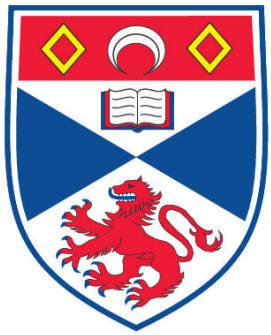
CHANGES IN GENE EXPRESSION, LIPID CLASS AND FATTY ACID COMPOSITION ASSOCIATED WITH DIAPAUSE IN THE MARINE COPEPOD *CALANUS FINMARCHICUS* FROM LOCH ETIVE, SCOTLAND

Katie Alice Jennie Hill

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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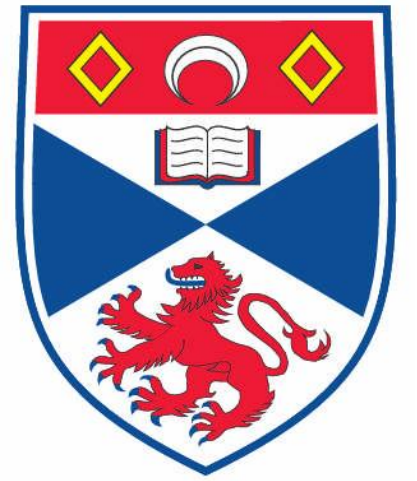

A thesis submitted for the degree of Doctor of Philosophy

University of St Andrews

April 2009

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ABSTRACT

Zooplankton are the major primary consumers in pelagic ecosystems, providing the principal pathway for energy transfer from primary production to higher trophic levels. The marine copepod *Calanus finmarchicus* is an important component of the pelagic food web in the North Atlantic and peripheral ecosystems, and forms an essential dietary component of a number of commercially important fish. As part of its life cycle, many *C. finmarchicus* overwinter in a diapause phase (a dormant overwintering phase where development is suppressed in adaptation to the seasonal food supply) at depths of 500 to 2000 m, but little is known about the triggers that initiate and terminate diapause, or the internal processes associated with these triggers. Understanding these processes is important, given that subtle changes in the environmental conditions which may affect diapause could have consequences for the entire *Calanus*-based ecosystem. In this study I took advantage of relatively easy access to a deep (> 100 m), isolated population of *C. finmarchicus* in Loch Etive (a sea loch on the west coast of Scotland) to sample *Calanus finmarchicus* monthly between April 2006 and June 2007 and measure lipid dynamics and gene expression associated with diapause. Chapter 1 of this thesis provides a general introduction to diapause and *Calanus finmarchicus*, Chapter 2 reports on the population of *C. finmarchicus* in Loch Etive, Chapter 3 reports changes in the lipid class and fatty acid composition of individual copepods, Chapter 4 reports on differential gene expression between diapausing and active *C. finmarchicus* and Chapter 5 provides a general discussion and puts this research into context. This study provides some initial insight into possible gene expression patterns, but further work is needed to attribute specific gene expression patterns with initiation and termination of diapause.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: General Introduction

This thesis considers aspects of diapause, an overwintering strategy, in the marine copepod *Calanus finmarchicus*. Copepods may be the most numerous metazoans on Earth (Schminke, 2007), and the pelagic marine ecosystems which they inhabit are the most voluminous on Earth, encompassing the entirety of the water column from the surface to near bottom and spanning from the tropics to the poles. Zooplankton play a key role as the major grazers in these ecosystems, providing the principal pathway for energy transfer from primary production to consumers at higher trophic levels. Changes in zooplankton communities, caused by climate change or potentially by the harvest of copepods for human consumption can have wide ranging impacts (Wickstead, 1967; Richardson, 2008). Zooplankton play an important role in shaping the extent and pace of climate change as they are sensitive to subtle environmental changes as well as being more directly involved, as the oceans ability to draw CO_2 down from the atmosphere to the seabed relies partially on the biological pump (Hays et al., 2003; Richardson, 2008). The calanoid copepods have been the most successful of all copepods in colonising all parts of the pelagic environment in both marine and freshwater (Mauchline, 1998), and many are key species in the ecosystems which they dominate, such as *Calanus finmarchicus.*

1.1 *Calanus finmarchicus*

The copepod *Calanus finmarchicus* (Gunnerus) is a vital component of the pelagic food web in the North Atlantic and peripheral ecosystems. *Calanus spp.* form up to 90% of the mesozooplankton biomass in these areas (Marshall and Orr, 1957; Conover, 1988; Longhurst and Williams, 1992, Mauchline, 1998; Bonnet et al., 2005) and notably form an essential dietary component of the larval, juvenile and adult

stages of a number of commercially important fish species such as herring, mackerel and cod (Conover et al., 1995; Runge and de LaFontaine, 1996; Kaartvedt, 2000). Whilst, for logistic reasons, much previous work focussed on coastal systems, in the past decade or so several international research programs such as the EU programs ICOS (e.g. Heath, 1999; Heath and Jónasdóttir, 1999) and TASC initiative (e.g. Tande and Miller, 2000), US GLOBEC (e.g. Wiebe et al., 2001) and the NERC Marine Productivity thematic program in the UK (e.g. Irigoien et al., 2003; Heath et al., 2008) have studied *C. finmarchcius* in the open ocean, where its lifecycle is played out in a dynamic and highly seasonal three dimensional environment driven by food availability, temperature and photoperiod regimes (Heath et al., 2000b; Speirs et al. 2006). As a consequence of these and earlier studies, much is now known about its physiology, natural history and spatial distribution. The life cycle of *C. finmarchicus* involves metamorphosis through six naupliar stages and five copepodite stages before moulting to the adult stage (Fig 1.1). Prior to moulting to the adult stage, the life cycle of *C. finmarchicus* often involves a diapause phase, a dormant overwintering phase where development is suppressed in adaptation to the seasonal food supply (Hirche, 1996). During copepodite stages CIV and CV in late summer and autumn most individuals initiate diapause (see section 1.2 below), sink out of the surface waters and overwinter in deep water. In late winter through to spring of the following year animals terminate dormancy and migrate to the surface to feed and reproduce (Hirche, 1996). The timing and duration of the overwintering period varies among locations across the range of *C. finmarchicus* (e.g. Planque et al., 1997; Hind et al., 2000; Heath et al., 2000a, 2008).

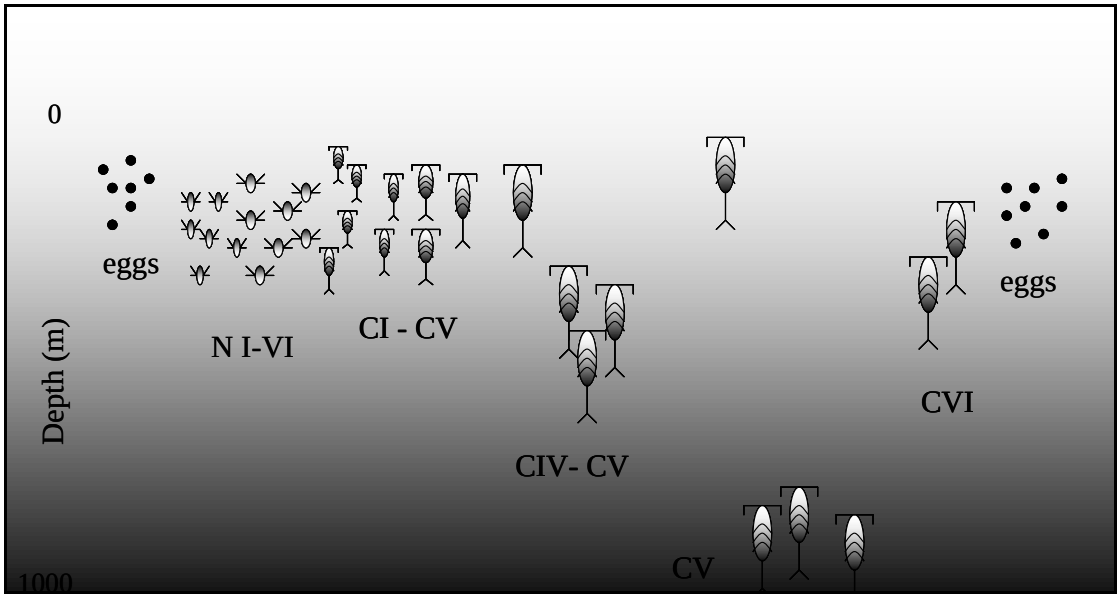

Fig 1.1 Life cycle of *Calanus finmarchicus* showing naupli stages N1-NVI, copepodite stages CI-CV and adult stage CVI.

The distribution of *C. finmarchicus* in the Northeast Atlantic has shifted northwards in the last 50 years due to climate change and the effects of the North Atlantic Oscillation (Fromentin and Planque, 1996; Planque and Batten, 2000; Beaugrand et al., 2002; Beaugrand, 2003; Bonnet et al., 2005) which have also caused the congeneric species *Calanus helgolandicus* to shift northwards in its distribution (Bonnet et al., 2005). *C. helgolandicus* have been reported to have been advected as far as the Farøe Islands (Lindeque et al., 2004), and are co-occurring with *C. finmarchicus* in the waters of the Northeast Atlantic and the North Sea (Williams and Conway, 1980; Planque and Fromentin, 1996; Lindeque et al., 2004). In the areas where they exist together, the two species have different seasonal timing of maximum abundances (Beaugrand, 2003), and replacement of *C. finmarchicus* by *C. helgolandicus* in some areas has had implications on the feeding, growth and subsequent recruitment of predators of *Calanus finmarchicus* (Beaugrand et al., 2003). Successful fish recruitment is highly dependant on synchronisation with pulsed planktic availability (Cushing, 1990; Beaugrand et al., 2003) and *C. finmarchicus*

abundance peaks in the spring, corresponding with the spawning of the Atlantic cod, whereas *C. helgolandicus* abundance peaks in the autumn. Since the late 1980s *C. finmarchicus* has been virtually absent from the North Sea, and there has been reduced zooplankton abundance in the spring and summer that has affected cod recruitment in this area. Consequently *C. finmarchicus* has been the subject of many modelling studies intent on predicting the response of *C. finmarchicus* to further climatic change, such as the warming and freshening of the North Atlantic (Levitus et al., 2001) and the consequences of this response to the *Calanus*-centred ecosystem.

Despite the large number of studies on the biology and ecology of *C. finmarchicus*, many gaps in our knowledge remain, particularly associated with the triggers causing the induction and termination of diapause. The cellular mechanisms associated with the induction and termination of diapause are largely unknown due to the difficulty of replicating diapause in the laboratory, and the fact that diapausing *C. finmarchicus* appears to terminate diapause when collected (Campbell et al., 2004). An understanding of the cellular processes associated with the induction and termination of the diapause phase would provide a more detailed understanding of the environmental physiology of *C. finmarchicus*, and may provide some insight into the physical and biological factors controlling diapause, which in turn may be used to provide modelling studies with more accurate predictions of when *C. finmarchicus* in a particular location may be entering diapause and the duration of the dormancy.

1.2 Diapause

Diapause and quiescence are both forms of dormancy (Dahms, 1995), and the two terms are often used interchangeably within the literature. There are, however, notable

physiological differences between each state and the terms are not synonymous. Diapause is generally considered to be an endocrine-mediated response to specific environmental cues, resulting in arrested development and reduced metabolic activity during a specific stage of metamorphosis (Dahms, 1995; Hirche 1996). Quiescence, on the other hand, is thought to be a spontaneous reaction to a local environmental driver resulting in a state that is reversible, such as impeded growth (Dahms, 1995; Hirche, 1996). For example, encysted embryos of the brine shrimp *Artemia franciscana* respond to anoxic periods in hypersaline lakes by entering quiescence and can withstand anoxia at room temperature for four years (Hand and Podrabsky, 2000). This is a spontaneous reaction to the lack of oxygen in their environment and the dormancy would not otherwise occur. However, the corn borer *Sesamia nonagroides* produce 2-4 generations of young before a reduction in photoperiod causes exposed larvae to enter diapause at a specific developmental stage, leading to reduced metabolism until an increase in photoperiod causes them to terminate diapause and continue to develop (Eizaguirre et al., 2005).

1.3 Diapause in *Calanus finmarchicus*

The overwintering state of calanoid copepods is poorly described, but *Calanus finmarchicus* is thought to undergo true diapause during overwintering. This is characterised by arrested development at a specific developmental stage - CV, seasonal migration to below 100 m (Speirs et al., 2005), reduced RNA:DNA ratio, sluggish behaviour, reduced metabolism (15-30% of active level), and cessation of feeding (Hirche, 1983, 1989, 1996; Miller et al., 1991; Wagner et al., 1998). Many aspects of insect diapause are similar to overwintering of *Calanus* sp., and the endocrine control of diapause in insects is well documented: various hormones have

been implicated, depending on species and the phase in their life-cycle during which they enter diapause (e.g. Highnam and Hill, 1977; Lee and Denlinger, 1997; Singtripop et al., 2000; Munyiri and Ishikawa, 2004; Zhang et al., 2004). While it has not been established that diapause in *Calanus* spp. is controlled by the endocrine system, Carlisle and Pitman (1961) noticed the presence of a large granular secretion of neurosecretory cells in active individuals of *C. finmarchicus* that appeared to be secreted prior to diapause.

It is generally accepted that *Calanus finmarchicus* overwinter in a diapause phase as an adaptation to life at high latitudes where there is a seasonal food supply (Marshall and Orr, 1955; Hirche, 1996), but there is controversy surrounding other possible ecological and physiological advantages to entering diapause. As the copepods arrest their development and reduce their metabolic rate, it is assumed that they cease to feed. Hirche (1983) measured considerably lower respiration rates from individuals of *C. finmarchicus* in diapause compared to active individuals, although Grigg and Bardwell (1982) observed only very small metabolic variations between diapausing and active individuals and suggested instead that diapause was merely an adaptation to synchronise reproduction with phytoplankton productivity, with no suppressed metabolism. In order for the animals that Grigg and Bardwell (1982) monitored to survive months without feeding, they must have taken on sufficient stores prior to entering diapause to successfully overwinter. Indeed *C. finmarchicus* does accumulate lipids, mainly in the CIII, CIV and CV stages of development, although the largest lipid accumulation occurs in stage CV (Lee et al., 2006), and by the time of descent into deep waters the CV copepodites have usually built up a large lipid reserves in a prominent oil sac (Fig 1.2) (Irigoien, 2004).

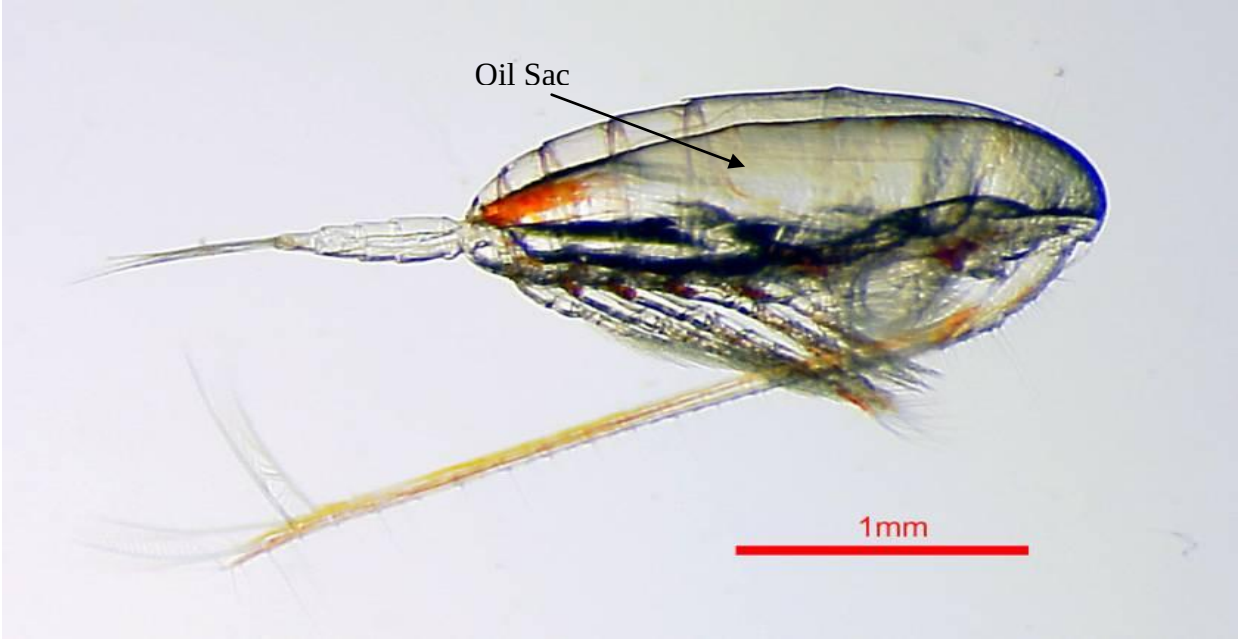

Fig 1.2 Calanus *finmarchicus* stage CV with prominent oil sac[1]

Lipids are stored mainly as high-calorie wax esters (see Lee et al., 2006 for a review). The use of the lipid store for sustaining metabolic processes during diapause is debated: decreases in the lipid store during diapause have been reported to be as low as 5% and as high as 70 % (Hirche, 1983; Hopkins et al., 1984; Jónasdóttir, 1999; Heath et al., 2008). Lipid stores could be preserved during diapause to fuel early egg production in the spring. Ascent of females has been observed prior to the spring bloom, and some adult females may still have enough left of the lipid stores after overwintering to use lipids for egg production before the advent of the spring bloom (Niehoff et al., 1999; Richardson et al., 1999). Eggs hatching prior to the spring bloom are thought to have an advantage to those that hatch during or post bloom (Varpe et al., 2007), as the peak food demands of the offspring coincide with the high food availability during bloom conditions, enabling rapid growth and development in a food limited ecosystem as per the Cushing match-mismatch hypothesis (Cushing,

[1]From: http://www.sintef.no/Projectweb/Calanus---home/ [accessed 30/03/09].

1990). It has also been suggested that diapausing copepods may use protein as an energy source during overwintering in order to preserve lipid stores for reproduction (Hirche, 1996; Jónasdóttir, 1999). However Evanson et al. (2000) observed a noticeable decline in the lipid stores during overwintering of *Neocalanus plumchrus* and hypothesised that protein, not lipid stores, may be used to fuel egg release.

Another role for lipids in the life cycle of *Calanus finmarchicus* that has been debated is buoyancy regulation. Visser and Jónasdóttir (1999) speculated that the stored wax esters should become denser more rapidly with increasing depth and decreasing temperature than seawater. Thus, depending on its relative composition, a copepod that is positively buoyant in warmer surface waters may become neutrally buoyant in deeper cold waters. Therefore the lipid content will determine the depth at which that animal will settle during diapause. There is, however, some controversy surrounding this 'buoyancy determines depth' hypothesis. Campbell and Dower (2003) suggest that the composition of lipids would have to be very finely regulated in order for the organism to achieve neutral buoyancy because the high compressibility of lipids makes any depth position of neutral buoyancy unstable, and the buoyancy force is highly sensitive to changes in chemical composition of the organism. Lipids are probably used by *C. finmarchicus* as an energy reserve during times of low food supply, and are likely to play a role in buoyancy regulation, although the exact mechanisms are yet to be clarified.

The physical conditions such as temperature, salinity and dissolved oxygen at the overwintering depth will have ecological implications, affecting mortality and reproduction of the population. The overwintering depth varies substantially between

locations, mostly ascribed to physical factors such as light, currents and temperature (Miller et al., 1991; Hirche, 1996; Irigoien, 2004), but ecological factors such as predator field are also likely to have a role in shaping vertical distribution (Kaartvedt, 2000). Animals must also overwinter below the convective mixed layer to avoid being returned to the surface prematurely during diapause (Irigoien, 2004). The cold temperature at depth of the overwintering habitat is also likely to be important, as internal energy resources essential for development and maturation of the gonads should last longer in cold water (Kaartvedt, 1996). *C. finmarchicus* appears to overwinter at a range of temperatures, typically from 4 to 12 °C (Durbin et al., 1995; Gislason and Astthorsson, 2000; Dale et al., 2001), however Hirche (1991) observed that the temperature ranges at which *C. finmarchicus* overwinter in the Greenland Sea encompassed –1 to +3 ^{o}C. He suggested that temperature preference may form the basis of depth selection for *C. finmarchicus*, with depth distribution decreasing with latitude. However, maximum abundance of *C. finmarchicus* is at sea surface temperatures of 6 to 10^{o}C (Helaouët and Beaugrand, 2007) and populations overwintering in shelf basins and in fjords may overwinter in temperatures of up to 11^{o}C (Sameoto and Herman, 1990). The depth in these fjords and basins is often constrained and the high temperatures and shallow waters may not be the optimum choice for overwintering. The fact that *C. finmarchicus* can overwinter in these locations suggests considerable plasticity of *C. finmarchicus* in coping with local fluctuating physical factors, implying that the physical conditions may not be the most important factors for successful overwintering.

Diapause stages as a predator avoidance mechanism have been documented among several copepods in freshwater systems (Hairston and Bohonak, 1998). *Calanus*

finmarchicus generally overwinters at depths of 500-2000 m in the open ocean (Hirche, 1996) and these dark habitats are thought to provide shelter from visual predators. By dispersing vertically in this way and reducing mobility it is also thought that *C. finmarchicus* reduces encounters with non-visual predators such as jellyfish (Kaartvedt, 1996). In the Norwegian Sea, *C. finmarchicus* are the favoured prey of many fish, and enter diapause relatively early compared to other populations. In this region, predation risk at diapause depth increases with time, because of the arrival of planktivorous fish that migrate to the region to spawn (Kaartvedt, 2000). The relatively early descent to diapause in the Norwegian Sea (from June onwards; Hirche, 1996) is quite likely to be a direct result of the increased predation risk, because descent occurs at a time when plenty of food is still available in the surface waters. Kaartvedt (1996) also suggests that the unusual distribution patterns seen in some medium deep fjords where overwintering *C. finmarchicus* seem to congregate between 200-300 m, and not at the maximum depth, may be controlled by predation. In these fjords where mesopelagic fish are permanently present, overwintering *C. finmarchicus* are likely to become aggregated at intermediate depths - below the predators in the surface waters and above those planktivorous fish that may feed at the bottom.

1.4 Possible cues for diapause induction and termination

The specific environmental cues that result in induction and termination of diapause have not yet been identified for *C. finmarchicus*. Several factors are hypothesised to be responsible for the onset of diapause. Photoperiod is a cue for induction and termination of diapause in many species of insect (Tauber and Tauber, 1981). Commonly, insects that overwinter in diapause have a critical photoperiod threshold

below which all individuals in a population enter diapause and above which diapause is terminated (Xue et al., 2002). Photoperiod (coupled with temperature) is also an important cue in the switch of production from subitaneous eggs (eggs that hatch immediately) to diapause eggs by cyclopoid copepods in freshwater systems (Hairston and Kearns, 1995), and for cyclopoid copepods with a late-copepodite diapause phase (Watson and Smallman, 1971). Aspects of diapause in copepods have been shown to be very similar to insect diapause, and seasonal changes in photoperiod are often invoked as possible cues to initiate and terminate diapause in marine copepods (e.g. Grigg and Bardwell, 1982). Although photoperiod has been used as a triggering signal in modelling studies for *Calanus* sp. dormancy strategy (e.g. Fiksen, 2000), field observations and simulation experiments examining the onset of dormancy do not support the hypothesis that dormancy is triggered by photoperiod alone (Hind et al., 2000; Johnson et al., 2008), as individuals appear to enter dormancy over a period of a month or more (Johnson et al., 2008). Photoperiod as a cue for termination of dormancy is more probable. Traditionally photoperiod has been thought unlikely to be a cue for termination of overwintering in *C. finmarchicus* because the light signal at the depths where *C. finmarchicus* over-winter (~ 500-2000 m) is typically very small (Campbell et al., 2004). However Berge et al. (2008) recorded diel vertical migration (DVM) of zooplankton during the polar night when irradiance values could not be detected by standard irradiance meters, but did not attribute the DVM response in the polar night to internal biological clock mechanisms as DVM did not occur on some nights, suggesting that zooplankton may be very sensitive to very small changes in light (Berge et al., 2008). Models attempting to link photoperiod with termination of diapause have contradictory evidence. Hind et al. (2000) propose that photoperiod cannot explain the timing of emergence from diapause observed of geographically

distinct populations of *C. finmarchicus*. However Speirs et al. (2005, 2006) suggested that the observed synchrony in the spring emergence of overwinterers in the Norwegian Sea cannot be explained by internal mechanisms such as a biological clock due to mixing of individuals with different life strategies via advection during diapause, but that a critical photoperiod would allow synchronous emergence from diapause.

Temperature may be an important cue in aquatic ecosystems, where animals are buffered from short-term fluctuations in temperature change by the thermal inertia of water (Hairston and Kearns, 1995). However, there is little variation in temperature throughout the year at the depths at which *C. finmarchicus* overwinters in the open ocean (Campbell et al., 2004); therefore temperature is unlikely to be a cue to terminate dormancy in *C. finmarchicus*. If temperature is involved in induction of diapause, then it is likely to be part of a combination of seasonal cues such as temperature and photoperiod, as observed in freshwater copepods (Watson and Smallman, 1971) and resting egg dormancy (Johnson, 1979; Hairsten and Kearns, 1995).

Copepods have not yet been induced to exhibit diapause under laboratory conditions. Campbell et al. (2004) suggested that pressure could be the missing stimulus. During the process of being collected, the copepods received enough stimulation to break diapause and continue their development - Campbell et al. (2004) proposed a reduction in pressure (as animals are brought to the surface in nets) could be the trigger to break dormancy. Very few studies have touched on the effect of pressure on the physiology of overwintering copepods, however Rice (1962) observed small

changes in the behaviour of *Calanus finmarchicus* in responses to small changes in pressure such as are associated with diel vertical migration. A 'biological clock' or an internal timer has also been proposed as a cue for induction and especially termination of diapause (Miller et al., 1991; Williams-Howze and Coull, 1992). Physiological changes such as the developing gonad have been proposed to act as an internal timer initiating termination - as soon as the developing female gonad has reached their final maturation state in CV, the animal is stimulated to moult (Diel and Tande, 1992). Such mechanisms are very difficult to test for when diapause cannot be initiated in laboratory conditions.

The role of lipids as energy stores and as possible buoyancy regulators has been previously discussed; however the potential additional role of lipid accumulation by *C. finmarchicus* as part of the mechanism of initiation and termination of diapause has recently been considered (Rey-Rassat et al., 2002; Irigoien, 2004; Hassett, 2006; Johnson et al., 2008). This 'lipid accumulation window hypothesis' (Johnson et al., 2008) is based on individuals only being able to enter diapause if they have accumulated sufficient lipid stores to sustain metabolism and to support moulting and gonad maturation costs on emerging from diapause (Rey-Rasset et al., 2002). Accumulation of this threshold, likely to be 25-50 % of dry weight (Rey-Rasset et al., 2002; Irigoien, 2004), would trigger physiological responses, which are likely to be hormone mediated (Irigoien, 2004). If this threshold is not obtained the individual does not prepare for or enter diapause (Johnson et al., 2008). Lipid depletion during diapause has also been hypothesised to act as an endogenous timer; individuals whose lipid stores are depleted below a certain critical level are forced to terminate diapause (Miller et al., 1991; Hirche, 1996; Ohman et al., 1998; Visser and Jónasdóttir, 1999;

Irigoien, 2004; Saumweber and Durbin, 2006; Johnson et al., 2008). However, another trigger terminating diapause is likely to operate for those animals who sustain lipid stores above a critical level through the whole winter.

1.5 Endocrine control of diapause

If diapause is a physiological response initiated either by external environmental stimuli, or by accumulation of a threshold lipid level, it is likely to be under endocrine control. Hormones can transduce environmental signals via hormone receptors to affect gene transcription in target tissues, and thus provide a link between environmental changes and consequential gene expression. Hormones are often multifunctional, and are now thought to co-ordinate the integrated expression of multiple traits across environmental conditions.

Many aspects of insect diapause are similar to overwintering of *Calanus finmarchicus*, and the endocrine control of diapause in insects is well documented (e.g. Highnam and Hill, 1977; Lee and Denlinger, 1997; Singtripop et al., 2000; Denlinger, 2002; Munyiri and Ishikawa, 2004; Zhang et al., 2004). Several key hormones serve as regulators of diapause, but precisely which hormones are involved depends on the species and developmental stage (see review in Denlinger, 2002). Insect development and reproduction are regulated by two lipoidal hormones, the sesquiterpenoid juvenile hormone (JH) and the steroid ecdysone (Highnam and Hill, 1977; Gade et al., 1997; Gilbert et al., 2000; Spindler-Barth and Spindler 2003; Riddiford et al., 2001). Ecdysone also regulates the moult cycle in decapod crustaceans (e.g. Rotlland et al., 2000; Styrishave et al., 2004). The regulatory effects of ecdysteroids are concentration-dependent (Johnson, 2003); typically during

moulting the ecdysteroid titre in the haemolymph peaks during the pre-moult stages and then decreases as ecdysis occurs. After ecdysis, the ecdysteroid titre is maintained at basal levels (see review in Chang, 1995). Johnson (2003; 2004) provides the only studies documenting ecdysteroid titre through the moult cycle in calanoid copepods. She found that in *Calanus pacificus* ecdysteroid titre was low after moulting, increases to a peak during pre-moult and decreases again before ecdysis. This pattern was evident despite high ecdysteroid variability. A similar ecdysteroid secretion pattern has been observed in decapod crustaceans (e.g. Lachaise et al., 1993; Chang, 1995; Rotlland et al., 2000; Styrishave et al., 2004). Johnson (2003) also demonstrated a difference between ecdysteroid levels in diapausing and active individual *C. pacificus*. Animals in diapause had significantly lower ecdysteroid levels than active individuals. During diapause, development (and consequently moulting) is suppressed, and low ecdysteroid titres in surface populations of *C. pacificus* can indicate preparation for diapause (Johnson, 2004). In some species of Lepidopteran insects that enter larval diapause, a high JH titre at diapause initiation has been documented (Yin and Chippendale, 1973; Agui, 1977). JH appears to prevent the release of ecdysone for larval growth and pupation and stimulate initiation and maintenance of diapause in several species of lepidopteran insects such as the yellow-spotted longicorn beetle *Psacothea hilaris* (Munyiri and Ishikawa, 2004), the Mediterranean corn borer *Sesamia nonagroides* (Eizaguirre et al., 2005) and the European corn borer, *Ostrinia nubilalis* (Chippendale and Yin, 1973; Bean and Beck, 1980, 1983). In these species JH titre drops when diapause is terminated, allowing for ecdysone to be released and for development to continue (Chippendale and Yin, 1973; Bean and Beck, 1980, 1983; Munyiri and Ishikawa, 2004; Eizaguirre et al., 2005). JH appears to have a different role, however, in the larval diapause of the lepidopteran

bamboo borer *Omphisa fuscidentalis* as Singtripop et al. (2000) reported that application of a JH analog terminates larval diapause in this species. In addition, in most cases of adult insect diapause, it is the absence of JH that induces diapause and activation of the *corpus allatum*, the gland that secrets JH, terminates diapause (see review in Denlinger, 2002).

The sesquiterpenoid hormone methyl farnesoate (MF) is the crustacean version of insect JH III (Fig 1.3), differing only by the absence of a hypoxide group (Homola and Chang, 1997). Irigoien (2004) proposed that MF could be involved in diapause regulation in *Calanus finmarchicus* by interacting with the hormone ecdysone to control development through the moulting process. The precise role of MF in crustacean physiology is unclear, although the known functions of MF in crustaceans are generally similar to the functions of JH in insects.

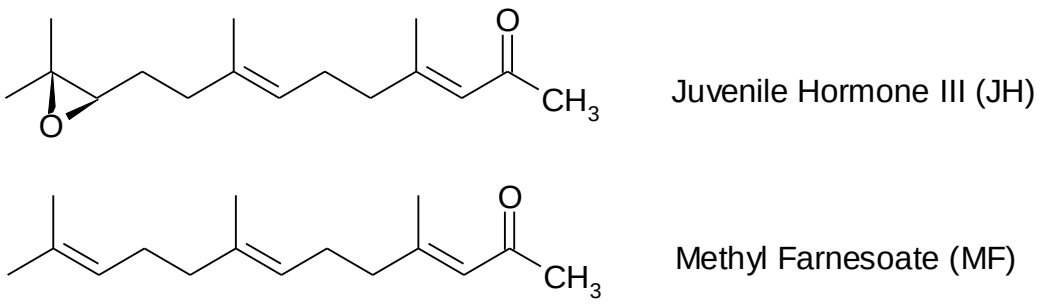

Fig 1.3 Chemical structure of juvenile hormone III and methyl farnesoate.

MF is known to be a multifunctional hormone involved in some aspects of reproduction (Rodreguez et al., 2002; Nagaraju et al., 2004), morphogenesis (Rotllant et al., 2000), the regulation of moulting (Homola and Chang, 1997; Tamone et al., 1997; Nagaraju et al., 2004) and to act as a juvenile hormone in barnacle cyprids (Smith et al., 2000). Irigoien (2004) suggested that diapause induction could be linked

to the lipid stores taken on by *C. finmarchicus* and diapause induction may be initiated by the concentrations of MF accumulated as a fatty acid in the lipid store. As discussed, JH has a different role in diapause processes in different species of insects, apparently triggering diapause in some species and terminating it in others (Chippendale and Yin, 1973; Bean and Beck, 1980, 1983; Denlinger, 2002; Munyiri and Ishikawa, 2004; Eizaguirre et al., 2005; Singtripop et al., 2000). However, coupled with ecdysteroids JH is involved in many aspects of insect development and MF is likely to be involved in some way, with regulating diapause in *C. finmarchicus*.

1.6 The aims and goals of this study

Given the importance of *Calanus finmarchicus* within the pelagic ecosystem of the North Atlantic and peripheral seas and the susceptibility of *C. finmarchicus* populations to climate change, it is important to understand the internal processes involved in regulating diapause: subtle changes in the environmental conditions which may affect diapause could have consequences for the entire *Calanus*- based ecosystem. This study takes advantage of relatively easy access to a deep (> 100 m), isolated population of *C. finmarchicus* in Loch Etive (a sea loch on the west coast of Scotland, see Chapter 2) to sample *C. finmarchicus* over an annual cycle and to study aspects of lipid dynamics and gene expression associated with diapause.

Specifically the objectives are:

Chapter 2: To provide detailed understanding of *C. finmarchicus* population dynamics within Loch Etive, to ascertain when the population enters

and terminates diapause and to monitor the abundance of *C. helgolandicus.*

Chapter 3: To measure the changes in total lipid, lipid class and fatty acid composition in *C. finmarchicus* over a seasonal cycle in Loch Etive, to provide further understanding if lipid accumulation is associated with initiation or termination of diapause in *C. finmarchicus*.

Chapter 4: To isolate and characterise target genes thought to be associated with the endocrine control of diapause in *C. finmarchicus.* To measure gene expression of these target genes, associated with MF and ecdysteroids, over a seasonal cycle, aiming to link expression of one or more target genes with diapause initiation or termination.

CHAPTER 2: *Calanus finmarchicus* in Loch Etive

2.1 INTRODUCTION

Loch Etive, a sea loch situated north of Oban on the west coast of Scotland (Fig 2.1), was chosen as the main sampling location for my investigations into the lipid dynamics and genetic basis of diapause in *Calanus finmarchicus* described in later Chapters of this thesis. As background to these studies, it is important to understand the dynamics of the *Calanus* population there: that is the subject of this Chapter. Loch Etive contains deep basins (>100 m), which make it representative of the deeper open ocean environments that *C. finmarchicus* also inhabits (e.g. Jaschnov, 1970; Conover, 1988). *C. finmarchicus* is the dominant mesozooplankton species in Loch Etive and are accessible there for sampling year round, which makes a time series of samples much easier to obtain from there than from the open ocean. Consequently Loch Etive is a good test bed for more difficult to obtain open ocean studies (deYoung et al., 2004). Loch Etive has atypical hydrodynamic conditions compared to other sea lochs on the west coast of Scotland. Like many sea lochs it is a glacially scoured fjord with an entrance sill at the Falls of Lora (Fig 2.1), however this sill is narrow (200m) and very shallow compared to other lochs (Fig 2.2), with a depth of only 7 m below mean high water (MHW; Edwards and Sharples, 1985) which reduces the internal tidal range of the loch to 2.0 m compared with an external range of 4.0 m (Edwards and Edelsten, 1977). Loch Etive is split into two main basins, an upper and lower basin (Fig 2.1), separated by the Bonawe sill (13 m below MHW, Nørgaard-Pedersen et al., 2006).

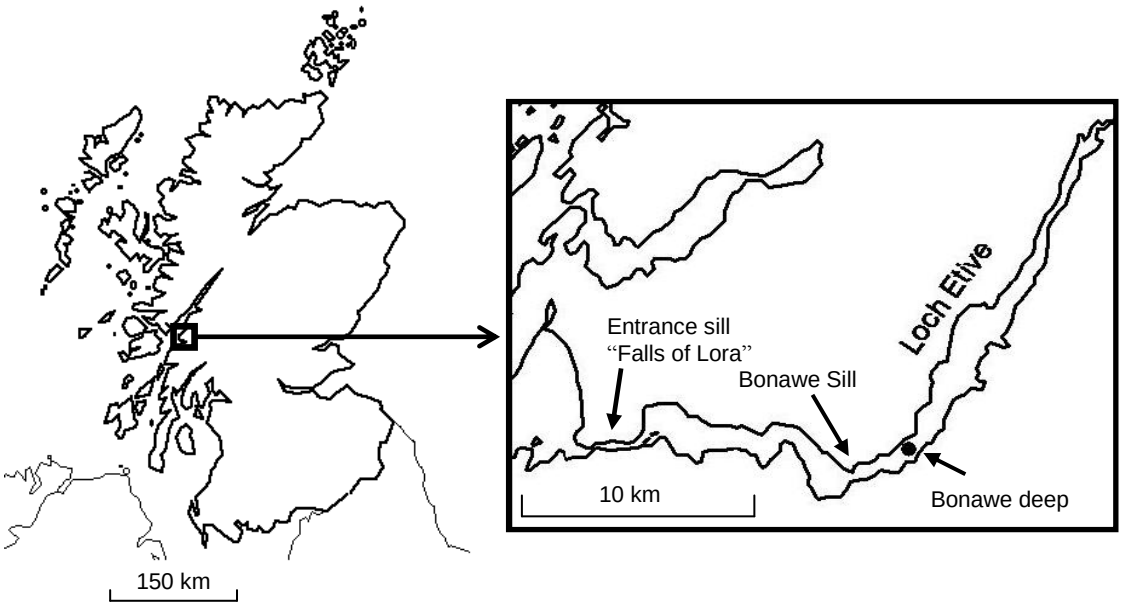

Fig 2.1 Situation of Loch Etive illustrating the two shallow sills and the sampling site in the Bonawe deep.

Loch Etive's large catchment area of 1400 km^2 (Wood et al., 1973) brings a very large freshwater influx compared to other Scottish sea lochs. The high freshwater influx determines much of the hydrogeography of the loch since this, together with the restrictions to the water exchange with the Firth of Lorne, means that the salinity of the surface layer is markedly reduced, from between 30 and 3 in the lower basin (Wood et al., 1973) and averaging 10 in the upper basin (McKee et al., 2002). The 'Bonawe deep' (max depth 150 m; Fig 2.2) within the upper basin was the focus of collection of *Calanus finmarchicus* for this study (Fig 2.1). The Bonawe sill only allows the exchange of surface water, exchange of the deep water within the Bonawe deep only occurs intermittently (on average every 16 months; Edwards and Edelsten, 1977) and will only occur during periods of reduced freshwater input and cold surface water, which causes the density of the surface water to exceed a critical value and a turbulent plume of dense salty water will flow from the lower basin into the upper basin renewing the bottom water (Edwards and Edelsten, 1977; Austin and Inall,

2002). Renewal or overturning events change the properties of the deep basin water rapidly, particularly by increasing dissolved oxygen concentrations (e.g. from 0.9 mg l^{-1} to 9.5 mg l^{-1} after a renewal event in May 2000; Austin and Inall, 2002). This means the residence time of the water in the Bonawe deep, which is dependant on renewal events, may extend up to 30 months (Edwards and Edelsten, 1977). In the stagnant bottom water present in the Bonawe deep when renewal events have not occurred, dissolved nutrients can accumulate and oxygen levels can become depleted (Austin and Inall, 2002).

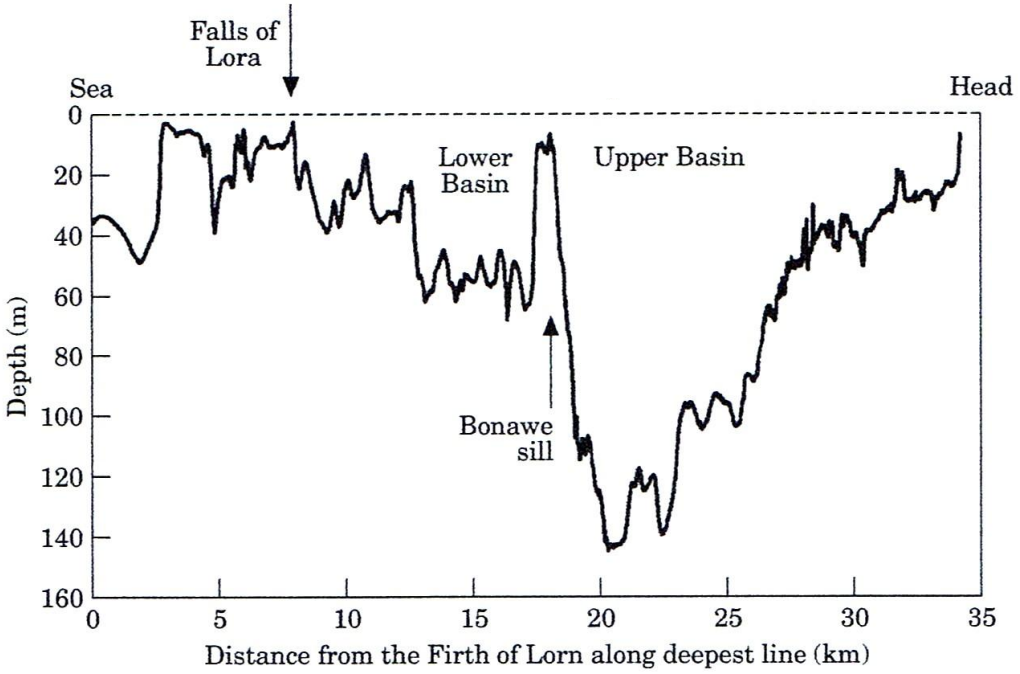

Fig 2.2 Bathmetry profile of Loch Etive taken along the line of deepest water. From Overnell et al. (2002).

The Bonawe deep (Fig 2.1) contains an isolated population of *Calanus finmarchicus*, the dominant meso-zooplankton species (Mauchline, 1987). The population in Loch Etive has become isolated as the latitudinal distribution of the species in the eastern Atlantic was pushed northwards (Beaugrand et al., 2002) and consequently *C. finmarchicus* in the coastal waters outside the Loch have declined in numbers and

been replaced by the congeneric species *Calanus helgolandicus* as the dominant meso-zooplankton species in the waters outside the Loch (Bonnet et al., 2005). Inside Loch Etive there are low numbers of *C. helgolandicus* within the inner basin, however as yet none have been reported within the upper basin and the Bonawe deep (Fig 2.3). Loch Etive's unusual hydrodynamic conditions are thought to be part of the reason that enables the population of *C. finmarchicus* to persist in the loch and are what have prevented *C. helgolandicus* from becoming established.

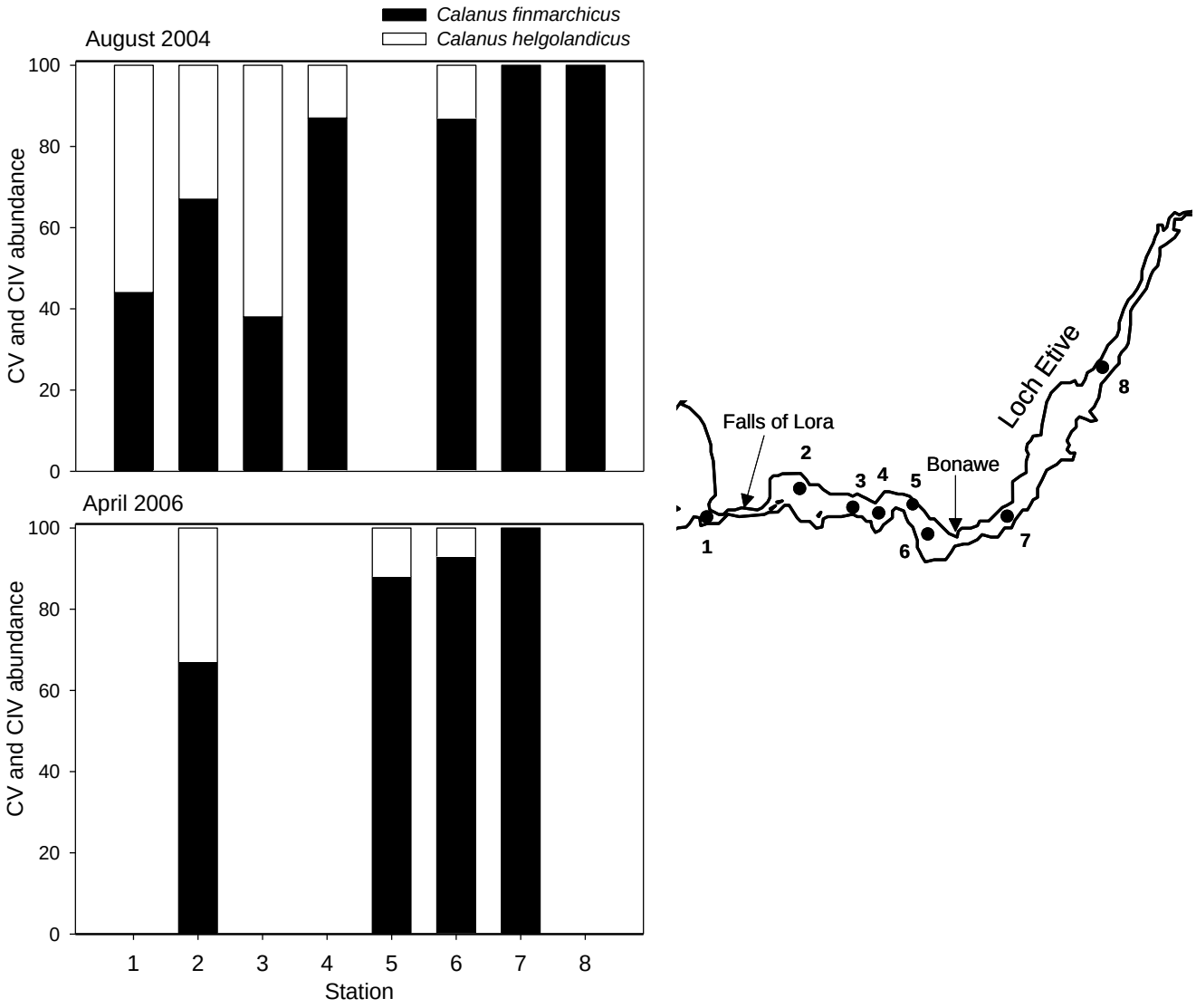

Fig 2.3 Percentage abundance of stage CV and CVI *C. finmarchicus* and *C. helgolandicus* in 8 stations through Loch Etive in 2004 and 2006. Station 7 is within the Bonawe deep. Data provided by Kathryn Cook, Fisheries Research Services Aberdeen.

2.2 MATERIALS AND METHODS

Fifteen monthly sampling trips were conducted between April 2006 and June 2007 at the Bonawe deep site in Loch Etive (Fig 2.1) from the RV *Seol Mara*. Hydrographic profiles were obtained using a Seabird 19 CTD and were taken during every month except May 2007. A 200 µm mesh, 1 m diameter ring net was used to take two vertical net hauls from 140 m. Onboard, the contents of the first net haul were preserved in 70% ethanol and the contents of the second net haul were kept live in a cool box whilst transported from the collection site back to the Scottish Association for Marine Sciences (SAMS) Dunstaffnage Marine Laboratory (within 2 hrs after capture), where they were sorted live, flash-frozen in liquid nitrogen and stored at -80^{o}C until further analysis. From July 2006 to June 2007 a 25 l Niskin bottle was used to collect water samples every 10 m (below 40 m) through the water column. Collected water was then passed through a 250 µm sieve and any *Calanus* in the sample were preserved in 70% ethanol onboard. A non parametric one way Analysis of Variance (ANOVA) was used to test if the distribution of *Calanus finmarchicus* by depth by month varied significantly.

The ethanol-preserved zooplankton from the vertical net haul were sorted to enable enumeration of *Calanus* spp. and separate moult stages. The sample was split using a Folsom plankton splitter up to 1/32 of the original sample, depending on the volume of plankton. The volume of *Calanus* sp. in the sample varied from 3 m^{-3} in April 2007 to 580 m^{-3} in August. Two subsections were analysed from each month using a compound microscope. All adult male and female *Calanus* individuals were identified to species level. *C. finmarchicus* CV and females were distinguished from *C. helgolandicus* by microscopic examination of the head shape and the curvature of the

inner edge of the basal segment of the fifth leg, whilst male *C. finmarchicus* were distinguished from *C. helgolandicus* by the relative lengths of the endopod and exopod of the fifth leg (Heath et al., 2000b; Fig 2.4). Due to the large number of CV in the samples, and the relatively rare occurrence of *C. helgolandicus*, only 200 individuals, picked at random, were identified to species level. All *Calanus* sp. copepodites CIV-CI present were counted.

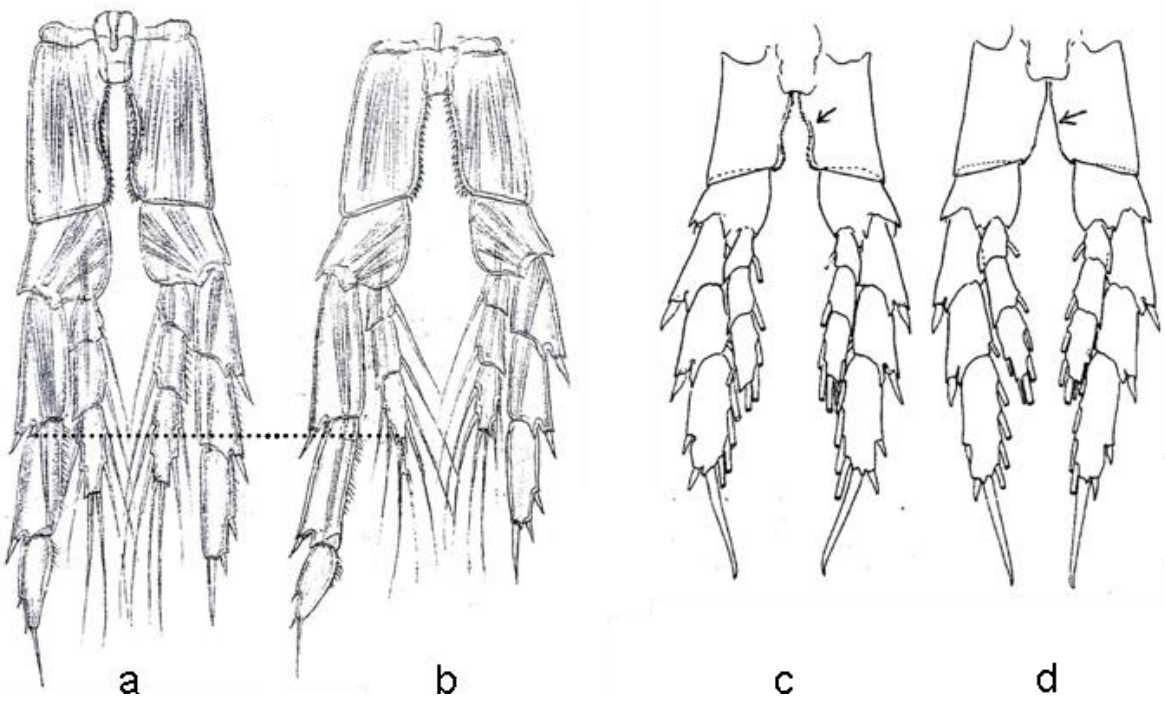

Fig 2.4 Fifth leg of **a.** *Calanus finmarchicus* male and **b.** *Calanus helgolandicus* male, the dotted line illustrates the longer endopod relative to the exopod in *C. finmarchicus* males, taken from Sars (1903); **c.** *C. helgolandicus* female distinguished by the curvature of the inner edge of the basal segment of the fifth leg (see arrow) compared to **d.** *C. finmarchicus*, from Fleminger and Hulsemann (1977).

The sampling in Loch Etive was conducted at varying times and dates each month, although as close together as possible, meaning possible changes in light intensity. As the migrations of some zooplankton species appear to follow isolumes - layers of constant light intensity (Mauchline, 1980), I wanted to compare the depth distribution of *C. finmarchicus* in Loch Etive as a function of irradiance at depth, as well as over

time. Irradiance values were collected every five minutes during the sampling period at SAMS each month, 15 km away from the Bonawe deep, and the mean values from a specific sampling time were used to calculate irradiance at depth using the Beer-Lambert law:

Equation 1 $$E_z = E_0.e^{-k_D.z}$$

where $\mathbf{E_z}$ is irradiance at a given depth **z**, $\mathbf{E_0}$ is irradiance at the surface, and $\mathbf{k_D}$ is the diffuse attenuation coefficient. In the upper basin of Loch Etive k_D is typically between 0.3-0.4 during the spring (Mckee et al., 2002), so a value of 0.35 was used.

2.3 RESULTS

2.3.1 Physical properties of the Bonawe deep

During all months the water column was stratified. The depth of the thermocline varied from 40 to 70 m from April 2006 to August, shallowed to around 30 m from September to February and deepened again to between 40 and 50 m in March and June 2007 (Fig 2.5). The temperature of the water column was more variable in the surface layer above the thermocline than below. Temperatures at 10 m rose from 7.4 ^{o}C in April 2006 to 13.9 ^{o}C in September. In October and November temperatures in the surface layer remained between 11.4 and 13.3 ^{o}C, but during December and January there was a deep layer of cold freshwater on the surface, shown by the decrease in salinity which caused a drop in temperature to between 4.7 and 9.5 ^{o}C in the first 15 m (Fig 2.5). The temperature of the water column below the thermocline was much more stable. From April 2006 to August it was in the range 7.4 to 8.8 ^{o}C, but from September to June 2007 the temperature of the bottom layer was warmer, in the range 10.9 to 12.6 ^{o}C (Fig 2.5).

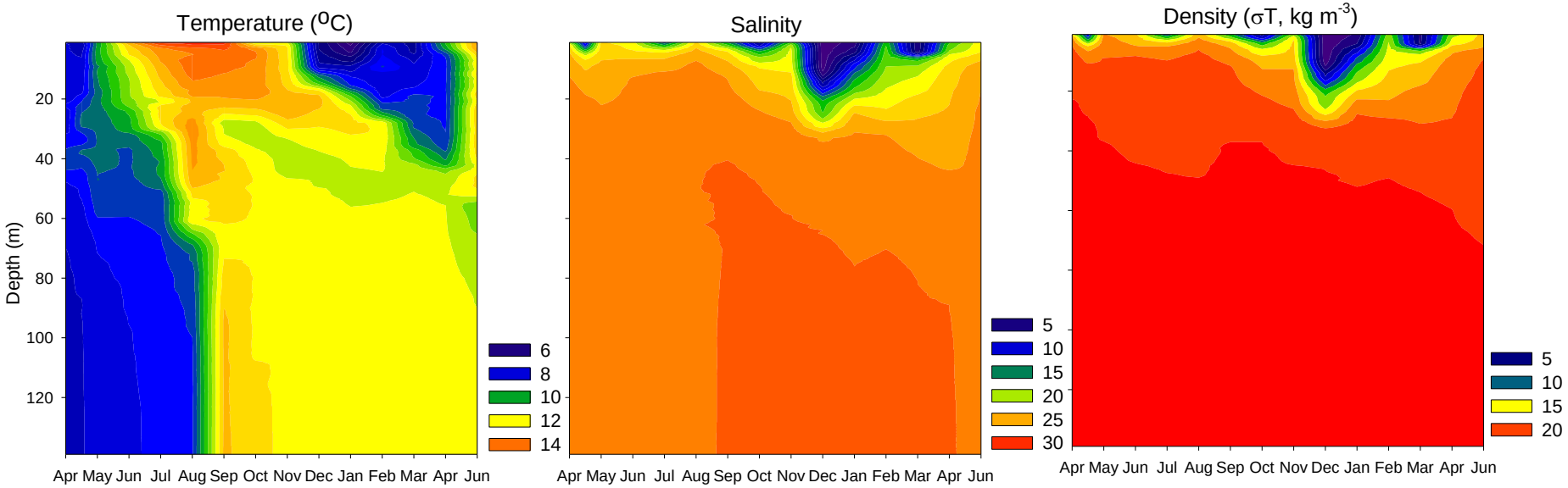

Fig 2.5 Temperature, salinity and density profiles of the Bonawe deep over the sampling period.

The salinity of the water column was variable above 40 m. In the first 10 m of the water column salinity was below 10 in October, December, January and March, indicating large volumes of freshwater had runoff into Loch Etive during these months. The density of the surface waters was also very variable due to the variations in freshwater input (Fig 2.5). Salinities in the deep basin below 80 m were very stable, between 26.0 and 26.1 from April 2006 to August, but in September the salinity below 50 m increased to 26.8 – 27.0. From September to June 2007 this deep water continued to be more saline than pre-September but did reduce in salinity to an average of 26.4 below 80 m by June 2007. Between 15 and 40 m the salinity of the water column is still influenced by the amount of freshwater runoff, reaching stable salinities at varying depths depending on the volume of runoff. This is particuarly evident during December and January when there was a large frehwater influx and the salinity did not reach 26.6 until 59 m in December and 68 m in January. Denisty of the bottom water appears to stay stable in the range 20.0 to 20.3 σT despite the change in temperature and salinity before and after September. The increase in salinity and temperature of the bottom water in September indicates that the bottom water may have been renewed between sampling in August and September.

2.3.2 Abundance of *Calanus finmarchicus* in Loch Etive

Total numbers of *Calanus finmarchicus* were highest (228 to 547 individuals m^{-3}) from July through to February and during May and June 2007 (Fig 2.6). Total numbers were reduced to between 2 and 113 individuals m^{-3} during April, May and June 2006 and to 119 and 10 individuals m^{-3} in March and April 2007 respectively. CV were much more abundant than any other stage in the net samples taken from June to March, reaching a maximum of 538 individuals m^{-3} in August. In April 2006

the adult stages, CVI males and females, made up a larger composition (50 % CVIf, 22% CVIm) of *C. finmarchicus* in the net samples, however they then made up a combined percentage of less than 18% of the total numbers in May and June 2006, and made up < 2 % of the total numbers of *C. finmarchicus* from July through to November (Fig 2.6). In December the only stage recorded in the net samples was CV. Adult females began to appear again in the net samples in January, but made up < 3 % of total numbers and < 6% of total numbers in February. By March and April 2007, however, adult CVI stages made up 45-50 % of *C. finmarchicus* in the water column, this dropped to 15-20% in May and June 2007.

CIV copepodites made up 12% and 44% of the net samples in April and May 2006 respectively, but less than 2% from June to September 2006. None were observed in the nets from September to March, but stage CIV made up 12% of the net sample the following April, 3% in May and 1% in June (Fig 2.6). Copepodites CI-CIII were under sampled by the 200 µm mesh, however no copepodites were observed in the net samples from October to March when they were observed during all other months. Individuals of *Calanus helgolandicus* were present in the net samples, however less than 4% of all stage CV and CVI individuals were *C. helgolandicus* in any given month (Fig 2.7).

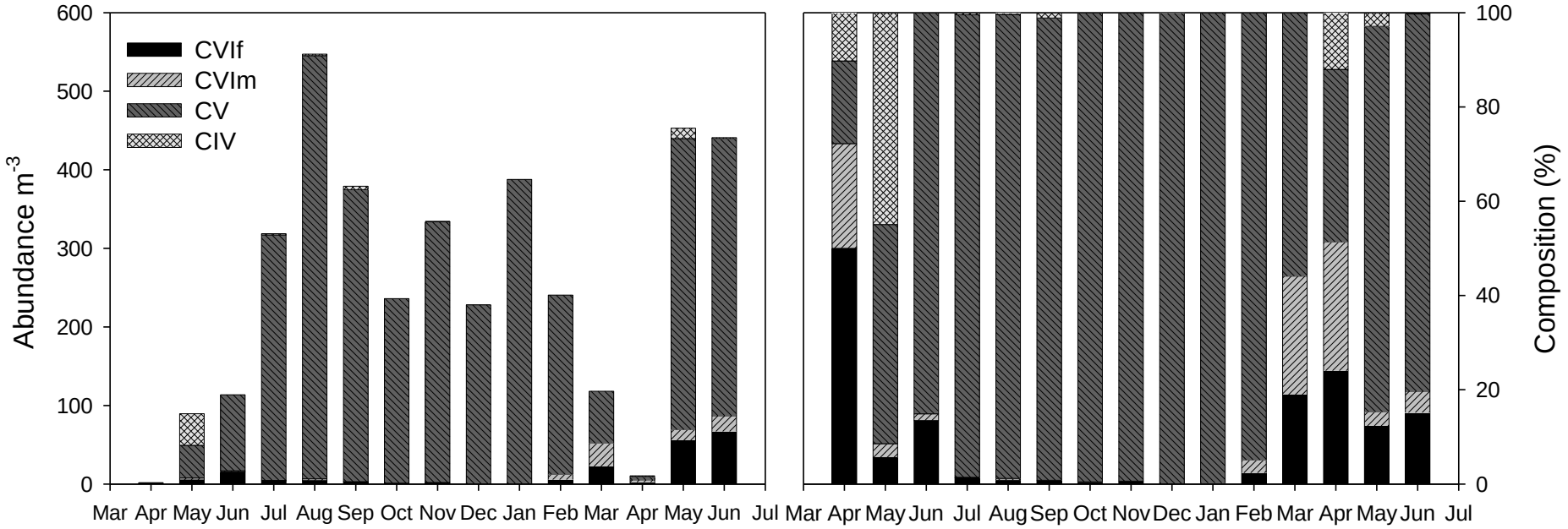

Fig 2.6 Abundance and relative composition of *Calanus finmarchicus* at the Bonawe deep.

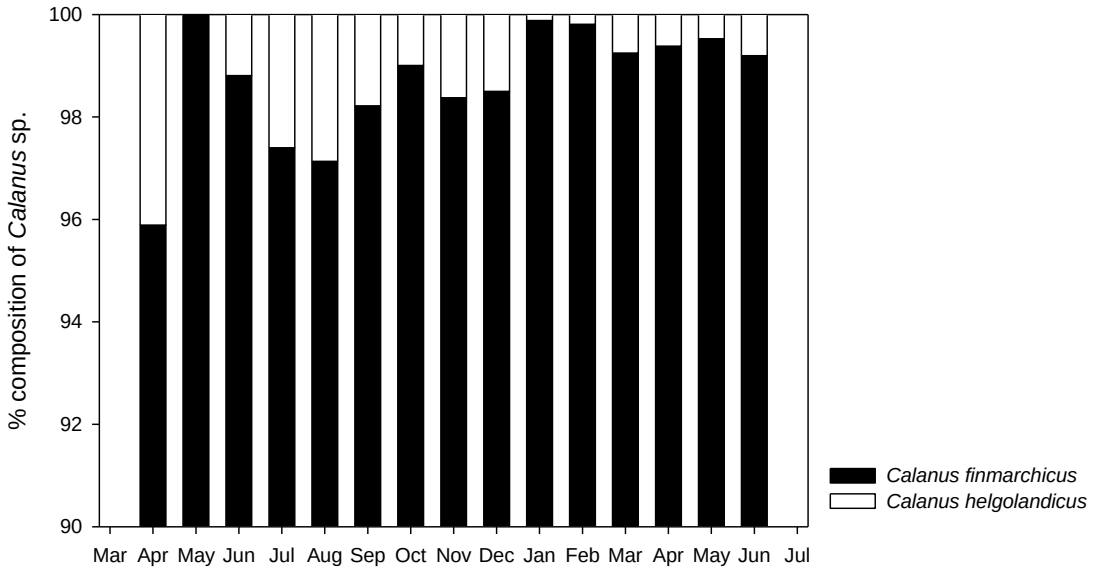

Fig 2.7 Composition of *Calanus* sp. in the net samples.

The total number of *Calanus finmarchicus* caught in the 25l Niskin bottles in any one month varied from 26 in March to 110 in November (Fig 2.8). During April 2007 only 6 individuals in total were caught from 11 Niskin bottles at 11 different depths, probably due to the decrease in abundance of *C. finmarchicus* in April 2007. The depth distribution of six individuals of *C. finmarchicus* is not likely to be representative of the whole population and, as the idea behind the depth profile was to observe any decrease in population depth which may be associated with diapause and by April it is likely that the population is active again, the data from April and June 2007 were not included. From July to November more than 75% of individuals were caught between 70 and 110 m (Fig 2.8) and less than 19% were caught below 110 m. In December and January more than 30% of animals were caught below 110 m, and by February and March more than 75 % of the individuals were again caught between 70 and 110 m.

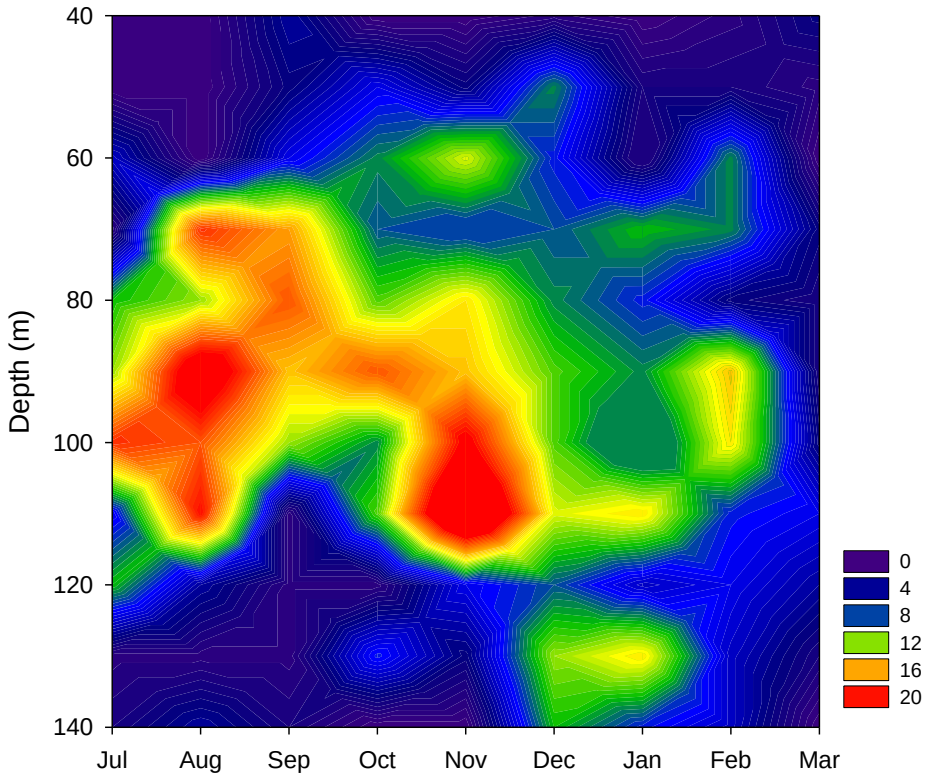

Fig 2.8 Numbers of *Calanus finmarchicus* individuals at 10 m depth intervals

To check that the depth distribution shown above was not unduly influenced by fluctuating light levels, depths were converted to irradiance levels at depth, scaling the measured surface illumination at the time the samples were taken by the attenuation value as per equation 1 (Fig 2.9). Loch Etive has a high CDOM (coloured dissolved organic matter) which strongly attenuates blue light in the water column (Mckee et al., 2002). Solar irradiance is strongly attenuated and below 100 m is in the range 4.9 x 10^{-13} to 8.3 x 10^{-20} (Fig 2.9). Despite undetectable levels of irradiance driving diel vertical migration in zooplankton in the Arctic (Berge et al., 2008), the seasonal variability in depth distribution does not appear to be a function of varying light intensity of the animals in winter in the Bonawe deep ($P<0.05$, non parametric ANOVA).

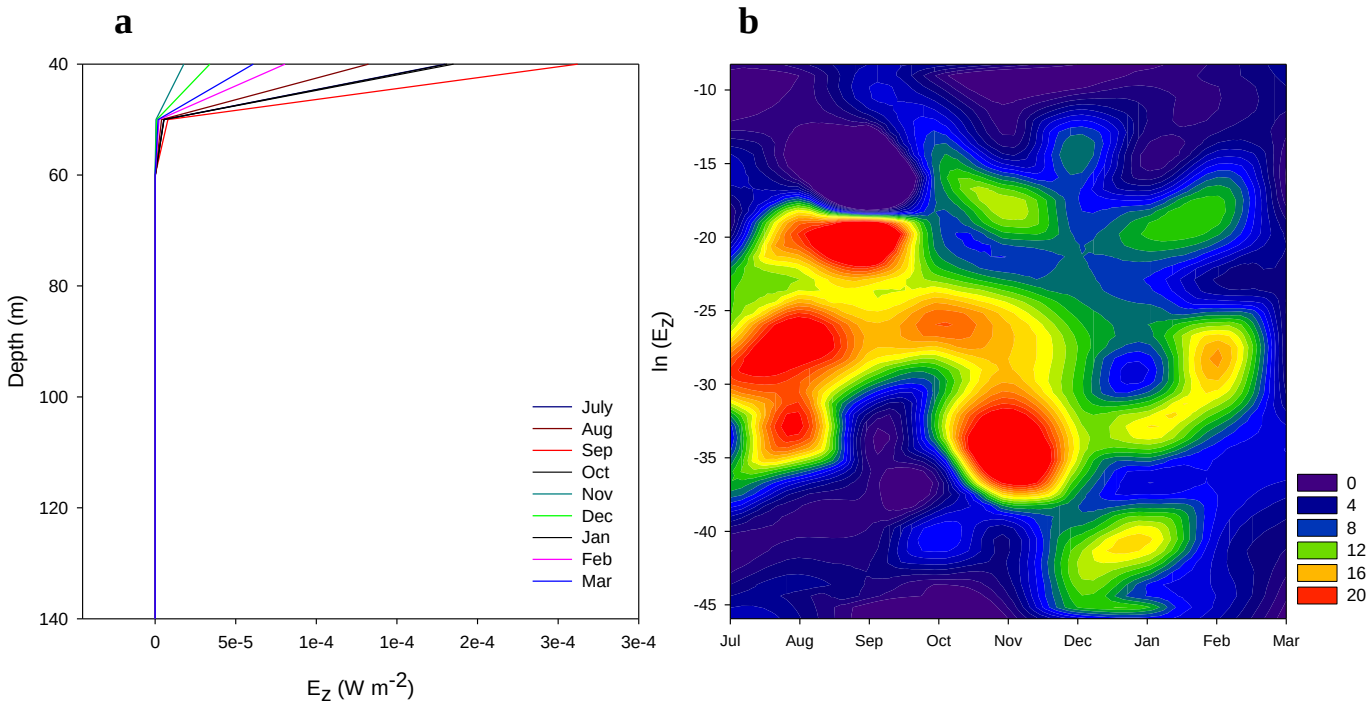

Fig 2.9 a. Irradiance at depth from 40-140 m. **b**. percentage of *Calanus finmarchicus* caught from July to March at a given depth against irradiance at a given depth.

2.4 DISCUSSION

Due to the shallow sill at Bonawe, the water properties of the Bonawe deep are altered only through local diffusion within the basin, convective overturning within the basin or by a deep water renewal event (Austin and Inall, 2002). Renewal events in Loch Etive generally result in rapid deep-water property changes of typically 1 unit of salinity and warming or cooling of 1 to 2^{o}C (Edwards and Edelsten, 1977; Austin and Inall, 2002). During the present study in Loch Etive, a deep water renewal event may have occurred between sampling in August and September, when the temperature of the bottom water increased from 8 to 12^{o}C - the temperature of the surface waters - and there was near homogeneity in the temperature of the water column at the point of sampling in September. The salinity of the bottom water has also increased slightly

during this time, indicating that the bottom water may have been refreshed. The relatively high surface salinity observed in August also suggests that freshwater runoff into the loch was low at this time. In shallow-silled fjords, deep water renewal generally occurs during periods of low freshwater run off (Edwards and Edelsten, 1977; Austin and Inall, 2002). However, during the period of my study in Loch Etive, no corresponding increase in the density of the surface waters prior to the overturn, to a point at which surface water density exceeds that of bottom water change is seen, unlike that observed by Edwards and Edelsten (1977). However, this is likely to have occurred in less than a month, and was probably missed between the two sampling points.

The only other report of *Calanus finmarchicus* in Loch Etive is provided by Mauchline (1987) and refers to data from 1972, 1978 and 1979. He reports that *C. finmarchicus* copepodites were most abundant, approximately between 15,000 to 35,000 individuals in the period April, May and June of 1978, followed by a surge in numbers of stage CV *C. finmarchicus* in May and June reaching a maximum of 17,000 individuals and an increase in the adult stages to a maximum of 5000 in May, June and July of those years. In August 1978 numbers of CV and adults appeared to dip to less than 1000 adult individuals and 3000 CV individuals, and few adults were present in the net samples from August onwards but CV showed a large increase to more than 40,000 individuals in September and numbers of CV remained above 15,000 until February 1979 (Mauchline, 1987). Through the rest of 1979 the seasonal pattern was the same as 1978 (Mauchline, 1987). As shown in the present study, the seasonal cycle of *C. finmarchicus* abundance in Loch Etive during 2006-07 still appears to be much the same, although the maximum number of CV appears to be in

August as opposed to July and there is no dip in numbers after the summer maximum. The large decline in numbers of the population in April 2006 and 2007 is matched in the data available from 1978 and 1979 from Mauchline (1987) and appears to be due to reproduction, there is a large decline in adults and CV, with a large increase in copepodite numbers in April and May in 2006-7 and 1978-9 (Mauchline, 1987). A decrease in numbers of adult and CV stages in April to June, to be replaced by high numbers of CI-IV copepodites is also common in the open ocean and is thought to be linked to the spring bloom (Head et al., 2000; Heath et al., 2000b). The potential overturning event in Loch Etive between sampling in August and September does not seem to have affected the population much directly, as numbers of *C. finmarchicus* in September were still high. The warm temperature of the bottom water did not appear to prevent animals from overwintering in Loch Etive. *C. finmarchicus* have been observed overwintering in temperatures up to 11^{o}C in coastal waters outside the UK (Williams and Conway, 1988; Parsons and Lalli, 1988).

If animals are diapausing in Loch Etive, it is likely that they would be doing so in December. A greater percentage of individuals were caught below 110 m in December and January than the rest of the year (Fig 2.8). Few adults were present in the net samples in October and November; and no adults were present in the net samples in December, although they were once again present in January albeit it in small numbers (Fig 2.6). This is also consistent with data presented by Mauchline (1987). The animals would have been suppressing development at stage CV and overwintering in a dormant state, which suggests they are diapausing, potentially entering diapause sometime between sampling during November and December and beginning to emerging from diapause in January - February, by which point it is likely

that most of the population has become active again. The data from this study and from Mauchline (1987) indicate that *C. finmarchicus* does not appear to overwinter as stage CIV in Loch Etive, unlike in the open ocean (Heath et al., 2004). The data from the present study and from Mauchline (1987) also show that no summer secondary maximum in copepodites was recorded in either 1978 or 1972. This indicated that *Calanus finmarchicus* in Loch Etive do not have more than one generation during a year; most of the population suppresses development upon reaching the CV stage and overwinters instead of moulting directly to adult and reproducing, producing multiple generations. No estimates of primary production exist from the upper basin of Loch Etive, but Wood et al. (1973) estimated gross annual primary production to be 70 C m^{-2} yr^{-1} in the lower basin. This is much lower than the estimate of 145 C m^{-2} yr^{-1} in the North Sea (Moll, 1998) and 648 C m^{-2} yr^{-1} for the coastal areas of the North East Atlantic (Sathyendranath et al., 1995). Primary production in Loch Etive is thought to be limited by the high coloured dissolved organic matter content in the freshwater layer (Mckee et al., 2002). This low production is likely to be the reason that only one generation of *C. finmarchicus* is produced in Loch Etive per season. Heath et al. (2000b) have also linked single-generation populations of *C. finmarchicus* with proximity to an overwintering site, such as the Faroe-Shetland channel.

The question of why *Calanus helgolandicus* has not become established in Loch Etive, as it has in the adjacent coastal waters, has become more pertinent because a few individuals of *C. helgolandicus* were found in the Loch during this study, in the lower basin (Fig 2.3) and in the Bonawe deep (Fig 2.7). Is it simply the limited advection into the Bonawe deep by the two shallow sills at the Falls of Lora and Bonawe? In the present study no increase in numbers of *C. helgolandicus* were

observed after the potential deep water renewal event, when they may be advected in, than before September despite the temperature increase of the bottom water to 12^{o}C after the deep water renewal event being a more favourable temperature for *C. heloglandicus* than *Calanus finmarchicus* (Helaouët and Beaugrand, 2007). Etive is a relatively harsh environment, with low salinity that is extremely variable in the surface waters, strong stratification and oxygen depletion in the stagnant bottom layer (Austin and Inall, 2002). *C. finmarchicus* is generally located in oceanic regions with lower stratification, and lower temperatures than *C. helgolandicus*, however *C. finmarchicus* has a greater tolerance interval to temperature and salinity than *C. helgolandicus* (Helaouët and Beaugrand, 2007). Is it perhaps this greater tolerance to temperature and salinity fluctuations which enables *C. finmarchicus* to graze within the surface waters where the animals are exposed to severe fluctuations in temperature and salinity driven by freshwater runoff, as well as being exposed to rapid temperature and salinity changes when a deep water renewal event occurs that allows them to outcompete their congeneric rivals? Future monitoring of the population in Loch Etive may answer some of these intriguing questions.

The isolated population of *C. finmarchicus* in Loch Etive has enabled the collection of a time series of *C. finmarchicus* individuals, which have a life-cycle comparable to that of open ocean populations near overwintering sites, without the associated difficulties of collecting a time series in these open-ocean environments and has provided an archive of samples that have enabled studies into some potential genetic and physiological bases of diapause (Chapters 3 and 4) which may be applied to open ocean populations.

CHAPTER 3: Variation in lipid class and fatty acid composition of *Calanus finmarchicus* over a seasonal cycle in Loch Etive, Scotland.

3.1 INTRODUCTION

Lipids are produced and stored in oil sacs by many copepods (Fig 3.1). The reasons for accumulation are debated, as described in Chapter 1, but lipids enable several aspects of the ecology of *Calanus finmarchicus* to be investigated: this is the subject of this chapter. Lipid accumulation by *C. finmarchicus* is important for survival in diapause through the food-sparse winter (Lee et al., 1970; Lee and Hirota, 1973; Hirche, 1996; Jónasdóttir, 1999).

Fig 3.1. *Calanus* sp. illustrating the orange lipid stores[2].

Lipids are stored by calanoid copepods mainly as wax esters (WE) and to a lesser extent triacylglycerols (TAG) (see Lee et al. 2006 for a review). In diapausing *C. finmarchicus*, the composition of this lipid store has been measured to be as high as 75-90% WE (Kattner and Krause, 1987; Kattner and Hagen, 1995; Jónasdóttir, 1999). The WE in *C. finmarchicus* are comprised of long-chain fatty acids and alcohols, which have a high calorific value (approximately 7909-9737 kJ mol^{-1}; Albers et al. 1996) as well as thermal expansion and compressibility properties which enable

[2] http://www.sfos.uaf.edu/research/arcdiv/watercolumn/copepod/calanus_marshallae.html

diapausing copepods to achieve neutral buoyancy in cold, deep waters, minimising the energy they need to expend to remain at depth during diapause (Jónasdóttir, 1999).

How much of the lipid store is actually depleted during the overwintering period is debated: Jónasdóttir (1999) calculated that only a small percentage (~5%) of the stored lipids are consumed during overwintering, while other studies have shown a considerable decrease in dry weight (40-70%) well in advance of termination of diapause (Hirche, 1983; Hopkins et al., 1984; Heath et al., 2008), chiefly due to lipid and carbon catabolism well in advance of termination of diapause. Preservation of some lipid throughout diapause is thought to be advantageous as lipid may be used to fuel egg production before the advent of the spring bloom (Niehoff et al., 1999, Richardson et al., 1999; Varpe et al., 2007), enabling rapid growth and development in the bloom as per the Cushing match-mismatch hypothesis (Cushing, 1990). This states that matches or mismatches in time and space between phytoplankton, zooplankton and fish larvae leads to year on year variability. A match occurs when the biomass of fish larvae and their planktonic prey overlaps, a mismatch occurs when there is an extensive temporal difference between pytoplankton/zooplankton and fish larvae abundance. *C. finmarchicus* females arriving at the surface post diapause with depleted reserves would need to feed in the bloom before spawning, leaving larvae to hatch towards the end of the bloom and suffer in a food limited system.

Lipid accumulation may act as a trigger for diapause. Several authors have put forward theories known as the 'lipid accumulation window hypothesis' (Johnson et al., 2008), which argues that individuals must accumulate sufficient lipid stores, exceeding a threshold value (25-50% of dry mass; Rey-Rasset et al., 2002) in order to

trigger a physiological response that results in diapause. Individuals that do not reach this threshold do not enter diapause and remain in the surface waters over winter (Pasternak et al., 2001; Rey-Rasset et al., 2002; Irigoien, 2004; Saumweber and Durbin, 2006; Johnson et al., 2008). Several studies have reported that animals caught in deeper water in winter contain larger lipid stores than individuals caught in the surface waters, consistent with this hypothesis (Jónasdóttir, 1999; Miller et al., 2000; Pasternak et al., 2001; Hasset, 2006). In the Southern Ocean the copepod *Calanoides acutus* has alternate life strategies, diapausing as CIV or CV but "choosing" to either take one or two years to accumulate lipids, gain maturity and reproduce (Pasternak et al., 1994; Drits et al., 1994; Tarling et al., 2004). It is possible that insufficient lipid stores may be the reason for choosing a 2 year strategy. In addition to the 'lipid accumulation window hypothesis' theory, metabolism of stored lipids during diapause could be part of the mechanism involved in dormancy termination, i.e. a copepod might become active again when it has depleted its lipid reserve beyond a certain threshold level (Miller et al., 1991; Hirche, 1996; Ohman et al., 1998; Visser and Jónasdóttir, 1999; Irigoien, 2004; Saumweber and Durbin, 2006). Thus, data on the dynamics of lipid storage over a seasonal cycle may provide insight into the potential link of lipid accumulation with diapause initiation and lipid metabolism with diapause termination in *C. finmarchicus* from Loch Etive and elsewhere.

In addition to providing insight into diapause initiation, lipid analysis can also be instructive for food web analysis. Fatty acid trophic markers (FATMs) have been used in marine ecosystems to follow energy transfer and to study predator-prey relationships (e.g. Falk-Petersen et al., 2004; Daalsguard et al., 2003; Petursdottir et al., 2008). Large pelagic copepods incorporate dietary fatty acids relatively unchanged

into storage lipid (Lee et al., 1971). The use of FATMs to characterise feeding on different taxonomic groups has been well established, e.g. the assignment of 16:1(n-7) and 20:5(n-3) to diatoms (Nichols et al., 1991; Viso and Marty, 1993; Daalsguard et al., 2003) and 18:4(n-3) and 22:6(n-3) to dinoflagellates (Graeve et al., 1994; Daalsguard et al., 2003). Odd and/or branched fatty acids (OBFA) and (n-7) and (n-9) monounsaturates are considered to be markers of microbial assimilation in crabs and gastropods (Pranal et al., 1996) and cladocerans (Desvilettes et al., 1994), however OBFA may not be useful in tracking the transfer of microbial prey to *Calanus* spp. as they made up <1% of total fatty acids in *Calanus glacialis* (Stevens et al., 2004b). Copepods feeding herbivorously generally contain higher proportions of polyunsaturated fatty acids (PUFA) than copepods feeding omnivorously (Falk-Petersen et al., 1987; Graeve et al., 1994) and from this, Stevens et al. (2004a) developed an omnivory index - the unsaturation coefficient (UC) – that could be used to distinguish microbial dietary intake in *Calanus glacialis*. UC is the ratio of the polyunsaturated wax ester to the total wax ester and assimilation of microbial material is marked by very low values of UC (Stevens et al., 2004a). Fatty acids provide information on the dietary intake and food constituents that lead to the sequestering of lipid reserves over a long period of time (Dalsgaard et al., (2003). Fatty acids thus provide a long term, integrated view of diet and feeding. Dietary changes, marked by FATM, will provide information into what *C. finmarchicus* in Loch Etive are grazing on over a seasonal cycle, what fatty acids are sequestered into the lipid stores and whether the animals are feeding during the winter, particularly during December when they are thought to be in diapause. This study, as far as I am aware, is the first study to measure lipid class and fatty acid composition of *Calanus finmarchicus* in

Loch Etive and is the first use of individual animals to study lipid class using scanning densitometry.

3.2 MATERIALS AND METHODS

3.2.1 Animal Collection

The monthly time series of stage CV *Calanus finmarchicus* collected in Loch Etive from April 2006 to June 2007 (described in Chapter 2 of this thesis) was used for lipid analysis. All the animals used had been transported by boat from the collection site back to the Scottish Association for Marine Sciences Dunstaffnage Marine Laboratory (within c. 2 hrs after capture), sorted live, flash-frozen in liquid nitrogen and stored at -80^{o}C until further analysis.

3.2.2 Extraction of lipids

Lipids were extracted using a modified version of the Folch method (Folch et al., 1957). Lipids were extracted separately from 10 individual CV *Calanus finmarchicus* and also from a separate, pooled bulk sample containing 20 animals for every point in the time series. Prior to lipid extraction, each sample was placed in a pre-weighed glass vial and the wet weight of each sample measured, after which the animals were freeze-dried for 4 hours in order to obtain the dry weight of each sample. To extract the lipids, chloroform: methanol solution (2:1 v/v; 500 µl to individual samples, 2 ml to bulk samples) was added to each vial and the samples were incubated in a refrigerator (~4^{o}C) for at least 16 hours (Webster et al., 2006) before 0.88% (w/v) potassium chloride solution (125 µl to individual samples, 500 µl to bulk samples) was added, the samples mixed and centrifuged at 1500 x g for 2 min. The organic layer was carefully removed into another pre-weighed glass vial, dried under nitrogen

and desiccated at room temperature until constant weight. Total lipid was re-dissolved in small amounts of chloroform and kept at –20^{o}C until further analysis.

3.2.5 Determination of lipid class

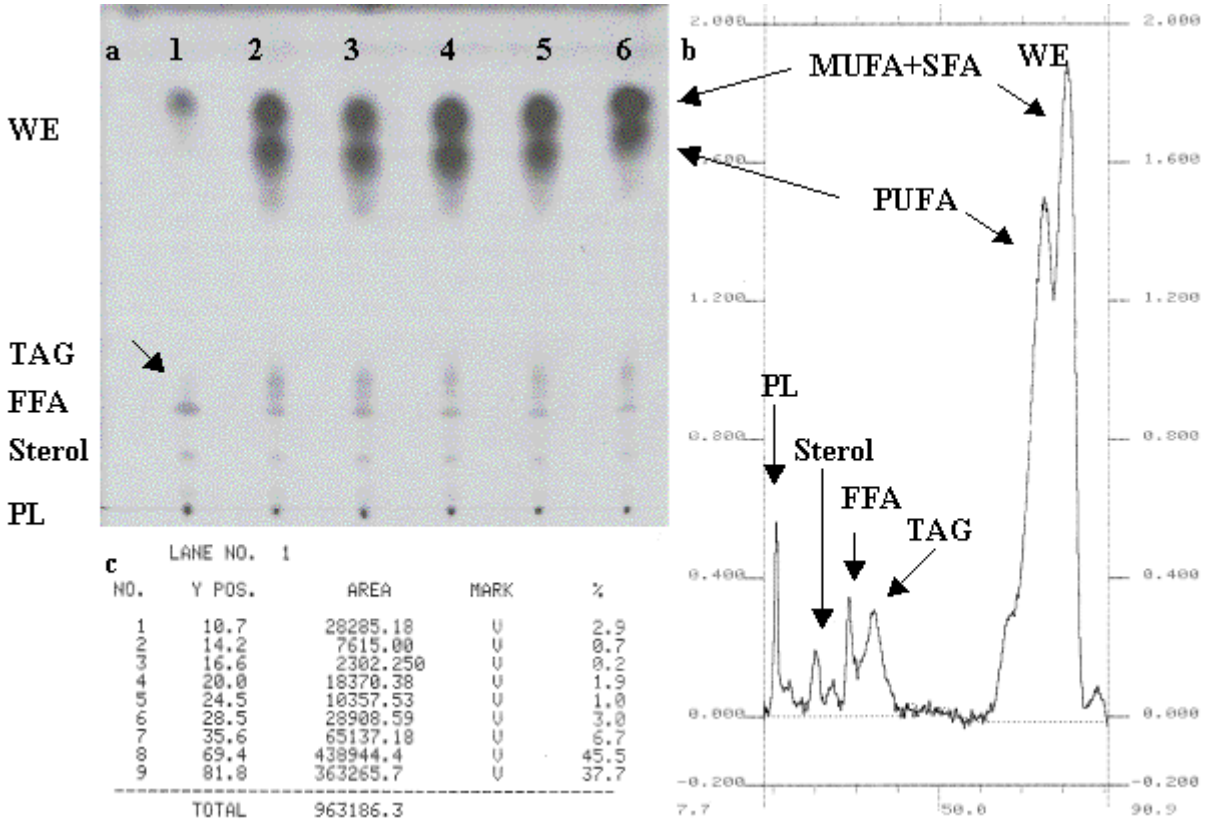

Fig 3.2 Example of **a**. high performance TLC plate showing the fractionation of total lipid into polar lipid (PL), sterol, free fatty acids (FFA), triacylglycerol (TAG) and wax ester (WE) including polyunsaturated (PUFA), saturated (SFA) and monounsaturated (MUFA) wax ester from six samples; **b**. the graph produced by the analysis of sample 6 from this plate from scanning densitometer; **c**. the numerical area of the peaks shown in b calculated by the machine.

Total lipid (15 µg from individual samples, 150 µg of bulk samples) was split into individual lipid classes by thin layer chromatography (TLC) on high performance 10 × 10 × 0.25 cm TLC plates of silica gel in a hexane:diethyl ether:acetic acid (18:2:0.2 v/v) solvent system. The plates were then sprayed with 8% (v/v) phosphoric acid

containing 3% (w/v) copper acetate solution, followed by heating at 160^{o}C for 13 min. Lipid class was determined by scanning densitometry, the separate lipid classes being identified by comparison with known standards (Fig 3.2). 10 individual samples from each month were initially analysed, however the number of replicates used for final analysis of lipid class varied between 4-10 replicates per month (Fig 3.3). The unsaturation coefficient (UC, proportion of polyunsaturated to total wax ester) was calculated for all samples in which the scanning densitometry could separate the two peaks (e.g. Fig 3.2).

3.2.4 Fatty Acid analysis

All of the available total lipid samples (79 individual samples and 15 bulk samples) were used for fatty acid analysis. An aliquot of 40 µg total lipid was analysed for fatty acid content from the bulk samples, whereas the whole total lipid sample remaining after lipid class analysis was used from the individual samples. Total lipid was re-dried under nitrogen and by desiccation before the addition of toluene (150 µl to individual samples, 450 µl to bulk) and the methylation reagent (methanol:sulphuric acid 99:1 v/v; 300 µl to individual, 900 µl to bulk) added. An internal standard 23:0 was added to each sample (5 µg to individual and 10 µg to bulk) and the glass vials were purged with nitrogen before the lids were attached. The samples were heated at 50^{o}C for 16 hours (Christie, 1982). On removal the samples were allowed to cool to room temperature before milliQ water (200 µl individual, 1 ml bulk) was added to each sample. On addition of hexane: diethyl ether (1:1 v/v) (300 µl to individual, 1 ml to bulk) the samples were mixed and centrifuged and the upper organic layer was transferred to a clean glass tube. This step was repeated and 2% (w/v) sodium bicarbonate (100 µl to individual, 500 µl to bulk) was added to the combined upper

organic layer. After mixing and centrifugation at 1500 x g the solvent was evaporated under nitrogen. The unpurified fatty acid methyl esters (FAMES) were dissolved in a small amount of hexane and stored at –20^{o}C until purification by thin layer chromatography. Prior to the application of the samples, the plates were dried under nitrogen and hexane (30 µl to individual, 100 µl to bulk) was added. The plates were developed in an hexane: diethyl ether: acetic acid (45:5:0.5) solvent system, after which they were sprayed lightly with dichloroflurescien stain and desiccated briefly. Fatty acid methyl esters (FAMES) were visualised under UV light and marked out by hand with a pencil. FAMES were scraped off the plate and dissolved in 2 ml hexane: diethyl ether (1:1 v/v), mixed and 1 ml 2% (w/v) sodium bicarbonate was added before centrifugation. The aqueous layer was removed to a clean vial and the solvent was evaporated under nitrogen and by desiccation, after which 20 µl hexane was added to the tubes and the sample was transferred to a pre-weighed glass vial, dried under nitrogen and desiccated until constant weight when total purified FAMES could be weighed. These were then re-dissolved in a small amount of hexane and stored at –20^{o}C until analysed on a TRACE 2000, Thermo Electron gas chromatograph (GC). The GC was equipped with on column injection, a Stabilwax column (Restek 30m x 0.32 mm i.d.) and hydrogen was used as the carrier gas.

The peak area corresponding to each fatty acid provided by the GC was used to calculate the percentage relative fatty acid composition using the peak area of the added internal standard, 23:0. Some results had to be discarded because of degradation of a few lipid samples. The number of replicate samples used for further analysis is shown in Table 3.1.

3.2.5 Data analysis

Prior to statistical analysis, percentage data were transformed using an arc sine square root transformation to normalise the data. To identify significant differences in lipid class composition and the UC between months, a one-way ANOVA followed by *post hoc* multiple comparison (Tukey's test) was performed using the Sigmaplot software. To identify samples that had similar lipid profiles, principal component analysis (PCA) was performed using the PRIMER 6 program. Cluster analyses, using the same program (based on Bray-Curtis similarity and complete linkage cluster), were performed to identify similar samples. A Students' t-test was used to test for significant differences in relative fatty acid composition between clusters.

3.3 RESULTS

The proportion of lipid classes within the total lipid from *Calanus finmarchicus* individuals showed considerable variation between and within months (Fig 3.3). Individual total lipid varied from 6.7% of dry mass of one individual sampled in May 2007 to 81% of dry mass in one individual sampled in August (Fig 3.3). Total lipid peaked in March (a mean of 58% of dry mass) and CV containing the smallest mean lipid stores were from April in both 2006 and 2007 (24.5% and 23.8% of dry mass respectively). There was a significant decrease in total lipid from a mean of 57% in October to 32% in December ($p<0.05$, one way ANOVA, Tukey's Test). From December to March total lipid had significantly increased to 58% ($p<0.05$). Wax ester was the largest component of the lipid stores in *C. finmarchicus* in all months, peaking in October (mean 88.6%, Fig 3.3). Individuals from April 2006 had significantly less WE content than all other months except June 2006 and May 2007 (mean 52%; $p<0.05$). The individuals collected during this month (April 2006) appear

to be split into two groups in terms of lipid class composition. Two individuals had large lipid stores (40-60% of dry mass), with a WE content of 80-90%, and four individuals had small lipid stores (12-25% lipid of dry mass) with a WE content of only 15-42% (Fig 3.3). The rest of the total lipid was composed of a larger relative proportion of polar lipids (19-25%), free fatty acids (19-37%) and TAG (3-10%) than animals with a larger percentage of wax esters (>60%) from any month (Fig 3.3; $p<0.05$). One individual from May 2007 with a smaller total lipid store (43% of dry mass) was also high in FFA (30.8% of total lipid), Sterol (8%) and PL (8.25%). Apart from the low % WE group of individuals from April 2006, the proportion of polar lipids did not vary significantly in relation to composition of the total lipid store over the time series ($p>0.05$) and was in the range 2-8% throughout (Fig 3.3). Relative composition of sterol in the lipid store is stable in the range 1.6-3.3% from animals collected in all months apart from April 2006 and May 2007 where it is significantly higher (means 6% and 6.3% respectively, $p<0.05$) (Fig 3.3). The triacylglycerol (TAG) component peaks in both June 2006 (11%) and June 2007 (8.2%) but otherwise remains in the range 1.5-7% (Fig 3.3).

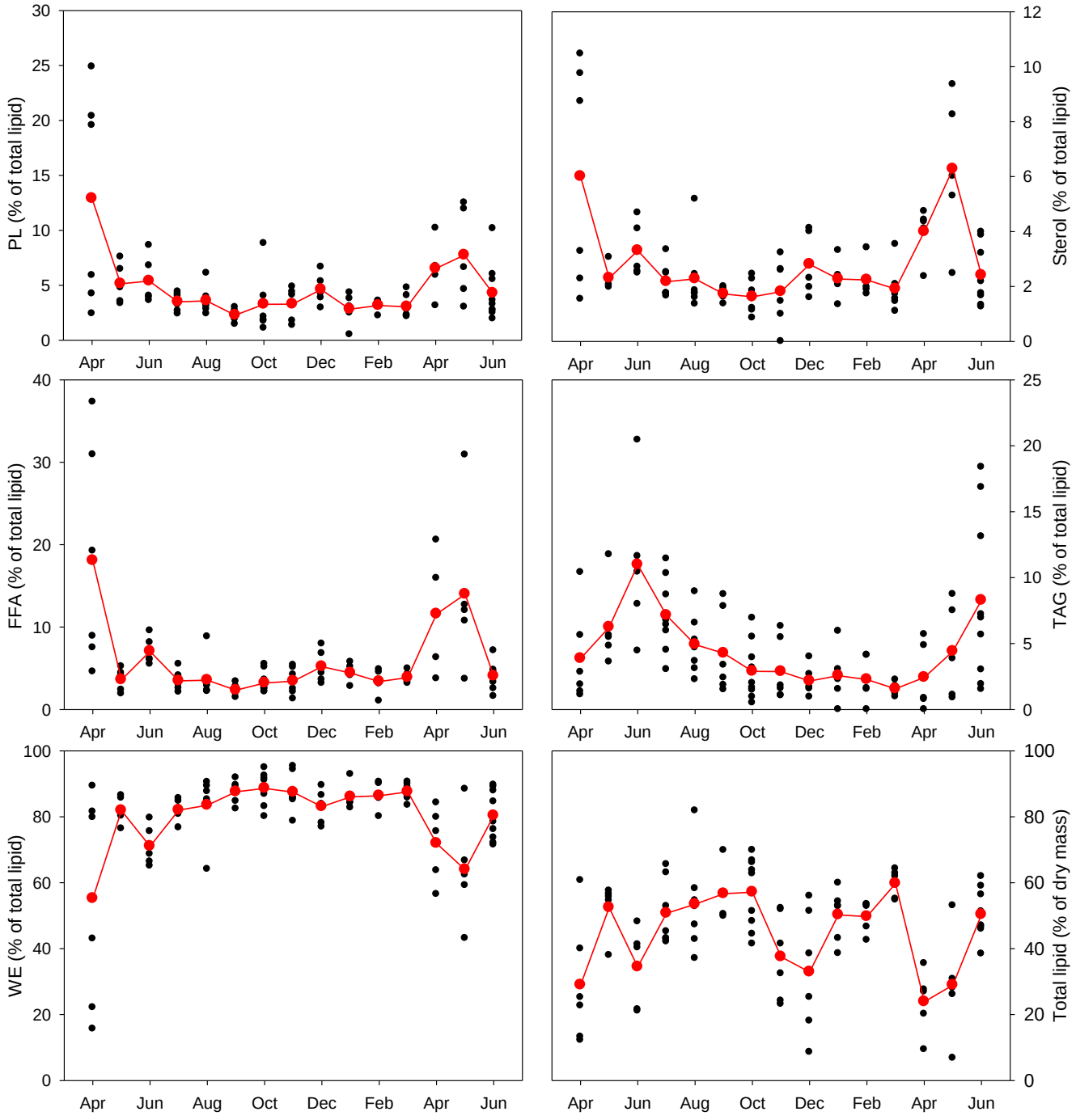

Fig 3.3. Total lipid and lipid class content of *Calanus finmarchicus* over a seasonal cycle. **a.** polar lipid (PL) content; **b.** sterol content; **c.** free fatty acid (FFA) content; **d.** triacylglycerol (TAG) content; **e.** wax ester (WE) content; **f.** total lipid content as a % of dry mass. Black circles represent data from individual copepods; red circles and line represent mean values.

The mean percentage of the relative composition of fatty acids through the time series is shown in Table 3.1. Principal fatty acids across all months were 14:0, 16:0, 16:1(n-7), 20:5(n-2) and 22:1(n-11). The diatom markers 16:1(n-7) and 16:2 (Nichols et al., 1991; Viso and Marty, 1993; Daalsguard et al., 2003) were abundant consistently throughout the year, but the markers 16:4(n-1) and 20:5(n-3) were more abundant during May, June, July, August, September and October 2006 and June 2007. Levels of the dinoflagellate marker 18:4(n-3) were elevated during May, June and July 2006 and May and June 2007, although the other dinoflagellate marker 22:6(n-3) (Graeve et al., 1994; Daalsguard et al., 2003) was highly abundant during April 2006 but made up a relatively small percentage of the total fatty acids during the rest of the year (Table 3.1).

Odd and/or branched fatty acids (OBFA) make up <1% of total fatty acids during any month (Stevens et al., 2004b) and so this index was not used to determine feeding strategy. The ratio of the sums of polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) also provide an indication of feeding strategy (Cripps and Atkinson, 2000). In the present study, regression analysis revealed moderate correlation between an increase in SFA with a decrease in PUFA (R^2=0.673, $p<0.01$) from *C. finmarchicus* in Loch Etive (Fig 3.4) as expected from normal lipid extractions. The ratio of PUFA:SFA was smallest during February, March and April 2007, significantly smaller than during May, June, July, August 2006 and June 2007 (Students' t-test, $p<0.05$; Table 3.1). However the unsaturation coefficient (UC; Stevens et al., 2004a) was not significantly different between any months ($p>0.05$, one way ANOVA) and no bacterial markers such as 18:1(n-7) (Stevens et al., 2004a) appear to be more abundant.

	Apr 06 n=4	May 06 n=5	Jun 06 n=5	Jul 06 n=6	Aug 06 n=7	Sep 06 n=5	Oct 06 n=9	Nov 06 n=5	Dec 06 n=5	Jan 07 n=5	Feb 07 n=4	Mar 07 n=3	Apr 07 n=4	May 07 n=2	Jun 07 n=8
14:0	14.1 ± 8.8	16.1 ± 6.6	15.9 ± 3.7	19.2 ± 3.2	17.0 ± 3.0	20.6 ± 4.8	17.9 ± 3.9	21.5 ± 6.6	20.6 ± 5.2	21.0 ± 2.8	26.4 ± 2.4	26.8 ± 0.8	19.5 ± 9.0	16.7 ± 4.4	15.1 ± 5.4
15:0	0.9 ± 0.5	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.3	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	1.1 ± 0.4	1.0 ± 0.3	0.7 ± 0.1
16:0	16.0 ± 5.3	11.0 ± 1.4	12.2 ± 2.0	11.8 ± 2.5	13.4 ± 3.5	10.8 ± 1.0	12.2 ± 1.8	13.0 ± 2.6	14.0 ± 2.0	12.6 ± 1.7	12.6 ± 3.4	11.3 ± 0.7	16.3 ± 6.5	19.6 ± 7.6	11.3 ± 2.3
16:1(n-7)	9.3 ± 3.1	10.4 ± 1.2	10.1 ± 1.5	11.1 ± 1.5	12.1 ± 1.4	12.7 ± 1.9	14.9 ± 1.6	13.2 ± 2.1	15.5 ± 3.5	14.0 ± 2.3	15.4 ± 1.7	16.3 ± 0.8	10.2 ± 6.2	8.7 ± 3.1	10.0 ± 1.4
16:2	3.1 ± 0.8	2.5 ± 0.4	2.7 ± 0.3	2.9 ± 0.2	2.8 ± 0.3	3.1 ± 0.4	3.4 ± 0.4	2.9 ± 0.5	3.1 ± 0.7	3.5 ± 0.4	3.5 ± 1.2	3.7 ± 0.6	2.7 ± 1.0	4.2 ± 1.8	3.5 ± 1.4
17:0	0.5 ± 0.4	0.5 ± 0.8	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ±0.0	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.2	0.0 ± 0.1	0.1 ± 0.1
16:3	0.8 ± 0.5	7.2 ± 1.6	6.1 ± 1.3	6.3 ± 1.3	6.2 ± 1.1	5.7 ± 1.5	4.3 ± 1.9	3.9 ± 1.0	2.1 ± 1.0	2.7 ± 0.8	3.2 ± 5.6	1.4 ± 1.2	0.5 ± 0.3	3.1 ± 2.8	6.3 ± 1.7
16:4(n-1)	0.4 ± 0.3	3.4 ± 1.4	3.5 ± 1.8	3.0 ± 1.0	2.9 ± 1.1	2.7 ± 0.8	1.4 ± 0.9	1.1 ± 0.5	0.3 ± 0.3	0.5 ± 0.4	0.7 ± 1.2	0.4 ± 0.2	0.1 ± 0.1	1.4 ± 2.5	3.7 ± 1.4
18.0	4.4 ± 3.5	1.7 ± 0.8	2.3 ± 1.2	1.5 ± 0.6	2.2 ± 2.1	1.2 ± 0.2	1.6 ± 0.6	1.8 ± 0.7	2.2 ± 1.8	1.3 ± 0.5	1.2 ± 0.3	1.4 ± 0.2	4.2 ± 2.8	3.6 ± 2.7	1.4 ± 0.4
18:1(n-9)	4.4 ± 1.7	3.8 ± 1.1	3.7 ± 1.1	3.0 ± 1.3	3.8 ± 0.7	3.2 ± 0.6	3.9 ± 0.9	4.2 ± 0.8	3.9 ± 1.8	3.9 ± 0.7	4.5 ± 1.2	4.0 ± 0.7	4.9 ± 1.7	5.3 ± 2.0	3.5 ± 1.2
18:1(n-7)	1.8 ± 0.8	0.9 ± 0.3	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.6	0.8 ± 0.9	0.7 ± 0.1
18:2(n-6)	0.9 ± 0.2	1.3 ± 0.6	1.1 ± 0.3	1.0 ± 0.4	1.2 ± 0.3	0.8 ± 0.9	0.8 ± 0.4	1.4 ± 0.9	3.2 ± 4.5	1.0 ± 0.2	1.4 ± 0.6	0.8 ± 0.2	1.0 ± 0.7	0.8 ± 0.7	1.4 ± 0.4
18:3(n-9)	0.5 ± 0.2	1.4 ± 0.8	1.1 ± 0.5	1.0 ± 0.3	1.2 ± 0.5	0.7 ± 0.4	0.7 ± 0.3	1.4 ± 1.1	1.3 ± 0.4	0.8 ± 0.3	1.2 ± 0.6	0.6 ± 0.3	0.7 ± 0.6	0.7 ± 0.6	1.7 ± 0.8
18.4(n-3)	1.1 ± 0.8	5.6 ± 27	6.7 ± 1.4	5.8 ± 0.5	5.2 ± 0.8	4.5 ± 1.0	3.1 ± 0.9	3.6 ± 1.9	1.7 ± 0.6	1.8 ± 0.8	1.3 ± 1.7	1.3 ± 0.6	0.4 ± 0.2	9.4 ± 7.3	7.7 ± 2.4
20:0	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.2	0.2 ± 0.1
20:1(n-9)	4.6 ± 2.5	4.4 ± 0.8	3.6 ± 0.9	4.4 ± 1.2	3.5 ± 1.8	3.0 ± 1.4	3.7 ± 1.7	4.3 ± 2.2	7.4 ± 3.1	6.0 ± 3.1	5.5 ± 3.0	5.2 ± 3.4	6.4 ± 3.5	4.4 ± 1.0	3.7 ± 1.2
20:1(n-7)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.3	0.1 ± 0.1	0.0 ± 0.1	0.6 ± 1.4	0.6 ± 1.7	0.0 ± 0.1	0.1 ± 0.1	0.7 ± 1.6	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
20:4(n-6)	0.3 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.4 ± 0.3	0.4 ± 0.1	0.3 ± 0.2	0.2 ± 0.3	0.5 ± 0.1
20:4(n-3)	0.5 ± 0.3	1.1 ± 0.4	0.9 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	1.3 ± 2.0	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.6	1.0 ± 0.2
20:5(n-2)	9.8 ± 5.5	12.2 ± 3.2	13.4 ± 2.8	12.9 ± 1.7	11.3 ± 4.1	12.4 ± 2.1	11.0 ± 3.0	8.3 ± 1.8	6.3 ± 1.6	8.1 ± 2.1	5.9 ± 4.6	6.3 ± 2.9	5.0 ± 1.8	6.5 ± 6.3	13.1 ± 1.6
22:0	2.9 ± 6.2	0.1 ± 0.1	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.3	0.6 ± 0.6	0.3 ± 0.7
22:1(n-11)	8.3 ± 5.6	9.5 ± 2.9	7.7 ± 2.2	8.3 ± 1.0	9.6 ± 1.8	10.1 ± 2.2	11.8 ± 2.8	11.2 ± 4.6	10.2 ± 5.3	12.9 ± 2.1	11.1 ± 4.6	12.1 ± 2.9	16.3 ± 5.4	8.1 ± 4.3	7.6 ± 1.5
22:1(n-9)	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.7 ± 0.2	0.5 ± 0.2	0.7 ± 0.2	0.5 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	0.4 ± 0.3	0.5 ± 0.4	0.9 ± 0.6	0.4 ± 0.4	0.4 ± 0.1
22:1(n-7)	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.2
22:5(n-2)	1.0 ± 0.7	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	1.3 ± 1.3	0.8 ± 0.4	0.6 ± 0.1	0.6 ± 0.3	0.7 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.4	0.8 ± 0.2
22:6(n-3)	12.8 ± 9.2	4.0 ± 1.7	4.5 ± 3.1	2.6 ± 2.0	2.4 ± 1.9	2.3 ± 1.7	2.6 ± 1.7	3.0 ± 2.2	2.5 ± 1.9	3.7 ± 1.4	1.9 ± 0.7	3.1 ± 1.5	5.6 ± 4.4	2.9 ± 3.4	4.9 ± 2.7
24:2(n-9)	0.6 ± 0.6	0.2 ± 0.3	0.3 ± 0.3	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.4 ± 0.2	0.6 ± 0.3	0.4 ± 0.3	0.6 ± -.3	1.0 ± 0.7	0.4 ± 0.6	0.5 ± 0.7
∑ PUFA	31.8 ± 4.2	40.2 ± 3.5	41.7 ± 3.8	38.1 ± 3.7	35.6 ± 3.2	35.2 ± 3.4	30.4 ± 3.0	28.0 ± 2.3	22.5 ± 1.8	24.6 ± 2.3	20.7 ± 1.7	19.5 ± 1.8	18.0 ± 1.9	30.5 ± 2.9	45.0 ± 3.8
∑ SFA	39.1 ± 6.7	30.1 ± 6.5	31.6 ± 6.6	33.7 ± 7.6	33.7 ± 7.2	33.9 ± 7.9	33.0 ± 7.3	37.8 ± 8.5	38.3 ± 8.4	36.2 ± 8.3	41.4 ± 10.1	41.1 ±10.1	42.3 ± 8.3	41.8 ± 8.5	29.1 ± 6.3
∑ UC	N/A	0.61 ± 0.05	0.64 ± 0.08	0.58 ± 0.04	0.54 ± 0.02	0.54 ± 0.03	0.46 ± 0.07	0.47 ± 0.05	0.38 ± 0.06	0.41 ± 0.06	0.44 ± 0.1	0.33 ± 0.08	N/A	0.49 ± 0.07	0.53 ± 0.09

Table 3.1 Relative composition of fatty acids (mean % of total fatty acids) in total lipid of CV *C. finmarchicus* from Loch Etive over a seasonal cycle. PUFA are polyunsaturated fatty acids, SFA are saturated fatty acids and UC is the unsaturation coefficient.

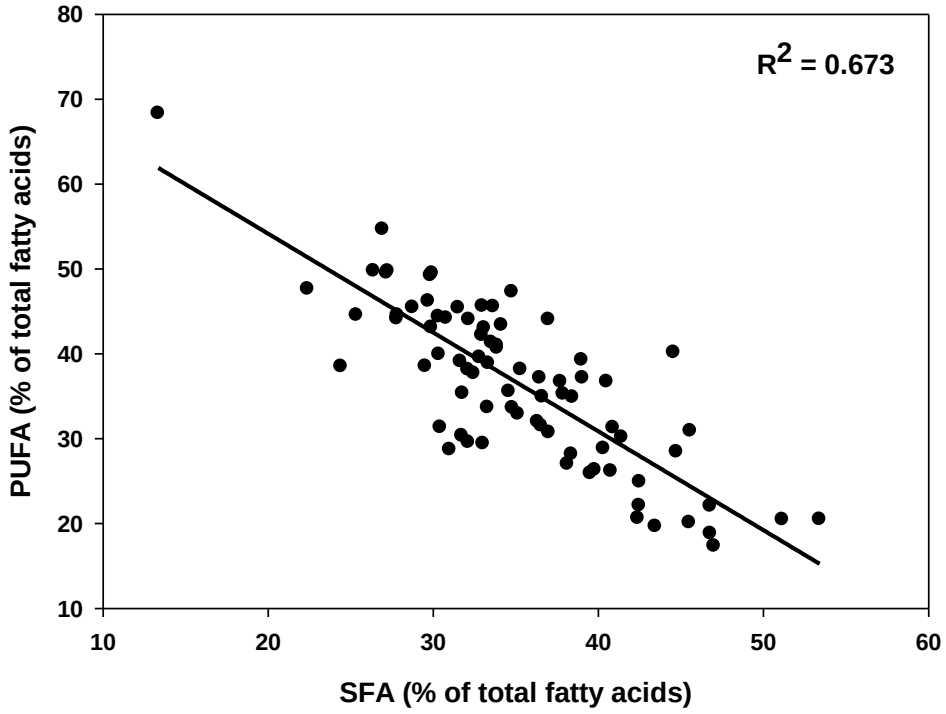

Fig 3.4. Regression of the sum of the polyunsaturated fatty acids (PUFA) against the sum of saturated fatty acids (SFA).

The lipid class and relative fatty acid contributions of all 79 individual and 15 bulk *Calanus finmarchicus* lipid samples from the 15 months were subjected to principal component analysis (PCA). The first two principal components accounted for 61.8% of the total variation within the data set. 22:6(n-3), 20:5(n-2), 18:4(n-3), 16:4(n-1), TAG, FFA, sterol and PL made a significant (>0.1) positive contribution to PC1, while 22:1(n-11), 16:1(n-7), 14:0 and WE made a significant negative contribution (Fig 3.5). WE, TAG, 20:5 (n-2), 18:4 (n-3), 18:0 and 16:4(n-1) made a significant positive contribution to PC2, whilst 22:6(n-3), 22:1(n-11), 20:1(n-9), 18:1(n-9), 16:0, FFA, sterol and PL made a significant negative contribution (Fig 3.5).

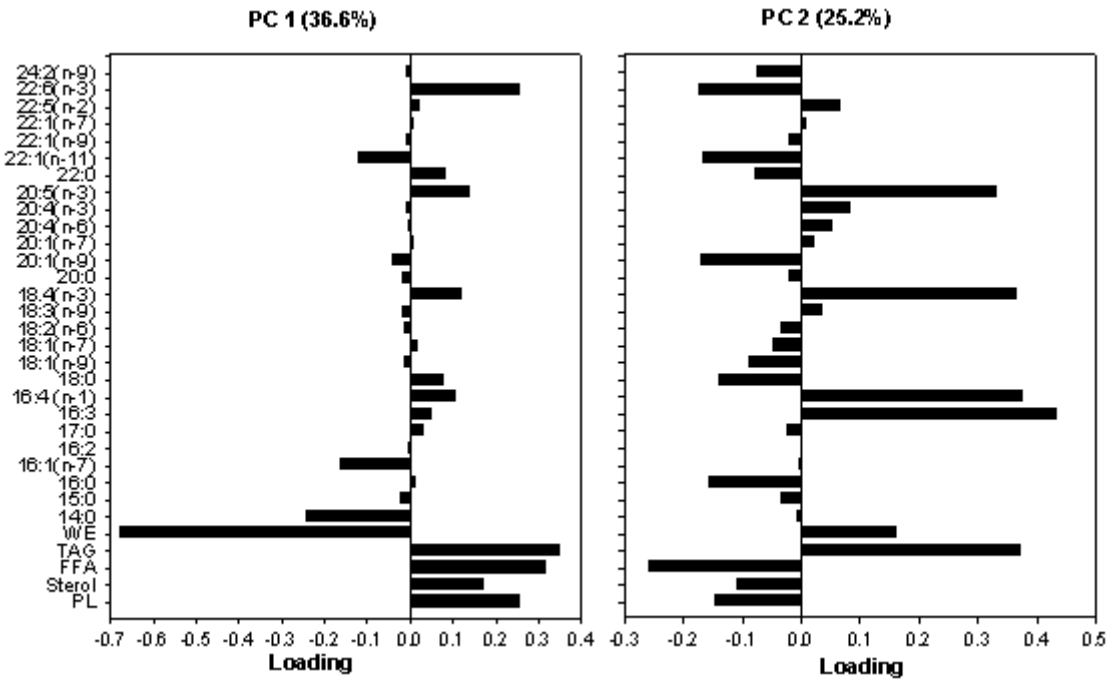

Fig 3.5. Variables affecting PC1 (left) and PC2 (right) in the principal component analysis.

Ordination of all the copepods on PC1 and PC2 followed by cluster analysis split the samples into two main groups (Fig 3.6). Cluster 1, termed the spring-summer cluster, contained the individual *C. finmarchicus* collected in May, June, July, August and September 2006 and May and June 2007. Cluster 2, termed the autumn-winter cluster, contained individuals collected in November, December, January, February and March. Samples collected in October were split between the two clusters, and samples collected in April 2006 and 2007 were entirely separate from either of the aforementioned clusters and not clustered together themselves. Two smaller groups were also formed, one within cluster 1 grouped together by slightly higher relative percentages in PL, sterol and FFA and the other between clusters 1 and 2 which appears to contain mostly samples from September and October although outliers from spring-summer and autumn-winter are also present. Samples in the spring-summer cluster have significantly higher relative percentages of the fatty acids 16:3, 16:4(n-1), 18:4(n-3) and 20:5(n-3) and a significantly lower percentage of 20:1(n-9)

than samples from the autumn-winter cluster (Students' t-test, $p<0.01$). Samples from April 2006 have significantly higher amounts of 22:6(n-3) and significantly less of 18:4(n-3) than both of the clusters, and significantly less of 16:4(n-1) than the spring-summer cluster ($p<0.05$) and had a significantly lower WE content than both of the clusters ($p<0.05$, one way ANOVA, Tukey's Test) which is the variable that has separated these samples from the clusters. One sample from May 2007 has also been separated from the clusters, this was also a sample with a low % WE.

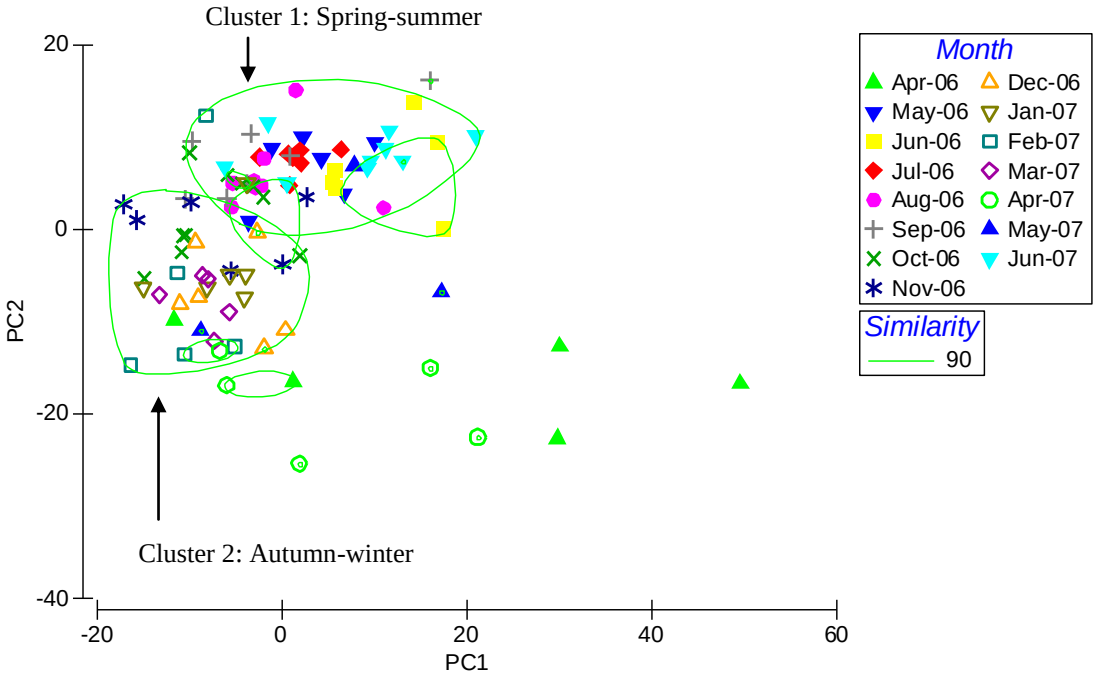

Fig 3.6. Ordination plot by non-metric multidimensional scaling (MDS) and principal components (PCA) showing clustering of samples.

3.4 DISCUSSION

Wax esters formed on average >80% of the total lipid stored by *Calanus finmarchicus* in Loch Etive. This is a similar finding to some previous studies (Kattner and Krause, 1987; Kattner and Hagen, 1995; Jónasdóttir, 1999). The high variability in lipid content evident between individuals in this study (Fig 3.3) has also been reported for animals elsewhere (Båmstedt, 1988; Madsen et al., 2008) and is probably due to population plasticity in response to environmental changes, variation in metabolic rates and feeding abilities and the mechanisms of diapause. Total lipid was high from May 2006 to October (Fig 3.3), characteristic of animals preparing for diapause (Irigoien, 2004). Animals collected in Loch Etive in December (thought to be in diapause, see Chapter 2) had 50% smaller lipid stores than animals collected in October, which suggests lipid stores are being used up during overwintering. This value is considerably more than the 5% that Jónasdóttir (1999) calculated from animals overwintering in the Farøe-Shetland Channel. Other studies have also recorded a decrease in lipid stores in copepods during diapause (e.g. Hopkins et al., 1984; Evanson et al., 2000; Saumweber and Durbin, 2006). In the present study, the animals collected in December potentially may have been approaching the end of diapause and utilising part of the lipid store for gonad development prior to completing the moult to the adult stage and ascending (Lee et al., 2006). The decrease in lipid stores from October to December and the appearance of some adult females in the net samples in January (Fig 2.6), which indicates that some animals have already begun to emerge from diapause during this month, fits with the theory that animals may terminate diapause when the lipid store declines below a certain threshold level (Miller et al., 1991; Hirche, 1996; Ohman et al., 1998; Visser and Jónasdóttir, 1999; Irigoien, 2004; Saumweber and Durbin, 2006). The decrease in total lipid from

October to December observed in *C. finmarchicus* collected from Loch Etive could also have been due to the utilisation of the lipid store to sustain metabolism through diapause (Saumweber and Durbin, 2006). TAG was a minor component of the lipid content in the present study and peaked in June 2006 and June 2007, indicating recent feeding activity (Håkanson, 1984). The small amounts of TAG present in individuals from Loch Etive in the winter months October-March suggests that the animals were not feeding intensively at that time, which may be expected given the reduced levels of primary production likely then (Wood et al., 1973). However it is not possible without further study to determine if the copepodites ceased feeding in December: a measure of feeding activity, such as gut content analysis, would be required for this.

Under certain conditions such as low availability of preferred prey, *Calanus* spp. can form a link between the microbial food web and higher trophic levels (Runge and LaFontaine, 1996). In my study the ratio of PUFA: SFA was smallest during February, March and April 2007, significantly less than in the spring-summer months. However the UC did not change significantly between months and no other bacterial markers appeared to be more abundant during February, March and April 2007. This indicates that during this period *C. finmarchicus* in Loch Etive had not assimilated more microbial material, suggesting further that they had not switched to microbial prey which may be expected as the winter phytoplankton standing crop is not thought to be particularly low in Etive when compared to the open ocean (Wood et al., 1973).

The dietary fatty acids present in *Calanus finmarchicus* did appear to vary through the year in Loch Etive. PCA analysis split the year into a spring-summer cluster and an autumn-winter cluster (Fig 3.6). The individuals within the spring-summer cluster

appeared to have a diet rich in diatoms and dinoflagellates, indicated by much higher abundances of the diatom markers 16:4(n-1) and EPA, and the dinoflagellate marker 18:4(n-3), than the autumn-winter cluster. The individuals from the autumn-winter cluster still contained diatom and dinoflagellate markers, but these made up a significantly smaller portion of the total fatty acids. The reduction in the abundance of diatom and dinoflagellate markers in the total fatty acid profile from spring-summer to autumn-winter may be a reflection of utilisation of these fatty acids from the storage lipid, either to be used in gonad development or synthesised into other fatty acids. The natural succession of phytoplankton from diatoms to flagellates in coastal waters is mirrored with a decrease in the 16:1/16:0 ratio (Jeffries et al., 1970). In this study, the abundance of the diatom marker 16:1(n-7) did not change significantly through the year, thus the ratio of 16:1(n-7): 16:0 did not change significantly. Diatoms appear to make up part of the diet of *C. finmarchicus* throughout the year in Loch Etive, consistent with the study by Wood et al. (1973) who reported that the dominant diatom species in the lower basin of Loch Etive was *Skeletonema costatum*, which persisted in abundance throughout the year, rarely declining to concentrations less than 10^4 cells l^{-1}.

The differences in lipid composition which separated one group of four individuals sampled in April 2006 from other samples were characterised by low total lipid content, low relative WE content and high relative FFA, sterol, and PL (Fig 3.3). The small WE content suggests that these animals have utilised their lipid stores. This has not occurred in the other two individuals collected in April 2006 that show the more usual high percentage composition of WE and smaller amounts of FFA, PL and sterol. The animals collected during April 2006 had significantly higher levels of the fatty

acid 22:6 (n-3) (Docosahexaenoic acid, DHA) than animals from the other months. DHA is a trophic marker for dinoflagellates (Dalsgaard et al., 2003), but it is also a component of membrane lipids in marine organisms (Jain et al., 2007). Severe starvation in copepods is characterised by major losses of storage relative to structural lipid (Lee et al., 1970), which may result in elevated levels of membrane lipids such as DHA (Lee et al., 1971). DHA is also thought to act as an antioxidant when a cell is subjected to oxidative stress such as starvation (Mukherjee et al., 2004). The low lipid content, low storage lipid (WE) and high structural lipid (sterol, FFA and PL) component of the four individuals from April 2006 may indicate that these animals were starved, possibly indicating late emergence from diapause. The slightly elevated levels of TAG found in two of these four individuals may indicate recent feeding in these animals (Håkanson, 1984).

The role of lipid accumulation in diapause initiation in *C. finmarchicus* is still unknown. Evidence supporting the 'lipid accumulation window hypothesis' is based solely on observations; it has not yet been possible to persuade *C. finmarchicus* to enter diapause in the laboratory and the link between lipid accumulation and diapause is consequently very difficult to prove. The decrease of total lipid during diapause in *C. finmarchicus* from Loch Etive fits with the theory that animals may terminate diapause when the lipid store declines to a certain level (Miller et al., 1991; Hirche, 1996; Ohman et al., 1998; Visser and Jónasdóttir, 1999; Irigoien, 2004; Saumweber and Durbin, 2006) and suggests that *C. finmarchicus* are utilising a significant amount of reserves during diapause. Whether or not lipids are the trigger mechanism for some CV to enter diapause and some to remain in the surface waters over winter, there will likely still be hormonal and genetic processes involved in determining the switch to

diapause and these may be a better target for investigation (Tarrant et al., 2008). The link between lipid accumulation and a specific genetic or hormonal signal may provide evidence to support or reject the lipid accumulation window hypothesis.

CHAPTER 4: Cloning of the retinoid X receptor (RXR) and gene expression patterns associated with diapause in *Calanus finmarchicus*

4.1 INTRODUCTION

Diapause in copepods is probably controlled by a host of mostly unknown physiological and cellular mechanisms with an associated characteristic gene expression pattern. Previous studies have looked at physiological causes and effects of diapause (e.g. Lee and Hirota, 1973; Hirche, 1983, 1996; Jónasdóttir, 1999; Visser and Jónasdóttir, 1999; Tande and Miller, 2000; Campbell et al., 2004; Irigoien, 2004; Heath et al., 2004) but no gene expression patterns have yet been resolved. This chapter addresses this issue.

The initial publication of *c*. 6000 expressed sequence tags (ESTs) on the GenBank[3] database in 2007 was a huge step forward for gene expression studies in *Calanus finmarchicus*. At the time of writing, there are now *c*. 11,000 *C. finmarchicus* EST sequences deposited in GenBank and they have already formed the base of gene expression studies (Hansen et al., 2007, 2008a, 2008b; Tarrant et al., 2008; Christie et al., 2008). There has only been one study so far documenting gene expression associated with diapause in *C. finmarchicus*: Tarrant et al. (2008) looked at diapausing 'deep-water' copepods and compared gene expression of six genes with 'shallow-water' *C. finmarchicus* caught at the same time and location, which they assumed to be active animals, i.e. not in diapause. Three genes that are associated with lipid synthesis, transport and storage (Tarrant et al., 2008) were found to be expressed at higher levels in active copepods, compared to diapausing ones, while

[3] http://www.ncbi.nlm.nih.gov/ [acessesed 27/03/09]

expression of a gene encoding ferratin, a protein associated with preventing lipid oxidation, was observed to be higher in diapausing copepods. Apart from this study, little is known about the genes that may regulate diapause in *C. finmarchicus* or any copepod species.

In most insect species, development and reproduction are regulated by the sesquiterpenoid juvenile hormone (JH) and the steroid ecdysone (Highnam and Hill, 1977; Gade et al., 1997; Gilbert et al., 2000; Spindler-Barth and Spindler 2003; Riddiford et al., 2001) and these two hormones are potential candidates for diapause regulation in *C. finmarchicus.* The crustacean version of JH, methyl farnesoate (MF) appears to have similar functions in crustaceans to that of JH in insects; MF is involved in regulating reproduction (Rodreguez et al., 2002; Nagaraju et al., 2004), morphogenesis (Rotllant et al., 2000), and the moulting cycle (Homola and Chang, 1997; Nagaraju et al., 2004). MF could be involved in diapause regulation in *Calanus finmarchicus* by interacting with the hormone ecdysone to control development through the moulting process (Irigoien, 2004). In decapod crustaceans MF is synthesised in the mandibular organ from farnesoic acid by the enzyme S-adenosyl-L-methionine farnesoic acid *O*-methyl transferase (FAMeT, Fig 4.1) (Wainwright *et al*., 1998).

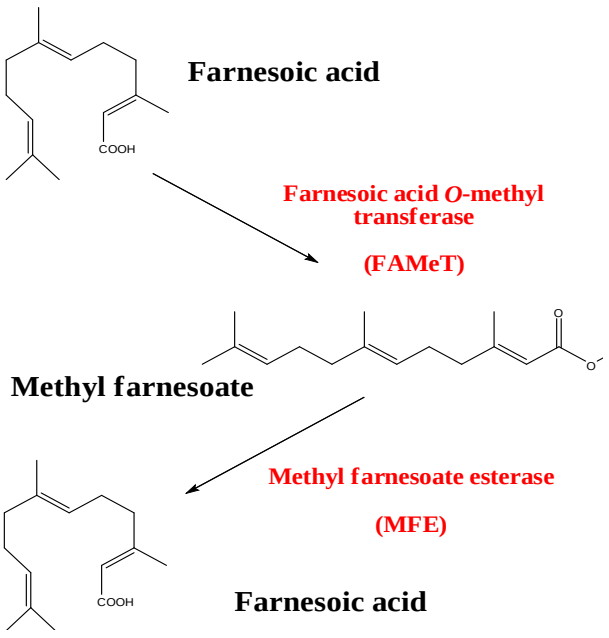

Fig 4.1 Biological pathway illustrating the enzymes involved in synthesis and metabolism of MF.

The ecdysteroids (primarily the active form 20-hydroxyecdysone) elicit their regulatory response by binding to the ecdysteroid receptor (EcR; LeBlanc, 2007). EcR is a nuclear hormone receptor, the full nucleotide sequence of which has been characterised in many insect species and in some crustaceans (*Celuca pugilator*, GenBank accession number AAC33432; *Carcinus maenas*, AY496928; *Gecarcinus* l*ateralis*, AAT77808; *Litopenaeus vannamei*, AAQ2460). Partial EcR mRNA transcripts have been deposited in GenBank for *C. finmarchicus* (ABQ57403, Tarrant et al., 2008). EcR coordinates arthropod development and metabolism, by regulation of gene transcription in association with the retinoid X receptor (RXR; Spindler-Barth and Spindler, 2003). RXR is a multifunctional nuclear hormone receptor, present in vertebrates and invertebrates (Oro et al., 1990). It functions as a transcriptionally active receptor either alone or with other nuclear receptors in a ligand dependant or independent manner (Mangelsdorf and Evans, 1995). RXR contains a DNA binding domain and a ligand binding domain, as seen in all nuclear hormone receptors (Germain et al., 2006). There is much more understanding of the mechanisms of RXR action in the vertebrates than in the invertebrates. In many vertebrates RXR functions with retinoic acids (RAs) to regulate various processes such as development, differentiation and homeostasis (Evans, 1988). There are many active forms of RA that bind to RXR in vertebrates: geometric isomers, hydroxlated forms and epoxidised forms are all known to be active *in vivo* (Marill et al., 2003). RXRs have been identified in vertebrates as important factors necessary for efficient binding to DNA of several members of the nuclear hormone receptor family, by forming heterodimers (Germain et al., 2006). In vertebrates, RXR can also form homodimers *in vitro* that can bind to DNA, suggesting the existence of RXR-specific signalling (Mangelsdorf et al., 1991). Jones et al. (2006) suggested that the same principles potentially applied

to arthropods as well, using MF/JH as an example. As with RA, several variations in the structure of MF/JH have been reported (Gadot et al., 1987; Mauchamp et al., 1999; Darrouzet et al., 1997). The insect RXR equivalent, ultraspiricle (USP) has been shown to bind JH in *Drosophila melanogaster* but with low affinity - 100 times lower than expected for a nuclear receptor, but enough to cause physiological effects and transcriptional activity (Jones and Sharp, 1997; Jones et al., 2001; Xue et al., 2002). However Jones et al. (2006) showed that MF bound to *D. melanogaster* USP with nearly a 100-fold higher affinity than JH and, at times, MF production was detected to be at much higher rates than JH. Barchuk et al. (2004) demonstrated that downregulation of USP gene expression delayed pupal diapause in honeybees. Expression of RXR, or the RXR/EcR complex, could potentially regulate transcription leading to the regulation of diapause in *C. finmarchicus*. EcR would be expected to be upregulated in the months the copepods are "active" prior to diapause, but to be downregulated during diapause. RXR may be expected to also be up-regulated prior to diapause if it is acting as a heterodimer allowing efficient binding to DNA by the EcR/RXR complex. If RXR is separately involved in regulating transcription leading to maintenance of diapause, potentially by the binding of MF, then it may be expected to act differently during diapause. MF may be involved in initiation or termination of diapause. If MF acts as JH functions in several species of lepidopteran insects, sustaining larval diapause, MF concentration would be expected to build prior to initiation of diapause and to remain high until the trigger for termination of diapause is received and MF titre would drop (Chippendale and Yin, 1973; Bean and Beck, 1980, 1983; Munyiri and Ishikawa, 2004; Eizaguirre et al., 2005). If MF acts as JH does during adult diapause of many insect species, MF titre would drop prior to diapause, be absent during diapause and build up slowly prior to

termination and release of ecdysone (Denlinger, 2002). Thus if MF is potentially a ligand for RXR, RXR expression may either be up-regulated during diapause, with an associated decrease in expression prior to emergence from diapause, or down-regulated with an associated increase in expression prior to emergence and release of ecdysone.

In insects three types of peptides that effectively inhibit JH synthesis have been characterised: A-type allatostatins (A-type ASTs); B-type ASTs and C-type ASTs (Stay and Tobe, 2007). To date, only A-type ASTs - peptides possessing the carboxy(C)-terminal motif –YXFGL/I amide- have been identified in crustaceans (Duve et al., 1997, 2002; Dircksen et al., 1999; Fu et al., 2005; Yin et al., 2006 Christie et al., 2008). Christie et al. (2008) identified a gene encoding an A-type allatostatin in *Calanus finmarchicus*. Few functional studies of ASTs in crustaceans have been conducted, however Kwok et al. (2005) suggested that the regulation of sesquiterpenoid production might be one such function. Thus A-type ASTs may regulate the production of MF, perhaps inhibiting synthesis on termination of diapause. The expression of the gene encoding the A-type AST identified by Christie et al. (2008) may also provide an indictor that MF is potentially involved in diapause.

In this study it was initially attempted to characterise the mRNA transcript of the enzyme involved in synthesising MF - FAMeT, but no part of the mRNA transcript could be isolated. After the publication of *c*. 6000 ESTs in the Genbank database in 2007, which did not include FAMeT, the RXR mRNA transcript from *C. finmarchicus* was characterised and expression of *RXR*, *EcR* and *A-type AST* genes was measured over a seasonal cycle using real-time quantitative PCR.

4.2 MATERIALS AND METHODS

4.2.1 Animal collection

All *Calanus finmarchicus* used for genetic analysis were in the stage CV. Animals from two locations were used: a time series of *C. finmarchicus* CV from Loch Etive, the collection of which has been described in Chapter 2 of this thesis, and animals overwintering in the Farøe-Shetland Channel (Table 4.1) from December 2006. The samples collected in Loch Etive were preserved in two ways; firstly some animals were preserved on board the RV *Seol Mara* in RNA*later*® (Ambion, Warrington, UK), however the stage of these animals could not be determined before preservation. Secondly, animals were transported back to the Scottish Association for Marine Sciences Dunstaffnage Marine Laboratory and identified live; CVs were separated into vials and flash-frozen in liquid nitrogen. Farøe-Shetland Channel animals were collected from various depths at three locations using an ARIES net (Dunn et al. 1993), from a cruise aboard the FRV Scotia undertaken by FRS Marine Laboratory, Aberdeen (Cruise 1906S, 2006). On this cruise zooplankton were sorted on ice immediately after recovery of the net. *Calanus* spp. CV were removed and flash-frozen in liquid nitrogen. Samples were stored in liquid nitrogen onboard the ship, then stored at –80^{o}C on return to the laboratory.

Date collected	Station coordinates	Sample depth (m)
16/12/2006	60^{o} 29.00’ N 04^{o} 26.00’ W	528
16/12/2006	60^{o} 29.00’ N 04^{o} 26.00’ W	579
16/12/2006	60^{o} 29.00’ N 04^{o} 26.00’ W	851
18/12/2006	61^{o} 35.00’ N 04^{o} 15.00’ W	920
18/12/2006	61^{o} 35.00’ N 04^{o} 15.00’ W	325
18/12/2006	61^{o} 28.00’ N 03^{o} 42.00’ W	3.5
18/12/2006	61^{o} 28.00’ N 03^{o} 42.00’ W	948

Table 4.1 Coordinates and depths from which *C. finmarchicus* CV were collected in the Farøe-Shetland channel.

4.2.2 Isolation of total RNA and cDNA synthesis

Total RNA was isolated from stage V individual *Calanus finmarchicus* from Loch Etive that had been sorted live at the Dunstaffnage Marine Laboratory and flash-frozen in liquid nitrogen. Total RNA was isolated by homogenising individual copepods in 50 µl of TRI Reagent® (Sigma-Aldrich, Poole, UK) and incubated for 5 min at room temperature (RT). To each sample 20 µl of chloroform were added and, after shaking, the samples were incubated at RT for 10 min. The RNA in the aqueous phase was removed by centrifugation at 12,000 × g for 15 min at 4°C and mixed with 50 µl of 100% isopropyl alcohol. The samples were then further incubated for 10 min at RT, and re-centrifuged under the same conditions. The supernatant was discarded and the RNA pellet washed with 100 µl 75% ethanol. After further centrifugation for 5 min at 7500 × g, the excess ethanol was removed and the RNA pellet air-dried for about 10 min. The pellet was then dissolved in 10 µl of DEPC-treated water and stored at –70°C until use. Total RNA was quantified using a nanodrop by measuring absorbance at 260 and 280 nm. Only samples with an absorbance ratio (260 nm/280 nm) between 1.7-2.0 were used. Further quality checks were made by running aliquots of denatured RNA on a 1% agarose gel to examine for degraded samples. cDNA was synthesised by incubating 2 µg of extracted RNA at 70°C for 10 minutes with 2 µg oligo dT and 1 µl 10 mM dNTP mix (both Promega, Southampton, UK). To each sample 1 µl M-MLV enzyme, 2 µl M-MLV buffer, 0.1 µl RNAsin (all Sigma-Aldrich) and DEPC-treated water to a total volume of 20 µl were added and all were incubated first at 37.5°C for 1 hour, and then at 70°C for 10 min. The resulting cDNA was stored at –20°C until use.

4.2.3 Attempted characterisation of FAMeT in *Calanus finmarchicus*

Using the BLAST[4] tool, FAMeT amino acid sequences from 10 crustacean and 5 insect species were retrieved (Table 4.2) and aligned (Fig 4.2). Conserved regions within the sequences were used to design degenerate primers (Table 4.3) to isolate the FAMeT sequence from *Calanus finmarchicus*.

Crustacea	**GenBank Accession No.**	**Insecta**	**GenBank Accession No.**
Cancer pagurus	AAR00732	*Drosophila melanogaster*	NP611544
Metapenaeus ensis	AAK28535	*Aedes aegypti*	ABF18366
Homarus americanus	AAA67081	*Tribolium castaneum*	XP970560
Penaeus monodon	ABA86955	*Apis mellifera*	XP623146
Scylla serrata	ABA86954	*Belgica antarctica*	ABF72903
Portunus pelagicus	AAZ40198		
Thenus orientalis	ABA86962		
Cherax quadricarinatus	ABA86960		
Litopenaeus vannamei	AA222181		

Table 4.2 FAMeT sequences obtained from GenBank.

Primer name	**Sequence**
FAMeT F1	5' GCAAGGGCGGCGANKGNGARCC 3'
FAMeT F2	5' AARGTNGAYACNCCNGAYAT 3'
FAMeT F3	5' GMNGARTAYMGNGARTTYTGG 3'
FAMeT F4	5' GCNCAYGAYKGYCAYRTNGC 3'
FAMeT F5	5' GGNACNGAYGARAAYAARGARTA 3'
FAMeT F6	5' TTYATHGGNGSNTGGGARGGNGC 3'
FAMeT F7	5' CAYTAYGGNTAYWSNACNGGNTGG 3'
FAMeT F8	5' GARRTNTTCATYGGNGGNTGG 3'
FAMeT R1	5' CGGATGGCGGAGTGYTRTTY 3'
FAMeT R2	5' CGNSWRTGYTGRTTYTCCCA 3'
FAMeT R3	5' TTCCAYTCNGGRTCNGTCCA 3'
FAMeT R4	5' TTRTANGTNARRARTCYTCNGT 3'
FAMeT R5	5' AAYTTNCKYTCYTCYTCRCARCA 3'
FAMeT R6	5' TCNCCNYCYTTNCCNAC 3'

Table 4.3 Degenerate primers used in the attempt to amplify a fragment of FAMeT.

[4] http://blast.ncbi.nlm.nih.gov/Blast.cgi [accessed 28/03/09]

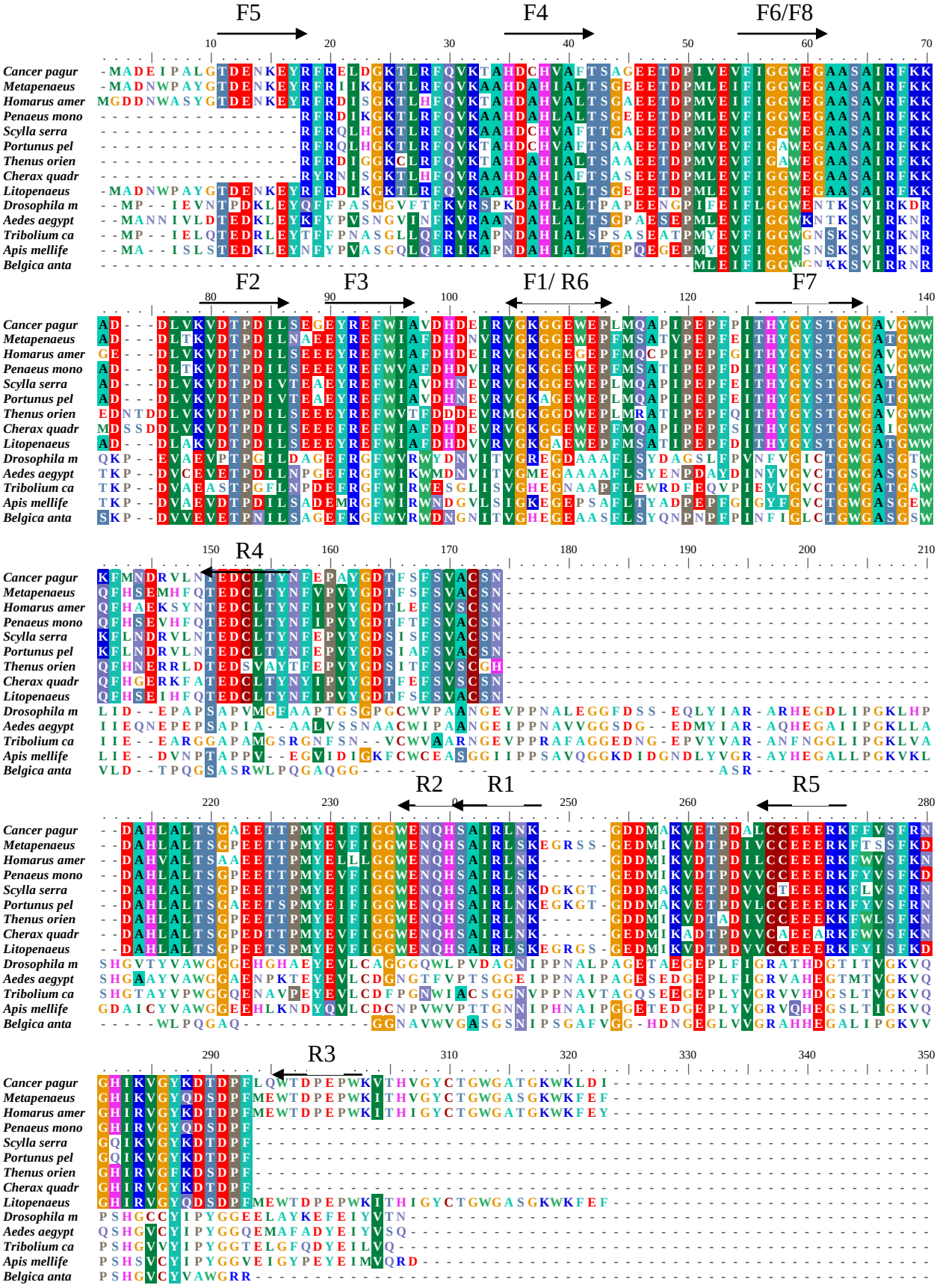

Fig 4.2 Alignment of the FAMeT protein sequences from Crustacea and Insecta. Conserved amino acids are shown by blocks of the same colour. Primer sites are shown by arrows.

Despite many changes in primer combinations and PCR cycling conditions, a product could not be amplified by PCR. Possibly the primers were too degenerate to amplify FAMeT. When the 6000 EST's from *C. finmarchicus* were deposited in GenBank in March 2007, no sequence resembling FAMeT was contained within them, however two of these EST's (accession numbers: EL666291; EL666280) were identified as similar to a juvenile hormone esterase (JHE) from the ladybird *Harmonia axyridis*. JHE metabolises juvenile hormone in insects (Kamita et al., 2003). MF esterases have been isolated in crustaceans (Nagaraju, 2007) however, unfortunately, there was no sequence information available for crustacean MF esterases in the databases. Despite the similarity between the *C. finmarchicus* sequences and the JHE sequences, it could not be proven that this enzyme specifically targets MF and not any other carboxyl esters, and so this enzyme cannot be used to link MF with diapause. However, an EST similar to RXR in other Crustacea was present and the potential involvement of RXR and EcR in regulating diapause made RXR a better target for investigation.

4.2.4 Characterisation of RXR in *Calanus finmarchicus*

Using the BLAST tool, a 624 bp *Calanus finmarchicus* EST published in GenBank (EL965886) similar to *RXR* in other Crustacea was found. The deduced amino acid sequence of this EST was aligned using the Clustal W software (Chenna et al., 2003) with known *RXR* mRNA sequences from crustaceans and insects obtained from GenBank (Fig 4.3).

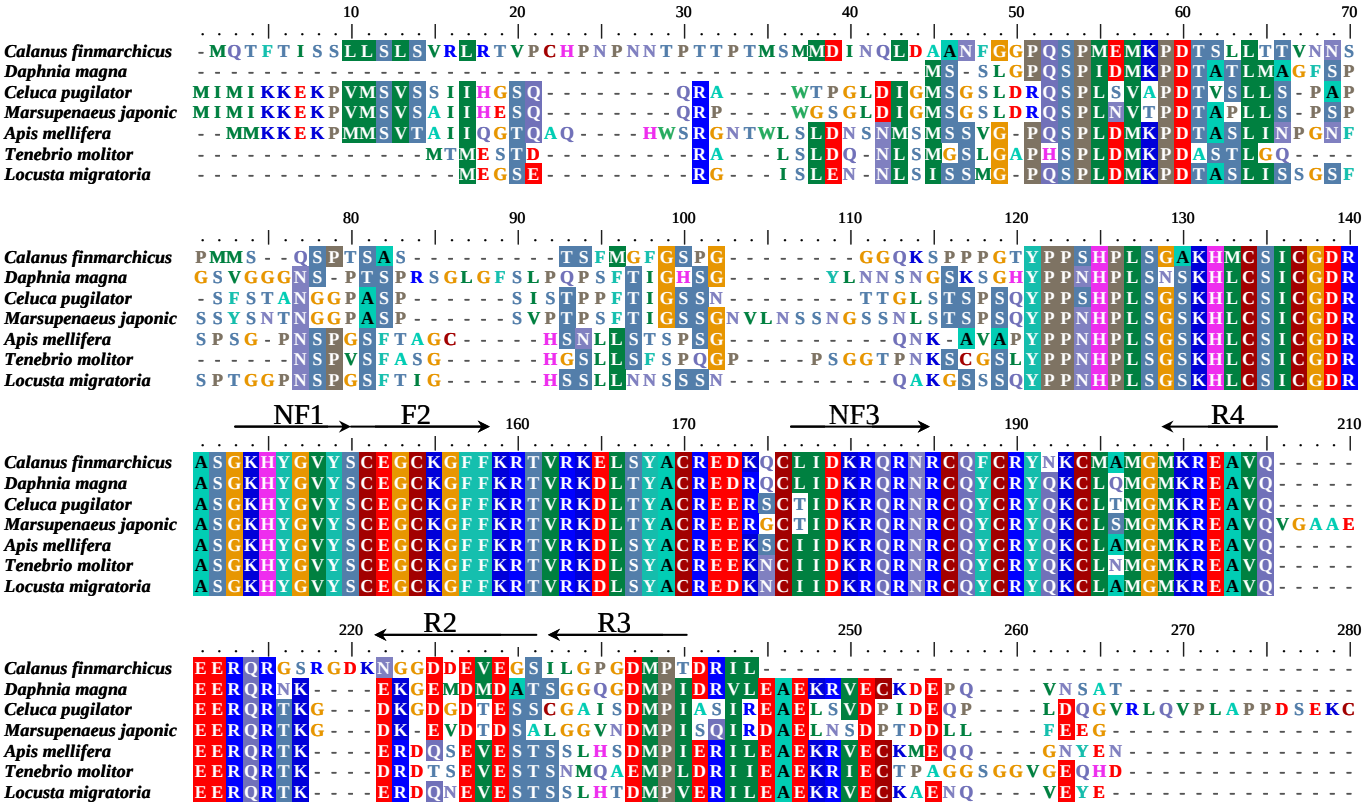

Fig 4.3 Alignment of the *C. finmarchicus* EST EL965886 and RXR protein sequences from Crustacea and Insecta. Conserved amino acids are shown by blocks of the same colour. Primer sites are shown by arrows.

This EST was used to design specific primers for PCR (RXR F2 and R2; Fig 4.3, Table 4.4), which were then used in PCR to amplify a 225 bp product from *C. finmarchicus* cDNA. PCR conditions were: 1 cycle at 94^{o}C for 4 min followed by 35 cycles of 30 sec at 94^{o}C, 30 sec at 48^{o}C and 1 min at 72^{o}C with a final extension of 4 min at 72^{o}C. Each reaction contained 0.5 µl of *C. finmarchicus* cDNA, 0.25 µl of 25 pmol µl^{-1} each of RXR F2 and RXR R2 primers, 1 unit (0.2 µl) GoTaq Polymerase, 5 µl of 5x GoTaq buffer, 0.5 µl dNTP mix (all Promega) and 18.3 µl sterile water. After PCR a ~ 200 bp product was identified on a 1.5% agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Crawley, U.K).

RXR F2	5’ TGTGAGGGCTGTAAGGGTTT 3’
RXR NF1	5’CAAGCACTATGGTGTTTACTCCTGT 3’
RXR NF3	5’GATTGACAAGAGGCAGAGGAAC 3’
RXR R2	5’TTCCCTCCACTTCATCATCC 3’
RXR R3	5’GCATGTCACCAGGTCCAAGG 3’
RXR R4	5’CCTGCACTGCCTCTCTCTTCATC 3’

Table 4.4 Primers designed to amplify the 225 bp RXR *C. finmarchicus* cDNA product and the 3’/5’ RACE products.

The isolated 200 bp fragment was cloned using a TOPO TA cloning kit (Invitrogen, Paisley, UK; pCR 2.1 TOPO vector, TOP 10 *Escherichia coli*) following the manufacturer’s instructions. Bacterial colonies containing ~200 bp inserts were cultured overnight in LB broth (2.5 % w/v, pH 7; BD, Oxford, UK) at 37^{o}C, and the cDNA-containing plasmids were isolated using High Pure Plasmid Isolation Kit (Roche, Welwyn Garden City, UK) following the manufacturer’s instructions and sequenced by Eurofins MWG using M13 vector-specific primers. The deduced amino acid sequence of the fragment was aligned to the deduced amino acid sequence of the 624 bp *C. finmarchicus* EST and published RXR protein sequences in insects and crustaceans using the Clustal W software (Chenna et al., 2003) and showed homology to those sequences (Fig 4.4).

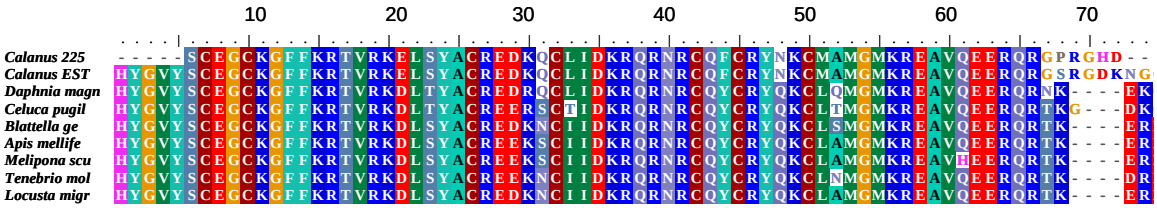

Fig 4.4 Alignment of the 202 bp fragment of *C. finmarchicus* cDNA with the *C. finmarchicus* EST EL965886 and RXR protein sequences from Crustacea and Insecta. Conserved amino acids are shown by blocks of the same colour.

4.2.5 3’ and 5’ RACE (Rapid Amplification of cDNA Ends)

RACE was performed using a 5’/3’ RACE kit, 2nd Generation (Roche). The isolated 202 bp *Calanus finmarchicus* RXR sequence was used to design specific primers (Fig 4.3, Table 4.4). 3’ RACE was performed first, 2 µg Total RNA from an individual *C. finmarchicus* stage CV collected in Loch Etive during February 2008 was used with kit reagents for first-strand synthesis. The resulting cDNA was used in a PCR reaction with RXR NF1 primer (Table 4.4) and the anchor primer provided in the kit. The running conditions were: 1 cycle of 94°C for 4 min, 35 cycles of 30 sec at 94°C, 30 sec at annealing temperature of 55°C and 2 min at 72°C finished with 1 cycle of 7 min at 72°C. A nested PCR was then performed using RXR NF3 (Table 4.4) with the anchor primer provided and 1 µl of 1:20 diluted PCR product from the first-round PCR, using the same PCR conditions. PCR reagents and concentrations for all RACE PCR reactions were the same as described in section 4.2.4. A ~1400 bp product was identified on a 0.8% agarose gel. As previously this product was gel-extracted using the QIAquick Gel Extraction Kit (Qiagen) and cloned with a TOPO vector kit (Invitrogen). Five positive clones were randomly selected for sequencing by Operon MWG. A 1387 bp sequence with homology to RXR in other Crustacea was identified using a BLAST search. 5’RACE was performed following the kit protocol, 2 µg of Total RNA from the same sample as that used in 3’ RACE was used with RXR R3 for first strand synthesis of RNA. The products were cleaned up using the High Pure PCR Product Purification Kit (Roche), before poly(A) tailing of the first strand cDNA, followed by PCR amplification of the dA-tailed cDNA using the anchor primer provided with the kit and RXR R2 (Table 4.4.) The PCR conditions used were: 1 cycle of 94°C for 4 min, 35 cycles of 30 sec at 94°C, 30 sec at annealing temperature of 53°C and 1.5 min at 72°C finished with 1 cycle of 7 min at 72°C. A

nested PCR was then performed using RXR R4 (Table 4.4) with the anchor primer provided and 1 µl of 1:20 diluted PCR product from the first-round PCR. The PCR conditions used were the same except the annealing temperature was modified to 59^{o}C. Several products around the expected size (~300 bp) were identified, gel-extracted and cloned as with 3' RACE. Five randomly selected positive clones were sequenced by Operon MWG and returned a 352 bp sequence with homology to RXR in other Crustacea. From the sequences obtained from both 3' and 5' RACE a putitative full-length 1759 bp cDNA sequence of RXR was deduced, along with a complete amino acid sequence, which was then compared to published sequences using the BLAST tool (Fig 4.5).

4.2.6 Phylogenetic analysis

The full-length *Calanus finmarchicus* RXR amino acid sequence was aligned to known *RXR* sequences from Crustacea, Chelicerata, Insecta and Cnidara (Table 4.5, Fig 4.5); all derived from the NCBI's GenBank database using the Clustal W software. The amino acids forming the separate domains of *RXR* were also all aligned separately from the same sequences. Percentage similarity to *C. finmarchicus RXR* was calculated from these alignments using the BIOEDIT program using the identity algorithm. The alignment of the ligand-binding domain of *C. finmarchicus RXR* was used for phylogenetic analysis and analysed using the neighbour-joining method by Clustal X with 1000 bootstrap repetitions.

	Species	GenBank Accession number
Crustacea	*Daphnia magna*	ABF4729
	Celuca pugilator	AAC32789
	Marsupenaeus japonicus	AB295493
	Gecarcinus lateralis	AAZ20369
Insecta	*Tenebrio molitor*	CAB75361
	Locusta migratoria RXR I	AAQ55293
	Amblyomma americanum	AAC15589
	Aedes aegypti	AAG24886
	Drosophila melanogaster	NP_476781
	Bombyx mori	NP_001037470
	Apis mellifera	AAP33487
Vertebrata	*Homo sapiens RXR α*	ABB96254
	Danio rerio RXR α	NP_571292
	Xenopus laevis	AP51128
	Gallus gallus RXR γ	NP_990625
Cnidaria	*Tripedalia cystophora*	AF091121

Table 4.5 Protein sequences from species of Crustacea, Chelicerata, Insecta and Cnidara used for comparison and phylogenetic analysis with the *C. finmarchicus* RXR protein sequence.

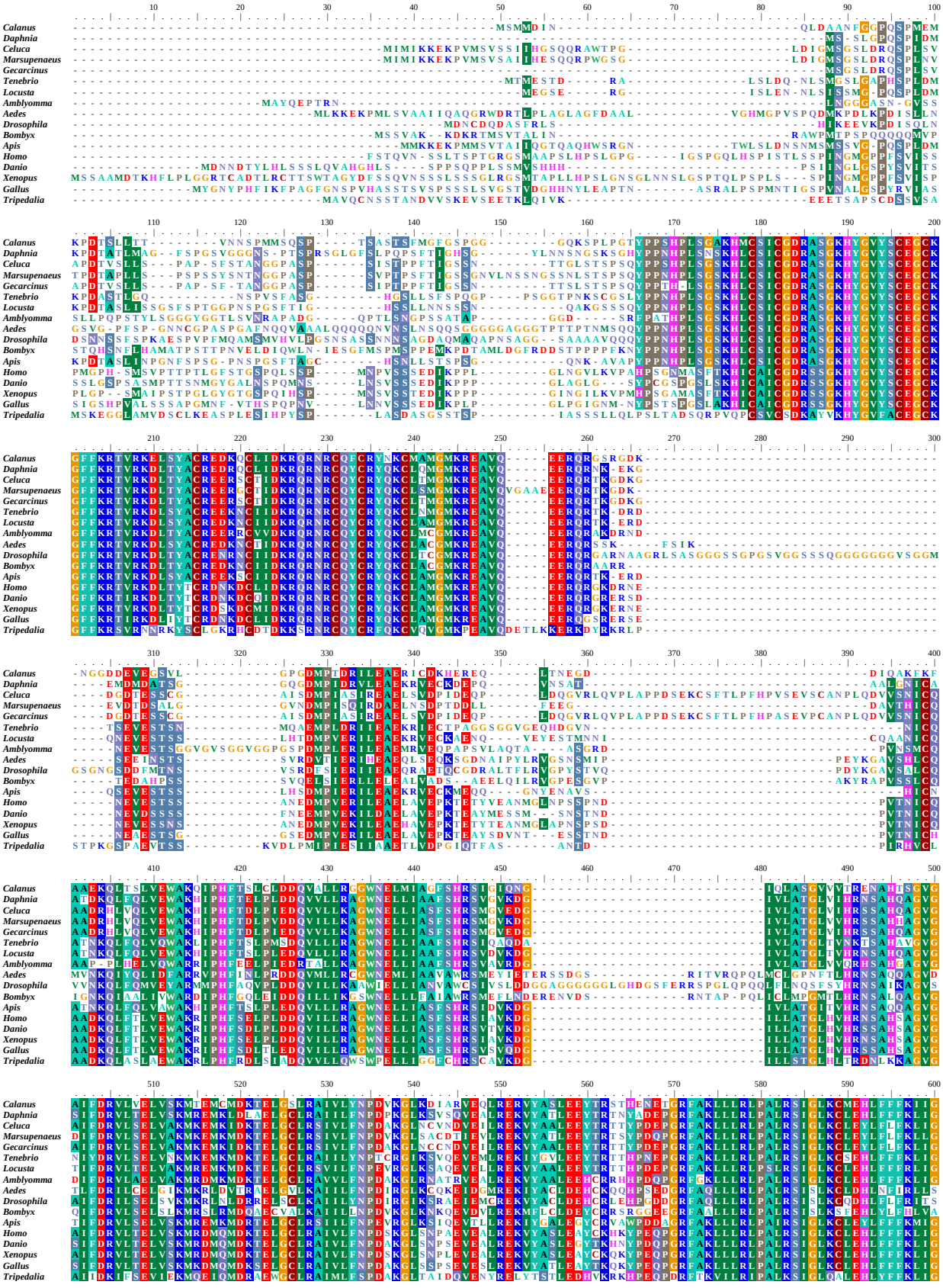

Fig 4.5 Alignment of the putitative full-length deduced open reading frame *C. finmarchicus* RXR sequence with the twelve RXR protein sequences from Crustacea, Chelicerata, Insecta and Cnidara. Amino acids conserved between sequences are shown by blocks of the same colour.

4.2.7 Quantitative Real-Time PCR

Total RNA was extracted as described in section 4.2.2 above. Samples from Loch Etive that had been preserved on board the vessel in RNA*later*® (Ambion) were used instead of CVs that had been taken to the laboratory before being flash-frozen. These animals could not be identified to stage on the vessel, but were identified in RNA*later*® just before RNA extraction. This identification, which took c. 1 min, did not appear to affect RNA quality- only samples with an absorbance ratio (260 nm/280 nm) between 1.7-2.0 were used and further quality checks were made by running aliquots of denatured RNA on a 1% agarose gel to examine for degraded samples. To minimise the impact on analysis of individual variation in gene expression groups of ten animals were pooled and the total RNA from the pool extracted, as above, using proportionally larger quantities of reagents (i.e. 150 µl TRI Reagent®, 40 µl chloroform, 100 µl 100% isopropanol and 200 µl 75% ethanol) were used to extract RNA from the pools of ten animals. Only RNAs of good quality as indicated by gel electrophoresis and with 260/280 nm absorbance ratios between 1.7-2.0 nm were used. Before reverse transcription 1.5 µg of total RNA was incubated at 37^{o}C for 30 min with 1.5 units (1.5 µl) RNase-free DNase and 1.5 µl 10x DNase reaction buffer in a 10 µl reaction volume. After 30 min 1.5 µl of DNase stop solution (all from Promega) was added to the samples and they were incubated at 70^{o}C for 10 min. Ten microlitres of this solution was then used to create cDNA as described previously in section 4.2.2.

Relative expression of mRNA transcripts was measured in the three target genes *RXR*, *EcR* and *A-type AST*. Two endogenous controls or 'housekeeping' genes (*16s rRNA* and *elongation factor A1α*) were chosen to normalise target gene quantities. Both

have been used for real-time PCR of copepod genes by other workers. For example, Tarrant et al. (2008) successfully used *16s rRNA* as a housekeeping gene for their study between deep diapausing and shallow *Calanus finmarchicus* from Georges Bank, USA. Hansen et al. (2008b) used elongation factor A 1α (*EFA 1α*) as the housekeeping gene for toxicological studies of gene expression study in *C. finmarchicus*. Expression of EFA 1α mRNA was stable between samples. For relative quantification of mRNA from the three target genes (*RXR*, *EcR* and *A-type AST*) and the endogenous controls (*16s rRNA* and *EFA 1α*) using SYBR Green technology, a set of primers for each gene had to be designed.

The primers for *RXR*, *EcR* and *A-type AST* were all designed using the integral Primer Express software with the sequence detector ABI Prism 7000™ (Applied Biosystems, Warrington, UK). Primer pairs were designed for use with the universal cycling conditions of the ABI Prism 7000™. These potential primer pairs were then scrutinised for their likelihood of producing primer dimers or non-specific amplification. Primers for *RXR* were designed using the functional part of the mRNA sequence obtained from the 3' RACE. Primers for *EcR* and *A-type AST* were designed from the sequences available from the GenBank database (EcR: EF583877; AST: EU000307), which fitted within the parameters required. All primer product lengths were between 50-150 bp (Applied Biosystems, 2008). Table 4.6 shows the sequences of all the primers used. Primers for *16s rRNA* were taken from Tarrant et al. (2008), and the primers for *EFA 1α* from Hansen et al. (2008b).

Primer name	Sequence	Product size (bp)
qPCR RXR F1	5' GAACTGGCACCTGTTCCTCT 3'	
qPCR RXR R1	5' GGGTTGTAAGGGGTTCTTCA 3'	109
qPCR AST 120	5' AACAACAGTAATGGCTTGCACTATGA 3'	
qPCR AST R	5' TGTCAGTATCAGGTCCATCTTCTCC 3'	55
qPCR EcR F	5' GACATTGCTGCTAAGAATTGTGCTA 3'	
qPCR EcR R	5' TCACACTTGGATGCTCAAACTCTC 3'	139
qPCR 16s rRNA F	5' AAGCTCCTCTAGGGATAACAGC 3'	
qPCR 16s rRNA R	5' CGTCTCTTCTAAGTCCCTGCAC 3'	114
qPCR EFA 1α F	5' CTCCGACTCCAAGAACAAGC 3'	
qPCR EFA 1α R	5' AATATGGGCGGTGTGACAAT 3'	127

Table 4.6 Primer sequences used in real-time quantitative PCR.

Reactions were conducted in 20 µl reactions, each containing 2 µl of 25 ng ul^{-1} cDNA, 2 µl of 3 pmol $µl^{-1}$ each primer, 10 µl Precision™ Master Mix with ROX (Primer Design Ltd, Southampton, UK) and 4 µl sterile water. All samples, including no template controls for each primer set were processed in 96 well plates. Four replicates of each sample and no template control were run. Each replicate consisted of cDNA synthesised from Total RNA extracted from one pool of ten animals. Each gene was run on a separate 96-well plate along with triplicate standards consisting of serially 10-fold diluted cDNA. The cDNA used for the standard samples was the same across all plates and was syntesised from a pool of ten animals collected from Loch Etive in September 2006.

The universal cycling conditions for the ABI Prism 7000™ Sequence Detection System were used, which are: 1 cycle of 50°C for 1 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec, 60°C for 1 min. An initial validation experiment was run with standard curves to test the efficiency of each primer pair, including the two endogenous control genes 16s rRNA and EFA 1α (Table 4.8). As the efficiencies varied slightly between each gene (Table 4.8), it was decided to use the relative

standard curve method to quantify samples. This uses a set of relative standards on each plate for each gene, from which the unknown samples are quantified, accounting for primer efficiency (Applied Biosystems, 2008). The quantity of these unknown samples is expressed relative to a calibrator sample. The specifity of primers was routinely checked by running aliquots of samples on 3% agarose gels, and by using dissociation protocols. The results from both consistently showed amplification of a single product of the expected size.

4.2.8 *Calanus helgolandicus*

At time of collection, *Calanus helgolandicus* had not been found in the Bonawe deep (Fig 2.3), and so all *Calanus* spp. were assumed to be *C. finmarchicus*. When it became apparent, however, that some *C. helgolandicus* individuals were present in the Bonawe deep (Fig 2.7), it was attempted to use genetic markers to identify the individuals (Bucklin et al., 1999; Lindeque et al., 1999) by isolating DNA from the interphase after RNA in the aqueous layer had been removed during extraction (section 4.2.2). However it was difficult to reliably extract DNA of sufficient quality to use DNA markers in PCR to identify the individual, so instead primers designed for use on cDNA to identify *C. finmarchicus* by Hill et al. (2001) were used to determine if individual copepods were *C. finmarchicus.* These primers were LCO-1490 and COI-2011 (Hill et al., 2001). This would not exclude *C. helgolandicus* from the pooled samples used for qPCR, but the risk of *C. helgolandicus* being present in the pools of ten individuals was deemed to be small due to few *C. helgolandicus* being found in the Bonawe deep (Fig 2.7).

4.2.9 Data Analysis

The relative expression of target gene mRNA was calculated by initially quantifying the unknown samples using the standard curve. Variation between the samples was calculated using the variation co-efficient (Applied Biosystems, 2008). The *RXR*, *EcR* and *A-type AST* sample masses were averaged and then normalised by dividing by the geometric mean of the sample masses of the endogenous controls, *16s rRNA* and *EFA 1α*. The normalised target values were then divided by the calibrator sample to calculate the fold-difference in mRNA expression between the calibrator and the samples. Although specific packages such as the REST software (Pfaffl et al., 2002) have been designed to analyse data from real-time PCR, they only allow for comparison between a control and treatment group, and not statistical differences between gene expression over a temporal scale. Thus, using the Sigmaplot software package, a one-way ANOVA was used to look for significant differences in expression between samples program followed by *post hoc* multiple comparison (Tukeys test). All data sets passed the Kolmogorov-Smirnov normality test, and the RXR and A-type AST data sets passed the Leverne median test for equal variance run by Sigmaplot, but the EcR data required log transformation to pass the Leverne median test for equal variance prior to running the one-way ANOVA. Patterns of gene expression were investigated by principal component analysis using the Primer 6 program.

4.3 RESULTS

4.3.1 Characterisation of RXR in *Calanus finmarchicus*

Following sequencing of five positive clones from each of the 3' and 5' RACE experiments, a 1759 bp putative full-length cDNA encoding RXR was constructed. Fig 4.6 shows the complete nucleotide region encoding a protein of 405 amino acids, with a deduced molecular weight of 44,894 Da. No isoforms were found in the fragments sequenced in the 5 random clones. All five regions characteristic of a nuclear hormone receptor are present in the amino acid sequence: - the N-terminal regulatory domain (A/B domain, amino acids 1-77), the DNA binding domain (C domain, amino acids 78-147), the hinge region (D domain, amino acids 148-214), the ligand binding domain (E domain, amino acids 215-371) and the C-terminal domain (F domain, amino acids 372-405). Each region was aligned and compared to known RXR sequences from Crustacea, Chelicerata, Insecta and Cnidara (Table 4.7). The putitative mRNA and protein sequences were submitted to GenBank (Accession No. FJ874901).

```
1    C TCC CTG TCT GTG CGC CTG AGG ACC GTG CCG TGC CAC CCC AAC CCC AAC AAC ATC CCC AAC   61
62     ACC CCC ACT ATG TCC ATG ATG GAT ATA AAC CAG CTG GAC GCT GCA AAC TTT GGC GGC CCT   121
                   M   S   M   M   D   I   N   Q   L   D   A   A   N   F   G   G   P    17

122    CAG AGC CCG ATG GAG ATG AAG CCA GAC ACG TCC TTG CTG ACC ACC GTG AAC AAC TCC CCC   181
18      Q   S   P   M   E   M   K   P   D   T   S   L   L   T   T   V   N   N   S   P    37

182    ATG ATG TCC CAG TCC CCC ACC TCA GCT TCA ACC TCC TTC ATG GGG TTT GGC TCA CCT GGA   241
38      M   M   S   Q   S   P   T   S   A   S   T   S   F   M   G   F   G   S   P   G    57

242    GGA GGG CAG AAG TCC CCT CTA CCT GGC ACC TAC CCC CCA TCC CAC CCC CTG TCT GGT GCC   301
58      G   G   Q   K   S   P   L   P   G   T   Y   P   P   S   H   P   L   S   G   A    77

302    AAG CAC ATG TGC AGT ATC TGT GGG GAC AGG GCC AGT GGA AAG CAC TAT GGT GTT TAC TCC   361
78      K   H   M   C   S   I   C   G   D   R   A   S   G   K   H   Y   G   V   Y   S    97

362    TGT GAG GGT TGT AAG GGT TTC TTC AAG AGG ACA GTC AGG AAG GAG CTT TCC TAT GCT TGC   421
98      C   E   G   C   K   G   F   F   K   R   T   V   R   K   E   L   S   Y   A   C   117

422    AGG GAA GAC AAG CAG TGC TTG ATT GAC AAG AGG CAG AGG AAC AGG TGC CAG TTC TGC AGG   481
118     R   E   D   K   Q   C   L   I   D   K   R   Q   R   N   R   C   Q   F   C   R   137

482    TAC AAC AAG TGC ATG GCC ATG GGG ATG AAG AGA GAG GCA GTG CAG GAG GAG AGG CAG AGA   541
138     Y   N   K   C   M   A   M   G   M   K   R   E   A   V   Q   E   E   R   Q   R   157

542    GGG TCC AGG GGG GAC AAG AAT GGG GGG GAT GAT GAA GTG GAG GGA AGC GTC CTT GGA CCT   601
158     G   S   R   G   D   K   N   G   G   D   D   E   V   E   G   S   V   L   G   P   177

602    GGT GAC ATG CCC ACT GAC AGG ATA CTG GAG GCA GAG AGG ATT TGT GAC AAA CAT GAG CGG   661
178     G   D   M   P   T   D   R   I   L   E   A   E   R   I   C   D   K   H   E   R   197

662    GAG CAG CTG ACT AAT GAG GGA GAT GAC ATC CAG GCA AAG TTT AAG TTT GCT GCA GAG AAA   721
198     E   Q   L   T   N   E   G   D   D   I   Q   A   K   F   K   F   A   A   E   K   217

722    CAG CTG ACC TCC TTG GTA GAG TGG GCC AAG CAG ATA CCT CAC TTT ACC AGC TTG TGT TTG   781
218     Q   L   T   S   L   V   E   W   A   K   Q   I   P   H   F   T   S   L   C   L   237

782    GAT GAT CAG GTG GCT CTC CTA AGG GGA GGC TGG AAT GAG TTG ATG ATT GCT GGG TTC AGC   841
238     D   D   Q   V   A   L   L   R   G   G   W   N   E   L   M   I   A   G   F   S   257

842    CAC AGA TCT ATT GGT ATT CAG AAT GGG ATC CAG CTT GCG AGT GGT GTG GTG GTG ACC AGG   901
258     H   R   S   I   G   I   Q   N   G   I   Q   L   A   S   G   V   V   V   T   R   277

902    GAG AAT GCT CAC ACT AGT GGG GTT GGA GCT ATC TTT GAC AGA GTC TTG GTG GAG CTG GTG   961
278     E   N   A   H   T   S   G   V   G   A   I   F   D   R   V   L   V   E   L   V   297

962    TCC AAG ATG ACG GAG ATG TGC ATG GAC AAG ACA GAG CTC GGC AGC TTG AGG GCC ATC GTC  1021
298     S   K   M   T   E   M   C   M   D   K   T   E   L   G   S   L   R   A   I   V   317

1022   CTC TAC AAC CCA GAT GTG AAG GGG TTG AAG GAC ATT GCC AGG GTG GAG CAG TTG AGG GAG  1081
318     L   Y   N   P   D   V   K   G   L   K   D   I   A   R   V   E   Q   L   R   E   337

1082   AGG GTG TAT GCC AGC CTG GAG GAA TAC ACC AGG TCC ACC CAT GAG AAT GAG ACA GGA AGG  1141
338     R   V   Y   A   S   L   E   E   Y   T   R   S   T   H   E   N   E   T   G   R   357

1142   TTT GCT AAG CTA CTG CTC AGA CTT CCA GCT TTG AGA TCA ATT GGA TTG AAG TGT ATG GAA  1201
358     F   A   K   L   L   L   R   L   P   A   L   R   S   I   G   L   K   C   M   E   377

1202   CAT CTT TTC TTT TTC AAA ATT ATT GGC GAG TCT GGT GCT GGT CTT GAC GCA CAC CTG TTC  1261
378     H   L   F   F   F   K   I   I   G   E   S   G   A   G   L   D   A   H   L   F   397

1262   GAC CTG CTA GAA CCG GCT GAT AAC TAG CTG GTG GTG ATT TGG ACA AGA GCT AGT TAG ACT  1321
398     D   L   L   E   P   A   D   N                                                   405

1322   AAG CCA AAT ACT ATG ACG CCC GGG GTG AAA TTG ATA ATG AAA ACT TTT ATG TTT TGA AAA  1381
1382   CTG CTT TAA AAG TTG ACT GGG AAA AGT TTT GGG CTA AAA TGA GAA TGT TTG ATT CCT GTT  1441
1442   TGA AGA GGT GCT ATT TTG GGT TTG AGT CTA CCC AGG GGA TTA CTA TAA TTT TGG AGG CAT  1501
1502   CTT CTA GTC TGT TTT TTA GAT TGT AAA TCT TAA ATC TTT GAA ATA TTT TCC AAG TTT TGA  1561
1562   CTT GCC AAC CTA TTA CCA CAA GTT TGC ATG AAG CCC AAA ACA ACT GTC TGT CGT CTC GCT  1621
1622   ACA GCT TAA TCT TCC TCC AAT TTT TAC AAT TTT AAG AAA ATT TCC AAT TAT ATA ATT GTA  1681
1682   ACT GAT CAA TTG AGA CAA TAA TCT ACA TTA TAA GTT TAT AAG TTC AGA AAT AAA ATT TGC  1741
1742   AAT GAA AAA AAA AAA AAA                                                         1759
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Fig 4.6 Nucleotide and deduced amino acid sequence of *C. finmarchicus* RXR isolated from cDNA fragments from 3' and 5' RACE. The DNA binding domain is highlighted in grey and the ligand binding domain in yellow.

	A/B N-domain	**C** DNA binding domain	**D** Hinge region	**E** Ligand binding domain	**Total**
D. magna	22.8	89.5	27.4	66.8	56.0
T. molitor	32.2	88.2	26.2	65.8	55.0
A. mellifera	20.0	89.5	27.9	62.5	51.2
C. pugilator	26.0	85.5	20.2	65.2	49.1
L. migratoria	27.8	90.8	28.4	66.3	56.1
A. americanum	17.6	82.9	29.3	61.4	44.9
G. lateralis	25.0	85.5	20.4	65.2	50.7
A. aegypti	18.5	89.5	15.6	40.0	35.6
D. melanogaster	19.2	84.2	12.8	40.8	35.0
B. mori	15.2	88.2	7.7	43.7	38.6
G. gallus	9.2	82.9	27.7	65.2	44.4
H. sapiens	8.8	84.2	27.9	67.4	47.0
D. rerio	11.5	81.6	29.2	66.3	45.6
X. laevis	7.5	82.9	29.4	66.8	42.8
M. japonicus	23.2	80.2	22.2	64.7	50.1
T. cystophora	6.7	53.1	21.5	48.4	34.7

Table 4.7 Percentage identity to *C. finmarchicus* of each domain forming the RXR protein sequence of sixteen species of Crustacea, Chelicerata, Insecta and Cnidara.

The *Calanus finmarchicus* RXR protein sequence shared the highest total identity with *L. migratoria RXR* (56.1%), followed by *D. magna* (56%) and *T. molitor* (55%). The DNA binding domain is highly conserved, with identity above 80% for all the Insecta, Crustacea and Chelicerata. Only the cnidarian *T. cystophora* has a low 53.1% identity to the *C. finmarchicus* DNA binding domain. In the ligand binding domain (LBD), *C. finmarchicus RXR* shows an identity of 61.4-67.4 with the Crustaceans *D. magna, C. pugilator, G. lateralis* and *M. japonicus*; the insects *T. molitor, A. melifera*, and *L. migratoria*; the chelicerate *A. americanum*; and the vertebrates *G. gallus, H. sapiens, D. rerio and X. laevis*. A significantly lower identity ($P<0.01$, Student's t-test) between 40-48.4% identity exists for the insects *A. aegypti*, *D. melanogaster* and *B. mori* as well as the cnidarian *T. cystophora*. The A/B domain and D domains are known to be regions of high sequence variability between species and % identity was

low, with *C. finmarchicus* RXR varying from 6.7-32.2% identity in the A/B domain, and 7.7-29.4% in the D domain to the 16 species.

In order to identify potential ligand preferences between phyla, phylogenetic analysis of the LBD of *C. finmarchicus* RXR was conducted with the 16 different species in Table 4.5 (Fig 4.7).

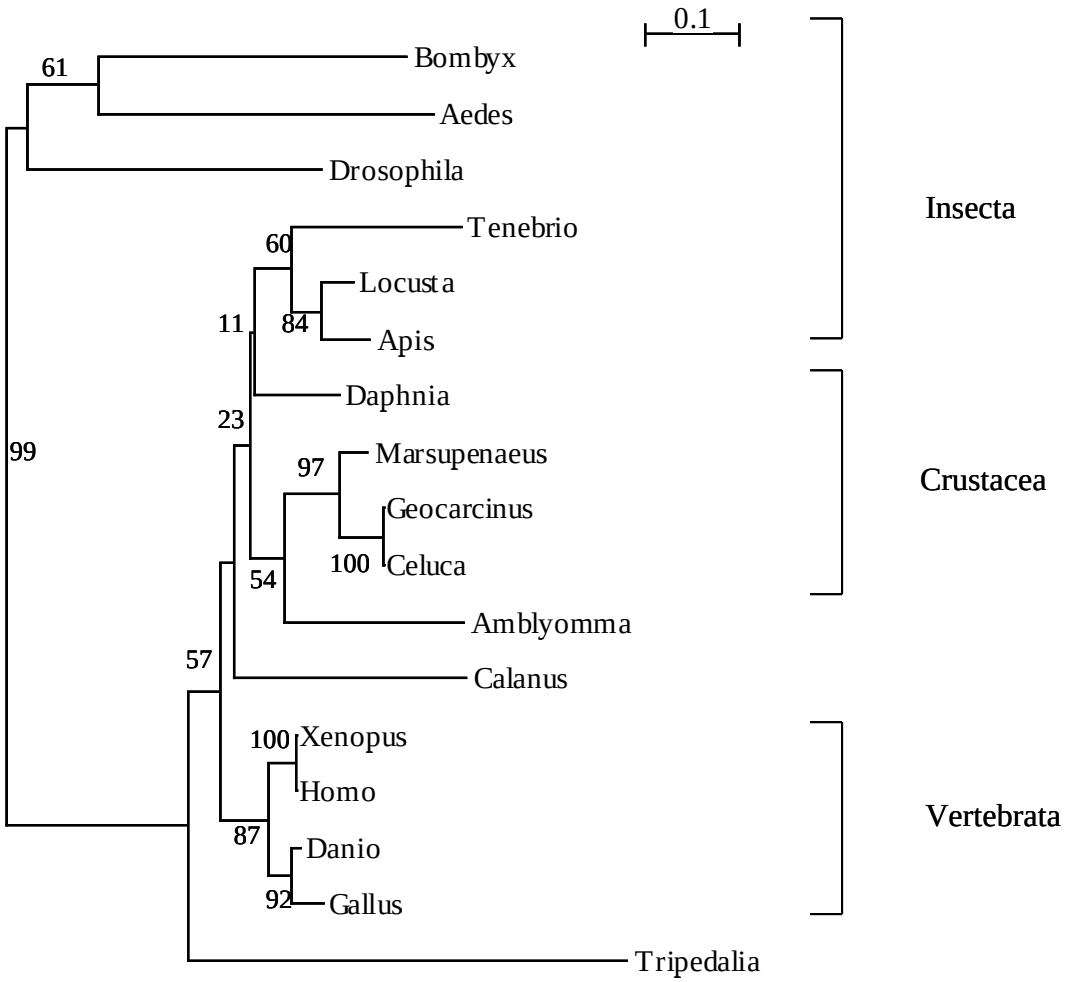

Fig 4.7 The phylogenetic tree for LBD of *RXR*/*USP*. The tree was drawn from the amino acid sequences of the LBD from *C. finmarchicus RXR* and the sixteen species in Table 4.5 using the neighbour-joining method. Numbers represent bootstrap values (%). The bar represents 0.1 substitutions per site.

Phylogenetic analysis produces three clades with the insects split between two (Fig 4.7). One clade contains the Dipterans *D. melanogaster* and *A. aegypti* and the Lepidopteran *B. mori*, collectively termed higher-order insects. A second clade, which includes *Calanus finmarchicus*, contains lower order insects and crustaceans, while the third contains the cnidarian *T. cystophora* and the vertebrates *G. gallus*, *H. sapiens*, *D. rerio* and *X. laevis* (Fig 4.7). These are the relationships: the higher order insects *D. melanogaster*, *A. aegypti* and *B. mori* emerged first, followed by the cnidarian *T. cystophora* followed by the vertebrates *X. laevis*, *H. sapiens*, *D. rerio* and *G. gallus*. The divergence of the rest of the species was more complicated. *C. finmarchicus* diverged separately from the rest of the Crustacea. The decapods *C. pugilator*, *G. lateralis* and *M. japonicus* are clustered together from a node from which the insect *A. americanum* also branched, however *D. magna* is on a separate node, from which diverges the second clade of Insecta containing the Hymenopteran *A. melilifera*, the Orthopteran *L. migratoria* and the Coleopteran *T. molitor*, collectively termed lower-order insects.

4.3.2 Quantitative real time PCR analysis

The amplification efficiency of each primer set measured in the initial validation experiments was calculated from the slope of the line generated by plotting the Ct value against the $\log_{10}$ of the dilution (Table 4.8). As the efficiency varied from 93%-110% between genes, the relative standard curve method was used to quantify samples. Expression of both housekeeping genes appeared stable over the time series, consequently the geometric mean of both *16s rRNA* and *EFA 1 α* was used to normalise the samples. Variation between the four replicates was in the range 0.008-3.9, under the recommended variation co-efficient of 4 (Applied Biosystems, 2008).

Gene primer set	Amplification efficiency
16s rRNA	93%
EFA 1α	97%
EcR	107%
RXR	110%
A-type AST	96%

Table 4. 8 Efficiency of each primer set.

There was little change in expression with depth of any of the target genes from the samples collected in the Farøe-Shetland Channel (Fig 4.8). All expression falls within a 2-fold range with no significant difference in expression between shallow and deep-water samples ($P > 0.05$, one way ANOVA). However a temporal change in expression of all the target genes from *C. finmarchicus* collected in Loch Etive (Fig 4.8) is evident. Expression of *RXR* from June to October was 2.2-2.8 fold (Fig 4.8), but decreases to 1.4 fold in November ($P<0.001$, one-way ANOVA, Tukey Test). RXR expression increases to 2.3 fold in December and by January expression of *RXR* is 3.6 fold, significantly higher than any other month ($P<0.001$). *RXR* expression is least in February, which is the calibrator sample, thus expression is 1 fold (Fig 4.8). From February to March expression increased to 2 fold, ($P<0.001$) and remained at similar levels (1.6-2 fold) to May. The change in expression of *EcR* involved much higher fold-differences in expression level than *RXR* or *A-type AST*. *EcR* expression followed much of the same pattern as *RXR*. Between June and October *Ecr* expression is in the range 18-22 fold and does not change significantly (Fig 4.8). *EcR* expression decreases significantly to 8 fold in November ($P<0.05$), and expression was least (1 fold) in December than any other month ($P<0.001$). In January *EcR* expression increases to 12-fold ($P<0.001$), and continues to increase to 16.9 fold in February and *EcR* expression remains high (26-28 fold) in the samples collected from March – May

(Fig 4.8). The expression of A-type AST mRNA is more variable. Expression of samples collected in June is 30 fold, significantly higher ($P<0.001$) than samples collected in July, where expression of *A-type AST* was 5 fold (Fig 4.8). Expression fluctuates in the range 8-23 fold from samples collected in August through to January when expression dropped significantly to 1-fold ($P<0.001$, Fig 4.8). Samples collected in February show 36-fold higher levels of expression than those taken in January ($P<0.001$) and expression of *A-type AST* remains in the range 36-46 fold from February to May (Fig 4.8).

4.3.4 Principal component analysis (PCA)

PCA was conducted for the three target genes *RXR*, *EcR* and *A-type AST*. The first principle component accounts for 78.5% of total variation and is dominated by *A-type AST* expression and *EcR* expression (Fig 4.9). The second component accounts for 20.9% of total variation and is dominated by *RXR* expression. December was separated by the cluster analysis from all the other months by the first and second principal components, indicating that the pattern in expression of the three genes in December was different to that of the other months.

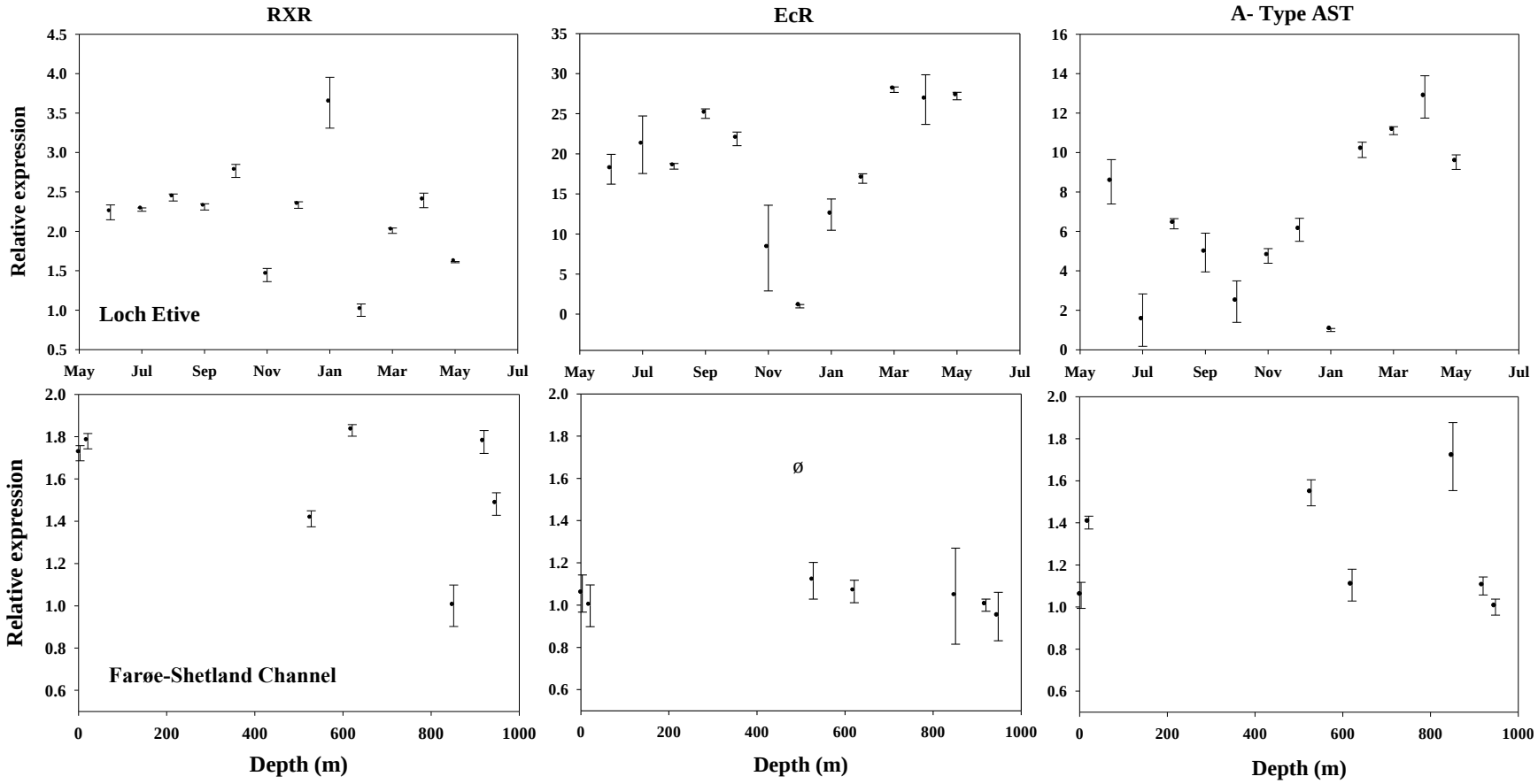

Fig 4.8 Expression of RXR, EcR and A-type AST mRNA normalised to 16s rRNA and EFA 1α from *C. finmarchicus* CV collected from Loch Etive (top) and the Farøe - Shetland Channel (below). The sample with the lowest expression was chosen as the calibrator value for each gene, all other samples were divided by this value to give fold-differences in relative expression between months/depths. Thus the calibrator has the arbitrary fold-difference of 1. Error bars represent standard deviation.

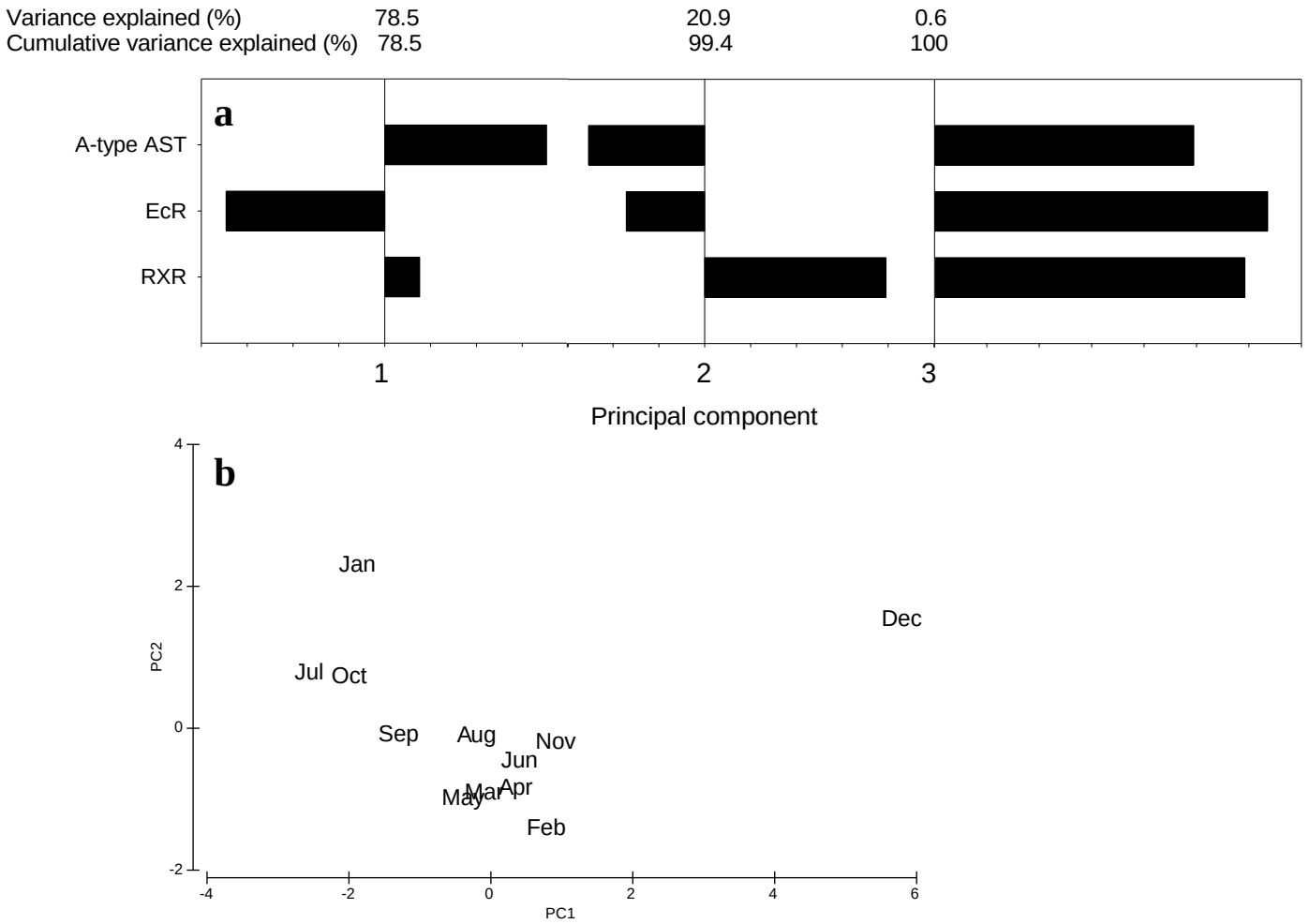

Fig 4.9 Plots from principal component analysis. **a**. Relative contribution of each gene to each PC. Bars represent eigenvectors, longer bars are components with a stronger relative contribution to that PC. Bars to the left of each line represent a negative influence, bars to the right a positive influence. **b**. Cluster plot of PC1 vs. PC2.

4.4 DISCUSSION

The full length mRNA of *Calanus finmarchicus* RXR characterised in this study was expected to share more sequence identity with the other crustacean sequences than those of the insects or vertebrates. In fact the Crustacea and the lower-order insects both have the highest sequence identity to the total *RXR* sequence in *C. finmarchicus*. Within the ligand-binding domain, crustacean *RXR* has been documented to be more similar to vertebrate *RXR* than to higher-order insect *USP* (Egea et al., 2000; Billas et al., 2001). The phylogenetic analysis of this study (Table 4.8, Fig 4.7) illustrates that the LBD of decapod crustacean *RXR* is closely related to lower insect *USP*s of *A. mellifera, L. migratoria, T. molitor* and *A. americanum*, but diverged from the Dipterans *D. melanogaster* and *A. aegypti* and the Lepidopteran *B. mori*. It has been well documented that the LBD's of USPs in the more advanced insect orders appear to have diverged from their RXR origins, possibly in relation to altered ligand specificity (Guo et al., 1998; Hayward et al., 1999; Riddiford et al., 2001, Asazuma et al., 2007). *C. finmarchicus* LBD is similar to the LBD of crustaceans, vertebrates and the less advanced insects than with the diverged Lepidopteran and Dipterans. However it does not share significantly more identity ($P>0.05$, Student's t-test) with the other crustacean *RXR* than that of the vertebrates, indicating that divergence of the LBD may also have occurred within the Crustacea.

It appears likely that there will not be one but several potential ligands for RXR in *Calanus finmarchicus*. The ligand with highest binding affinity for RXRα in vertebrates is 9-*cis* RA (Germain et al., 2006). RXR from the mollusc *Biomphalaria glabrata* binds 9-*cis* RA and transactivated transcription. RXR from the jellyfish *Tripedalia cystophora*, which shares 48% identity to *C. finmarchicus* in the LBD, and

the insect *L. migratoria,* which shares 66% identity to *C. finmarchicus* in the LBD have been shown to bind 9-*cis* RA (Kostrouch et al., 1998; Nowickyj et al., 2008). *D. melanogaster* USP which shares 40% identity with *C. finmarchicus* in the LBD does not bind 9-*cis* RA (Oro et al., 1990). The flour beetle *Tribolium castaneum* has a USP that is more similar to crustacean RXR than the USP of the higher-order insects, but also does not bind 9-*cis*-RA (Iwema et al., 2007). The natural ligands for nuclear hormone receptors are known to not fully occupy the cavity of the ligand-binding pocket and potentially the divergence in the LBD may be in the unoccupied space (Riddiford, 2008). Endocrine signalling via RA has not been demonstrated in crustaceans, however Hopkins (2001) showed that *RXR* levels are elevated in regenerating limb tissue of the crab *C. pugulator* on stimulation by 9-*cis* RA, leading to her assumption that endocrine signalling via RXR, potentially by 9-*cis* RA, does occur in crustaceans.

In insects, it has been well documented that EcR and USP/RXR form a heterodimer that coordinates development and metabolism and modifies the expression of a multitude of different genes in a tissue- and time- specific manner (Spindler-Barth and Spindler, 2003). USP has been considered as an orphan receptor without a ligand whilst acting as a heterodimer (Schubiger and Truman, 2000), but is thought to still be necessary for moulting as in *Drosophila melanogaster*, the USP null mutant cannot complete the moult to the second larval stage (Oro et al., 1992; Hall and Thummel, 1998). Riddiford (2008) suggests that USP may only be involved in binding JH or MF either in situations in which JH acts in the absence of 20E, or can perhaps modulate JH action both in the absence and presence of 20E by switching dimeric partners. In Crustacea, more variants of *EcR* and *RXR* exist than in insects (Chung et al., 1998;

Wu et al., 2004; Kim et al., 2005; Asazuma et al., 2007), and Wu et al. (2004) demonstrated that EcR and RXR form heterodimers in the absence of 20E *in vitro*, suggesting a role for the EcR/RXR heterodimer in processes other than moulting. USP has been shown to bind JH III in *D. melanogaster* but with low affinity- 100 times lower than expected for a nuclear receptor but enough to cause physiological effects and transcriptional activity (Jones and Sharp, 1997; Jones et al., 2001; Xue et al., 2002). Jones et al. (2006) showed that methyl farnesoate binds to *D. melanogaster* USP with nearly a 100-fold higher affinity than JH III, and at times MF production was detected to be at much higher rates than JH III. However, a high affinity JH receptor, Methoprene-tolerent (Met) has also been identified in *D. melanogaster* (Miura et al., 2005). The function of the capability of JH binding to two separate receptors has not been elucidated, however Riddiford (2008) suggested that potentially MET is the binding partner of JH in the cases where USP/RXR cannot bind JH such as in the beetles and less derived insects (Hayward et al., 2003). Clearly more research needs to be done. The search for potential ligands for RXR/USP has a long way to go still, even in the well-studied Insecta and Decapoda. Further study with *C. finmarchicus* RXR is needed to determine if MF, 9-*cis*-RA or another potential ligand bind to *C. finmarchicus* RXR with any affinity and if so, if they affect gene transcription.

During dormancy it is thought that any unnecessary physiological processes are maintained at basal levels (Hirche, 1996), and characteristic patterns of gene expression associated with diapause may be established. However, the present study shows there is no significant change in gene expression for any of the target genes between shallow and deep water *Calanus finmarchicus* CV collected in the Farøe-

Shetland channel. Two to four days prior to these samples being collected there was a major storm (force 10) and it is possible that there may have been some mixing of diapausing copepods to shallower waters. Thus animals caught in shallow waters assumed to be 'active' might have actually been disturbed diapausing animals, although some change in gene expression may still be expected. Without alternate analysis to determine if these animals were diapausing or not such as the aminoacyl-tRNA synthetases content (Yerba et al., 2006) or mid-gut epithelium histological changes (Bonnet et al., 2007), it is impossible to tell whether or not expression of the three target genes changes during diapause.

By contrast, over the time series of samples taken in Loch Etive, there is a definite change in temporal expression and an association of the target genes. As discussed in Chapter 2 of this thesis, December is when *Calanus finmarchicus* is thought to be diapausing in Loch Etive, as no adult animals were observed from the net samples during this month (Fig 2.6), and a high percentage of the population are deeper in the water column. In January it is thought that emergence from diapause has begun, as some adult male and female *C. finmarchicus* were found in the net samples in January, but not in large numbers, indicating that the majority of the population had yet to develop from CV. December was separated by the PCA analysis from the other months, signifying that the gene expression pattern was different when the animals are in diapause. The pattern of temporal expression of EcR supports this hypothesis. Johnson (2003) demonstrated a typical crustacean ecdysteroid pattern in *Calanus pacificus,* and documented ecdysteroid titre being significantly reduced during diapause when development is suppressed. In the present study in Loch Etive, EcR mRNA expression appears to be at its lowest during December when the animals are

assumed to be in diapause: ca 15-20 times lower than *EcR* expression from June to October and January to May when the animals may be assumed to be active and are preparing to moult to the adult stage. The 14-fold decrease in relative *EcR* expression from October to November indicates that some of the population may already be diapausing in November; but that *EcR* expression is still 8-fold higher than in December is indicative that some of the population is still active. The large increase in *EcR* expression from December to January supports the hypothesis that the population is emerging from diapause, or preparing to emerge, during this month. This pattern is similar to *EcR* expression before, during and after diapause in the flesh fly *Sarcophaga crassipalpis* (Rinehart et al., 2001) and the tobacco hornworm *Manduca sexta* (Fujiwara et al., 1995), but these findings are in contrast to those of Tarrant et al. (2008), who found that expression of *EcR* in *C. finmarchicus* was higher in the copepods appearing to be in diapause than the active individuals. However as these authors only sampled at one point in time, they had limited knowledge on the stage of diapause and the moult-status of the animals and it is possible that these animals were appearing to emerge.

The pattern of *EcR* expression over diapause in *Calanus finmarchicus* in Loch Etive does follow the expected pattern of ecdysteroid titre over diapause, and may be a good candidate for a marker of diapausing populations. Whether expression of *EcR* is co-ordinated with ecdysone secretion over diapause or is involved in other processes in *C. finmarchicus* requires further study. Asazuma et al. (2007) suggest that ecdysteroid titre was not the only target of expression of *RXR* and *EcR* in the prawn *Marsupenaeus japonicus*, as expression of *EcR* and *RXR* did not wholly co-ordinate with ecdysone secreation. By contrast, in the tick *Ornithodoros moubata EcR* and

RXR expression are closely synchronised with increases in ecdysone titres (Horigane et al., 2008). Also, in the fiddler crab, *Celuca pugilator,* expression of *EcR* was seen to increase during pre-moult with ecdysteroid titre (Chung et al., 1998). However these species do not enter diapause. Clearly, more studies are needed to explore possible links between ecdysteroid titre with expression of *EcR* over diapause in *C. finmarchicus*, to determine if *EcR* expression is wholly linked with ecdysteroids, or if it has a role in other pathways.

RXR proteins are essential players in several hormonal pathways because they form many heterodimers and can act as ligand-activated transcription factors (Germain et al., 2006). RXR may therefore affect transcription in *Calanus finmarchicus* in several ways, such as by binding a ligand and acting on its own as a homodimer (Mangelsdorf et al., 1991), or forming heterodimers with other nuclear hormone receptors, commonly EcR, and acting in a ligand independent manner and simply ensuring efficient DNA binding (Germain et al., 2006). In the present study the expression of *RXR* was not found to mirror that of *EcR* in *C. finmarchicus*, indicating that RXR may play another role than simply forming part of an EcR/RXR complex and regulation of the moult cycle, however over some parts of the year there is similarity in expression patterns. Initially *RXR* expression does follow the same outline as *EcR*, expression is relatively high from June to October, decreases significantly in November, and in January increases again. However, the significant drop in *RXR* expression in February is not what would be expected if RXR were acting exclusively as a heterodimer with EcR or regulating the moult cycle in a different way. The significant decrease in *EcR* expression in December is also not matched by *RXR*.

In the present study, the largest increase in expression of *RXR* in *Calanus finmarchicus* and the highest expression of *RXR* over the time-series is in January when the population is beginning to emerge from diapause. This is then followed by a drop in expression to its lowest in February. This indicates that RXR is involved in other processes than simply regulating the moult cycle because *EcR* expression is low in December and high in February. The pattern of expression of *RXR* from the decrease in expression in November when the population is preparing for/in diapause followed by an increase in expression in December and a further increase in January prior to a drop in February suggests that RXR may be involved in terminating diapause. In *Sarcophaga crassipalpis* expression of USP gradually declines at the onset of diapause until undetectable (Rinehart et al., 2001). Transcripts reappear late in diapause and are further elevated when diapause is terminated, suggesting it may be involved in the processes leading to termination of diapause (Rinehart et al., 2001). Expression of RXR does not disappear at any time during diapause in *C. finmarchicus*, but this is likely to be due to variation in diapause duration and the timing of termination within the population in Loch Etive.

A-type allatostatins potentially regulate MF production in *C. finmarchicus* (Christie et al., 2008). The relatively high *A-type AST* expression from August to December found in the present study may indicate that MF production is being inhibited. The significant drop in expression in January may potentially indicate that *C. finmarchicus* is synthesising MF as the animals terminate diapause. This correlates with the high increase in *RXR* expression in January. Certainly from February to May AST expression is significantly higher than in January, so MF synthesis may be suppressed again. Potentially MF may be the ligand for RXR and may be involved in termination

of diapause, however without measurement of MF in *C. finmarchicus* and determining a link with *RXR* expression, and *A-type AST* expression such explanations are conjectural. As discussed above however, the ligand may be MF, 9-*cis*-RA or something else entirely. More studies are required to determine if expression of *RXR* is always elevated on termination of diapause, if MF can bind to *C. finmarchicus* RXR with any affinity, and to measure MF titre directly through diapause in *C. finmarchicus*.

In summary, there is a pattern in gene expression associated with diapause in *Calanus finmarchicus* in Loch Etive. During diapause *EcR* expression is suppressed, as expected. *RXR* is up regulated in animals thought to be emerging from diapause, and *A-type AST* is down regulated during this time, potentially allowing synthesis of MF, which may be the ligand for RXR. This study provides some initial insight into possible gene expression patterns. However much more research is needed to determine if these patterns are the same between years in Loch Etive, and if *C. finmarchicus* diapausing in other locations have similar patterns in gene expression, before a characteristic pattern in gene expression can be attributed to diapause and emergence in this species.

CHAPTER 5: General Discussion

In order to understand some of the internal processes involved in diapause in *Calanus finmarchicus*, a 14 month time series of *Calanus finmarchicus* from Loch Etive was analysed for variation in lipid content and gene expression over the seasonal cycle. This has been the most comprehensive study of *C. finmarchicus* in Loch Etive since Mauchline (1983), and capitalised on the publication of 11,000 expressed sequence tags from *C. finmarchicus* in the GenBank database part way through my PhD studies. These tags were the first such information relating to functional proteins in *C. finmarchicus* to be published, and opened the door for genetic studies of *C. finmarchicus*.

My work has taken the novel approach to studying lipid class and fatty acid content of *individual* copepods and, so doing, has exposed some extreme variability between the lipid and fatty acid content of different individuals within months. This in turn has highlighted the potential importance of focussing on individuals when investigating theories for initiation of diapause such as the lipid window accumulation hypothesis: whole populations will likely be composed of individuals in many states of lipid accumulation, and this population variability clouds the physiological response of individual processes initiating or terminating diapause.

The changes in gene expression documented in this study show the down-regulation of EcR during diapause and the up-regulation of RXR towards the end of diapause and during termination. The isolation and publication in GenBank of the putative full length mRNA of RXR from *C. finmarchicus* will provide the starting point for more studies of the role of RXR in diapause and development of *C. finmarchicus*. In this

final Chapter I seek to set my work in the context of other studies and to discuss the opportunities for further research.

5.1. A summary of the life cycle of *Calanus finmarchicus* in Loch Etive

Within the Bonawe deep in Loch Etive, *Calanus finmarchicus* copepodites stages CI-CIV were present in my net samples from April to September, indicating that *C. finmarchicus* develops from the nauplii stages NI-N6 and the copepodite stages CI-CV from the spring and by late summer nearly all individuals have achieved CV. Numbers of *C. finmarchicus*, most abundantly stage CV, peak during the summer months of July and August when most individuals reach this stage. Total numbers of *C. finmarchicus* slowly decline from August to October, probably due to predation. By October, the remaining CV have accumulated lipids (accumulation occurs through the summer) and enter diapause between October and December, a state characterised by low expression of *EcR* in November and December. Lipid reserves were reduced by up to 50% during diapause and animals began to emerge from diapause and moult to the adult stage from January to February. Emergence was characterised by an increase in expression of *EcR and RXR*, and a drop in expression of *A-type AST*. Stage CV copepodites that still have sufficient lipid reserves then moult to adult stages and reproduce. Low primary production (estimated at 70 C m^{-2} yr^{-1} in the lower basin; Wood et al., 1973), limited by the high coloured dissolved organic matter content in the freshwater layer (Mckee et al., 2002), is likely to be the reason that only one generation of *C. finmarchicus* is produced in Loch Etive per season.

5.2. Gene expression of *C. finmarchicus* over a seasonal cycle

I put together a gene expression profile from three key genes possibly involved in the hormonal regulation of diapause, the ecdysteroid receptor *EcR*, the retinoid X receptor *RXR*, and a gene encoding an allatostatin potentially involved with the regulation of *RXR*, *A-type AST*. Animals which had been preserved in RNA*later*® immediately on collection were used to halt gene expression changes associated with collection. *EcR* in particular showed the largest changes in expression and may be used as a marker for diapause, as *EcR* expression was reduced when the animals were diapausing and increased when net samples indicated that the animals were beginning to emerge. The gene expression profile of animals sampled in December was distinct from the other months (separated by principal component analysis), further demonstrating that December, when most of the population appears to be in diapause, has a different population gene expression profile than other months when the population is active. The reduced expression of *EcR* during overwintering also indicates that the animals in Loch Etive do undergo 'true diapause', marked by suppressed development. In a study on *C. finmarchicus* in the Gulf of Maine done at the same time as my work, expression of *EcR* was not found to be significantly different between simultaneously-collected shallow (assumed to be active) and deep (assumed to be diapausing) populations of *C. finmarchicus* (Tarrant et al., 2008). This may be because the deep population was preparing to emerge and *EcR* had already begun to be expressed, as *EcR* is likely to be one of the first receptors to be switched on when the trigger for termination of diapause occurs, preparing the individual for moulting to the adult phase before migrating back to the surface waters. The study by Tarrant et al. (2008) used animals collected at one point of time only and individuals in the shallow population may include animals that had recently terminated diapause: differential

gene expression between shallow and deep animals collected at one point in time, instead of over a time series as in my work from Loch Etive, may not pick up changes in gene expression with diapause. This should be considered when using *EcR* as a marker for diapause without another indicator for diapause such as the aminoacyl-tRNA synthetases content (Yerba et al., 2006) or mid-gut epithelium histological changes (Bonnet et al., 2007). It is harder to attribute the changes in expression of *RXR* and *A-type AST* seen in my work with diapause, although the changes in *RXR* expression from June to November mirrored those of *EcR*, suggesting that *RXR* and *EcR* may possibly be acting as a dimer to control moulting and development in the active population. *RXR* showed an increase in December and a large increase in expression in January, combined with a drop in expression of *A-type AST* mRNA. In February this was followed by a drop in *RXR* expression to its lowest level and a corresponding increase in *A-type AST* expression. This may indicate that *RXR* is involved in emergence from diapause, expression building throughout the diapause period in response to an internal or external trigger for development, causing the increase in *EcR* expression in January. Expression of *USP* followed a similar pattern during pupal diapause in the fly *Sarcophaga crassipalpis* (Rinehart et al., 2001), however further study of *RXR* in *C. finmarchicus* is needed to support this theory.

5.3 Lipids as a trigger for diapause induction or termination?

I tried to address the potential role of lipids in diapause initiation and termination of *Calanus finmarchicus* by measuring total lipid content and lipid class and fatty acid composition over a seasonal cycle. In Loch Etive, *C. finmarchicus* accumulated lipids through the spring and summer months until October and appeared to utilise some 50% of these lipid reserves whilst in diapause during the winter: this level of

depletion is similar to that observed in other studies (Hopkins et al. 1984; Heath et al., 2008). Although not conclusive, the accumulation of lipid from July to October fits with the recently espoused 'lipid window accumulation hypothesis' (Johnson et al., 2008), as I believe *C. finmarchicus* in Loch Etive enter diapause between October and November, when the expression of *EcR* is reduced. The decrease of total lipid during diapause also fits with the theory that animals may terminate diapause when the lipid store declines to a certain level (Miller et al. 1991, Hirche 1996, Ohman et al. 1998, Visser & Jónasdóttir 1999, Irigoien 2004, Saumweber & Durbin 2006; Johnson et al., 2008). It has been estimated that the 'critical level' is 70 μg (Rey-Rasset et al., 2002); although another trigger for diapause termination is likely for those animals that sustain lipid stores above this critical level through the whole winter. My novel use of analysing lipid dynamics of single individuals (instead of the commonly used approach of analysing groups of ten or more animals that is usually adopted) highlighted the extreme variability between individual lipid dynamics. Lipid accumulation is dependant on the functional relationship between the production of wax esters and ambient food availability and temperature (Johnson et al., 2008) and different individual strategies may be appropriate, depending on the state of the individual such as body size, energy reserves and metabolic costs, all or some of which may affect the timing of diapause. The one or two year life history strategy of *Calanoides acutus* in the Southern Ocean is thought to be largely dependant on the lipid reserves accumulated by an individual. A one generation strategy is adopted by those individuals that accumulate sufficient lipid reserves to overwinter and reproduce in the following spring whereas those that do not accumulate enough lipids remain as stage CV and accumulate lipids over another season, before overwintering and then reproducing (Tarling et al., 2004). Energetic demands on individuals such as

unforeseen physiological stress in unfavourable environments caused by advection or a deep water renewal event in Loch Etive during diapause are also likely to vary considerably (Pepin and Head, 2009). Thus the continuing study of individual lipid content and physiological state is essential for understanding the role of lipids in diapause of *C. finmarchicus.* Lipid accumulation by *C. finmarchicus* is also of interest to parties harvesting or intent on harvesting copepods, either for human consumption (Wiborg, 1976), or for use in the aquaculture industry: against a background of decreasing conventional fish resources, and availability of fish oils for use in aquaculture, various countries including Norway are investigating the use of zooplankton as a food source (Olsen et al., 2004).

5.4 Implications of my research and scope for further research:

There is much scope for further research based on the results of this study. Key points include:

1. To identify other genes potentially involved in regulating diapause by using suppressive subtractive hybridization (SSH) to compare gene expression: adopting a similar approach to that of Tarrant et al. (2008), but applying SSH to a series of samples collected over a seasonal cycle may identify other suits of genes involved in diapause regulation.

2. To expose the role of *RXR* in diapause, which remains ambiguous. Measuring expression of *RXR*, *EcR* and *A-type AST* through copepodites stages CIII to adult in a cultured population of *Calanus finmarchicus*, members of which do not enter diapause and may be sampled more frequently, would perhaps show

the role of *RXR* and *A-type AST* in the normal reproductive cycle where development is not suppressed. It would be interesting to see if, under such conditions, *RXR* expression and *EcR* expression patterns were similar through development and thus, if they were acting together as a dimer complex in an active population. *RXR* may also be involved in the reproductive processes in *C. finmarchicus* and, by comparing expression in females at different stages of reproduction, e.g. females with barely visible developing gonads, fully developed gonads and post-spawning females, the role of RXR in the reproductive process may be elucidated. Additionally, the expression of *RXR* and *A-type AST* over the normal reproductive cycle may then be compared with expression over diapause obtained from this study and would possibly identify a relationship between reduced expression of *A-type AST* and an increase in expression of *RXR*.

3. To measure the expression of *EcR*, *RXR* and *A-type AST* from *C. finmarchicus* collected over a seasonal cycle in an open-ocean deep water environment where the animals are known to overwinter in large numbers, such as the Farøe-Shetland channel. There appeared to be no change in gene expression in animals collected during one point in time at different depths in the Farøe-Shetland channel, possibly due to all the individuals being in the same state of diapause, as *EcR* expression was not significantly different, despite depth differences. Differential gene expression may be more evident over a seasonal cycle, and changes in gene expression of populations known to be diapausing may identify if *RXR* may be involved in emergence from diapause prior to the expression of *EcR*.

4. To measure the titre of the potential ligand for *RXR*, methyl farnesoate (MF) from cultured *C. finmarchicus* over the normal reproductive cycle. MF could be isolated using gas chromatography-mass spectrometry (GC-MS), and quantified using a known amount of methyl farnesoate standard. The titre of MF secreted by *C. finmarchicus* is likely to be small, and consequently large numbers of animals may be needed to isolate the hormone. By using cultured animals, large numbers of live animals that may be easily identified to stage are available. If there are enough samples, this technique could then be applied to the time series of animals collected from Loch Etive, and it may be possible to link *RXR* expression with MF secretion.

5. To further test the lipid accumulation window hypothesis (Johnson, 2008), as well as to investigate the possible termination of diapause caused by the depletion of lipids below a critical level, lipid accumulation and lipid class composition in depth-determined individuals of *C. finmarchicus* should be sampled frequently, perhaps weekly, from August to March in Loch Etive, coupled with *EcR* expression studies to determine diapause state. This may also provide more information on the timing of diapause in Loch Etive.

5.5 Concluding remarks

Over the last 50 years, with the rise in greenhouse gas emissions, the oceans have become warmer and more acidic (Jackson et al., 2008). The warming of the sea surface has also increased stratification as warmer, lighter surface waters inhibit

mixing (Schmittner, 2005). An increase in stratification in the North Pacific has already caused a regime shift in the plankton communities in this area (McGowen et al., 2003). Combined with increased eutrophication, this inhibition of vertical mixing has led to a decrease in dissolved oxygen concentrations and the formation of anoxic 'dead zones' in some continental seas such as the Baltic (Diaz and Rosenberg, 2008). Climate models predict further warming and acidification of the ocean, with corresponding increases in stratification and decreases in dissolved oxygen content (Diaz and Rosenberg, 2008; Schmittner et al. 2008; Stramma et al., 2008). What is the likely impact of these changes on *Calanus finmarchicus*? *C. finmarchicus* is adapted to a cold oceanic environment, where high winter mixing is the norm and where surface nutrients and dissolved oxygen concentrations are high (Helaouët and Beaugrand, 2007). Increased stratification of the coastal waters over the summer months will reduce primary production at a time when nauplii and copepodites are abundant. This will constrain growth, may restrict the number of generations *C. finmarchicus* can produce in a year such is presently the case in Loch Etive, reduce abundance of *C. finmarchicus*, and possibly delay the initiation of diapause as individuals may take longer to accumulate requisite lipid stores. The increase in size and abundance of hypoxic zones will also reduce the habitat available to *C. finmarchicus* (Diaz and Rosenberg, 2008). The presence of hypoxic zones in the deeper waters in the summer months may also have an impact on diapause, either decreasing the diapause depth or delaying the onset of diapause. Increased acidification of the ocean may also reduce the hatching success of *C. finmarchicus* (Mayor et al., 2007). The persistence of *C. finmarchicus* in the relatively harsh environment of Loch Etive, where primary production is low (Wood et al., 1973), where temperatures can be high and where stratification exists in the upper basin

almost permanently, indicates that the species can adapt and survive in conditions atypical of the open ocean. Perhaps, however, it is the fact that there is little competition from *Calanus helgolandicus* in Loch Etive that has enabled *C. finmarchicus* to persist there. The shift in dominance from *C. finmarchicus* to *C. helgolandicus* in the North Sea that has already occurred is thought to have been triggered solely by temperature increases and associated changes such as increased stratification and decreased oxygen (Helaouët and Beaugrand, 2007). If sea temperatures continue to rise it is likely that the distribution of *C. finmarchicus* will move even further northwards. The potential large decreases in abundance and distribution of *C. finmarchicus* with increases in sea surface temperatures and ocean acidification will significantly reduce secondary production in regions such as the North Atlantic where it forms a large part of the biomass (Marshall and Orr, 1957; Conover, 1988; Longhurst and Williams, 1992; Mauchline, 1998) and will further affect the recruitment of commercial fish species such as herring, mackerel and cod. The sensitivity of zooplankton to subtle environmental changes makes them key markers of change in the ocean. Understanding the physiological and genetic basis of these adaptations provides understanding of the effects of these environmental changes and how further change will affect the pelagic ecosystem.

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