DEVELOPMENT OF CULTURE AND TOXICITY TESTING METHODS FOR THE FRESHWATER COPEPOD *BRYOCAMPTUS ZSCHOKKEI*

by

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ABSTRACT

The aim of this thesis was to evaluate the use of a meiofaunal copepod as a test species for assessing the developmental and reproductive effects of toxicants relevant to freshwater ecosystems. The harpacticoid copepod *Bryocamptus zschokkei* was chosen as a candidate test species as it possesses several attributes (widespread distribution, small size and fast development times) that are considered pre-requisites for toxicity test organisms, and has previously been shown to be a sensitive component of the stream community to contaminant exposure. Prior to conducting toxicity tests with *B. zschokkei*, studies were performed to evaluate the effects of water hardness and food quality on the development and reproduction of this copepod. These data were then used to define optimal culture conditions. *Bryocamptus zschokkei* was insensitive to water hardness at <150 mg l^{-1} (as $CaCO_3$), reflecting its range of tolerance in the field and suggesting the potential for toxicity testing across a range of hardness levels. Food quality affected development and reproduction: beech leaves (*Fagus sylvatica* L.), conditioned for 2 weeks supported optimal overall development to adult and reproduction of *B. zschokkei* and were chosen for use in subsequent culturing and testing regimes.

Development and reproduction assays for assessing the effects of environmental parameters on *B. zschokkei* were combined to produce the first full life-cycle toxicity test for a freshwater copepod. This life-cycle test was relatively quick (6 weeks at 20°C) and is highly reproducible. The effects of three reference chemicals, the trace metal zinc, the pesticide lindane, and the moulting hormone 20-hydroxyecdysone (20-HE), were measured using this toxicity test. There was no effect of 20-HE (0-269 µg l^{-1}) on the life cycle of *B. zschokkei*. *Bryocamptus zschokkei* was, however, relatively sensitive to zinc and lindane compared with other freshwater crustaceans although sensitivity depended on the chemical and the duration of exposure. A model of 'equiproportional development' was used to aid interpretation of the mechanism of toxicity of lindane, which was found to act by significantly prolonging the development time to adult. Reproductive endpoints (numbers of eggs and nauplii per female) were the most sensitive measure of zinc and lindane exposure, with lowest observed effect concentrations (LOEC) of 0.48 mg Zn l^{-1} and 32 µg lindane l^{-1}, respectively. An increase in abortion frequency, observed for these contaminants, may have potential as a biomarker of stress for this copepod. In conclusion, it is proposed that toxicity tests with *B. zschokkei* should be included in contaminant assessment procedures for freshwater systems as they would increase the choice and ecological relevance of current testing regimes.

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Chapter 1

General Introduction

1.1 The Role of Meiofauna in Stream Ecosystems

Lotic meiofauna include those animals which pass through a 500 μm mesh but are retained on a 63 μm mesh (Giere, 1993; Robertson *et al*, 2000a). Within the stream benthos, this biotic category incorporates a diverse range of species from a number of functional groups. Permanent meiofauna, such as copepods, ostracods, rotifers and micro-turbellarians, spend their entire life cycle within the meiofaunal component of the benthos. The early larval stages of macroinvertebrates, including chironomid and stonefly larvae, also fall within the meiofaunal size class and are known as temporary meiofauna. As well as having a smaller size, permanent meiofauna differ from macrofauna in that they have a relatively faster life cycle and complete their life cycle entirely within the stream benthos, whereas many of macroinvertebrates (insects) have a terrestrial adult stage. The meiofaunal component of lotic systems is abundant (densities of animals can reach up to 105 individuals ml^{-1} sediment), and has a high degree of bio-diversity (Ward *et al*, 1998; Hakenkamp & Morin, 2000). Although full species lists for stream meiobenthos are rare, existing data indicate that the meiofauna contribute to between 58 and 82% of the total species numbers (Robertson *et al*, 2000b). The role of benthic meiofauna in the functioning of stream dynamics is becoming increasingly well established (Robertson *et al*, 2000a). For example, it is recognised that meiofauna play an important role in benthic food web dynamics (Schmid-Araya & Schmid, 2000). Permanent meiofauna often feed on microflora including algae, bacteria and fungi either as filter or suspension feeders or by browsing the surface biofilm of rocks and leaves (Perlmutter & Meyer, 1991). In turn, the meiofauna, is an important food source for macroinvertebrate predators (Rundle & Hildrew, 1990; Lancaster & Robertson, 1995; Schmid & Schmid-Araya, 1997) and bottom-dwelling fish (Robertson, 1990; Rundle & Hildrew, 1992). In addition, meiofauna (at least locally) have an important role in benthic metabolism as well as nutrient and organic matter cycling (Hakenkamp & Morin, 2000).

Within the interstitial spaces of stream sediments, feeding and movement of meiofaunal species is thought to stimulate microbial activity by increasing the flow of oxygen and nutrients to biofilm layers and their associated microbial community. Metabolic waste produced by meiofauna is likely to be rich in nutrients such as nitrogen and phosphorous which can be utilised by microbial communities (Hakenkamp & Morin, 2000). Few studies have addressed these ecological roles, however, there is evidence that grazing by meiofauna increases the metabolism or density of microbes in lentic systems (Perlmutter & Meyer, 1991; Traunspurger *et al*, 1997; Hakenkamp & Morin, 2000). Some meiofaunal organisms influence stream sediment dynamics through bioturbation (Ward *et al*, 1998). The reworking and pelletisation of sediments, as they pass through the gut of meiofauna can both increase microbial activity (Hargrave, 1976) and change the sediment composition by increasing the grain size (Ladle & Griffiths, 1980; O'Doherty, 1985). As a result, meiofauna provide fine particulate organic matter (FPOM) to the stream system, which may be used as a food source for other stream invertebrates.

1.2 Pollution and Meiofauna

In view of the diversity and potential ecological significance of lotic meiofauna, it is becoming increasingly apparent that these communities need to be considered in holistic approaches to environmental monitoring. Meiofauna organisms are particularly vulnerable to the effects of contaminants because of their mode of life. For example, many pollutants have an affinity for particulate matter, therefore, sediments and pore waters often contain elevated concentrations of contaminants compared with surface waters (Salomons *et al*, 1987; Tessier & Campbell, 1987). Permanent meiofauna, living their entire lives within the interstitial spaces of the stream benthos, are particularly vulnerable to exposure to such sediment-bound contaminants. The role of meiofauna in bioturbation and food web dynamics means that they may also be influential in determining the ultimate effects, fate,

recycling and bioconcentration of contaminants within the stream system. The importance of incorporating the meiofauna into pollution monitoring of estuarine and marine systems has been recognised for many years (Coull & Chandler, 1992). Indeed, marine meiofauna have been found to be at least as sensitive as macroinvertebrates to contaminants in community level studies (Austen *et al*, 1989; Somerfield *et al*, 1994). The sensitivity of meiofaunal species may be related to the fact that they have relatively short generation times, high metabolism and fast growth rates compared with macroinvertebrates, and therefore react more quickly to environmental perturbation (Warwick, 1993; Burton, 1998). In marine systems, copepods and nematodes are the most widely used meiofaunal taxa in biomonitoring studies (Coull & Chandler, 1992). Analysis of marine benthic communities identified copepods as one of the most sensitive meiofaunal species (mainly compared with nematodes) to organic pollution (Raffaelli & Mason, 1981; Van Damme *et al*, 1984). Indeed, the ratio of nematode to copepod numbers has been proposed as an index of organic pollution, and has been found to be a useful tool for assessing organic pollution in sandy sediments (Raffaelli, 1987). The benthic meiofaunal communities of freshwater systems have received far less attention in environmental monitoring, but the available data support evidence from marine systems that copepods are relatively more sensitive to contaminants than other meiofaunal elements. For example, harpacticoid copepods, considered good bioindicators of oligotrophic lakes, are more sensitive to anoxia and eutrophy than other meiofauna in community studies evaluating the effects of organic loading on benthic communities (Särkkä, 1992; 1995). Analysis of the distribution of meiofaunal crustaceans in the groundwater and springs of southern Finland showed cyclopoid copepods (many species of which have the ability to enter diapause in response to negative environmental conditions) to be more tolerant of anthropogenic disturbance than harpacticoids (Särkkä *et al*, 1997). Recently, Burton *et al* (2001) evaluated the use of stream meiofauna as indicators of trace metal pollution and found that elevated copper, and

to a lesser extent zinc, were the major factors explaining the distribution of two harpacticoid copepods *Bryocamptus zschokkei* and *B. praegeri*. These harpacticoids were also highlighted as being particularly sensitive to elevated aluminium concentrations in Welsh streams (Rundle, 1993).

Meiofauna has been largely ignored in toxicity bioassessment of benthic freshwater systems, particularly in evaluating the effects of sediment contamination (Nebeker *et al*, 1984; Giesy & Hoke, 1989; Girling *et al*, 2000). Current international testing standards recommend several potential macrofaunal test species for assessing the effects of sediment associated contaminants, including chironomid larvae, amphipods, oligocheates and daphnids (ASTM, 1999a). While it is often suggested that the ecological relevance of a test species to the system to be protected should be an important consideration when choosing a test species, this rarely appears to be a major concern to those conducting regulatory tests (Cairns & Pratt, 1989). For example, the cladoceran *Daphnia magna* is used regularly in sediment testing despite its planktonic mode of life (Nebeker *et al*, 1984; Giesy & Hoke, 1989). Other animals also recommended as freshwater test species include the amphipod *Hyalella azteca*, which is not indigenous to British freshwater systems (ASTM, 1999a). The lack of concern over the ecological relevance of a test species perhaps reflects a toxicological approach to environmental assessment where availability and ease of culture are the main priorities of the test organism as opposed to an ecotoxicological approach where ecological relevance is the major concern (Chapman, 1995a).

1.3 Single-Species Tests

The relevance of single-species tests in environmental monitoring has often been questioned. For example, data from single-species toxicity tests cannot necessarily be extrapolated reliably to the field (Cairns *et al*, 1993). One reason for this is that

contaminants rarely occur in isolation but are often present as low concentration mixtures of contaminants. It is also advocated that the most 'sensitive' species in a community should be used in single-species tests to ensure that the entire community is being protected. It is now recognised, however, that different species respond differently to individual chemicals and that it is, therefore, impossible to choose the most 'sensitive' species within a community. In general, any species that has been found to be particularly sensitive to environmental perturbation is often difficult to maintain under artificial laboratory conditions (Maltby & Calow, 1989; Cairns & Pratt, 1989; Chapman, 1995a). Despite such reservations about their ecological relevance, it is generally accepted that single-species tests will continue to have a major role in environmental risk assessment and in regulating the effects of potentially harmful new chemical products (Cairns *et al*, 1993; Forbes & Forbes, 1994). Indeed, laboratory-based studies may be the only way in which the causes of effects observed in the field can be reliably verified (Chapman, 1995b). In addition, single-species tests have a role in determining the potential mechanism of action of contaminants. This has become particularly apparent from recent evidence of the potential effects of endocrine disrupting chemicals (EDCs). Recognition of the existence of EDCs has increased the emphasis on developing single-species tests with endpoints which may indicate perturbation of endocrine function and for species where full life-cycle tests can be carried out (deFur *et al*, 1999). It is, therefore, imperative that the tools used in toxicity evaluation are as reliable as possible and at least have some relevance to the systems that need to be protected (Chapman, 1995a). A number of critiques have evaluated the requirements, choice and application of single-species toxicity tests (Cairns & Pratt, 1989; Richardson & Martin, 1994; Chapman, 1995a; Power & McCarthey, 1997). The choice of a test organism will vary depending on the information required and the system that is to be protected. Some of the key criteria of a test organism are outlined below (adapted from deFur *et al* 1999; ASTM, 1999a):

- Laboratory culture - The ability to culture animals in the laboratory introduces an aspect of quality control to a toxicity test limiting the effects of previous exposure to contaminants, disease or parasites. It is also an advantage because animals of a defined age or life-stage are available all year round.
- Short generation time - This means that effects on different life-history parameters (e.g. development and reproduction) can be screened rapidly and makes full life-cycle or transgenerational studies more practical. In addition, shorter tests are likely to be less expensive.
- Small size - Small animals often have short generation times and require less space for their culture or for conducting tests. Small test systems also reduce the quantity of test compound that is required. In some cases, small size may be a disadvantage if bioaccumulation of chemicals or biomarkers of exposure are being measured as test end points.
- Multiple life-stages - Easily identifiable life-stages means that potentially more sensitive juvenile stages can be tested which may give a more accurate indication of the potential impact of a contaminant.
- Ecological or economic relevance - Organisms with a widespread distribution are often favoured as test species as this increases the geographical range over which a species might be considered representative.
- Relative sensitivity - In risk assessment, it is considered that if the most sensitive species is unaffected by a contaminant then the entire community will be protected. While the existence of a most sensitive species is a matter for debate (Maltby & Calow, 1989; Cairns & Pratt, 1989), it is imperative that, before recommending a particular test species, comparative information on the reliability of responses and discrimination between chemicals of interest is evaluated.
- Standardisation - Toxicity tests giving repeatable results under defined environmental

conditions both within and between laboratories increase their reliability and acceptance by regulatory bodies. Inter-laboratory calibration is used to verify standardisation between laboratories, but are typically only carried out for established test species.

1.4 Marine Harpacticoid Copepods as Model Test Organisms

In a review of the use of marine meiofauna for assessing the effects of contaminants in single-species tests more than half the tests were carried out using copepods (Coull & Chandler, 1992). Harpacticoid copepods possess many attributes that make them ideal as toxicity test organisms and have been used for evaluating the effects of marine contaminants for more than 20 years (Bengtsson, 1978). In general, they are small, have fast development times and are easily cultured under laboratory conditions. As discussed previously, harpacticoid copepods have been highlighted as being sensitive to pollution in community-level environmental assessment of marine systems (Section 1.2). Early studies, using harpacticoid copepods as test organisms, focused on the acute toxicity of contaminants (Bengtsson, 1978; Moraïtou-Apostolopoulou, 1978). Larval life-stages of harpacticoid copepods are recognised easily and potentially are more vulnerable to pollutant exposure than adults, therefore, the differential sensitivity of life-stages (nauplii and copepodids) to selected pollutants are often reported (Verriopoulous & Moraïtou-Apostolopoulou, 1982; Hutchinson & Williams, 1989; Green *et al*, 1996). The value of these tests is now recognised by regulatory bodies and acute test methods using the copepodid stages of *Tisbe battagliai* have been included as an international standard method (ISO, 1998). In fact, short-term tests (96 h) using the harpacticoid *T. battagliai* have been used for *in situ* bio-monitoring of effluents from a contaminant gradient in the German Bight (North Sea) in shipboard experiments (Williams, 1992). Astley *et al* (1999) also found that acute tests using *T. battagliai* were more sensitive as a biomonitor of pollution than more conventional biomarker techniques (measures of crab glutathione reductase activity and metallothionein

induction) in an assessment of contaminated sites from the Tees Estuary.

While acute toxicity tests have value as a screen for contaminant effects, it is now generally accepted that the long-term effects of a contaminant cannot be extrapolated from acute toxicity data (Cairns *et al*, 1993). Sub-lethal effects will also give more information on the mechanism of toxicity of a contaminant. Harpacticoid copepods were found to be ideal for use in chronic exposures, and methods have been developed to assess the sub-lethal effects of contaminants on both development to adult (Le Dean & Devineau, 1987) and reproduction of copepods (Bengtsson & Bergström, 1987; Chandler, 1990). More recently, the use of life-cycle tests which incorporate endpoints for development to adult and reproduction are being advocated in view of evidence for the potential endocrine disrupting effects of some chemicals (deFur *et al*, 1999; Hutchinson *et al*, 1999a, b). Such tests include aspects of embryonic, gonadal and larval development, each of which is subject to endocrine regulation. A number of chemicals, including those suspected of affecting both vertebrate (17β-oestradiol and diethylstilbesterol) and invertebrate (20-hydroxyecdysone, diflubenzuron) endocrine function, have already been screened in copepod life-cycle tests (Wright *et al*, 1996; Hutchinson *et al*, 1999a, b). It has been suggested that full life-table analysis be used in pollution studies. The life-cycle test is extended to obtain information on the age-specific survival, development and reproduction of a cohort of individuals which is used to calculate the intrinsic rate of natural increase for the population (Chapman, 1995a). Endpoints obtained from life-table studies may have greater ecological relevance than short-term toxicity tests because they consider effects on the whole population rather than on individuals and also have the potential to highlight differences between treatments even when individual variability is high (Bechmann, 1994). Full life-tables were used successfully to evaluate the effects of copper (Bechmann, 1994) on *T. furcata*, and nonylphenol

(Bechmann, 1999) and pentachlorophenol (PCP) on *T. battagliai* (Williams, 1997). Abbreviated life-table analysis has been incorporated into sediment testing regimes to assess the effects of chlorpyrifos on the infaunal harpacticoid *Amphiascus tenuiremis* (Green & Chandler, 1996). There is, however, evidence to suggest that increasing the length of the bioassay does not necessarily increase its sensitivity (Lotufo, 1997; Lotufo & Fleeger, 1997; Kovatch *et al*, 1999). It is difficult to argue against the use of harpacticoid copepods as model test organisms. For example, populations of *T. battagliai* were entirely suited for testing complex models of density dependence on the effects of exposure to pentachlorophenol (PCP) (Sibly *et al*, 2000). Test guidelines incorporating survival and reproduction of pelagic marine crustaceans including harpacticoid copepods have been identified as a priority for development by the Organisation for Economic Co-operation and Development (OECD, 1998a). In view of the applicability of harpacticoid copepods for marine pollution assessment, it is surprising that there is no equivalent representative test species from the freshwater meiofauna.

1.5 Freshwater Copepods as Test Organisms

Almost without exception, toxicity tests using freshwater copepods have been either acute or short-term chronic development assays. The majority of available data refer to the effects of trace metals where toxicity appears to be dependant on life stage (Burton, 1998), previous exposure (Lalande & Pinel-Alloul, 1986; Burton, 1998) or species (Lalande & Pinel-Alloul, 1986). In general, copepods appear to be amongst the most sensitive crustacean species to trace metal exposure, although they are generally more tolerant than daphnids (Baudouin & Scoppa, 1974; Notenboom *et al*, 1992; Burton, 1998). Mode of life and feeding strategies are probably important in dictating differential sensitivity both within and between species. For example, the predatory cyclopoid *Cyclops abyssorum* was more tolerant to zinc, copper, cadmium and mercury than the cyclopoid *Tropocyclops prasinus*

mexicanus or daphnids, which are both filter feeders (Baudouin & Scoppa, 1974; Lalande & Pinel-Alloul, 1986). Adult *Diaptomus clavipes* were found to be much more tolerant to acridine than daphnids. This increased tolerance was suggested to be due to the fact that on achieving adulthood, copepods cease to moult while cladocerans continue to moult through their life (Cooney & Gehrs, 1984). The fact that adult copepods do not moult may also explain the increased sensitivity of juvenile life stages of freshwater copepods to contaminants (Cooney & Gehrs, 1984; Burton, 1998; Willis, 1999). In contrast, the acute toxicity of synthetic pyrethroids to freshwater copepods appears to be in the same range as observed for cladocerans (Day, 1989). Calanoid and cyclopoid copepods from New Zealand lakes and the groundwater harpacticoid *Parastenocaris germanica* show relatively more sensitivity to the reference toxicant pentachlorophenol (PCP) compared with other freshwater invertebrates (Notenboom *et al*, 1992; Willis, 1999). Copepods also vary in their responses to contaminants depending on the part of the water body they inhabit. For example, it has been suggested that groundwater species may show enhanced sensitivity to some contaminants because of the additional stress of low oxygen conditions (Mösslacher, 2000). Mösslacher (2000) compared the relative sensitivity of groundwater species and related surface-living animals to inorganic nutrient enrichment as potassium chloride (KCl) and potassium nitrate (KNO_3). Tolerance of Crustaceans to KCl and KNO_3 displayed the following pattern, ostracods > copepods > isopods with the sensitivity of different Crustacean groups being more important than the influence of groundwater verses surface-living species. Alternatively, it has been suggested that the groundwater harpacticoid *Parastenocaris germanica* showed increased sensitivity to PCP because it is adapted to low oxygen groundwater conditions and that this chemical acts by uncoupling respiration (Notenboom *et al*, 1992).

Most other toxicity data for freshwater copepods originates from mesocosm studies where

the numbers of surviving adults and nauplii at selected time periods are used as an endpoint. Many of these studies do not distinguish between species of copepod, therefore, it is difficult to make statements about individual species sensitivity. This lack of species differentiation has been recognised as a drawback of mesocosm tests (Girling *et al*, 2000). The majority of these studies also concentrate on zooplankton rather than meiobenthic communities. In mesocosms, planktonic copepods have shown sensitivity particularly to exposure to insecticides including lindane (Fliedner & Klein, 1996; Peither *et al*, 1996), chlorpyrifos (Brock *et al*, 1992; Zrum *et al*, 2000), bifenthrin (Hoagland *et al*, 1993) and permethrin (Kaushik *et al*, 1985). Reports of differential sensitivity of different zooplankton are variable with a general trend towards large-bodied zooplankton showing greater sensitivity to pesticides (Hanazato, 2001). In many studies, however, copepods appear to be at least as sensitive to contaminant exposure as cladocerans (Kaushik *et al*, 1985; Hoagland *et al*. 1993; Gillespie *et al*, 1997). In the majority of mesocosms, where adult and naupliar sensitivity is compared, adults are usually less sensitive to toxicant exposure than larval stages, corroborating evidence from single-species tests (Brock *et al*, 1992). Considering the ecological relevance and practical applicability of freshwater copepods to toxicity testing regimes, it is surprising that more information for these taxa is not available. This lack of information is particularly remiss in view of their widespread use in mesocosm studies, as it is prudent to understand the response of individual species if contaminant effects and inter-species relationships in more complex systems (mesocosms and field studies) are to be fully understood (Rundle, 1993). Few studies have attempted to compare the responses to pollutants of individual species of copepod with respect to effects observed in mesocosms (Day, 1989; Burton, 1998; Willis, 1999). As the information currently available refers to different species and a range of contaminants, it is difficult to make generalised conclusions on the responses of freshwater copepods to pollutant exposure.

1.6 *Bryocamptus zschokkei* (Schmeil, 1893) as a Representative Meiofaunal Test Species

As discussed previously, the major requirements of a test species are their ecological relevance and relative sensitivity to contaminant exposure. *Bryocamptus zschokkei* was identified as a sensitive indicator of trace metal pollution from stream community analysis and, as such, was considered a key species for further investigation under controlled laboratory conditions (Burton, 1998). The acute and chronic (> 10 days) effects of copper were evaluated using survival, development to adult and fecundity as test endpoints. Chronic effects on survival of this copepod in toxicity tests appeared to reflect the declines observed in field populations, suggesting that this species may be highly relevant for assessing the effects of contaminants in stream sediments (Burton, 1998).

Bryocamptus zschokkei also possesses many other attributes that support its further development as a test organism. This harpacticoid has a widespread distribution with a range that extends over the entire holarctic (Rundle *et al*, 2000). It occurs in a range of habitats including lakes (Anderson & DeHenau, 1980), hyporheic streams, ponds and springs (Shiozawa, 1986; Rundle, 1993; Fryer, 1993), and brackish waters (Amhed-Abada, 2000). *Bryocamptus zschokkei* is considered a core constituent of stream microcrustacean communities, with densities of 8221 per m^2 being recorded in streams of southwest England (Burton, 1998). It is present all year round with peaks of abundance during late summer / autumn (Rundle, 1990; 1993). It favours well oxygenated, fast-flowing waters (Gurney, 1932; O'Doherty, 1985) although it has also been recorded at depths of up to 70 cm in well oxygenated interstitial sediments (Kowarc, 1992). *Bryocamptus zschokkei* is usually found associated with organic matter including leaf material and bryophyte flushes that allow shelter from high flow conditions (O'Doherty, 1985; Fryer, 1993). The distribution of *B. zschokkei* in the field is determined by both physiochemical parameters, pH and aluminium concentration (Rundle, 1990; Rundle & Ormerod, 1991; Rundle, 1993) and physical

parameters (they show a preference for grassy margins over riffles) (Rundle & Ormerod, 1991; Suren, 1992). While the distribution of this harpacticoid is relatively well characterised, other aspects of the basic biology of *B. zschokkei* are still unclear; for example, it is not known what this copepod feeds on in its natural environment. Detritus has been considered to be the major food source for sediment-dwelling harpacticoids, although there is increasing evidence (from marine studies) that copepods can feed selectively on individual particles (Marcotte, 1984). Algae, protozoa, fungi and bacteria have been proposed as potential food sources for *B. zschokkei* within the stream benthos (O'Doherty, 1985). It is also known that food utilisation by harpacticoid copepods may vary with season (Lee *et al*, 1976) or stage of development (Decho & Fleeger, 1988). A greater insight on how basic environmental parameters affect the life history of *B. zschokkei* is required if culture conditions are to reflect environmental conditions and in order to help interpret some of the more subtle effects of contaminants.

Bryocamptus zschokkei may also be important in terms of overall productivity in streams. For example, an estimate of dissolved organic carbon (DOC) production in a stream in South Carolina for *B. zschokkei* was 396 mg m^{-2} yr^{-1} based on copepod biomass of 22 mg dry mass m^{-2} (O'Doherty, 1985). This was similar to estimates of production of 500 mg m^{-2} yr^{-1} for the dominant leaf-shredding stonefly larvae *Peltopera maria* in the same stream system (Meyer & O'Hop, 1983). Although *B. zschokkei* is not always recorded at the densities recorded in South Carolina (22 mg dry mass m^{-2}), such high levels of DOC production by this copepod suggests that it may have a considerable influence on stream sediment productivity. Grazing by stream harpacticoids has also been shown to enhance microbial activity on the surface of leaf material, which could result in an increase in stream secondary production (Perlmutter & Meyer, 1991). In addition, meiofaunal crustaceans (including *Bryocamptus* spp.) have been found to contribute to the diets of both

macroinvertebrates (Lancaster & Robertson, 1995) and bottom-dwelling fish (Rundle & Hildrew, 1992).

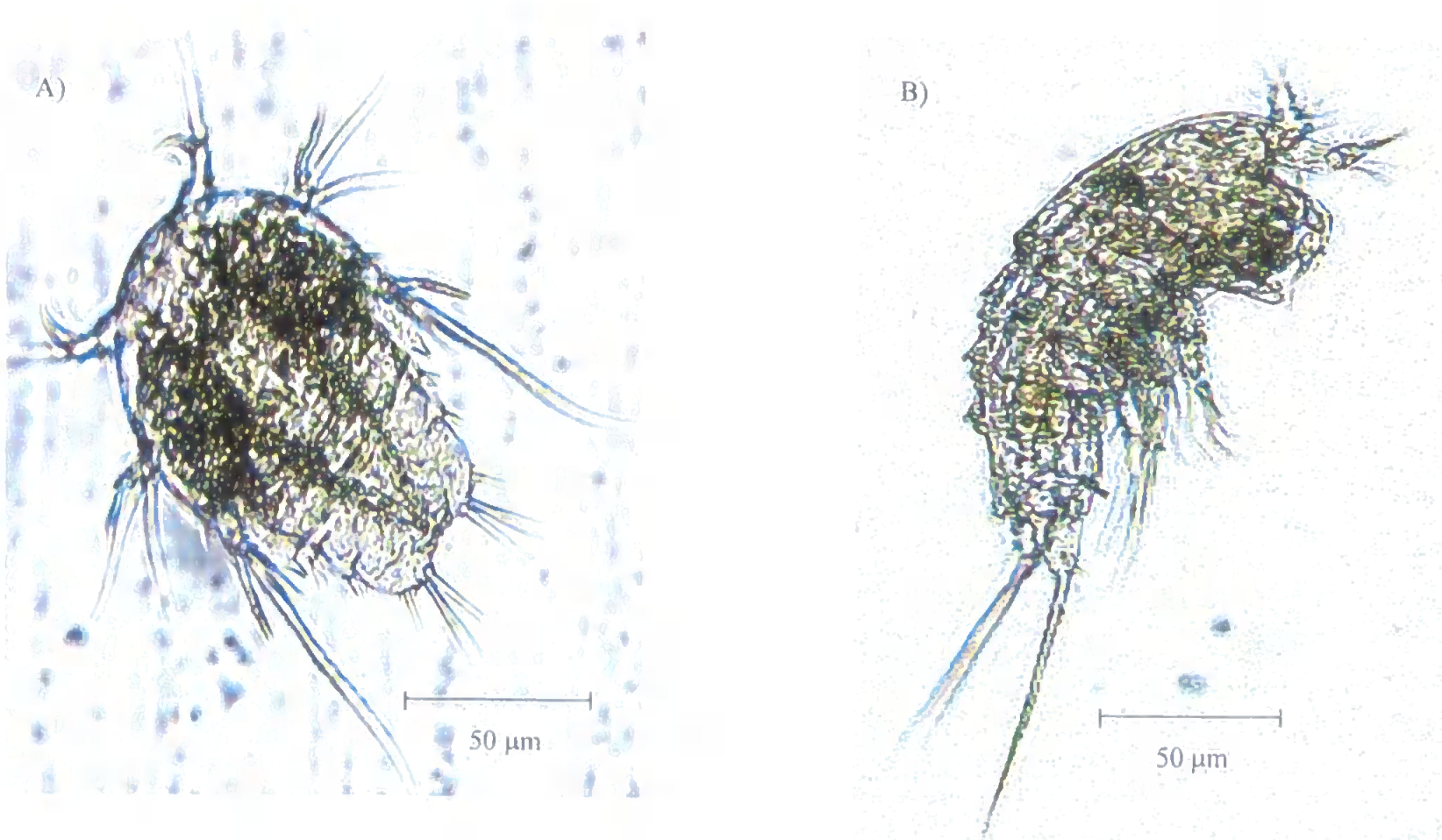
The ability to maintain a species in the laboratory is also a pre-requisite of a test organism. *Bryocamptus zschokkei* have been reared successfully in the laboratory on a diet of beech leaf material taken directly from a stream (O'Doherty, 1985; Burton, 1998). Other copepods in this genus which have been amenable to laboratory culture include *B. echinatus* (on a diet of decaying plant material) (Sarvala, 1979; Kowarc, 1990) and *B. minutis* (fed a mixed diet of protists, rotifers, detritus and benthic algae) (Monocov, 1972, 1976 cited in Sarvala, 1998).

Bryocamptus zschokkei demonstrates typical harpacticoid development, moulting through six naupliar stages (N1, N2, N3, N4, N5, N6) and five copepodid stages (C1, C2, C3, C4, C5), with the sixth copepodid moult corresponding to the adult (Sarvala, 1979). The naupliar stages are morphologically distinct from copepodid stages (Fig. 1.1). The life cycle of *B. zschokkei* is relatively short with reported development times to adult ranging from 25 to 35 days at 15°C, although individuals have been known to have a life span of up to 370 days at 18°C (O'Doherty, 1985; Burton, 1998). Burton (1998) ran developmental and reproductive toxicity assays using *B. zschokkei* which took six weeks at 15°C. As *B. zschokkei* is sexually reproducing, endpoints such as sex ratio or male and female abnormalities (e.g. intersex) could be incorporated into toxicity test designs. Males and females are easily differentiated, both from the distinct shape of their antennae and because of their sexual dimorphism; males are smaller, about 0.42 mm long while the larger females range from to 0.54-0.60 mm in length (Gurney, 1932). Pairing of copepods rarely occurs before the animals are fully mature (O'Doherty, 1985; personnel observation) when males are observed clasping the female by their caudal rami (Fig. 1.2). Fertilisation of broods

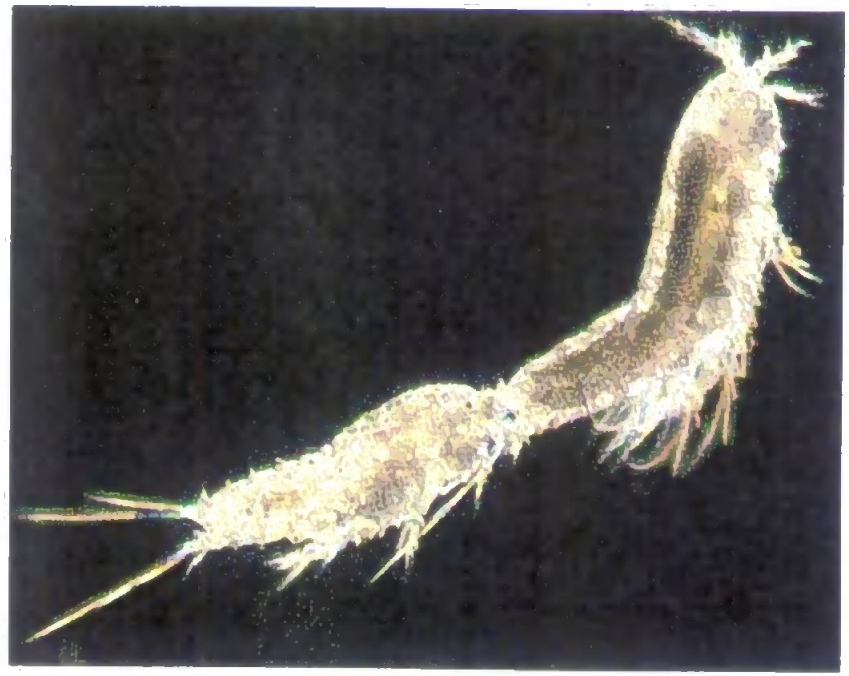
occurs via spermatophore-mediated transfer, from which multiple broods can be fertilised (O'Doherty, 1985). Once fertilisation has occurred, there is the potential to monitor reproduction of females, in isolation from males, at least for short periods of time. Females carry an external egg sac (Fig. 1.3) and, therefore, it should be possible to continually monitor any effects of potential stressors on each individual brood. *Bryocamptus zschokkei* has a life-history of continuous iteroparity in which a number of small broods of offspring (up to 40 offspring per brood) are produced over their entire life (O'Doherty, 1985, Rundle, 1993). Offspring production is less seasonal than other species and *B. zschokkei* is known to reproduce over the entire year in southern England (Rundle, 1990), therefore there should always be animals available for initiating cultures or conducting toxicity tests.

1.7 Outline of Thesis

It is apparent that a representative test species from the meiofauna of lotic systems would increase the choice and relevance of current testing regimes. The general aims of this thesis were, therefore, to evaluate the use of a meiofaunal copepod (*Bryocamptus zschokkei*) as an ecologically-relevant test species to measure developmental and reproductive effects of toxicants released into the freshwater environment. As the general biology of this copepod is relatively poorly understood (as with the majority of freshwater meiofauna) the first half of this thesis concentrates on optimising culture conditions for *B. zschokkei*, by developing testing regimes and evaluating the sub-lethal effects of environmental parameters (water hardness and detritus type). The effects of water hardness on the development to adult of *B. zschokkei* and general culture techniques are described in Chapter 2. This chapter also considers the applicability of two models of development (isochronal vs. equiproportional), that have been described previously for marine harpacticoid and freshwater calanoid copepods. Chapter 3 assesses the effects of water hardness on several reproductive parameters of *B. zschokkei* including embryonic development time and the numbers of eggs



igure 1.1: The morphologically distinct A) naupliar and B) copepodid stages of *Bryocamptus zschokkei*.



igure 1.2: Male *Bryocamptus zschokkei* (left) clasping the female (right) by her caudal rami.

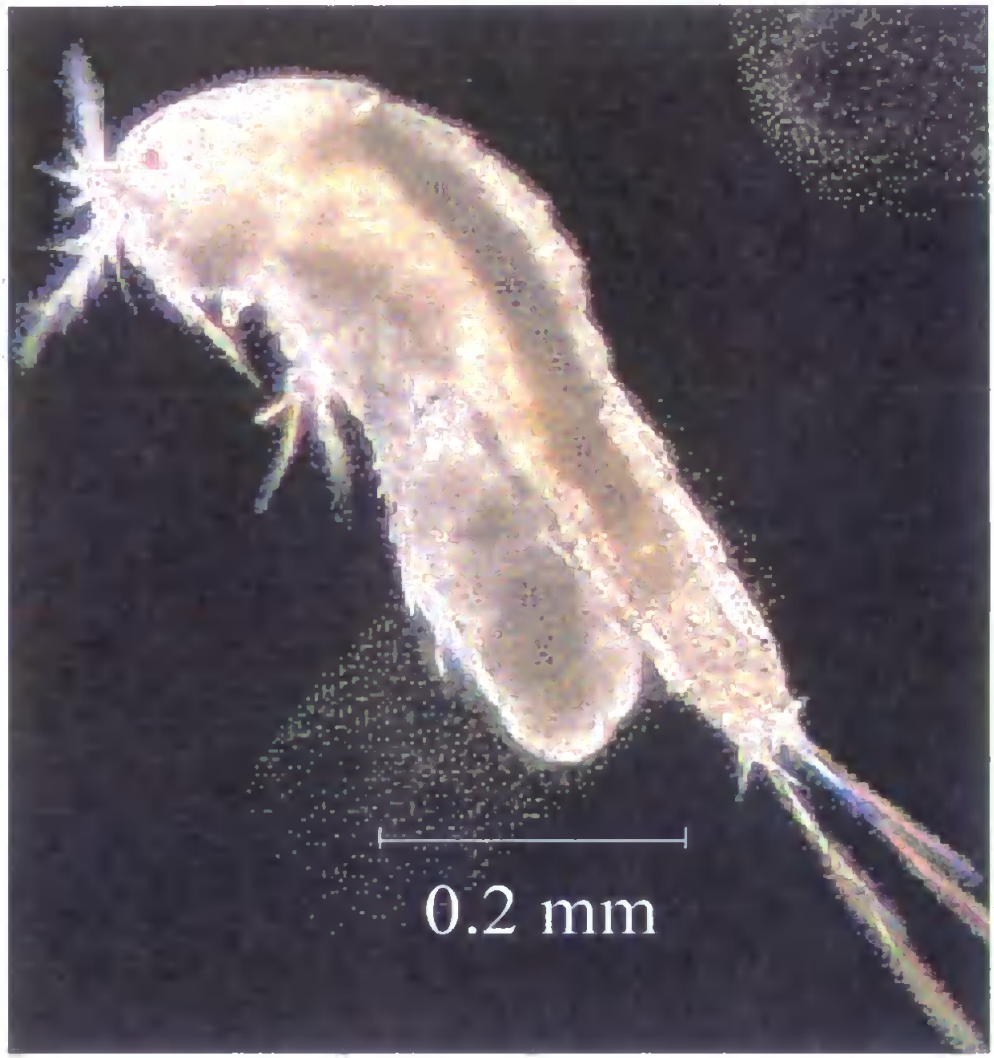


Figure 1.3: Female *Bryocamptus zschokkei* carrying her egg sac externally

and nauplii per female and reports baseline data on the reproduction of *B. zschokkei* under laboratory conditions. The effects of different types of detritus as potential food sources for *B. zschokkei* were considered in Chapter 4, by recording their effects on copepod development and reproduction in two separate experiments. Electron photo-micrographs were obtained using scanning electron microscopy for the different detrital types and the surface microflora was quantified using image analysis. The area of microbial coverage and density of bacteria on the detritus were used as an indication of food quality, suggesting the amount of potential food available for *B. zschokkei* to browse upon.

In the second half of this thesis, the development and reproduction bioassays described in

Chapters 2-4 are used in a single life-cycle test for assessing the effects of contaminants on *B. zschokkei*. As one of the major requirements of a test species is to have a database of their responses to a range of chemicals, the sensitivity of *B. zschokkei* to three potential toxicants (20-hydroxyecdysone, zinc and lindane) suspected of exerting their effects on developmental or reproductive parameters was evaluated. Chapter 5 considers the effects of the arthropod moulting hormone 20-hydroxyecdysone (20-HE), which might be expected to have an effect on copepod development, on *B. zschokkei*. The acute toxicity to different life-stages of *B. zschokkei* (nauplii, copepodid and adult) and chronic effects of zinc are considered in Chapter 6 incorporating recommended improvements to the life-cycle test design suggested in Chapter 5. The final experimental chapter reports the acute and chronic effects of the pesticide lindane to *B. zschokkei* (Chapter 7). Chapter 8 provides a critical evaluation of the potential of *B. zschokkei* for future use as a test organism.

Chapter 2

Influence of water hardness on the post-embryonic development of *Bryocamptus zschokkei*

2 Abstract

Prior to the use of any species in environmental monitoring it is important to understand the effects on their life history of natural variation in water chemistry before considering any potential influence of contaminants. The effect of water hardness (between 10 and 150 mg l^{-1} as $CaCO_3$), a key environmental variable in freshwater systems, on the survival and development times of *Bryocamptus zschokkei* are reported here. Excessive handling of the naupliar stages of *B. zschokkei* resulted in a significant reduction in their survival and increased development times compared with lower handling frequency. When handling was reduced, the post-embryonic development of *B. zschokkei* (17.9 days at 20°C) was relatively quicker than previous estimates for this species (30.3 days at 18°C, O'Doherty, 1985). Hardness had no effect on juvenile survival or development times. The data obtained were used to test the application of two copepod development models, isochronal (equal duration of each moult stage) vs. equiproportional (each moult stage represents a known proportion of the total development time) to the development of this copepod. *Bryocamptus zschokkei* exhibited equiproportional development, and more time was spent in the later copepodid stages (C4 and C5), irrespective of hardness. As some toxicants might be expected to disrupt the normal moulting pattern, this model of equiproportional development may have application in interpreting the mode of action of contaminants in toxicity tests.

2.1 Introduction

Before investigating the potential impact of contaminants on a species, it is essential to understand fully their biological response to natural variations in water chemistry. The development and growth rates of freshwater copepods are influenced by environmental conditions but most of the data are for calanoid and cyclopoid copepods (Hart 1990; Maier, 1994; Twombly *et al*, 1998), with similar information for harpacticoids being relatively

scarce (Sarvala, 1979). Indeed, details on the life-history strategies of harpacticoid copepods are limited (Rundle, 1993; Robertson, 2000). The response of ontogenetic development to increasing temperature has been characterised for a few species of freshwater harpacticoid (Sarvala, 1979, O'Doherty, 1985), but it is not known how this group responds to other environmental variables such as food quality or the ionic composition of the dilution water. Water hardness is one of the most important chemical parameters in shaping freshwater assemblages, and has been shown to correlate positively with shifts in the distribution and abundance of freshwater zooplankton (Tessier & Horwitz, 1990), stream macroinvertebrates (Wright *et al*, 1984) and benthic microcrustaceans (Rundle, 1990; Rundle & Hildrew, 1990; Fryer, 1993). Rundle & Ramsay (1997) demonstrated large-scale geographic differences in the community composition of stream microcrustaceans as a result of low mineral concentrations, regardless of pH and ionic concentration. Calcium concentrations also influence the density and life-history parameters of field populations of individual species of amphipods (Økland & Økland, 1985; Gibbons & Mackie, 1991; Meyran, 1997), cladocerans (Hessen *et al*, 1995) and microcrustaceans (Fryer, 1993). In crustaceans, calcium is considered an essential element and, after moulting, a rapid uptake of this mineral is required for hardening of the exoskeleton (Stevenson, 1985; Alstad *et al*, 1999). Calcium is obtained directly either from food or water. For example, the calcium content of the water flea *Daphnia magna* can be related directly to the chemical composition of their dilution medium (Cowgill *et al*, 1986). Development might, therefore, be vulnerable to perturbation as a result of changes in water hardness, which can alter the bioavailability of calcium. For example, field populations of the amphipod *Gammarus fossarum* were larger and had a longer moult cycle from water with high compared with low water hardness (Meyran, 1997). Delayed maturation of *D. magna* (Lewis & Maki, 1981) and low juvenile survival of the North American fairy shrimp,

Streptocephalus seali, have been observed in soft compared to hard water (Anderson & Hsu, 1990).

Development patterns, including information on the duration of individual moult stages of several species of calanoid and cyclopoid copepods have been documented (Hart, 1990; van den Bosch & Gabriel, 1994). Two models of copepod development have been proposed for these groups, 'isochronal' and 'equiproportional development'. Isochronal development is a life-cycle with equal duration of each of the various moult stages. This model appears to be rare for copepods (Hart, 1990). Equiproportional development is where the duration of each development stage is a fixed proportion of the total development time, and is independent of temperature and food availability. The latter model is thought to fit most copepods (Hart, 1990), however, neither model has been validated for freshwater harpacticoids.

In order to obtain a more thorough understanding of the life history of *B. zschokkei* in response to variation in water chemistry, and to define culture and testing regimes the aims of this chapter were to:

1. assess the effects of water hardness on the post-embryonic development times of this copepod,
2. obtain baseline data for development times to the first copepodid stage, to adult and for individual copepodid stages, and to
3. determine if models of 'isochronal' or 'equiproportional' development are applicable for this species of harpacticoid.

2.2 Materials and Methods

2.2.1 Copepod Cultures

Cultures were initiated using animals sampled on 28 July 1998 from the River Yealm at Hele Cross, Cornwood, Devon (grid reference 50°25'90N, 4°57'50W). Samples were taken using a kick net (63 μm mesh) throughout the stream, and passed through 500 μm and 63 μm sieves. The smaller biological fraction was transported back to the laboratory where copepods used to initiate cultures, were sorted individually under a dissecting microscope, using a Pasteur pipette. *Bryocamptus zschokkei* were maintained in 13.5 cm diameter 1 litre capacity glass crystallising dishes (cultures) which were covered with a loose fitting lid, to reduce evaporation. Approximately one hundred adults of mixed sexes were used to seed each stock population. Cultures were initially maintained in a constant temperature room at 18 (± 1.0)°C which was increased to 20 (± 1.0)°C after a period of acclimation. The light regime was 16 h light and 8 h dark with a 20 min dawn dusk transition time. Water from the stream where the copepods were sampled was used as the culture medium. This was filtered through a 0.2 μm mesh to remove protozoa, algae, fungi and small metazoans, and measurements of pH, hardness, alkalinity and the concentrations of water soluble heavy metals were made prior to use. Approximately 400 ml of water was added to each culture vessel giving a water depth of ca 5 cm. Partial replacement (250 ml) of media was carried out three times per week and the pH (240 pH meter, Corning, USA) and dissolved oxygen (58 meter, YSI Incorporated, USA) content of the water removed was obtained. Water temperatures and general observations on the condition of the animals, including mortalities and the number of dropped egg sacs, were recorded daily for the first three months (until cultures were established) after which observations were performed three times per week.

Initially, animals were provided a food source of naturally conditioned leaves taken from the stream where they were obtained (River Yealm, 28 July 1998). Beech leaves (*Fagus sylvatica* L.) were chosen as these have previously been shown to support growth and reproduction of *B. zschokkei* (O'Doherty, 1985; Burton, 1998). The leaves were frozen before use at -20°C for 48 h, in order to kill any associated fauna, but maintain the associated microbial community. Leaves were renewed when necessary (i.e. if the leaf material became shrivelled or degraded). A procedure for artificially conditioning leaf material was developed to increase standardisation (see Section 2.2.3). Cultures were maintained at 20 (± 1)°C which corresponds to maximum July temperatures in rivers where *B. zschokkei* has been sampled (Rundle, 1990). Rundle and Hildrew (1990) found that *B. zschokkei* was more abundant in warmer waters and it is well established that, within their tolerance range, harpacticoid development is accelerated at high temperatures (Sarvala, 1979; Hicks and Coull, 1983). A temperature of 20°C is also recommended for regulatory testing of other freshwater Crustacea by the American Society for Testing and Materials (1999a, b).

2.2.2 Conditioning Leaves

To standardise the feeding regime for *B. zschokkei*, copepods were supplied with artificially conditioned leaves. Beech leaves (*F. sylvatica* L.) were obtained from a single tree in Churston Woods, Brixham, Devon (grid reference 50°24'15N, 4°31'30W) on the 3 September 1998. Only whole leaves with no visual signs of pathogen infection, were used as a food source. The leaves were oven dried at 60°C for 48 h and then stored in a sealed polythene container at 20°C. Before use, leaves were rehydrated in Elendt's M4 medium (Elendt & Bias, 1990), which had been aged for at least seven days. As it takes about two weeks immersion in water for leeching of toxins and microbial colonisation of leaves to

occur (Allan, 1995), leaves were maintained in continuously aerated Elendt's medium for 14 days; the medium was replaced every seven days.

2.2.3 Test Medium

The advantages of culturing and testing Crustacea in a fully defined medium are well established (Cowgill *et al*, 1986; Vijverberg, 1989). A reconstituted medium also facilitates the manipulation of physiochemical parameters by allowing alteration of the concentrations of the mineral salts that contribute hardness and alkalinity. Elendt's M4 appears to be one of the most appropriate culture media for the maintenance of *Daphnia magna*, allowing long-term culture with no apparent signs of reduced viability or reproduction (Elendt & Bias, 1990; Bradley *et al*, 1993). Good reproducibility of toxicity tests between laboratories using this medium has been demonstrated and it is now recommended as a standard medium in regulatory test guidelines (OECD, 1998b). Modifications of Elendt's medium have been made for the culture and testing of the amphipod *Hyalella azteca* (Borgmann, 1996) and the marine copepod, *Acartia tonsa* (Ole Kusk & Wollenberger, 1999). Elendt's M4 medium was therefore chosen for use in the present experiment. Elendt's M4 has hardness of 200 mg l^{-1}, an alkalinity of 30 mg l^{-1} (both as $CaCO_3$), conductivity of 585 $\mu s\ cm^{-1}$ and pH 7.9. All these values are much higher than those recorded in the stream water from which *B. zschokkei* were obtained (Table 2.1). The mineral salts that contribute to water hardness and alkalinity in Elendt's M4 medium are $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KCl and $NaHCO_3$. For use with *B. zschokkei*, these salts were diluted using distilled water, prior to the addition of other essential elements to obtain a medium of the required hardness. In the investigations concerning water hardness (Chapters 2 & 3) each dilution water treatment is referred to in terms of its hardness, but it should also be noted that alkalinity, conductivity and pH also vary with treatment (Table 2.1). Experimental treatments comprised reconstituted water with mineral salts diluted to give a range of hardness conditions of 10, 50, 100 and 150

mg l^{-1} (expressed as $CaCO_3$) and a reference of filtered stream water (0.2 μm) acted as a control (Table 2.1). The stream water was collected from the River Yealm (Section 2.2.2). All culture media were aged for at least one week prior to use, after which, they were kept in the dark to prevent any excessive growth of micro-algae.

Table 2.1: Water quality parameters for the five hardness treatments

Treatment (expressed as nominal hardness)	Measured parameters			
	pH	Conductivity (μs cm^{-1})	Hardness (mg l^{-1} $CaCO_3$)	Alkalinity (mg l^{-1} $CaCO_3$)
Stream water (9.3)	6.72	063	9.3	4.4
10	7.05	043	10.7	6.4
50	7.44	163	56.3	13.8
100	7.70	297	108.3	19.2
150	7.81	443	155.7	26.8

2.2.4 Post-Embryonic Development

One hundred ovigerous females were separated from the stock population (Section 2.2.1) which had been maintained in a culture medium of filtered (0.2 μm) stream water for seven months. These females were kept in a loosely-covered crystallising dish containing stream water and pre-conditioned leaf material (half a leaf). Animals were monitored every 24 h and, if nauplii were present, the females were transferred into a new vessel containing fresh food and the same medium. This procedure was continued until there were about one hundred <24 h old nauplii, which were used to initiate each experiment. Individual nauplii were placed in 1 cm diameter polystyrene Cellwells™ (Dow Corning, Corning, NY, USA) containing 2 ml of medium of appropriate hardness and a 4 mm diameter leaf disc pre-conditioned in Elendt's medium for 14 days. At each water hardness there were 18 replicate nauplii, maintained in a temperature-controlled room at 20 (± 1.0)°C with a photoperiod of 16 h light:8 h dark, and a 20 min dawn-dusk transition time. A partial water replacement (50%) was carried out three times per week, and the dissolved oxygen content and pH of

the water were measured before discarding. In order to ensure an adequate supply of food, every 14 days, copepods were transferred into new Cellwells™ containing fresh media and a new leaf disc. Copepod survival, and the presence of moulted exuviae, recorded daily using a Wild M8 binocular microscope under darkfield illumination (magnification × 20), were used to calculate the duration of the combined naupliar stages (Dn), duration of each individual copepodid stage, the total copepodid development (Dc) and the time taken to reach the adult stage (N1-A). The ratio of males to females was also determined at the end of the experiment.

The early naupliar stages of *B. zschokkei* are transparent and very small; N1 is about 70 μm long (Sarvala, 1977). It was, therefore, difficult to locate the early naupliar stages without dislodging the animal from the leaf disc. Initially, the experiment was carried out using daily observations of the nauplii (high-handling frequency). Mortality of nauplii was high during this experiment and development to adult took longer than expected based on previous data (Burton, 1998). Consequently, a further experiment, with low-handling frequency was performed; nauplii were observed on day 1, to determine that they had been transferred successfully into the cell well, with the next observation on day 7 (low-handling frequency). Daily observations were then made until the end of the experiment. The 'high-handling frequency' experiment was run for 45 days because of the longer time taken for animals to develop to adult. The second 'low-handling frequency' experiment was terminated after 35 days.

2.2.5 Statistical Analyses

Fisher's Exact Test was used to isolate significant differences between treatments in copepod survival at the end of each experiment. All data were tested for normality (Shapiro-Wilks) and equality of variances (Bartlett's test) before applying analysis of

variance techniques. If data did not conform to these assumptions, then they were $\log_{10}$ transformed. One-way ANOVA was carried out to consider differences in the time taken to moult to C1 and adult, and the time taken to moult from C1 to adult, both between and within experiments. If the assumptions of ANOVA were still not met even after transformation, data were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Wilcoxon's test to highlight which treatments were different. To test the model of equiproportional development, two-way ANOVAs were carried out to identify any effects of handling frequency or hardness, stage and their interaction on arc-sine, square root transformed data of the proportion of the development time spent in each copepodid stage. Any data that were still not normally distributed were confirmed using the Kruskal-Wallis test. Chi Square tests were carried out to consider if hardness or handling frequency influenced sex ratio. All statistical tests were carried out to detect differences at the 5% level unless stated otherwise.

2.3 Results

2.3.1 Handling Frequency

In the 'low-handling frequency' experiment, survival was significantly higher than in the 'high-handling frequency' (Fisher Exact, $p<0.05$). At 'low-handling frequency', there was no mortality in the stream water treatment and survival ranged from 72-83% at all hardness levels (Table 2.2). When the handling frequency was high, fewer than 60% of copepods survived to day 28 and survival at day 45 (conclusion of experiment) ranged from 29% at 100 mg l^{-1} $CaCO_3$ to 59% at 10 mg l^{-1} $CaCO_3$ (Table 2.3). Development times were also significantly longer when the handling frequency was high compared with low (Kruskal-Wallis, $p<0.05$). For example, the mean (± 1SD) time taken to attain adulthood in the stream water treatment was 36.2 (± 4.1) days, when nauplii were observed daily and 17.9 (± 3.6) days when handling of nauplii was reduced, representing a halving of the development

times. The handling of nauplii had subsequent effects on overall development with longer copepodid development times, as indicated by high Dc/Dn ratios, at high-handling frequency compared with when less frequent observations (where Dc is the copepodid development time and Dn is the naupliar development time) (Tables 2.2 and 2.3).

2.3.2 Water Hardness

There were no significant effects of hardness on the survival of juvenile animals in either experiment (Table 2.2 & 2.3). There was also no significant difference in the combined naupliar and copepodid development times, or in the time taken to develop to adult in either experiment (Fig. 2.1). For males, development times were significantly longer compared with females (Student's t-test, $p<0.05$) (Fig. 2.2). If the duration of individual stages was considered, C5 was significantly longer than all the other stages, and stage C4 was longer than stages C2 and C3 irrespective of treatment (Fig. 2.3) (Kruskal-Wallis, $p<0.05$).

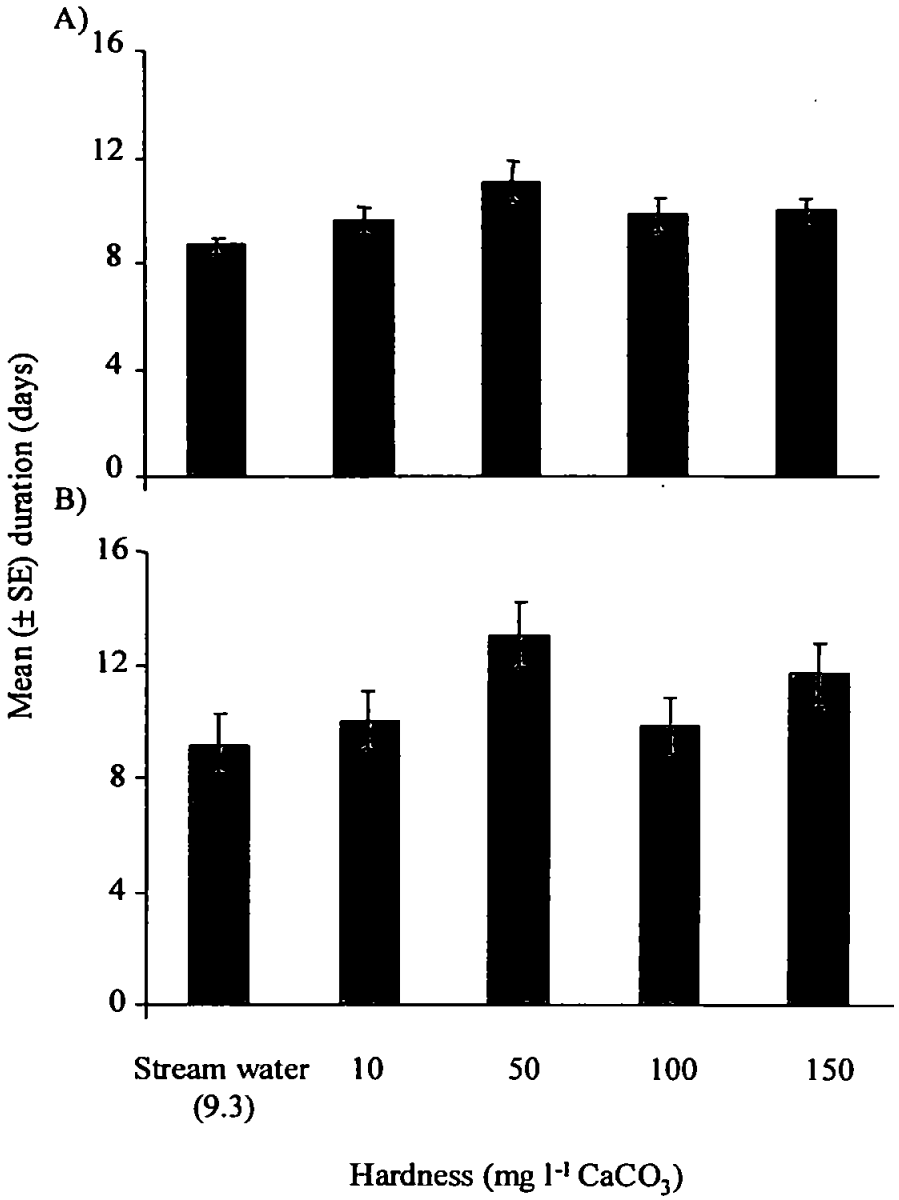


Figure 2.1: Effect of water hardness on A) naupliar development [Dn] and B) copepodid development [Dc] in *Bryocamptus zschokkei* at 'low-handling frequency'.
No significant difference between treatments; n= 14-18 nauplii per treatment.

Table 2.2: The development times (mean ± 1SD) of combined naupliar stages (Dn), individual (C1-C5) and combined (Dc) copepodid stages and hatching to adult (N1-A) for *Bryocamptus zschokkei* at different hardness with low handling frequency.
n is the number of survivors (from original 18) at the end of the experiment and n^a is the number of survivors that developed to adult stage within 35 days (end of experiment). The number in parentheses is the number of animals used to calculate the duration of each stage. Dc/Dn is the ratio of the copepodid stages and the duration of the naupliar stages which, if isochronal development is assumed, should be 0.83 (Hart, 1990). There were no significant differences in the development times of *B. zschokkei* at different water hardness.

Hardness ($mg\ l^{-1}\ CaCO_3$)	n	n^a	Dc/Dn	Dn	Individual copepodid stages					Dc	(N1-A)
					C1	C2	C3	C4	C5		
Stream water (9.3)	18	18	1.06	8.67 ± 1.24 (18)	1.72 ± 0.57 (18)	1.28 ± 0.57 (18)	1.22 ± 0.43 (18)	1.89 ± 0.58 (18)	3.11 ± 1.32 (18)	9.22 ± 2.56 (18)	17.89 ± 3.64 (18)
10	14	13	1.04	9.60 ± 1.96 (14)	1.93 ± 1.49 (14)	1.79 ± 1.12 (14)	1.64 ± 0.74 (14)	2.43 ± 1.83 (14)	3.15 ± 1.14 (13)	10.00 ± 4.00 (13)	19.31 ± 5.17 (13)
50	15	15	1.18	11.07 ± 2.96 (15)	2.13 ± 0.92 (15)	2.07 ± 1.10 (15)	1.93 ± 1.03 (15)	2.93 ± 1.44 (15)	4.00 ± 1.41 (15)	13.07 ± 5.08 (15)	24.13 ± 7.85 (15)
100	14	14	1.01	9.80 ± 2.54 (15)	2.00 ± 1.36 (15)	1.53 ± 0.92 (15)	1.64 ± 0.74 (14)	1.71 ± 0.99 (14)	3.43 ± 0.76 (14)	9.86 ± 3.18 (14)	19.36 ± 5.46 (14)
150	16	13	1.17	9.94 ± 2.21 (16)	2.28 ± 1.44 (16)	1.59 ± 0.92 (16)	2.38 ± 1.20 (16)	3.31 ± 2.24 (16)	2.85 ± 1.99 (13)	11.62 ± 5.25 (13)	21.08 ± 6.98 (13)

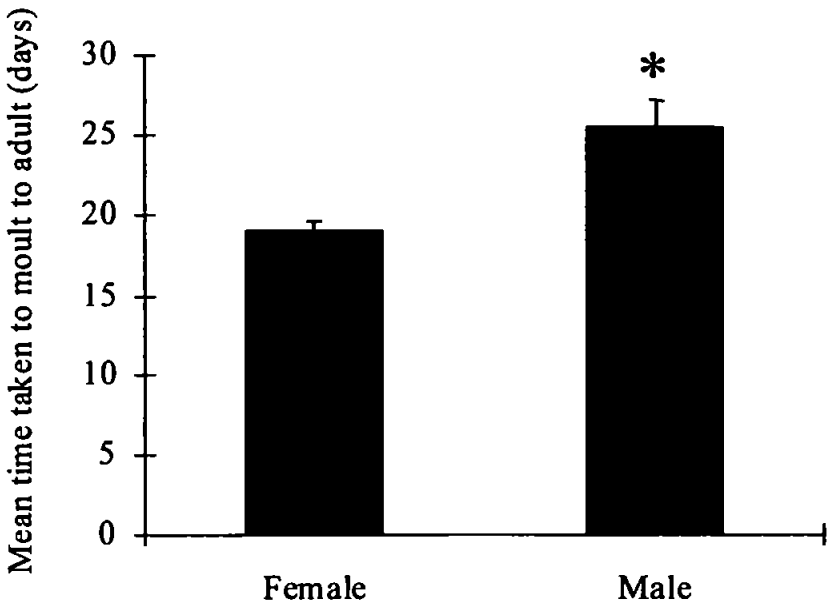


Figure 2.2: Time taken (mean ± 1SE) for male and female *Bryocamptus zschokkei* to moult to adult.
Data pooled from all five treatments in the low handling frequency experiment; n=56 females and 17 males. * Males take significantly longer to moult to adulthood (Student's t-test, $p<0.05$).

To test the model of 'equiproportional' development, a two-way ANOVA was carried out on the proportion of total development spent in each copepodid stage in the 'low-handling frequency' experiment (Table 2.4). This analysis confirmed that there was no effect of hardness on development but there was a significant difference in the proportion of time spent in each stage (Two-way ANOVA, $p<0.05$) and a significant interaction term (Two-way ANOVA, $p<0.05$) (Fig. 2.4). Bonferroni's multiple comparison tests demonstrated again that the proportion of time spent in each stage was significantly longer for the later copepodid stages (C4 and C5) than in stages C2 and C3. The results of a two-way ANOVA for the proportion of total development spent in each copepodid stage in the high vs. low frequency handling experiments (Table 2.5) showed a significant effect of handling, stage and on their interaction (Two-way ANOVA, $p<0.05$) (Fig. 2.5).

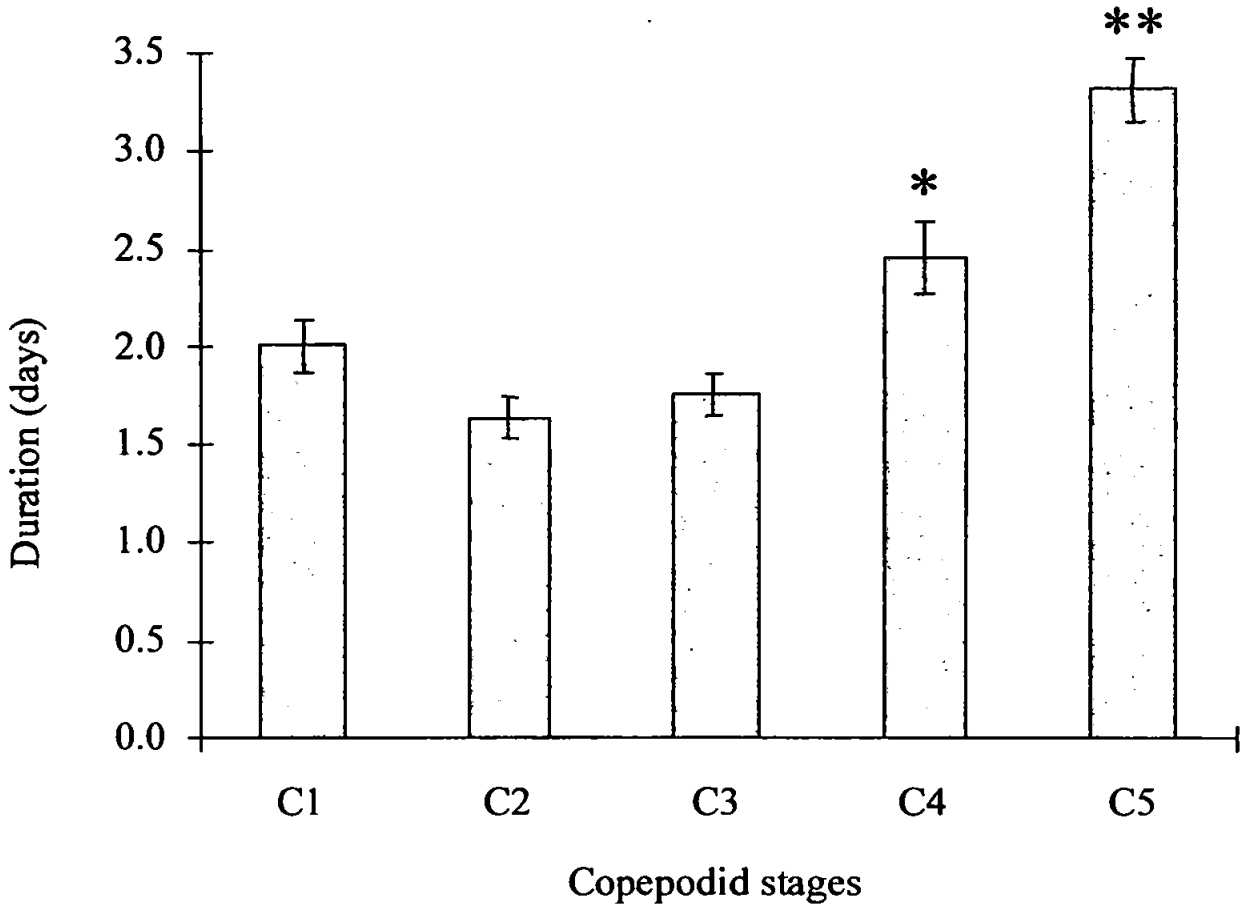


Figure 2.3: The mean (± 1SE) duration of individual copepodid stages for *Bryocamptus zschokkei*.
Data was pooled from all five treatments in the low handling frequency experiment.
** Indicates a significant difference from all other stages (Kruskal-Wallis, $p<0.05$).
* Indicates a difference from stages C2 and C3 (Kruskal-Wallis, $p<0.05$).

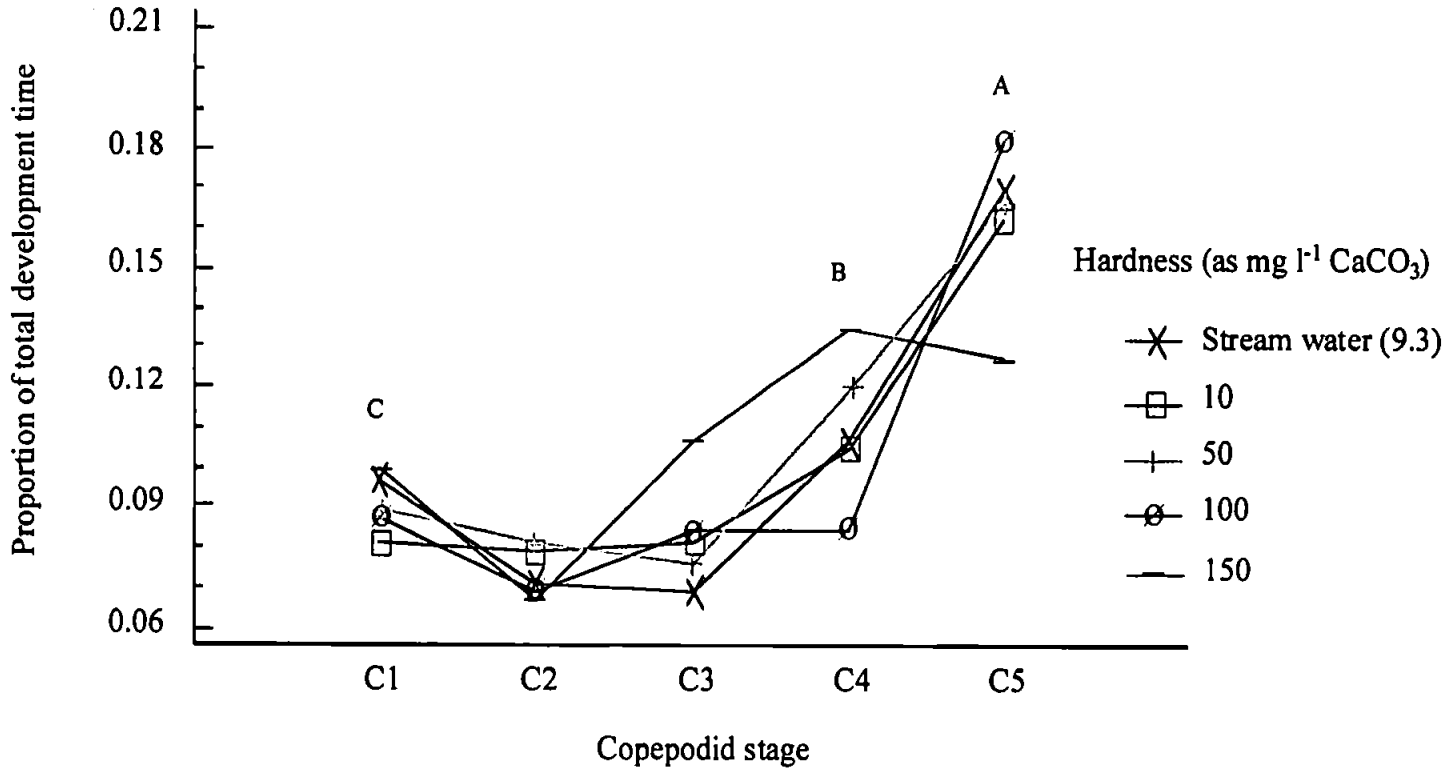


Figure 2.4: An interaction plot showing the proportion of total development spent in each copepodid stage at different hardness in the 'low handling frequency' experiment.
A significantly longer proportion of total development from stages C1-C4 is indicated by 'A', from C1-C3 by 'B' and from C2 by 'C'. A similar pattern of development is observed at all hardness levels and there was no significant effect of hardness on the proportion of time spent in each moult stage.

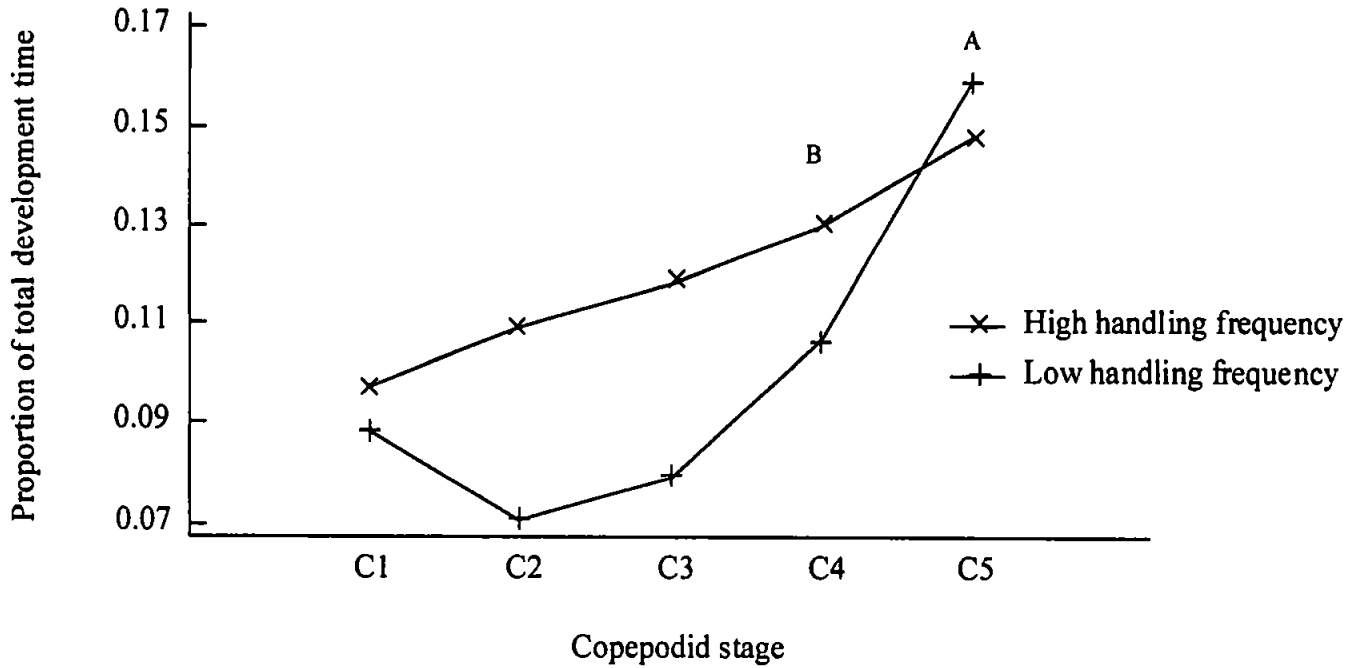


Figure 2.5: An interaction plot showing the proportion of total development spent in each copepodid stage high vs. low handling frequency.
A significantly longer proportion of total development from stages C1-C4 is indicated by 'A' and from C1-C3 by 'B'. The pattern of development is significantly different for animals exposed to high compared with low handling frequency with proportionally more of the total development time spent in early copepodid stages (Two-way ANOVA, $p<0.05$).

Table 2.4: The effect of the proportion of total development time spent in each moult stage and hardness on *Bryocamptus zschokkei* in the experiment with low handling frequency. This shows the results of a Two-way Analysis of Variance on arc sine, square root transformed data where * indicates significance at the 1% level.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Moult stage	2841.26	4	710.32	76.43	0.001*
Hardness	20.75	4	5.19	0.56	0.693
Interaction	468.66	16	29.29	3.15	0.001*
Residual	3159.89	340	9.29		
TOTAL	6625.06	364			

Table 2.5: The effect of the proportion of total development time spent in each moult stage and handling frequency on *Bryocamptus zschokkei*.
This shows the results of a Two-way Analysis of Variance on arc sine, square root transformed data where * indicates significance at the 1% level.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Moult stage	2245.62	4	561.41	50.99	0.001*
Handling	449.98	1	449.98	40.87	0.001*
Interaction	363.31	4	90.83	8.25	0.001*
Residual	6000.88	545	11.01		
TOTAL	9851.16	554			

2.3.3 Sex Ratio

There was no significant effect of water hardness on the sex ratio of fully developed adults at different hardness in either experiment but there was a significant effect of handling (χ^2 6.35 d.f. 1 $p<0.05$). In the 'high-handling frequency' experiment, the ratio of males to females did not differ significantly from an expected 1:1 sex ratio (χ^2 1.28, d.f. 2, $p>0.05$), whereas in the 'low-handling frequency' experiment, the sex ratio was consistently female biased (χ^2 21.07, d.f. 4 $p<0.05$) (Table 2.6).

Table 2.6: The proportion of male and female *Bryocamptus zschokkei* moulting to adult at different water hardness in two experiments with different handling frequencies.
* Indicates a significant female bias.

	Hardness (mg l^{-1} $CaCO_3$)				
High handling	Stream water (9.3)	10	50	100	150
Male	0.44	0.40	0.43	0.40	0.38
Female	0.56	0.60	0.57	0.60	0.63
Low handling					
Male	0.28	0.23	0.20	0.21	0.23
Female	0.72*	0.77*	0.80*	0.79*	0.77*

2.4 Discussion

2.4.1 Handling Frequency

When naupliar stages were exposed to high rather than low-handling frequency development times of *Bryocamptus zschokkei* doubled. This is probably due to increased movement, as a result of being dislodged from the leaf material during observations, which may be imposing additional metabolic costs to the nauplii. Increasing the time taken to moult to adult might be compensating for such increases in metabolism. The copepodid and adult body form is better adapted for movement during feeding and prey avoidance, and are likely to be better able to cope with suspension into the water column (Epp & Lewis, 1980). In fact, adult harpacticoids have even been found to actively migrate into the water column, usually as a result of high densities of animals in the benthos (Shiozawa, 1986; Palmer, 1992). This is also likely to be an important mechanism for dispersal (Palmer, 1992).

2.4.2 Water Hardness

No significant effects of different water hardness on the development times of *B. zschokkei* were observed between treatments. *Bryocamptus zschokkei* is a eurytopic species found in a range of habitats including lakes (Anderson & DeHenau, 1980), streams (Shiozawa, 1986;

Rundle, 1993), ponds and springs (Fryer, 1993), and brackish waters (Amhed-Abada, 2000). This harpacticoid is tolerant of both acidic and calcareous conditions and, in the UK, has also been recorded at hardness levels of between 0 and 115 mg l^{-1} (as $CaCO_3$) and within pH ranges of 4.3 and 8.3 (Rundle & Hildrew, 1990; Rundle, 1993; Fryer, 1993; Burton, 1998). In North America, *B. zschokkei* has been found in rivers with a hardness as high as 250 mg l^{-1} (Shiozawa, 1986). This suggests that the ability to tolerate water hardness in this copepod is not necessarily an adaptation of a specific population but is within the physiological range of responses of each individual. This contradicts evidence from studies on other freshwater crustaceans. For example, Meyran (1997) found an increase in development times, when *Gammarus fossarum* was transferred to water of higher hardness (from 13 to 80 mg l^{-1} Ca) despite the fact that hardness does not limit the distribution of this amphipod in the field. While hardness did not influence *B. zschokkei* in the present laboratory study other physiochemical parameters (pH and aluminium concentration) are known to affect the distribution and abundance of field populations of this copepod, as well as the composition of the associated microcrustacean community (Rundle & Hildrew, 1990; Rundle & Ormerod, 1991; Rundle, 1993). It seems likely therefore that water chemistry variables other than hardness are more important factors in determining the distribution of *B. zschokkei* in the field.

A further explanation for the lack of effect of water hardness on *B. zschokkei* is that other ions may be more influential than hardness in calcium regulation. It is well established that in crustaceans uptake of calcium during moulting is required for hardening of the exoskeleton (Stevenson, 1985; Alstad *et al*, 1999). Evidence from freshwater ostracods indicates that changes in alkalinity rather than hardness are more important in this calcification process and hence moulting. Mezquita *et al* (1999) found that water with low alkalinity (<70 mg l^{-1}) resulted in low survival and slower moulting rates of *Herpetocypris*

intermedia even when hardness was relatively high (93 mg l^{-1} Ca). Although alkalinity was varied with hardness in this study its concentration never exceeded 30 mg l^{-1} as $CaCO_3$ (Table 2.1). Low levels of alkalinity did not, however, appear to affect survival or reproduction in *Daphnia magna* or *Ceriodaphnia dubia* (Cowgill & Milazzo, 1991a), while *D. magna* does show delayed maturation water with low hardness (Lewis & Maki, 1981). Further work is therefore required in order to interpret fully the relationship between hardness, alkalinity and calcium uptake in crustaceans.

2.4.3 Copepod Development Times

Life-history data for freshwater harpacticoids are sparse, however, it is generally agreed that they have slower development than freshwater calanoid and cyclopoid copepods (Maier, 1990). In this study the post-embryonic development times for *B. zschokkei* (17.9 days at 20°C) are closer to those for benthic cyclopoid copepods [10-30 days (Sarvala, 1979; Hart, 1990; Robertson, 2000)] than for other benthic harpacticoids at the same temperature [30-40 days (Sarvala, 1979; Glatzel, 1990; Kowarc 1990)]. Most data available for freshwater harpacticoids are for animals adapted to cold waters, where development might be expected to be slower (Glatzel, 1990; Kowarc, 1990). Benthic harpacticoids from smaller water bodies (as opposed to lakes) or semi-terrestrial habitats have been observed to have shorter development times [15-25 days (Nielsen, 1966; Rouch, 1968, and references therein)]. More rigorous laboratory studies characterising the development of other benthic harpacticoids are, therefore, required before generalised conclusions can be made on how life-history characteristics of copepods may be related to their exploitation of a particular niche (Robertson, 2000).

Within their tolerance range to high temperatures it is widely accepted that copepod post-embryonic development time is accelerated by an increase in temperature (Hicks & Coull,

1983, Hart, 1990). The short development times, recorded for *B. zschokkei* in this study at 20°C, compared with results from lower temperatures (Table 2.7), are therefore to be expected. There are, however, a number of other factors that affect copepod development,

Table 2.7: Data from studies investigating the post-embryonic development of *Bryocamptus zschokkei.*
All development parameters refer to the mean ± standard error. NR - not recorded.

Parameter	O'Doherty, 1985	Burton, 1998 (Control data)	Current study (Stream water)
Temperature (°C)	18	15	20
Photoperiod (Light:Dark)	0:24	0:24	16:8
Light Intensity (K-Lux) Cosine	Dark	Dark	050
Naupliar development [Dn](days)	12.67 ± 0.8	9 ± 1.59	8.67 ± 0.29
Copepodid development [Dc](days)	NR	18.6 ± 0.56	9.22 ± 0.60
Total development [N1-A] (days)	30.38 ± 1.8	~ 27	17.9 ± 0.86
Dc/Dn	2.39	2.25	1.06

which may explain such high inter-species variability in the time taken to moult to adult. For example, development times may also vary between geographically-isolated populations of the same species as reported for the marine harpacticoid *Scottolana canadensis* (Lonsdale & Levinton, 1986). These differences may result from genetic and environmental adaptation or from laboratory conditions; for example, photoperiod influences post-embryonic development in copepods (Miliou, 1992). Previous estimates of the time taken by *B. zschokkei* to moult to key life-stages were made for animals maintained in total darkness (O'Doherty, 1985; Burton, 1998). In this study, a photoperiod of 16 h light and 8 h dark was chosen, as this is the lighting regime recommended for the culture and regulatory testing of other freshwater crustaceans (OECD, 1998b; ASTM, 1999a, b). These conditions are also more representative of those in the River Yealm at the time of collection. Food quantity and quality also affect post-embryonic development times of copepods including *B.*

zschokkei and this will be discussed later (Chapter 4). All these factors are likely to contribute to the observed differences in development times of isolated populations of *B. zschokkei*.

The ratio of copepodid to naupliar development times (Dc/Dn ratio) in the 'low-handling frequency' experiment ranged from 1.01-1.18 and are similar to those calculated for other freshwater harpacticoids [0.63-1.44 (Sarvala, 1979; Hart, 1990)]. Hart (1990) in his review on the patterns of development in copepods, considered evidence for 'isochronal development' (equal duration of each moult stages) and concluded that this type of development was rare in copepods.

If isochronal development were assumed, harpacticoid copepods which moult through six naupliar and five copepodid stages should have a Dc/Dn ratio of 0.83 (Hart, 1990). As the Dc/Dn ratio for *B. zschokkei* is greater than 0.83, this copepod does not appear to conform to isochronal development, assuming the food quality provided in this study is sufficient for optimal development. Evidence that development is not isochronal is also supported by the fact that the durations of the individual copepodid stages are not the same. The alternative model of equiproportional development, in which each moult stage should represent a known proportion of the total development time (Hart, 1990; van den Bosch & Gabriel, 1994) appears to be a more accurate representation of the development pattern of *B. zschokkei*. In this study, each copepodid stage of *B. zschokkei* did represent the same proportion of the total development time. Many freshwater calanoid and cyclopoid copepods also conform to this second model of development (Hart, 1990; Twombly, 1996). In this study, the duration of the final copepodid stages (C4 and C5) were longer than the other stages, which is a pattern associated with equiproportional development (Hart, 1990). This pattern has also been observed in marine harpacticoids where it has been attributed to a

greater food dependence of the late copepodid stages (Hicks & Coull, 1983). For planktonic copepods, it was proposed that this trait had developed as a result of selection for a life-history with less time spent in small, naupliar stages which are more vulnerable to predation (Hart, 1990). While benthic harpacticoids are probably less vulnerable to predation than the zooplankton (Lancaster & Robertson, 1995), there is still pressure for stream-living harpacticoids to become larger in order to reduce the risk of being washed downstream. High handling (but not water hardness) significantly altered the proportion of development spent in each life with more time being spent in smaller copepodid stages. This result supports the conclusion that the smaller life stages are more vulnerable to handling (Section 2.4.1) (Fig 2.4).

Equiproportional development predicts that development is independent of food quality and temperature (Hart, 1990) and hardness (this study) and, as such might have a potential role in determining the mode of action of contaminants in toxicity tests. For example, some pollutants may affect development indirectly by reducing feeding or increasing metabolic rates in which case overall development times would increase but should remain 'equiproportional'. Other contaminants would be expected to act by disrupting the moulting process directly, for example, the insect growth regulator diflubenzuron results in an inability in copepods to shed their exuvium during moulting (Wright *et al*, 1996). A mechanistic effect might be expected to alter the development time but also disrupt equiproportional development particularly where individual moult stages might show different degrees of sensitivity (Costlow, 1977; Wright *et al*, 1996).

2.4.4 Sex Ratio

In all treatments of the low-handling frequency experiment, the sex ratio of adults was biased towards females. A 1:1 ratio was observed in laboratory trials of *B. zschokkei*

allegheniensis, although copepods were not maintained in culture for long periods of time (O'Doherty, 1985). It is not unusual to observe female-biased populations of harpacticoids in the field (Hicks & Coull, 1983); Rundle (1993) found that males contributed 20-40% of the population of *B. zschokkei* in Welsh streams. A ratio of two females for each male was also found for *B. zschokkei* in an Austrian mountain stream (Kowarc, 1991). It is not known how such a female-biased sex ratio would affect natural population dynamics, because a single male can inseminate many females and, once inseminated, spermatophore-mediated sperm transmission enables each female to fertilise multiple broods (O'Doherty, 1985). Lower numbers of males may, however, result in males taking longer to locate females; delayed fertilisation in species of the marine harpacticoid genus *Tisbe* is known to result in lower fertility of females (Volkmann-Rocco, 1972). Skewed sex ratios are not unusual in laboratory cultures, although a bias towards males is more commonly observed (Bergmans, 1981; Green *et al*, 1995; Williams 1997). Hicks & Coull (1983) reviewed evidence of the causes of biased sex ratios in marine harpacticoids and found that temperature, food quality and quantity, density, crowding, light intensity and inbreeding had all been shown to influence the ratio of males and females present. It is not known if any of these factors would cause an imbalance in the cultures of *B. zschokkei* used in the present study.

The overall development times of the small number of males moulting to adult in the low-handling frequency experiment were longer than for females. Slower development times of males is unusual as male harpacticoids, being smaller than the females, tend to achieve adulthood earlier (Hart, 1990). Males and females have different life-history constraints; males are required to achieve maturity quickly so that they are ready to mate as soon as the females moult to adult. The number of eggs a female can carry increases with the size of the animal, therefore, a greater investment in growth for females is required to maximise this

potential (Allan, 1984). At higher temperatures, this difference in development times often becomes less apparent (Sarvala, 1979).

2.5 Summary

- Under defined laboratory conditions the post-embryonic development of *B. zschokkei* was quicker than previous estimates for this species and other freshwater harpacticoids.
- Development of *B. zschokkei* fits the model of equiproportional development, observed in calanoid and cyclopoid copepods. This model may have applications for determining the mode of action of contaminants in toxicity tests.
- Water hardness had no effect on either juvenile survival or development times of *B. zschokkei*.
- Increased handling of naupliar stages resulted in significantly higher mortality and prolonged development times.

Chapter 3

Influence of water hardness on reproductive parameters of *Bryocamptus zschokkei*

3 Abstract

As one of the most important chemical variables in freshwater systems, the effect of hardness was investigated on the reproductive output (numbers of eggs and viable nauplii per female) of *Bryocamptus zschokkei*. Newly mated pairs were exposed to hardness at 10, 50, 100 and 150 mg l^{-1} (as $CaCO_3$) for eight weeks; numbers of broods, eggs and viable nauplii produced by each female were recorded. No effect of hardness on any reproductive parameter was observed. The numbers of eggs and nauplii produced per female were elevated and the intrinsic rate of natural increase (r_m) was doubled compared with a previous estimate for this species. Abortion rate was also high in this copepod. If aborted eggs are a response to stress abortion rate may have application as an indicator of toxicant exposure.

3.1 Introduction

The importance of considering the effects of hardness, as a key environmental variable in determining the distribution and life-history patterns of freshwater Crustacea, was established in Chapter 2. While there was no effect of hardness (10 - 150 mg l^{-1} as $CaCO_3$) on the survival or post-embryonic development times of the harpacticoid *Bryocamptus zschokkei*, it cannot be assumed that there would be no effect on reproductive output. Indeed, reproduction in copepods is often more sensitive than development to environmental conditions, as revealed from studies of food quality (Williams & Jones, 1999) and toxicant exposure (Chandler, 1990). Reproduction in field populations of *B. zschokkei*, and the related harpacticoid *Bryocamptus praegeri,* was found to be highly sensitive to physiochemical parameters, with a trend towards smaller eggs and fewer clutches respectively at sites of low pH (Rundle, 1993). Moreover, in laboratory populations of *B. zschokkei* exposed to copper, a decrease in the numbers of offspring per female was observed at concentrations where there was no effect on development times (Burton, 1998).

While food quality has a major effect on reproduction of copepods (Williams & Jones, 1999; Twombly *et al*, 1998), it is well established that other factors, including temperature (McLaren, 1978; Abdullahi, 1990), food characteristics (Poulet *et al*, 1994; Ianora, 1998), time to first mating (Volkmann-Rocco, 1972) and remating success (Parrish & Wilson, 1978; Ianora *et al*, 1989), also have a significant role in determining the numbers of offspring produced. In addition, contaminant exposure can reduce fecundity in copepods (Bengtsson & Berström, 1987), by causing a shift in resource allocation, resulting in less energy available for the provision of eggs (Hicks, 1979). It is essential to understand how such variables influence reproduction in copepods because they are directly related to recruitment and ability of a population to replenish itself. Before considering the effects of a contaminant on a potential new test species it is, therefore, imperative to gain information on the variables that influence reproductive output. For example, it is prudent to assess the tolerance range of a species (or even particular strain) to water quality variables to avoid attributing their potential toxic effects to a contaminant (Cowgill, 1987).

Few studies have considered the effects of hardness on reproduction of freshwater Crustacea. Reduced fecundity has been observed in cladocerans in response to low calcium concentrations (Cowgill & Milazzo, 1991b; Hamza *et al*, 1998; Hessen *et al*, 2000). High levels of hardness, conductivity, sulphate and alkalinity were related to increased reproductive output of the amphipod *Hyalella azteca* (Gibbons & Mackie, 1991).

The aims of this chapter were to:

1) Gain baseline data on reproductive parameters (egg and nauplii production) of *B. zschokkei* under defined laboratory conditions.

2) Assess the effects of water hardness on fecundity.

3) To produce an abbreviated life-table using fecundity and developmental data obtained in Chapter 2.

3.2 Materials and Methods

3.2.1 Test Medium

The stream water and four reconstituted water treatments used in this experiment were identical to those used to investigate the effects of hardness on the post-embryonic development of *B. zschokkei* (Chapter 2).

3.2.2 Experimental Animals

Sixty ovigerous females from stock cultures (Chapter 2) were placed in a 13.5 cm diameter glass crystallising dish containing 400 ml of filtered stream water (0.2 μm) and two pre-conditioned beech leaves (*Fagus sylvatica* L.) (Chapter 2). These females were removed after one week, leaving a cohort of juveniles less than one week old. The juvenile copepods were observed daily until they achieved adulthood and began to form mating pairs; the latter were transferred to 2 cm diameter polystyrene Cellwells™ (Dow Corning, Corning, NY, USA) using a wide-bore glass pipette. Cellwells™ contained 3 ml of test medium and a 1.1 cm diameter beech leaf disc (pre-conditioned in Elendt's M4 medium for 14 days).

3.2.3 Experimental Design

Each treatment consisted of 14 pairs maintained in a temperature-controlled room at 20 (± 1°C) with a photoperiod of 16 h light:8 h dark and a 20 min dawn-dusk transition time. A partial water replacement (66%) was carried out three times per week, and the dissolved oxygen content and pH of the water was measured before the water was discarded. Daily observations of survival (animals which respond to gentle stimulation), and the presence of egg sacs and nauplii were made using a Wild M8 binocular microscope with darkfield

illumination (magnification, ×20). Every seven days, each pair was transferred to another Cellwell™ containing fresh media and a new leaf disc. Any nauplii that had hatched during that week remained in the old Cellwell™ and were counted as soon as possible. If more than one brood of nauplii was present, they were distinguished on the basis of size. Any eggs that had not hatched were counted, so that the overall hatching success (proportion of viable offspring) could be determined. This experiment was continued for eight weeks, by which time offspring production was low in all treatments. These observations were used to calculate: a) the embryonic development time (the time from egg sac release externally to hatching of nauplii); b) the inter-brood period (the time between egg sac releases); and c) the number of broods per female. Any aborted egg sacs were removed from the cell well and viewed under a high-powered inverted microscope to determine the number of eggs present. An abortion was considered to be an egg sac from which no viable offspring hatched.

3.2.4 Abbreviated Life Table

Abbreviated life tables were constructed by incorporating post-embryonic development data from the 'low handling-frequency' trials (Chapter 2) with measures of offspring number and adult survival from this experiment. The age-specific survival (l_x) and fecundity (m_x) were calculated using an estimate for the age of first reproduction in order to calculate the intrinsic rate of natural increase (r_m) (Fig. 3.1). The life-table calculations were based on the equation derived by Lotka (1925):

$$\sum l_x m_x e^{-rx} = 1$$

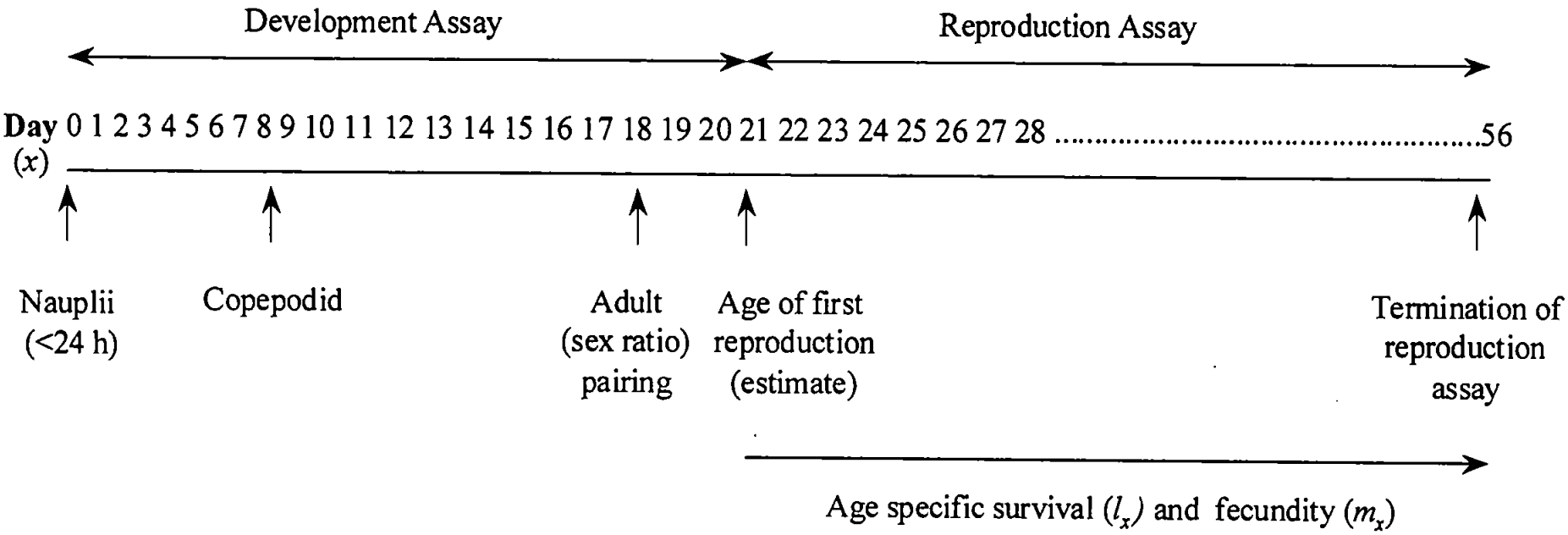


Figure 3.1: A summary of the life-history of *Bryocamptus zschokkei* indicating where data are obtained for incorporation into the abbreviated life-table calculations.

where:

l_x = the number of females on day x / the number of females at the start of the experiment (i.e. 14 females used to initiate the reproduction experiment),

x = age (days),

m_x = number of female offspring (nauplii) produced on day x / number of living females on day x (using survival data from the reproduction experiment only),

r = the intrinsic capacity for increase for the particular environmental conditions,

e = 2.17828 (a constant), and

U_x = $l_x m_x$ = number of female offspring (nauplii) produced on day x / number of females at the start of the life table (or in this case the reproduction experiment i.e. 14 females).

Ux, the realised fecundity, is the number of new born females produced per day per female from the previous generation. This can be used to calculate the factor by which the female population increases from one generation to the next, the net reproductive rate (R_o):

$$R_o = \sum l_x m_x = \sum U_x$$

As a result of the skewed sex ratios observed in the development assay, the same animals could not be followed throughout their entire lives. The data used for these abbreviated life-table calculations, therefore, incorporate data from two experiments (Fig. 3.1). For example, when calculating l_x (the number of females on day x divided by the number of females at the start of the experiment), the female survival data from the reproduction experiment only was used. Juvenile mortality was assumed to be zero (which was true for the stream water control and was not greater than 28% in any of the treatments) and was not incorporated into the model. The 'age of first reproduction' is estimated as this information was not obtained in either experiment. The reproduction experiment was terminated at eight weeks after which time offspring production may still have occurred but

only at a very low level. To compare results with previously published data, calculations of m_x assumed a 1:1 sex ratio for newborn animals. An estimate of m_x was also made using the female biased sex ratios (1:2.6) observed in the 'low handling-frequency' development assay (Chapter 2).

3.2.5 Statistical Analyses

Fisher's Exact test was used to identify any significant effects of hardness on copepod survival at the end of the experiment (day 56); contingency table procedures were used to investigate differences in male and female survival. Two-way ANOVA was carried out to assess the effects of hardness and time on the numbers of eggs and nauplii produced per female. One-way ANOVA was used to consider the effect of hardness on the number of broods per female, eggs and nauplii per brood, and embryonic development times. Bonferroni's multiple comparison procedure was used to differentiate between the means. Reproductive parameters were tested for normality and equality of variances before using analysis of variance techniques. If these assumptions for ANOVA were not met, the data were square root transformed. Data that still did not conform were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Mann Whitney tests to highlight which treatments were different at the 5% level.

3.3 Results

3.3.1 Survival

Survival was relatively high (>65%) in all treatments with no significant differences between the stream water control and the reconstituted water treatments up to day 56 (end of experiment) (Table 3.1). There was also no significant difference in the survival of males or females in any treatment.

Table 3.1: Effect of hardness on the reproductive parameters of *Bryocamptus zschokkei.*
n is the number of females per treatment, n_a is the number of females producing egg sacs and n_b is the number of females producing egg sacs from which viable offspring hatched. There was no significant difference between treatments.

Hardness (mg l^{-1} $CaCO_3$)	% Survival	n	n_a	n_b	Total number of broods	Total number of successful broods	Proportion aborted	Mean (± 1SD) number of broods per female (n)	Mean (± 1SD) eggs per brood (n_a)	Mean (± 1SD) nauplii per brood (n_b)
Stream water (9.3)	71.4	14	14	12	107	67	0.37	7.64 ± 2.13	10.47 ± 4.24	8.25 ± 4.97
10	78.6	14	14	14	103	65	0.37	7.35 ± 2.06	11.14 ± 4.04	8.38 ± 5.11
50	77.8	13	13	9	91	47	0.48	7.00 ± 3.00	9.63 ± 4.37	7.76 ± 4.26
100	67.9	14	14	12	84	59	0.30	6.00 ± 2.72	10.96 ± 4.59	10.44 ± 4.89
150	67.9	14	14	11	81	43	0.46	5.79 ± 2.33	9.86 ± 3.78	8.51 ± 4.66

3.3.2 Fecundity

There was no significant difference in the mean number of eggs or nauplii per female at any of the hardness levels (even when data for females that aborted all their egg sacs were excluded (Fig. 3.2). There was, however, a significant time effect (Two-way ANOVA, $p<0.05$) (Fig. 3.3), with the mean numbers of eggs and nauplii produced being highest in weeks three and four, this trend was most significant for naupliar abundance.

Hardness had no significant effect on the number of broods produced by each female (Table 3.1). The proportion of aborted egg sacs ranged from 0.30-0.46 depending on hardness (Table 3.1). There were significantly more egg sacs aborted from broods eight and nine compared with broods two to five (Kruskal-Wallis, $p<0.05$).

3.3.3 Brood Interval and Embryonic Development

Embryonic development took 3-7 days and did not vary with hardness (Table 3.2). Generally females produced a new egg sac within 24 h of hatching as long as the previous brood had not been aborted (i.e. median interval between subsequent broods < 1 day).

Table 3.2: Embryonic development times for *Bryocamptus zschokkei* at different hardness. There was no significant effect of hardness.

Hardness (mg l^{-1} $CaCO_3$)	Mean (± 1SD) brood duration (days)	n
Stream water (9.3)	4.85 ± 0.58	67
10	4.86 ± 0.62	65
50	4.91 ± 0.65	47
100	4.86 ± 0.63	59
150	4.80 ± 0.50	43

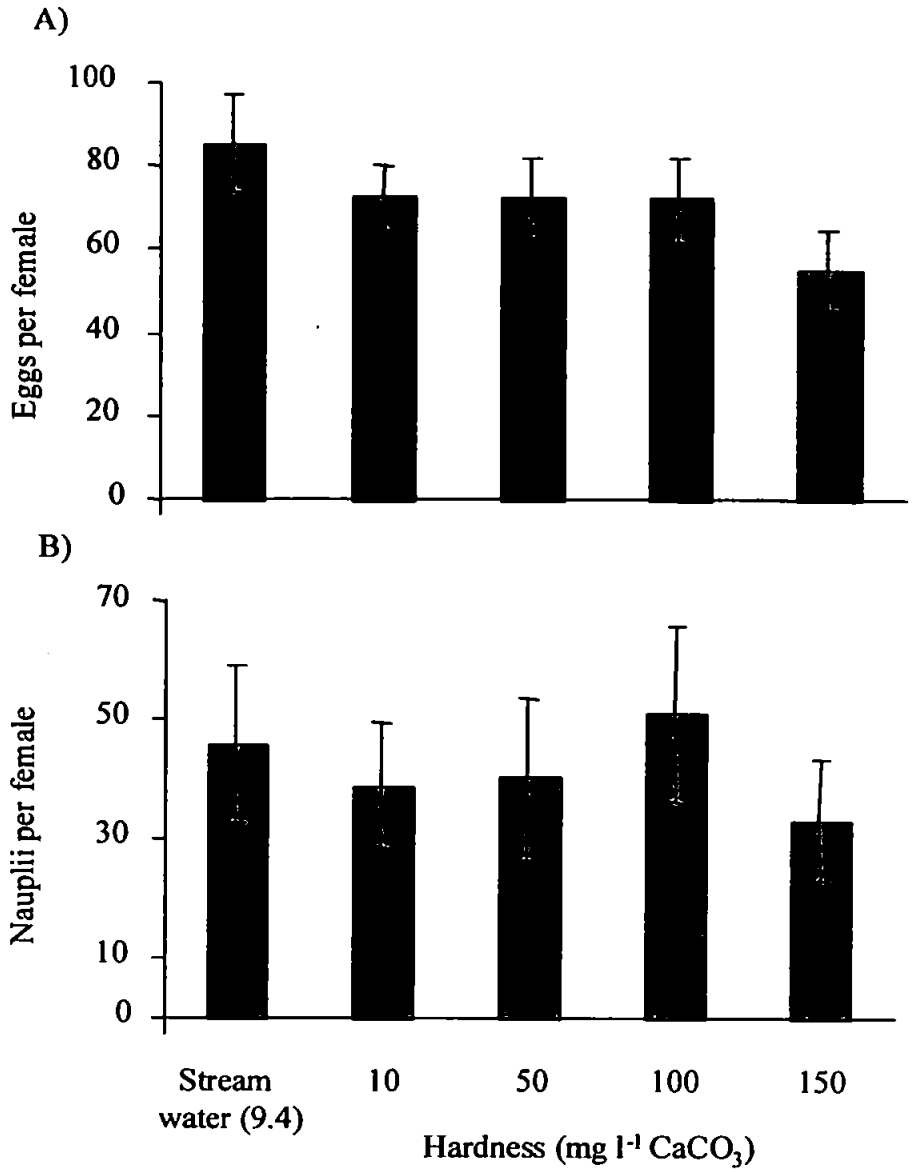


Figure 3.2: The effect of hardness on the mean (±1SE) number of A) eggs and B) nauplii produced by each female
These data exclude females that did not produce egg sacs from which viable offspring hatched. There were no significant differences between hardness levels.

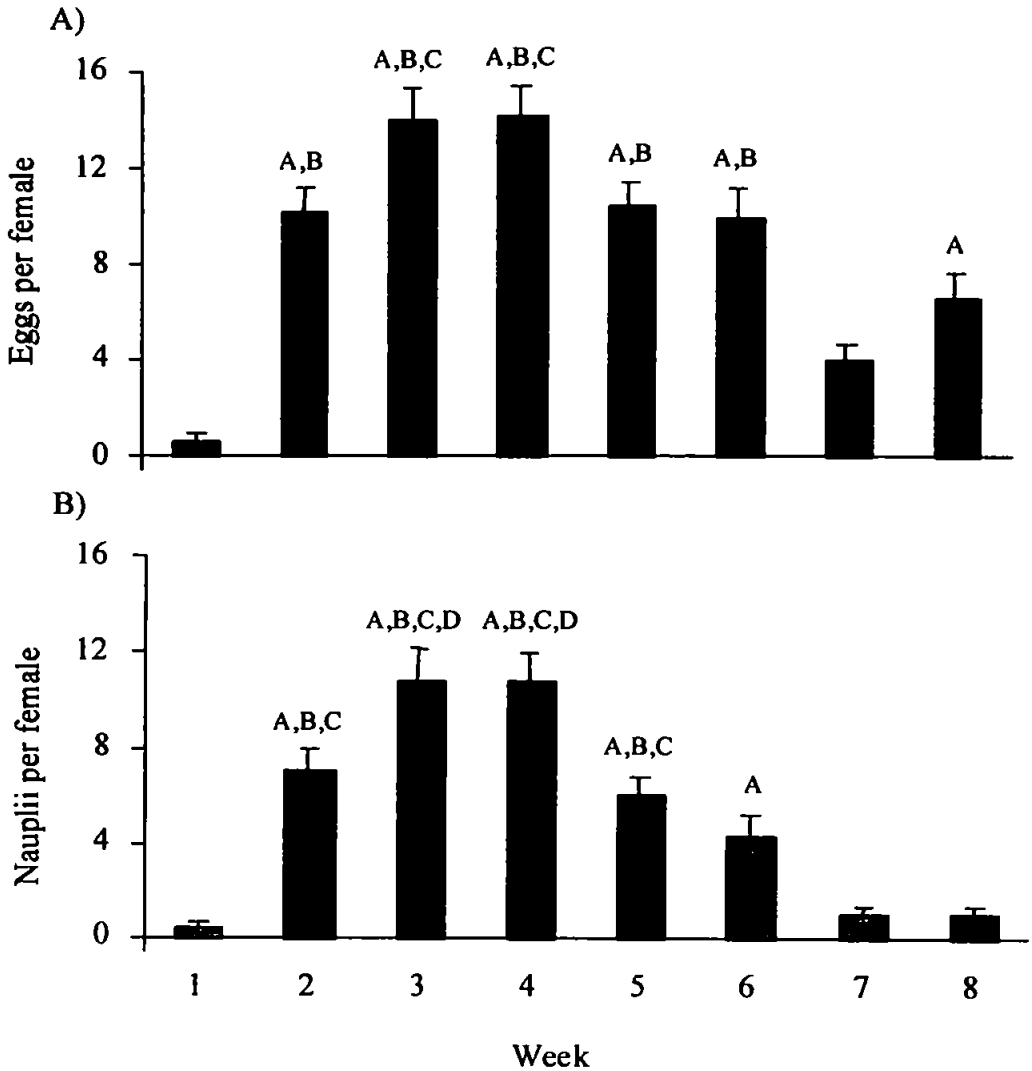


Figure 3.3: The mean (± 1SE) number of A) eggs and B) nauplii produced per female each week.
This incorporates data from all five hardness levels but excluded females that did not produce viable offspring. A significant increase in the numbers of eggs and nauplii from week 1 (A), week 7 (B), week 8 (C) and from weeks 2, 5 and 6 (D) are indicated (One-way ANOVA, $p<0.05$).

3.3.4 Abbreviated Life-Table

The intrinsic rate of increase (r_m) of *B. zschokkei* was highest in stream water and 100 mg l^{-1} $CaCO_3$ treatments (Table 3.3). However, values were not markedly different from the remaining treatments (± 27%) (NB could not test significance). The net reproductive rate (R_o) and cohort generation time (*Tcoh*) showed a similar pattern to r_m. For example, at 100

mg l^{-1} $CaCO_3$, there was the greatest increase in the abundance of newborn females (R_o) over the shortest generation time (T*coh*). These data were calculated assuming offspring with a 1:1 sex ratio, however, life-table calculations were also made incorporating the female-biased sex ratio (1:2.6) observed in the 'low handling-frequency' development assay (Chapter 2). The cohort generation time (T*coh*) remained the same but a large increase in r_m and R_o was observed reflecting an increase in the number of females available for reproduction. An increase of similar proportions may be expected in the other treatments where comparable sex ratios were recorded.

Table 3.3: Summary of 'Life-Table Analyses' for *Bryocamptus zschokkei* populations maintained under different environmental conditions.
This table includes life-table estimates for animals at each hardness exposure and an estimate for *B. zschokkei allegheniensis* (O'Doherty, 1985) which all assume a 1:1 sex ratio for offspring. The life-table data for the stream water control was also calculated using the 1:2.6 sex ratio for offspring as observed in the 'low handling frequency' development experiment (Chapter 3).

Hardness (mg l^{-1} $CaCO_3$) at 20 ± 1°C	Sex Ratio (male:female)	$R_o = \sum U_x$ = net reproductive rate per generation	T*coh* = cohort generation time (days)	r_m = intrinsic rate of natural increase
Stream water (9.3)	1:2.6	130.00	40.61	0.137
Stream water (9.3)	1:1	19.29	40.61	0.079
10	1:1	19.39	43.68	0.073
50	1:1	13.85	48.28	0.058
100	1:1	21.96	39.35	0.079
150	1:1	13.46	44.33	0.062
O'Doherty (1985) - 18°C	1:1	4.14	74.7	0.02

3.4 Discussion

3.4.1 Water Hardness

There were no significant differences in any of the fecundity parameters or life-table calculations between treatments of *B. zschokkei* raised at different hardness levels. In field populations of this copepod, reproduction has been shown to be sensitive to water physiochemistry, with a reduction in the numbers of gravid females and in the mean clutch size in streams of low pH and alkalinity and elevated aluminium concentrations (Rundle, 1990). Other freshwater crustaceans have shown a positive response to increased hardness in terms of demographic parameters (Gibbons & Mackie, 1991; Meyran, 1997). For example, increased reproductive output of field populations of *Hyalella azteca* was related to environmental variables including increased hardness, alkalinity and conductivity (Gibbons & Mackie, 1991). As clutch size and female body size are usually positively correlated for crustaceans, an increase in body size of *Gammarus fossarum* observed in lakes with high water hardness would be expected to result in higher levels of fecundity (Meyran, 1997). In laboratory studies, the cladocerans *Daphnia magna* and *Ceriodaphnia dubia* both showed impaired reproduction at concentrations less than 71 mg l^{-1} $CaCO_3$ (Cowgill & Milazzo, 1991a). Data on the effects of hardness are sometimes contradictory and, in another study, no effects of hardness were observed on *C. dubia* tested in reconstituted water in the range 40-180 mg l^{-1} $CaCO_3$ (Cooney *et al*, 1992). It has, therefore been argued that the observed negative effects of low water hardness on the survival and reproduction of cladocerans may be due the absence of essential trace minerals (for example, zinc and boron) in dilution waters rather than just the ionic composition of the media (Winner, 1989). In fact, it is still not clear which ions are most important for calcium regulation in Crustacea; recent evidence from studies on the ostracod *Herpetocypris*

intermedia suggests that alkalinity is more important than hardness as a limiting factor for moulting and calcification of the ostracod (Mezquita, 1999).

A further explanation for a lack of an effect of hardness is that the small size of *B. zschokkei* (adult female ca. 600 μm) reduces their demand for calcium. Selection for microcrustaceans and rotifers in American lakes with low hardness was hypothesised to result from a greater relative requirement for calcium by large-bodied cladocerans because of their increased size (Tessier & Horwitz, 1990). The calcium content of copepods is just 0.06% dry weight compared with 2.2 to 7.7% in daphnids (Cowgill, 1976; Havas, 1985; Yan *et al*, 1989).

3.4.2 Fecundity

Egg and offspring production in *B. zschokkei* were higher than has been observed for the American sub-species *B. zschokkei allegheniensis* (Table 3.4). Reproductive data for the latter species (O'Doherty, 1985), however, were collected over a comparatively longer time period and at a slightly lower temperature (35 weeks at 18 ± 1°C), compared with the present study. In both studies the reproductive period was comparable with nauplii production ceasing after eight to ten weeks. Burton (1998) found a similar number of offspring per *B. zschokkei* female to those observed in the present study, in laboratory maintained animals over a six week period, obtained from the River Lynher, south-west England. It is difficult to attribute the increase in numbers of eggs and nauplii per female observed in this study compared with previous estimates for this species to a single factor because of the range of variables that can influence reproduction. In this case the observed differences probably reflect the responses that occur between geographically isolated populations or sub-species (Lonsdale & Levinton, 1986) as well as the refined culturing methodology in the present study. For example, the food quality, photoperiod and temperature were all different to those used in previous studies, all of which are known to

influence harpacticoid reproduction (Miliou, 1992; Williams & Jones 1999). As reproduction is improved it suggests that the current culture methods can be adopted for further studies.

The mean numbers of eggs per brood observed in this study were lower than those recorded for field populations of *B. zschokkei*, where females sampled directly from the Upper River Tywi, mid-Wales were found to have clutches of about 20 eggs (Rundle, 1993). In field populations of *B. zschokkei* larger numbers of eggs were observed in late spring and early summer (Rundle, 1993). Lower productivity in this study, compared with field conditions may be linked to the lack of seasonality in temperature and food abundance as a result of standard laboratory conditions. Food quality is a major factor in determining the reproductive output of copepods (Hopp *et al*, 1997; Twombly *et al.* 1998). While a leaf litter diet supports rapid development times and good survival of *B. zschokkei* (Chapter 2), reproduction in marine harpacticoids is more sensitive to food limitation than growth or development (Williams & Jones, 1999).

Table 3.4: Data from studies investigating the reproductive parameters of *Bryocamptus zschokkei*.
All fecundity parameters are expressed, as the mean ±1SE and NR indicates data were not recorded.

Parameter	O'Doherty, 1985	Burton, 1998	Current study
Temperature (°C)	18	15	20
Photoperiod (Light:Dark)	0:24	0:24	16:8
Light Intensity (K-Lux) Cosine	Dark	Dark	050
Period of observations (weeks)	34	6	7
No. eggs per sac (range)	8-24	NR	10.47 ± 4.24 (2-24)
Embryonic development	4.38 ± 0.13	NR	4.85 ± 0.07
Eggs per female	122 ± 14	NR	85.5 ± 11.74
Nauplii per female	22 ± 7	58 ± 3.09	46.08 ± 8.30
Broods per female	18 ± 2	NR	7.64 ± 0.56

3.4.3 Embryonic Development

Copepod embryonic development times will depend on culture conditions (temperature and food quality), acclimation effects and geographical separation (Lonsdale and Levinton, 1985). The long-term previous thermal conditions of a population are considered the main cause of variation both within and between species (Herzig, 1983). In this study embryonic development for *B. zschokkei* (4.85 days) was comparable to previously data at the same temperature (4.38 days, O'Doherty, 1985) which probably reflect similar temperature regimes in the streams from which the different populations of copepods were obtained (i.e. 15°C). These estimates for embryonic development in *B. zschokkei* are also in the same range recorded for other freshwater harpacticoids (5-8.3 days, Sarvala, 1979) but are generally longer than observed in benthic freshwater cyclopoids (1-3 days, Maier, 1990;

Robertson, 2000) or marine harpacticoids (2-4 days, Lonsdale & Levinton, 1985). Differences in embryonic development times of copepods tend to be more pronounced at low temperatures (Herzig, 1983).

Usually, a new egg sac was released within 24 h of nauplii hatching. Such rapid release of new egg sacs has been observed for other freshwater harpacticoids and is considered a good indicator of an optimal food supply (Sarvala, 1979). Demographic models have demonstrated that an increase in the inter-clutch period can reduce the overall fecundity of copepod populations irrespective of mortality by reducing the time that the female is reproductively active (Ohman *et al*, 1996).

3.4.4 Individual variability

The variation in the numbers of eggs and nauplii produced by *B. zschokkei* in this study was not unexpected as this parameter is influenced by several life-history parameters (e.g. body size, time to first egg sac, embryonic development time, the interbrood period), each of which is subject to inherent variability (Båmstedt, 1988). In field populations of copepods, plasticity in life-history parameters appears to be an adaptation for exploiting different or changeable habitats (Allan, 1984; Båmstedt, 1988; Ohman *et al*, 1996). Twombly *et al* (1998) suggested that large differences in the offspring production of the freshwater calanoid *Boeckella triarticulata* were either a response to environmental uncertainty ('bet hedging') or that other environmental factors (such as interspecific interactions) were important in moulding their life-history strategies. Two natural populations of the freshwater copepod *Mesocyclops edax* showed heritable differences in life-history and they were related to their environment (Allan, 1984). Sarvala (1990) also found that populations of the freshwater harpacticoid *Paracamptus schmeili,* sampled from different depths of a lake, showed pronounced demographic differences shifting from a one to a three year life-

cycle. Natural variability in life-history responses of copepods is consistently reflected in laboratory-reared populations (Carlotti & Nival, 1991; Strawbridge *et al*, 1992; Bechmann, 1994; Twombly *et al*, 1998). Increase in variability of reproductive output is often associated with stress, including sub-optimal feeding regimes (Williams and Jones, 1999) and pollution exposure (Bechmann, 1994). Large differences in responses of individuals to the same parameter has implications for toxicity tests where a high degree of standardisation is a prerequisite and variability is considered a weakness in the test method (Calow, 1996). However, it has been argued that biological variability itself is a valuable parameter to be considered in population level tests (Depledge, 1990). As the success of a species is related to the attributes (e.g. development rate, fecundity) of the individuals that make up a population, some individuals will be better able to respond to environmental change than others (Depledge, 1990; 1994). Therefore, individual variability will actually determine how a population will survive after a change in environmental conditions, for example, contaminant exposure.

3.4.5 Life-Tables

Life-tables incorporate information on the development, reproduction and survival of a population and allow calculation of the intrinsic rate of natural increase of a population (r_m). The latter statistic is an ecologically relevant expression of the potential of a population to increase under defined conditions. Demographic parameters have been used to assess the influence of culture conditions on marine harpacticoid copepods (Williams, 1997) and full or abbreviated life-tables are being applied increasingly for considering the effects of toxicants (Allan & Daniels, 1982; Green & Chandler, 1996, Bechmann, 1999). An advantage of the life-table approach is that it considers the entire population and, therefore, gives an indication of an adverse effect, even if high individual variability prevents meaningful statistical analysis (Bechmann, 1994). Although the life-table described in this

study is abbreviated, it still incorporates the fundamental quantities of demographic analysis, such as measures of offspring number and the temporal patterns of births (Bergmans, 1984). As such, it is a useful tool for considering differences between treatments. Caution should be observed, however, when comparing with previously published data, as the same individuals were not used to obtain all the information contained in the life-table.

The values of r_m calculated using this life-table (with a 1:1 sex ratio) were more than twice as high as the most positive estimates made for *B. zschokkei allegheniensis* (O'Doherty, 1985). The main factor affecting r_m is the age of first reproduction (Allan & Daniels, 1982). As the estimate of development time used to calculate this value (Chapter 2) was almost half the time taken for the North American strain then an increase in r_m would be expected. This was also reflected in the cohort generation times of the two populations T*coh* (Table 3.3). Other factors that influence the capacity of a population to increase are the number of offspring per brood and the number of successful broods, both of which were higher in this study. Extrinsic factors such as temperature and food quality (see Chapter 4) are also known to influence r_m in harpacticoid copepods (Hicks & Coull, 1983; Williams, 1997).

Sex ratio also influences the life-table parameters (Table 3). For example, the factor by which the female population increased from one generation to the next (R_o) showed an 85% increase if the 1:2.6 (male: female) sex ratio observed in the 'low handling frequency' experiment and laboratory cultures (personal observation) is used. This reflects the higher number of females available for offspring production. As field populations of *B. zschokkei* (Kowarc, 1991; Rundle, 1993) are female biased calculating r_m using a 1:1 sex ratio may actually be an underestimation of population increase under environmentally realistic conditions.

3.4.6 Hatching Success

Female *B. zschokkei* carry their egg sacs externally and nauplii hatch from the egg sac while it is still attached (O'Doherty, 1985). In the current study a relatively high abortion frequency (30 to 48%) was observed depending on the treatment (Table 1). Aborted egg sacs have been detected previously in *B. zschokkei* maintained in the laboratory (O'Doherty, 1985; Burton, personal communication). There is no information available on the hatching success of this harpacticoid in the field. Rundle (1993), when sorting dead females taken directly from the stream, observed that some egg sacs detached easily which could be due to broods that were not viable, however, this occurred in less than 10% of cases.

Bryocamptus zschokkei can, via spermatophore mediated transfer, fertilise a number of broods from a single mating and an absence of remating may therefore be an explanation for the high abortion frequency (O'Doherty, 1985). Females isolated from males produce broods from which no viable offspring hatch, (personal observation) a phenomenon observed in other freshwater harpacticoids (Sarvala, 1979). A delay in fertilisation may therefore have caused the high proportion of aborted first egg sacs and, if the males were infertile, this may explain why some females never produced viable offspring. In this study, males were left with the females so that the absence of remating should not have been a problem. This theory, however, does not explain low hatching success of broods from which viable offspring did hatch. It has also been suggested that in copepod species where high sexual dimorphism exists (as is the case with *B. zschokkei*) the males may be weaker and therefore need more attempts to fertilise the female (Maier, 1994).

Published data reporting low hatching success or abortions in other harpacticoids are rare and there is little discussion of the implications. Sarvala (1979) showed that hatching success in other species of freshwater harpacticoids was found to be >90%, except in

Canthocamptus staphylinus, where <60% of eggs were viable (Sarvala, 1979). The marine benthic harpacticoid *Amphiascus tenuiremis* has been observed to produce nauplii for four weeks after which no juveniles were found despite continued egg production (Green *et al*, 1995). A mean hatching success of 57% was observed for *Longipedia* spp. fed on a diet of algae (Onbé & Kimoto, 1985) and discarded egg sacs have been observed as a stress response in *Scottolana canadensis* (Harris, 1977). Photoperiod and spectral composition caused an abortion rate of up to 57% in *Tisbe holothuriae* (Miliou, 1992) and *Tisbe furcata* females were observed to produce egg sacs from which no viable offspring hatched (Bechmann, 1994). Other authors have found poor juvenile survival when hatching success has been low (Feller, 1980), a feature that was not observed for *B. zschokkei* (Chapter 2). Nevertheless, if abortions in *B. zschokkei* occur as a response to stress it may be possible to utilise this phenomenon as an indicator of toxicant exposure.

Evidence for mechanisms to explain abortions in copepods comes mainly from work on calanoids. In *B. zschokkei*, an increase in abortion frequency and a reduction in hatching success were both observed with time, egg viability in calanoid copepods is also age dependent (Ianora, 1998). High embryonic mortality in subtemperate calanoid copepods was attributed, until recently, entirely to remating success or cannibalism (Ianora *et al*, 1989). There is now evidence that other factors influence hatching success including anoxia (Jonasdottir & Kiørboe, 1996) or food quality, for example lack of essential nutrients (Ianora & Poulet, 1993) or the presence of deleterious chemical compounds (Ianora, 1998). Food quality has also been shown to affect spermatophore production in calanoid copepods (Ianora & Poulet, 1993), so the possibility of low male fertility being the cause of abortions should not be discounted. Unfertilised oocytes are not an ecologically sensible strategy (Ianora & Poulet, 1993). An explanation of low hatching success would be desirable,

particularly as other life-history parameters (e.g. fast development times and short brood intervals) suggest good culture conditions.

3.5 Summary

- There was no effect of hardness on any of the reproductive parameters measured for *Bryocamptus zschokkei*.
- The number of eggs and nauplii produced under defined laboratory conditions were higher than those observed previously for this species resulting in a doubling in the estimate of the intrinsic rate of population increase (r_m).
- High biological variability in reproductive parameters probably reflects the ability of this species to respond to natural environmental changes
- While abortion frequency in this copepod is high, their occurrence as a response to stress might be useful as an indicator of toxicant exposure.

Chapter 4

Effect of detritus quality on the development and reproduction of *Bryocamptus zschokkei*

4 Abstract

Allocthonous inputs of leaf litter and woody debris drive the trophic dynamics of woodland streams. Detritus [decomposing leaf material in the form of coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM)] is the most important source of food for many aquatic invertebrates and the interaction between detritus and macroinvertebrate detritivores has been extensively studied. There is, however, very little information on how meiofauna influence detritus processing in streams, despite their abundance and diversity within the stream benthos. This study considers the effects of three types of detritus (beech leaves (*Fagus sylvatica*), oak leaves (*Quercus robur*) and stream derived FPOM) and leaves conditioned for different lengths of time (two vs. six weeks) on the development and reproduction of the freshwater harpacticoid *Bryocamptus zschokkei*. Grazing on leaf discs and the food available to copepods reared on these diets was determined using a scanning electron microscope (SEM). Grazing by *B. zschokkei* was found to reduce the area of microbial colonisation on beech leaves suggesting that they are feeding on microbial biofilms rather than on the detrital material itself. Oak leaves supported a larger microbial community than beech but there was no effect observed on the development or reproduction of *B. zschokkei* reared on either food source. At the scale that *B. zschokkei* is feeding the microbial community on both these detritus types is probably sufficient to support fast development and high offspring production for this copepod. FPOM was a lower quality food source for this copepod, resulting in significantly longer development times and lower brood production for *B. zschokkei*. Diatoms present on beech leaves conditioning for 6 weeks, may have contributed to the diet of *B. zschokkei*. Significantly higher hatching success and shorter embryonic development times were observed on leaves conditioned for six compared with two weeks although development to

adult was prolonged. This may be related to differential feeding of larval and adult life-stages.

4.1 Introduction

Allocthonous inputs of coarse particulate organic matter (CPOM), in the form of leaf litter and woody debris, drive the trophic dynamics of woodland streams (Giller & Malmqvist, 1998). Once submerged in the water column, leaf material undergoes weight loss through leeching of soluble molecules such as amino acids, carbohydrates and phenolics (Suberkropp *et al*, 1976). This is followed by microbial colonisation, which is dependent on the leaf species (Petersen & Cummins, 1974), time in the stream (Suberkropp & Klug, 1976), and on chemical conditions such as nutrient content (Webster & Benfield, 1986) and pH (Burton *et al*, 1985; Chamier, 1987). The activities of these microbial colonisers result in decomposition. In the early stages of decomposition (<6 weeks), fungi (mainly aquatic hyphomycetes) are the dominant microbes (Bärlocher & Kendrick, 1973; Suberkropp & Klug, 1976; Chamier & Dixon, 1983) but, after 6-12 weeks (depending on the leaf species), CPOM will have been broken down to fine particulate organic matter (FPOM, size range 45-1000 μm) which is too small to support fungal hyphae. At this stage, bacterial biomass becomes proportionally more important than the fungi and the degradation process is complete (Suberkropp & Klug, 1976).

In freshwater systems decomposing leaf material in the form of CPOM and FPOM is referred to as detritus and is the most important source of food for many aquatic invertebrates. The interaction between detritus and detritivores is the most extensively studied trophic pathway within stream systems (Allan, 1995). The nutritional quality and palatability of detritus (particularly CPOM) to many detritivores is dependent on the degree

of conditioning, where conditioning is related to the amount of microbial colonisation and leaf degradation. A preference for leaves at an optimal stage of conditioning (maximum colonisation by microbes) has been observed for several detritivore species (Arsuffi & Suberkropp, 1984; Suberkropp & Arsuffi, 1984). Indeed, many stream invertebrates can differentiate between areas of leaf with different degrees of conditioning or even between species of fungi (Arsuffi & Suberkropp, 1985; Graça *et al*, 1993a, b). For example, feeding preferences of the amphipod *Gammarus pseudolimneaus* were related to the species of fungi colonising the leaf rather than to the identity of the leaf species (Bärlocher & Kendrick, 1973). Bacteria also contribute a significant component of the diet of some detritivores including blackflies (Edwards & Meyer, 1987) and mayflies (Edwards & Meyer, 1990). Leaf conditioning is enhanced by microbes releasing enzymes which breakdown structural carbohydrates (cellulase and hemicellulase) and it has been proposed that rather than the attached microbes, it is the leaf material itself, that contribute the major energy source for detritivores (Cummins & Klug, 1979; Lawson *et al*, 1984). Detritus is, therefore, a complex food resource and its quality will be determined by the amount of conditioning and degradation of the leaf material but also on the feeding ecology of the species itself.

It is often difficult to define whether detritivores are generalist or specialist feeders or which aspect of detritus (bacteria, fungi or the leaf itself) a particular species obtains most of its energy from. Food preference and stable isotope experiments indicate what animals consume and process. However, the key to understanding the value of food quality is the response of individuals in terms of life-history traits. Demographic parameters reflect the ability of a particular food to support long-term population growth (Chen & Folt, 1993), and may be used to interpret the energetic costs and benefits of living in a particular habitat (Stanko-Mishic *et al*, 1999). For example, *Asellus aquaticus* fed diets of unconditioned and

conditioned leaf material had significantly higher growth rates and fecundity on the latter diet (Graça *et al*, 1993b). Unconditioned leaf material can, therefore, be considered a low quality diet for *A. aquaticus*. This is not, however, a general conclusion as *Gammarus pulex* fed on the same two diets showed no significant difference in growth or fecundity (Graça *et al*, 1993b). Survival and growth of some detritivores even varies depending on the species of fungus that they are fed (Arsuffi & Suberkropp, 1985). More information on how different sources of detritus influence demographic parameters (such as development and reproduction) of detritivores would give a greater understanding of interspecies exploitation of food resources (Graça *et al*, 1993b; Stanko-Mishic *et al*, 1999).

Despite the large interest in macroinvertebrate detritivore-detritus interactions, very little is known of the influence of meiofauna in the detritus processing of streams, even though meiofauna and protozoans are thought to play an important role in food web dynamics forming a trophic link between detritus and macroinvertebrates (Borchardt & Bott, 1995; Hackenkamp & Morin, 2000; Swan & Palmer, 2000). There is evidence to suggest that meiofaunal distribution in lotic systems is linked with organic matter (Swan & Palmer, 2000). For example, in colonisation experiments of interstitial sediments a positive relationship was observed between the amount of sediment, and harpacticoid and nematode abundance, suggesting that meiofaunal feeding may be related to the presence of FPOM (Schmid-Ayara, 2000; Schmid-Araya & Schmid, 2000). Field studies have also shown that leaf packs with the highest microbial biomass support the greatest abundances of several meiofaunal groups (Palmer *et al*, 2000). The species-specific distribution of some microcrustaceans (including *Bryocamptus zschokkei*) has also been found to vary with the amount and size structure of organic material (Shiozawa, 1986; Robertson *et al*, 1995). Further evidence for meiofauna consuming detritus comes from laboratory studies which

show that stream harpacticoids (*Attheyella* spp) will graze selectively on detritally-associated bacteria, resulting in a reduction in bacterial density and biomass (Perlmutter & Meyer, 1991). There is a high biodiversity in the meiofauna of the stream benthos and therefore specialisation in feeding might be expected for species that co-exist (Robertson *et al*, 2000b). The harpacticoid *Bryocamptus zschokkei* often occurs with species of *Attheyella* in the interstitial sediments of streams and it has been proposed that algae, protozoa, fungi and bacteria are all potential food sources for *B. zschokkei* in its natural environment.

The general aims of this chapter are to consider the life-history responses of *B. zschokkei* when reared on different detrital diets. The potential food available to *B. zschokkei* on each diet was assessed using scanning electron microscopy (SEM). Specific aims were to assess whether:

1) Harpacticoid grazing influences the microbial community of beech leaves.
2) Detritus type [i.e. beech leaves (*Fagus sylvatica*), oak leaves (*Quercus robur*) or FPOM] influences copepod development and reproduction.
3) Detritus quality (i.e. leaves conditioned for two vs. six weeks) influences copepod development and reproduction.

4.1 Materials & Methods

4.1.1 Grazing Experiment

To assess the effects of harpacticoid grazing on the microbial community associated with leaf litter, scanning electron micrographs of leaf discs grazed by different densities of copepods were compared. Leaf discs (1 cm diameter) were cut from beech leaves that had been laboratory conditioned for two weeks in Elendt's medium (see Chapter 2). A leaf disc was added to each of eighteen 2 cm diameter polystyrene Cellwells™ (Dow Corning,

Corning, NY, USA), with 3 ml of Elendt's M4 medium (100 mg l^{-1} $CaCO_3$). Eight adult female copepods were added to each of six wells (low density), 16 females were each introduced into a further six wells (high density), and six wells contained no animals (controls). Copepods were allowed to graze the leaves for seven days. A partial water replacement (66%) was carried out every second day. On day seven, the copepods were removed and a smaller (0.4 cm diameter) disc was obtained from the leaves for microbial counts.

4.1.2 Scanning Electron Microscopy

Leaf discs from both the grazing and demographic experiments were fixed in 2.5% Gluteraldehyde (Agar Scientific Ltd., 66a Cambridge Road, Stanstead, Essex) for 2 h. They were rinsed in 0.1M Cacodylate buffer, and dehydrated serially through 30, 50, 70, 90, 100% and 100% dry ethanol for 15 mins each, and transferred in ethanol to a critical point drier. Once dry, the discs were stored in a desicator for up to two weeks before observation. A layer of gold coating was applied to the surface of the specimen using an EMITECH K550 splutter coating unit prior to observation under a JOEL 5300 scanning electron microscope. Images were taken at ×200 to measure the overall microbial coverage and diatom density, and ×2000 to obtain bacterial densities. At each magnification 18 leaf discs were used. The area of leaf colonised by microbial flora was measured at ×200 using an image analysis programme calibrated to measure 1.03 μm per pixel (Leica Quantimet 570). The density of bacteria (×2000) and diatoms (×200) were estimated from images by manual counting.

4.1.3 The Effects of Detritus Type on Demographic Parameters

A beech leaf diet (*F. sylvatica*) is known to support development and reproduction of *B. zschokkei* (Chapters 2 & 3). Oak leaves (*Q. robur*), however, were common in the stream from which copepods were obtained to initiate the cultures used in this thesis. As detritus quality related to leaf species (Bärlocher & Kendrick, 1973), oak leaves were chosen as an alternative food source for *B. zschokkei*. Fine particulate organic matter (FPOM) is considered as the third type of detritus. In view of the small size of *B. zschokkei* and the fact that it inhabits interstitial sediments, where FPOM is the major organic component, FPOM may be considered a more appropriate food source for this copepod.

In September 1998, beech leaves were collected from Churston Woods, Brixham, Devon (grid reference 50°24'15N, 4°31'30W). In March 1999, oak leaves were taken from a tree in close proximity to the River Yealm site (Hele Cross, Cornwood, Devon, grid reference 50°25'90N, 4°57'50W) where copepods were collected for laboratory cultures (see Chapter 2). Only whole leaves with no visual sign of pathogen infection were used as a food source. All leaves were oven dried for 48 h at 60 °C and stored in a sealed polythene container at 20 °C until required for conditioning. Preliminary experiments showed that there was no significant difference in the colonisation by microbes of beech leaves conditioned in stream water or artificial Elendt's M4 medium (Table 4.1) (Elendt & Bias, 1990). Copepods fed leaves conditioned in either stream water or Elendt's M4 medium also did not differ significantly in terms of their life-history responses (Table 4.2). Therefore, all leaves were conditioned for two weeks by immersion in continuously aerated Elendt's M4 medium, which was replaced once per week.

Table 4.1: The effects of two different conditioning medium (stream water and artificial Elendt's M4 medium) on the microbial colonisation of beech leaves.
There was no significant effect of conditioning medium.

Microbial colonisation	Stream water conditioned	Elendt's M4 medium conditioned
Mean ± 1SD area of total microbial colonisation (μm^2)	$2.79 \times 10^4 \pm 1.24 \times 10^4$ (n=17)	$3.20 \times 10^4 \pm 1.26 \times 10^4$ (n=17)
Mean ± 1SD density of bacteria (per cm^2)	$2.19 \times 10^6 \pm 1.28 \times 10^6$ (n=17)	$2.66 \times 10^6 \pm 1.67 \times 10^6$ (n=17)

Table 4.2: The effects of two different conditioning medium (stream water and artificial Elendt's M4 medium) on the life history parameters of *Bryocamptus zschokkei*.
There was no significant effect of conditioning medium.

Life-history parameter	Stream water conditioned	Elendt's M4 medium conditioned
Mean ± 1SD development time to adult (N1-A) (days)	19.47 ± 3.66 (n=15)	18.83 ± 0.94 (n=12)
Mean ± 1SD no. eggs per female	100.89 ± 41.21 (n=9)	116.75 ± 26.08 (n=8)
Mean ± 1SD no. nauplii per female	58.00 ± 53.02 (n=9)	54.13 ± 36.57 (n=8)

FPOM was obtained from the River Yealm sampling site (see above and Chapter 2). Kick samples of organic material were collected by disturbing the bottom sediments at several points in the stream into a 63 μm mesh. This material was then passed through 500 μm and 63 μm sieves. The FPOM retained on the 63 μm sieve was returned to the laboratory and frozen at – 20°C for 48 h to kill the metazoans but to maintain the associated microflora. The organic fraction was separated from small stones and mineral particles by elutration, and stored at 20°C in the laboratory in continuously aerated Elendt's M4 medium.

4.1.4 The Effects of Detritus Quality on Demographic Parameters

To assess the influence of detritus quality on the demographic parameters of *B. zschokkei*, copepods were reared on a food source of beech leaves that had been conditioned for either

2 or 6 weeks in continuously aerated Elendt's M4 medium (medium replaced once per week).

4.1.5 Demographic Parameters

Trials were carried out in a temperature-controlled room at 20 (± 1) °C with a photoperiod of 16 h light: 8 h dark, and a 20 min dawn-dusk transition. Temperature was recorded daily. For all treatments, the dilution medium was Elendt's M4 medium modified to give a hardness of 100 mg l^{-1}, an alkalinity of 30 mg l^{-1} (both as $CaCO_3$), a conductivity of 290 µs cm^{-1} and a pH of 7.7 (Chapters 2 & 3). Partial water replacements (66%) were carried out three times a week and the dissolved oxygen content and pH of the media was measured before discarding. Development to adult was measured from the nauplius stage as described in Chapter 2. Fifteen nauplii (<24 hour old) per treatment were maintained individually in 1 cm diameter polystyrene Cellwells™ (Dow Corning, Corning, NY, USA) containing 2 ml of dilution medium and a 4 mm diameter disc of appropriately conditioned leaf material or 0.1 ml of FPOM suspension. As this study was concerned with food quality not quantity, all food was fed in excess so that the difference in surface area of the leaf material and FPOM should not influence the results. Animals were transferred into new Cellwells™ containing fresh medium and a new leaf or FPOM every 14 days. On day 1 of the experiment, nauplii were observed, to determine if they had been transferred successfully, but were not examined again until day 7, as handling of the naupliar stages significantly delays the development time to adult in *B. zschokkei* (Chapter 2). Copepod survival and the presence of moulted exuviae were recorded daily, and used to calculate the duration of the combined naupliar stages (Dn), individual copepodid stages, combined copepodid stages (Dc) and the total time taken to reach the adult stage (N1-A). The ratio of the time taken to develop through copepodid verses naupliar stages (the Dc/Dn ratio) was

also calculated, as this is considered to be a good indicator of food quality in copepods (Hart, 1990; Williams, 1997). Sex ratio, determined by size and the morphology of the antennae, was recorded at the end of the experiment.

The starting point for the reproduction trials was newly mated pairs (Chapter 3). Fourteen pairs were used per treatment, each placed in a 2 cm diameter polystyrene Cellwell™ containing 3 ml of Elendt's medium and a 1.1 cm diameter leaf disc or 0.2 ml of FPOM suspension (food in excess). Daily observations of the presence of egg sacs and nauplii were used to calculate: a) the embryonic development time (the time from which the egg sac was released externally to the time nauplii hatched), b) the inter-brood period (the time taken for a female to release a new egg sac after the previous one had hatched) and c) the number of broods per female. Aborted egg sacs were removed from the cell well and viewed on an inverted microscope (× 40) to determine the number of eggs present. For the purpose of this study, an aborted egg sac was defined as an egg sac from which no viable offspring hatched. Every seven days, each copepod pair was transferred to a new Cellwell™ containing fresh media and a new leaf disc. Nauplii that had hatched during that week remained in the old Cellwell™ and were counted as soon as possible. If more than one brood of nauplii was present, they were distinguished on the basis of size. Eggs that had not hatched were also counted, so that the overall hatching success could be determined. This experiment was continued for eight weeks. Daily observations of copepods were carried out using a Wild M8 binocular microscope under darkfield illumination (× 20-40 magnification).

Data for construction of abbreviated life-tables were derived from post-embryonic development times, and the measures of adult survival and offspring production from the reproduction experiment (see Chapter 3). As the same animals were not followed through

their entire life-cycle, approximations of the age specific survival (l_x) and the age of first reproduction are used. These parameters give estimates of the intrinsic rate of natural increase (r_m) and the net reproductive rate (R_O), allowing comparisons between treatments and with the previous data on *B. zschokkei* obtained in Chapter 3.

Leaves used in the feeding experiments were also observed under the SEM so that the food available to copepods fed on different diets could be characterised. To compare different types of detritus and detritus quality, 18 leaf discs (0.4 cm diameter) were obtained from four leaves of each leaf litter diet. Leaves were observed under the SEM as described in Section 4.2.2.

4.1.6 Statistical Analysis

Fishers Exact Test (Finney, 1963) was used to identify differences in copepod survival at the conclusion of development (day 28) and reproduction (day 50). Before applying analysis of variance techniques, all data were tested for normality and equality of variances using Bartlett's test. Data not conforming to these assumptions were log10 transformed. One-way ANOVA was used to test for differences between treatments in both 'grazing' (zero vs. low vs. high density) and detritus type (beech vs. oak vs. FPOM) trials using Bonferroni's multiple comparison procedure to discriminate between the means. If the assumptions for ANOVA were not met even after transformation, data were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Mann Whitney tests to highlight which treatments were different. Students t-tests, assuming either equal or unequal variances, were carried out detect differences between 2 or 6 weeks conditioning.

4.2 Results

4.2.1 Grazing Experiment

High and low density grazing by copepods reduced significantly the area of leaf covered by microbes compared with the control (zero grazing) (One-way ANOVA, $p<0.05$) (Fig. 4.1a). There was no significant difference in the area of microbial coverage of leaves with high or low copepod grazing. Although the overall microbial community was reduced in the presence of copepods there was no significant effect on the density of bacteria, in fact there was a trend towards increasing numbers of bacteria with increased grazing pressure (Fig. 4.1b).

4.2.2 The Influence of Different Detritus Types

4.2.2.1 Microbial Community

Visually, the microbial community present on the oak leaves was very different from that found on the beech leaves. For example, the numerically dominant fungal spore on the beech leaves was not observed on oak (Fig. 4.2). This difference in surface flora was reflected in the area of microbial coverage, which was significantly higher on oak compared with beech (Student's t-test, $p<0.05$) (Fig. 4.3a). The density of bacteria was also significantly higher on oak than beech leaves (Student's t-test, $p<0.05$) (Fig. 4.3b). No quantitative data were collected for FPOM as it was difficult to process defined quantities of this material for observation under the SEM. Nevertheless, photomicrographs of FPOM under the SEM demonstrated low bacterial densities and limited biofilm development (Fig. 4.4).

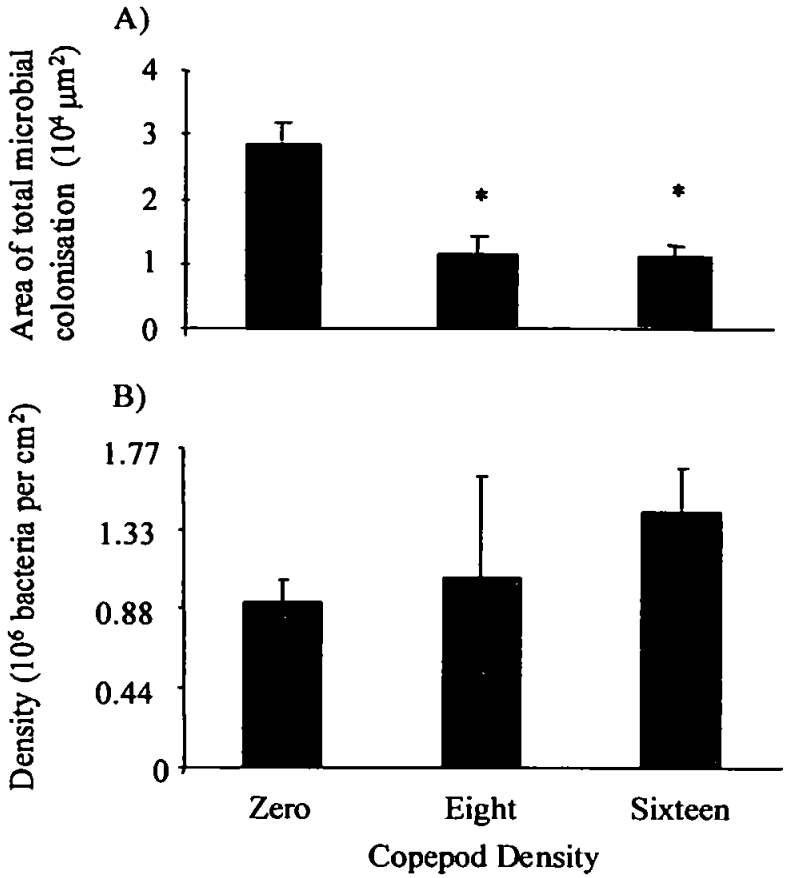


Figure 4.1: The microbial community on the surface of leaves grazed by eight or sixteen copepods for seven day, compared with controls (zero grazing).
A) shows the mean (± 1SE) area of microbial coverage (10^4 µm^2) on the surface of leaves and B) is the mean (± 1SE) density of bacteria (10^6 bacteria per cm^2). n =18 leaf discs per treatment and * indicates a significant difference between treatments (One-way ANOVA, $p<0.05$).

4.2.2.2 Development

There was no significant difference in the development times for nauplii or copepodids reared with oak and beech leaves, however, development of each life-history stage took significantly longer on FPOM compared with the leaf litter diets (One-way ANOVA, $p<0.05$) (Fig. 4.5). The Dc/Dn ratio, calculated for copepods on these three diets, was similar (Table 4.3). Survival during the development to adult is also shown in Table 4.3. Survival was 100% only on the beech leaf diet, and no difference between the three treatments during development to adult was observed (Fisher Exact, $p>0.05$).

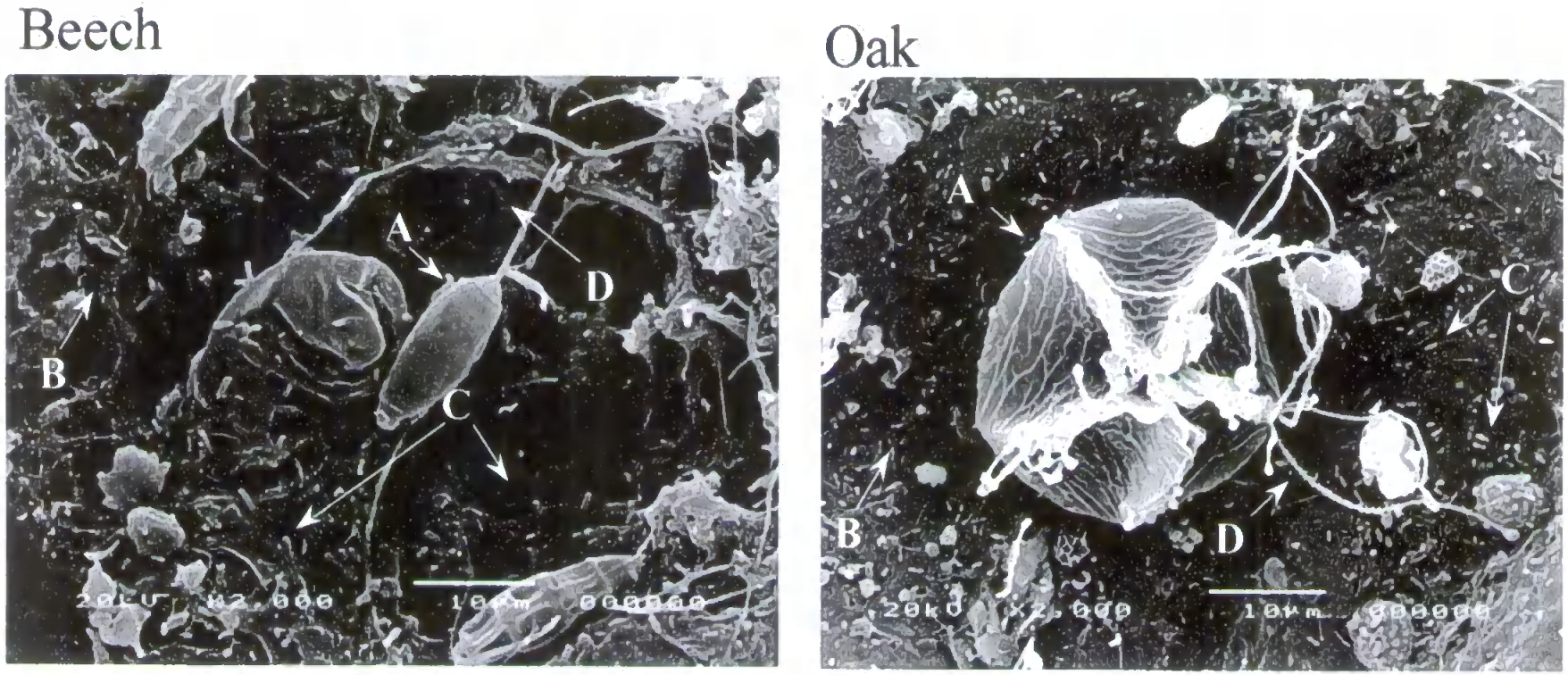


Figure 4.2: Electron micrographs (×2000) of the surface different types of detritus (beech vs. oak).
A) Fungal spore (N.B. different species of fungal spore on beech and oak leaves), B) polysaccaride matrix, C) bacteria cells and D) fungal hyphae. Scale bar represents 10 μm.

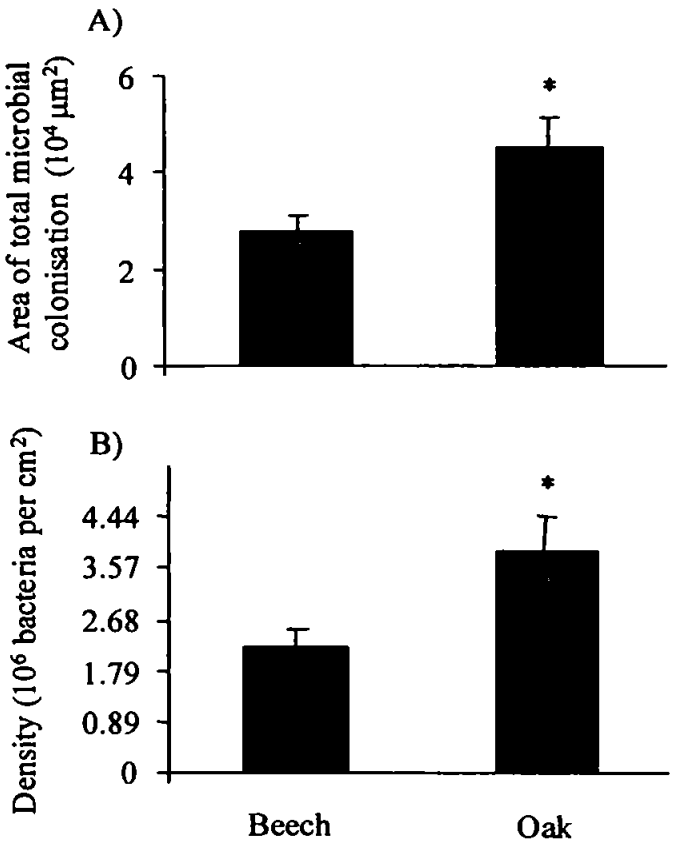


Figure 4.3: The microbial community on the two types of leaves (beech and oak) used to consider the effects of different detritus types on *Bryocamptus zschokkei*
A) shows the mean (± 1SE) area of microbial coverage (10^4 µm^2) on the surface of leaves and B) is the mean (± 1SE) density of bacteria (10^6 bacteria per cm^2). n =18 leaf discs per treatment and * indicates a significant difference between treatments (Student's t-test, $p<0.05$).

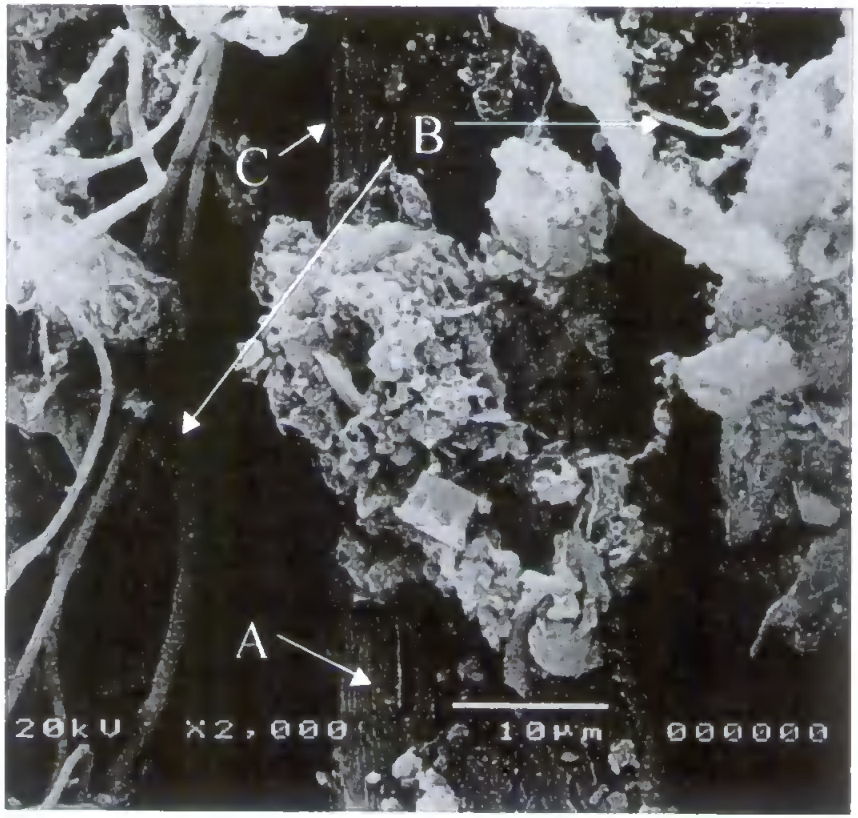


Figure 4.4: Electron micrograph (×2000) of fine particulate organic matter (FPOM)
A) bacteria cells, B) fungal hyphae and C) detrital material. Scale bar represents 10 µm.

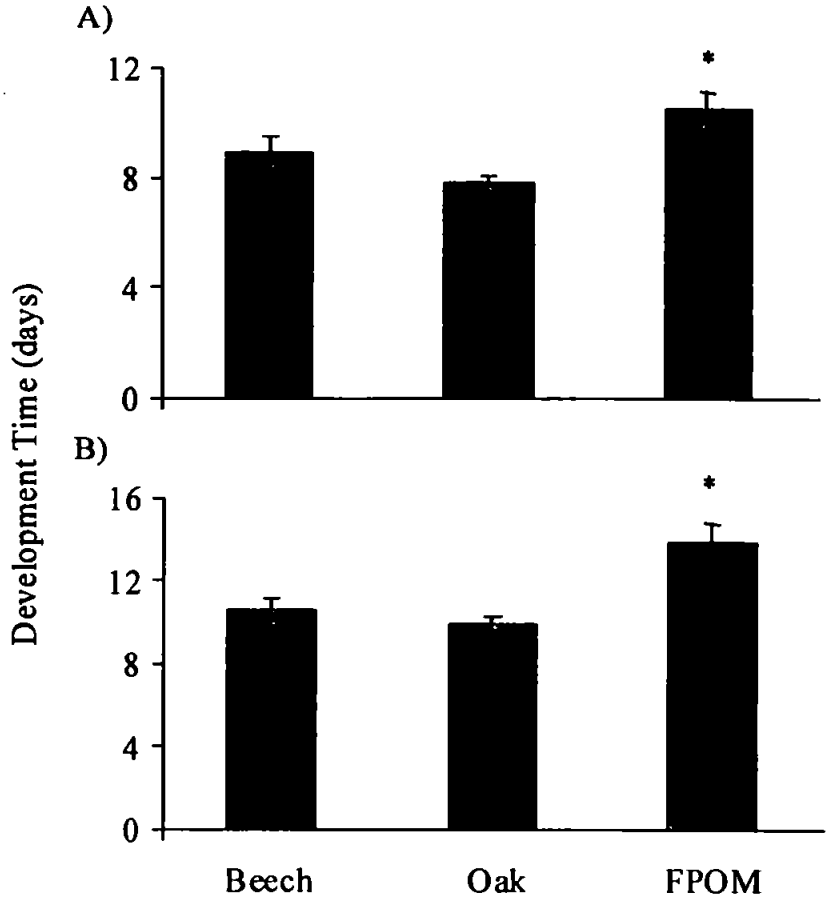


Figure 4.5: The mean (± 1SE) effect of different detritus types on the time taken for A) naupliar development (Dn) and B) copepodid development (Dc) of *Bryocamptus zschokkei*. n = 11-15 copepods per treatment. * Indicates a significant difference from beech and oak (One-way ANOVA, $p<0.05$).

Table 4.3: The mean ± 1SD development time to adult of *Bryocamptus zschokkei* reared on different types of detritus.
n = number of survivors (from original 15) at the end of the experiment and n^a = number of survivors that developed to adult stage within 28 days (end of experiment). Of the animals moulting to adult (n^a), the proportion that were male and female is shown. # Indicates mortality post development to adult and ## indicates that one animal did not achieve adulthood before the experiment was terminated. * Indicates a significant difference (Kruskal-Wallis, $p<0.05$)

Detritus type	n	n^a	Male	Female	Dc/Dn	Development to adult n^a (days) (N1-A)
Beech	15	15	0.13	0.87	1.18	19.47 ± 3.66
Oak	$10^{\#}$	14	0.14	0.86	1.27	17.79 ± 1.76
FPOM	$12^{\#\#}$	11	0.45	0.55	1.32	24.18 ± 4.09*

4.2.2.3 Reproduction

Only four females fed on FPOM produced egg sacs from which viable offspring hatched and the mean number of broods and eggs per female were significantly reduced compared with animals fed on the leaf litter diets (One-way ANOVA, $p<0.05$) (Table 4.4). Copepods on oak or beech leaves showed no significant differences in the numbers of egg or nauplii per female (Table 4.4). The mean length of the interval between successive broods was prolonged significantly (3.5 days) on the FPOM diet (One-way ANOVA, $p<0.05$) (Table 4.5). There were, however, no significant differences in the embryonic development times between the copepods reared on three types of detritus (Table 4.5). There was no significant difference in adult survival on any of the diets (Table 4.6). A decrease of 60% in the intrinsic rate of natural population increase (r_m) was observed for copepods fed FPOM compared with the beech leaf food source. Life-table parameters were similar for copepods fed on either oak or beech (Table 4.7).

Table 4.4: The effect of three different types of detritus on reproductive parameters, the number of eggs per brood and the number of broods, eggs and viable nauplii per female produced by *Bryocamptus zschokkei*.
n = the original number of females per treatment, n_a = the number of females producing egg sacs and n_b = the number of females producing egg sacs from which viable offspring hatched. * Indicates a significant difference from other treatments (One-way ANOVA, $p<0.05$).

Detritus type	n	n_a	n_b	**Mean ± 1SD eggs per brood**	**Mean ± 1SD number of broods per female (n_a)**	**Mean ±1SD number eggs per female (n_b)**	**Mean ± 1SD number nauplii per female (n_b)**
Beech	14	12	9	11.01 ± 4.52 n=89	8.08 ± 3.18	100.89 ± 41.21	58.00 ± 53.02
Oak	14	14	8	12.83 ± 4.35 n=76	7.14 ± 2.66	94.38 ± 67.90	61.38 ± 79.20
FPOM	14	12	4	8.10 ± 3.46* n=30	4.00 ± 1.71*	32.25 ± 20.95*	25.75 ± 17.59

Table 4.5: The effect of three different types of detritus on the embryonic development time and the interval between successful broods (from which viable nauplii have hatched) and aborted broods produced by female *Bryocamptus zschokkei*.
* Indicates a significant difference from other treatments (One-way ANOVA, $p<0.05$)

	Embryonic development		Successful broods			Aborted broods		
Detritus type	Mean ±1SD brood duration (days)	n	Mean ±1SD brood interval (days)	Median brood interval (days)	n	Mean ±1SD brood interval (days)	Median brood interval (days)	n
Beech	4.07 ± 0.96	55	1.06 ± 2.16	0	52	3.83 ± 3.17	3	30
Oak	4.02 ± 0.83	33	0.54 ± 1.12	0	35	4.63 ± 3.64	4	52
FPOM	3.50 ± 1.09	12	4.50 ± 3.37*	4	10	6.38 ± 5.25*	5	29

Table 4.6: The number of male and female (from original 14) *Bryocamptus zschokkei* reared on different types of detritus surviving to the end of the reproduction experiment (day 50).

Detritus type	Males	Females
Beech	11	13
Oak	11	9
FPOM	11	11

Table 4.7: Summary of 'Life-Table Analyses' for *Bryocamptus zschokkei* populations fed different types of detritus and detritus of different quality (two vs. six weeks conditioning). Life-tables assumed a 1:1 sex ratio for offspring production. For animals reared on oak or beech R_o and r_m might therefore be expected to be higher because of the female skewed sex ratios. Caution should be observed when comparing with previously published data as these life-tables were constructed using data from two separate experiments and therefore did not follow the same individuals over the entire time period.

Detritus type 20 (± 1) °C	**Sex Ratio (male:female)**	**$R_o = \sum U_x$ = net reproductive rate per generation**	**T*coh* = cohort generation time (days)**	**r_m = intrinsic rate of natural increase**
Beech	1:4	18.68	42.96	0.077
Oak	1:4	17.54	44.72	0.073
FPOM	1:1	3.68	48.01	0.028
Conditioning time 20 (± 1) °C				
Two weeks	1:4	18.68	42.96	0.077
Six weeks	1:4	27.46	43.95	0.084

4.2.3 The Influence of Detritus Quality

4.2.3.1 Microbial Community

Diatoms were only recorded on leaves conditioned for six weeks (mean (± 1SE) = 0.87×10^4 (± 0.11×10^4) diatoms per cm^2. A significantly lower density of bacteria was recorded for leaves conditioned for six compared with two weeks (Student's t-test, $p<0.05$) (Fig. 4.6b).

There was no difference in the area of microbial coverage between the two conditioning times (Fig. 4.6a). Beech leaves conditioned for two and six weeks are shown in Figure 4.7.

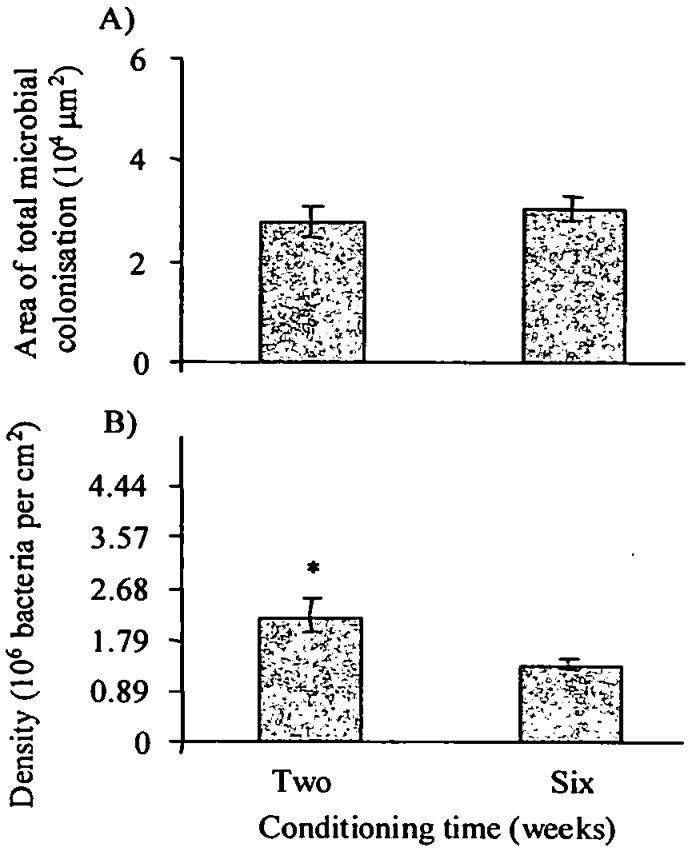


Figure 4.6: The microbial community on leaves conditioned for different lengths of time (two vs. six weeks).
A) shows the mean (± 1SE) area of microbial coverage (10^4 µm^2) on the surface of leaves and B) is the mean (± 1SE) density of bacteria (10^6 bacteria per cm^2). n =18 leaf discs per treatment. * Indicates a significant difference between treatments (Student's t-test, $p<0.05$).

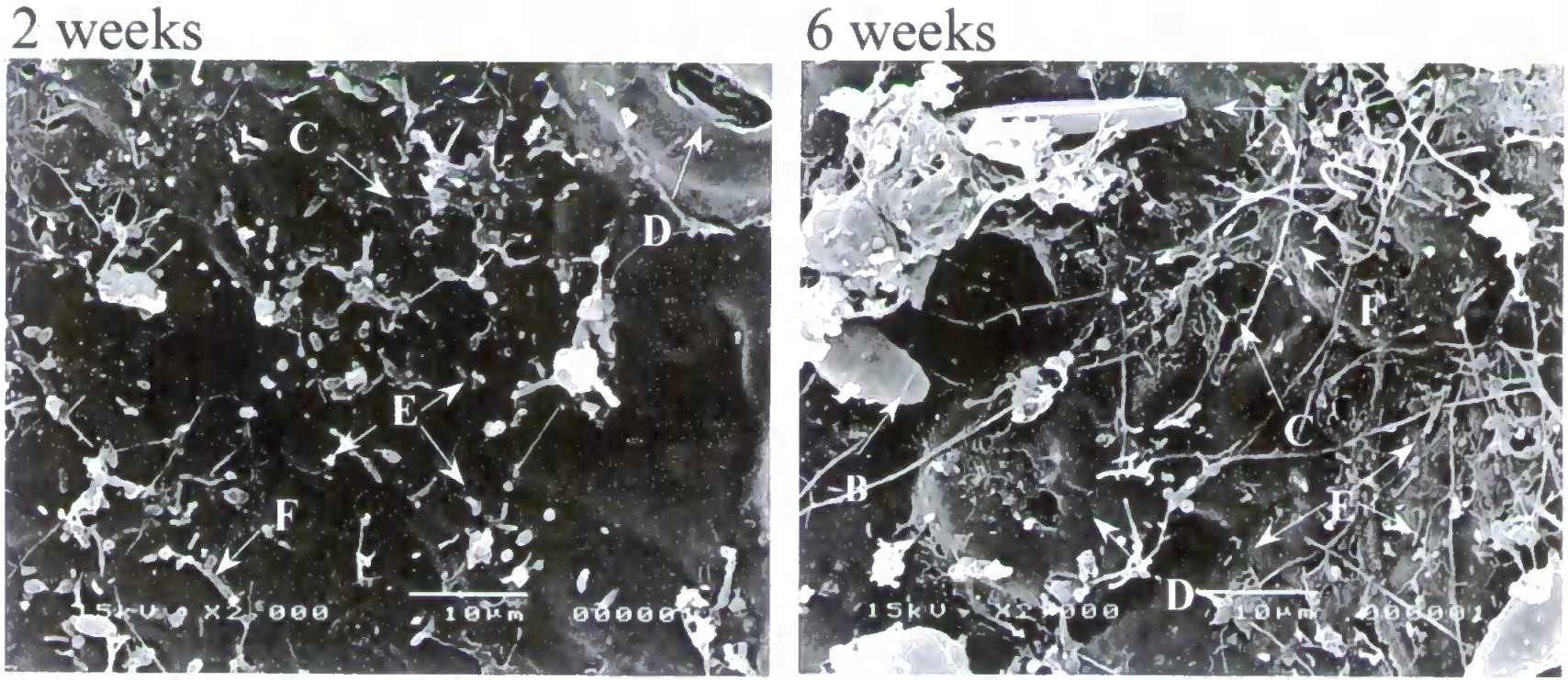


Figure 4.7: Electron micrographs (×2000) of the surface of leaves conditioned for two or six weeks.
A) diatom B) fungal spore C) polysaccaride matrix, D) leaf stomata, E) bacteria cells and F) fungal hyphae. Scale bar represents 10 µm.

4.2.3.2 Development

There was no significant difference in naupliar development (Dn) for the two conditioning times but the time taken for copepodid development (Dc) was, however, significantly longer in animals fed leaves conditioned for six verses two weeks (Student's t-test, $p<0.05$) (Fig. 4.8). This was reflected in a high Dc/Dn ratio (1.40 as opposed to 1.18) for copepods reared on leaves conditioned for the lesser time. Analysis for individual stages indicated that the second copepodid stage (C2) was significantly prolonged in animals fed leaves conditioned for six weeks (Student's t-test, $p<0.05$) (Table 4.8).

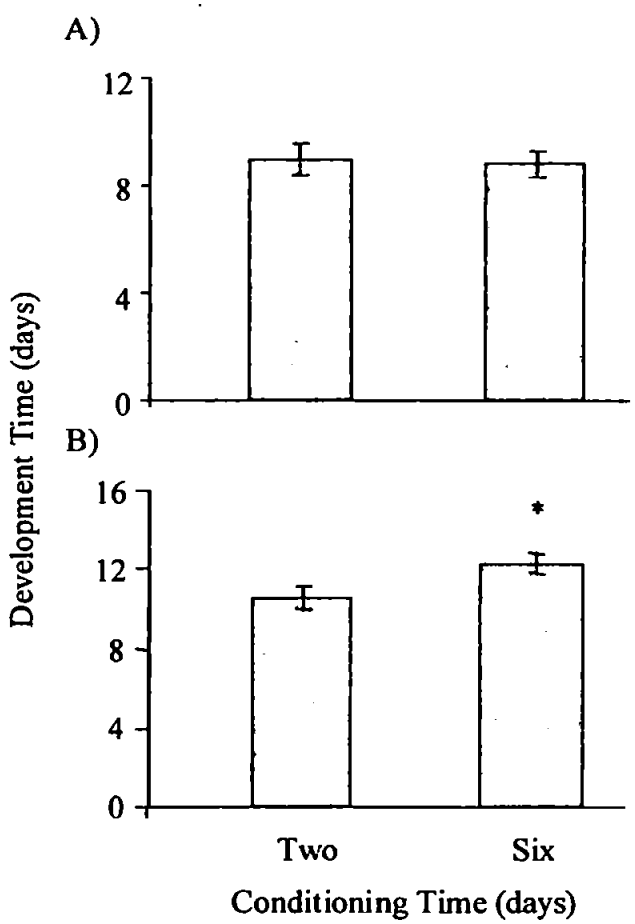


Figure 4.8: Effect of leaves conditioned for different lengths of time (two vs. six weeks) on the time taken mean (± 1SE) for A) naupliar development (Dn) and B) copepodid development (Dc) of *Bryocamptus zschokkei*.
n = 13-15 per treatment. * Indicates a significant difference (Student's t-test, $p<0.05$).

Table 4.8: The development times (mean ±1SD) of combined naupliar stages (Dn), individual and combined copepodid stages (Dc), and hatching to adult (N1-A) for *Bryocamptus zschokkei* fed on beech leaves conditioned for two or six weeks.
n = number of survivors (from original 15) at the end of the experiment and n^a = number of survivors that developed to adult stage within 28 days (end of experiment). The number in brackets is the number of animals used to calculate the duration of each stage. * Indicates a significant difference between the two treatments (Student's t-test, $p<0.05$).

Conditioning				Naupliar	Individual copepodid stages					Copepodid	Hatching
time (weeks)	n	n^a	Dc/Dn	Stages (Dn)	C1	C2	C3	C4	C5	Stages (Dc)	to Adult (N1-A)
Two	15	15	1.18	8.93±2.28 *(15)*	1.87±0.52 *(15)*	1.47±0.52 *(15)*	1.80±0.56 *(15)*	2.07±0.88 *(15)*	3.33±1.68 *(15)*	10.53±2.67 *(15)*	19.47±3.66 *(15)*
Six	13	13	1.40	8.79±1.85 *(14)*	2.23±0.60 *(13)*	2.54±0.52 *(13)* *	2.31±0.63 *(13)*	2.31±0.95 *(13)*	2.92±0.49 *(13)*	12.31±1.60 *(13)* *	21.08±1.85 *(13)*

4.2.3.3 Reproduction

Hatching success was significantly higher in copepods reared on leaves conditioned for the longer period and fewer non-viable eggs were produced per female on leaves conditioned for six compared with two weeks (Student's t-test, $p<0.05$) (Fig. 4.9). A higher mean number of nauplii per female were recorded for leaves conditioned for six as opposed to two weeks but there was high variability resulting in the difference being not significant (Table 4.9). Embryonic development times were significantly shorter in females fed on beech leaves conditioned for 6 weeks than those fed on leaves with less conditioning (Student's t-test, $p<0.05$) (Fig. 4.10). Despite differences in individual demographic parameters, life-table analysis showed that there was very little difference in the intrinsic rate of increase (r_m) or the net reproductive rate per generation (R_o) for animals fed beech leaves conditioned for two or six weeks (Table 4.7).

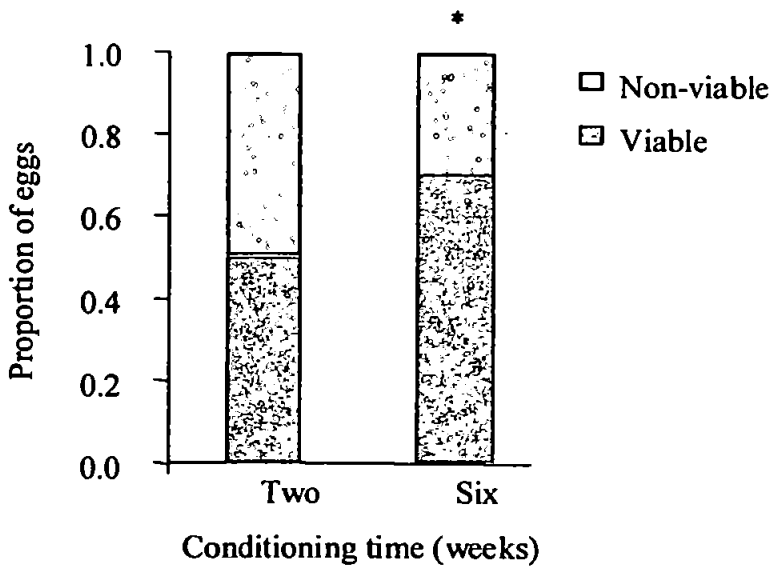


Figure 4.9: The proportion of viable and non-viable eggs produced by female *Bryocamptus zschokkei* fed leaves conditioned for different lengths of time (two vs. six weeks). n=9-10 females per treatment. * Indicates a significant difference (Student's t-test, $p<0.05$)

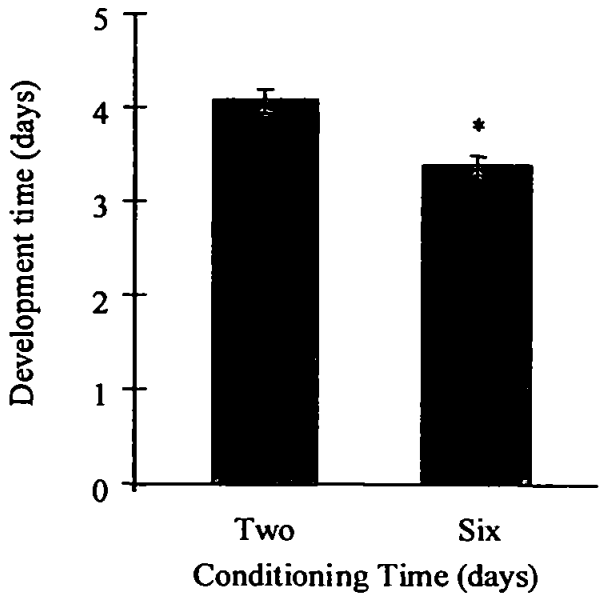


Figure 4.10: The effect of conditioning time (two vs. six weeks) on the mean (± 1SE) embryonic development time of egg sacs of *Bryocamptus zschokkei*.
n =55 (two weeks) and 62 (six weeks). * Indicates a significant difference (Student's t-test, $p<0.05$)

Table 4.9: The effect of conditioning time on reproductive parameters, the number of eggs per brood and the number of broods, eggs and viable nauplii per female produced by *Bryocamptus zschokkei*.
n = the original number of females per treatment, n_a = the number of females producing egg sacs and n_b = the number of females producing egg sacs from which viable offspring hatched. There was no significant effect of conditioning time.

Conditioning time (weeks)	n	n_a	n_b	Mean ± 1SD eggs per brood	Mean ± 1SD number of broods per female (n_a)	Mean ± 1SD number eggs per female (n_b)	Mean ± 1SD number nauplii per female (n_b)
Two	14	12	9	11.01 ± 4.52 n=89	8.08 ± 3.18	100.89 ± 41.21	58.00 ± 53.02
Six	14	14	10	11.61 ± 5.94 n=94	7.93 ± 4.01	102.60 ± 69.11	76.70 ± 77.77

4.3 Discussion

4.3.1 Grazing Experiment

The extensive biofilm on stream sediments is thought to contribute either exclusively or in part to the diet of the lotic meiofauna (Hakenkamp & Morin, 2000; Schmid-Araya & Schmid, 2000). Qualitative assessment of leaves grazed by the freshwater harpacticoid (*Attheyella* sp.) showed that increased copepod density (up to 10 per 1.1 cm diameter leaf disc) and grazing time (up to 14.5 days) was accompanied by a reduction in the amount of fungi and organic debris on the leaf surface (Perlmutter & Meyer, 1991). The present study provides the first quantitative evidence that the harpacticoid *B. zschokkei* consumes microbes from the biofilm of leaf surfaces. For example, the area of beech leaves covered by microbes was reduced as a result of copepod grazing by around 60% over seven days. This supports evidence from marine systems that meiofauna are grazing on microbial biofilms rather than on the detrital material itself (Marcotte, 1984). Grazing pressure by harpacticoids in the studies described exceeds levels observed in the field under normal conditions but reflect levels which have been observed if competition from aquatic insects is removed, for example as a result of pesticide exposure (Cuffney *et al*, 1984; Perlmutter & Meyer, 1991). This suggests that when locally abundant, *B. zschokkei* and other harpacticoids will have a significant role in the productivity of the stream benthos.

Grazing by *Attheyella* sp. reduced the density of detritally associated bacteria by up to 58% (Perlmutter & Meyer, 1991). In the current study, despite the reduction in microbial cover as a result of copepod grazing, the density of bacteria was not significantly influenced by the presence of *B. zschokkei* (Fig. 4.2). This difference in resource utilisation by these two harpacticoid species suggests a degree of feeding selectivity. Perlmutter & Meyer (1991) showed that *Attheyella* sp. could distinguish between different types of bacteria, preferring

the larger rod-shaped cells. A degree of specialisation might be expected as these, and several other harpacticoid species, often co-exist within the stream benthos (Rundle & Hildrew, 1990; Rundle & Ormerod, 1991). It would appear that, at least for adult *B. zschokkei*, bacteria may be a less important component of the diet than for other harpacticoid copepods.

Despite a large decrease in the microbial coverage on the leaf surface, due to grazing by *B. zschokkei* there was no effect of grazing on bacterial density even at high copepod numbers. These results suggest that microbial activity may be stimulated by the activities of the copepods. It has been suggested that movement and grazing by interstitial meiofauna increases the oxygen and nutrients to biofilm layers, enhancing microbial proliferation (Hakenkamp & Morin, 2000). Also, the removal of organic matter by copepod feeding may increase the area of leaf available for the bacteria to colonise (Hargrave, 1970; Gerlach, 1978). *Bryocamptus zschokkei* is also known to generate relatively large amounts of faecal material, likely to be high in nitrogen and phosphorous, as well as production of dissolved organic carbon (DOC) (O'Doherty, 1985; Brown, personal observation), both of which would provide additional food for microbes (O'Doherty, 1985).

4.3.2 The Influence of Different Types of Detritus

There were no significant differences in any of the life-history characteristics of *B. zschokkei* reared on oak or beech leaves, in spite of the fact that microbial coverage was higher on oak compared with beech (Fig. 4.2 & 4.3). Oak leaves are consistently more palatable than beech to macroinvertebrate detritivores (Kaushik & Hynes 1971; Iverson, 1974). Growth of the trichopterans *Sericostoma personatum* (Iverson, 1974) and *Potamophylax cingulatus* (Otto, 1974) was retarded on beech compared with other leaf species; the amphipod *G.*

pulex also shows a low preference for conditioned beech leaves (Nilsson, 1974; Friberg & Jacobsen, 1994). The low palatability of beech leaves may result from their poor nutrient content (Iverson, 1974; Kaushik & Hynes, 1971) and may also be due to high levels of condensed tannins which appear to inhibit cellulose breakdown in this leaf species (Friberg & Winterbourn, 1996). Such factors may explain the lower microbial coverage on beech compared with oak. In addition, leaf texture can also affect colonisation by microorganisms. Beech leaves are smoother than other leaf species (including oak) and, as a result, support lower densities of microbes (Friberg & Winterbourn, 1996). In the current study, high densities of bacteria were observed in the crevices on the surface of the oak leaves, which may help explain the significantly higher densities of bacteria on oak compared with beech leaves.

Despite differences in leaf species and different colonisation of microbes, there appears to be no nutritional benefit to be derived by *B. zschokkei* from the oak leaf treatment. One possible explanation for the lack of response in the demographic parameters to the potential difference in the nutritional quality of these diets is that the copepods are compensating for poor nutritional quality by consuming more of the low quality diet. Friberg & Jacobsen (1999) found this was the response of the shredding caddis *S. personatum* to poor food quality. If *B. zschokkei* increased their foraging rate on the poorer diet, a trade off in terms of reproductive output might be expected with females putting more energy into finding food than offspring production; however, such a trade off was not observed in this study. It is well established that macroinvertebrate detritivores can distinguish between differentially conditioned parts of a leaf and can select the most productive areas on which to feed (Arsuffi & Suberkropp, 1985). Meiofauna, however, forage at a much smaller scale and may be more efficient at selecting high quality food. It seems that there is sufficient

microbial colonisation on the surface of beech leaves to support rapid development and reproduction of *B. zschokkei* on a diet, which might for other species be considered low quality.

Prolonged development and a reduction in the numbers of broods and eggs per female were observed on the FPOM diet compared with the leaf litter treatments. Low survival has been observed previously for *B. zschokkei* that were fed on a diet of fine detrital particles as opposed to leaf litter (O'Doherty, 1985). The development of the chironomid larvae *Paratendipes albimanus* was not supported on a diet of stream-derived FPOM (Milton-Ward & Cummins, 1979). Although measures of detritus quality are often inconsistent, there is evidence to suggest that FPOM is a lower quality food than course particulate organic matter (CPOM) (Webster *et al*, 1999). Lower rates of microbial metabolism have been measured on stream-derived FPOM compared with CPOM (Peters *et al*, 1987; 1989). As results from the current grazing experiment suggest that *B. zschokkei* is feeding on the surface biofilm, the absence of microbial colonisation on the surface of FPOM (Fig. 4.4) is probably the main factor affecting the life-history parameters on this diet. It may be that the particle size of FPOM is to small to support fungal hyphae or substantial biofilm production (Golladay & Sinsabaugh, 1991). Treating the FPOM to use in these laboratory trials, for example killing the meiofauna, may also have reduced the palatability of this food source. For example, meiofauna may improve the quality of FPOM by stimulating microbes and through the addition of faecal material (Hackenkamp & Morin, 2000). A preference for CPOM might also have been predicted from field studies in which the distribution of *B. zschokkei* has been correlated with high sediment modal weight (Shiozawa, 1985; Robertson *et al*, 1995).

Despite the low food quality of FPOM for *B. zschokkei*, enough nutritional benefit was derived to support development (albeit with extended development) and good survival (Table 4.3). Extended development times might be an adaptation to help avoid over exploitation of resources when conditions are food limited (Hicks, 1979). Detritus, as a major food resource, is characterised by low persistence and patchy distribution (Townsend, 1989), and animals may alter their life-history strategies in response to changes in detrital quality. Within the stream environment, *B. zschokkei* may be able survive for long periods on a poor quality diet. Input of higher quality detritus results in prolonged development times and a reproductive strategy that is highly flexible to food availability (e.g. by increasing the number of broods produced).

4.3.3 The Influence of Detritus Quality

While postembryonic development of the copepodid stages of *B. zschokkei* is prolonged on beech leaves conditioned for six weeks, the embryonic development times were shorter and overall hatching success was significantly higher compared with animals fed leaves conditioned for two weeks. This increase in offspring production suggests that leaves conditioned for longer are of higher food quality, corroborating the conclusion from numerous other studies for macroinvertebrates (Kaushik & Hynes, 1971; Iverson, 1974; Bird & Kaushik, 1985; Graça *et al*, 1993b).

The main difference in the microbial community of beech leaves conditioned for six rather than two weeks was the presence of diatoms on the former. The leachate released from beech leaves during early conditioning has been shown to inhibit algal growth (Friberg & Winterbourn, 1996). In this study leachate released from the beech leaves during conditioning may be delaying the colonisation of microalgae such as diatoms. It is not

known whether *B. zschokkei* is utilising the microalgae directly as a food source. Diatom frustules have been found in the gut contents of this copepod taken directly from the field (personal observation), but it is not known if they are of any nutritional value (Hicks & Coull, 1983). There is an increasing amount of evidence to suggest that detritivores show a high degree of feeding plasticity and will supplement their diets or even show a preference for alternative food sources. Such foods tend to be of high nutritional value and include fresh macrophytes and microalgae (Jacobsen & Sand-Jensen, 1994, 1995; Friberg & Jacobsen, 1994; Ledger & Hildrew, 2000). A microalgae source improved the survival and production of two estuarine copepods otherwise maintained on a detrital diet (Heinle *et al*, 1977). Microalgae also appear to provide essential fatty acids to the diets of some marine harpacticoids (Weiss *et al*, 1996). Assimilation efficiencies of different food types would be required to determine the trophic role (generalist vs. specialist) of this primary consumer (Mihuk, 1997).

Copepodid development took significantly longer in animals fed on beech leaves conditioned for six rather than two weeks. The relationship between total naupliar and copepodid development (Dc/Dn ratio) has been used as an index of food availability during development (Hart, 1990; Williams 1997). A decrease in the Dc/Dn ratio is associated with an increase in food supply because naupliar stages tend to be less dependant on food than copepodid stages (Hart, 1990). The Dc/Dn ratios of 1.40 and 1.18 recorded for copepods fed leaves conditioned for six and two weeks, respectively, suggests the former may have been food limited. Extended development was also observed in this copepod on a diet of FPOM, which appears to be as a result of low microbial colonisation. While improved hatching success and short embryonic development times suggest that beech leaves conditioned for six weeks are a high quality food for *B. zschokkei*, larval life stages are to

some extent food limited. One explanation is that adult and juveniles are consuming different food types. Calanoid and cyclopoid copepods often exhibit ontogenetic shifts in feeding behaviour (Chow-Fraser & Wong, 1986; Santer & van den Bosch, 1994). Decho & Fleeger (1988) showed that the marine harpacticoid *Nitocra lacustris* did not start to ingest diatoms until they were C2 or C3, prior to which they consumed exopolymer exudates and bacteria. As the density of bacteria was lower on leaves conditioned for six rather than two weeks it might be that juveniles were food limited. Naupliar stages of *B. zschokkei* range in total length from 79-160 μm (Sarvala, 1977) and copepodid 248-536 μm (personal observation). Small life-history stages would be unlikely to consume diatoms [length, mean (± 1SD)=23.2 (± 3.7) μm] or fungal spores 14.7 (± 1.0) μm while bacteria 1.7 (± 0.8) μm would be easy to handle. The second copepodid stage contributes most to the extended development of *B. zschokkei*, suggesting that there were not enough bacteria available to support increased movement and metabolism of this development stage while larger copepodids could exploit other available food resources.

4.4 Summary

- The overall area of microbial cover on beech leaves was reduced in the presence of *B. zschokkei* while the density of bacteria was not affected. The data suggests that *B. zschokkei* may be selectively feeding on the microbial community as suggested previously for other freshwater harpacticoids.
- Oak leaves showed a higher area and diversity of microbial colonisation compared with beech. The high degree of microbial colonisation on oak leaves was, however, not reflected in food quality as no significant differences in demographic parameters were observed in *B. zschokkei* fed on these diets.

- Prolonged development to adult, and low level reproduction of copepods fed on FPOM, suggests that this is a low quality food for *B. zschokkei*.
- Beech leaves conditioned for six weeks supported greater hatching success and reduced embryonic development times than on leaves conditioned for two weeks but copepodid development was longer on the former diet. The presence of diatoms on the leaves conditioned for six weeks may provide additional nutrients to the diet of this copepod supporting the increase in reproductive output. Larval life stages would probably be too small to utilise this food source.

Chapter 5

The effects of the crustacean moulting hormone 20-hydroxyecdysone on the development and reproduction of *Bryocamptus zschokkei*

5 Abstract

Bryocamptus zschokkei was exposed to the crustacean moulting hormone 20-hydroxyecdysone (20-HE) in a full life-cycle toxicity test. This moulting hormone has been recommended as a reference (positive control) for evaluating relevant endpoints for chemicals with the potential to disrupt endocrine function in crustaceans. There was no significant effect of 20-HE on any of the measured endpoints (i.e. development to adult, sex ratio or numbers of eggs or nauplii per female), which probably reflects a greater tolerance of *B. zschokkei* to 20-HE compared with other crustaceans. The effects of 20-HE to *B. zschokkei* may also have been reduced by the presence of leaf discs, therefore, in subsequent long-term studies using this copepod as a test organism, measurement of the amount of test substance present in the dilution water is recommended. A solvent effect was also observed, with significantly prolonged copepodid (Dc) and total (N1-Adult) development times in the solvent (methanol) control compared with the control. Methanol may act as an additional carbon source for the microbial community on the leaf surface and, in turn, may affect food availability to *B. zschokkei*. This is the first time a full life-cycle toxicity test has been conducted for a freshwater harpacticoid copepod, however, before the potential use of this test for assessing toxicants effects can be fully evaluated, further investigations are required which incorporate some recommended modifications to the test protocol.

5.1 Introduction

In recent years, there has been growing concern over the effects of anthropogenic compounds, which at low levels have the ability to disrupt normal endocrine function in animals (Colborn *et al*, 1993). While most of the evidence to date comes from studies on vertebrates, there is growing concern that invertebrates may also be vulnerable to the effects of endocrine disruption (ED) (see recent reviews by Pinder *et al*, 1999; deFur *et al*, 1999). The endocrine systems of arthropods (insects and crustaceans) are the best defined

of the aquatic invertebrates (Pinder *et al*, 1999). While vertebrate-type hormones, including oestrogens and androgens have been detected in arthropods; a functional role for them has not been established (Pinder *et al*, 1999). However, arthropods do possess non-vertebrate peptide hormones, (including the juvenile hormones and ecdysteroids), that are known to be vulnerable to perturbation by certain contaminants. In fact, many pesticides are designed specifically to target the endocrine systems of insects; examples include methoprene and tebufenozide, which are juvenile hormone and ecdysone agonists, respectively. As crustaceans possess similar endocrine systems to insects, they might also be vulnerable to the effects of such chemicals. In addition, there are examples of effects in field populations of crustaceans consistent with ED. These include the occurrence of a high incidence of intersexuality (>90%) in several species of marine harpacticoid associated with a sewage outfall in the Firth of Forth, Scotland (Moore & Stevenson, 1991; 1994) and intersex lobsters *Homarus americanus* from sites in Nova Scotia, Canada (Sangalang & Jones, 1997). It was also suggested that a dramatic reduction in the proportion of males in daphnid populations in Lake Mendota (USA) since 1945 could be due to ED effects on male development (Dodson & Hanazato, 1995).

The steroid hormone 20-Hydoxyecdysone (20-HE) is the physiologically active moulting hormone in arthropods (Skinner, 1985). As a result of this, 20-HE has been recommended as a reference chemical for considering relevant endpoints for measurement in studies on the potential effects of endocrine disrupting chemicals (EDCs) (Hutchinson *et al*, 1999a, b; deFur *et al*, 1999). Hormonal control of moulting in Crustacea is the subject of several reviews although most of the information available comes from data on higher crustaceans, particularly decapods (Quackenbush, 1986; Charmantier *et al*, 1997; Fingerman, 1997). Despite the lack of information on hormone control in lower crustaceans, a role in moulting for 20-HE has been identified in cirripedes (Clare, 1997), malacostracans (Suzuki *et al*,

1996) and cladocerans (Bodar *et al*, 1990). In general, ecdysone is synthesised in ecdysial glands known as Y-organs and then secreted into the haemolymph where it is converted into the active form 20-HE. This hormone has also been found in the ovaries and embryos of some crustaceans, suggesting that 20-HE may also have a role in regulating reproduction (Suzuki *et al*, 1996; Subramoniam, 2000). Exogenous 20-HE is active in an *in vitro* ecdysteroid receptor-based screen derived from the B_{II} blood cell line of *Drosophila melanogaster* (B_{II} Assay) showing agonistic activity at 100 $\mu g\ l^{-1}$ 20-HE (Dinan *et al*, 2001). *In vivo* studies have also shown that exogenous exposure to 20-HE affects moulting of decapods (Freeman & Costlow, 1984), moulting and settlement in cirrepedes (Freeman & Costlow, 1983), the sex ratio and offspring production of cladocerans (Bodar *et al*, 1990; Peterson *et al*, 2001), and survival and offspring production of copepods (Hutchinson *et al*, 1999a, b).

In an effort to define cause and effect for potential EDCs there has been increased emphasis for laboratory tests that include endpoints such as sex ratio and morphological abnormalities. The use of 'life-cycle' tests has also been advocated to highlight particular life stages or endpoints vulnerable to perturbation by hormone mimics (deFur *et al*, 1999). It is recommended that life-cycle tests incorporate aspects of embryo, larval and gonad development and reproduction. A life-cycle test developed for the marine harpacticoid *Tisbe battagliai* has been used successfully to evaluate the effects of some vertebrate steroid hormones (oestrone, 17β-oestradiol, and 17α-ethynylestradiol) alongside 20-HE as a reference chemical (Hutchinson *et al*, 1999a). This present study combines the development and reproduction assays for *Bryocamptus zschokkei* (see Chapters 2-4) to produce the first full life-cycle toxicity test for a freshwater harpacticoid.

The general aim of this chapter is to consider how the affects of elevated concentrations of 20-HE might be manifest during the life-cycle of *B. zschokkei* with a view to using this chemical as a reference for the potential effects of ED. The specific aims for this chapter were to assess the:

1. Influence of chronic exposure to 20-HE on the long-term survival, development and reproduction of *B. zschokkei*.

2. Potential of a full life-cycle test for *B. zschokkei* adapted from protocols for considering the effects of water hardness on development (Chapter 2) and reproduction (Chapter 3).

5.2 Materials and Methods

5.2.1 Test Substance

20-Hydroxyecdysone (20-HE), minimum purity 95% was obtained from Sigma, Poole, Dorset, UK. Test solutions were prepared using the methods described by Hutchinson *et al* (1999a). A primary stock solution of 5 mg l^{-1} 20-HE was prepared by dissolving the test compound in methanol (a solvent carrier) and stored in an amber vial at –4°C. A secondary stock was prepared by the addition of an appropriate volume of primary stock to 200 ml of dilution water whilst stirring. Individual test solutions (100 ml volumes) were obtained by diluting known volumes of secondary stock with dilution water. In each test solution, the solvent concentration was made up to 0.1 ml l^{-1} methanol. All glassware was acid washed and then leached in dechlorinated water prior to use. For this study, the dilution water was modified Elendt's M4 medium (Elendt & Bias, 1990) as used in copepod cultures, with a hardness of 100 and an alkalinity of 30 (both as mg l^{-1} $CaCO_3$), conductivity was 290 µs cm^{-1} and pH 7.7. The composition of the dilution water complies with water quality guidelines recommended by the American Society of Testing and Materials (1999a) for the

testing of freshwater benthic invertebrates. The dilution water was aged for 7 days prior to use and aerated continuously.

5.2.2 Chronic Toxicity

The exposure concentrations used in this study were within the same range as observed to cause effects on moulting in the cladoceran *Daphnia magna* (Bodar *et al*, 1990), and on survival and reproduction in the marine harpacticoid *Tisbe battagliai* (Hutchinson *et al*, 1999a). The exposure concentrations consisted of a control, a solvent control, and 0.87, 2.7, 8.7, 26.9, 86.5 and 269 μg l^{-1} 20-HE. All experimental work was carried out in a temperature-controlled room at 20 (± 1)°C and a photoperiod of 16 h light: 8 h dark with a 20 min dawn-dusk transition. Temperature was recorded daily. Copepods were supplied with a 4 cm diameter beech leaf disc (*Fagus sylvatica* L.) as a source of food; discs were replaced every 14 days. Test solution was replaced (75%) three times per week and the dissolved oxygen concentration and pH of the media were measured before discarding.

The effects of 20-HE on the development and reproduction of *B. zschokkei* were measured in a single experiment. Nauplii (≤ 24 h old) were used to initiate the experiments and allowed to develop to adult. The nauplii were obtained from ovigerous females (approximately 100) which had been isolated from stock populations and maintained for 24 h in a 100 cm diameter crystallising dish containing Elendt's M4 medium (100 ml) and a 4 cm diameter beech leaf disc. After 24 h, ovigerous females were removed leaving only newly-hatched nauplii. The experiment was initiated with 40 nauplii per treatment, maintained individually in 1 cm diameter polystyrene Cellwells™ containing 2 ml of test solution and a 4 mm diameter beech leaf disc. Every 14 days, the animals were transferred into new Cellwells™ containing fresh medium and a new leaf disc. Nauplii were observed on day 1 of the experiment to determine if they had been transferred successfully but not

again until day 7 because excessive handling of naupliar stages can significantly delay the development time to adult of *B. zschokkei* (see Chapter 2). Subsequently, daily observations of copepods were carried out using a Wild M8 binocular microscope under darkfield illumination (magnification, ×20-40). Copepod survival and the presence of moulted exuviae were recorded daily, and used to calculate the duration of the combined naupliar (Dn) and copepodid (Dc) stages, and the total time taken to moult to adult. The ratio of males to females was also recorded. To consider the effects of 20-HE on reproduction once copepods had achieved adulthood (approx. 20 days), females and males were combined in a 2:1 ratio to match the observed female bias. After pairing, Cellwells™ containing females were monitored for the presence of egg sacs and nauplii. These data were used to calculate the embryonic development time (the time from which the egg sac was released externally to the time nauplii hatched), and the number of eggs and nauplii per female. Every seven days, females were transferred to a new Cellwell™ and nauplii that had hatched during that week were retained in the old vessel and counted. If more than one brood of nauplii was present, they were distinguished on the basis of size. Non-viable eggs were also counted. The duration of this experiment was six weeks, which in the dilution water control, was enough time for copepods to develop to adult and for females to produce an average of three broods.

5.2.3 Statistical Analysis

Differences between treatments in survival at 21 and 42 days were analysed using Fisher's Exact test (Finney, 1963). Chi square tests were used to consider differences in the sex ratio of copepods moulting to adult. All data were tested for normality (Shapiro-Wilks) and for equality of variances (Bartlett's test) before using analysis of variance techniques. If data did not conform to these assumptions, then they were $\log_{10}$ transformed. One-way ANOVA was used to test for differences between 20-HE exposures in development times, and

numbers of eggs and nauplii per female using Bonferroni's multiple comparison procedure to discriminate between the means. If the assumptions for ANOVA were not met, data were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Mann Whitney tests to highlight which treatments were different.

5.3 Results

5.3.1 Long-Term Survival

There was no significant difference in mortality between the solvent control and control at either day 21 or 42. Mortality was not exposure dependent and significantly lower survival, compared with the control, was observed for all 20-HE exposures (Fisher's Exact Test, $p<0.05$) apart from 269 $\mu g\ l^{-1}$ 20-HE at day 21 and all exposures except 2.7 and 269 $\mu g\ l^{-1}$ at day 42 (Fig. 5.1). At both days 21 and 42, there was no significant difference in mortality between the solvent control and any of the 20-HE exposures. After 21 days, most animals had achieved adulthood and, thereafter, there was no significant mortality in any of the treatments.

5.3.2 Development

There was a significant increase in the development times of copepodids (Dc) and from nauplius to adult (N1-Adult) for *B. zschokkei* in the solvent control compared with the control (Student's t-Test, $P<0.05$). There was, however, no difference in the development times for nauplii (Dn), copepodids (Dc) or from nauplius to adult (N1-Adult) in the solvent control compared with any of the 20-HE exposures (Fig. 5.2). There was also no difference in the ratio of males to females between treatments (χ^2 6.87, d.f. 4, $p=0.14$; Table 5.1) even though only two males moulted to adult at 269 $\mu g\ l^{-1}$ 20-HE and there were no males at 8.7 $\mu g\ l^{-1}$ 20-HE.

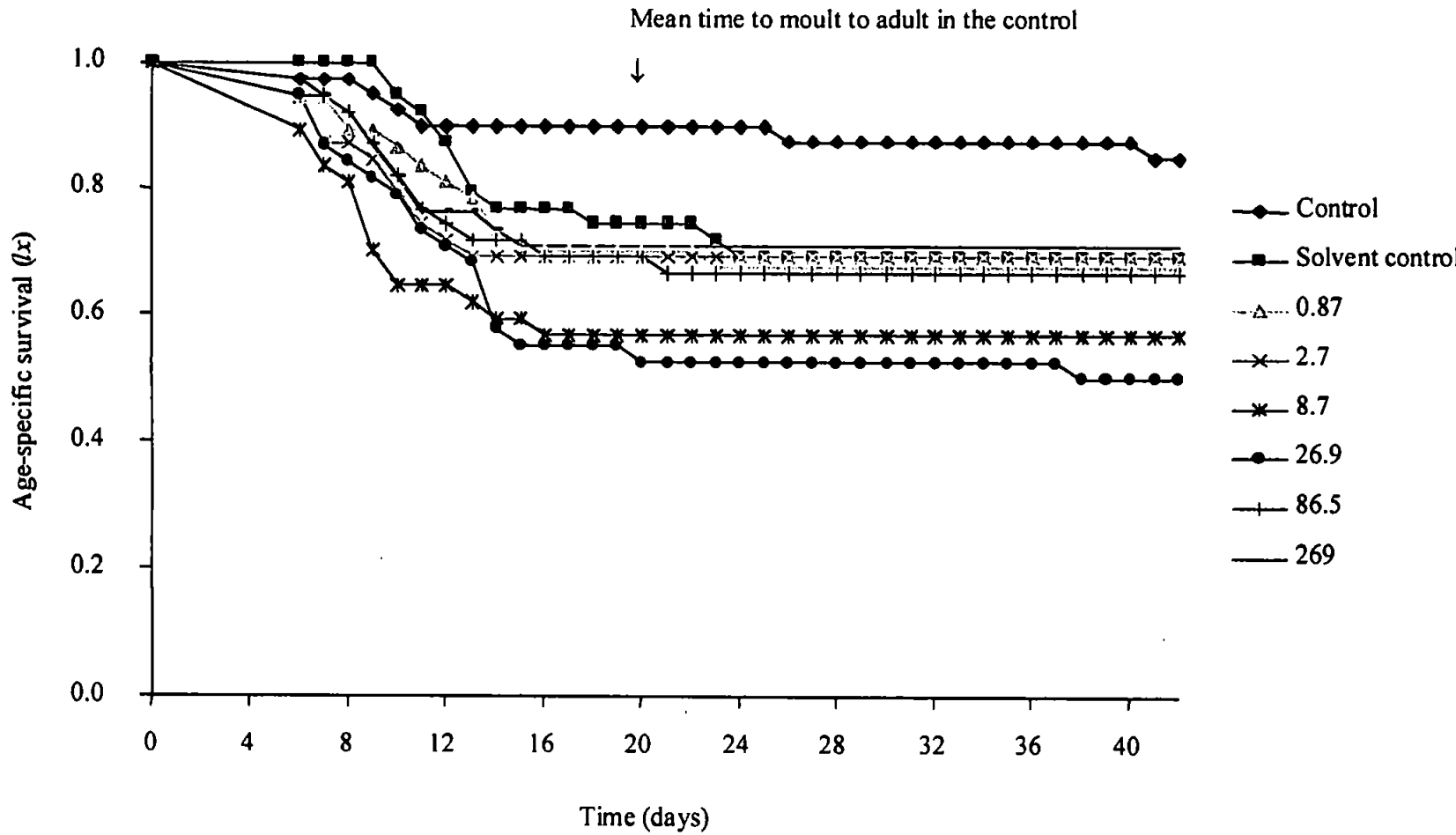


Figure 5.1: The effect of 20-HE on the age-specific survival (*lx*) of *Bryocamptus zschokkei*.
At day 21 there was significant mortality in all 20-HE treatments except the highest (269 µg l^{-1}) from the control but not compared with the solvent control (Fisher's Exact Test, $p<0.05$); n=37-40 per treatment.

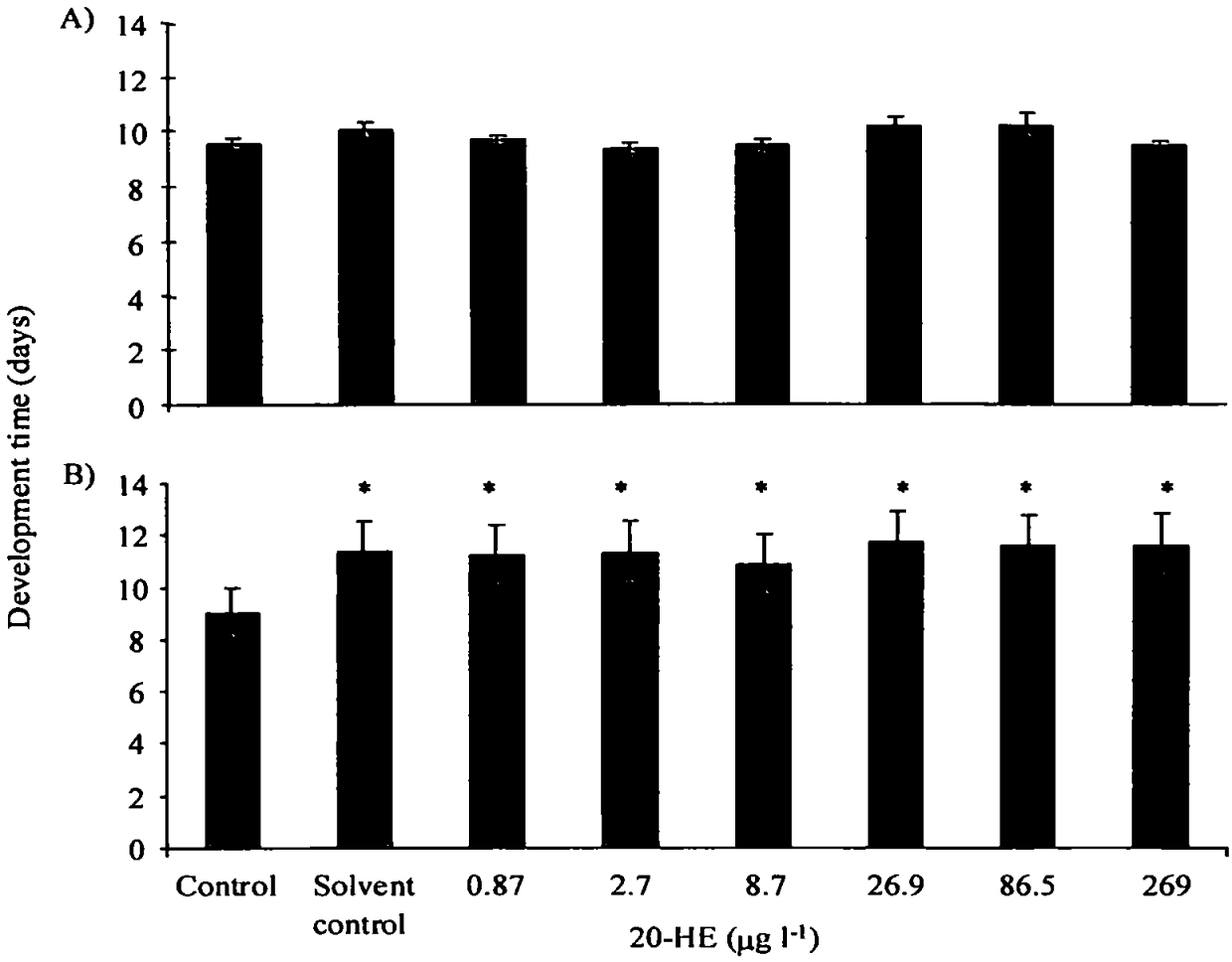


Figure 5.2: Effect of 20-HE on the time taken (mean ± 1SE) for A) naupliar development (Dn) and B) copepodid development (Dc).
* Indicates significantly longer development times compared with the control (Kruskal-Wallis, $p<0.05$); n=19-35 animals moulting to adult per treatment.

5.3.3 Reproduction

There was no significant difference between treatments in the time to release the first egg sac (Table 2) or in the numbers of eggs and nauplii per female in any of the 20-HE exposures compared with the controls (Fig. 5.3). There was no egg production at 8.7 µg l^{-1} 20-HE because there were no males present. The numbers of viable nauplii produced per female at the highest concentration of 20-HE (269 µg l^{-1}) were 45 and 39% lower compared with the control and solvent control respectively. As there were only two males moulting to adult and two females producing viable offspring at 269 µg l^{-1} 20-HE, a significant difference was not detected. Embryonic development times did not differ significantly between treatments (Table 5.2).

Table 5.1 The mean ± 1SD development times for combined naupliar (Dn) and copepodid stages (Dc), and from hatching to adult (N1-A) for *Bryocamptus zschokkei* exposed to 20-HE.
The numbers of males and females moulting to adult in each treatment are also shown. n = number of original animals and n^a = number of animals that survived to achieve adulthood. The number in brackets is the number of animals used to calculate the duration of each stage. * Indicates a significant difference between the control and solvent control (Student's t-test, $p<0.05$). There was no effect of 20-HE compared with the solvent control.

20-HE ($\mu g\ l^{-1}$)	n	n^a	Dc/Dn	Sex ratio of copepods		Development time (days)		
				Male	Female	Dn	Dc	(N1-A)
Control	40	35	0.95	11	24	9.51 ± 1.38 (35)	9.03 ± 0.86 (35)	18.54 ±1.72 (35)
Solvent control	39	28	1.14	5	23	10.04 ± 2.64 (28)	11.43 ± 1.79 (28)*	21.46 ± 2.62 (28)*
0.87	37	25	1.17	6	19	9.64 ± 1.04 (25)	11.24 ± 1.42 (25)	20.88 ± 2.09 (25)
2.7	39	27	1.21	5	22	9.33 ± 0.83 (27)	11.33 ± 1.82 (27)	20.67 ± 2.13 (27)
8.7	37	20	1.15	4	36	9.43 ± 1.08 (21)	10.86 ± 1.68 (21)	20.29 ± 2.43 (21)
26.9	38	19	1.15	0	19	10.21 ± 1.47 (19)	11.74 ± 2.76 (19)	21.95 ± 2.76 (19)
86.5	39	26	1.14	6	20	10.23 ± 2.30 (26)	11.62 ± 2.26 (26)	21.85 ± 2.77 (26)
269	38	27	1.22	2	25	9.46 ± 0.69 (28)	11.59 ± 1.39 (27)	21.04 ± 1.56 (27)

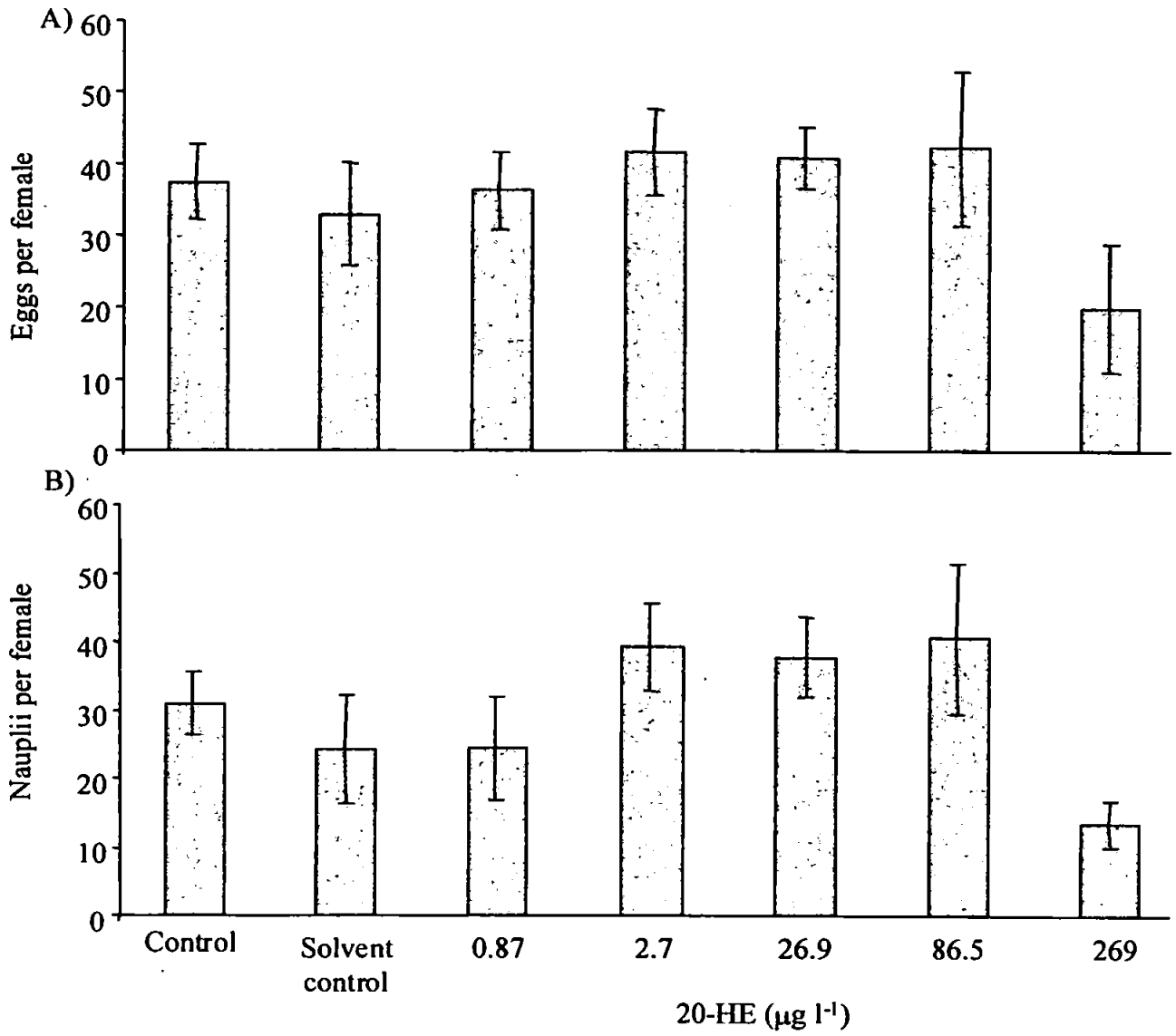


Figure 5.3: The mean ± 1SE A) eggs and B) nauplii per female at different 20-HE exposures.
There was no significant effect of 20-HE; n=2-18 per treatment.

Figure 5.3: The mean ± 1SE A) eggs and B) nauplii per female at different 20-HE exposures.
There was no significant effect of 20-HE; n=2-18 per treatment.

Table 5.2: The effect of 20-HE on the number of broods produced by *Bryocamptus zschokkei*, the time to first egg sac and on the embryonic development time of each brood.
n is the number of females per treatment, n_a is the number of females producing egg sacs and n_b is the number of females producing egg sacs from which viable offspring hatched. There is no significant effect of 20-HE.

20-HE (µg l^{-1})	n	n_a	n_b	Total no. of broods	Total no. of successful broods (n_s)	Proportion of aborted broods	Mean ± 1SD time to first egg sac (n_a)	Mean ± 1SD embryonic development time (days) (n_s)
Control	22	20	18	59	42	0.29	26.85 ± 3.18	3.09 ± 0.69
Solvent control	10	9	7	25	15	0.40	29.44 ± 3.32	3.07 ± 0.49
0.87	13	11	8	29	16	0.45	29.82 ± 3.95	3.38 ± 0.62
2.7	10	10	8	22	18	0.18	30.80 ± 4.16	3.14 ± 0.41
26.9	8	8	7	23	18	0.22	31.00 ± 1.85	3.03 ± 0.46
86.5	11	9	5	22	13	0.41	31.11 ± 4.76	3.08 ± 0.49
269	4	3	2	6	3	0.50	27.67 ± 2.52	3.00 ± 0.00

5.4 Discussion

5.4.1 Effects of 20-Hydroxyecdysone

There was no effect of 20-HE on development times for *B. zschokkei* over the exposure range chosen for this study. In view of evidence from previous studies on the effects of 20-HE, an excess of this hormone would be expected to result in hyperecdysonism, where an animal is unable to regulate haemolymph levels of 20-HE (Bodar *et al*, 1990; deFur *et al*, 1999). Hyperecdysonism occurs in the presence of persistent 20-HE (or chemicals that mimic 20-HE) due to an agonistic effect on the ecdysone receptor, often manifest as accelerated moulting in Crustacea (McConaugha & Costlow 1981; 1987; Freeman & Costlow 1984; Clare *et al*, 1992). Impaired moulting was observed in *Daphnia magna* exposed to 480 $\mu g\ l^{-1}$ 20-HE with animals failing to shed their exuviae and sometimes living in more than one moult sac before dying (Bodar *et al*, 1990). Survival of the marine harpacticoid *Tisbe battagliai* during development was significantly reduced at 269 $\mu g\ l^{-1}$ 20-HE (Hutchinson *et al*, 1999a). Reports on the effects of 20-HE are, however, variable with no significant effect on naupliar development of the marine copepod *Arcartia tonsa* being recorded at exposures up to 4 $mg\ l^{-1}$ 20-HE (Andersen *et al*, 2000). A potential role for ecdysteroids (including 20-HE) in regulating reproduction in Crustacea has also been suggested (Suzuki *et al*, 1996; deFur *et al*, 1999; Subramoniam, 2000) and reproductive parameters appear to be more sensitive to exposure to 20-HE than effects on development. For example, a decrease in offspring production has been observed in *D. magna* at 480 $\mu g\ l^{-1}$ 20-HE (Bodar *at al*, 1990), *Daphnia pulex* at 100 $\mu g\ l^{-1}$ 20-HE (Peterson *et al*, 2001) and *T. battagliai* at 26.9 $\mu g\ l^{-1}$ 20-HE (Hutchinson *et al*, 1999 a, b). In the present study, the number of nauplii per female *B. zschokkei* was lower at 269 $\mu g\ l^{-1}$ 20-HE than observed in the controls and in other exposures. It is, however, impossible to attribute this to a toxic effect of 20-HE because of the low numbers of males moulting to adult in this treatment.

Crustaceans are able to regulate haemolymph levels of 20-HE either through the production of a moult inhibiting hormone (MIH), which acts on the target tissues of ecdysteroids blocking their effects, or they may regulate production of 20-HE through a negative feedback mechanism (Charmantier *et al*, 1997). It would appear that, in *B. zschokkei*, ecdysteroid activity could be regulated over the concentration range tested. Therefore, the most likely explanation for a lack of effect of 20-HE in *B. zschokkei* is that the nominal exposure range chosen was too low for effects on development or reproduction to be apparent within the chosen test system. Due to practical constraints, no chemical analysis was obtained for the present study. There is, however, approximately 40% loss of 20-HE compared with nominal exposures over three days from seawater maintained in 3 cm diameter Cellwells™ at 20°C in the absence of any additional organic matter (Hutchinson, personal communication). The presence of organic matter (i.e. a leaf disc) would be expected to reduce further the toxicity of 20-HE as observed for both zinc (Chapter 6) and lindane (Chapter 7). It may also be that micro-organisms present on the surface of the leaf disc have the capacity to degrade 20-HE. The presence of a leaf disc increases the ecological relevance of the test environment by reflecting more closely field conditions where organic matter will strongly influence the toxicity of a particular contaminant. The microbial flora on the surface of the leaf disc also increases the complexity of the test creating in effect a microcosm. It is therefore imperative that detailed chemical analysis is obtained in future studies using this test system so that a true representation of the compound being tested can be obtained.

5.4.2 Solvent Effects

A significant increase in copepodid development (Dc) and total development times (N1-Adult) of *B. zschokkei* was observed in all treatments containing the carrier solvent methanol compared with the control. An exposure-independent increase in mortality in the

20-HE treatments compared with the control, but not with the solvent control, was also indicative of a solvent effect. The carrier solvent, methanol, did not exceed 0.1 ml l^{-1} in any of the treatments which is the maximum solvent concentration recommended in regulatory guidelines for testing of freshwater invertebrates (ASTM, 1999a, b). Methanol was also the carrier solvent in previous studies on *Daphnia magna* (Bodar *et al*, 1990) and *Tisbe battagliai* (Hutchinson *et al*, 1999a, b) exposed to 20-HE and no solvent effects were observed in these studies. The use of water-miscible solvents such as methanol, ethanol and acetone are, however, known to stimulate undesirable growth of micro-organisms in some test systems (Granmo *et al*, 1989; Willis, 1998; ASTM, 1999a). The test system used in this study included a leaf disc from which *B. zschokkei* is known to obtain food (Chapter 4). Methanol may be acting as an additional source of carbon for microbes and, as such, influencing the microbial population on the surface of the leaf disc. A change in the microbial flora of the leaf surface could alter the quality of the leaf as a food source for *B. zschokkei*. For example, significantly prolonged development times have been observed for *B. zschokkei* fed on leaf discs of low detritus quality where quality was related to the degree of microbial coverage on the leaf surface (Chapter 4). In the current study, the leaf discs were replaced once every 14 days (based on protocols used to consider water hardness and detritus quality), and this may have been long enough for the microbial community to respond to the presence of a solvent. In a subsequent study, where *B. zschokkei* were exposed to lindane with methanol as a solvent carrier, the leaf disc was replaced every 7 days and no solvent effects were observed (see Chapter 7). It is recommended, therefore, that in further tests, with *B. zschokkei* the leaf discs be renewed regularly.

5.4.3 Full Life-Cycle Test

Based on the published literature this was the first time a full life-cycle toxicity test has been carried out for a freshwater harpacticoid. In six weeks, *B. zschokkei* developed to adult and

produced approximately three broods of offspring, therefore, incorporating embryonic, larval and gonadal development. The assay was sensitive enough to detect solvent effects on the development time to adult but over the concentration range tested no effects of 20-HE were observed. While this study demonstrates that a full life-cycle test can be carried out using *B. zschokkei*, further work is required before the utility of this bioassay can be fully evaluated. For example, the sensitivity of *B. zschokkei* to a range of chemicals should be evaluated to establish if this copepod also exhibits relatively high tolerance to other contaminants. The suggested recommendations for more detailed chemical analysis and more frequent renewal of leaf discs should also be incorporated into any further tests with this species.

5.5 Summary

- There was no effect of 20-HE on the development or reproduction of *B. zschokkei* over the nominal concentration range tested (8.7-269 $\mu g\ l^{-1}$ 20-HE). It is possible that the presence of a leaf disc reduced the toxicity of 20-HE to *B. zschokkei* or that breakdown of this hormone was accelerated by micro-organisms present on the leaf surface. Detailed chemical analysis is therefore recommended when carrying out any subsequent toxicity testing using these protocols.
- A significant increase in copepodid (Dc) and total development times (N1-A) of *B. zschokkei* was observed in the methanol control compared with the control. This solvent effect may be an indirect effect on the growth of micro-organisms on the leaf surface due to methanol providing an additional carbon source. More frequent renewal of leaf discs is recommended for further testing with this species.
- Further work is required incorporating the above recommendations before the potential of this life-cycle test can be fully evaluated.

Chapter 6

The effects of zinc on the development and reproduction of *Bryocamptus zschokkei*

6 Abstract

The lethal and sublethal effects of zinc to the freshwater harpacticoid *Bryocamptus zschokkei* were measured in this study. Larval life-stages (nauplii and copepodids) were more sensitive than adult females to acute zinc exposure with a 96 h LC50 (± 95% CL) of 0.62 (0.52-0.73) mg Zn l^{-1} for copepodids in the absence of food. The toxicity of zinc to adult females was significantly reduced in the presence of a leaf disc. In chronic exposures, long-term survival (≥ 10 days) and reproductive parameters (numbers of egg and nauplii per female, and embryonic development times) showed similar sensitivity to zinc with significant mortality, reduced offspring production and prolonged embryonic development times observed for copepods at measured concentrations of 0.48 mg Zn l^{-1}. Based on acute and chronic toxicity data *B. zschokkei* is more tolerant to zinc than cladocerans, but has a similar sensitivity range as peracarid crustaceans (*Gammarus pulex* and *Asellus aquaticus*) which are "standard" aquatic organisms used in the generation of water quality guidelines.

6.1 Introduction

Copepods have been widely used for studying the effects of pollutants in marine systems (Bengtsson & Bergström, 1987; Williams, 1992; Green *et al*, 1995) and possess many attributes that are advantageous for toxicity test organisms, including short life-cycles, small size and ease of culture in the laboratory. More importantly, test organisms should be relevant to the environment that needs to be protected. The use of the freshwater harpacticoid *Bryocamptus zschokkei* as a bioassay organism for lotic systems was proposed after this copepod was identified as a sensitive indicator of copper toxicity in rivers of Southwest England (Burton *et al*, 2001). Preliminary laboratory trials showed that adult survival and reproduction of *B. zschokkei* were influenced by exposure to copper and that for accurate evaluation of developmental effects, there was a requirement for more refined methodologies (Burton, 1998). More detailed measurements during reproduction are

needed to determine the mode of toxicity of the contaminant. Standardised protocols for *B. zschokkei* have been described (Chapters 2-4) to evaluate how natural environmental parameters (i.e. water hardness and food quality) affect development and reproduction. These methods have been adapted in order to develop chronic toxicity testing protocols for this copepod (Chapter 5). Zinc was chosen for evaluation in this study as this trace metal (in association with copper) is known to affect the distribution and abundance of *B. zschokkei* in the field (Burton *et al*, 2001). Concentrations of zinc in surface and pore waters of metal-impacted rivers occur at levels which are lethal to some freshwater crustaceans (Burton *et al*, 2001).

There is a relatively extensive database documenting the lethal effects of zinc on freshwater biota. For example, 48 h LC50s for daphnids have been recorded at exposures as low as 40 μg Zn l^{-1} (Eisler, 1993). With the exception of daphnids, freshwater copepods appear to be amongst the most sensitive species of crustacean to this metal (Boudouin & Scoppa, 1974; LaLande & Pinel-Alloul, 1986; Notenboom *et al*, 1992). Growth and development of crustaceans appears to be sensitive to sublethal zinc exposure (Biesinger & Christensen, 1972; Maltby *et al*, 1990; Conradi & Depledge, 1999), but data for copepods are scarce. Studies of the chronic effects of zinc to copepods have tended to focus on reproductive output rather than development (Sunda *et al*, 1987; Verriopoulos & Hardouvelis, 1988), despite the fact that copepod development has been shown to be vulnerable to perturbation by trace metals such as copper and cadmium (D'Agostino & Finney, 1974; Moraïtou-Apostolopoulou *et al*, 1983). Reproduction in crustaceans is very sensitive to zinc and results in decreased egg production in marine copepods at sublethal exposures (Sunda *et al*, 1987; Verriopoulos & Hardouvelis, 1988). A significant reduction in offspring production has been observed in daphnids at concentrations as low as 25 μg Zn l^{-1} in soft water (Paulauskis & Winner, 1988).

This study had the general aim of reporting the lethal and sub-lethal responses of *B. zschokkei* to zinc. Specific aims were to assess:

1. the effects of acute exposure to zinc on different life-stages (nauplii, copepodids and adult females),
2. how the presence of food influenced acute zinc toxicity, and
3. the influence of chronic exposure on long-term survival (>10 days), development and reproduction.

6.2 Materials and Methods

6.2.1 Test Chemical

Zinc, added as zinc sulphate ($ZnSO_4.7H_2O$), was obtained from Fisher Scientific Ltd. Primary stocks were prepared by dissolving the test compound in dilution water. Individual test solutions (100 ml volumes) were prepared by diluting the appropriate volume of the aqueous stock solution with dilution water. The dilution water, Elendt's M4 medium (Elendt & Bias, 1990), was the same as that used in the cultures. Elendt's M4 medium was diluted to give a hardness of 100, an alkalinity of 30 (both as mg l^{-1} $CaCO_3$), conductivity of 290 $\mu s\ cm^{-1}$ and pH 7.7. All glassware was acid washed and leeched in dechlorinated water for 24 h prior to use. The concentration of zinc in water was analysed using a Perkin Elmer 1100B atomic absorption spectrophotometer (AAS) accurate to 0.01 mg Zn l^{-1}. The measured concentration of zinc in the chronic exposures was calculated using time-weighted means by the following equation:

$$\frac{(x_1 + y_1 * D1) + (x_2 + y_2 * D2) + \ldots\ldots\ldots + (x_n + y_n * Dn)}{2\sum D1, D2 \ldots\ldots\ldots Dn}$$

Where x is the measured concentration at the start of each renewal period, y is the measured concentration at the end of each renewal period and D is the number of days in the renewal period.

6.2.2 Acute Toxicity

Test concentrations for acute toxicity tests were chosen on the basis of range finding experiments, and consisted of a dilution water control and exposures of 0.32, 0.56, 1.0, 1.8, 3.2 and 5.6 mg Zn l^{-1}. The acute toxicity of zinc up to 96 h was calculated for three life-stages (nauplii, copepodid and non ovigerous adult females) in the absence of food, and for non ovigerous adult females in the presence of a food source (a pre-conditioned beech leaf disc). All experimental work was carried out in a temperature-controlled room at 20 (±1)°C and a photoperiod of 16 h light: 8 h dark, with a 20 minute dawn / dusk transition. The temperature in the test-vessels was recorded daily using a thermometer.

Ovigerous females (approximately 100) were transferred from cultures to a 100 cm diameter crystallising dish, with a 4 cm diameter beech leaf disc (*Fagus sylvatica* L.) as a food source, over a 24 h period. All females were then removed, leaving nauplii (≤ 24 h old) for initiating the exposure experiments. To obtain copepodids (stage C1–C3) and non-ovigerous adult females, ovigerous females were separated from the cultures as described for the nauplii. Leaf discs and media were changed three times per week for 10 or 25 days in order to obtain copepodids and adults respectively.

At each zinc exposure, there were four replicates, each of five animals, per 2 cm diameter polystyrene Cellwell™ (Dow Corning, Corning, NY, USA); this was set up for all three life-stages (nauplii, copepodid and non ovigerous adult females) and for non ovigerous females in the presence of a leaf disc. Each Cellwell™ contained 3 ml of the appropriate test

solution. The treatments exposing adult females in the presence of a food source contained a 1 cm diameter beech leaf disc (*F. sylvatica* L.) which had been pre-conditioned for two weeks in Elendt's medium. At the beginning of the experiment, 10 ml of each test solution was retained in a glass vial for chemical analysis. After 48 h exposure, 2.4 ml of test solution from each Cellwell™ was replaced. To assess if the presence of leaf discs influenced zinc concentrations in the test vessels, the concentration of zinc in the test solution (9.6 ml) was measured for treatments with adult females both in the presence and absence of leaf discs. Media removed from the vessels containing nauplii and copepodids were used to obtain pH and dissolved oxygen concentrations before they were discarded.

For this experiment, the measurement of effect was mortality. Each replicate was observed for the presence of dead animals every 24 h. A copepod was considered to be dead if it did not respond to gentle mechanical stimulation after 10 seconds. Using these data, it was possible to calculate an LC50 value.

6.2.3 Chronic Toxicity

The following logarithmic gradient of exposure concentrations was used (Rochinni *et al*, 1982), based on acute toxicity values: control (dilution water) and 0.032, 0.056, 0.1, 0.18, 0.32, 0.56 and 1 mg Zn l^{-1}. All experimental work was carried out under the same conditions as acute exposures. For all treatments, the dilution water was Elendt's M4 medium. Test solutions were replaced (75%) three times per week, and the dissolved oxygen concentration and pH of media were measured before discarding. Exposure concentrations were analysed for zinc once per week, using 20 ml of the test solution before and after renewal (old and new solution). The timings of weekly sampling of water for zinc analysis were varied to incorporate both two and three day intervals in the water changes.

Developmental and reproductive responses of *B. zschokkei* to zinc were measured in a single exposure experiment. The development time was based on the time taken for nauplii (≤ 24 h old) to moult to adult. Forty nauplii (obtained as described in Section 6.2.2), maintained individually in 1 cm diameter polystyrene Cellwells™ containing 2 ml of test solution and a 4 mm diameter beech leaf disc, were used at each zinc exposure. Daily observations were carried out using a Wild M8 binocular microscope under darkfield illumination (magnification, × 20-40). Every 7 days, the copepods were transferred into new Cellwells™ containing fresh medium and a new leaf disc. Nauplii were observed on day 1 of the experiment to determine if they had been transferred successfully but not again until day 7 because excessive handling of naupliar stages can significantly delay the development time to adult of *B. zschokkei* (see Chapter 2). Subsequent daily observations of copepod survival, and the presence of moulted exuviae, were used to calculate the duration of the combined naupliar (Dn) and copepodid (Dc) stages and the total time taken to moult to adult. The number of males and females was also recorded. As soon as the final moult had occurred (after approx. 20 days) copepods were paired so that reproductive parameters could be considered. Females and males were combined in a 2:1 ratio to match the observed female bias. As fertilisation of broods occurs via spermatophore mediated transfer, multiple broods can be fertilised from a single mating, therefore, when a female was observed to have produced an egg sac she was transferred to a separate cell well and monitored in isolation. The presence of egg sacs and nauplii were recorded daily and used to calculate the embryonic development time (i.e. the time from egg sac release to hatching), the inter-brood period (the time taken for a female to release a new egg sac after the previous one had hatched) and the number of broods per female. Aborted egg sacs were removed from the cell well and viewed under a high powered inverted microscope to determine the number of eggs present. Every seven days, animals were transferred to a new Cellwell™ and nauplii that had hatched during that week were retained in the old vessel and counted. If more than

one brood of nauplii was present, they were distinguished on the basis of size. Non-viable eggs were also counted, so that the overall hatching success could be determined. The duration of this experiment was six weeks, sufficient time for control copepods to develop to adult and for females to produce an average of three broods.

6.2.4 Statistical Analysis

The acute toxicity of zinc after 48 and 96 h was calculated for all life-history stages using the moving average angle method (Stephan, 1977). In the chronic exposures, significant differences between treatments in survival at days 21 and 42 were identified using Fisher's Exact test (Finney, 1963). All data were tested for normality (Shapiro-Wilks) and for equality of variances (Bartlett's test) before using analysis of variance techniques. If data did not conform to these assumptions, they were $\log_{10}$ transformed. One-way ANOVA was used to test for differences in development times and egg and nauplii production between zinc exposures using Bonferroni's multiple comparison procedure to discriminate between the means. If the assumptions for ANOVA were not met, data were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Mann Whitney tests to highlight which treatments were different.

6.3 Results

6.3.1 Analytical Chemistry

As loss of zinc between renewal periods was greater than 20% in the presence of leaf material, mean measured zinc concentrations (Table 6.1) were used to calculate LC50 values. The measured concentration of zinc in the chronic exposures is shown in Table 6.2. Zinc was elevated compared with nominal concentrations in the dilution water control and at low zinc exposure concentrations (0.032-0.18 mg Zn l^{-1}). The dilution medium used in both acute and chronic exposures was therefore re-analysed for zinc (using a 1 litre sample)

at the end of the experiment. This confirmed that the dilution water contained only trace levels of zinc (0.0015 mg Zn l^{-1}).

6.3.2 Acute Toxicity

Zinc toxicity increased with time for all life-stages with larval life-stages (nauplii and copepodids) being up to three times more sensitive than adult females (Table 6.3). The presence of leaf material reduced the toxicity to adult females of zinc by 35% compared to females tested in the absence of a leaf disc.

6.3.3 Long-term Survival

During development, mortality was concentration-response related (Fig. 6.1). After 21 days, mortality was significantly higher at 0.48 and 0.78 mg Zn l^{-1} compared with the control (Fisher's Exact Test, $P<0.05$). At 0.78 mg Zn l^{-1} all copepods died by day 28. In all other exposures most copepods had achieved adulthood by day 21 and after which there was no significant mortality.

Table 6.1: Measured concentrations of zinc in the acute exposures both in the absence and presence of leaf material.

Nominal conc. of zinc (mg Zn l^{-1})	Mean (± 1SD) measured conc. of zinc (mg Zn l^{-1}) n=2	Mean (± 1SD) measured conc. of zinc + Leaf (mg Zn l^{-1}) n=2
Control	0.08 (± 0.07)	0.08 (± 0.06)
0.32	0.32 (± 0.02)	0.29 (± 0.05)
0.56	0.53 (± 0.02)	0.45 (± 0.10)
1	0.90 (± 0.04)	0.73 (± 0.21)
1.8	1.60 (± 0.00)	1.23 (± 0.43)
3.2	2.85 (± 0.07)	2.40 (± 0.55)
5.6	4.85 (± 0.07)	4.30 (± 0.61)

Table 6.2: The time weighted mean measured zinc concentrations calculated from chronic exposures.

Nominal conc. of zinc (mg Zn l^{-1})	Time weighted conc. of zinc (mg Zn l^{-1})	% of nominal
Control	0.024	
0.032	0.058	+ 45
0.056	0.081	+ 31
0.100	0.134	+ 25
0.180	0.213	+ 15
0.320	0.303	-5
0.560	0.485	-13
1.000	0.779	-22

Table 6.3: The 48 and 96 h LC50 (± 95% CL) values for three life-stages of *Bryocamptus zschokkei* exposed to zinc in the absence of food and for adult females exposed in the presence of leaf material.
NR = not recorded.

Life stage	**LC50 (± 95% CL)**	
	48 h	**96 h**
Nauplii	1.38 (1.17-1.69)	0.92 (0.78-1.09)
Copepodid	0.94 (0.76-1.14)	0.62 (0.52-0.73)
Adult	2.89 (2.46-3.41)	2.07 (1.73-2.57)
Adult + Leaf	NR	3.23 (2.99-3.65)

6.3.4 Development

There was no significant effect of zinc on the duration of naupliar development (Dn), copepodid development (Dc), or on the total development time to adult (N1-Adult) (Kruskal-Wallis, P>0.05) (Table 6.4). Apart from two animals, all copepods moulted to

adult within the 42 day duration of this experiment. The ratio of males to females was independent regardless of treatment (χ^2 11.11, d.f. 6, $p>0.05$; Table 6.4).

6.3.5 Reproduction

The number of eggs and nauplii per brood was significantly lower at 0.48 mg Zn l^{-1} (i.e. the highest concentration where animals survived to reproduce) compared with the control (One-way ANOVA, $p<0.05$) (Fig. 6.2). This zinc exposure was the only one to result in naupliar mortality during hatching. Fewer eggs and nauplii per female were observed at zinc exposures greater than 0.13 mg Zn l^{-1} compared with the control but, because of high variability and low numbers of females achieving reproductive status in these treatments, no significant difference was detected (One-way ANOVA, $p>0.05$) (Table 6.5). Egg sac abortions increased from the control (20%) in zinc exposures above 0.13 mg Zn l^{-1} (39 - 62%), but there was no clear pattern between treatments (Table 6.5). For example, although significantly more egg sacs were aborted at 0.13 and 0.3 mg Zn l^{-1} than in the control, there were no differences at 0.21 and 0.48 mg Zn l^{-1} compared with the control (χ^2 21.31 d.f. 6, $p<0.05$). Embryonic development time at 0.48 mg Zn l^{-1} was significantly longer than the control and compared with zinc exposures up to 0.21 mg Zn l^{-1} (Kruskal-Wallis, $p<0.05$) (Fig. 6.3).

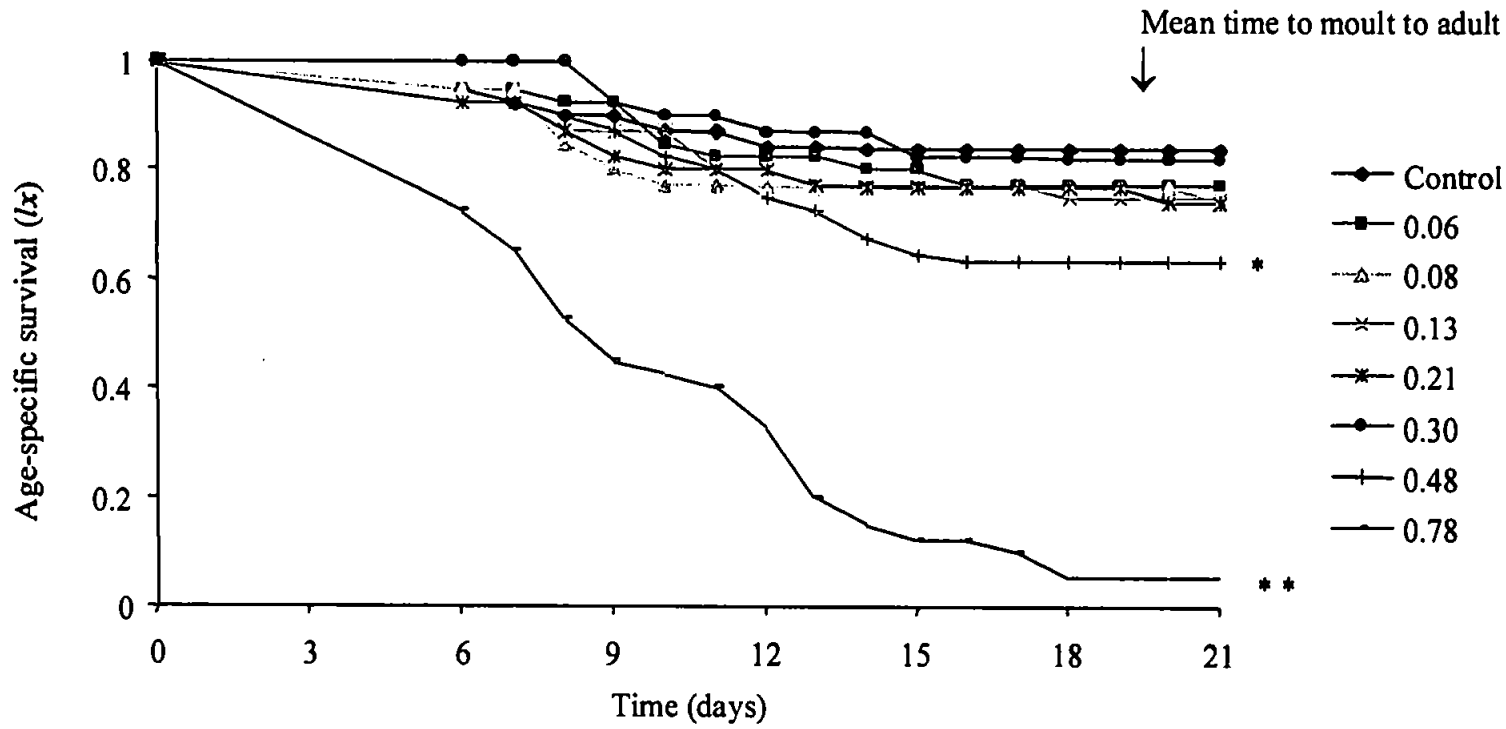


Figure 6.1: The effect of zinc (mg Zn l^{-1}) on survival of *Bryocamptus zschokkei* during development to adult.
* Indicates a significant difference from the control (Fisher's Exact Test, $p<0.05$) and * * indicates a significant difference from the control (Fisher's Exact Test, $p<0.01$); n=38-40 per treatment.

Table 6.4: The development times (mean ±1SD) for combined naupliar (Dn) and copepodid stages (Dc), and hatching to adult (N1-A) for *Bryocamptus zschokkei* exposed to zinc.
The numbers of males and females moulting to adult in each treatment are also shown. n = number of original animals and n^a = number of animals that survived to achieve adulthood. The number in brackets is the number of animals used to calculate the duration of each stage. # Indicates that two other animals survived the 42 day duration of the experiment but did not moult beyond C5 during this time. There was no significant difference between treatments.

Mean measured conc. zinc (mg Zn l^{-1})	n	n^a	Dc/Dn	Sex of copepod		Development time (days)		
				Male	Female	Naupliar stages (Dn)	Copepodid stages (Dc)	Hatching to adult (N1-A)
Control	38	32	1.00	11	21	9.321±0.68 (34)	9.34±1.18 (32)	18.66±1.43 (32)
0.06	40	31	1.06	18	13	9.70±0.98 (33)	10.29±3.16 (31)	20.03±3.16 (31)
0.08	38	28	0.99	13	15	9.70±1.88 (30)	9.61±1.23 (28)	19.07±1.23 (28)
0.13	40	29	1.00	15	14	9.84±1.19 (31)	9.83±1.26 (29)	19.59±1.90 (29)
0.21	39	28	1.04	11	17	9.63±1.16 (30)	10.00±1.41 (28)	19.61±2.23 (28)
0.30	39	30#	0.98	7	23	9.77±2.33 (31)	9.57±1.07 (30)	19.10±2.73 (30)
0.48	38	24	0.98	7	17	10.10±1.88 (29)	9.92±1.21 (24)	20.00±2.84 (24)

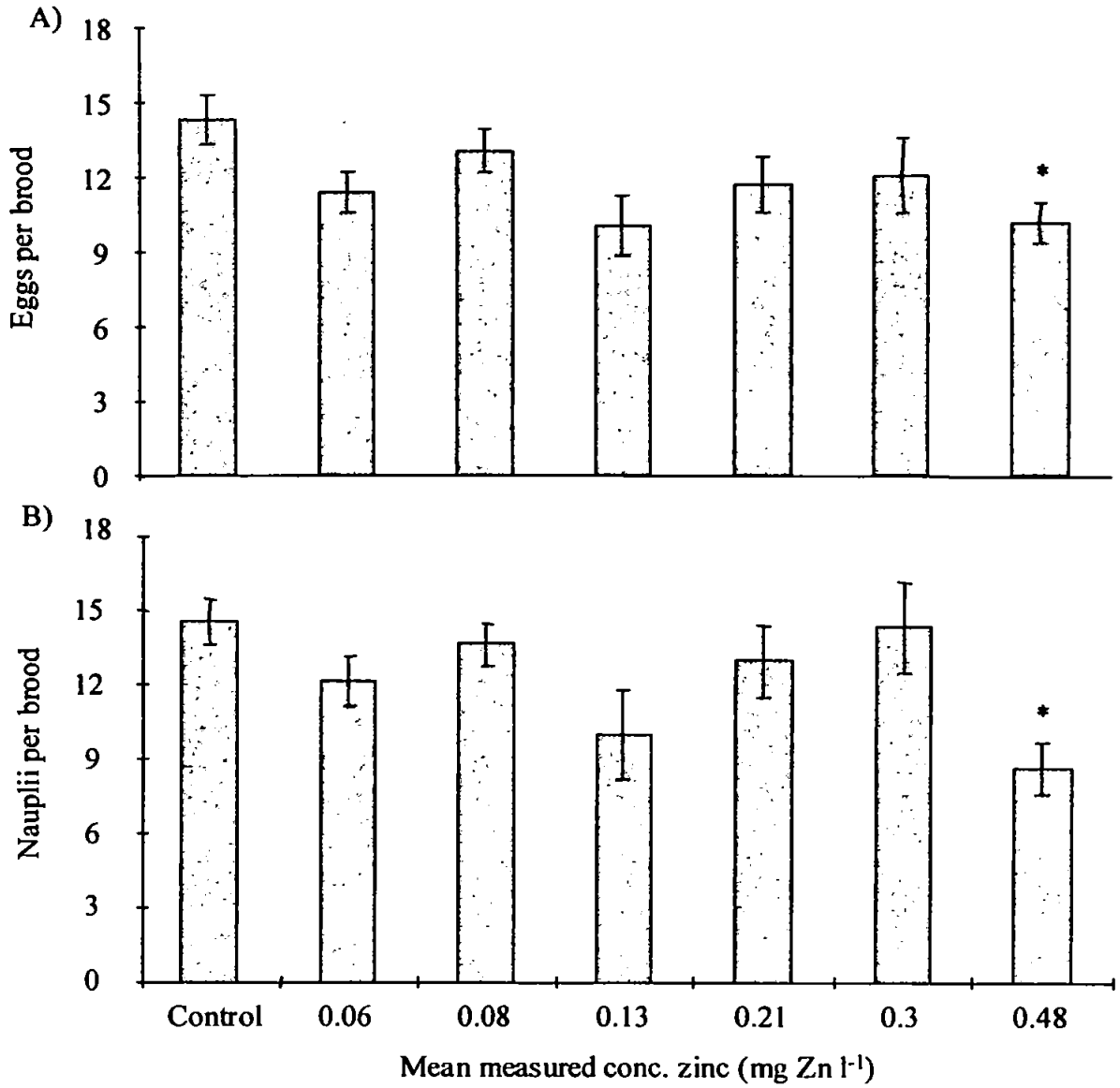


Figure 6.2: The mean ±1SE number of A) eggs and B) nauplii per brood at different zinc exposures.

* Indicates a significant difference from the control (One-way ANOVA, $p<0.05$); n=7-15 females per treatment.

Table 6.5: The effect of zinc on the number of broods produced by *Bryocamptus zschokkei* and on the number of eggs and nauplii per female.
n = the number of females per treatment, n_a = the number of females producing egg sacs and n_b = the number of females producing egg sacs from which viable offspring hatched. * Indicates a significant difference from the control, and from 0.06 and 0.08 mg Zn l^{-1} (χ^2 21.31 d.f. 6, $p<0.05$).

Mean measured conc. zinc (mg Zn l^{-1})	n_a	n_b	Total number of broods	Total no. of successful broods	Proportion of aborted broods	Mean (±1SD) no. broods per female (n_b)	Mean (±1SD) no. eggs per female (n_b)	Mean (±1SD) no. nauplii per female (n_b)
Control	20	15	50	40	0.20	3.00 ± 1.31	41.87 ± 24.80	38.73 ± 24.78
0.06	14	12	49	35	0.29	4.08 ± 1.16	39.92 ± 19.84	35.33 ± 20.58
0.08	14	13	48	34	0.29	3.31 ± 1.32	38.92 ± 23.15	35.61 ± 21.29
0.13	13	7	25	12	0.52*	2.43 ± 0.98	19.86 ± 9.70	17.14 ± 11.19
0.21	16	12	40	22	0.45	3.08 ± 1.00	28.91 ± 18.36	24.64 ± 18.73
0.30	15	7	34	13	0.62*	2.71 ± 1.25	29.71 ± 21.30	26.57 ± 22.12
0.48	16	12	38	23	0.39	2.75 ± 1.14	22.83 ± 10.59	15.41 ± 9.74

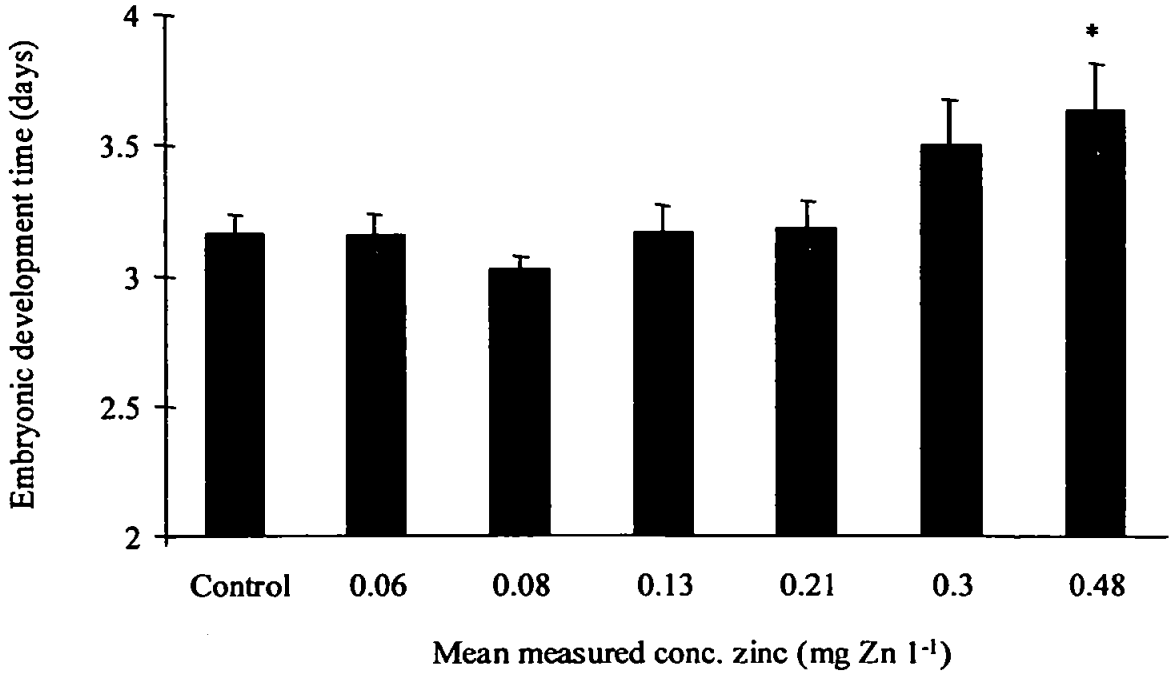


Figure 6.3: The mean (±1SE) effect of zinc on the time taken for embryos to hatch.
* Indicates a significant difference from the control and from 0.06-0.21 mg Zn l^{-1} (Kruskal-Wallis, $p<0.05$); n= 7-15 females per treatment.

6.4 Discussion

6.4.1 Acute Toxicity

Naupliar and copepodid stages of *Bryocamptus zschokkei* were more sensitive to acute zinc exposure than adult females. This corroborates previous data on the toxicity of trace metals to marine copepods (Verriopoulos & Moraïtou-Apostolopoulou, 1982; Sunda *et al* 1987; Verriopoulos & Moraïtou-Apostolopoulou, 1989; Bechmann, 1994; Hutchinson *et al*, 1994) and for larval *B. zschokkei* exposed to copper (Burton, 1998). The vulnerability of early life-history stages to contaminant exposure compared with adults has been attributed partly to the high surface area to volume ratio of the smaller stage, which increases the area over which a toxicant can be absorbed. In the case of trace metals, increased sensitivity is related also to the moult cycle (Naylor *et al*, 1990). As crustaceans get older, they may be able to mobilise more trace metals within their exoskeleton, thereby, reducing body concentrations (Bertine & Coldberg, 1972; Verriopoulos & Hardouvelis, 1988). There is

also evidence that the detoxification mechanisms present in adult Crustacea are not properly developed in larval stages resulting in less effective regulation of metal ions (Bernard and Lane, 1964; Bryan, 1971; Bryan & Hummerstone, 1971).

The presence of a leaf disc in the test vessel reduced the toxicity of zinc to adult females. As the hydrated cation is the most bioavailable zinc ion, complexation with organic matter (such as leaf material) will reduce its availability and, as a result, its toxicity (Rainbow *et al*, 1993). For example, humic acids reduced the toxicity of zinc to *Daphnia magna* (Paulauskis & Winner, 1988) and the presence of macroalgae (*Ulva* spp.) increased the tolerance of the marine harpacticoid *Tisbe holothuriae* to zinc (Verriopoulos & Moraïtou-Apostolopoulou, 1989). Leaf material accumulated zinc in a concentration dependent manner and at a higher rate than gammarids exposed at the same concentration (Maltby & Crane, 1994). Fungal colonisation of leaf material will also adsorb metals via organic ligands on the surface of their mycelium (Duddridge & Wainwright, 1980; Abel & Barlöcher, 1984). Uptake of zinc by crustaceans is, however, often via their food. For example, approximately 50% of zinc accumulated by marine copepods was due to ingestion of algae (Wang & Fisher, 1998). *T. holothuriae* showed high mortality when zinc was supplied via contaminated macroalgae (Verriopoulos & Moraïtou-Apostolopoulou, 1989). Therefore, while a reduction in the toxicity of zinc in the presence of organic matter occurs during short-term exposures, uptake of zinc through dietary mechanisms may represent a major route of exposure to zinc in long-term studies. This must be taken into consideration when carrying out chronic exposures or when attempting to extrapolate back to field conditions.

When considering the acute toxicity of a contaminant, comparisons with previously published data must be made with caution because a number of factors can alter the

tolerance of a population. For example, physiochemical parameters, especially water hardness and pH, affect the toxicity of zinc by altering the concentration of the toxic free metal ion available to the test organism. In water with low water hardness and pH [typical for *B. zschokkei* (Rundle & Hildrew, 1990; Fryer, 1993)], greater sensitivity to zinc might be expected. It is also well established that continued exposure to metals can infer a degree of tolerance. For example, the sensitivity of the copepod *Tropocyclops prasinus mexicanus* to zinc was increased by an order of magnitude when individuals were obtained from a pristine water rather than a polluted lake (LaLande & Pinel-Alloul, 1986). *Bryocamptus zschokkei* previously exposed to copper were less sensitive to this metal in laboratory trials than copepods from pristine sites (Burton, 1998). Although a number of factors can influence the acute toxicity of zinc some general comparisons with other freshwater Crustacea can be considered. For example, other freshwater copepods have similar LC50s when acutely exposed to zinc as *B. zschokkei*, although *B. zschokkei* was more sensitive to zinc than the predatory cyclopoid *Cyclops abbyssorum* (Table 6.6). *Bryocamptus zschokkei* also appears to be more sensitive to zinc than *Gammarus pulex* and *Asellus aquaticus*. The latter are larger freshwater Crustacea often used as standard test organisms for water quality guidelines and both are representative species of stream macro-benthos (Martin & Holdich, 1986; Abel & Green, 1981; Naylor *et al*, 1990). Cladocerans, however, appear to be the most sensitive crustacean group to zinc with 96 h LC50s (over a range of water hardness values) between 42-655 mg l^{-1} (Attar & Maly, 1982). Daphnids were also found to be much more sensitive to acute copper exposure than *B. zschokkei* (Burton, 1998). As copepods and cladocerans have very different life histories this may influence the mode of action of a toxicant and hence the relative sensitivity to different chemicals. For example, while cladocerans continue to moult once attaining adulthood copepods have a determinate number of moults with the final moult being the adult (Cooney & Gehrs, 1984). Crustacea are likely to be more vulnerable to contaminants during moulting. Moulting may, however,

also be a mechanism for excreting contaminants that have been secreted into the exoskeleton. However, as daphnids are rarely present in running waters (Rundle & Ramsay, 1997), their relevance for use in protecting such systems may be open to debate (Cairns, 1986; Gray, 1989; Burton, 1998).

6.4.2 Long-term Survival

For *B. zschokkei* survival from nauplius to adult appears to be at least as sensitive to zinc as the other parameters (development times and reproductive parameters) measured in the chronic exposures. Exposure to 0.78 mg Zn l^{-1} resulted in 100% mortality after 28 days. Survival was also significantly reduced at 0.49 mg Zn l^{-1} compared with the control. These data support the acute toxicity data and highlight the sensitivity of larval life-stages to zinc exposure, particularly as no effects of zinc were observed on adult survival during this experiment. Zinc has also been found to reduce the longevity of both *D. magna* (Winner, 1981) and the marine harpacticoid *Tisbe holothuriae* when exposed over their entire life cycle (Verriopoulos & Hardouvelis, 1988). In view of this, it might be that further effects on the survival of *B. zschokkei* would be observed if exposures were continued over the entire life of the copepod. It would, however, be time consuming to test this hypothesis for *B. zschokkei* as they have a relatively long life cycle surviving for up to 370 days at 18 °C (O'Doherty, 1985).

Control mortality did not exceed 10% during development to adult (21 days). This complies with limits set by standardised test guidelines requiring at least 80% survival of benthic freshwater invertebrates during 10 day tests (ASTM, 1999a). High naupliar mortality is a potential problem associated with chronic toxicity tests using freshwater copepods (Willis, 1999). Control mortality of 28 and 65% has been reported for naupliar stages of *Calamoecia lucasi* and *Boeckella delicata,* respectively, in an 8 day development assay

(Willis, 1999). The high survival observed in this and previous experiments with *B. zschokkei* (Burton, 1998) reflect satisfactory culture and testing regimes.

6.4.3 Development

There was no effect of zinc exposure on the time taken for *B. zschokkei* to develop from nauplius to adult. There is evidence that copper and cadmium disrupt development of marine harpacticoids (D'Agostino & Finney, 1974; Moraïtou-Apostolopoulou *et al*, 1983) but no comparable data for zinc. Moulting frequency was, however, reduced in the isopod *Porcellio scaber* when fed zinc contaminated food (Drobne & Strus, 1996) and growth (or scope for growth) of Crustacea has been retarded in response to zinc exposure (Biesinger & Christensen, 1972; Maltby & Naylor, 1990; Maltby *et al*, 1990; Conradi & Depledge, 1999). Impairment of growth in amphipods exposed to zinc appears to be due to a reduction in feeding rates (Maltby *et al*, 1990; Weeks, 1992). While growth and development in copepods are thought to be independent processes, they often respond in a similar manner to environmental conditions (Twombly & Tisch, 2000). Development in *B. zschokkei* was sensitive to low food quality (Chapter 4), therefore, had zinc caused a depression in feeding rate, it would have been expected to be reflected by an extension of the development times.

Table 6.6: The acute toxicity of zinc to selected freshwater invertebrates.
Unless otherwise stated, LC50 values are based on nominal concentrations of the free zinc ion and hardness and alkalinity are expressed in terms of mg l^{-1} as $CaCO_3$.

Species	Life stage	Metal salt	Test conditions	Exposure time (h)	LC50 (± 95% CL) (mg Zn l^{-1})	Ref.
Bryocamptus zschokkei	**Adult**	**$ZnSO_47H_2O$**	**20°C, Hardness 100, pH 7.7**	**96**	**2.07 (1.73-2.57)**	**This study**
	Nauplii		**Alkalinity 30**	**96**	**0.92 (1.73-2.57)**	
Tropocyclops prasinus mexicanus	Adult	$ZnCl_2$	18°C, Hardness 120, pH 7.10 Alkalinity 3.8-4.4	48	2.93 (2.86-3.00)	LaLande & Pinel-Alloul, 1986
Parastenocaris germanica	Adult	$ZnSO_47H_2O$	10.5°C, Hardness 0.79 mmol l^{-1}	96	1.7 (1.4-2.0)	Notenboom *et al*, 1992
Cyclops abbyssorum	Adult	$ZnSO_47H_2O$	Calcium 0.46 meq l^{-1}, pH 7.2	48	5.5 (4.5-6.8)	Baudouin & Scoppa, 1974
Eudiaptomus padanus	Adult		Alkalinity 0.58 meq l^{-1}	48	0.50 (0.39-0.72)	
Daphnia hyalina	Adult			48	0.04 (0.03-0.05)	
Daphnia magna	Adult	$ZnSO_47H_2O$	20-24°C, Hardness 240	48	0.92	Hall *et al*, 1986
Ceriodaphnia dubia	≤ 48 h	$ZnSO_47H_2O$	25°C, Hardness 280-300,	48	0.36 (0.26-0.50)	Schubauer-Berigan *et al*,
Hyalella azteca	7-14 days		pH 7-7.5, Alkalinity 225-245	96	1.50 (1.20-1.90)	1993
Lumbriculus variegatus	Adults			96	>5.00	
Asellus aquaticus	Adults	$ZnSO_47H_2O$	13°C, Hardness 45-55,	96	18.2 (12.1-25.1)	Martin & Holdich, 1986
Crangonyx pseudogracalis	Adults		pH 6.7-6.8, Alkalinity 40-60	96	19.8 (15.1-24.5)	
Gammarus pulex	Adults (fed leaf discs)	$ZnSO_47H_2O$	13.5°C, Hardness 112-116, pH 8.12, Alkalinity 253-257	96	(1-2)	Crane, 1995

6.4.4 Reproduction

Zinc exposure at 0.48 mg Zn l^{-1} caused a significant reduction in the numbers of eggs per brood for *B. zschokkei*. Decreased egg production appears to be a characteristic response to zinc exposure and has been observed in the snail *Biomphalaria glabrata* at 0.5 mg Zn l^{-1} (Münzinger & Guarducci, 1988) and in *Daphnia magna* at between 0.025-0.1 mg Zn l^{-1} (Biesinger & Christensen, 1972; Paulauskis & Winner, 1988). The marine harpacticoid *Nitocra spinipes* also showed a reduction in the numbers of viable offspring at zinc exposures between 0.17 and 0.43 mg Zn l^{-1} (Bengtsson & Bergström, 1987). A significant reduction in the numbers of females producing egg sacs by another harpacticoid, *Tisbe holothuriae*, exposed to concentrations of zinc greater than 7 μg Zn l^{-1}, became more pronounced after two and three generations (Verriopoulos & Hardouvelis, 1988). Maltby & Naylor (1990) observed an increase in the number of broods aborted by *G. pulex* exposed to zinc but there was no effect on offspring production of broods not aborted. The abortion of broods was attributed to a shift in the resource allocation of females due to a reduction in the energy available to provision eggs. The lower numbers of eggs per brood observed in *B. zschokkei* is probably also due to a reduction in resource allocation by females. This is supported by a significant increase in the proportion of broods aborted by *B. zschokkei* at some zinc exposures above 0.13 mg Zn l^{-1}.

Zinc also affected reproduction of *B. zschokkei* through a lethal effect on the brood itself, causing a decrease in the number of viable offspring produced. At exposures of 0.48 mg Zn l^{-1}, the reduction in viable offspring was associated with prolonged embryonic development times and naupliar mortality during hatching. Copepods carry their broods externally within a brood sac that protects the embryos from contaminants. Direct contact of vulnerable

naupliar life-stages to zinc may have been compounded by an increase in metabolic activity during hatching.

In this study, chronic effects on reproduction were only observed at high zinc concentrations, at which lethal effects on juvenile life-stages were also observed; a lowest observed effect concentration (LOEC) of 0.49 mg Zn l^{-1} was obtained for both these endpoints. This was also within the range at which zinc was acutely toxic to copepodids. Although the presence of leaf material reduces the toxicity of zinc to *B. zschokkei* and makes comparisons with other species more difficult to interpret, this experimental design will reflect more closely conditions in the field. The sensitivity of cladocerans to acute exposure has resulted in them being the focus of the majority of chronic studies using zinc, where effects on brood size have been observed at concentrations as low as 0.025 mg Zn l^{-1} in soft water (Paulauskis & Winner, 1988). In general, freshwater crustaceans are less sensitive than cladocerans with sublethal effects of zinc on *Gammarus pulex* being recorded between 0.3-0.5 mg Zn l^{-1} (Maltby *et al*, 1990; Crane, 1995).

The levels of zinc toxic to *B. zschokkei* exceed the recommended UK Environmental Quality Standards (8-50 μg Zn l^{-1} depending on the water hardness) (Hunt & Hedgecott, 1992), however, trace metals are still locally elevated in some river systems. For example, in a recent survey of streams in Southwest England, surface water zinc varied between 0.05 and 0.87 mg Zn l^{-1} at twelve different sites (Burton, 1998). Analysis of the meiofaunal community at these sites indicated that restriction in the distribution and abundance of *B. zschokkei* was due mainly to copper contamination often in combination with elevated levels of zinc (Burton *et al*, 2001). The presence of *B. zschokkei* (albeit at reduced densities) in sediments with measured concentrations of interstitial zinc at levels observed to cause lethal

effects in this study (0.95 and 1.08 mg Zn l^{-1}) reflects the potential for this copepod to develop resistance to this heavy metal.

6.5 Summary

- Nauplii and copepodids were more sensitive to zinc than adult females corroborating data for other crustaceans exposed to this metal.
- The presence of a leaf disc reduced the toxicity of zinc to adult females.
- In the chronic exposures, survival during development and reproductive parameters (reduced egg and nauplii production and prolonged embryonic development times) showed equal sensitivity to zinc resulting in a lowest observed effect concentration of 0.48 mg Zn l^{-1}.
- Whilst *B. zschokkei* is more tolerant to zinc than *Daphnia magna*, the former species shows similar acute and chronic sensitivities as other freshwater Crustacea (*Gammarus pulex* and *Asellus aquaticus*) commonly used for generation of water quality guidelines.

Chapter 7

The effects of lindane on the development and reproduction of *Bryocamptus zschokkei*

7 Abstract

The effects of lindane were assessed at nominal concentrations between 3.2 - 3200 μg l^{-1} on the survival, development times and reproduction of the freshwater harpacticoid *Bryocamptus zschokkei*. This copepod was highly tolerant to acute lindane exposure, with more than 50% of animals surviving 96 h exposure to 3200 μg l^{-1} lindane. Using data from chronic exposures, a 10 day LC50 of 241μg l^{-1} lindane (95%CL 141-440) was calculated which is an order of magnitude greater than lethal effects observed for other freshwater Crustacea. There were also sub-lethal effects of lindane, with development times being significantly longer at 100 μg l^{-1} lindane compared with the controls. Development of *B. zschokkei* remained equiproportional (i.e. the same proportion of the total development time was spent in each moult stage) regardless of lindane exposure. It appears, therefore, that increased development times may be explained by of shifts in metabolic activity due to stress imposed by toxicant exposure, rather than to a direct effect of lindane on the moulting process. Reproductive effects of lindane, observed at 32 μg l^{-1}, included fewer eggs and viable offspring per female compared with the solvent control. At low concentrations of lindane (3.2 and 10 μg l^{-1}), a significant stimulation in reproductive output was observed compared with the control and the solvent control. This appears to be a hormesis response but is also discussed in view of recent evidence that suggests that chemicals, known to be oestrogenic in vertebrates (such as lindane), also cause an increase in offspring production in some invertebrates at low doses.

7.1 Introduction

Lindane (gamma hexachlorocyclohexane) is a broad-spectrum organochlorine insecticide that has been widely used in agriculture for crop and seed protection, in forestry against pests on cut logs, and in public health to suppress insect borne diseases (Sang *et al*, 1999). Lindane has also been added to shampoos and lotions to control for scabies and lice in

humans (Bintein & Devillers, 1996). While the use of lindane is restricted in Europe, and is now subject to a complete EC Member State ban (ENDS, 2000a), it is still widely used in many countries, including India and Mexico (Li *et al*, 1999). As a result of its volatile nature and persistence in the environment, lindane has been distributed globally where it can be detected in the air, surface waters and within organisms (Walker *et al*, 1999). Although its use has been gradually phased out, lindane still persists in aquatic systems at levels known to cause chronic effects in non-target species. For example, in Greece where the use of lindane has been restricted since 1975, it is still detected in surface waters at levels up to 0.15 $\mu g\ l^{-1}$ (Miliadis, 1994, Piperidou *et al*, 1994; Albanis *et al*, 1995). In the UK, concentrations of lindane in rivers and estuaries have declined due to a reduction in its use but it is still present in major rivers at concentrations up to 0.025 $\mu g\ l^{-1}$ lindane (Robson & Neal, 1997; Zhou *et al*, 1998; Power *et al*, 1999).

There is a large database on the aquatic toxicology of lindane to non-target invertebrates. The response to acute lindane exposure can be very variable. For example, reports of the 48 h LC50 of lindane to *Daphnia magna* vary between 0.48-3.8 $mg\ l^{-1}$ (Macek *et al*, 1976; Randall *et al*, 1979; Gliwicz & Sieniawska, 1986). Such variation in the acute toxicity of lindane to *D. magna* probably reflects different degrees of tolerance between strains of this cladoceran (Baird *et al*, 1989). Many other species are highly sensitive to lindane; for example, for freshwater amphipods, 96 h LC50s are in the range 12.9-79 μgl^{-1} lindane (Taylor *et al*, 1991; Blockwell *et al*, 1999a) and between 9.6-34 μgl^{-1} lindane for insects (Taylor *et al*, 1991; Shultz & Liess, 1995). In chronic studies, growth of the freshwater amphipods *Gammarus pulex* and *Hyalella azteca* have been significantly depressed at 6.1 and 13.5 μgl^{-1} lindane respectively (Blockwell *et al*, 1996; 1999b). Lindane at 250 μgl^{-1} was, however, required to detect a significant effect on growth of the cladoceran *D. magna*

(Ferrando *et al*, 1995). Reproductive behaviour of amphipods is also affected by lindane with a significant reduction in the numbers of precopulatory pairs and gravid females at 13.5-17.7 μgl^{-1} lindane (Macek *et al*, 1976; Blockwell *et al*, 1999a). There has also been recent concern over the potential of lindane to disrupt the endocrine systems of humans and wildlife. Lindane has been identified as being weakly oestrogenic both *in vitro* and *in vivo* (Flouriot *et al*, 1995; Petit *et al*, 1997). It has also been reported as being anti-oestrogenic, blocking the response of oestrogen-dependent cells to oestrodiol (Cooper *et al*, 1989). As a result of its oestrogenic activity, this insecticide has been identified as a priority candidate, endocrine disrupter in terms of exposure concern in a draft list drawn up by the European Commission (ENDS, 2000b). In addition, lindane has been shown to act as an ecdysone agonist in an *in vitro* ecdysone receptor assay, raising concerns that this pesticide may disrupt the endocrine function of insects (Dinan *et al*, 2001). As moulting in Crustacea is also regulated by ecdysones, perturbation of development might also be predicted as a result of lindane exposure.

Toxicity testing regimes for the freshwater harpacticoid *Bryocamptus zschokkei* have been developed in this thesis and used to consider developmental and reproductive effects of both natural environmental conditions and perturbation by contaminants. For example, prolonged development times were observed in response to excessive handling and low food quality (Chapters 2 & 4). A decrease in the number of eggs and nauplii per female were observed for *B. zschokkei* exposed to zinc (Chapter 6). These protocols are, therefore, used in this study with the general aim of considering the effects of different concentrations of lindane on the lethal and sublethal responses of *B. zschokkei*. Specific aims were to assess:

1. The effects of acute exposure to lindane on different life-stages (nauplii, copepodids and adult females).

2. How the presence of a leaf disc in the test system influenced acute toxicity.
3. The influence of chronic exposure to lindane on long-term survival, development time and reproduction.

7.2 Materials and Methods

7.2.1 Test Substance

The test substance in this study was radiolabelled for the purpose of chemical analysis. Hexachlorocyclohexane Gamma-Isomer (lindane) and radiolabelled (^{14}C) lindane were both obtained from Sigma, Poole, Dorset, UK. The purity of lindane and ^{14}C-lindane was specified as 99% (w/w) and 95% (w/w), respectively. Radiolabelled lindane had a specific activity of 109 KBq mg^{-1}. For each exposure, individual stock solutions were prepared in methanol, using equal quantities of radiolabelled (hot) ^{14}C-lindane made up to the appropriate concentrations with non-labelled material (cold). Test solutions were prepared by the addition of 10 µl of aqueous stock solution to 100 ml of dilution medium whilst stirring. The concentration of the carrier solvent methanol in the test solutions did not exceed 0.1 ml l^{-1}.

7.2.2 Acute Toxicity

Nominal test concentrations of 320, 560, 1000, 1800 and 3200µg l^{-1} lindane were chosen on the basis of published data describing the effects of lindane on other freshwater Crustacea (Green *et al*, 1986; Taylor *et al*, 1991; Ferrando *et al*, 1992). A logarithmic scale was used for the exposure range to put emphasis on effects at the lower end of the range (Rochinni *et al*, 1982). A control (dilution water) and solvent control (made up to 0.1 ml l^{-1} methanol) were also included. Dilution water was Elendt's M4 medium (Elendt & Bias, 1990) diluted to give a hardness of 100 mg l^{-1} as $CaCO_3$, an alkalinity of 30 mg l^{-1} as $CaCO_3$, conductivity

of 290 μs cm^{-1} and pH 7.7. The acute toxicity of lindane up to 96 h was calculated for three life stages of *B. zschokkei* (nauplii, copepodids and non ovigerous adult females) in the absence of food using the methods described in Chapter 6. The acute toxicity of lindane to non ovigerous adult females was also determined for females in the presence of a 1 cm diameter beech leaf disc (*Fagus sylvatica*). All experimental work was carried out in a temperature-controlled room at 20 (± 1) °C and a photoperiod of 16 h light: 8 h dark with a 20 minute dawn dusk transition. Temperature was recorded daily. The measured endpoint of the acute experiments was mortality. Each replicate was observed every 24 h for the presence of dead animals. A copepod was considered to be dead if it did not respond to gentle mechanical stimulation after 10 seconds. In addition to mortality, observations of the following behaviours were recorded: 1) how many animals required mechanical stimulation and 2) for exposure experiments conducted in the presence of a leaf disc, the number of females on the surface of the leaf disc.

Water samples from both new and old test solutions were obtained for analysis of water quality parameters (pH and dissolved oxygen) and chemical analysis by liquid scintillation counting (LSC). Three replicate 10 ml samples of new test solution were retained in glass scintillation vials prior to each renewal for chemical analysis. To assess the difference in dissolved lindane concentration in Cellwells™, with and without a leaf disc, duplicate 9.6 ml samples were taken from wells not containing a leaf, a third sample was taken from wells containing leaf discs and analysed separately. Only one renewal of test solution was made over the 96 h duration of this test. At the end of the assay, the lindane accumulated in the leaf discs was determined by combustion followed by LSC (see Section 7.2.5). The "time-weighted" mean concentrations of lindane at each exposure were calculated as described in Chapter 6.

7.2.3 Chronic Toxicity

The chronic effects of lindane on development and reproduction were assessed using the methods described in Chapter 6. Treatments included a control (dilution water), solvent control and nominal exposure concentrations of 3.2, 10, 32, 100, 320, 1000 and 3200 $\mu g\ l^{-1}$ lindane. This exposure range overlaps the range chosen for acute exposures because a short-term lethal effect was not observed in the acute experiment. The range also includes lower lindane concentrations than used in the acute exposures to incorporate concentrations, which relate to published toxicity data for other freshwater Crustacea (Ferrando *et al*, 1995; Blockwell *et al*, 1999b). Three replicate 10 ml samples of new test solution were retained in glass scintillation vials for LSC before each renewal. Prior to discarding old test solution, three replicate 9.6 ml samples were obtained from each exposure to determine the concentration of lindane present. Water was renewed three times per week, therefore, the timings of these analyses were noted so that two and three day intervals between water changes could be taken into account.

7.2.4 Chemical Analysis

On termination of the acute assay, leaf discs were transferred to individual pre-weighed "combustocones" (Camberra Packard) and allowed to dry for 1 h at 60°C. The dry weight (mg) was then measured prior to combustion in a Packard D306 sample oxidiser (Camberra-Packard). In this process, ^{14}C in the sample is oxidised to $^{14}CO_2$, trapped in a "Carbosorb" (Camberra Packard) and mixed with "Permafluor E" scintillator (Camberra Packard). To obtain a count for radioactivity, and hence calculate the amount of lindane that had adsorbed to the leaf disc, the vial containing the trapped $^{14}CO_2$ was then analysed by liquid scintillation counting (LSC). The concentration of lindane in water samples was also determined by LSC. An equal volume of Opti-phase Hisafe 3 scintillator (Fisher Scientific Ltd.) was added to water samples from the acute and chronic assays. The

samples were equilibrated to ambient temperature for twenty minutes and counted in a Camberra Packard 2500 TR tri-Carb spectrometer (Packard Instruments Ltd.) for 10 mins or 1.6×10^5 counts whichever was reached first.

7.2.5 Statistical Analysis

A Student's t-test was performed to assess differences in lindane concentrations in treatment water in the presence and absence of leaf material. Differences between treatments in the number of copepods associated with leaf discs in the acute exposures were analysed using chi-square tests. In the chronic experiment, the lethal concentration of lindane was calculated after 10 days using the moving average angle method (Stephan, 1977). For chronic exposures, differences between treatments in survival at days 21 and 42 were analysed using Fisher's Exact test (Finney, 1963). All data were tested for normality (Shapiro-Wilks) and for equality of variances (Bartlett's test) before using analysis of variance techniques. If data did not conform to these assumptions, they were log transformed. One-way ANOVA was used to test for differences between lindane exposures using Bonferroni's multiple comparison procedure to discriminate between the means. If the assumptions for ANOVA were not met, data were analysed using Kruskal-Wallis analysis of variance by ranks, followed by Mann Whitney tests to highlight which treatments were different. To test whether copepods exposed to lindane still conformed to a model of 'equiproportional' development (Chapter 2) a two-way ANOVA was carried out on arc-sine, square root transformed data of the proportion of total development time spent in each copepodid moult stage. Any data that were still not normal were confirmed using the Kruskal-Wallis test.

7.3 Results

7.3.1 Analytical Chemistry

Measured lindane concentrations in the test vessels were within 20% of the nominals in both the acute and chronic exposures (Table 7.1), therefore, all data are referred to in terms of the nominal concentration. In the acute exposures, the concentration of lindane did not differ significantly in the presence or absence of food (Student's t-test, $p>0.05$), despite leaf discs accumulating lindane in a concentration-dependant manner (Table 7.1). The time-weighted mean concentrations of lindane were not more than 20% different from nominals (Table 7.2).

7.3.2 Acute Toxicity

Lindane did not result in more than 50% mortality of any life stage of *B. zschokkei* within 96 h, therefore, LC50 values could not be calculated. All animals exposed to lindane showed limited activity, often not moving unless gently stimulated. This response was unpredictable, therefore, it could not be used to calculate an EC50 (effective concentration) (for example, some animals that were stimulated after 48 h would not require stimulation at 96 h). In treatments where a leaf disc was present, significantly fewer animals were found on the leaf disc at all lindane concentrations compared with the control and solvent control (96 h, χ^2 22.28 d.f. 6 $p<0.05$) (Fig. 7.1). Only 40% of copepods were recorded on the leaf litter at the highest concentration of 3200 $\mu g\ l^{-1}$ lindane whereas in both the controls more than 90% were on the leaf discs.

Table 7.1: Mean measured concentrations of lindane (0, 48 and 96 h) in the acute exposures, in the presence and absence of leaf material and the concentration of lindane accumulated in the leaf discs after 7 days exposure.
There was no significant difference in the water concentration of lindane in the presence or absence of a leaf disc.

Nominal conc. of lindane ($\mu g\ l^{-1}$)	Mean (± 1SD) measured conc. of lindane ($\mu g\ l^{-1}$) n=3	Mean (± 1SD) measured conc. of lindane + leaf ($\mu g\ l^{-1}$) n=3	Measured conc. of lindane accumulated in leaf discs ($mg\ kg^{-1}$ dry wt)
Control	<2	<2	<7.6
Solvent control	<2	<2	<7.1
320	310 (± 1.7)	297 (± 2.5)	257
560	520 (± 2.5)	500 (± 3.5)	436
1000	910 (± 4.3)	875 (± 5.0)	772
1800	1650 (± 8.2)	1600 (± 10.0)	1520
3200	2880 (± 17.3)	2775 (± 25.2)	2457

Table 7.2: The time-weighted mean measured lindane concentrations calculated in chronic exposures

Nominal conc. of lindane ($\mu g\ l^{-1}$)	Time weighted conc. of lindane ($\mu g\ l^{-1}$)	Percentage of nominal
Control	<2	-
Solvent control	<2	-
3.2	3.5	+8.6
10	9.7	-3.0
32	30.0	-6.3
100	89.9	-10.1
320	297.4	-7.1
1000	832.5	-16.8
3200	3575.0	+10.5

7.3.3 Long-term Survival

Despite the lack of lethal effects in acute exposures, long-term survival was significantly reduced in the chronic experiments at exposures ≥320 μg l^{-1} lindane compared with the control and solvent control (Fisher's Exact Test, P<0.05). At 1000 and 3200 μg l^{-1} lindane, all animals had died by day 13 and at 320 μg l^{-1} by day 42 (Fig. 7.2). Significant mortality was also observed at 100 μg l^{-1} lindane after 21 days compared with the control (Fisher's Exact Test, p<0.05) but not with the solvent control. A 10 day, LC50 of 241 μg l^{-1} (95%CL of 141-440) was determined for the naupliar stages using these data. Except for 320 μg l^{-1} lindane, where all five animals moulting to adult died before the end of the experiment, there was no further significant mortality for adults.

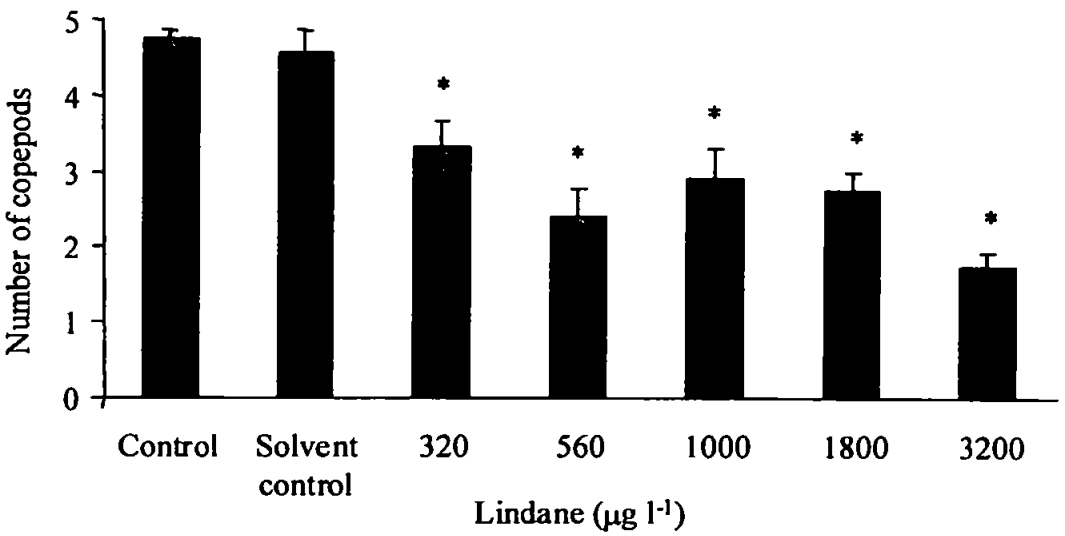


Figure 7.1: The mean (± 1SE) number of females found on the leaf disc in acute toxicity tests.
The data were pooled from observations at 48, 72 and 96 h.
* Indicates a significant difference from the control and the solvent control (96 h, χ^2 22.28 d.f. 6 p<0.05); n =20 copepods at each exposure

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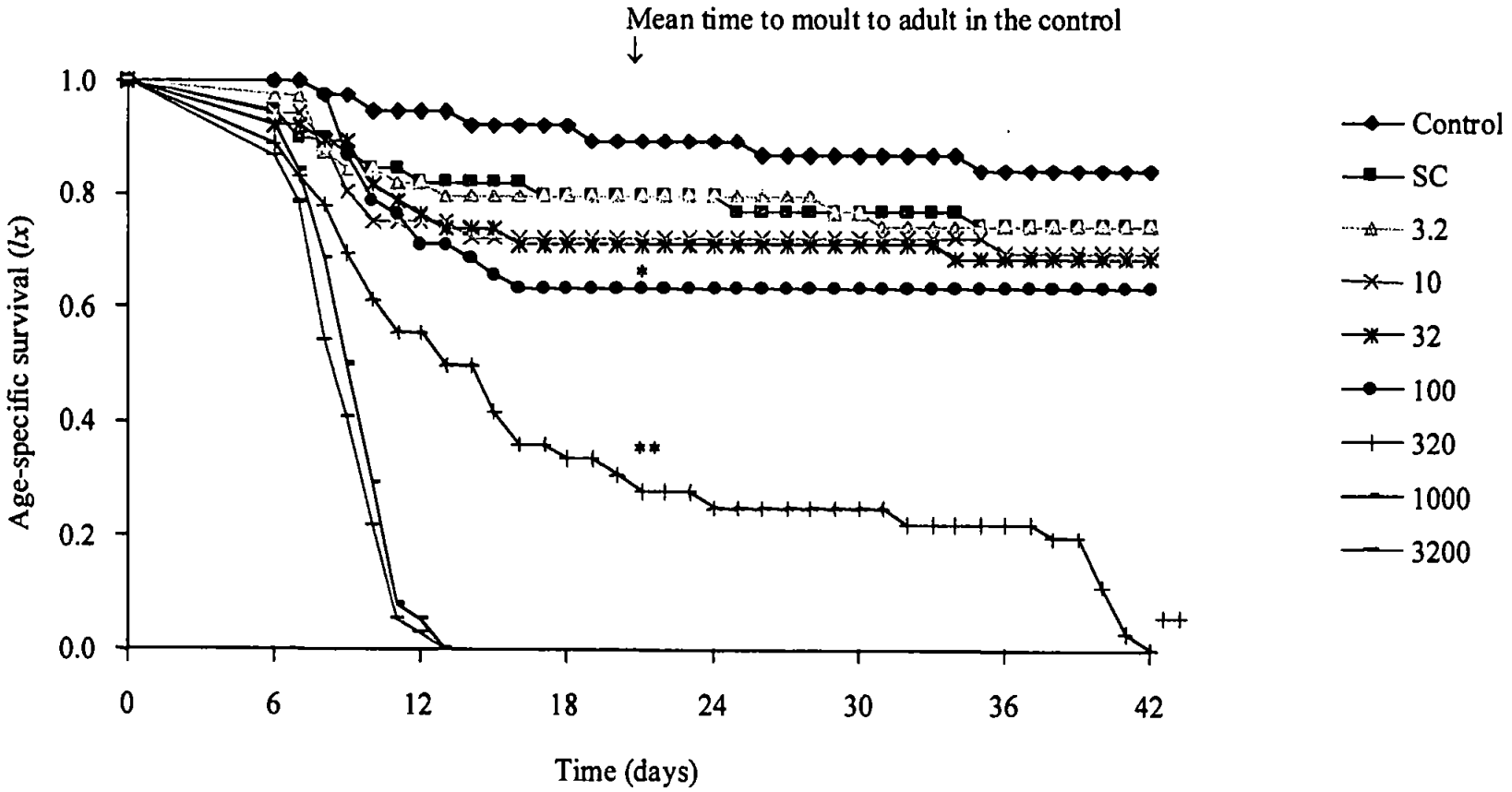

Figure 7.2: The effect of lindane (µg l^{-1}) on the age-specific survival (*lx*) of *Bryocamptus zschokkei*.
** Indicates a significant difference from the control and solvent control and * from the control at day 21 (Fisher's Exact Test, $p<0.05$). ++ Indicates a significant difference from the control and solvent control at day 42 (Fisher's Exact Test, $p<0.05$); n=38-40 for each treatment.

Table 7.3: The mean ± 1 SD development times for combined naupliar (Dn) and copepodid stages (Dc), and hatching to adult (N1-A) for *Bryocamptus zschokkei* exposed to lindane.
The numbers of males and females moulting to adult in each treatment are also shown. n = number of original animals and n^a = number of animals that survived to achieve adulthood. The number in brackets is the number of animals used to calculate the duration of each stage. * Indicates a significant difference from all other treatments (Kruskal-Wallis, $p<0.05$).

Nominal conc. of lindane (µg l^{-1})	n	n^a	Dc/Dn	Sex ratio of copepods		Development time (days)		
				Male	Female	Dn	Dc	(N1-A)
Control	38	33	0.95	11	22	9.94 ± 1.45 (35)	9.45 ± 2.80 (33)	19.36 ± 3.32 (33)
Solvent control	39	30	0.83	9	21	10.69 ± 2.64 (32)	8.87 ± 0.68 (30)	19.53 ± 2.80 (30)
3.2	39	31	0.91	11	20	9.68 ± 1.14 (31)	8.77 ± 0.67 (31)	18.45 ± 1.41 (31)
10	36	25	0.90	7	18	10.11 ± 1.85 (27)	9.08 ± 0.91 (25)	18.80 ± 1.38 (25)
32	38	26	0.94	8	18	10.30 ± 1.86 (27)	9.70 ± 1.07 (26)	20.00 ± 2.08 (26)
100	38	21	1.00	6	15	11.44 ± 2.63 (25)	11.48 ± 2.11 (21)*	22.57 ± 3.44 (21)*

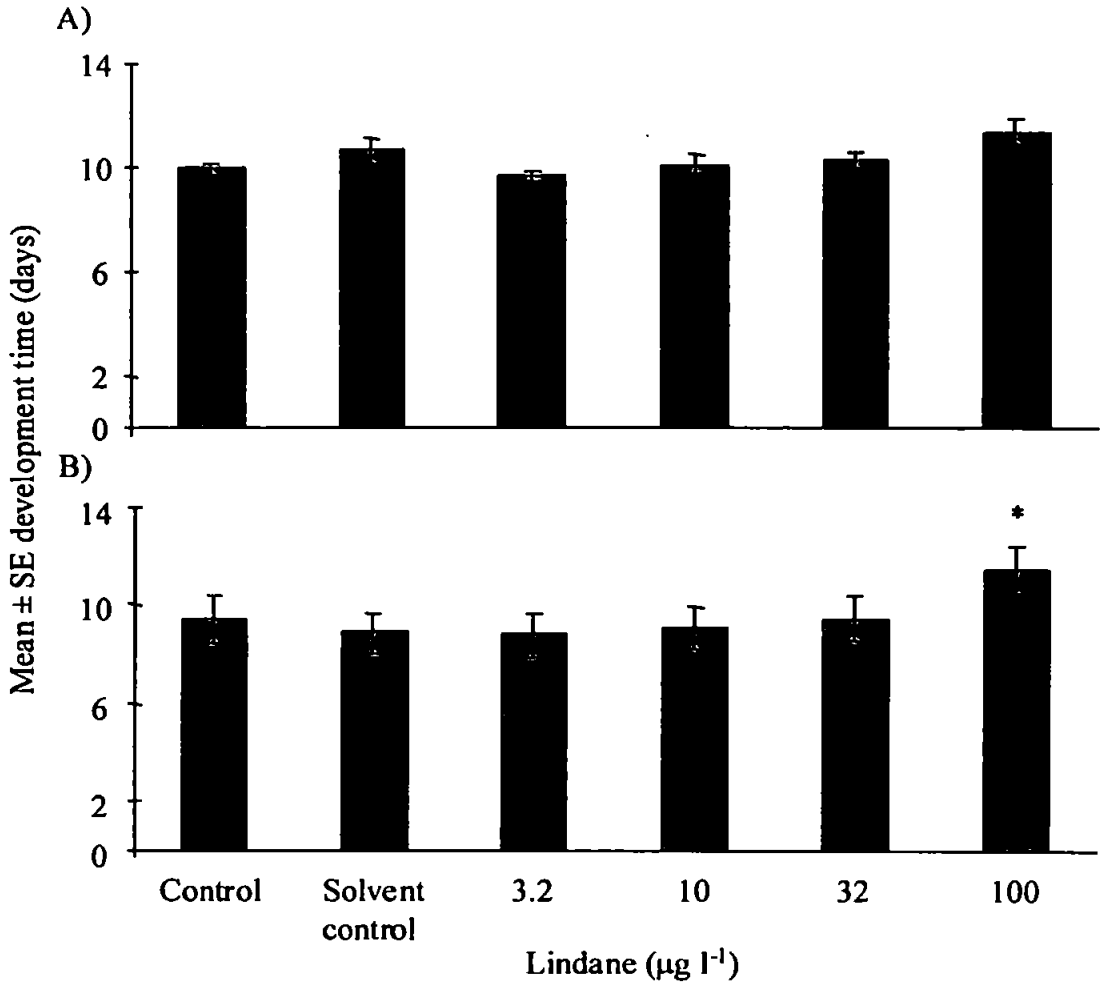

Figure 7.3: Effect of lindane on the time taken (mean ± 1SE) for A) naupliar (Dn) and B) copepodid development (Dc).
* Indicates a significant difference from all other treatments (Kruskal-Wallis, $p<0.05$); n = 21-35 for each treatment.

7.3.4 Development

Development times for copepodids (Dc) and from nauplius to adult (N-A) were significantly prolonged at 100 µg l^{-1} lindane compared with both the controls and lower lindane exposures (Table 7.3; Fig. 7.3) (Kruskal-Wallis, $p<0.05$). For copepods exposed to higher lindane concentrations, development times could not be calculated because of the high mortality in these treatments. Although there was a significant effect of lindane on the development time to adult of *B. zschokkei*, there was no significant change in the proportion of development time spent in each moult stage regardless of lindane concentration (Table 7.4).

Table 7.4: The effect of the proportion of total development time spent in each moult stage and lindane exposure on *Bryocamptus zschokkei*.
This shows the results of a Two-way ANOVA on arc sine, square root transformed data where * indicates significance at the 1% level.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Moult stage	4803.18	4	1200.79	162.50	0.001*
Lindane	75.64	5	15.128	2.05	0.07
Interaction	379.61	20	18.98	2.57	0.001*
Residual	5948.69	805	7.39		
TOTAL	11477.0	834			

7.3.5 Reproduction

The mean time taken for adult females to produce their first brood was significantly longer (by approximately 5 days) at 100 μg l^{-1} lindane compared with the controls (Kruskal-Wallis, $p<0.05$) (Table 7.5). The number of fertile females in this treatment was also significantly reduced compared with the controls with only three of the twelve paired females producing viable offspring (χ^2 14.17 d.f. 2 $p<0.05$) (Table 7.5). This treatment was excluded from further analysis because there were not enough animals for meaningful statistical analysis.

There was no significant difference between the control and the solvent control in any of the reproductive parameters measured (Student's t-test, $p>0.05$). At 32 μg l^{-1} lindane, the numbers of eggs and nauplii per female were reduced by 44 and 60% respectively compared with the control but a significant decrease was only observed with respect to the solvent control (One-way ANOVA, $p<0.05$) (Fig. 7.5). The proportion of broods aborted was significantly higher at 32 μg l^{-1} lindane compared with lower lindane exposures and both controls (χ^2 18.97 d.f. 4, $p<0.05$) (Table 7.5). At low concentrations of lindane, increased

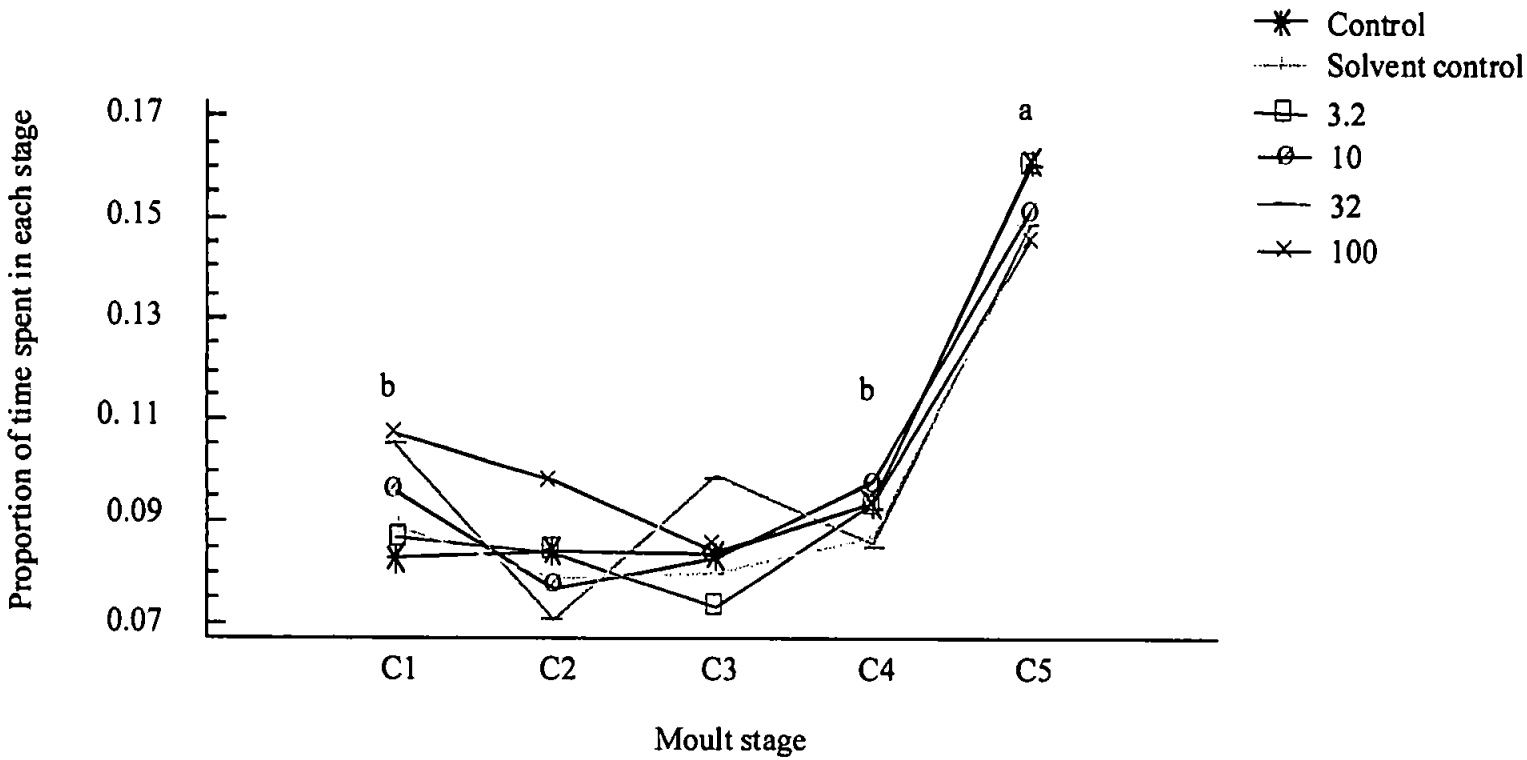

Figure 7.4: The proportion of the total development time spent in each molt stage in the controls and at four lindane ($\mu g\ l^{-1}$) exposures.
'a'- indicates a significantly longer stage duration compared with all other stages and 'b'- a significantly longer stage duration compared with C2 and C3 (Two-way ANOVA, $p<0.05$). A similar pattern of development is observed at all hardness levels and there was no significant effect of lindane on the proportion of time spent in each moult stage; n=25-33 for each treatment.

offspring production was observed with significantly more viable nauplii at 10 μg l^{-1} compared with the control and solvent control (51 and 38% less nauplii per female respectively) (One-way ANOVA, $p<0.05$). A significant increase in the number of eggs per female (37 and 45% at 3.2 and 10 μg l^{-1} respectively) was also observed compared with the control (One-way ANOVA, $p<0.05$).

A stimulation of offspring production was reflected in the number of eggs and viable nauplii per brood which were significantly lower in the control and 32 μg l^{-1} lindane compared with 10 and 3.2 μg l^{-1} lindane (Kruskal Wallis, $P<0.05$) (Table 7.5). There was no significant difference in the number of broods per female compared with either of the controls, although significantly more broods were observed at 3.2 and 10 μg l^{-1} lindane compared with 32 μg l^{-1} lindane (Table 7.5) (One-way ANOVA, $p<0.05$). The embryonic development time was similar at all treatments (Table 7.6).

7.4 Discussion

7.4.1 Acute Toxicity

Bryocamptus zschokkei was highly tolerant of acute exposure to lindane compared with other freshwater invertebrates (Table 7.7). It is difficult to explain the resistance of this copepod to short-term lindane exposure, particularly in view of the susceptibility of other freshwater Crustacea. Generally, lindane acts via the stomach through ingested food, and affects the nervous system. It is possible that this copepod is reducing uptake of lindane by not consuming food even when it is available. This explanation is supported by the behavioural changes exhibited by *B. zschokkei* where less animals were found on the leaf discs (from which they are known to obtain food, see Chapter 4) compared with the control

Table 7.5: The effect of lindane on the number of broods produced by *Bryocamptus zschokkei*, time to first egg sac and on the numbers of eggs and nauplii per female.

Where n is the number of females per treatment, n_a is the number of females producing egg sacs and n_b is the number of females producing egg sacs from which viable offspring hatched. Where, [A] (χ^2 31.85 d.f. 5, $p<0.05$) and [B] (Kruskal-Wallis, $p<0.05$) are significantly different from all other treatments. A significant increase with respect to the control, solvent control and 32 $\mu g\ l^{-1}$ lindane is indicated by [E], with respect to the control and 32 $\mu g\ l^{-1}$ lindane by [D] and with respect to just 32 $\mu g\ l^{-1}$ lindane by [C] (Kruskal-Wallis, $p<0.05$).

Nominal conc. of lindane ($\mu g\ l^{-1}$)	n	n_a	n_b	Total no. of broods	Total successful broods	Proportion of aborted broods	Mean ± 1SD time to first egg sac (n_a)	Mean ± 1SD no. of broods per female (n_a)	Mean ± 1SD no. eggs per brood (n_b)	Mean ± 1SD no. nauplii per brood (n_b)
Control	19	17	14	59	37	0.37	25.65 ± 3.72	3.53 ± 1.42	10.46 ± 5.44	10.20 ± 5.67
Solvent control	15	15	13	55	34	0.38	25.25 ± 3.79	3.44 ± 1.26	13.49 ± 5.80	14.06 ± 5.75
3.2	20	18	17	76	56	0.26	23.06 ± 3.89	4.22 ± 1.26 [C]	14.18 ± 6.56	14.20 ± 6.70
10	16	14	13	60	44	0.27	25.00 ± 2.83	4.29 ± 0.99 [C]	16.63 ± 5.82	17.70 ± 4.71
32	14	12	8	35	12	0.66 [A]	26.93 ± 4.08	2.69 ± 1.32	10.37 ± 5.36	7.42 ± 6.19
100	12	8	3	21	5	0.76 [A]	32.22 ± 4.29 [B]	2.33 ± 0.71	11.38 ± 5.60	11.00 ± 5.07

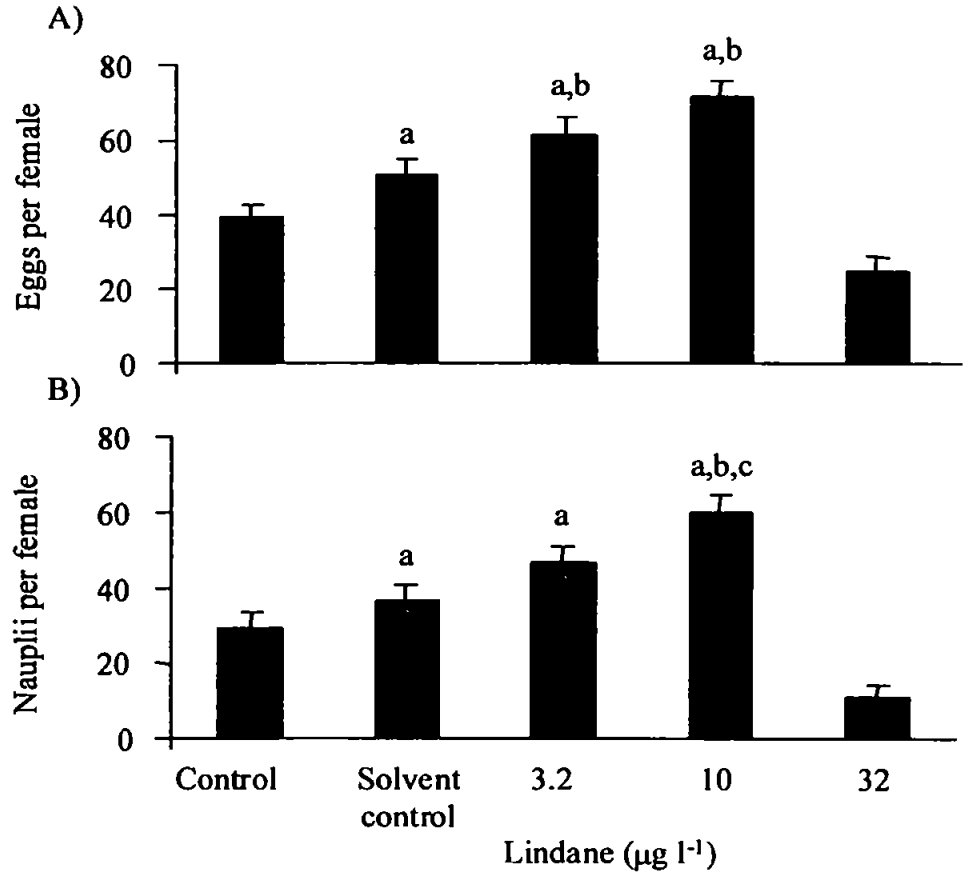

Figure 7.5: The mean (± 1SE) number of A) eggs and B) nauplii per female *Bryocamptus zschokkei* producing viable offspring at different exposures of lindane.
A significant difference from 32 µg l^{-1} is indicated by 'a', from the control by 'b' and from the solvent control by 'c' (One-way ANOVA, $p<0.05$); n=8-17 females for each treatment).

Table 7.6: Embryonic development times for *Bryocamptus zschokkei* at different lindane exposures.
There was no significant effect of lindane on development times.

Nominal conc. of lindane (µg l^{-1})	Mean ± 1SD brood duration (days)	n
Control	3.26 ± 0.62	39
Solvent control	3.25 ± 0.57	34
3.2	3.13 ± 0.49	52
10	3.15 ± 0.43	44
32	3.38 ± 0.53	12
100	3.00 ± 0.29	7

animals. Chandler (1990) also reported unusually high lethal tolerance of marine harpacticoids to the pesticide fenvalerate compared with other Crustacea, which was attributed to a lack of uptake via the stomach. *Bryocamptus zschokkei* did show low activity at all acute lindane exposures and movement was often so limited that fungi started to grow on the animals. Despite limited activity, the copepods did respond to gentle mechanical stimulation. The ability to reduce energy utilisation by restricting movement probably also contributed to the ability of *B. zschokkei* to withstand high lindane exposures.

7.4.2 Long-Term Survival

Nauplii (<24 h old) were able to withstand exposure to lindane up to 3200 μg l^{-1} for more than six days before significant mortality was observed. A 10 day LC50 of 241 μg l^{-1} (95%CL of 141-440) for nauplii is more than an order of magnitude greater than observed for juvenile amphipods (7-9.8μg l^{-1} lindane) or chironomid larvae (13 μg l^{-1} lindane) (Taylor *et al*, 1991; Blockwell *et al*, 1999a). In this experiment, any significant mortality occurred during the larval life stages (nauplii and copepodids), with no significant mortality for adults. Low mortality of adults compared with juveniles was also observed for *B. zschokkei* exposed to zinc (Chapter 6) and copper (Burton, 1998) and for other crustaceans exposed to lindane (Taylor *et al*, 1991; Blockwell *et al*, 1999a).

7.4.3 Development Times

Growth and development of freshwater Crustacea is known to be sensitive to lindane and disruptive effects have been observed at concentrations of between 6.1–250 μg l^{-1} (Ferrando *et al*, 1992; Blockwell *et al*, 1996; 1999b). While effects on the development times from nauplius to adult were observed for *B. zschokkei,* they were only manifest at the highest concentration for which data were available, 100 μg l^{-1} lindane, reflecting an apparent tolerance to lindane of this copepod relative to other Crustacea.

Table 7.7: The acute toxicity of lindane to selected freshwater invertebrates. Hardness is expressed in terms of mg l^{-1} as $CaCO_3$.

Species	Life-stage	Test conditions	Nominal/ measured	Exposure time (h)	LC50 ± 95% CL (µg l^{-1})	Ref.
Bryocamptus zschokkei	**Adult and nauplii** **Nauplii**	**20±1°C, hardness 100, pH 7.8**	**nominal**	**96** **240**	**> 3200** **241 (141-440)**	**This study**
Gammarus pulex	2^{nd}-3^{rd} moult	12±1°C, hardness 151, pH 6.8-7.2	measured	96 240	79 7	Taylor *et al*, 1991
Chironomous riparius	2^{nd} instar	20±1°C, hardness 151, pH 6.8-7.2	measured	96 240	34 13	
Gammarus pulex	Adult	15±2°C, hardness 160-228, pH 7.0-7.6	nominal	96	34 (25-46)	Abel, 1980
Gammarus pulex *Asellus aquaticus* *Chironomous riparius*	Adult Adult 4^{th} instar	11±1°C, hardness 92.9, pH 7.5-8.0	measured	96	225 375 235	Green *et al*, 1986
Hyalella azteca	0-7 days	21.9°C, hardness 105, pH 7.9	measured	96 240	12.9 (8.9-18.8) 9.8 (7.3-13.3)	Blockwell *et al*, 1999a
Daphnia magna	< 24 h			48	516 (480-551)	Randall *et al*, 1979
Daphnia magna	Juvenile	22±1°C, hardness 250, pH 7.9	nominal	24	1640 (1150-1780)	Ferrando *et al*, 1992

Bryocamptus zschokkei has been found to conform to a model of equiproportional development where each moult stage represents a certain proportion of the total development time (Chapter 2). In copepods, this type of development is independent of processes such as food quality and temperature which affect metabolism (Hart, 1990). It is therefore hypothesised that contaminants, which affect metabolic processes directly, would have no effect on 'equiproportional' development whereas chemicals exerting an effect on moulting directly would disrupt the normal moulting pattern. This theory has been tested for *B. zschokkei* exposed to lindane where it was found that although overall development times were longer at 100 $\mu g\ l^{-1}$ lindane than in the controls, there was no significant difference in equiproportional development. If lindane was having a mechanistic affect on the developmental process, it might be expected to interrupt the normal moulting pattern, particularly if each moult stage exhibits a different degree of sensitivity (Costlow, 1977; Wright *et al*, 1996). For example, copepods exposed to the insect growth regulator diflubenzuron had difficulties in shedding their exuviae (Wright *et al*, 1996). The use of a copepod growth model is of particular interest when considering lindane as this insecticide is known to act as an ecdysone agonist in an *in vitro* ecdysteroid receptor assay (Dinan *et al*, 2001). As development in copepods is mediated via the ecdysteroid receptor, effects on these hormonal processes would be expected to be manifest in the pattern of moulting. As lindane did not disrupt the moulting pattern of *B. zschokkei*, it seems likely that the observed increase in development time is as a result of changes in metabolic processes and not due to endocrine disruption. Zou & Fingerman (1997) did not observe any effects of lindane on moulting of *D. magna* that could be attributed to endocrine disruption. The model ecdysone agonist 20-hydroxyecdysone also had no effect on the development times of *B. zschokkei* (Chapter 5). It is well established that, in response to contaminant exposure, animals are subject to increased metabolic demands for maintaining homeostasis, causing

the redirection of energy to other physiological processes (Maund *et al*, 1992; Blockwell *et al*, 1999b). Lindane is known to reduce the feeding rate of *Gammarus pulex* (Blockwell *et al*, 1998) and the filtering rate of *Daphnia magna* (Gliwicz & Sieniawska, 1986). Reduced feeding would result in less energy available for growth. Whilst the feeding rate of lindane-exposed *B. zschokkei* has not been considered in the present study, fewer lindane-exposed copepods were found on the surface of leaf discs (on which this copepod is known to feed, see Chapter 4) compared with control animals in the acute exposures.

7.4.4 Reproduction

The number of eggs and viable offspring produced per female, were the most sensitive endpoints for lindane exposure to *B. zschokkei*, with lower numbers of both observed at 32 $\mu g\ l^{-1}$ lindane compared with the solvent control. There was also a significant increase in the proportion of broods aborted at 32 $\mu g\ l^{-1}$ lindane compared with lower exposure levels and the controls. This supports previous evidence for egg sac abortions being a stress response in *B. zschokkei*. For example, *B. zschokkei* exposed to zinc also showed an increase in abortion frequency at high zinc exposures (Chapter 6). Aborting broods may mean that females can allocate more energy to physiological processes such as metabolism of the toxicant rather than to the provision of eggs (Maltby & Naylor, 1990). It may be that measuring abortion frequency in *B. zschokkei* will have application as a biomarker of pollution exposure (Chapter 2).

Other life-history endpoints (survival and development times) considered in this study suggest a high degree of tolerance of *B. zschokkei* to lindane compared with other freshwater Crustacea. Effects on reproduction at 32 $\mu g\ l^{-1}$ lindane are, much lower than those observed for *D. magna* at 250 $\mu g\ l^{-1}$ lindane (Ferrando *et al*, 1995) and similar to

effects observed for *Hyalella azteca* (Amphipoda) at 13.5 μg l^{-1} lindane (Blockwell *et al*, 1999b). The lowest observed effect concentration (LOEC) of 32 μg l^{-1} lindane for *B. zschokkei* is also consistent with results from mesocosm experiments where reduced densities of freshwater copepods have been reported between 2 - 200 μg l^{-1} lindane (Lay *et al*, 1987; Neugebaur-Büchler *et al*, 1991; Peither *et al*, 1992; Fliedner & Klein, 1996).

Of particular interest in this study was the significantly higher numbers of nauplii per brood at low lindane exposures compared with the control (3.2 and 10 μg l^{-1} lindane) and solvent control (10 μg l^{-1} lindane). This phenomenon, where stimulation by a toxicant is observed at the lower end of the dose response curve, is termed hormesis and has been widely documented in pharmacological and toxicological studies (Stebbing, 1982). The result of this phenomenon is a beta dose response curve (Fig. 7.6). A database of studies in which hormesis has been documented shows that common endpoints for low-dose enhancement include offspring and egg production (as observed in this study), and that the response is most often observed for heavy metals, insecticides and radiation (Calabrese & Baldwin, 1999). The potential implications of this phenomenon are now being recognised. For example, physiological 'hormetic' stimulation may be responsible for insect outbreaks after pesticide applications (Morse, 1998). Evidence from studies on 'growth' hormesis suggest that hormesis is an adaptive response to the inhibitory effects of agents at high concentrations which results in an overcompensation of control mechanisms (Stebbing, 1998). In a recent review, the implications of hormesis have been considered in terms of life-history traits and ecological risk assessment (Forbes, 2000). The available data suggests that stimulation of one life-history trait would be expected to result in a trade off with other traits, ultimately compromising overall fitness of the individual.

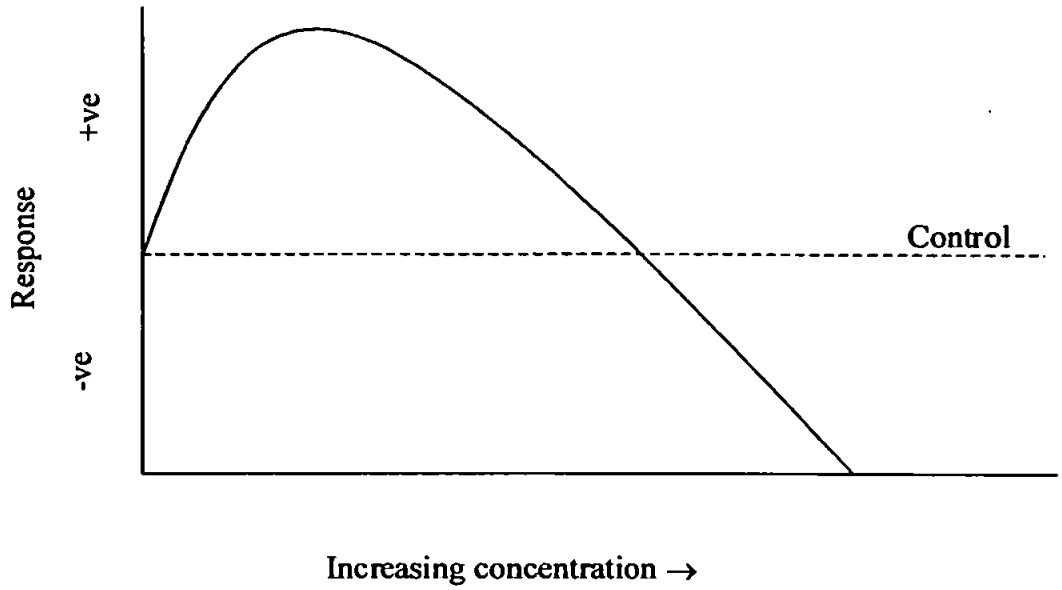

Figure 7.6: A beta-shaped dose response curve which shows stimulation at the lower end of the curve, indicative of a hormesis response (adapted from Stebbing, 1982).

For example, in the presence of environmental stress a female may put more effort into offspring production than into her own survival so that the next generation will be present to exploit any improvement in natural conditions. Forbes (2000) concluded that stimulation of individual life-history traits (i.e. reproduction or growth) might have evolved as an adaptation to maintain fitness in a changeable environment. The stream benthos in which *B. zschokkei* inhabits is a complex environment characterised by the presence of a transient and patchy food resource, detritus. *Bryocamptus zschokkei* appears to have adapted a number of traits for surviving life within this heterogeneous environment such as tolerance of changes in environmental conditions (Chapters 2 & 3), variability in offspring production (see Chapter 3) and omnivory (see Chapter 4). The ability to shift energy resources in order to maximise survival from an evolutionary perspective (as characterised by a hormesis response) may be a further adaptation of *B. zschokkei* for successfully exploiting these interstitial stream sediments.

Recent evidence on the effects of endocrine disrupting chemicals suggests that oestrogenic chemicals cause a specific 'hormetic' response manifest in the reproductive parameters of

invertebrates. This effect is of particular interest in this study as lindane is known to display weak oestrogenic activity (Flouriot *et al*, 1995; Petit *et al*, 1997). Oehlmann *et al* (2000) reported morphological alterations of the prosobranch snail *Marisa cornuarietis* exposed to the xeno-oestrogens bisphenol A and octylphenol, which were related to a massive stimulation in oocyte and spawning mass production. These females have been termed 'superfemales'. There is also evidence for stimulation in reproductive output in crustaceans exposed to low doses of xeno-oestrogens. For example, the marine copepod *Acartia tonsa* exposed to both bisphenol A and 17β-oestrodiol showed significant increases in reproduction which were attributed to an oestrogenic effect on female sexual maturation and egg production (Andersen *et al*, 1999). Alkylphenol exposed female daphnids showed malformations based on the morphological differences between males and females, which was associated with an increase in reproduction (Gerritsen *et al*, 1998). The amphipod *Corophium volutator* (Brown *et al*, 1999) and the copepod *Tisbe battagliai* (Bechmann, 1999) each showed beta shaped dose response curves when exposed to 4-nonylphenol but this stimulation in offspring production was not significant. More mechanistic evidence is required before such increase in reproductive output in crustaceans can be directly attributed to endocrine disruption. It should be noted that there is no evidence of oestrogens having a functional role in arthropods (deFur *et al*, 1999).

From the current data, it is not possible to conclude that the stimulation in reproduction of *B. zschokkei* in this study was due to the oestrogenic properties of lindane. There does not appear to be evidence for low-dose increases in reproduction for other Crustacea exposed to this insecticide (Ferrando *et al*, 1995; Blockwell *et al*, 1999b). It may be that the tolerance of this copepod to the direct toxicity of lindane results in subtle sublethal effects becoming more apparent. If this were true, it has implications for choosing test species as it suggests that the most sensitive species might not necessarily give the most information on

how a contaminant is exerting its toxicity. Whatever the reason for this stimulatory response, it provides an interesting paradigm for risk assessors, as an increase in reproductive output cannot be considered detrimental to the fitness of the individual species (Andersen *et al*, 1999; Forbes, 2000). It may not be beneficial, however, particularly if the individual is making a trade off in some respect (e.g. longevity or maternal input into individuals of the next generation) and overall population fitness is compromised. Alternatively, if other species in a community do not respond in the same fashion a sudden increase in numbers as a result of low dose stimulation in reproduction could result in single species dominance of natural populations.

7.5 Summary

- *Bryocamptus zschokkei* is highly tolerant of short-term exposure to lindane with a 96 h LC50 > 3200 μg l^{-1} lindane.
- Long-term survival and development times of this copepod are affected at 100 μg l^{-1} lindane. Using the model of equiproportional development (Chapter 2) prolonged development times were probably due to shifts in metabolic activity due to stress imposed by toxicant exposure.
- Abortion frequency was higher at 32 μg l^{-1} lindane compared with controls. Females produced significantly fewer eggs and nauplii at 32 μg l^{-1} lindane compared with the solvent control and lower lindane exposures (3.2 and 10 μg l^{-1} lindane). Chronic sensitivity to this insecticide is within the range observed for other freshwater Crustacea.
- Stimulation in the numbers of eggs and nauplii per female was observed at low lindane exposures (3.2 and 10 μg l^{-1}) compared with the controls, and was considered to be a hormesis response.

Chapter 8

General Discussion

8 Discussion

The main aim of this thesis was to evaluate the freshwater meiofaunal copepod *Bryocamptus zschokkei* as a test species for assessing the developmental and reproductive effects of contaminants to lotic systems. *Bryocamptus zschokkei* was amenable to laboratory culture and a suite of toxicity testing procedures was developed for this copepod, including tests for assessing the effects of environmental parameters (water hardness or food quality) or contaminants on development to adult (Chapter 2), reproduction (Chapter 3), and the full life-cycle (Chapters 5-7). In addition, acute toxicity tests for multiple copepod life-stages have been described (Chapters 6-7). This final chapter aims to review critically the applicability of *B. zschokkei* as a test organism with respect to the key requirements of a test species (Section 1.3) and in relation to established testing protocols for assessing contaminant effects in freshwater systems.

8.1 Laboratory Culture

There are several obvious advantages to being able to culture a test organism in the laboratory, not least of which is the ability to obtain large numbers of animals of a defined age for conducting toxicity tests. A culture also avoids pre-exposure of animals to contaminants, disease or parasites. Water quality and nutrition are the key parameters in determining successful culture conditions for copepods (Vijverberg, 1989). Within this thesis, the effects of water hardness (Chapters 2 & 3) and detritus quality (Chapter 4) on the development and reproduction of *B. zschokkei* were established and used to define the optimal culture conditions for this copepod. *Bryocamptus zschokkei* was insensitive to changes in water hardness up to 150 mg l^{-1} (as $CaCO_3$) with no significant effects of hardness being observed on either development or reproduction (Chapters 2 & 3). The tolerance of *B. zschokkei* to water hardness in the laboratory reflected the range of calcium levels at which populations have been recorded in the field. The ability of *B. zschokkei* to

adapt to different calcium concentrations also means that this copepod can be considered a relevant test organism for water bodies with different hardness levels.

The four different types of detritus [Beech leaves (*Fagus sylvatica*) conditioned for 2 or 6 weeks, oak leaves (*Quercus robur*) conditioned for 2 weeks or stream derived fine particulate organic matter (FPOM)] supplied to *B. zschokkei* as a food source supported development to adult and reproduction of this copepod (Chapter 4). Laboratory conditioned beech leaves conditioned for 2 weeks maintained optimal overall development times to adult and offspring production of this copepod compared with the other detritus types. A low maintenance culture system was, therefore, established for *B. zschokkei* using reconstituted water of modified Elendt's M4 medium (hardness of 100 mg l^{-1} as $CaCO_3$), and a food source of beech leaves laboratory conditioned for 2 weeks prior to use. Survival in the cultures was high and was reflected in the toxicant exposure experiments where mortality did not exceed 20% in any of the controls over the 6-week duration of the tests (Chapters 5-7). These levels of mortality are in line with regulatory test guidelines for 10 day toxicity tests using other freshwater benthic invertebrates (ASTM, 1999a). The cultures of *B. zschokkei* also provided large numbers of ovigerous females all year round and they produced nauplii (<24 h old) for initiating life-cycle toxicity tests.

8.2 Small Size and Short Generation Time

This thesis describes the first full life-cycle bioassay for a freshwater copepod. The maintenance and toxicity testing regimes described for *B. zschokkei* require limited amounts of space. In fact, a life-cycle test including multiple treatments can be carried out in conventional incubators or within a meter square of bench space. The tests also require only small amounts of test chemical, for example, less than 200 ml of test solution per treatment at each renewal. This is important when a test compound is highly toxic (less material to

dispose of, expensive or is in short supply.

Bryocamptus zschokkei took a mean (± 1SD) of 17.9 (± 3.6) days to develop to adult at 20°C (Chapter 2). A development bioassay for this copepod can, therefore, be carried out in three weeks, while a full life-cycle test, which also includes the time taken for females to produce three broods, takes six weeks (Chapters 5-7). These toxicity tests are faster than previous tests described for *B. zschokkei*, where, a development to adult bioassay took 20-30 days and a three brood reproduction test took 6 weeks at 15°C (Burton, 1998). With the exception of cladocerans, the duration of the life-cycle test with *B. zschokkei* compares favourably with other freshwater crustaceans recommended as toxicity test species (deFur *et al*, 1999). Survival and growth toxicity tests using freshwater amphipods such as *Hyalella azteca* and *Gammarus pulex* have been considered a priority by the OECD Test Guidelines Program. Most amphipods, however, have a generation time of at least 5 weeks at 20°C, which means that a full life-cycle test for these species may be time consuming and impractical (OECD, 1998a; deFur *et al*, 1999). The generation time of freshwater isopods, such as *Asellus aquaticus* also used in the generation of water quality guidelines, takes several weeks at 12°C (Peeters *et al*, 2000). Standard methods for conducting life-cycle tests for three species of cladocerans (*Daphnia magna*, *D. pulex* and *Ceriodaphnia dubia*) take 3 weeks at 20°C, providing information on contaminant effects on both development and reproduction (OECD, 1998b; ASTM, 1999b). Such rapid bioassays are one of the main reasons why cladocerans are so widely used in toxicity bioassessment. Indeed, despite their planktonic mode of life and occurrence primarily in waters with low flow conditions, cladocerans are even recommended as sediment test organisms (Nebeker *et al*, 1984; Geisy & Hoke, 1989; ASTM, 1999a). Cladocerans are also parthenogenic and therefore toxicants are likely to have a different mode of action in these animals compared with sexualy

reproducing organisms. The differential responses of male and female animals cannot be assessed in a bioassay using Daphnids (Cooney & Gehrs, 1984). A bioassay using *B. zschokkei* would provide a relatively quick and more ecologically relevant alternative to cladocerans for determining the toxicity of contaminated freshwater sediments.

The life-cycle test described for *B. zschokkei* is similar in duration to combined development and reproduction tests using the benthic marine harpacticoids *Amphiascus tenuiremus* and *Microarthridion littorale,* which take 5-6 weeks at 20°C. Toxicity tests using these detritivores are already widely used in marine sediment bioassessment (Green *et al*, 1995; Green & Chandler, 1996; Kovatch *et al*, 1999). The life cycles of infaunal detritivores are, however, considerably longer than those of some epibenthic marine harpacticoids; for example, a full life-cycle test for *Tisbe battagliai* (incorporating reproductive data for three broods) takes just three weeks at 20°C (Hutchinson *et al*, 1999a, b). The different development time of these marine harpacticoids (and *B. zschokkei*) reflects their relative diets and different life-history strategies. *Tisbe battagliai* consumes a mainly algal diet which is considered to provide a higher quality food source than detritus, the major food available to *B. zschokkei or A. tenuiremis* within either the freshwater or marine benthos respectively (Hicks & Coull, 1983; O'Doherty, 1985). It is imperative that toxicity testing regimes include species from different functional feeding groups, habitat types and with different modes of life, as such animals are likely to respond differently to contaminant exposure.

The majority of bioassays using freshwater copepods incorporate short-term development tests considering toxicant effects on development to the first copepodid stage (N1-C1) (Cooney & Gehrs, 1984; Willis, 1999). A full life-cycle toxicity test has not been described

for any other freshwater copepod, other than *B. zschokkei*. Development assays at 20°C for two species of calanoid (*Boeckella delicata* and *Calamoecia lucasi*) and two species of cyclopoid copepod (*Mesocyclops leuckarti* and *Diaptomus clavipes*) took approximately 10 days, which would be the same duration as an equivalent test using *B. zschokkei* (Cooney & Gehrs, 1984; Willis, 1999). Low control survival has also been reported as a problem in toxicity tests using some freshwater copepods (Willis, 1999). Alternative test species representative of permanent meiofaunal communities would include cyclopoid copepods, which generally have relatively shorter embryonic development times and larger clutch sizes than stream harpacticoids (Robertson, 2000). The generation times of many cyclopoids (10-30 days at 20°C) are, however, within the same range as those recorded for *B. zschokkei* (ca. 20 days at 20°C) (see Chapter 2). There is also evidence to suggest that, in benthic communities, cyclopoid copepods may be less sensitive than harpacticoids to adverse environmental conditions. For example, cyclopoid copepods were relatively more tolerant to trace metal contamination than harpacticoids in a community-level survey of streams in Southwest England (Burton *et al*, 2001). Many cyclopoid species are also tolerant of acidic (Rundle & Hildrew, 1990) or eutrophic conditions (Särkkä, 1992). The ability of cyclopoid copepods to enter a resting stage in response to stress may increase their tolerance range to adverse environmental conditions (Särkkä, 1992). Other meiofaunal species such as ostracods or mites, which have been identified as being sensitive to copper contamination (Burton *et al*, 2001), may prove difficult to culture in the laboratory. Some nematode worms were also found to be sensitive to trace metal contamination but individual species can be difficult to identify (Burton, 1998).

8.3 Multiple Life-Stages

It is well established that larval life stages of copepods are generally more sensitive to contaminant exposure than adults (Verriopoulos & Moraïtou-Apostolopoulou, 1982;

Bechmann, 1994; Williams, 1997). This was also the case for *B. zschokkei* where naupliar and copepodid stages were more sensitive to both zinc and lindane exposure than adult copepods (Chapters 6 & 7). Techniques for obtaining larval copepods for initiating toxicity tests have been described (Chapters 2 & 6). The sensitivity of larval stages to contaminants makes them ideal for use in short-term toxicity tests. The development to adult bioassay described in this thesis (Chapter 2) could be abbreviated to produce a 10-day test (at 20°C) to consider contaminant effects on the time taken to develop to the first copepod stage (N1-C1). In view of the fact that *B. zschokkei* was tolerant of acute (96 h) but sensitive to 10-day lindane exposure, a naupliar development assay is likely to be a more relevant screen for toxicant effects than an acute toxicity test (≤96h) (Chapter 7). Abbreviated development assays have been described for other freshwater copepods (Cooney & Gehrs, 1984; Willis, 1999) and a test which incorporates copepodid development of the marine harpacticoid *Tisbe battagliai* is included as a standard method for toxicity assessment (ISO, 1998)

8.4 Ecological Relevance

The requirement for a test organism representative of the meiofauna was discussed in the Introduction (Chapter 1). *Bryocamptus zschokkei* was considered to be an ideal candidate species for developing as a test species because of its widespread distribution, presence in a wide range of habitats and apparent sensitivity to trace metal contamination in the field (Burton *et al*, 2001). Copepods are also amongst the most species diverse and abundant components of freshwater benthic communities (Robertson *et al*, 2000b). *Bryocamptus zschokkei* can, therefore, be considered to be a highly relevant test species for use in assessing the effects of contaminants on benthic freshwater communities in the United Kingdom. Particularly when alternative recommended test organisms include non-indigenous species such as *Hyalella azteca* or members of the zooplankton, such as, *Daphnia magna* and *Ceriodaphnia dubia* (ASTM, 1999a)

The ecological relevance of the life-cycle test for *B. zschokkei* was increased by the addition of a leaf disc as a food source for the copepod. Allocthonous inputs of leaf material drive the trophic dynamics of woodland stream systems (Giller & Malmqvist, 1998) and detritus and its associated microbial flora are considered the major food source available to *B. zschokkei* in the natural environment (O'Doherty, 1985; Chapter 4). The presence of a leaf disc in the test design, therefore, increases the relevance of the test by reflecting field conditions more closely, in effect creating a "microcosm". The addition of a leaf disc means that the toxicity test will not only highlight the direct toxic effects of a particular contaminant on *B. zschokkei* but may also indicate indirect effects of toxicant exposure. For example, the significant increase in the development time to adult of *B. zschokkei*, observed at low solvent (methanol) concentrations, was attributed to an indirect effect on the growth of the microbial community on the leaf disc (Chapter 5). The acute toxicity of zinc was reduced in the presence of a leaf disc (Chapter 6) and adsorption of lindane onto leaf material was concentration dependant (Chapter 7). The association of *B. zschokkei* with leaf material was also used as a behavioural endpoint for acute lindane exposure (Chapter 7). The presence of leaf material within the test system, therefore, appears both to increase the relevance of the test and to give an indication of the complex interactions between organic matter, the test species and contaminant exposure. It should be noted that to understand fully how a contaminant is behaving within the test system detailed chemical analysis and measurement of the concentration of test substance in the dilution water and if possible in the leaf material is essential.

8.5 Relative Sensitivity

Sensitivity to a range of contaminants is a particular concern for regulatory bodies needing to define a threshold level of response for contaminants, and is considered a key

requirement of a toxicity test organism (ASTM, 1999a). The sensitivity of *B. zschokkei* to exposure to each of three chemicals, the moulting hormone 20-hydroxyecdysone, the trace metal zinc and the pesticide lindane, on the life-cycle has been discussed (Chapters 5-7). *Bryocamptus zschokkei* showed variable tolerance depending on the toxicant, being completely insensitive to 20-hydroxyecdysone (Chapter 5) but exhibiting sensitivity comparable with other freshwater crustaceans to zinc and lindane (Chapters 6 & 7). The duration of exposure was also important in determining the relative sensitivity of this copepod. For example, *B. zschokkei* is highly tolerant of acute (96 h) lindane exposure but relatively sensitive to chronic (6 week) lindane exposure compared with other freshwater crustaceans (Chapter 7). These results support a general view that a 'most sensitive' species does not exist but that species respond differently depending on the contaminant and its mode of action (Maltby & Calow, 1989; Cairns & Pratt, 1989). In view of such inter-species differences in response to toxicant exposure, an expansion of current testing regimes to include novel species such as *B. zschokkei* should be encouraged. Furthermore, species that may be more tolerant to certain toxicants might have application for ranking contaminated sites, where a more sensitive species might not be present (Traunspurger & Drews, 1996).

As well as gaining information on the threshold of response and sensitivity of test organisms to different contaminants, toxicity tests can also be used gain information on the mechanism of effect of a particular toxicant. Daily observations of *B. zschokkei*, while labour intensive, meant that development times of individual moult stages could be determined accurately. Using information on individual stage duration, *B. zschokkei* was found to conform to a development model of "equiproportional development" where each moult stage is a known proportion of the total development time, irrespective of processes that affect metabolism

such as temperature and food quality (Chapter 2). It was proposed that this development model might be useful in determining whether a toxicant is exerting its effect directly on the moulting process or via an indirect effect of reducing the energy available for growth. The model of "equiproportional development" was tested for lindane, which was the only toxicant in this thesis found to increase the development time to adult of *B. zschokkei* (Chapter 7). When exposed to lindane, *B. zschokkei* still conformed to "equiproportional development", suggesting that the increase in development time to adult was a result of shifts in metabolic processes. The model of "equiproportional development" may, therefore, be useful as a tool for evaluating the mode of action of a toxicant on the moulting process. Indeed, such models may have particular application when considering the effects of endocrine disrupting chemicals (EDCs). As moulting in crustaceans is under hormonal control, the effects of EDCs might be expected to be manifest on the moulting process directly, which should be highlighted using the model of "equiproportional development". Further studies are required both using *B. zschokkei* and other copepods before the utility of this model as a tool for highlighting toxicant effects on the moulting process can be fully evaluated.

Daily observations of egg and offspring production can also be made for *B. zschokkei* as the female carries her egg sac externally. These observations enable general conclusions on how toxicants exert their effects on the reproductive processes of this copepod. For example, mortality of nauplii during hatching suggested that the significant decrease in offspring production of *B. zschokkei* exposed to zinc was due to a direct toxic effect on the larvae (Chapter 6). Significant increases in egg and offspring production in *B. zschokkei* exposed to low doses of lindane also highlighted the potential of a hormesis response (Chapter 7). In addition, increased abortion frequency was observed in copepods exposed to zinc and

lindane (Chapters 6 & 7). Abortions have been reported as a stress response in the marine harpacticoids *Scottolana canadensis* and *Tisbe holothuriae* (Harris, 1977; Miliou, 1992). An increase in abortion frequency in the amphipod *Gammarus pulex* at elevated zinc exposures was attributed to females shifting their resources to their own maintenance rather than to the provision of eggs (Maltby & Naylor, 1990). While further studies are required to determine if increased abortions are a general response of *B. zschokkei* to toxicant exposure, abortion frequency may have application as a biomarker of stress for this copepod (Chapters 6 & 7).

8.6 Standardisation and Reproducibility

The main objective of a toxicity test is to predict, with confidence, the concentration at which an adverse biological effect can be measured. Inter-individual variability in the responses of different endpoints to a contaminant can lead to problems with the reproducibility of a test and may also result in an effect not being detected because of too much background noise (Bradley, 1993; Forbes & Forbes, 1994). The life-cycle test using *B. zschokkei* showed variation between individuals to different extents, depending on the endpoint being measured (Table 8.1). The time taken for naupliar (Dn) and copepodid development (Dc), and development to adult (N1-A) for control *B. zschokkei* at 20°C is highly conserved between individuals as indicated by low CVs (Table 8.1). Low variability means that toxicant effects on development were detectable even when there was only a small increase in development time; indeed significantly prolonged development times were observed as a result of exposure to methanol (carrier solvent) (Chapter 5) and lindane (Chapter 7). There is also a high degree of reproducibility in copepod development times between different experiments. For example, there was no significant difference in the time taken to moult to adult (N1-A) in control *B. zschokkei* from the three life-cycle tests described in Chapters 5, 6 and 7 (Kruskal-Wallace, $p<0.05$) (Fig. 8.1).

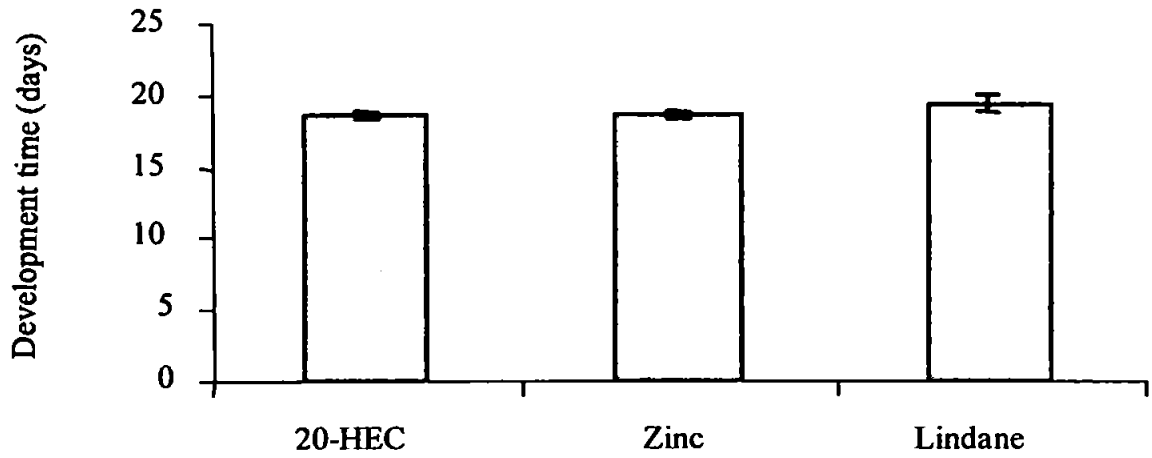

Figure 8.1: The time taken (mean ± 1SE) to develop to adult (N1-A) in *Bryocamptus zschokkei* control animals from three separate life-cycle toxicity tests.
20-HEC (Chapter 5), zinc (Chapter 6) and lindane (Chapter 7). There was no significant difference between treatments (Kruskal-Wallis, $p<0.05$); n=32-35 for each treatment.

Reproductive endpoints showed more difference in overall variation from those measured during development (Table 8.1). For example, traits such as time to first egg sac and the embryonic development time show much less variation than the numbers of eggs or nauplii per female (Table 8.1). Such differences in the variability of different life-history traits have been observed in other species. For example, asexually and sexually reproducing populations of the brine shrimp *Artemia* were each found to exhibit considerable differences in the mean CV of different traits (% CV from 7-151) (Browne *et al*, 1984; Forbes & Depledge, 1996). The intrinsic inter-individual variability in offspring production observed for *B. zschokkei* appears to be a phenomenon reflected in many laboratory reared populations of copepods (Carlotti & Nival, 1991; Bechmann, 1994; Twombly *et al*, 1998).

Table 8.1: The mean (± 1SD) percent coefficient of variation (CV) for different life-history traits measured for *Bryocamptus zschokkei* in life-cycle toxicity tests.
The means are based on control animals from three life-cycle tests; 20-HE (Chapter 5), zinc (Chapter 6) and lindane (Chapter 7). n is the number of animals in each experiment used to calculate the CV.

Trait measured	Mean (± 1SD) CV (%)	n
Naupliar development (Dn) (days)	12.15 (± 4.17)	34-35
Copepodid development (Dc) (days)	17.23 (± 10.80)	32-35
Development to adult (N1-A) (days)	11.37 (± 5.09)	32-35
Time to first brood (days)	13.36 (± 1.36)	14-18
Embryonic development time (days)	18.44 (± 4.17)	39-42
Eggs per female	56.32 (± 3.69)	14-18
Nauplii per female	60.73 (± 3.00)	14-18

The reasons for such variability in reproductive parameters and its implications for toxicity testing are discussed in Chapter 3. Despite high variability in the numbers of eggs and nauplii produced per female, these parameters were very sensitive endpoints for contaminant exposure. For example, the lowest observed effect concentrations (LOEC) for both zinc (Chapter 6) and lindane (Chapter 7) were based on significant decreases in the numbers of eggs per female. These endpoints were also used to highlight a significant stimulation in egg and offspring production at low doses of lindane (Chapter 7). As well as being a sensitive indicator of toxicant exposure, the numbers of eggs and nauplii per female showed high reproducibility between intralaboratory tests. There was no significant difference in the numbers of eggs or nauplii per female *B. zschokkei* in control animals from the three life-cycle tests described in this thesis (Chapters 5-7) (One-way ANOVA, $p<0.05$) (Fig. 8.2). These results suggest that the endpoints measured in the life-cycle test for *B. zschokkei* are sensitive and reproducible and that this test would, therefore, have application

for discriminating between a range of toxicants. Further validation of this life-cycle test would include intercalibration exercises to determine its reproducibility when carried out by different operators or with different copepod populations.

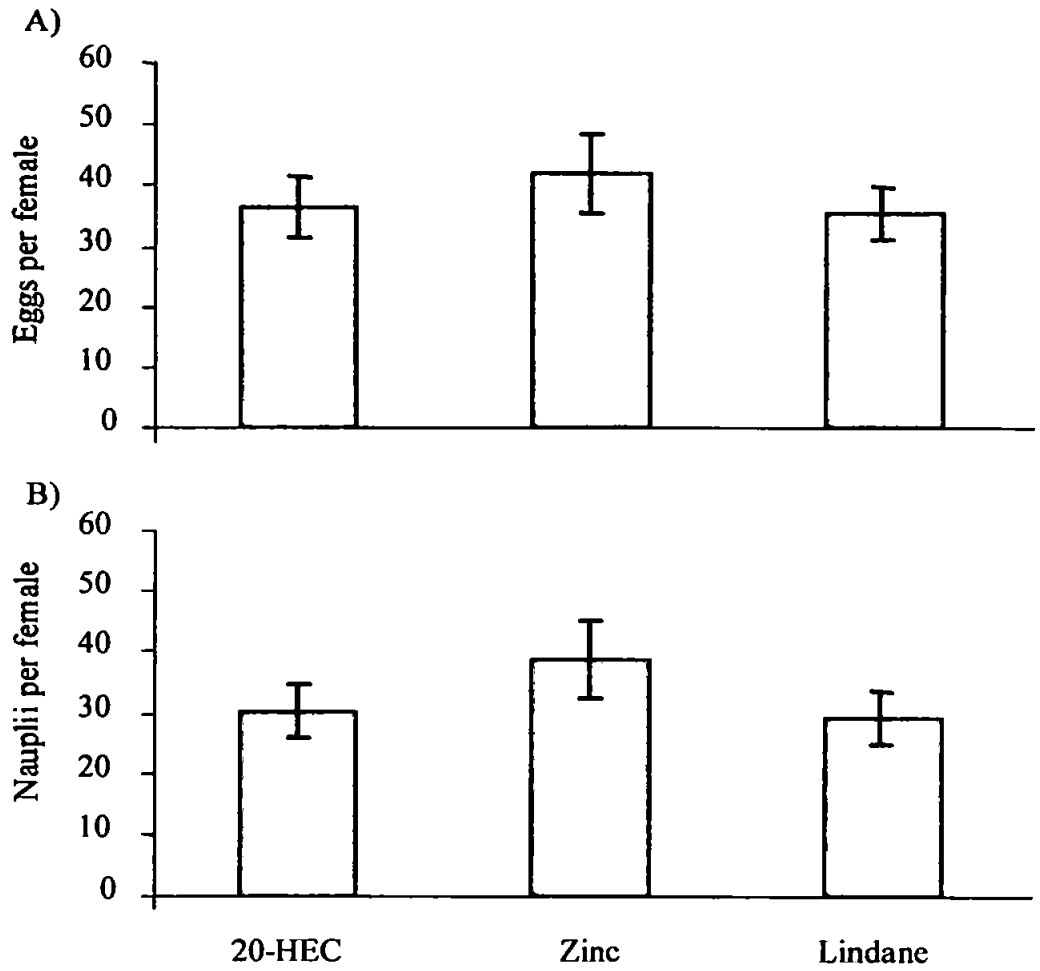

Figure 8.2: The mean ± 1SE A) eggs and B) nauplii per female in *Bryocamptus zschokkei* control animals from three separate life-cycle toxicity tests.
20-HEC (Chapter 5), zinc (Chapter 6) and lindane (Chapter 7). There was no significant difference between treatments (One-way ANOVA, $p<0.05$); n=14-17 for each treatment.

8.7 Conclusions and Recommendations

The freshwater harpacticoid *B. zschokkei* possesses many of the attributes (small size, fast development times and multiple life stages) that make its marine counterparts such ideal toxicity test organisms. In addition to these traits, *B. zschokkei* has proved amenable to low-maintenance laboratory culture. Relatively quick, standardised and highly reproducible

toxicity testing protocols, including a full life-cycle test, have been established for this copepod and used to demonstrate the sensitivity of *B. zschokkei* to chronic exposure to two common contaminants (zinc and lindane) compared with other freshwater crustaceans. Most importantly, this copepod is a representative test species from the meiofauna and, due to its widespread distribution, is relevant for assessing contaminant effects on the benthos of a range of freshwater habitats. The evidence provided in this thesis suggests that bioassays using *B. zschokkei* should be considered by risk assessors to supplement current testing procedures increasing the choice and relevance of regulatory procedures by including a representative meiofaunal test species. A bioassay using *B. zschokkei* might also be developed further to provide an ecologically relevant alternative to cladocerans in freshwater sediment toxicity evaluation.

Life-cycle tests have been recommended for assessing the effects of chemicals with the ability to disrupt hormone function in invertebrates (deFur *et al*, 1999; Hutchinson *et al*, 1999a, b). The rigorous design of the life-cycle test using *B. zschokkei* described in this thesis enables both the application of a development model of "equiproportional development" and detailed evaluation of how toxicants exert their effect on reproductive parameters. These observations increase the potential of this bioassay to emphasise the mechanism of toxicity of contaminants, and highlight perturbation of development or reproduction, which might be indicative of endocrine disruption. Further work is required, (e.g. screening chemicals suspected of causing perturbation of endocrine function) to validate fully the model of "equiproportional development" and to evaluate the potential of this test as a screen for endocrine disrupting chemicals.

8.8 Summary

- *Bryocamptus zschokkei* can be maintained easily under laboratory conditions to provide sufficient numbers of animals of known background to conduct large scale life-cycle toxicity tests
- The first full life-cycle toxicity test for a freshwater copepod has been described using *B. zschokkei*. The duration of this test (6 weeks at 20°C) is similar to that of equivalent toxicity tests using the benthic harpacticoids *Amphiascus tenuiremus* and *Microarthridion littorale,* which are widely used in marine sediment bioassessment. Abbreviated toxicity tests using *B. zschokkei* can be adapted easily from the methods described in this thesis.
- A major advantage of *B. zschokkei* as a test organism is that it can be considered representative of several different freshwater habitats over a wide geographical range. Providing a leaf disc as a food source for *B. zschokkei* enhances the ecological relevance of the test system.
- The relative sensitivity of *B. zschokkei* to different toxicants depends on the contaminant and on the duration of exposure. Characterisation of the responses of this copepod to other toxicants will allow more generalised conclusions to be made on its overall sensitivity to pollution exposure.
- A development model proposing "equiproportional development" has been validated for *B. zschokkei*. Using this model it may be possible to distinguish between toxicants that exert an effect directly on the moulting process and effects on development that occur as a result of shifts in metabolic processes. Such a model has application when considering the effects of potential endocrine disrupting chemicals, which might be expected to cause perturbation of the hormonal processes controlling moulting.
- As female *B. zschokkei* carry their egg sacs externally, detailed observations of toxicant

effects on the brood itself and hatching of larval stages can be evaluated. The numbers of eggs and nauplii per female were found to be sensitive endpoints for zinc and lindane exposure. An increase in abortion frequency was also observed in response to these contaminants. Abortion frequency appears to be a useful biomarker of stress for this copepod.

- The variability of different life-history traits used as endpoints for toxicity tests are relatively low (CV, 12-61%). Reproductive parameters (numbers of eggs and nauplii per female) show a high degree of variability compared with measured developmental parameters but were the most sensitive endpoints for both zinc and lindane exposure.
- The life-cycle test for *B. zschokkei* described in this thesis is standardised and highly reproducible. No significant differences were observed in either development or reproductive parameters between control animals from three different bioassays.
- The use of a life-cycle test using *B. zschokkei* is recommended for future development both to supplement present testing regimes and as an ecologically relevant alternative to current sediment test organisms.

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