but the latter happened over a larger range of pH (6.54 - 6.27 *cf* 7.04 - 6.86 in the former

conditions). In experiments carried out on *N.puber*, blood pH data for the hypoxic groups show a slight alkalosis overall with increase in enrichment concentration. Under normoxic conditions acidosis is brought about by low enrichment (NE200) initially, a higher level of enrichment (NE400 and NE1200) leads to alkalosis compared with non-enriched conditions. Statistical analysis of all the blood pH data revealed significant differences (oneway ANOVA; P=0.0072; Appendix 3.6), but significant differences were not found with comparisons of the normoxic or hypoxic data only (P>0.05 - Appendix 3.7 & 3.8). Comparisons of all the *Necora* final water pH data did give significant differences (oneway ANOVA; P=0.0126; Appendix 3.2), comparison of the normoxic or hypoxic groups only, showed no significance (P>0.05 - Appendix 3.7 & 3.8).

Table 6.6a and b give data for urea analyses made for water and haemolymph samples taken during the course of these experiments. The haemolymph levels found for *C.pagurus* were very variable, (P>0.05, oneway ANOVA of all the data, Appendix 3.2) and for comparison of the normoxic groups alone (P>0.05, oneway ANOVA, Appendix 3.3), however significant differences were found in the data relating to hypoxic conditions (P=0.000, oneway ANOVA, Appendix 3.4) due to differences between the non-enriched and the most enriched (HE1200) conditions (Tukey multiple comparison test, Appendix 3.4). Statistical differences were found in the data from water analyses of urea for *C.pagurus* (P=0.000, oneway ANOVA for all experimental groups, Appendix 3.4), this may be due to zero values of urea which were found in the hypoxic experiments. In the experiments undertaken with *L.puber* analyses for urea in the media were not measured, or were zero, and statistical analysis of this data was therefore not undertaken. Haemolymph levels of urea were statistically significant for comparisons of all the data, and comparisons of normoxic and hypoxic groups only (oneway ANOVA, P=0.000, P=0.0013 and P=0.000 respectively, Appendices 3.6-3.8). Among the normoxic experiments the differences were due to the high urea

levels in the haemolymph of the NE400 group compared with the NE200 group (Tukey multiple comparison test, Appendix 3.7). In the hypoxic experiments, low levels of urea found in the haemolymph of the HE1200 group caused the statistical differences found (Tukey multiple comparison test, Appendix 3.8). No clear trends were therefore found in the urea data.

6.4. DISCUSSION

6.4.1. Excretion Rates and Blood Ammonia

The mean weight-specific net ammonia efflux rates found in these studies for *C.pagurus* ranged from 0.02 μmol NH_4 g^{-1} hr^{-1} (N) to 0.18 μmol NH_4 g^{-1} hr^{-1} (NE1200) (Table 6.1a) and for *N.puber* 0.17 μmol NH_4 g^{-1} hr^{-1} (NE400) to -1.23 μmol NH_4 g^{-1} hr^{-1} (HE1200). The values which relate to normoxia and non ammonia-enriched seawater conditions are in accord with those given elsewhere for *C.pagurus* (Chapter 5) and other published values for decapods (*Cancer irroratus*; Kormanik and Evans, 1984: *Nephrops norvegicus*; Hosie *et al*, 1991; Hagerman *et al*, 1990; Table 1.7 and Table 5.2). Animals commonly showed periods of an hour or more where an apparent net uptake, or no apparent net efflux occurred. This was observed under all conditions tested and was more frequent in the ammonia-enriched media. Three experimental groups with *C.pagurus* had no individuals which demonstrated an uptake - normoxic, normal conditions and 200 μmol NH_4 l^{-1} enriched conditions (N and NE200) and hypoxic conditions only (H) - Table 6.2a. In the *N.puber* studies, animals which did not demonstrate any periods of net ammonia uptake after four hours of the experimental conditions were in the hypoxia only (H) and lowest enriched, hypoxic (HE200) groups. Preliminary investigations involving vigorous aeration of media with high and low ammonia concentrations demonstrated very small alterations to dissolved ammonia (NH_4) values. These observations on apparent (often transient) net uptake are in accord with those of Hosie *et al*, (1991) where similar studies to these were undertaken using *Nephrops norvegicus*.

Mean haemolymph ammonia concentrations found (Table 6.1a and b) ranged from 112.8 to 237.4 μmol ammonia l^{-1} (from the HE400 and HE200 groups respectively) for *C.pagurus* and 134.4 to 443.8 μmol ammonia l^{-1} (from H and HE400 groups respectively) for *N.puber* and compare with normoxic values given in Chapter 5 (Table

5.1) and Table 1.9, and for other decapod species recently quoted in the literature - 87.7 to 212.2 μmol l^{-1} (Hosie *et al*, 1991) and 130 ± 63 μmol l^{-1} (Hagerman et al, 1990). These concentration values convert to relatively small weight-specific blood ammonia content values for the animals in these experiments Table 6.3. These weight-normalised net efflux and blood content values can be used to estimate the blood ammonia turnover times and to compare different experimental conditions. Ammonia enrichment of the medium and periods of hypoxia are physiological stresses which *C.pagurus* and *N.puber* encounter commonly during live transport and marketing. Reports of mortalities are made from time to time with this method of transportation, but, these experiments show that such conditions are withstood by the animals, at least for short periods of six hours. Some vivier journeys, however, may last for more than 24h. Nothing, however, is known of the longer-term consequences which are of importance in the context of the extended-live husbandry of product under commercial conditions

6.4.2. Processes Involved in Excretion

Branchial ammonia efflux involves a possible ionic exchange mechanism using a Na^+/NH_4^+ exchange pump and transepithelial diffusion of ammonia as the free form and/or the ionic form in fish and crustaceans (Regnault 1987). The present data provide no information which helps resolve the involvement of any Na^+/NH_4^+ exchange mechanism in these species, but does offer support for the contention that flux occurs along a pNH_3 or $[NH_4^+]$ gradient by diffusion (Table 6.4a and b). Diffusion may also allow NH_4^+ to be taken up by the animals when exposed to ammonia-enriched media, since both a negative ratio (*ie* <1) and an uphill gradient for the blood:medium $[NH_4^+]$ ratio were found (Table 6.5a and b). Cameron (1986) reported that measured increases in blood ammonia occurred by reversal of NH_4^+ gradients and by downhill pNH_3 gradients from medium to animal. Transepithelial potentials were not measured here, but were found to have very little effect on NH_4^+

movements in *Cancer irroratus* exposed to ammonia-enriched media (Kormanik and Evans 1984). The final pNH_3 gradients calculated for the present data were downhill from the blood to the external medium in all cases (Table 6.5a and b). In ammonia-enriched media this gradient can provide the only means by which branchial efflux of ammonia may be effected (Table 6.5a and b). The NH_3:NH_4^+ proportions during efflux depend on the relative amounts of the two ammonia species in the blood and external medium (which is determined by pH in both cases) and their relative permeabilities across the gill, the free ammonia (NH_3) being more permeable to biological membranes than NH_4^+ (Warren, 1962).

NH_3 and NH_4^+ calculated data are presented in Table 6.5a and b and show that a net uptake of NH_4^+ may contribute to blood ammonia. Cameron (1986) found that blood ammonia levels in *Callinectes sapidus* exposed to high ammonia concentrations in the medium, needed to increase above external levels before efflux was re-established. In the present experiments it is not possible to determine to what extent uptake is ocurring and to what extent blood NH_4^+ concentrations may be due to the accumulation of metabolically-produced ammonia. The 6 h duration of these experiments involving enriched media may not have been sufficient for the haemolymph ammonia concentrations to reach levels where ammonia efflux down concentration gradients were re-established. In normal conditions (N) for both species weight-specific ammonia efflux rates are high relative to the weight-specific blood content, inhibition of ammonia efflux accross the gills would need to proceed for only a short period of time in order to bring about large changes in blood ammonia concentration, unless protein metabolism is also inhibited. The blood concentration changes that were measured (Table 6.3a and b) under ammonia-enriched conditions were more modest than would be expected for a simple case of inhibition of branchial efflux and this may indicate that ammonia production at the tissue level - or release from the tissues to the haemolymph - was much reduced during enrichment, even

under normoxia; or that detoxifying processes (ammonia removal from blood and tissues) were ocurring.

6.4.3. Effects of Hypoxia

In molluscs, ammonia production apparently is not affected by any metabolic shut-down during anaerobiosis (de Vooys & de Zwann 1978; Bishop *et al*, 1983) but this has since been shown not to be so (Widdows & Shick, 1991). Anaerobic ammonia excretion under short-term anoxia has been found in fish (van Waarde, 1983) and in similar anoxia experiments, lactate was produced in the tissues of *Carassius auratus* (Thillart & Kesbeke, 1978). Although no significant increase in circulating lactate levels was found in adult *Cancer pagurus* by Bradford & Taylor (1982) increases of blood lactate levels have been reported in response to hypoxia and emersion in *Cancer* (Uglow *et al*, 1986; Chapter 5). Ammonia fluxes measured under hypoxia in these studies may have occurred during anaerobiosis and involved ammonia production under anaerobiosis since haemolymph lactate levels have also been known to accumulate whilst the animals are in vivier conditions (Chapter 3), which these experiments aim to represent.

6.4.4. Acid-base Status and Detoxification Processes.

In both *C.pagurus* and *N.puber* the normoxic experimental groups showed an overall slight metabolic alkalosis (*cf* the normal normoxic groups - final column, Table 6.1a and b), while the hypoxic groups showed a slight acidosis. The water final pH showed a trend of gradual decrease over the normoxic and hypoxic conditions, except in the case of hypoxic conditions with enrichment (200 μmol NH_4 l^{-1} - HE200) and hypoxia with 400 μmol NH_4 l^{-1} enrichment (HE400) for *C.pagurus* and *N.puber* respectively. Alkaline conditions increase the NH_3 component of ammonia in the blood (demonstrated in Cameron 1986) this component is considered to be the more toxic

since it can move across biological membranes more quickly (Kormanik & Cameron, 1981a; Warren, 1962). Such blood pH changes (alkalosis) have also been shown to increase the haemocyanin oxygen affinity under hypoxic conditions (McMahon et al, 1978; Burnett, 1979). The experiments here, were carried out under normoxia and the measured alkalosis may have been a response to the ammonia-enriched conditions. The production of urate was suggested to be a possible factor in increasing the oxygen affinity of haemocyanin under hypoxia in *Carcinus maenas* (Lallier *et al*, 1987) and *Penaeus japonicus* (Lallier & Truchot, 1989). Such measurements were not made here although determinations of urea production in the haemolymph were undertaken. Spaargaren (1982) suggested that urea production, from elevated blood NH_4^+ concentrations under medium ammonia-enrichment, may serve as a convenient detoxification process. His *Carcinus* experiments were carried out over many days but the present experiments were carried out over periods of hours and it is unlikely that high levels of urea would accumulate in this short time. Periods of comparable length, therefore, require to be tested and will equate with the duration of many live transport journeys. Nitrogenous compounds (NH_4^+) in the haemolymph serve to reduce the oxygen affinity of haemocyanin, in *Notostomus gibbosus* and *Cancer magister* (Sanders *et al*, 1992). This effect in *C.magister* was observed at a lower pH range, but was lessened at higher pH - ranges of pH in the present studies for *C.pagurus* and *N.puber* are similar to those measured by Sanders *et al*, (1992). Blood acidosis (*cf* hypoxia alone experiment) ocurred with hypoxia and enriched medium conditions for both species (Table 6.1a and b). Water pH overall decreased with ammonia enrichment under hypoxia, which may have caused the pH decrease in the haemolymph. Such an effect was found in freshwater crustaceans subjected to acidic conditions (pH 3.8) for moderate periods (24h) also leading to an increase in ammonia excretion (Mauro & Moore, 1987). Blood acidosis reduces the proportion of the NH_3 (more toxic) component of blood ammonia, in high external ammonia conditions and so may also aid the removal of ammonia. The overall effect measured on the

organisms in the present investigations, across the whole experimental series, may be due to responses to the level of hypoxia experienced. Mauro & Malecha (1984) reported that blood pH increased at oxygen tensions between 80 and 40 torr and decreased below levels of 15 torr, when lactate production also ocurred in *Macrobrachium rosenbergii*. The initial response was due to ventilatory changes ocurring in the animals which evoked a pH increase by loss of carbon dioxide from the blood. In these experiments, and comparable studies undertaken with *Nephrops norvegicus* (Hosie *et al*, 1991), despite differences in blood pH with experimental condition, a relative alkalinity of the haemolymph to the medium always ocurred. Howell *et al*, (1973) suggested that such a relative alkalinity was maintained by manipulation of pCO_2 and HCO_3^-. It is unclear whether such mechanisms are involved here, and further work is needed to understand more fully the effect of ammonia enrichment on *Necora puber* and *Cancer pagurus*.

Table 6.1a. *Cancer pagurus*: Summary of ammonia efflux data, water and blood ammonia levels and pH data. P_WO_2: partial pressure of oxygen in water (torr). Values are means (±SEM) for n=6 individuals in each case.

Experimental conditions	Weight (g)	Weight-specific net ammonia efflux (μmol g^{-1} h^{-1})	Water		Blood	
			final ammonia conc (μmol l^{-1})	final pH	final ammonia conc (μmol l^{-1})	final pH
Normoxia (P_WO_2=155 torr)	522.00 (73.13)	0.02 (0.00)	23.68 (1.39)	7.04 (0.03)	129.55 (12.55)	7.66 (0.07)
Enrichment (200μmol l^{-1})	527.82 (69.40)	0.14 (0.05)	264.16 (10.87)	6.98 (0.05)	218.03 (27.59)	7.75 (0.05)
Enrichment (400μmol l^{-1})	519.37 (72.12)	0.01 (0.06)	399.52 (17.53)	6.91 (0.09)	139.93 (14.17)	7.72 (0.04)
Enrichment (1200μmol l^{-1})	501.61 (80.47)	0.18 (0.14)	1192.88 (10.94)	6.86 (0.06)	222.28 (22.98)	7.66 (0.05)
Hypoxia (P_WO_2=24 torr)	512.04 (78.51)	0.02 (0.00)	37.33 (2.78)	6.54 (0.02)	134.71 (24.40)	7.82 (0.04)
Enrichment+hypoxia (200μmol l^{-1} ,P_WO_2=24 torr)	506.80 (76.49)	0.04 (0.03)	227.72 (7.45)	6.62 (0.06)	237.39 (25.97)	7.76 (0.03)
Enrichment+hypoxia (400μmol l^{-1},P_WO_2=24 torr)	509.41 (78.51)	0.03 (0.02)	444.83 (8.05)	6.43 (0.03)	112.83 (35.81)	7.75 (0.03)
Enrichment+hypoxia (1200μmol l^{-1},P_WO_2=24 torr)	526.20 (70.64)	0.00 (0.10)	1194.49 (14.55)	6.27 (0.08)	198.83 (42.27)	7.61 (0.12)

Table 6.1b. *Necora puber*: Summary of ammonia efflux data, water, blood ammonia and pH data. P_WO_2: partial pressure of oxygen in water (torr). Values are means (±SEM) for n=6 individuals in each case.

Experimental conditions	Weight (g)	Weight-specific net ammonia efflux (μmol g^{-1} h^{-1})	Water		Blood	
			final ammonia conc (μmol l^{-1})	final pH	final ammonia conc (μmol l^{-1})	final pH
Normoxia (P_WO_2=155 torr)	80.83 (5.78)	0.03 (0.01)	16.76 (0.96)	7.50 (0.46)	269.36 (49.36)	7.63 (0.06)
Enrichment (200μmol l^{-1})	91.22 (4.00)	0.05 (0.10)	230.08 (14.89)	6.95 (0.07)	310.98 (35.41)	7.53 (0.06)
Enrichment (400μmol l^{-1})	85.99 (3.95)	0.17 (0.10)	406.01 (10.80)	6.87 (0.07)	165.64 (39.83)	7.67 (0.02)
Enrichment (1200μmol l^{-1})	83.45 (5.18)	-0.22 (0.27)	1215.19 (52.17)	6.89 (0.06)	396.47 (101.63)	7.66 (0.07)
Hypoxia (P_WO_2=24 torr)	83.25 (4.45)	0.04 (0.01)	19.62 (1.07)	6.69 (0.06)	443.81 (119.75)	7.82 (0.02)
Enrichment+hypoxia (200μmol l^{-1} ,P_WO_2=24 torr)	86.05 (7.28)	0.14 (0.03)	239.18 (7.62)	6.62 (0.04)	194.01 (29.27)	7.84 (0.05)
Enrichment+hypoxia (400μmol l^{-1},P_WO_2=24 torr)	85.99 (5.28)	0.07 (0.06)	416.35 (9.81)	7.76 (0.05)	134.41 (19.28)	7.76 (0.05)
Enrichment+hypoxia (1200μmol l^{-1},P_WO_2=24 torr)	86.24 (3.53)	-1.23 (1.37)	1235.39 (19.05)	6.62 (0.01)	207.12 (46.26)	7.62 (0.11)

Table 6.2a. *Cancer pagurus*: Variability in net ammonia efflux within and between experimental groups, showing number of individuals which displayed net efflux over 3 hours and incidence of hourly measurements where decreases in medium ammonia were found. In all cases, second set of values are number of measurements made.

Experimental conditions	Individuals	Incidence net ammonia efflux
Normoxia (P_wO_2=155 torr)	0/6	4/24
Enrichment (200μmol l^{-1})	0/6	0/12
Enrichment (400μmol l^{-1})	1/6	12/24
Enrichment (1200μmol l^{-1})	1/6	12/24
Hypoxia (P_wO_2=24 torr)	0/6	7/24
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	1/6	5/12
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	1/6	8/12
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	4/6	10/24

Table 6.2b. *Necora puber*: Variability in net ammonia efflux within and between experimental groups, showing number of individuals which displayed net efflux over 3 hours and incidence of hourly measurements where decreases in medium ammonia were found. In all cases, second set of values are number of measurements made.

Experimental conditions	Individuals	Incidence net ammonia efflux
Normoxia (P_wO_2=155 torr)	1/6	8/12
Enrichment (200μmol l^{-1})	3/6	4/12
Enrichment (400μmol l^{-1})	2/6	5/12
Enrichment (1200μmol l^{-1})	3/6	8/12
Hypoxia (P_wO_2=24 torr)	0/6	3/12
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	0/6	6/12
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	5/6	7/12
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	3/6	6/24

Table 6.3a. *Cancer pagurus*: Weight-specific blood ammonia contents and their replacement rates $\left(\frac{\text{Mean weight-specific blood content}}{\text{Mean weight-specific efflux rate}} \times 60\right)$

Blood volume has been taken as 30% of fresh body weight. Values are given as means (±SEM) for n=6 individuals in each case.

Experimental conditions	Blood ammonia		Replacement-rate time (mins)
	gross (μmol)	Weight-specific (μmol g^{-1})	
Normoxia (P_wO_2=155 torr)	20.02 (3.45)	0.04 (0.00)	120.00
Enrichment (200μmol l^{-1})	35.02 (6.97)	0.07 (0.01)	30.00
Enrichment (400μmol l^{-1})	22.66 (7.60)	0.04 (0.00)	240.00
Enrichment (1200μmol l^{-1})	34.29 (8.70)	0.07 (0.01)	23.33
Hypoxia (P_wO_2=24 torr)	21.33 (6.05)	0.04 (0.01)	120.00
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	37.81 (10.04)	0.07 (0.01)	30.00
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	16.63 (4.88)	0.03 (0.01)	60.00
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	29.78 (5.78)	0.06 (0.01)	0.00

Table 6.3b. *Necora puber*: Weight-specific blood ammonia contents and their replacement rates $\left(\frac{\text{Mean weight-specific blood content}}{\text{Mean weight-specific efflux rate}} \times 60\right)$

Blood volume has been taken as 30% of fresh body weight. Values are given as means (±SEM) for n=6 individuals in each case.

Experimental conditions	Blood ammonia		Replacement-rate time (min)
	gross (μmol)	Weight-specific (μmol g^{-1})	
Normoxia (P_wO_2=155 torr)	6.31 (0.89)	0.08 (0.01)	160.00
Enrichment (200μmol l^{-1})	8.64 (1.22)	0.09 (0.01)	108.00
Enrichment (400μmol l^{-1})	4.43 (1.28)	0.05 (0.01)	17.65
Enrichment (1200μmol l^{-1})	10.29 (3.09)	0.12 (0.03)	Net uptake
Hypoxia (P_wO_2=24 torr)	11.16 (2.92)	0.13 (0.04)	195.00)
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	4.90 (0.61)	0.06 (0.01)	25.71
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	3.50 (0.59)	0.04 (0.01)	34.29
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	5.43 (1.36)	0.06 (0.01)	Net uptake

Table 6.4a. *Cancer pagurus*: Relative rates of efflux of ammonia (J_{amm}) with reference to normoxic rates (100%). J^1_{amm} = normoxic flux, J^2_{amm} = calculated flux. pNH_3: partial pressure of NH_3

Experimental conditions	Predicted flux (J2amm%) based on:		Actual excretion rate
	pNH_3 gradient	NH_4^+ gradient	
Normoxia (P_WO_2=155 torr)	-	-	100% (J^1_{amm})
Enrichment (200μmol l^{-1})	233%	-41%	70%
Enrichment (400μmol l^{-1})	84.9%	-247.7	50%
Enrichment (1200μmol l^{-1})	29501%	-1133.5%	900%
Hypoxia (P_WO_2=24 torr)	395%	1.8%	100%
Enrichment+hypoxia (200μmol l^{-1} ,P_WO_2=24 torr)	345%	6.7%	200%
Enrichment+hypoxia (400μmol l^{-1},P_WO_2=24 torr)	156%	-317%	150%
Enrichment+hypoxia (1200μmol l^{-1},P_WO_2=24 torr)	10%	-948%	0%

Table 6.4b. *Necora puber*: Relative rates of efflux of ammonia (J_{amm}) with reference to normoxic rates (100%). $J1_{amm}$ = normoxic flux, J^2_{amm} = calculated flux. pNH_3: partial pressure of NH_3

Experimental conditions	Predicted flux (J2amm%) based on:		Actual excretion rate
	pNH_3 gradient	NH_4^+ gradient	
Normoxia (P_wO_2=155 torr)	-	-	100% (J^1_{amm})
Enrichment (200µmol l^{-1})	100%	32%	166%
Enrichment (400µmol l^{-1})	54%	-96%	567%
Enrichment (1200µmol l^{-1})	100%	-4c-4%	-750%
Hypoxia (P_wO_2=24 torr)	278%	167%	75%
Enrichment+hypoxia (200µmol l^{-1} ,P_wO_2=24 torr)	130%	-19%	450%
Enrichment+hypoxia (400µmol l^{-1},P_wO_2=24 torr)	59%	-113%	233%
Enrichment+hypoxia (1200µmol l^{-1},P_wO_2=24 torr)	45%	-328%	-4100%

Table 6.5a. *Cancer pagurus*: Blood:medium ratios of NH_3 and NH_4^+ gradients. Values are means for n=6 individuals in each case.

Experimental conditions	Blood:medium ratio of		Gradient (μmol l^{-1})		pNH_3 gradient (μtorr)
	NH_3	NH_4^+	NH_3	NH_4^+	
Normoxia (P_wO_2=155 torr)	24.43:1	5.56:1	+0.75	+105.1	+11.33
Enrichment (200μmol l^{-1})	5.26:1	1.45:1	+1.75	-44.02	+26.43
Enrichment (400μmol l^{-1})	2.66:1	0.35:1	+0.64	-260.39	+9.62
Enrichment (1200μmol l^{-1})	176.8:1	4.2e-9:1	+220.9	-1191.5	+3342
Hypoxia (P_wO_2=24 torr)	66.2:1	3.5:1	+2.96	+94.42	+44.81
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	15.09:1	1.04:1	+2.58	+7.09	+39.11
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	6.05:1	0.24:1	+1.08	-333.17	+17.70
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	4.25:1	0.16:1	+1.11	-996.76	+16.75

Table 6.5b. *Necora puber*: Blood:medium ratios of NH_3 and NH_4^+ gradients. Values are means for n=6 individuals in each case.

Experimental conditions	Blood:medium ratio of		Gradient (μmol l^{-1})		pNH_3 gradient
	NH_3	NH_4^+	NH_3	NH_4^+	(μtorr)
Normoxia (P_wO_2=155 torr)	69.33:1	16:1	+1.78	+250.8	+26.97
Enrichment (200μmol l^{-1})	6.16:1	1.35:1	+1.79	+79.10	+27.14
Enrichment (400μmol l^{-1})	3.23:1	0.41:1	+0.97	-241.3	+14.63
Enrichment (1200μmol l^{-1})	2.28:1	0.32:1	+1.78	-820.49	+26.95
Hypoxia (P_wO_2=24 torr)	309.6:1	22.48:1	+4.95	+419.2	+74.95
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	14.03:1	0.80:1	+2.28	-47.45	+34.50
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	5.20:1	0.32:1	+1.05	-282.99	+15.87
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	1.78:1	0.2:1	+0.81	-823.17	+12.20

Table 6.6a. *Cancer pagurus*: Urea: blood and water concentrations. Values are means (±SEM) for n=6 individuals in each case.

Experimental conditions	Blood urea (mg dl^{-1})	Water urea (mg dl^{-1})
Normoxia (P_WO_2=155 torr)	5.52 (4.90)	0.54 (0.14)
Enrichment (200μmol l^{-1})	1.65 (0.39)	0.18 (0.18)
Enrichment (400μmol l^{-1})	1.01 (0.19)	0.78 (0.17)
Enrichment (1200μmol l^{-1})	1.20 (0.29)	1.95 (0.33)
Hypoxia (P_WO_2=24 torr)	0.13 (0.13)	0.00 (0.00)
Enrichment+hypoxia (200μmol l^{-1} ,P_WO_2=24 torr)	0.03 (0.03)	0.00 (0.00)
Enrichment+hypoxia (400μmol l^{-1},P_WO_2=24 torr)	1.91 (0.39)	-
Enrichment+hypoxia (1200μmol l^{-1},P_WO_2=24 torr)	1.28 (0.26)	-

Table 6.6b. *Necora puber*: Urea: blood and water concentrations. Values are means (±SEM) for n=6 individuals in each case.

Experimental conditions	Blood urea ($mg\ dl^{-1}$)	Water urea ($mg\ dl^{-1}$)
Normoxia (P_wO_2=155 torr)	0.71 (0.19)	0.00 (0.00)
Enrichment (200μmol l^{-1})	0.29 (0.21)	0.00 (0.00)
Enrichment (400μmol l^{-1})	2.22 (0.33)	0.00 (0.00)
Enrichment (1200μmol l^{-1})	1.66 (0.46)	-
Hypoxia (P_wO_2=24 torr)	2.74 (0.00)	0.00 (0.00)
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	3.28 (0.17)	0.00 (0.00)
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	2.65 (0.35)	0.00 (0.00)
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	0.37 (0.37)	-

Chapter 7

General Discussion

The data in this thesis comprise a comprehensive body of information relating to the physiology and the live marketing of the crab *Necora puber* and *Cancer pagurus*. Both of these species are currently the subject of a thriving export market between the UK and mainland Europe. Despite the rapid growth of this trade in recent years there has been little attempt made to describe it in any detail, to examine possible physiological impairment caused by various procedures and to gather information relating to the ecophysiology of the animals in their natural habitats. A few studies have been made to identify particular points in the marketing chain where the animals (= product) are vulnerable to bad practices (Quinlan, 1984; Whyman *et al*, 1985; MacMullen *et al*, 1986; Uglow *et al*, 1986). Despite this, little effort has been made to utilise such information to suggest improvements of existing methods.

Opportunity has been taken to examine all aspects of the marketing chain from first-landing to point of final sale to continental dealers. This has shown that such chains can be extremely complex, may involve several intermediary dealers and entail several occasions when the product is handled, subjected to aerial exposure or kept in small static volumes of seawater which rapidly become fouled with waste products from the animals. Significantly, the studies have revealed a considerable lack of awareness by dealers and employees of the potential hazards many of the procedures may have on their product. Protocols are not standardised and, usually, have been derived *via* a series of *ad hoc* methods which normally work (often), rather than *via* a systematic evaluation to produce a series of optimal procedures.

7.1. Physical Damage

In their natural environment crustaceans can autotomise limbs in response to predation or as a result of physical damage (Warner, 1977; McVean, 1982). The normal occurrence of limb loss in a natural population of *Necora puber* was studied by Norman & Jones (1991) who found that the majority of mature individuals sampled had no limb loss (67.2% and 71.2% for males and females respectively - combined 'n' was 1079). Few individuals had more than two or three limbs missing and the relationship of more than one limb missing with crab size was positive. These observations were related to the ecology and behaviour of this organism in the natural environment. The effects of such limb loss can relate to a reduction in intrinsic quality relative to organisms of a similar age due to the effect on growth, in the normal habitat of the crab, since metabolic resources may be diverted to regeneration of an autotomised limb (Hartnoll, 1982). Studies on *Cancer pagurus* demonstrated that missing limbs lead to smaller growth increments at moult (Bennett, 1973). This may confer further disadvantages on the organism, such as loss of competitive edge.

It is reasonable to consider that physical damage to individual crabs during live transport will affect their intrinsic quality from a scientific viewpoint, as well as in the commercial market. Incidence of damage is likely to be far higher in marketing as greatly increased chances of physical damage occur (as described in Chapter 4 of this thesis) due to mutual aggression of the organisms, handling *etc.* In the context of live marketing a fundamental difference lies between a 'damaged' limb and one that has been autotomised since in the latter case there is no blood loss and less likelihood, therefore, of the animal dying in transit. While damage data has been collected during this work and demonstrates the effect on perceived 'quality' from the market (Chapter 4), the physiological stress effect on the organisms concerned is still somewhat unquantifiable due to the lack of background information. Data on the physiological

and biochemical status of the organisms from different stocks and in different seasons as well as a full field study where biochemical data and damage data are collected in tandem during live transport would provide appropriate background material. Such data would also help explain the mortality of intact individuals, commonly found (Table 4.1 - 4.5, Chapter 4) An ocurrence which was also found during testing of live transportation methods for the Dungeness crab (*Cancer magister*) (Barnett, *et al*, 1973). The development of practices to reduce damage to the 'product' at all stages of the marketing chain and thereby reduce stress is also favoured.

Handling itself is known to cause stress - indicated by an increase in (aquatic) oxygen consumption in *Cancer pagurus* and *Maja squinado* (Aldrich, 1975), and also cardiac arrest (an indicator of stress) in other cancrid species (Florey & Kriebel, 1974). Such stress can be reduced by careful handling of crabs at sea (Thomas, 1969; Edwards & Early, 1972), repairing damaged limbs (in the case of *Cancer pagurus* - Chapter 3) and during holding (Whyman *et al*, 1985). Rigorous selection and minimal disturbance of the animals during transport (Barnett *et al*, 1973), especially in air (Otwell & Webb, 1977; Whiteley & Taylor, 1986; Taylor & Whiteley, 1989), avoidance of transfer between vehicles during the journey (MacMullen *et al*, 1986) and care when unloading the consignment at destination (MacMullen *et al*, 1986; Uglow *et al*, 1986; Whiteley & Taylor, 1986) all serve to reduce stress to the organisms concerned.

7.2. Aerial exposure/Emersion

During the live marketing of crustaceans the animals are subject to aerial exposure for varying lengths of time at several stages of the marketing chain (Chapter 3). Investigation of mortalities or survival of commercial species which have undergone aerial exposure shows that deaths can be high *eg* 77% after 24h, at 80% RH and 16°C in *Necora puber* (Johnson & Uglow, 1985a) compared with 15.4% after 24h, at 80%

RH and 16°C in *Cancer pagurus* (Whyman *et al*, 1985) and about 30% after 4d at 100% RH (fog system) and 6°C in *Cancer magister* (Barnett *et al*, 1973) and 16.7‰ after 4h at 80-95% RH (obtained by covering the crab in an open basket with moist burlap) and 10°C in *Callinectes sapidus* (Otwell & Webb, 1977). Median resistance times were determined for lobsters *Homarus americanus* acclimated to various temperatures (0, 10 & 20°C) to be between 4 and 8°C in aerial and moist conditions. Continuous sprays of seawater or exposure to different air/oxygen mixtures did not increase survival; feeding of the animals reduced survival (McLeese, 1965). During the present studies a 'mist system' used for live transport (Chapter 3) resulted in high mortalities. The investigations carried out by Barnett *et al*, (1973) demonstrated a lessened mortality of *Cancer magister* held in trays partially flooded with recirculated seawater at 6°C (*cf* from 30% using a fog/mist system to 10%); such rates were similarly sustained during road testing of this system on a 3 and 4 day journey. A partial emersion response of the intertidal prawn *Palaemon elegans* to hypoxic conditions was found to be effective in providing higher levels of oxygenation of the blood and a reduction in lactate levels than prawn which were totally emersed (Taylor & Spicer, 1988). Similar results of a reduction in haemolymph lactate leves (*cf* emersed crabs) were found in a partial emersion cascade system using *Cancer pagurus* in laboratory trials (Hosie & Uglow, unpublished).

The accumulation of lactate, accompanied by haemolymph acidosis is documented in many crustaceans (*Carcinus maenas* Truchot, 1975; *Cancer productus* Defur & McMahon, 1984a & b; *Panulirus argus* Vermeer, 1987; *Palaemon elegans* Taylor & Spicer, 1988; *Homarus vulgarus* Taylor & Whiteley, 1989). Haemolymph constituents measured during laboratory simulations of typical live transport procedures on aerial exposure and aerial holding conditions demonstrated elevated blood glucose and lactate leves (*cf* normal aquarium levels), in both *Necora puber* (Whyman *et al*, 1985; Uglow *et al*, 1986) and *Cancer pagurus* (Uglow *et al*, 1986). Circulating levels of

glucose and lactate were reduced upon re-immersion in fully aerated, clean seawater, however reduction did not reach normal levels in *Necora* after 10h re-immersion (16.3 mg 100 ml^{-1} *cf* 4.86 mg 100 ml^{-1} for lactate and 8.26 mg 100 ml^{-1} *cf* 1.56 mg 100 ml^{-1} for glucose) (Uglow *et al*, 1986). Elevation of glucose and lactate levels (*cf* holding pond levels) were found in crabs during aerial transport or held in air prior to loading into holding ponds or vivier tanks (Uglow *et al*, 1986; Chapter 3). *eg* for lactate 40 25 mg 100 ml^{-1} after >6h exposure and 57.45 mg 100 ml^{-1} after dry transport of about 18h *cf* 17.08 mg 100 ml^{-1} for *Cancer pagurus* (Table 3.2) and 11.67-20.20 mg 100 ml^{-1} after raising from keep pots *cf* 6.25 mg 100 ml^{-1} in holding ponds (post-vivier transport) (Table 3.5). The circulating levels were clearly reduced on return of the animals to water where the supply was fresh and well aerated.

The removal of an organism from its normal aquatic environment prevents exchange across the gills, in the case of ammonia, a waste product, an accumulation would be expected; especially since the ambient water aids the removal of ammonia from the organism across the gills (Chapter 1). Specimens of adult and juvenile *Cancer productus* were found to have an increase in blood ammonia of 60 μM NH_4^+ and 106 μM NH_4^+ (juveniles and adults repectively) after 3 - 4h emersion (Defur & McMahon, 1984b). Up to 2h of emersion caused a near doubling of blood ammonia from 7.22 mg ml^{-1} to 13.77 mg ml^{-1} in *Panulirus argus* (Vermeer, 1987). The data presented in Chapter 5 for blood ammonia are initial time series information on laboratory investigations of the effects of aerial exposure in adult and juvenile *Cancer pagurus*. A clear increase with time of exposure is observed in both adults and juveniles (at 58.7 μmol l^{-1} h^{-1} and 77.1 μmol l^{-1} h^{-1} over 8 hours in adult and juvenile crabs repectively). Elevated levels of blood ammonia (*cf* aquarium conditions) were found in both *Cancer* (431.2 μmol l^{-1} *cf* Table 6.1a) and *Necora* (upto 575.6 μmol l^{-1} *cf* Table 6.1b) held in air prior to loading into vivier tanks (Table 3.5) a constant increase with time (25 μM h^{-1}) in blood ammonia was found in juvenile *Cancer pagurus* on aerial

exposure for up to 18h following a decrease after the first 2 hours (Regnault, 1992). The ammonia production during emersion in these animals was calculated to be some 15% - 30% less than anticipated due to a reduced metabolism in air (attributed to a decrease in oxygen consumption compared with immersed rates) which is implied in the literature (cited in Regnault, 1992) that metabolism in the emersed organism occurs at about 30% of the normal level. This reduction was attributed to storage of the ammonia, in this instance increased amino acid production in muscle was suggested, based on elevated GDH activities with emersion (Regnault, 1992). An apparent reduction of ammonia production (*cf* that predicted, Table 7.1) was measured in the haemolymph of emersed animals for the present investigations (Chapter 5), relating to a 13.4% and 47.3% of that predicted in juveniles and adults repectively. When re-immersion occurred, however, the subsequent excretion rate over 45 minutes was elevated (Table 5.3) *cf* normal rates (Table 5.1) and indicated that an actual ammonia production of around 87% and 53% above the haemolymph measured changes occurred for juveniles and adults respectively. These increases had occurred over the 4¾ hours of emersion and subsequent efflux measurements on re-immersion (Table 7.1). The ammonia production figures are based on efflux measurements over 45 minutes only. Possible sites of ammonia 'storage' during emersion include the gill chamber and water present has been shown to incur an increased ammonia content during emersion in *Cancer* and *Necora* (Hemingway, 1992; Hickling, 1993) and be responsible for large increases in ambient ammonia levels after re-immersion. A reduction in aerobic metabolism has been shown to occur on emersion *eg* 13.6% in *Cancer pagurus* and 20.2% in *Necora puber*, (Uglow *et al*, 1986) and 19% in *Necora puber* (Johnson & Uglow, 1985a) (all figures relative to water oxygen consumption measurements). Indeed, DeFur & McMahon (1984a) found that oxygen uptake in adult and juvenile *Cancer productus* could not be maintaned during emersion. The anaerobic production of ammonia (as found in fish (van Waarde, 1983)) by the tissues during emersion could not be ruled out since lactate increases have been measured in

juvenile *Cancer pagurus* from 30 minutes post-emersion (Table 5.4) and much earlier than suggested by Regnault (1992) at 24h, based on the LDH activities of cheliped muscle. The argument that a bound form of ammonia, as amino acids, may contribute to the storage of excess ammonia during emersion (Regnault, 1992) is not corroborated by present data relating to adult *Cancer pagurus*, since blood ammonia levels after only 45 minutes post-re immersion were lower than normal aquarium levels (Table 5.3 and 5.1), however, juvenile blood ammonia was elevated at this time (*cf* aquarium levels). The possibility that amino acids may act as ammonia storage in juvenile *Cancer* exists since they will take a longer time period to break down and the limited cell volume regulatory capacity of juvenile *Cancer* compared with adults (Wanson *et al*, 1983) may pre-dispose this stage of *Cancer pagurus* to utilisation of such a mechanism. Alternatively free amino acids may confer other advantages to juvenile *Cancer* on emersion (Regnault, 1992).

7.3. Hypoxia

The effects of hypoxia in terms of respiration and oxygen consumption on crustacean species of commercial importance have been studied by several authors (*Homarus gammarus* Spoek, 1974; *Homarus americanus* McMahon & Wilkins, 1975; *Homarus vulgaris* Butler *et al*, 1978; *Cancer pagurus* Bradford & Taylor, 1982; *Nephrops norvegicus* Hagerman & Uglow, 1985). Hypoxic conditions are imposed on Crustacea during live marketing, at a moderate level in holding ponds (50%-90%) and at much higher levels in vivier tanks (84% to 46% over 84h) (Whyman *et al*, 1985). The organisms may also be subject to slight changes in salinity or water temperature, exposed to 'bad' water quality, or are recovering from other stresses imposed (*eg* aerial exposure, handling) leading to an increased oxygen demand in the animals at a time when oxygen availability is low (see Table 3.1 Chapter 3). Additionally the animals are packed using a high animal to water ratio (1:1 for *Cancer pagurus* and 1:2 for *Necora*

puber), which will rapidly reduce water oxygen levels (64% to 14% over 24h in a single crate of *Necora* - laboratory simulations -Whyman *et al*, 1985) despite constant aeration of the water (Chapter 3).

In some crustacean species the oxygen consumption is dependant upon the external oxygen tension (P_WO_2) over the complete range (conformation) but in others the oxygen consumption is independant of the oxygen tension down to a critical (P_C) oxygen tension (regulation) (Herreid, 1980). Herreid (1980) has further defined factors which affect P_C. Those pertaining to live marketing, include an increase in P_C with temperature, temperature effects can also convert a regulating organism into a conformer; P_C depends on the activity of the animal, fettered lobsters (*Homarus gammarus* became conformers (*cf* regulators) (Spoek, 1974) and P_C depends on the amount of heamocyanin (respiratory pigment) in the blood. *Cancer* exhibits respiratory independance down to oxygen tensions of 60-80 torr (Bradford & Taylor, 1982).

Ammonia efflux studies under hypoxic conditions have been undertaken with a few crustacean species; *Nephrops norvegicus* (Hagerman *et al*, 1990; Hosie *et al*, 1991), *Penaeus semisulcatus*, *Macrobrachium malcolmsonii* (Laxminarayana & Kutty, 1982), *Cancer pagurus* and *Necora puber* (present studies). In *Nephrops norvegicus*, acclimated excretion rates were found to be linear and negative with respect to oxygen tension (P_WO_2=16-155 torr) exposed to long term hypoxia (up to 3 weeks) (Hagerman *et al*, 1990). Imposed, short term hypoxic conditions demonstrated a variation in excretion rate which was not significantly different in *Cancer pagurus* or *Necora puber* (Chapter 6), and increased (0.05 to 0.11 μmol g^{-1} h^{-1}) or decreased (0.15 to 0.09 μmol g^{-1} h^{-1}) markedly in *Nephrops norvegicus* depending on the acclimated temperature (6°C and 12°C respectively) (Hosie *et al*, 1991). The implications during live transport are that ammonia excretion of around normal levels

are likely, especially since the animals will be subject to the additional stresses of crowding in the vivier tanks and the motion of the lorry.

The production of lactate for anaerobic energy production during hypoxic conditions is well documented in Crustacea (Bridges & Brand, 1980a, b; Mauro & Malecha, 1984; Hagerman & Szaniawska, 1986; Johnson & Uglow, 1985a, b, 1987; Lallier *et al*, 1987; Taylor & Spicer, 1987; Hagerman *et al*, 1990). Lactate production was observed in *Necora puber* subjected to 25% P_wO_2 for 4h (from 5.72 to 7.05 mg $100ml^{-1}$) and these levels did not return to normoxic levels after 4h of fully aerated conditions in laboratory experiments (Whyman *et al*, 1985). Higher levels than these were found in crabs at various stages during vivier transportation for *Cancer* and *Necora* (Table 3.2 and 3.5) *cf* normal, normoxic values of 5-7 mg $100ml^{-1}$ and 4.5 mg $100ml^{-1}$ for *Cancer* and *Necora* respectively.

Values for haemolymph ammonia concentrations during hypoxia have recently been given in the literature for *Nephrops norvegicus* (Hagerman *et al*, 1990; Hosie *et al*, 1991) and for *Cancer pagurus* and *Necora puber* (Chapter 5 and 6). In *Nephrops norvegicus* a reduction in blood ammonia with respect to normoxic conditions was found in short term experiments (Hagerman *et al*, 1990; Hosie *et al*, 1991), while a general reduction in blood ammonia with time in long term experiments was found (Hagerman *et al*, 1990). Overall a reduced metabolism with hypoxia is implied. Similar results for juvenile and adult *Cancer* were found (Chapter 5), although some variation from this trend was seen, depending on conditions, in both *Cancer pagurus* and *Necora puber* (Chapter 6). Warren & Schenker (1960) found that exposing mice which had been administered ammonium chloride, to low levels (13%) of oxygen, greatly increased its toxicity. Although the LD_{50} did not change, life was prolonged with 99% oxygen. These findings probably have significant implications for the live trade of crustaceans.

7.4. Ammonia Enrichment of the Media

During the commercial practices of live transport, high levels of ammonia accumulate in the vivier tanks of consignment lorries (over 6 mmol l^{-1}; Table 3.6) due to ammonia excretion, bacterial activity and the presence of decaying food remains or dead animals [0.02 µmol l^{-1} to 0.72 µmol l^{-1} of ammonia were produced at 10°C and 14°C over 24h in a tank of seawater by one dead crab (*Cancer pagurus*) - Uglow *et al*, 1986]. Very high haemolymph ammonia levels are also found in animals during consignment (over 4 mmol l^{-1} in *Cancer pagurus* and *Necora puber* on one 48h journey - Table 3.5) due to metabolic accumulation and, possibly, uptake from the surrounding medium, as has been reported in other commercial crustacean species (*Callinectes sapidus* Cameron, 1986; *Nephrops norvegicus* Hosie *et al*, 1991; *Cancer pagurus* and *Necora puber*, Chapter 6).

Investigations of the effect of elevated medium levels of ammonia on ammonia excretion have been used to investigate mechanisms by which ammonia efflux may take place in Crustacea (Kormanik & Cameron, 1981b; Kormanik & Evans 1984; Cameron 1986; Hosie *et al*, 1991; Chapter 6) and fish (Fromm & Gillette, 1969; Maetz, 1972, 1973; Cameron & Heisler, 1983). The diffusional permeability of tissue such as the gill to NH_3 (the unionised form of ammonia) is high enough to account for a significant fraction of ammonia excretion but the diffusional permeability of NH_4^+ is also considerable (Kormanik & Cameron, 1981a). Evans & Cameron (1986) confirm that NH_3 and NH_4^+ diffusion takes place as well as basolateral (serosal) Na^+/NH_4^+ exchange in gill ammonia transport. The present studies demonstrate the permeability of the gill in *Cancer pagurus* and *Necora puber* to both NH_3 and NH_4^+ diffusion (Chapter 6). These, and observations from commercial studies demonstrate that internal levels of ammonia accumulate (or uptake occurs) under enriched conditions.

Uptake of ammonia will eventually cause toxicity. LC_{50} values determined for the lobster *Homarus americanus* (Young-Lai *et al*, 1991) equated to 12,000 μmol l^{-1} total ammonia and 191 μmol l^{-1} as the NH_3 component (calculated from 219 mg l^{-1} and 3.25 mg l^{-1} respectively). An application factor of 0.1 was used to determine 'safe' values for lobster holding conditions; this translates to 1,250 μmol l^{-1} above which levels often rise during live transport (Table 3.5, Chapter 3). Levels of total ammonia measured during live transport studies (Chapter 3) have not reached these levels found to be toxic to lobsters. The maximal NH_3 calculated component (using the Henderson-Hasselbalch equation, Chapter 6 and a pK value of 9.77 (Seager *et al*, 1988)) for the total ammonia measured during enrichment experiments carried out on *Cancer pagurus* and *Necora puber* (Chapter 6) were 0.27 μmol l^{-1} and 0.65 μmol l^{-1} respectively in the external medium. Similarly, calculated maximal haemolymph (internal) NH_3 concentrations found during these experiments were 1.92 μmol l^{-1} for *Cancer* and 4.13 μmol l^{-1} for *Necora*. None of these values reached those found to be toxic to adult *Homarus americanus* (Young-Lai *et al*, 1991). What seems to be apparent is that these organisms are tolerant to levels of ammonia (especially NH_3) much greater than that to which they are normally exposed. The question of what damage, if any, is incurred during sub-lethal exposure is raised. In *Homarus americanus* it was considered that ammonia in the external medium might affect the Na^+/NH_4^+ transport mechanism by permanently or temporarily imparing sodium transport sites. Chen & Nan (1993) considered that prolonged ammonia exposure may damage the respiratory and excretory system of the shrimp, *Penaeus chinensis*.

7.5. Live Transport

The physiological responses of organisms to individual investigations of live transport are described in Chapter 3 of this thesis and on other occasions by Whyman *et al* (1985) and Uglow *et al* (1986). The effects of conditions during the actual journey

result in elevated haemolymph levels of lactate, glucose and ammonia (Chapter 3, Table 3.1 - 3.9; Uglow *et al*, 1986) in the organisms concerned and these effects are reproduced in laboratory simulations (Whyman *et al*, 1985; Chapters 5 & 6). Accumulation of these substances is brought about by stressors, such as emersion and handling at various stages of the marketing chain. The effects of handling and return to water of organisms has been investigated. Hunter (in press) found that handling of *Crangon crangon* significantly elevated their blood ammonia levels and ammonia excretion rates. Such an effect on larger Crustacea would have serious implications for water quality in a live transport situation. This effect may be related to higher oxygen consumption, which was found after handling or activity in *Cancer pagurus* (Aldrich, 1975; Uglow *et al*, 1986). The effects of activity may be similar to the imposed stresses of the live transport journey and over-crowding in vivier tanks and may induce anaerobic metabolism (Burke, 1979; McDonald *et al*, 1979; Carlsson & Gade, 1986; Whyman *et al*, 1985). Hypoxia (see previously), under these conditions will further contribute to lactate production (Laxminarayana & Kutty, 1982; Carlsson & Gade, 1986).

Anecdotal reports from dealers involved in live transport practices, indicate that animals often travel with reduced mortalities if they are kept for a few days in holding ponds prior to transportation. Investigations of the effect of starvation and hunger on ammonia excretion indicate that recent feeding brings about an elevation in excretion (*eg* Regnault, 1981; Nelson & Kropp, 1985). Food remains from the gut contribute to ammonia loading of the water (MacMullen *et al*, 1986); this is one of the reasons that laboratory ammonia efflux measurements are made in a post-feeding phase (at least 24h after feeding). Oxygen consumption rates also increase after feeding (Ansell, 1973; Aldrich, 1975), this would contribute to a rapid deterioration in water quality in a live transport situation.

Recommendations to dealers involved in live transport include the importance of settling of the animals in water and holding of the animals for at least 1 to 2 days prior to loading for vivier trasnport. The recovery of animals after emersion is also important, to avoid further stress and also to allow any ammonia, glucose and lactate, which may have accumulated to be released or metabolised. Handling should be kept to a minimum, especially once transportation has begun. Recovery of the animals in fresh seawater after transportation is also important (>12h may be required for nomal haemolymph levels to reach those found in the aquarium, *eg* >1 mmol l^{-1} ammonia was found in *Cancer pagurus* and *Necora puber* following overnight recovery in holding ponds after vivier transport).

During the course of these studies ammonia efflux was found to be related to body mass. An ability to predict ammonia loading of a given volume of water would be advantageous, especially where filters are used in the system. With reference to live transport, filters may be designed to remove ammonia in a recirculating system, for example in a system such as the cascade method, described in Chapter 3. Turnover rates for a number of organisms are given in Table 7.2. These can be seen to be very rapid in some cases (minutes, or proportions of a minute). Since these rates can be related to body weight they can also serve as a 'tool' for determining the ammonia production of different organisms involved in live transport, or in aquaculture.

7.6. Further work

These studies have indicated where further research is warranted. One of the principal areas in this context is concerned with 'scrubbing' the dissolved ammonia from seawater. Such a system, of course, can be done in a variety of ways including the use of biofilters and ozonation systems. However, what is needed is a low-technology, light (transport - cost implications) system which is able to cope with the quantities of ammonia produced in the vivier tanks and holding ponds described in this work. The work has two fundamental targets:- a) the 'in transit' system which would aim to reduce the rate and extent of dissolved ammonia build up during vivier transport; b) coping with the intensive 'in pond' long-term storage that is currently being practiced for high value species such as lobsters.

The former system would have immediate advantages to the trade. The improved delivery of quality product to the continent, with a reduced delivery of poor quality product and with a subsequent decreased 'in pond' mortality. Secondly the delay in the build-up of potentially lethal ammonia levels would allow longer-distance transport and allow penetrations to potentially lucrative markets such as Italy or Portugal.

A further line of study relates to the development of new 'dry' systems of transporting product so that it may be air-freighted to distant markets (*eg* Japan). An extension of this, of course, would be to minimise the volume of water needed for vivier lorry transport of product to continental destinations.

As there appears to be a recognisable link between exposure time to high dissolved ammonia levels and subsequent mortalities in the days following delivery, there is clearly a need for a more detailed and systematic examination of ammonia and its toxicity. Such studies should include examination at the tissue and cellular levels - utilising material collected in 'the field' this work, logically, should investigate the secretion of ammonia from the tissues to the haemolymph and the modifying effects of high blood ammonia concentrations. Such studies are conspicuous by their absence in the literature - even those studies that pertain to fish have little to say about this topic.

Finally, there is need for more information on the organoleptic aspects of the live marketing of crustaceans. There is virtually an absence of acceptable data in the literature that pertain to any possible changes to product taste or texture that may occur during live transport at high ammonia concentrations, or during prolonged storage (perhaps without feeding) in advance of the seasonal market.

Such studies, it is hoped, will follow-on from this work.

Table 7.1. *Cancer pagurus*: Estimation of ammonia production of animals during emersion for 4 hours, based on blood ammonia measurements alone and comparison with production rates estimated from re-immersion efflux rates.

	Ammonia produced over 4 hours (Table 5.5)	Measured ammonia increase in haemolymph over 4 hours (calculated from Table 5.1)	Apparent reduction in blood ammonia production (%) based on measured amount	Difference in actual ammonia production, as a proportion of predicted changes in blood ammonia (%) *
Adult	427.6	202.3	47.3	52.7
Juvenile	2465.2	329.3	13.4	86.6

* = ((col A - col B)/ col A) x 100

Table 7.2 Replacement times of ammonia in different species of Crustacea. Source of information given in parenthesis.

Species	Weight (g)	Replacement time (min)
Crangon crangon (Hunter, 1991)	0.9	0.77-2.45
Palaemonetes varians (Hunter, 1991)	0.4	0.49-0.59
Nephrops norvegicus (Hosie *et al*, 1991)	112.19	56
Cancer pagurus (adult) (present studies)	522.0	120
Cancer pagurus (juvenile) (present studies)	111.32	52.2
Necora puber (present studies)	80.83	160

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SUMMARY

1. Descriptions of the practices in the marketing chain of live crabs from first landing to sale at dealers' premises on the continent were given. The two species involved in these studies were brown crab (*Cancer pagurus*) and the velvet swimming crab (*Necora puber*). Typical holding facilities and different types of transport methods were described for vivier consignments, in which the animals are transported alive with seawater (or high humidity situations), in refrigerated containers.

2. Investigations have been made of blood constituents and their changes in response to different stresses during actual live transport. Assessments of the extent of physical damage on crabs during live transport have also been studied. Laboratory investigations were made of the effect of some common stressors during live transport, namely that of emersion, hypoxia and high ammonia levels of the water in which the crabs are held and their subsequent effect on blood ammonia levels and excretion rates.

3. During live transport a number of stressors were identified including mechanical stress due to handling and physical damage; physiological stress due to emersion and deleterious water quality (including hypoxia and high ammonia conditions); and thermal stress during emersion and due to vivier water being at a different temperature to normal ambient temperatures for the animal and similarly when offloading at dealers' holding ponds.

4. The effects of a conventional vivier tank journey for *Cancer pagurus* (from Benbecula, western Scottish Isles to Santander, N Spain), where the animals are transported in large tanks of aerated and air chilled seawater, did not bring about any trends of change in blood total protein and haemocyanin contents, although in relative terms (*cf* total protein) a reduction of haemocyanin was detected over the full duration

of the vivier journey. Periods of emersion lead to elevated levels of blood lactate (*eg* 40.2 mg/100ml after >6h) which decreased on re-immersion (17.1 mg/100ml). Comparative journeys of vivier transported and aerially transported crabs demonstrated a lower mean lactate level for the former (63.3 mg/100ml and 20.3 mg/100ml vivier and emersed crabs respectively).

6. A vivier journey using aerated seawater tanks from S Wales (UK) to Audierne (France) sampled both *C.pagurus* and *N.puber*. Total protein levels in the blood did not change significantly during the journey for either species (*eg* 57.28 mg/ml to 49.33 mg/ml for *C.pagurus* and 41.05 mg/l to 41.57 mg ml for *N.puber*). Lactate levels were elevated for *C.pagurus* after 48 hours (from around 27.59 mg/100ml for freshly caught animals to 53.2 mg/100ml), however such an increase in lactate did not occur for *N.puber* (freshly caught animals 20.20 mg/100ml and 6.25 mg/100ml after transport) reflecting normal levels, probably due to immersion. Significant results were found in the blood ammonia concentrations, increases from around 400 µmol/l to 1485.45 µmol/l during the journey in *C.pagurus* and 189.76 µmol/l to 4565.57 µmol/l in *N.puber*. These levels were reduced to 1549.7 µmol/l and 1315.61 µmol/l overnight in holding ponds respectively.

7. A novel cascade method of holding crabs and for vivier transport was investigated. The holding method, over two weeks resulted in decreased total protein concentrations (20-25% from 41.75 to 34.24 mg/ml) and elevated lactate levels (29.3 to 58.22 mg/100ml), but little change in glucose concentrations (2.11 to 2.4 mg/100ml). Glucose levels in consigned crabs were elevated (7.99 mg/100ml) and were less, but elevated after overnight recovery in holding ponds (6.13 mg/100ml). Total protein levels were not significantly different, although lactate levels were elevated (124.81 mg/100ml) after the journey and were lessened after recovery in holding ponds (47.17 mg/100ml). Blood ammonia levels were also reduced after overnight in holding ponds (from 511.44 to 81.18 µmol/l). Changes of vivier water occurred during transport and

the highly toxic nitrite was detected in the vivier water prior to a water change - this may indicate the incidence of hypoxia during transport.

8. Analyses were made of crabs at the end of a journey using a novel mist system. Ammonia haemolymph levels were high at 1629.9 µmol/l and lactate levels were elevated at 83.96 mg/100ml. The ammonia concentration of the water used for the mist system was also relatively high at the end of the journey (3680.77 µmol/l) compared with water ammonia levels during conventional vivier journeys (from 1941 to 6107 µmol/l).

9. Physical damage affects the quality of a given consignment in terms of the continental dealers' requirements, and therefore also affects its commercial worth. Aspects of physical damage were quantified which had occurred during consignment loads from different parts of the UK to Spain and France. Increased incidences of recent damage (which causes bleeding and may lead to mortality and a reduction of water quality in the vivier) were found to occur where consignments were transferred between vivier lorries as part of the normal marketing practice. Increased damage was also found on longer vivier journeys; in this and the latter case, the evidence was based on comparisons with crabs after first landing. The time of year may have a bearing on the incidence of damage and the level of mortalities due to damage as well as the initial handling practices of catchers in the region where the vivier consignment originated. A feature of all consignments studied was the incidence of mortality (or rejected moribund animals) of intact individuals, which ranged from 29-20 % of the load to 0%. This phenomenon requires further research for explanation.

10. The effects of hypoxia on juvenile and adult *C.pagurus* was variable , but overall with >4 hours hypoxia (P_wO_2=16 torr) resulted in a decrease in blood ammonia (174 to 97 µmol/l and 71 to 50 µmol/l in adults and juveniles respectively). Blood acidosis also occurred with this time period of hypoxia.

11. Blood ammonia increased with time of emersion (15°C, RH80%) in *C.pagurus*. from 174 µmol/l in aquarium animals to 644 µmol/l after 8 hours in adults and from 71 µmol/l to 2185 µmol/l after 12 hours in juveniles. Acidosis also occurs with emersion and the relative proportion of ammonia in the free form (NH_3) decreases.

12. Weight-specific ammonia excretion (or efflux) rates were significantly higher in juvenile *C.pagurus* than in adults (P=0.000). The excretion rates within each group (0.034 µmol/g/h for adults and 0.181 µmol/g/h for juveniles at 15°C) were reduced with hypoxia (0.029 µmol/g/h at P_wO_2=16 torr and 0.128 µmol/gh at P_wO_2=39 torr, in adults and juveniles respectively), but the results were not significantly different. Efflux rates are size dependant, demonstrated by a linear relation between efflux rates and weight where $\log y = 0.176 - 1.001 \log x$ ($r = -0.77$).

13. Blood ammonia levels of 283.8 µmol/l and 92.1 µmol/l and efflux rates (1.17 µmol/g/h and 0.2 µmol/g/h were elevated after 4 hours emersion (15°C, RH80%) compared with aquarium levels for juvenile and adult *C.pagurus* respectively. The higher levels and efflux rates in the juvenile animals resulted in significant differences from the adult data (P=0.046). weight-specific blood ammonia levels were calculated after 4 hours emersion to be 0.028 µmol/g and 0.085 µmol/g for adult and juvenile crabs respectively.

14. *In situ* investigations carried out with juvenile *C.pagurus* on the shore demonstrated much higher resting blood ammonia levels and ammonia efflux rates than animals acclimatised to aquarium conditions (1582.5 µmol/l and 0.46 µmol/g/h respectively). The calculated weight-specific blood ammonia of these animals was 0.4 µmol/g. An emersion period of 30 minutes resulted in blood ammonia levels of 3284.67 µmol/l, although the measurements were variable, a mean weight-specific blood ammonia of 0.82 µmol/g was calculated; haemolymph ammonia levels returned to 1590.03 µmol/l after re-immersion and a weight-specific blood ammonia of 0.4

µmol/g. Ammonia efflux rates of 3.56 µmol/g/h were measured after 30 minutes emersion. Lactate levels of these animals increased from 30 to 117.1 mg/100ml during 30 minutes emersion.

15. The effect of normal, very low levels of ammonia and enriched ammonia concentrations of 200, 400 and 1200 µmol/l under both normoxia - P_wO_2=155 torr and hypoxia - P_wO_2=24 torr had no significant statistical effect (P>0.05) on ammonia efflux rate in *C.pagurus* and *N.puber*. The efflux rates were very variable, including individuals with no net efflux or even apparent uptake over the time course of the experiment.

16. The blood ammonia levels of *C.pagurus* and *N.puber* were very variable under normal, very low levels, and enriched ammonia concentrations at 200, 400 and 1200 µmol/l under normoxia - P_wO_2=155 torr and hypoxia - P_wO_2=24 torr, however, statistical differences (P<0.05) were found in the normoxic groups due to higher blood ammonia levels in the individuals of *C.pagurus* at 1200 µmol/l enrichment, similarly a statistical difference (P<0.05) was found in the hypoxic groups for *N.puber* due to effects of hypoxia alone (P_wO_2=24) and hypoxia with 400 µmol/l enrichment. Calculated blood ammonia content values and weight-specific blood ammonia content data showed significance for the same comparisons as blood ammonia data alone for *C.pagurus* and *N.puber* respectively.

17. Mean weight normalised data for efflux rates and weight-specific blood ammonia contents in the two species were used to produce estimates of the time required for a complete replacement of blood ammonia (assuming a steady production and efflux rate). Under normal, normoxic, P_wO_2=155, conditions the values for *C.pagurus* at 120 minutes and *N.puber* at 160 were some of the longest replacement times calculated. Values due to the different treatments depended on the experimental conditions and were variable.

18. Calculations of the fluxes of the two different forms of ammonia (NH_3 and NH_4^+) across the gills were made in order to determine the form in which ammonia efflux occurs by diffusion across the gill surface in these crabs. The situation in normoxic conditions was used for comparison with calculated, predicted changes in NH_3 or NH_4^+ flux due to the conditions imposed. For both species the better estimate was that based on the partial pressure of NH_3 (or pNH_3 gradient), except in enriched conditions of 200 μmol/l for *C.pagurus* and in *N.puber* at an enrichment of 1200 μmol/l under both normoxic and hypoxic conditions.

19. Ratios of NH_3 and NH_4^+ were calculated between the haemolymph and external medium and demonstrated the high blood:medium gradients of ammonia present under normal conditions. The ratios of $[NH_4^+]$ were <1:1 for both species in nearly all conditions of enrichment, indicating a possible uptake of ammonia due to a downhill gradient of NH_4^+ from medium to blood. Under hypoxia the pNH_3 gradient was negatively related to enrichment in both species of crab, with significant differences in the data due to differences between the non-enriched and most highly enriched groups ($P<0.05$). The pNH_3 gradient under situations where the ratio of $[NH_4^+]$ were <1:1 indicates that ammonia efflux may occur downhill along the pNH_3 gradient alone. Under normoxic conditions the pNH_3 gradient data were highly variable, however significant differences ($P<0.05$) were found in both species due to a single experiment where high efflux along the pNH_3 gradient under enrichment occurred, indicating that this mode of excretion can be used under such conditions.

20. Under all experimental conditions a blood alkalosis with respect to the medium was maintained. Under normoxic and ammonia enriched conditions the blood pH was variable in both crab species, but was very close to non-enriched conditions at the most extreme enrichment conditions - 1200 μmol/l. Hypoxia initially caused a blood alkalosis and a subsequent increased acidosis effect was observed with increased enrichment, the highest enrichment level (1200 μmol/l) with hypoxia (P_WO_2=24)

caused blood acidosis with respect to the normoxic, normal situation in both *C.pagurus* and *N.puber*.

21. Measurements of urea in the haemolymph and medium during experiments of hypoxia and ammonia enrichment were highly variable. No trends of accumulation in the blood of *C.pagurus* or *N.puber* were found with enriched conditions.

22. In the light of observations in the field, laboratory investigations and the results of water and haemolymph analyses undertaken during commercial transportation of *C.pagurus* and *N.puber*, recommendations have been made to reduce the stress effects at points along the marketing chain of live crabs.

APPENDIX 1 Damage data pertaining to Chapter 4.

Appendix 1.1. *Cancer pagurus*. Damage data – freshly-landed crab.

	CATCH DATA - various trips				
ANIMAL SOURCE	Islay - Port Ellen	Kyle -from boat	Wales - Hobb Pt	Wales - FG1	Wales - FG2
Species	Cancer %	Cancer %	Cancer %	Cancer %	Cancer %
DAMAGE CATEGORY	October	May	January	January	January
Dead/moribund	0	0	0	2	0
Intact alive	27	61	83	60	80
Intact dead	0	0	0	2	0
Hen	14	68	56	48	80
Cock	86	32	44	52	20
DAMAGE RECENT					
Damage only	4	1	0		
1 leg	43	11	0	4	4
2 legs	14	0	0	0	0
3 legs	0	0	0	0	0
4 legs	0	0	0	0	0
Cripple x2	4	0	0	4	0
Loss only					
1 leg	29	9	6	10	0
2 legs	25	3	0	0	0
3 legs	0	0	0	0	0
4 legs	0	1	0	0	0
Cripple x2	0	4	0	0	0
DAMAGE OLD					
Damage only					
1 leg	4	0	0	14	0
2 legs	14	0	0	0	0
3 legs	0	0	0	0	0
4 legs	0	0	0	0	0
Cripple x2	11	0	0	0	0
Loss only					
1 leg	4	5	11	14	12
2 legs	0	0	0	0	0
3 legs	0	0	0	6	0
4 legs	0	0	0	0	0
Cripple x2	0	8	0	6	0
OLD & RECENT					
limbs	0	3	0	2	0
cripples	0	0	0	0	0
OTHERS					
soft	32	0	0	0	0
black spot	32	0	0	4	4
berried female	4	0	6	4	0
small	0	5	0	0	0
light	29	9	0	0	0
large	0	3	0	0	0
regenerated legs	7	9	0	4	0
nicking damage	0	0	0	0	0
Number in sample 'n'	28	79	18	50	25

Appendix 1.2. *Cancer pagurus* . Damage data at stages during vivier trip from Scotland to Santander.

ANIMAL SOURCE	Vivier - Scotland to Spain					
Species	HH - from Scotld		Vivier to Madrid		Falmouth/Ch Isles to Madrid	
	Cancer %		Cancer %		Cancer %	
DAMAGE CATEGORY						
	ALIVE	**DEAD**	**ALIVE**	**DEAD**	**ALIVE**	
Dead/moribund	0	4	0	15	0	
Intact alive	34	0	13	0	42	
Intact dead	0	2	0	2	0	
Hen	83	3	75	11	72	
Cock	13	1	10	4	28	
DAMAGE RECENT						
Damage only	1	0.5	0.5	0.5	1	
1 leg	1	2	13	5	5	
2 legs	4	0.5	2	0.5	1	
3 legs	1	0	0.5	0.5	0	
4 legs	1	0	0	0.5	0	
Cripple x2	1	0	0.5	0	0	
Loss only						
1 leg	12	1	3	3	0	
2 legs	7	1	1	1	0	
3 legs	2	0.5	0	1	0	
4 legs	1	0	0.5	0.5	0	
Cripple x2	3 +	0.5	0	0.5	0	
DAMAGE OLD						
Damage only					0	
1 leg	4	0.5	13	1	32	
2 legs	0.5	0	17	0.5	3	
3 legs	0	0	0.5	0.5	0	
4 legs	0	0	0	0	0	
Cripple x2	0.5	0	1	0.5	2	
Loss only						
1 leg	13	0.5	20	3	21	
2 legs	5	0	8	2	5	
3 legs	1	0.5	3	0.5	0	
4 legs	0.5	0	2	0.5	1	
Cripple x2	1	1	5 +	0.5	2	
OLD & RECENT	4	0.5	2	2		
limbs	4	0	4	0	3	
cripples	0.5	0	0.5	5	0	
OTHERS						
soft	0	2	9	1	1	
black spot	2	2	0.5	0	1	
berried female	0.5	0.5	0	0	0	
small	34	0	23	3	0	
light	0.5	0	0	0.5	0	
large	8	0	8	2	17	
regenerated legs	6	0.5	2	1	8	
nicking damage	0		0	0.5	0	
Number in sample 'n'		618		574	98	

Appendix 1.3. *Cancer pagurus* . Damage data collected in Santander and Madrid. Animals mostly sourced from Scotland.

ANIMAL SOURCE	Spanish Data - vivier Jouneys Santander 1 from Scotland		Santander 2 from Scotland		Santander 3 from Scotland	
Species	Cancer %		Cancer %		Cancer %	
DAMAGE CATEGORY	**ALIVE**	**DEAD**	**ALIVE**	**DEAD**	**ALIVE**	**DEAD**
Dead/moribund	0	29	0	19	0	9
Intact alive	44	0	41	0	54	0
Intact dead	0	9	0	4	0	4
Hen	71	26	78	18	81	7
Cock	1	1	4	1	9	1
DAMAGE RECENT						
Damage only			0.5			
1 leg	2	2	6	1	3	1
2 legs	1	0.5	1	0.5	1	0
3 legs	0.5	0	0	0.5	0.5	0.5
4 legs	0.5	0	0	0.5	0	0.5
Cripple x2	0.5	0.5	1	0.5	0	0.5
Loss only						
1 leg	6	0.5	12	2	10	1
2 legs	2	4	5	4	3	1
3 legs	0	1	1	1	1	0.5
4 legs	0.5	1	0	1	0.5	0.5
Cripple x2	6	0.5 +	51 +	1	4 +	1
DAMAGE OLD						
Damage only					0.5	0
1 leg	0.5	0.5	1	0	1	0
2 legs	0.5	0	0.5	0	1	0
3 legs	0	0	0	0	0	0
4 legs	0	0	0	0	0	0
Cripple x2	0	0	0	0	0.5	0
Loss only						
1 leg	6	2	7	0.5	10	1
2 legs	2	1	3	0.5	3	2
3 legs	1	0.5	1	0.5	1	0
4 legs	0	0.5	0.5	0	0.5	0
Cripple x2	1	1	1	0.5	2 +	1
OLD & RECENT	1	1	2			
limbs	1	0.5	3	0	1	0.5
cripples	0	0	0	0	3	0.5
OTHERS						
soft	0	0	0	0	0	0
black spot	0	0	0	0	0	0
berried female	0	0	0	0	0	0
small	0	0	0	0	0	0
light	0	0	0.5	2	0	0
large	0	0	0	0	0	0
regenerated legs	0	0	0	0	0	0
nicking damage	0	0	0	0	0	0
Number in sample 'n'	492		511		640	

Appendix 1.3 continued. *Cancer pagurus* . Damage data collected in Santander and Madrid. Animals mostly sourced from Scotland.

ANIMAL SOURCE Species	Santander 4 from Scotland Cancer %		Santander 5 from Scotland Cancer %		Madrid-from S'der Cancer %	French Load Cancer %
DAMAGE CATEGORY						
	ALIVE	DEAD	ALIVE	DEAD	ALIVE	ALIVE
Dead/moribund	0	14	0	11	0	0
Intact alive	64	0	50	0	3	35
Intact dead	0	4	0	3	0	0
Hen	78	12	80	9	63	81
Cock	7	2	10	1	3	19
DAMAGE RECENT						
Damage only	0.5	1	0.5	1	3	
1 leg	4	17	6	0.5	9	16
2 legs	1	7	2	0.5	6	0
3 legs	1	2	1	0	0	0
4 legs	0	1	0	0	0	0
Cripple x2	1	4 +	1	0.5	0	0
Loss only						
1 leg	1	48	14	3	18	13
2 legs	0.5	2	5	1	9	0
3 legs	0.5	0.5	2	0.5	3	0
4 legs	0	0.5	0.5	0.5	0	0
Cripple x2	0	0.5 +	2	1 +	0	3
DAMAGE OLD						
Damage only						
1 leg	1	7	1	0	3	0
2 legs	0.5	1	0	0	0	3
3 legs	0	0.5	0	0	0	0
4 legs	0	0	0	0	0	0
Cripple x2	0	0.5	0	0	3	0
Loss only						
1 leg	0	1	3	1	18	26
2 legs	0	0	1	0.5	0	10
3 legs	0	0.5	1	0	0	0
4 legs	0	0	0.5	0	0	0
Cripple x2	0	0	2	0.5	3	0
OLD & RECENT						3
limbs	1	0.5	0.5	0.5	0	0
cripples	2	1	0.5	0.5	3	0
OTHERS						
soft	0	0	0	0	0	0
black spot	0	0	0	0	0	0
berried female	0	0	0	0	0	0
small	0	0	0	0	0	0
light	0	0	0	0	0	0
large	0	0	0	0	0	0
regenerated legs	0	0	0	0	0	0
nicking damage	0	0	0	0	0	0
Number in sample 'n'	414		428		30	31

Appendix 1.4. *Cancer pagurus* . Damage data collected from animals held in "cascade" system and during novel cascade vivier trip.

ANIMAL SOURCE Species	Kyle to Roscoff - Cascade Journey Catch data Cancer %	Reject from cascade holding		Roscoff 'DOA'	
DAMAGE CATEGORY	(As previous)				
	ALIVE	**ALIVE**	**DEAD**	**ALIVE**	**DEAD**
Dead/moribund	0	0	95	10	90
Intact alive	61	5	0	3	0
Intact dead	0	0	2	0	13
Hen	68	5	56	0	60
Cock	32	0	40	10	30
DAMAGE RECENT					
Damage only	1	0	10		
1 leg	11	0	13	3	3
2 legs	0	0	2	0	0
3 legs	0	0	0	0	0
4 legs	0	0	0	0	0
Cripple x2	0	0	22	0	0
Loss only					
1 leg	9	0	20	3	10
2 legs	3	0	5	0	0
3 legs	0	0	0	0	0
4 legs	1	0	0	0	0
Cripple x2	4	0	24	0	0
DAMAGE OLD					
Damage only			0	0	
1 leg	0	0	0	0	3
2 legs	0	0	0	0	3
3 legs	0	0	0	0	0
4 legs	0	0	0	0	0
Cripple x2	0	0	0	0	0
Loss only					
1 leg	5	0	15	3	13
2 legs	0	0	7	0	17
3 legs	0	0	0	0	7
4 legs	0	0	2	0	7
Cripple x2	8	0	2	0	0
OLD & RECENT					7
limbs	3	0	12	0	0
cripples	0	0	5	0	0
OTHERS					
soft	0	0	0	0	7
black spot	0	0	0	0	3
berried female	0	0	0	0	3
small	5	0	0	0	3
light	9	0	0	0	7
large	3	0	0	0	17
regenerated legs	9	0	0	0	0
nicking damage	0	0	10	0	17
Number in sample 'n'	79	41		30	

Appendix 1.5. *Cancer pagurus and Necora puber* . Damage data collected from animals during a vivier journey from South Wales to Audierne.

	S Wales to Audierne vivier trip - data on arrival			
ANIMAL SOURCE	Audierne viv	Audierne o/n in hp	Audierne viv	Audierne o/n in hp
Species	Necora %	Necora %	Cancer %	Cancer %
DAMAGE CATEGORY	**ALIVE**	**ALIVE**	**ALIVE**	**ALIVE**
Dead/moribund	0	0	0	0
Intact alive	20	20	40	40
Intact dead	0	0	0	0
Hen	20	20	50	50
Cock	80	80	50	50
DAMAGE RECENT				
Damage only				
1 leg	30	30	10	0
2 legs	20	20	0	10
3 legs	0	10	0	0
4 legs	0	0	0	0
Cripple x2	10	0	0	0
Loss only				
1 leg	40	30	30	30
2 legs	10	10	0	0
3 legs	10	10	10	10
4 legs	0	0	0	0
Cripple x2	10	10	0	0
DAMAGE OLD				
Damage only				
1 leg	0	0	0	0
2 legs	0	0	0	0
3 legs	0	0	0	0
4 legs	0	0	0	0
Cripple x2	0	0	0	0
Loss only				
1 leg	0	0	0	0
2 legs	0	0	0	0
3 legs	0	0	0	0
4 legs	0	0	0	0
Cripple x2	0	0	0	0
OLD & RECENT				
limbs	0	0	0	0
cripples	0	0	0	0
OTHERS				
soft	0	0	0	20
black spot	0	0	10	0
berried female	10	10	0	30
small	10	10	30	0
light	0	0	0	30
large	30	20	30	0
regenerated legs	10	0	0	0
nicking damage				
Number in sample 'n'	10	10	10	10

APPENDIX 2 Statistical analyses undertaken for data presented in Chapter 5.

Appendix 2.1 Comparison (one-way ANOVA) of data from *Cancer pagurus*.under experimental stresses sustained during live transport.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Adult		
Blood ammonia (Aquaria, emersed and hypoxia)	P=0.0000	9 3 4 2 10 8 7
Blood pH (Aquaria, emersed and hypoxia)	P=0.0000	7 8 3 2 4 10 9
Juvenile		
Blood ammonia (Aquaria, emersed and hypoxia)	P=0.0000	6 1 12 5 13 11 14 15
Blood pH (Aquaria, emersed and hypoxia)	P=0.0000	15 6 14 13 5 12 11 1

Key:

Adult		Juvenile	
Experimental group	Group number	Experimental group	Group number
Normal aquarium	2	Normal aquarium	1
Long term hypoxia	3	Short-term hypoxia	5
Short term hypoxia	4	Long-term hypoxia	6
8 hours emersion	7	4 hours emersion	11
4 hours emersion	8	0.5 hours emersion	12
1 hour emersion	9	1 hour emersion	13
0.5 hours emersion	10	8 hours emersion	14
-	-	12 hours emersion	15

Appendix 2.2 Comparison (one-way ANOVA) of efflux rate data taken at different temperatures and hypoxia levels.

Variable	P-value	Significance
Efflux rate (All adult groups - Table 5.2)	P=0.5608	NS
Efflux rate (All juvenile groups -Table 5.2)	P=0.9613	NS
Efflux rate - comparison of whole efflux rate data set	P=0.0000	Adult data significantly different from juvenile data

Appendix 2.3. T-test of data for measurements taken from adult and juvenile *Cancer pagurus* after 4 hours emersion

Variable	P-value
Blood ammonia	P=0.046
Efflux rate	P=0.041

Appendix 2.4. Comparison (one-way ANOVA) of data collected from shore-based experiments using juvenile *Cancer pagurus* (n=6 in all cases)

Variable	P-value	Significance
Blood ammonia level	P=0.5255	NS
Lactate	P=0.0000	31 32 30 33

Key:

Experimental group	Group Number
0.5 hours emersion and bled	30
Immersed and bled	31
Immersed normal efflux rate measurement and bled	32
0.5 hours emersion, efflux rate measurement and bled	33

APPENDIX 3 Statistical analyses undertaken for data presented in Chapter 6.

Appendix 3.1 *Cancer pagurus*. Experimental groups used in statistical comparisons.

Experimental conditions	Group
Normoxia (P_wO_2=155 torr)	1
Enrichment (200μmol l^{-1})	3
Enrichment (400μmol l^{-1})	5
Enrichment (1200μmol l^{-1})	6
Hypoxia (P_wO_2=24 torr)	7
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	8
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	9
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	10

Appendix 3.2 *Cancer pagurus*. Comparison of all experimental groups.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.4970	NS
Blood ammonia	P=0.0000	9 1 7 5 10 3 6 8
Blood ammonia content	P=0.2250	NS
Weight-specific blood ammonia content	P=0.0072	9 1 7 5 10 3 6 8
Blood pH	P=0.3001	NS
Water pH	P=0.0000	10 9 7 8 6 5 3 1
Turnover	P=0.4320	NS
pNH_3	P=0.0000	5 1 10 9 3 8 7 6
Blood urea	P=0.4702	NS
Water urea	P=0.0000	7 8 3 1 5 6

Appendix 3.3. *Cancer pagurus*. One-way ANOVA. Comparison of experimental groups under normoxia only.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.3783	NS
Blood ammonia	P=0.0045	1 5 3 6
Blood ammonia content	P=0.2597	NS
Weight-specific blood ammonia content	P=0.0045	1 5 3 6
Blood pH	P=0.6096	NS
Water pH	P=0.2237	NS
Turnover	-	NS
pNH_3	P=0.0000	5 1 3 6
Blood urea	P=0.5354	NS
Water urea	P=0.0001	3 1 5 6

Appendix 3.4 *Cancer pagurus*. One-way ANOVA. Comparison of experimental groups under hypoxia only.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.9654	NS
Blood ammonia	P=0.0530	NS
Blood ammonia content	P=0.1796	NS
Weight-specific blood ammonia content	P=0.0530	NS
Blood pH	P=0.1803	NS
Water pH	P=0.0009	10 9 7 8
Turnover	P=0.4764	NS
pNH_3	P=0.0153	10 9 8 7
Blood urea	P=0.0000	8 7 10 9
Water urea	-	-

Appendix 3.5 *Necora puber*. Experimental groups used in statistical comparisons.

Experimental conditions	Group
Normoxia (P_wO_2=155 torr)	1
Enrichment (200μmol l^{-1})	2
Enrichment (400μmol l^{-1})	3
Enrichment (1200μmol l^{-1})	4
Hypoxia (P_wO_2=24 torr)	5
Enrichment+hypoxia (200μmol l^{-1} ,P_wO_2=24 torr)	6
Enrichment+hypoxia (400μmol l^{-1},P_wO_2=24 torr)	7
Enrichment+hypoxia (1200μmol l^{-1},P_wO_2=24 torr)	8

Appendix 3.6 *Necora puber*. Results of one-way ANOVA statistical tests. Comparison of all experimental groups.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.5406	NS
Blood ammonia	P=0.0131	7 3 6 8 1 2 4 5
Blood ammonia content	P=0.0239	7 3 6 8 1 2 4 5
Weight-specific blood ammonia content	P=0.0131	7 3 6 8 1 2 4 5
Blood pH	P=0.0072	2 8 1 4 3 7 5 6
Water pH	P=0.0126	7 6 8 5 3 4 2 1
Turnover	P=0.2039	NS
pNH_3	P=0.0161	8 3 7 4 1 2 6 5
Blood urea	P=0.0000	2 8 1 4 3 7 5 6
Water urea	-	-

Appendix 3.7 *Necora puber*. Results of one-way ANOVA statistical tests. Comparison of experimental groups under normoxia only.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.3496	NS
Blood ammonia	P=0.1023	NS
Blood ammonia content	P=0.1502	NS
Weight-specific blood ammonia content	P=0.1023	NS
Blood pH	P=0.3256	NS
Water pH	P=0.2231	NS
Turnover	P=0.1561	NS
pNH_3	P=0.8415	NS
Blood urea	P=0.0013	2 1 4 3
Water urea	-	-

Appendix 3.8 *Necora puber*. Results of one-way ANOVA statistical tests. Comparison of experimental groups under hypoxia only.

Variable	P-value	Significant groups (Tukey - multiple comparison)
Efflux rate	P=0.4517	NS
Blood ammonia	P=0.0185	7 6 8 5
Blood ammonia content	P=0.0200	7 6 8 5
Weight-specific blood ammonia content	P=0.0185	7 6 8 5
Blood pH	P=0.0868	NS
Water pH	P=0.2310	NS
Turnover	P=0.7101	NS
pNH_3	P=0.0050	8 7 6 5
Blood urea	P=0.0000	8 7 5 6
Water urea	-	-

APPENDIX 4 Other publications.

Reports and papers published by the author during the period of studies for this thesis.

Marine Biology 110, 273–279 (1991)

Marine Biology

Some effects of hypoxia and medium ammonia enrichment on efflux rates and circulating levels of ammonia in *Nephrops norvegicus*

D.A. Hosie [1], R.F. Uglow [1], L. Hagerman [2], T. Sondergaard [2] and K. Weile [2]

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Abstract. Eutrophication has been reported for autumn months in regions of the Kattegat/Skagerrak, causing stress to bottom-living organisms. The present studies, undertaken in April (1989), investigated the effects of hypoxia and high ammonia levels in the burrowing decapod *Nephrops norvegicus* (L.). The net ammonia efflux rates and circulating ammonia levels at 6 and 12 °C, at normoxia [partial pressure of O_2 in the water (torr), $P_wO_2 = 155$ torr)] and hypoxia $P_wO_2 = 24$ torr) in normal seawater and ammonia-enriched (300 µmol ammonia l^{-1}) seawater were examined. The hourly weight-specific efflux rates were very variable and in all groups included some individuals which showed periods of no net efflux, or even a net uptake of ammonia. At each temperature, net efflux-rate differences due to treatments were not significant ($P > 0.05$; ANOVA, in all cases) and only the differences between the net efflux rates of the normoxic groups were significantly affected by temperature ($P < 0.05$; ANOVA). Circulating ammonia levels were also variable, and at 6 °C the ammonia-enriched groups had significantly higher weight-specific blood ammonia content values than the normoxic group ($P < 0.05$ in both cases). A net uptake of ammonia occurred in ammonia-enriched conditions – probably along a reversed NH_4^+ gradient, as downhill pNH_3 gradients were maintained in all groups – and may represent the only means by which some branchial efflux of ammonia could proceed.

Introduction

Ammonia is the principal end-product of protein metabolism in decapod crustaceans. It is transferred from the tissues to the haemolymph, from where it is excreted across the surface of the gills (>95%) and in the urine (up to 2%) to the (usually) more dilute external medium. Efflux rates were found to be directly related to temperature in *Carcinus maenas* (Needham 1957), in some penaeid species (Spaargaren et al. 1982) and in *Pandalus platyceros* (Quarmby 1985). Spaargaren (1982) found that the 6 d acclimated blood-ammonia levels of *C. maenas* were strongly regulated by means of a variable flux of NH_4^+ and, in high external ammonia conditions, high [NH_4^+] efflux was supplemented by urea formation. Regnault (1986) found that ammonia excretion of *Crangon crangon* was not influenced by external ammonia concentrations up to 87 µmol l^{-1}.

The proportion of ammonia excreted via the urinary bladders is very small: 1 to 2% of the total amount produced in *Callinectes sapidus* (Cameron and Batterton 1978), <2% in *Jasus edwardsii* (Binns and Petersen 1969), and negligible in *Carcinus maenas* (Harris and Andrews 1985). Because of its relatively small contribution to the total amonia efflux (cf. branchial excretion) a special study of urinary ammonia was not included in the present study.

Nephrops norvegicus (L.) is a burrowing decapod which forms the basis of many commercially valuable fisheries in European waters. It is also a species which is being considered for regular, bulk, live consignments by road from Scotland and Scandinavia to markets principally in France, Spain and Italy. Such "vivier" (tank) transport-systems utilise high biomass-to-water ratios (1 : 1), and journeys may take 2 to 3 d. Very high (1000 to 2000 µmol l^{-1}) external ammonia concentrations can develop, along with moderate to severe hypoxia during this time (MacMullen et al. 1986).

In some areas of its natural environment in the Kattegat/Skagerrak region, *Nephrops norvegicus* may be subjected to prolonged periods (weeks) of strong hypoxia resulting from agricultural runoff-induced eutrophication and water stratification (Hagerman and Pihl-Baden 1988). Furthermore, under normoxic conditions, sediment pore-water ammonia concentrations of 200 to 300 µmol l^{-1} have been noted (Hopkinson 1987). Under hypoxia, an ammonia efflux from the sediment of 100 µmol m^2 h^{-1} was found by Kristensen (1984). Combined with the effluxes of ammonia by *N. norvegicus* itself, it is likely that there are periods when very high concentrations of ammonia may occur in the burrows of this species. Abnormally high ammonia concentrations

and hypoxia may also occur during the live marketing of *N. norvegicus*. The present study is designed to provide more information on the effects of such ecological and commercial-marketing conditions on ammonia effluxes in *N. norvegicus*.

Materials and methods

Specimens of *Nephrops norvegicus* (L.) were creel-caught in the northern Kattegat during April (1989). The decapods (48.6 to 244.8 g fresh weight) were maintained at the Helsingør (Denmark) laboratory in running, aerated seawater (10°C; 30 to 32‰ S) for at least 7 d before being used in experiments. Plastic cylinders were placed in the stock tanks to act as substitute burrows for the decapods. The aquaria were kept under low light intensities and the decapods reacted clearly to visual stimuli in the laboratory.

Using groups of 8 individuals, net ammonia flux was measured at two temperatures (6 and 12°C), at two oxygen concentration levels (normoxia, partial pressure of oxygen in water, $P_wO_2 = 100\%$ 155 torr; and $P_wO_2 = 15\%$ 24 torr) and at two ammonia concentrations (normal seawater, <5 μmol l^{-1}) and seawater enriched by adding ammonium chloride solution to normal seawater to bring it to a concentration of 300 μmol ammonia l^{-1}, this being a common "vivier tank" ammonia concentration. The decapods were allowed to acclimate for at least 36 h to the intended experimental temperature before being left to settle overnight in individual plastic aquaria with 5 litres of normal, static seawater and aeration. A complete change of water was made the following morning and the decapods were left for 2 h to dissipate any excess haemolymph ammonia that might have built up overnight. The water was then carefully siphoned from each aquarium and replaced with 5 litres of fresh, normal or enriched seawater of the appropriate temperature. If necessary, oxygen levels were lowered at this time by purging with nitrogen gas until the required P_wO_2 level was obtained; water P_wO_2 levels were subsequently monitored using a Yellow Springs-53 portable oxygen meter. During normoxia experiments, the individual aquaria were aerated. The decapods were left to settle for a further 2 h before the first water sample (~1 ml) was taken at time $t = 0 (t_0)$. Further water samples were taken every hour for four consecutive hours (i.e., a 3 h excretion period, $t_0 - t_3$). In the hypoxia experiments, P_wO_2 values were checked regularly.

A haemolymph sample was taken from each individual 30 min after the last water sample had been taken. Haemolymph samples (max. 70 μl) were obtained from the base of the fourth or fifth peraeopod by inserting a hypodermic needle ventrally through the arthrodial membrane. This method was considered less stressful than previously used methods (Hagerman and Uglow 1985). The pH of the haemolymph was measured immediately after taking the sample, and the sample was diluted in saline (9 g l^{-1} NaCl) for measurement of blood ammonia.

Ammonia concentrations were measured using a modified flow-injection/gas-diffusion technique (Clinch et al. 1988). This consists briefly of a carrier stream of 0.01 *M* NaOH separated by a polytetrafluoroethylene (PTFE) gas-permeable membrane from a 0.5 g l^{-1} concentration of bromothymol blue solution. The ammonia released by the strong base diffuses across the PTFE membrane and alters the pH and colour of the bromothymol blue which is detected colorimetrically. This technique, originally designed for ammonia measurement in freshwater is very sensitive. In seawater, sensitivity is more variable due to precipitation of salts in the carrier stream which eventually affect the membrane permeability. During these experiments the limit of detection was 0.5 to 1.0 μmol l^{-1} for both seawater and blood analyses using 0.25 ml or 0.5 ml sample injection volumes. For each experiment, the actual sample volume was kept constant. All solutions were prepared using fresh, double-distilled water. Blood samples (0.05 ml, diluted 1:20 in saline, 9 g l^{-1} NaCl) or water samples were injected directly into the NaOH carrier stream.

The outputs from the detector were displayed on a potentiometric recorder (Phillips PM 8252A) and were measured in relation to peak heights of ammonium sulphate standards. A series of standards in the ranges 10 to 50 and 300 to 400 μmol ammonia l^{-1} were prepared for the normal and ammonia-enriched experiments, respectively. Loss of sensitivity was checked by injecting a 40 or 340 μmol l^{-1} standard, respectively, at regular intervals. The standard solutions were made up with the stock experimental water as used at the start of the experiment i.e., fresh seawater, enriched seawater or, for the haemolymph samples, saline (9 g l^{-1} NaCl).

pH measurements of seawater and haemolymph were made using a BMS 3MK2 suction sampler and Radiometer PHM73 pH meter equipped with a G299A pH probe.

The total ammonia present comprised free ammonia (NH_3) and the ionised form (NH_4^+) in ratios that depend on the pH and the pK of the medium. As described by the Henderson-Hasselbalch equation,

$$pH = pK + \log([NH_3]/[NH_4^+]) . \quad (1)$$

The $[NH_3]$ can be calculated as follows:

$$[NH_3] = \frac{[NH_3] + [NH_4^+]}{1 + 10^{(pK - pH)}} \quad \text{(Armstrong et al. 1978)} . \quad (2)$$

The pK values were taken from values given for seawater by Bower and Bidwell (1978) and the blood osmotic concentrations were taken to be similar to that of seawater. From the calculated value of $[NH_3]$, the appropriate value of pNH_3 was obtained:

$$pNH_3 = [NH_3] \times (22.09/\alpha) , \quad (3)$$

where α = Bunsen solubility coefficient = 1.46, given by Washburn (1928; cited in Kormanik and Cameron 1981 a).

Where appropriate, data on efflux rates were subjected to analysis of variance using SPSS [Subprogram(s) of the Statistical Package for the Social Sciences] on a PC (Norušis 1986, manual, p. A1–H11).

Results

The weight-specific net ammonia-efflux rates of groups of animals *Nephrops norvegicus* ($n = 8$) in normal, normoxic media (N), normal, hypoxic media (H), ammonia-enriched, normoxic (EN) and ammonia-enriched, hypoxic (EH) media are given in Table 1. These data show the rates to be very variable, and each group included some individuals with no apparent net efflux, or even an apparent net uptake, over one or more hours (Table 2).

Considering each temperature separately, the individual variability of the efflux rate was sufficient to mask any significant differences between the mean values of all the experimental groups ($P > 0.05$; ANOVA). Comparison of net efflux rates of similarly-treated groups revealed a significant difference due to temperature only in the case of the normoxic groups ($P < 0.05$; ANOVA). The comparisons of the remaining groups (H, EN and EH) at the two temperatures were not significant.

The mean values of blood ammonia concentrations at the end of the experiments are also included in Table 1. Again, individual variability was large in all groups. Comparing N with H, N with EN and EN with EH groups at 6°C, only the N with EN comparison was significant ($P > 0.05$). At 12°C only the EN with EH group comparison was significant ($P < 0.01$). Respiratory alkalosis resulted after 6 h of hypoxia, and enrichment was also accompanied by a rise in blood pH. There ap-

Table 1. *Nephrops norvegicus*. Summary of ammonia efflux data, water, blood ammonia and pH data. P_wO_2: partial pressure of oxygen in water (torr). Values are means (±SEM) for $n=8$ individuals in each case

Experimental conditions	Weight (g)	Weight-specific net ammonia efflux (μmol g^{-1} h^{-1})	Water		Blood	
			final ammonia conc (μmol l^{-1})	final pH	final ammonia conc (μmol l^{-1})	final pH
Temperature = 6 °C						
Normoxia	112.19 (14.71)	0.05 (0.03)	7.22 (1.06)	7.51 (0.05)	133.37 (14.58)	7.76 (0.04)
Hypoxia (P_wO_2 = 24 torr)	134.81 (24.69)	0.11 (0.01)	11.15 (2.09)	7.54 (0.05)	87.75 (32.74)	7.91 (0.03)
Enrichment (300 μmol l^{-1})	121.64 (10.91)	−0.03 (0.04)	313.82 (3.68)	7.23 (0.06)	212.16 (24.54)	7.85 (0.02)
Enrichment + hypoxia (P_wO_2 = 24 torr, 300 μmol l^{-1})	105.9 (15.48)	−0.04 (0.02)	313.88 (2.76)	7.41 (0.10)	190.91 (43.41)	7.98 (0.03)
Temperature = 12 °C						
Normoxia	130.93 (11.42)	0.15 (0.01)	20.28 (4.70)	7.10	170.59 (32.18)	7.44
Hypoxia (P_wO_2 = 24 torr)	130.93 (11.42)	0.09 (0.02)	14.96 (1.78)	7.26 (0.05)	112.33 (28.80)	7.88 (0.03)
Enrichment (300 μmol l^{-1})	110.29 (21.48)	−0.33 (0.34)	260.81 (19.43)	7.03 (0.07)	201.40 (15.46)	7.93 (0.02)
Enrichment + hypoxia (P_wO_2 = 24 torr, 300 μmol, l^{-1})	122.34 (15.99)	0.07 (0.06)	297.70 (2.36)	7.33 (0.04)	129.60 (16.94)	8.04 (0.03)

Table 2. *Nephrops norvegicus*. Variability in net ammonia efflux within and between experimental groups, showing number of individuals which displayed net efflux over 3 h and incidence of hourly measurements where decreases in medium ammonia were found. In all cases, second set of values are number of measurements made

Experimental conditions	Individuals	Incidence
Temperature = 6 °C		
Normoxia	7/8	10/24
Hypoxia (P_wO_2 = 24 torr)	8/8	9/24
Enrichment (300 μmol l^{-1})	4/8	10/24
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	2/8	12/24
Temperature = 12 °C		
Normoxia	8/8	3/24
Hypoxia (P_wO_2 = 24 torr)	8/8	2/24
Enrichment (300 μmol l^{-1})	7/8	12/24
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	4/8	9/24

pears to be no relationship between blood pH and blood ammonia concentration, in agreement with the finding of J. P. Truchot (personal communication to M. Regnault). This suggests that ammonia, in ionised or unionised form, plays no significant role in the blood acid-base chemistry of decapods.

The rough estimate that blood volume = 0.30 ml g^{-1} fresh body weight – an approximation based on the blood volume estimates given for other decapods (e.g. Nicol 1967, Spaargaren 1972, Prosser 1973, Gleeson and Zubkoff 1977) – was used to estimate the blood ammonia-content data and the weight-specific blood ammonia-content data in Table 3. Analysis of variance of these data revealed that at 6 °C the ammonia-enriched groups had significantly higher mean weight-specific blood content values than the normoxic group ($P<0.05$; ANOVA in each case). At 12 °C, the only comparison that reached a significant level was that between the (EN) and the (EH) groups ($P<0.01$).

The weight-normalised data for efflux rates and blood-ammonia contents were used to produce estimates of the time required to effect a complete replacement of the blood ammonia (assuming a steady efflux rate and a cessation of ammonia production) at each of the experimental conditions (Table 3). Analysis of variance of the data revealed no significant differences between the normoxic and hypoxic non-enriched media values at either temperature ($P>0.05$ in each case). However, as expected, the differences between the normal and enriched groups were significant at each temperature.

The Fick equation allows a calculation of NH_3 or NH_4^+ flux due to the partial pressure or pNH_3 gradient (data from Table 5) or the NH_4^+ concentration difference across the gills, as follows:

$$J_{NH_3} = D_{NH_3} \cdot A \cdot \alpha \cdot \Delta P_{NH_3}/x\,, \qquad (4)$$

where J_{NH_3} = total ammonia flux (mol s^{-1}); D_{NH_3} = diffusive coefficient of the membrane (m^2 s^{-1}); A = surface

Table 3. *Nephrops norvegicus*. Blood ammonia replacement rates (calculated as time for complete replacement of blood ammonia) derived from weight-specific excretion rate and expressed as percentage of weight-specific blood ammonia content (μmol g^{-1} fresh wt). Blood volume was taken to constitute 30% of fresh weight. Values are means ($\pm$SEM) for $n=8$ individuals in each case

Experimental conditions	Blood ammonia		Replacement-rate time (min)
	gross (μmol)	weight specific (μmol g^{-1})	
Temperature = 6 °C			
Normoxia	4.43 (0.073)	0.039 (4×10^{-3})	56 (27.9)
Hypoxia (P_wO_2 = 24 torr)	2.83 (0.59)	0.021 (9×10^{-3})	18 (5.7)
Enrichment (300 μmol l^{-1})	9.00 (2.08)	0.074 (7×10^{-3})	Net uptake
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	7.82 (2.27)	0.074 (0.02)	Net uptake
Temperature = 12 °C			
Normoxia	6.78 (1.55)	0.052 (9×10^{-3})	26.6 (7.3)
Hypoxia (P_wO_2 = 24 torr)	4.86 (1.37)	0.037 (8×10^{-3})	25.6 (4.8)
Enrichment (300 μmol l^{-1})	7.17 (1.98)	0.065 (4×10^{-3})	Net uptake
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	5.02 (1.12)	0.041 (5×10^{-3})	Net uptake

area of exchange (m^2); α = Bunsen solubility coefficient of ammonia (ml NH_3 l^{-1} $torr^{-1}$); ΔP_{NH_3} = partial pressure gradient of NH_3 (torr); x = barrier thickness (m) or

$$J_{NH_4^+} = D_{NH_4^+} \cdot A \cdot (C^{in}_{NH_4^+} - C^{out}_{NH_4^+})/x\,, \qquad (5)$$

where $J_{NH_4^+}$ = total ammonia flux (mol s^{-1}); and ($C^{in}_{NH_4^+} - C^{out}_{NH_4^+}$) = concentration gradient (mol m^{-3}). If NH_3 diffusion is the dominant mode of excretion, the measured ammonia flux should be predicted by changes in the pNH_3 gradient; similarly if NH_4^+ diffusion is the dominant mode of excretion, the measured ammonia flux should be predicted by the NH_4^+ concentration gradient. Kormanik and Cameron (1981 a, b) used the ratio of two such estimates relating to different experimental conditions to predict changes in the net ammonia flux attributable to the changes in conditions. Using J_{NH_3} or $J_{NH_4^+} = J_{amm}$:

$$\frac{J^1_{amm}}{J^2_{amm}} = \frac{\Delta P^1_{NH_3}}{\Delta P^2_{NH_3}}\,, \qquad (6)$$

or

$$\frac{J^1_{amm}}{J^2_{amm}} = \frac{(C^{1\,in}_{NH_4^+} - C^{1\,out}_{NH_4^+})}{(C^{2\,in}_{NH_4^+} - C^{2\,out}_{NH_4^+})}\,. \qquad (7)$$

Normoxic (N) net-efflux values at 6 or 12 °C were used as J^1_{amm} estimates in Eqs. (6) and (7) to produce predictions

Table 4. *Nephrops norvegicus*. Relative rates of efflux of ammonia (J_{amm}) with reference to normoxic rates (100%). J^1_{amm} = normoxic flux, J^2_{amm} = calculated flux (Eqs. 6 and 7). pNH_3: partial pressure of NH_3

Experimental conditions	Predicted flux (J^2_{amm}%) based on:		Actual excretion rate
	pNH_3 gradient	NH_4^+ gradient	
Temperature = 6 °C			
Normoxia	–	–	100% (J^1_{amm})
Hypoxia (P_wO_2 = 24 torr)	100%	60%	220%
Enrichment (300 μmol l^{-1})	100%	−83%	−60%
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	120%	−99%	−80%
Temperature = 12 °C			
Normoxia	–	–	100% (J^1_{amm})
Hypoxia (P_wO_2 = 24 torr)	185%	54%	60%
Enrichment (300 μmol l^{-1})	267%	−34%	−220%
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	195%	−133%	47%

Table 5. *Nephrops norvegicus*. Blood:medium ratios of NH_3 and NH_4^+ and calculated pNH_3 and NH_4^+ gradients. Values are means for n = 8 individuals in each case

Experimental conditions	Blood:medium ratio of		Gradient (μmol l^{-1})		pNH_3 gradient (μtorr)
	NH_3	NH_4^+	NH_3	NH_4^+	
Temperature = 6 °C					
Normoxia	43:1	22:1	+0.9	+127.8	+11.0
Hypoxia (P_wO_2 = 24 torr)	27:1	16:1	+0.7	+76.1	+11.5
Enrichment (300 μmol l^{-1})	3:1	0.7:1	+1.0	−105.7	+12.0
Enrichment + hypoxia (P_wO_2 = 24 torr) 300 μmol l^{-1})	3:1	0.6:1	+1.0	−126.8	+14.0
Temperature = 12 °C					
Normoxia	26:1	14:1	+0.8	+164.9	+10.7
Hypoxia (P_wO_2 = 24 torr)	33:1	6.5:1	+1.4	+88.9	+19.8
Enrichment (300 μmol l^{-1})	7:1	0.8:1	+2.5	−56.6	+28.6
Enrichment + hypoxia (P_wO_2 = 24 torr 300 μmol l^{-1})	2.4:1	0.4:1	+1.4	−186.7	+20.9

of flux for the other experimental conditions. These estimates, along with the actual measured values, are given in Table 4. In all cases except that of 6 °C (H), the better estimate is that based on the $[NH_4^+]$ concentration gradient.

Normal seawater has very low ammonia levels compared to those in the blood, and this is reflected by the high blood:medium ratios of $[NH_3]$ and $[NH_4^+]$ found (Table 5). Such ratios became substantially reduced in the ammonia-enriched groups and the NH_4^+ ratios became <1:1. Despite the differences in the experimental conditions at each temperature, the pNH_3 gradient changed little, but with some evidence of a trend to increase in 12 °C enriched conditions. This contrasts with the NH_4^+ gradients which, at each temperature, became negative (or reversed) in enriched conditions. Clearly too, hypoxia effects a diminution of the NH_4^+ gradients at both temperatures in normal media.

Discussion

Excretion rates and blood ammonia

The mean weight-specific net ammonia-efflux rates for *Nephrops norvegicus* in this study ranged from -0.04 (net uptake) to 0.15 μmol g^{-1} h^{-1} (Table 1). These values, which relate to non ammonia-enriched seawater conditions, are in accordance with those given elsewhere for *N. norvegicus* (Hagerman et al. 1990). The decapods commonly underwent periods of an hour or more with no apparent net efflux, or even an apparent net uptake. This was observed under all conditions tested, was more frequent at 6 than at 12 °C, and was most common in the ammonia-enriched media. Not all individuals showed a net ammonia efflux at the end of 3 h, as demonstrated by the group mean-efflux data of the 6 °C (EN) and (EH) groups and the 12 °C (EN) groups. Preliminary tests involving vigorous aeration of media with high and low ammonia concentrations yielded very small alterations to dissolved ammonia values. These observations on apparent net uptake are supported by several other studies in progress in our laboratories. Armstrong et al. (1981) concluded that net uptake in *Macrobrachium rosenbergii* was a transitory response to hyperosmotic stress and possibly caused by a reversal of the Na^+/NH_4^+ exchange system. In the present context, a hyperosmotic stress was not involved, but *N. norvegicus* is a very weakly regulating species (cf. *M. rosenbergii*) which may be able to use ammonia in the context of ionic regulation.

Mean haemolymph ammonia concentrations recorded (Table 1) ranged from 87.7 to 212.2 μmol ammonia l^{-1} and compared well with the normoxic values for this species of 280 μmol l^{-1} (Robertson 1961) and 130 ± 63 μmol l^{-1} (Hagerman et al. 1990). These high concentration values, however, convert to relatively small weight-specific content values (mean = 0.05 μmol ammonia g^{-1} fresh wt) for the individuals in the present experiments. The weight-normalised net efflux and blood content values can be used to estimate the blood ammonia replacement times and to compare different experimental conditions. At the end of the 6 h time period of the present experiments, ammonia uptake still occurred in the groups in the ammonia-enriched media (i.e., individuals were still not acclimated to the medium ammonia levels). However, in the normal media, replacement times between 18 and 56 min occurred (Table 3). These are relatively slow replacement times compared with ca. 2 min for *Crangon crangon*, as similarly calculated by D. A. Hunter and R. F. Uglow (unpublished data). Ammonia enrichment of the medium and periods of hypoxia may not be uncommon in the natural environment of burrowing species such as *Nephrops norvegicus*. Hagerman and Pihl-Baden (1988) found that *N. norvegicus* is able to survive periods of days or even weeks of eutrophication-induced severe hypoxia in the Kattegat. Such resistance to adverse conditions has commercial implications, since the bulk transport of live crustaceans (crabs/lobsters) in lorry-based "vivier" tanks frequently result in medium ammonia levels >1000 μmol ammonia l^{-1}.

The 6 h duration of our experiments involving enriched media may not have been sufficient for the haemolymph ammonia concentrations to reach levels where net effluxes down concentration gradients were established. Cameron (1986) found that blood ammonia levels in *Callinectes sapidus* exposed to high ammonia concentrations in the medium, needed to increase above external levels before efflux was re-established; this occurred by reversal of NH_4^+ gradients and by normal, downhill pNH_3 gradients. In the present case, a net uptake of NH_4^+ may have contributed to blood ammonia (Table 5), but it is not possible to determine to what extent the contribution, if any, is derived from uptake and to what extent it may derive from the accumulation of metabolically-produced ammonia. Weight-specific ammonia efflux rates, however, are high relative to the weight-specific blood content; therefore, branchial efflux inhibition would not need to proceed for a long period of time in order to effect substantial changes in blood ammonia concentration unless protein metabolism were also inhibited. The blood concentration changes that did occur under ammonia-enriched conditions were more modest than would be expected for a simple case of inhibition of branchial efflux, and this may indicate that ammonia production at the tissue level – or release from the tissues to the haemolymph – was much reduced during enrichment, even under normoxia, or that detoxifying processes (ammonia removal from blood and tissues) were occurring.

Effects of hypoxia

In molluscs, ammonia production is not apparently affected by any metabolic shut-down during anaerobiosis (Bishop et al. 1983). Anaerobic ammonia excretion under short-term anoxia has been found in fish (van Waarde 1983) and, in similar anoxia experiments, lactate was produced in the tissues of *Carassius auratus* (Thillart and Kesbeke 1978). An increase in circulating lactate levels was found in *Nephrops norvegicus* under similar conditions of hypoxia to those used here (Bridges and Brand

1980), so it is possible that the ammonia release measured under hypoxia in the present study may partly result from anaerobic metabolism.

Processes involved in excretion

Regnault (1987) reviewed nitrogen excretion in crustaceans and pointed out that branchial ammonia excretion in fish and crustaceans comprises a possible ionic exchange mechanism involving a Na^+/NH_4^+ exchange pump and transepithelial diffusion of ammonia as the free form and/or the ionic form. The present data (Table 4) provide no information which helps resolve the involvement of any Na^+/NH_4^+ exchange mechanism in *Nephrops norvegicus*, but does offer support for the contention that flux mainly occurs along a pNH_3 or $[NH_4^+]$ gradient by diffusion, in agreement with Kormanik and Cameron (1981 a, b). Diffusion may also allow NH_4^+ to be taken up by the decapods during exposure to ammonia-enriched media, since both a negative ratio (i.e., <1) and an uphill gradient for the blood:medium $[NH_4^+]$ ratio were found (Table 5). Transepithelial potentials were not measured here, but were found to have very little effect on NH_4^+ movements in *Cancer irroratus* exposed to ammonia-enriched media (Kormanik and Evans 1984). In all cases, the final pNH_3 gradients were downhill from the blood to the external medium. In ammonia-enriched media this gradient can provide the only means by which branchial efflux of ammonia may be effected. The $NH_3:NH_4^+$ proportions during efflux depend on the relative amounts of the two ammonia species in the blood and external medium and their relative permeabilities, the free ammonia being more permeable than NH_4^+. Using the present data for normoxia, it is estimated that 35 to 40% or 60 to 70% of the net ammonia efflux in *N. norvegicus* would be attributable to NH_4^+ [depending on whether the gill is 100 or 300 times more permeable to NH_3 than NH_4^+: Oelert et al. 1968 (cited in Cameron 1986), Kormanik and Cameron 1981 a, Evans 1986]. Such values are in broad agreement with those given for *Callinectes sapidus* by Kormanik and Cameron (1981 a) and for several species of fish by Evans (1986).

Metabolic implications and detoxification processes

All experimental groups showed a slight metabolic alkalosis (cf. the relevant normoxic groups in last column of Table 1). As well as increasing the toxic NH_3 component of ammonia in the blood, this would also have increased the haemocyanin oxygen affinity (McMahon et al. 1978, Burnett 1979, McMahon, 1988). It is possible that urate production also ocurred under hypoxia, which may have additionally increased haemocyanin affinity (Lallier et al. 1987, Lallier and Truchot 1989). Spaargaren (1982) has suggested that urea production from elevated blood NH_4^+ concentrations under medium ammonia-enrichment, may also serve as a convenient detoxification process. We shall examine such possibilities in further studies on *Nephrops norvegicus* and other species, involving more prolonged exposures to hypoxia and medium ammonia enrichment.

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ASPECTS OF BLOOD PHYSIOLOGY AND AMMONIA EXCRETION IN *NEPHROPS NORVEGICUS* UNDER HYPOXIA

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Abstract—1. Circulating haemocyanin, glucose, lactate, and ammonia concentrations and ammonia excretion rates of *Nephrops norvegicus* were studied in normoxia and in various hypoxia levels for periods up to 3 weeks.

2. Increases in circulating glucose and lactate took place in oxygen tensions $P_wO_2 = <30$ Torr only, indicating aerobic metabolism down to this oxygen tension.

3. In moderate hypoxia (half saturation), *N. norvegicus* synthesised haemocyanin; in more severe hypoxia some haemocyanin catabolism occurred.

4. Mean normoxic blood ammonia concentration was 130 μM NH_4^+ /l with large individual variation. Blood ammonia levels decreased with time in both normoxia and hypoxia showing a lowered metabolic rate (activity level) over the experimental period. In short term experiments, blood ammmonia levels decreased in hypoxia.

5. Mean ammonia excretion rate was 0.16 μM NH_4^+ g/wet wt/hr under normoxia and excretion rate showed a negative, linear relationship to external oxygen tension.

INTRODUCTION

Some benthic marine species can tolerate lowering of their external medium oxygen tensions if such decreases proceed sufficiently slowly. Such adaptations may involve the synthesis of extra blood pigment and a general decrease in metabolic level. Rapid decreases in medium oxygen levels may be lethal to the same species. Highly mobile species such as fish may show a limited tolerance and adaptational response to hypoxia and thus contrast with many less mobile or burrowing species. *Nephrops norvegicus* (L) belongs to the latter category as it has been shown to possess relatively good adaptation abilities (Hagerman and Uglow, 1985).

Field measurements in the Kattegat in 1986 revealed areas where bottom waters were $P_wO_2 = <10$ Torr and the *Nephrops* caught appeared to be starving, moribund and with very low haemocyanin concentrations (Hagerman and Baden, 1988). *Nephrops* leaves its burrows at oxygen tensions $P_wO_2 = <40$ Torr and at still lower tensions it becomes inactive, haemocyanin levels decrease and they become moribund.

In marine crustacea, the end product of protein metabolism is ammonia which is released to the haemolymph from the metabolising tissues and is then excreted to the external medium across the surface of the gills. The effect of hypoxia on ammonia excretion in crustaceans has received relatively little attention and the published data are somewhat equivocal—possibly because of initial sampling differences, in terms of nutritional status and recent handling stresses, amongst the animals used (see Regnault and Aldrich, 1988). Most laboratory studies have dealt with only short periods of experimental hypoxia (hours) but in areas such as the Kattegat, periods of eutrophication-induced hypoxia may prevail for several weeks at a time. Information on the effect of both short- and long-term periods of hypoxia appears to be appropriate if a better understanding of the responses of *Nephrops* is to be sought.

The aim of the present work was to assess the effect of short- and long-term hypoxia on nitrogen metabolism, as measured by ammonia production (circulating ammonia levels) and excretion, in *Nephrops*. In addition, the general laboratory behaviour of the animals was assessed in normoxia and in hypoxia.

MATERIALS AND METHODS

Specimens of *Nephrops norvegicus* were creel-caught at depths of 30–40 m near the Marine Biological Station, Kristineberg, Sweden during 1988 and 1989. The animals were transported in aerated seawater by road to Helsingør, Denmark and then kept in aquaria supplied with running sea water (10°C, 30‰, S). The aquaria were supplied with sand substratum, several centimetres deep, and 10 cm diameters of red plastic tubing, bisected longitudinally, which acted as "burrows" for the animals. The animals were fed once each week on a mixture of bivalve (*Mytilus*) flesh and shrimps.

Two series of experiments were made. In one, groups of 10 animals, of roughly similar size, were each exposed to a particular oxygen tension for a period of 3 weeks. The experimental oxygen tensions were $P_wO_2 = 155$ Torr (normoxia), 78, 47, 31 and 16 Torr—corresponding to 100, 50, 30, 20 and 10% oxygen saturation respectively. The particular tensions were obtained by bubbling the media with fixed ratios of nitrogen gas and air. Tensions were

monitored continuously *via* an YSI 5739 electrode connected to an YSI 58 oxygen meter and a Philips PM 8252A recorder. All experiments were made at 10°C and the experimental aquaria were draped with black plastic sheeting to minimise the stresses of the holding room lighting and optical stimuli. Throughout these experiments, the oxygen tensions varied within $P_wO_2 \pm 5$ Torr.

Water samples (1.0 ml) for ammonia determinations (excretion) were taken daily at fixed times and analysed immediately. This series of experiments measured the total contributions of all 10 animals in each case. Blood samples (300 μl from each animal) were taken via a hypodermic syringe inserted through the arthrodial membrane at the base of a walking leg. Blood samples were collected once each week and were analysed for ammonia concentration (production), lactate, glucose and haemocyanin concentrations.

The second series of experiments comprised short-term (5 hr) exposures to particular oxygen tensions. Groups of 8 *Nephrops* were allowed to acclimatise for 16 hr in individual aquaria supplied with 5 l of seawater. The water was then changed (5 l) and the oxygen tension brought to the required level ($T = 0$). At 1 hr intervals, water samples (1 ml) were collected for ammonia analyses. After 5 hr the water was again renewed with aerated normal seawater and at $T = 24$ a further water sample was taken as an estimate of the recovery performance. These short-term experiments were carried out using water tensions of $P_wO_2 = 155$, 47 or 16 Torr. In addition to the water samples taken, haemolymph samples (100–150 μl) were taken at times $T = 0$, 2.5 and 5 hr for estimations of blood ammonia concentrations.

Biochemical analyses

Haemolymph lactate was measured using Boehringer–Mannheim Test Kit 139084 and haemolymph glucose using Sigma diagnostic procedure No. 510-A. Haemocyanin levels were estimated using the method described by Hagerman and Uglow (1985) and using a millimolar extinction coefficient of 17.65. Measurements were made using a Unicam SP1800 UV-spectrophotometer.

Ammonia concentrations in water or blood samples were made using a flow-injection gas-diffusion system modified after Clinch *et al.* (1988). Samples (100–250 μl) were injected into a carrier stream of 0.01 M NaOH. Bromothymol Blue (0.5 g/l redistilled water) was used as the indicator. A set of dilution standards was freshly prepared each day from a mother solution of 5 mM $(NH_4)_2SO_4$ (= 0.0335 g in 500 ml redistilled H_2O). Standards were injected at regular intervals to monitor any possible changes in sensitivity that may have occurred—as does happen when seawater samples are injected over a period of time.

As a control measure, seawater samples, aerated and non-aerated, were examined at regular intervals for ammonia concentration. No evidence of any significant loss of ammonia was found to occur over a 6 hr period so this potential source of variability was not further considered in these studies.

RESULTS

Haemocyanin levels

Figure 1 shows the data on haemocyanin levels at the various oxygen tensions. The normoxia (control) groups showed no significant change during the course of the experiment whereas the moderate hypoxia of $P_wO_2 = 78$ Torr induced a slight synthesis of haemocyanin which was most evident between days 7 and 14 and amounted to an increase of 0.012 mM/l/day. At the lower tensions, the animals were observed to be inactive and they did not feed. Haemocyanin decreased in each of these groups at rates which were clearly related to the severity of the hypoxia. All the animals were still alive at the end of the 3 weeks in the $P_wO_2 = 47$ and 31 Torr groups and they showed a clear synthesis of haemocycanin in the subsequent normoxic recovery period. At $P_wO_2 = 16$ Torr, 50% of the animals had died within 2 days and the survivors showed a decrease in haemocyanin even after this short time.

Glucose levels

The data obtained for circulating glucose in the experimental and control (normoxia) groups are shown in Fig. 2. Glucose levels show considerable inter- and intraspecific variation with basal levels for active, but otherwise unstressed, decapods commonly being 5–20 mg/100 ml (Johnson, 1985). Here the control group showed a very stable blood glucose level throughout the experimental period. This contrasts with the other groups which, with the exception of the $P_wO_2 = 16$ Torr group, showed a progressive decrease in blood glucose over the course of the experiment. The $P_wO_2 = 16$ Torr group survivors showed a marked hyperglycaemia with levels approximately doubling during the 2 days that they could be sampled.

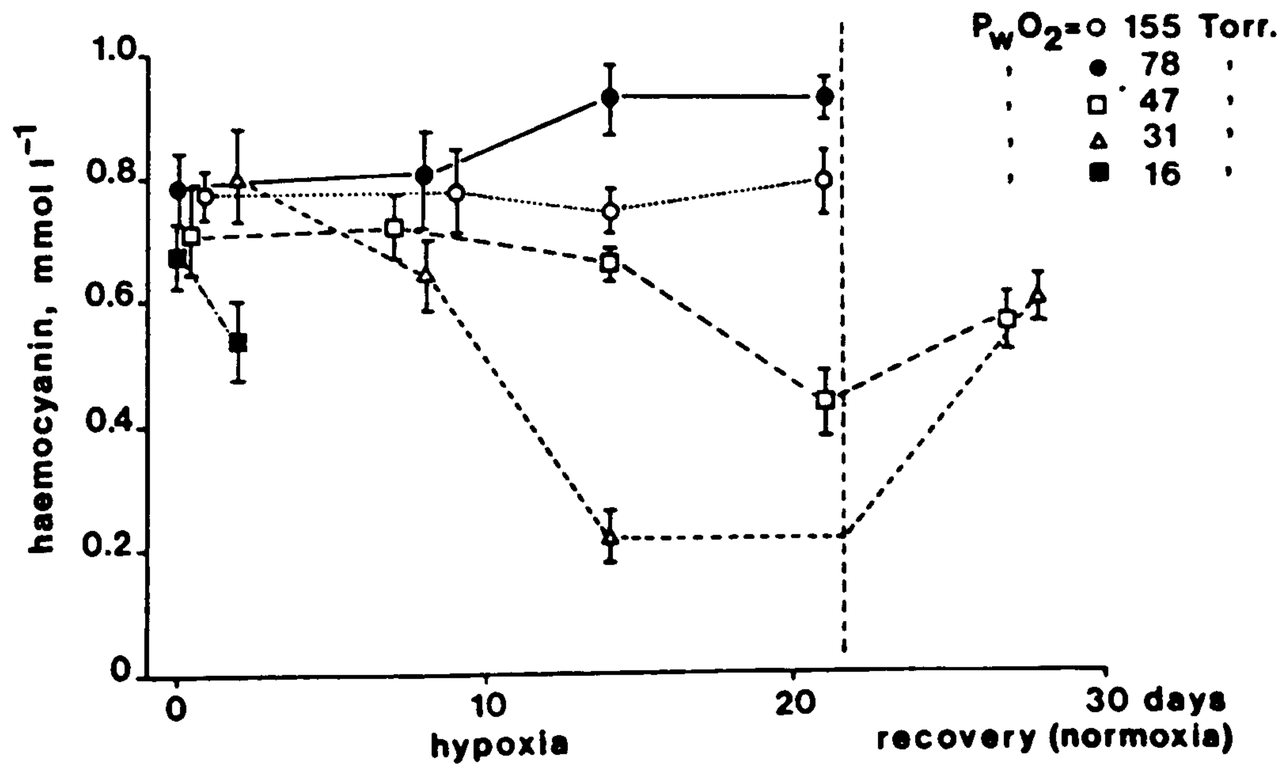

Fig. 1. *Nephrops norvegicus*. Haemocyanin concentrations (mmol/l ± S.E.M.) during exposure to hypoxia for 21 days and subsequent recovery in normoxia.

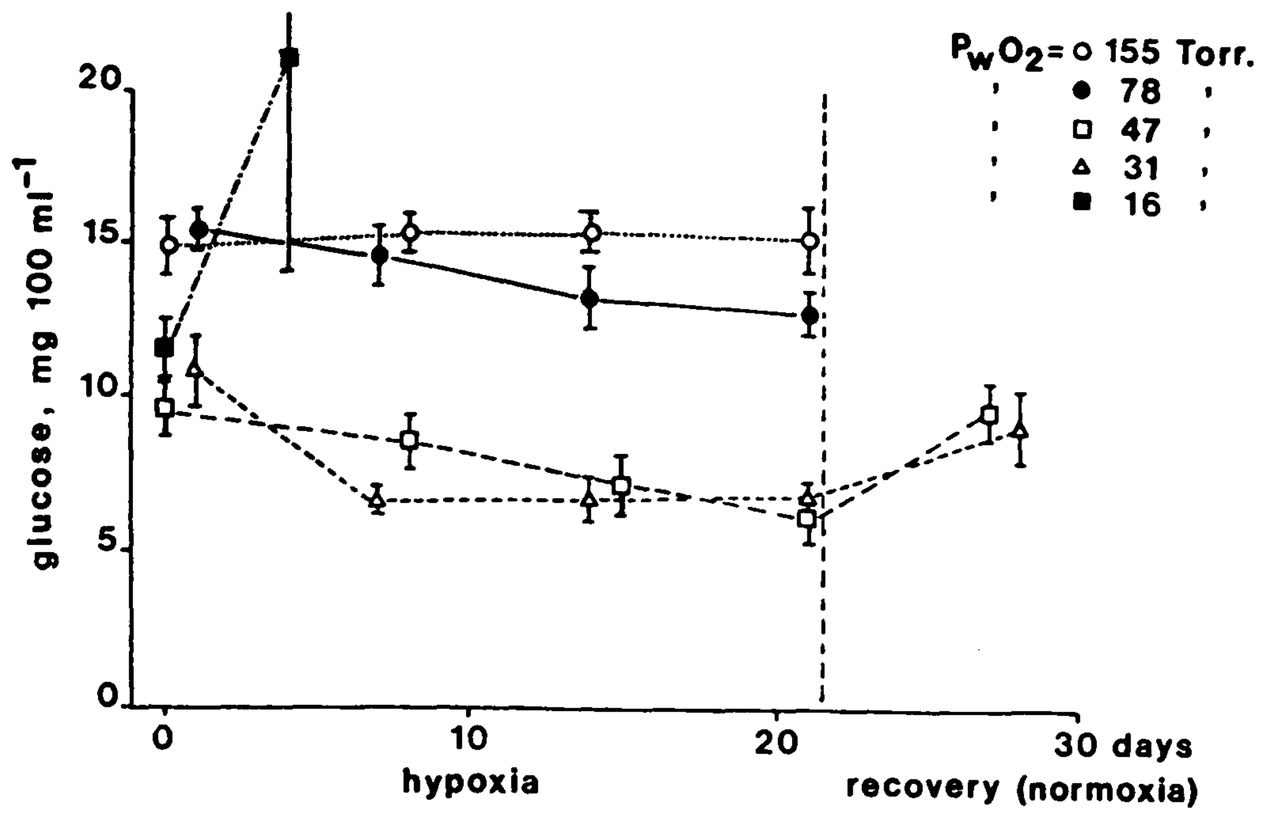

Fig. 2. *Nephrops norvegicus*. Circulating glucose levels (mg/100 ml ± S.E.M.) during exposure to hypoxia for 21 days and subsequent recovery in normoxia.

Haemolymph lactate levels

Lactate was accumulated only in the $P_wO_2 =$ 16 Torr group survivors and these showed an increase from the normoxic mean value of 1.75 ± 0.51 mg/100 ml ($n = 6$) to 63 ± 2.23 mg/100 ml ($n = 5$) at day 2. Again it was not possible to keep the animals alive for more than 2 days at this oxygen tension. The groups held at other oxygen tensions all kept normoxic levels throughout the course of the experiment.

Blood ammonia levels

Circulating ammonia levels showed a large individual variability as well as showing a temporal change in mean values (Table 1). The normoxic mean regulated value was 130.4 ± 9.66 μM NH_4^+/l ($n = 50$) but, as the data in Table 1 show, there was considerable group-to-group variation which tended to obscure the subsequent effects of the hypoxic treatments. When the data were converted to relative values (% change from $T = 0$ days) all the mean values of the surviving groups at $T = 21$ days were found to have decreased by roughly the same amount (Table 1).

Ammonia excretion rates

The weight-specific ammonia excretion rates, calculated as the mean of groups ($n = 10$ each) held at a particular oxygen tension for 21 days are shown in Fig. 3. Excretion rate was found to have an approximately linear negative relationship to the external oxygen tensions over the range $P_wO_2 = 16$ Torr to $P_wO_2 = 155$ Torr.

The short-term experiments (5 hr at any particular oxygen tension) yielded much higher excretion rates at normoxia than were found in the 21-day experiment (0.35 μM NH_4^+/g wet wt/hr as opposed to 0.16 μM NH_4^+/g wet wt/hr). The values obtained at the hypoxic levels were similar in the two groups of experiments.

DISCUSSION

For some crustacean species, the synthesis of haemocyanin under moderate hypoxia, can be taken to indicate that such conditions are tolerable, i.e. the animals are active and feeding (Hagerman, 1986). The haemocyanin synthesis found here in the long-term experiments show that *Nephrops norvegicus* can cope with moderate hypoxia for extended periods. The rates of synthesis found here were lower than those measured by Hagerman and Uglow (1985) for *Nephrops* but this may be because the animals used in these studies had higher initial haemocyanin concentrations. The decrease found in haemocyanin levels at strong hypoxia also accords with earlier observations; the animals having emerged from their burrows were inactive and not feeding (Hagerman,

Table 1. *Nephrops norvegicus*. Haemolymph ammonia concentration in absolute units (NH_4^+/l, ±S.E.M., $n = 10$) and relative (% change) in normoxia and during exposure to 3 week hypoxia

P_wO_2 Torr	Days of exposure 0	7	14	21
155	121.29 ± 9.89 100%	154.49 ± 8.98 127.4%	67.11 ± 6.36 90.23%	86.13 ± 4.45 71.01%
78	176.41 ± 20.41 100%	170.89 ± 15.12 96.9%	109.44 ± 15.09 62.0%	136.91 ± 5.24 77.6%
47	97.07 ± 7.14 100%	24.98 ± 2.02 25.7%	57.24 ± 2.61 59.0%	55.63 ± 3.55 57.3%
31	162.58 ± 33.44 100%	113.97 ± 8.40 70.1%	124.12 ± 3.16 76.3%	108.83 ± 10.29 66.9%
16	89.47 ± 9.60 100%	67.18 ± 2.05* 75.1%		

*$n = 5$, measured at day 2.

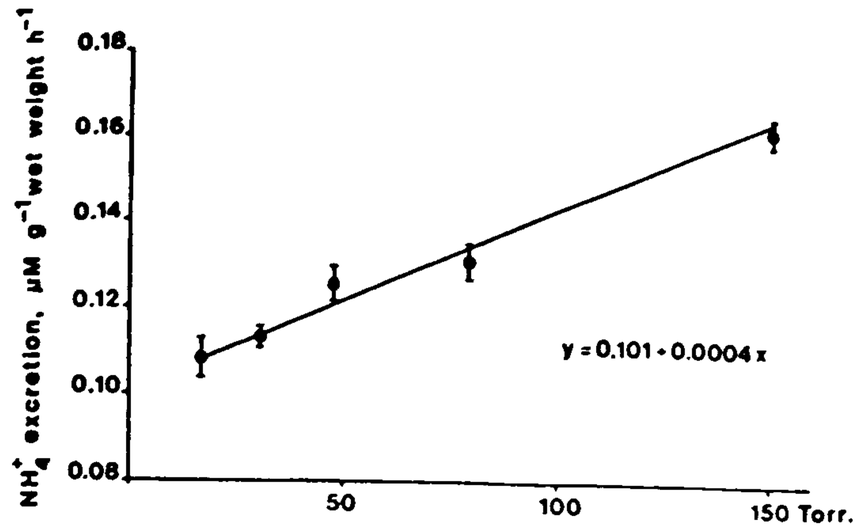

Fig. 3. *Nephrops norvegicus*. Ammonia excretion rates (μM/g wet wt/hr) in normoxia and during 3 weeks exposure to hypoxia.

Table 2. *Nephrops norvegicus*. Haemolymph ammonia concentrations (μM NH_4^+/l. ±S.E.M., n = 8) in normoxia and during hypoxia

P_wO_2	Haemolymph ammonia Start conc.	5 hr exposure	% Deviation
Normoxia (155 Torr)	110.76 ± 3.11	163.25 ± 4.02	+47.39
Hypoxia (47 Torr)	57.88 ± 7.37	40.60 ± 8.28	−29.85
Hypoxia (16 Torr)	72.82 ± 6.97	60.93 ± 4.09	−16.33

1986; Hagerman and Baden, 1988; Mangum, 1990). This was observed here at P_wO_2 = 16 Torr and after about 10 days at P_wO_2 = 31 Torr.

The accumulation of anaerobic metabolites does not occur as long as aerobic metabolism can be maintained. Hyperglycaemia induced by an increased glycogen utilization and an accumulation of circulating lactate became obvious in these studies only in the P_wO_2 = 16 Torr group. The extent of the circulating glucose increase found was similar to that found for other crustacean species exposed to severe hypoxia (Taylor and Spicer, 1987; van Aardt, 1988).

Bridges and Brand (1980) showed an increase of circulating lactate to *ca.* 80 mg/100 ml when *Nephrops* was exposed to sudden hypoxia of P_wO_2 = 20 Torr for 5 hr. The present data showed an accumulation up to 63 mg/100 ml after 2 days at P_wO_2 = 16 Torr. To a certain extent, the accumulation rate can be related to metabolism and hence to overt activity (Hagerman and Szaniawska, 1986, 1990) and it is reasonable to assume that the lower accumulation rates found here (*cf.* those of Bridges and Brand, 1980 animals) reflect lower metabolic levels resulting from the provision of a substratum and an artificial "burrow". Also small deviations in salinity might cause great changes in the hypoxic accumulation rate of lactate (Johnson and Uglow, 1987).

The blood ammonia concentration data highlight the importance of experimental conditions and procedures in experiments of this type. The 50% increase in mean circulating ammonia levels seen in the short-term experiments (Table 2) are due, probably in large part, to metabolic increases induced by the general handling and blood sampling of the animals. Increased energy demands result in lowered cellular energy content and a consequent increase in ADP. Both functions of GDH are activated by ADP, but GDH in oxidative function may act as an ATP regenerating system in order to restore the energy charge. An activation of GDH in oxidative direction causes an increased ammonia production (Batrel and Regnault, 1985). This could be the reason for increased blood ammonia in short-term experiments in normoxia. Hence, the slow, progressive decreases of circulating ammonia found in the long-term experiments probably reflect a gradual adaptation to the experimental surroundings. The comparatively low stable ammonia levels, taken in conjunction with the stability of the circulating glucose and haemocyanin values measured in the long-term groups, have been taken here as evidence that the animals are well adapted to such conditions. A decrease in ammonia production and excretion, followed by a rapid increase in blood urate, was observed by Lallier *et al.* (1987) when *Carcinus maenas* was exposed to P_wO_2 = 40 Torr for 24 hr. Thus, in this case a shift to another metabolic pathway (urate production) or a transformation of ammonia to urate seemed to occur. This would also be a possible explanation to decreases in blood ammonia and excretion during exposure to hypoxia.

The normoxic blood ammonia values published for other crustaceans (Table 3) show a large interspecific variability and values are generally much higher than those found here. Some of this variability may be attributed to genuine interspecific differences, to differences in physiological condition of the animals or to technical difficulties hitherto in measuring accurately the ammonia concentrations in small sample volumes. The present results show also that undesigned handling stresses are probably responsible for considerable intraspecific variability of circulating ammonia.

The weight specific ammonia excretion values are also lower than those found for other species [e.g. 0.49 μM NH_4^+/g wet wt/hr for *Carcinus maenas* (Spaargaren, 1982); 1.10 μM NH_4^+/g wet wt/hr for *Callinectes sapidus* (Mangum *et al.*, 1976)]. Even if the excretory rate is size-dependent (Regnault, 1987), weight-for weight, *Nephrops* has lower excretion rates than other species.

Taken together, the excretion rate and blood concentration data allow speculation on the rates of ammonia fluxes in this species. If the approximate blood volume of *Nephrops* is taken to be 30% of the fresh body weight (as pertains in other species, e.g.

Table 3. Normoxic haemolymph ammonia concentrations (μM NH_4^+/l. ±S.D.) in various crustaceans

Nephrops norvegicus	130 ± 63	(n = 50)	This investigation
N. norvegicus	280		Robertson (1961)
Callinectes sapidus	330 ± 20	(n = 11)	Mangum *et al.* (1976)
C. sapidus	785 ± 55	(n = 6)	Kormanik and Cameron (1981)
Carcinus maenas	945 ± 170	(n = 12)	Binns (1969b)
C. maenas	250 ± 50	(n = 26)	Harris and Andrews (1985)
Cancer irrotatus	379 ± 59	(n = 7)	Kormanik and Evans (1984)

Binns, 1969a; Spaargaren and Kraay, 1973) then a 100 g animal would have a blood volume of 30 ml. A circulating ammonia concentration of 120 μM NH_4^+/l becomes equivalent to a blood content of 3.6 μM NH_4^+. With a normoxic excretion rate of 0.160 μM NH_4^+/g wet wt/hr the example animal would excrete 7.5% of the blood content each minute (turnover rate). Complete cessation of excretion, or even altered excretion rates are thus able to effect rapid and substantial alterations to the circulating ammonia levels and may prove to be a rapid method of stabilising blood pH changes.

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SEA FISH INDUSTRY AUTHORITY
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AN ASSESSMENT OF DAMAGE AND MORTALITY OF THE BROWN CRAB DURING VIVIER TRANSPORT

Technical Report No. 294

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SUMMARY

Two consignments of live crab have been studied to investigate damage and mortality between consignments of brown crab exported from the UK to spain.

The consignment from the Hebrides was first transported by vivier lorry to N. Wales where it was off loaded, examined and repacked for onward shipment by a Spanish buyer's vivier lorry to Spain via France. In Spain the crab were again off loaded, inspected and repacked and sent in another vivier truck to the main wholesale fish market in Madrid. Each leg of this journey took 36 to 40 hours.

The second consignment was sent from the South Coast of England to Madrid and this journey also took 30-40 hours.

Although there are many variables to be considered the two samples were believed to be fairly typical consignments. Mortality in the Hebridian consignment on arrival in Spain was higher than the South Coast consignment and this was attributed to poorer selection of animals at the point of dispatch. There is an undoubled need for training here especially in the understanding of the transportation process and the requirements of the Spanish markets. Where the fishery only engages in crab fishing intermittently, or in an uncoordinated way, this is most apparent.

The South Coast consignment benefitted from better selection and the lack of intermediate handling and repacking. Even amongst intact (undamaged) animals there was a higher mortality in the Hebridian consignments indicating other influences - possibly condition or seasonal factors which will need further investigation.

The report suggests protocols for both the catchers and the buyers to ensure that only animals which have a reasonable chance of survival and selected and thereafter are treated in a way that improves these chances.

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LIVE HANDLING AND TRANSPORT OF CRUSTACEAN SHELLFISH: AN INVESTIGATION OF MORTALITIES

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SEA FISH INDUSTRY AUTHORITY
Industrial Development Unit

Technical Report No. 280 January 1986

LIVE HANDLING AND TRANSPORT OF CRUSTACEAN SHELLFISH: AN INVESTIGATION OF MORTALITIES

1. INTRODUCTION AND BACKGROUND

The investigations described in this report are the most recent of a series of studies aimed at solving problems associated with the transport and marketing of live crustaceans such as crab.

The context within which these studies have been initiated by SFIA, is that of an expanding industry in a state of some uncertainty. The UK crab fishery is prosecuted in a number of regions and is based on several sub-stocks of the target species. The market opportunities for crab and for crab products are also distinct and the various regional fisheries have developed with traditional specialities in certain market sectors.

Now, however, there is a universal tendency for any producer to try to broaden the market appeal of his products. These moves have introduced a number of problems to the crab industry and it is to these that SFIA has addressed itself. The aim, overall, is to give each of the major regional fisheries the same chance of having a broad-based and demand-led stimulus to its development.

This particular series of studies started in 1983/84 with the development of a live trade in velvet crab (Liocarcinus puber) from Scotland to continental Europe which was plagued with heavy mortalities. SFIA commissioned a study by the University of Hull to find the underlying reasons and establish a methodology for such investigations.

That study started just as the Hebridean fishery for the brown crab (Cancer pagurus) was diversifying from pure processing into live export. With that development came further, inexplicable mortalities which were all the more puzzling because they were occuring with companies which had a long and successful history of transporting English brown crab alive and in good condition to both home and continental markets.

The significance of excess mortality in this context is that it devalues the commodity in two ways. First there is a straightforward cash loss equivalent to the percentage mortality; secondly the region of production starts to acquire a poor reputation and foreign buyers will start paying less and looking for alternative supplies. Already in 1985 there has been a significant loss of markets by producers in the Hebrides as a result of a series of instances of mass mortality in their consignments to Europe. The countries involved include Spain, France and Holland.

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