Offspring size, provisioning and performance as a function of maternal investment in direct developing whelks

By

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Abstract

Initial maternal provisioning has pervasive ecological and evolutionary implications for species with direct development, influencing offspring size and energetic content, with subsequent effects on performance, and consequences in fitness for both offspring and mother. Here, using three sympatric marine intertidal direct developing gastropods as model organisms (*Cominella virgata*, *Cominella maculosa* and *Haustrum scobina*) I examined how contrasting strategies of maternal investment influenced development, hatchling size, maternal provisioning and juvenile performance.

In these sympatric whelks, duration of intra-capsular development was similar among species (i.e. 10 wk until hatching); nonetheless, differences in provisioning and allocation were observed. *Cominella virgata* (1 embryo per capsule; ~3 mm shell length [SL]) and *C. maculosa* (7.7 ± 0.3 embryos per capsule; ~1.5 mm SL) provided their embryos with a jelly-like albumen matrix and all embryos developed. *Haustrum scobina* encapsulated on average 235 ± 17 embryos per capsule but only ~10 reached the hatching stage (~1.2 mm SL), with the remaining siblings being consumed as nurse embryos, mainly during the first 4 wk of development. Similar chronology in the developmental stages was recognizable among species. Higher growth rates and evident juvenile structures became clear by the second half of development and larval characteristics were less frequently observed. Even after 10 weeks of encapsulation and despite emergent crawling juveniles, some hatchling *H. scobina* still retained "larval" traits, suggesting that this nurse embryo-based provisioning could result in intra-capsular asynchrony of development, and that female of this species would be able to bet-hedge in a higher extent compared with female *C. maculosa* or *C. virgata*.

Maternal investment in newly laid egg capsules differed among the three study species. The structural lipids phospholipid (PL) and cholesterol (ST) and the energetic lipids aliphatic hydrocarbon (AH), triglycerides (TG), diglycerides (DG) and free fatty acids (FFA) occurred in all three species. Only eggs (and also hatchlings) of the multi-encapsulated embryos *C. maculosa* and *H. scobina* were provisioned with the energy lipids wax ester (WE) and methyl ester (ME), suggesting an interesting similarity with pelagic larvae of other invertebrates and fish where those lipid classes have also been recorded. Despite differences in hatchling size, the small *H. scobina* had significantly higher amounts of the energy storage lipid TG compared with *C. maculosa* and *C. virgata*, suggesting interesting trade-offs between offspring size and offspring energy

resources. *H. scobina* was the only species that suffered a complete depletion of FFA during development (5th wk), suggesting an additional role of this energetic lipid during the early stages of development. Differences in the amount of lipids among newly laid capsules and siblings within capsules were also detected within species. In both species with multiple embryos per capsule, *C. maculosa* and *H. scobina*, these differences were largely explained by variation in TG and PL, enhancing the important role of the major structural (PL) and energy (TG) lipids during the early stages of these whelks, and also providing an integrative approach for evaluating maternally-derived lipids on a per-individual basis in direct developing species with contrasting provisioning and offspring size.

Because in direct developers maternal provisioning to the embryos is the primary source of nutrition until offspring enter juvenile life, differences in performance should be closely related with initial provisioning, which in turn may reflect maternal nutritional conditions. Field-based surveys and manipulative experiments in the laboratory showed that different maternal environments (i.e. locations and sites) and contrasting offspring size influenced juvenile performance in different ways for *C. virgata* and *C. maculosa*. Despite the large differences in conditions and available resources between the Wellington Harbour and the nearby South Coast, the two locations did not influence the hatchling size of either species, and the most important source of variation was at the smallest scale (i.e. among sites), with substantial variation also occurring within and among females. Between and within species differences in hatching size reflected juvenile performance when fed, regardless of whether subjected to desiccation stress. When starved however, species-specific and size differences in performance were less significant. As has been described for many taxa, large offspring often perform better than small conspecifics; however, because this performance is likely to be context-dependent, understanding the importance of the different scales of variation is crucial for determining how variation in size reflects an organism's performance.

Despite the long recognized role of intra-specific variation in offspring size in mediating subsequent performance, the consequences of inter-specific variation in per-offspring maternal investment for co-occurring taxa have been rarely examined in a predator-prey context. Manipulative experiments in the laboratory with hatchling and juvenile *C. virgata* and *C. maculosa* revealed that vulnerability of their early life-stages to common crab predators (i.e. the shore crab *Cyclograpsus lavauxi*) is highly size-

dependent. When predator size was evaluated, small crabs were unable to eat hatchlings of either whelk species. Medium and large shore crabs consumed both prey species; however, hatchlings of *C. virgata* were less vulnerable to predation by medium crabs than large ones, and *C. maculosa* were equally vulnerable to both sizes of crabs. In hatchlings of both prey species the shell length and shell thickness increased over time; however, only *C. virgata* reached a size refuge from predation after two months post-hatch. Results showed that vulnerability to predators can be mitigated by larger sizes and thicker shells at hatch; nonetheless, other species-specific traits such as juvenile growth rates, may also play key roles in determining the vulnerability of hatchling and juvenile snails when exposed to shell-crushing predators.

Overall, these findings suggest that when defining offspring size, provisioning and performance relationships, many context-dependent scenarios are likely to arise. Therefore examining the early life-history stages of direct developing whelks with contrasting maternal investment under an integrative morphological, physiological and experimental approach, allowed a better understanding of how these complex relationships arises and how mediated the species life-history in terms of offspring size, maternal provisioning and subsequent juvenile performance.

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Chapter 1

General Introduction

1.1 Maternal effects on offspring traits

For most multicellular organisms, one of the main reproductive traits is the production of gametes with different sizes, e.g. ova are large and sperm are small, and thus, differential investment has led to mothers and fathers playing very different roles (Marshall et al. 2008). Mothers provide offspring with their necessary nutritional requirements, but in most organisms, maternal decisions also have diverse implications. For example, a mother's choice of oviposition site can be based on different environmental characteristics, ranging from temperature and moisture regimes to food quality and levels of competition that offspring are likely to experience. Given these close associations between offspring and mother, maternal effects have been recognized as the most important determinant of an offspring's initial phenotype and fitness (Bernardo 1996a). For over 20 years, maternal effects and their consequences have been subject to intense study in plants, insects and terrestrial vertebrates, but these effects have received far less attention in marine systems (Marshall et al. 2008). Because of the different reproductive strategies, and thus, varied ways in which mothers provisions their offspring, marine invertebrates provide a good model system for evaluating different questions regarding maternal effects and their possible consequences for offspring condition and performance.

The life cycles of most benthic marine invertebrate species includes a pelagic larval stage that may spend days, weeks or even months in the plankton before larvae metamorphose into benthic juveniles (Young 2002). These free-living dispersive stages may be feeding, by acquiring food during their development (planktotrophic) or non-feeding (lecithotrophic), relying entirely on yolk placed in the egg by the mother (Hill 1991; McEdward & Janies 1993; Young 2002). Other reproductive strategies lack dispersive larval stages and the individuals develop to the juvenile stage entirely within egg masses, egg capsules, or brood chambers (aplanktonic), although they often pass through a distinctly larval morphology. However, it is generally believed that free-living

larvae are primitive in marine invertebrate life histories and that the loss of larval stages is a derived condition (see Strathmann 1978; Pechenik 1999).

Regardless of the inter-specific ways to release gametes, larvae, and in some cases fully-formed juveniles into the surrounding environment, a common trait in every taxon that has been studied is propagule size or offspring-size variation (Bernardo 1996b; Marshall et al. 2008). Offspring size can affect every stage of a marine invertebrate's life history and has pervasive effects on performance, even in species for which maternal provisioning accounts for only a small proportion of larval nutrition (e.g. planktotrophs) (Marshall et al. 2008). Studies on the early-life history stages of several invertebrate species have demonstrated that larger sizes are often correlated with better performance in terms of feeding rate, duration of planktonic period, settlement rate, growth rate and survival (see Spight 1976a; Gosselin 1997; Moran & Emlet 2001; Phillips 2002; Marshall et al. 2003; Marshall & Keough 2004; Emlet & Sandro 2006).

The level of provisioning that an individual receives from its mother can have far-reaching consequences for subsequent performance and phenotype via 'carry-over' effects on later life stages. Many of these carry-over effects are thought to be mediated by variation in larval energetic reserves such that larger offspring have more energetic reserves than smaller offspring. Thus initial differences in size and provisioning can have consequences that may persist into the juvenile or even to the adult, thereby affecting the performance and phenotype of individuals through their lives (Wendt 2000; Phillips & Gaines 2002; Phillips 2004; Marshall et al. 2008). Traditionally, we have viewed marine invertebrate populations as being strongly affected by the quantity of larvae entering a population; however, intra-specific variation in offspring traits also means that the quality of larvae may have equally important effects describing the condition that a pool of settlers have when they recruit into the adult population (Hunt & Scheibling 1997; Pechenik et al. 1998; Phillips 2002; Marshall & Keough 2008a). For several species of marine invertebrates, reduced egg size, lipid content, or larval food typically leads to smaller, more poorly conditioned larvae, which subsequently become juveniles with reduced growth rates and/or lower survival (reviewed in Phillips & Gaines 2002). During this recruitment period, most invertebrate larvae are limited in their ability to feed and rely on endogenous reserves, primarily lipids and proteins, to fulfill their energetic needs (for examples see: Rodriguez et al. 1990; Hereas et al. 1998; Videla et al. 1998; García-Esquivel et al. 2001; Sewell 2005; Phillips 2006; Prowse et al. 2008, among others).

Offspring size is not the only, and sometimes not even the best, indicator of offspring quality (McEdward & Carson 1987; McEdward & Morgan 2001). To evaluate the physiological conditions of early life stages, studies have often assessed an index based on neutral lipid or triglycerides (TG) content (see Sewell 2005). It has long been recognized that lipids are the major metabolic energy reserves during the early life-history stages of many animal groups, being rapidly depleted by embryos and larvae during development (Byrne et al. 2008a). While the structural lipids (e.g. phospholipids and cholesterol) are the main component of all cellular membranes, the energy-storage lipids, and especially triglycerides, are the reserves that fulfill offspring needs immediately after hatch (Sewell 2005; Byrne et al. 2008a; Guay et al. 2011). In this sense, TG is recognized as the most important lipid class since is the primary endogenous energy reserve fueling the basal metabolism in several species of marine invertebrates, including mollusks and echinoderms (e.g. Gallager et al. 1986; Moran & Manahan 2003; Sewell 2005; Falkner et al. 2006; Pernet et al. 2006).

In mollusks it is well known that the greatest reliance on stored energy reserves is during embryogenesis and metamorphosis, when the feeding larvae lack a functional mechanism for particulate feeding (i.e. Jaeckle & Manahan 1989; Rodriguez et al. 1990; Whyte et al. 1987; Whyte et al. 1990; Heras et al. 1998; Videla et al. 1998; Labarta et al. 1999; Moran & Manahan 2003). Similarly, research in echinoid larvae suggest that from the total lipid classes, the energetic lipid triglyceride (TG) is rapidly depleted during prefeeding development, while structural lipids (i.e. phospholipids, PL) remain relatively stable as they are used to construct the larval body (Sewell 2005; Byrne et al. 2008b). Although a range of studies have explored maternal provisioning in marine invertebrates with planktonic dispersal stages, there is an important knowledge gap regarding the maternally-derived resources during early stages of non-dispersive direct developing species.

1.2 Ecological implications

In marine systems, offspring quality variation can be viewed as a filter on the number of individuals that pass through each stage. If offspring quality is relatively high, then more of the offspring may become successful recruits than if offspring quality is relatively low (Marshall & Keough 2008a). Studies across a variety of taxa and habitats

have demonstrated that individuals that start life at smaller sizes are more likely to lose in intra- or inter-specific interactions or be negatively affected by any density-dependent interaction compared with larger conspecifics [e.g. gastropods (Spight 1976a; Gosselin 1997; Moran & Emlet 2001), mussels (Phillips 2002; Phillips & Gaines 2002), barnacles (Thiyagarajan et al. 2003), ascidians (Marshall et al. 2003, 2006) and bryozoans (Marshall & Keough 2008b)].

These consequences of offspring size have the potential to dramatically change population dynamics, since initial size *per se* could later modify specific traits of juvenile individuals within a given population (reviewed by Marshall & Keough 2008a). In a classical example, Spight (1976a) found that larger intertidal snail hatchlings performed better than small conspecifics because they were able to better tolerate physical stress (e.g. desiccation), travel further to find food or shelter, have a larger food supply (e.g. larger prey can be taken), or be susceptible to fewer predators. Although there are data from a variety of studies demonstrating these size-mediated intra-specific effects, inter-specific effects for species with similar life-histories are less well explored. Additionally, given coincident seasonal reproduction, early life stages of many taxa co-occur in benthic habitats, and are therefore vulnerable to the same threats. If there are size-dependent or species-specific responses, those traits may therefore influence the subsequent community structure of early recruits.

1.3 Studies in direct developers

Direct developing species lack free-living larval stages that can affect (through larval feeding) the relationship between juvenile provisioning and original maternal provisioning; therefore, it has been suggested that the relationships among offspring provisioning, size, quality and performance are likely to be strongest and most consistent for these species (Marshall & Keough 2008a). Interestingly, different strategies for increasing per-offspring maternal investment have been reported in direct developing taxa, including nurse eggs (Spight 1976b), consumption of developing or non-developing siblings (see Cubillos et al. 2007), intragonadal sibling cannibalism (Byrne 2006), and even partial predation on maternal body parts (reviewed in Marshall & Keough 2008a). The variation of such supplementary food provided to the embryo can be important in determining offspring size, and it has been suggested that mothers

with more control of the provisioning for individual offspring would be more likely to adaptively adjust the size of their offspring according to local conditions (see Marshall et al. 2008). For example, in the absence of extraembryonic material offspring may exhibit low intra-specific variation in size, and to influence their hatchling size, selection would have to act on egg size, albumen availability or growth (see Rivest 1983). Unlike this pattern, species that provides embryos with varying kinds of extraembryonic food usually exhibit larger variations in hatchling size, resulting for example from differential provisioning among capsules or different number of embryos per capsule (Chaparro et al. 1999; Lloyd & Gosselin 2007). In such species, competition among capsule mates for limited resources within the microenvironment of the capsule may therefore be predicted to generate high variation in hatchling size when variable consumption of resources (i.e. nurse eggs) by feeding embryos occurs (Spight 1976b; Rivest 1983; Collin 2003).

Although such studies have provided valuable insights into maternal allocation of resources in either laboratory or field conditions, a more integrative approach is still needed in order to evaluate how maternally-derived resources mediate offspring size and juvenile performance in species with different reproductive strategies and under similar environmental conditions.

1.4 Study species

Molluscan gastropod whelks are a large and diverse group of direct developers, in which the development occurs within benthic egg capsules and the hatchlings often emerge as juveniles into the same environment as their parents (Pechenik 1999). The species considered in this study are the predatory whelk *Haustrum scobina* (Neogastropoda: Muricidae) and the scavenging whelks *Cominella virgata* and *Cominella maculosa* (Neogastropoda: Buccinidae).

Haustrum scobina is abundant in rocky grounds from sheltered to exposed shores in New Zealand, but it may also occur on soft shores with sufficient hard substrata (Morton & Miller 1968). The predatory nature of *H. scobina* and its ability to drill through the shell of oysters, little black mussels and barnacle prey has been well documented (Stewart & Creese 2004), playing an important role in determining the lower limit of distributions of barnacles (reviewed in van der Sman 2007). *Cominella*

virgata and *C. maculosa* are commonly found on moderately sheltered rocky reefs down to the sublitoral fringe; however, both may occur on soft shores where hard substrata such as dead bivalve shells are available (Morton & Miller 1968). Although both species are scavengers, only *C. virgata* has been reported occasionally feeding on living snails by drilling (Stewart & Creese 2004).

Recent studies on these three whelk species have suggested that although the variation in adult food supply did no affect subsequent hatchling size, it did affect offspring performance (van der Sman 2007). However, the large variability in hatchling size that occurred within clutches and individual capsules in both high and low food treatments, suggests that hatchling size may not be the best method to quantify reproductive allocation in these species, and other juvenile traits such as growth rates need to be considered for evaluating provisioning and performance (van der Sman et al. 2009). Explicit studies between offspring size and other indices of offspring quality are therefore still necessary, and probably the best way to evaluate the quality of hatchlings and juveniles is in terms of energetic reserves or lipid provisioning. If these kind of approaches can be undertaken in these direct developing species, it will be possible to understand for the first time how variation in energetic reserves for offspring originate or are mediated by maternal traits or experiences, the scales at which variation occurs, and the ecological consequences of variation in energetic reserves for juveniles.

1.5 Aims and structure of this thesis

Despite accumulating evidence in marine species show that juvenile survival can depend on offspring size, there are still unresolved questions about the relationship between offspring size and quality at hatch or metamorphosis (i.e. as measured by lipid content), and further, how these traits influence the performance of juveniles (i.e. growth and survival). Therefore, the main objective of this study is to evaluate maternal provisioning, offspring size, offspring quality and juvenile performance in three species of intertidal whelks with different reproductive strategies: *C. virgata* (1 embryo per capsule), *C. maculosa* (multiple embryos per capsule) and *H. scobina* (multiple embryos per capsule and intra-capsular nurse embryo consumption).

Integrative measurements of offspring or hatchling quality have not been explored in this system or for marine direct developers in general. Therefore, I used a suite of field

collections and manipulative experiments to examine different questions on the early life-history of these three whelk species based on the following structure: In Chapter 2, I provide a detailed description of the packaging strategy and intra-capsular development observed in these three species in order to present the morphological basis associated with the concominant questions regarding provisioning and performance. In Chapter 3, I describe a modified protocol to identify and quantify different lipid classes on a per-individual basis in early stages of direct developing whelks using the Iatroscan thin-layer chromatography-flame ionization detector (TLC/FID) system. In Chapter 4, I apply this methodology by examining inter- and intra-specific differences in maternal provisioning (lipids) on egg capsules and hatchlings of the three species, together with the dynamic of lipid classes over development. In Chapter 5, I used manipulative experiments with the two congeneric *Cominella* species to examine how different maternal environments (i.e. high vs low productive sites) mediate the hatchling size and performance of juveniles under different environmental stressors (i.e. desiccation and starvation). This chapter is published in Marine Ecology Progress Series (Carrasco et al. 2012). In Chapter 6, I also used a manipulative approach with the same two species to evaluate how inter-specific vulnerability to predators is mediated by early-juvenile traits (including size) at different stages post-hatch (i.e. 1day, 1 month and 2 months). This chapter is published in Invertebrate Biology (Carrasco & Phillips 2012). Each of these chapters has been written as an individual manuscript (exept chapter 3); therefore, there might be redundancy in some of the information provided. Finally, in Chapter 7, I discuss and summarize the main findings of this thesis.

Chapter 2

Encapsulation and developmental traits of three New Zealand neogastropods with contrasting embryo packaging and maternal provisioning

2.1 Abstract

Early life stages of three co-occurring whelks from New Zealand waters, *Cominella virgata*, *C. maculosa* and *Haustrum scobina*, were studied in order to compare their oviposition patterns, capsule morphology and intra-capsular development. Clutches observed over time in the field varied in loss rates and causes of mortality: *H. scobina* seemed to be more vulnerable to bacterial infection (identified by a purple coloration) and predation (rasped or drilled) than the *Cominella* species. Duration of intra-capsular development was similar among species (i.e. 10 wk until hatching); nonetheless, differences in provisioning and allocation were observed. *Cominella virgata* (1 embryo per capsule) and *C. maculosa* (7.7 ± 0.3 embryos per capsule) provided their embryos with a jelly-like albumen matrix and all of embryos within the capsule developed. *Haustrum scobina* encapsulated 235 ± 17 embryos per capsule but only 10 on average reached the hatching stage, with the remaining siblings being consumed as nurse embryos during the first 4 wk of development. Hatchling morphology and mean size varied among species: *C. virgata* had a "double whorled" shell of 2.7 mm length (SL) on average, whereas *C. maculosa* and *H. scobina* had "single whorled" shells of 1.6 mm and 1.2 mm SL on average, respectively. Trade-offs between size at hatching and hatchling number also occurred in the latter two species. Inter-specific differences in capsular and juvenile traits described here will likely have major ecological consequences during the early-life history stages of these co-occurring direct-developing snails.

2.2 Introduction

Encapsulation of developing embryos within maternally-derived structures such as gelatinous egg masses or benthic egg capsules is widespread in many aquatic animals, including chondrichthyan fish, amphibians, mollusks and many polychaete worms (Pechenik 1979; Rawlings 1994; Rawlings 1999; Kamel et al. 2010a). This kind of parental protection therefore, has evolved independently in several phylogenetically diverse groups in response to the risks associated with embryonic development, and has also been shown to have a major evolutionary impact on the reproductive success and habitat expansion of many taxonomic groups (Rawlings 1994).

The retention of eggs within tough multi-laminated capsules or elaborate gelatinous masses is energetically costly, and requires special morphological and physiological specializations of the female's reproductive anatomy; therefore, survival benefits to the developing embryos (even with no parental care) should be considerable (reviewed by Pechenik 1979). In marine gastropods, benthic egg capsules have been well documented among the whelk families Muricidae, Buccinidae, Nassariidae, Melongenidae, Fasciolariidae, Volutidae and Ranellidae (D'Asaro 1991, 1993, 1997, 2000; Penchaszadeh et al. 1999; Gallardo et al. 2012), and are thought to occur in most marine neogastropod mollusks, from deep-sea areas to the upper levels of the intertidal zone (Rawlings 1999).

Capsule structures varies dramatically within this group, and differences in size, shape, complexity and chemical composition are apparent within families, among closely-related species and even among populations of a single species (see Rawlings 1999). Although their primary function is assumed to be protection of the developing embryos against the many sources of mortality generated in a hostile benthic environment (e.g. desiccation, wave action, bacterial attack, UV radiation, predation and salinity stress; reviewed in Dumont et al. 2008; Segura et al. 2010; Roche et al. 2011), the capsule morphology should not restrict embryonic nutrition, gas exchange or excretion, to promote successful development and emergence of hatchlings (Pechenik 1999).

Given the suite of stressors present in natural conditions, encapsulating structures may also vary as a result of adaptive responses, reflecting the outcome of different selective pressures acting during embryonic development in the intertidal habitats in which they are spawned [e.g. desiccation, osmotic stress, and ultraviolet (UV) radiation,

Rawlings 1999]. Furthermore, capsular traits such as volume or height can be consistently related with offspring traits (e.g. size or number) and therefore, differences in such patterns may have major ecological implications (for examples see: Spight 1976a; Rivest 1983, 1986; Pechenik et al. 1984; Chaparro et al. 1999; Saglam & Duzgunes 2007; Averbuj & Penchaszadeh 2010; Chatzinikolaou & Richardson 2010; Lahbib et al. 2011).

Within capsules, patterns of maternal provisioning and mechanisms of embryonic nutrition may also play key roles in determining traits such as size at hatching. For example, species that produce all normally developing eggs of similar size produce offspring of a relatively uniform size; to influence their size, selection would have to act on egg size, albumen availability or embryonic growth (see Rivest 1983). An alternative strategy in some prosobranch gastropods is the production of some eggs which do not go on to complete development, but instead provide nutrition for siblings. These include non-developing nurse eggs (oophagy), fertilized zygotes and embryos (adelphophagy) or even viable siblings (cannibalisms) to be consumed by other embryos during their encapsulated development. In such species, competition among capsule mates for limited resources within the microenvironment of the capsule is therefore predicted to generate high variation in hatchling size when variable consumption of nurse eggs by feeding embryos occurs (Spight 1976b; Rivest 1983; Collin 2003).

By mediating size at hatchling, those maternally-derived resources are major determinants of the subsequent juvenile performance in terms of growth, survival and energy content, with positive offspring size effects being extended further into the offspring's life (see Marshall & Keough 2010). From an energetic point of view, it has been suggested that direct development is an advanced reproductive mechanism, particularly in those cases in which development is fueled by nurse eggs, favoring a fast embryonic development and reducing the hatchling period (reviewed in Chaparro & Pashke 1990). Although marine benthic mollusks display a range of reproductive strategies, it has generally been recognized that the production of pelagic eggs that develop into the planktotrophic trochophore and veliger larvae is the ancestral condition (Pechenik 1999). Consequently, an important step in the evolution of reproductive strategies took place with the acquisition of internal fertilization and the different ways of embryo packaging where development occurs (i.e. gelatinous envelops or elaborated egg capsules; see Gallardo et al. 1992).

Since particular capsular morphologies and differences in maternal provisioning may interact to mediate specific offspring traits, and because both characteristics are the foundation of many ecological questions, this chapter adds to the current knowledge on neogastropod encapsulation and development while also aims to provide a comparison into the early-life history stages of three co-occurring whelks from New Zealand waters: *Cominella virgata*, *C. maculosa* and *Haustrum scobina*.

My objectives for these three species were to: 1) compare their egg-laying characteristics and capsule morphology, 2) evaluate possible threats affecting their capsules in the field, 3) provide a detailed description of their encapsulated development, 4) evaluate the relationship between capsule volume and hatchlings traits (i.e. size and number) in the species that encapsulate multiple embryos per capsule, and 5) discuss this information in the context of the evolutionary aspects of embryo provisioning. I hypothesize that, first: smaller capsules (i.e. *C. virgata* and *H. scobina*) will be more vulnerable to field conditions; second: hatching size will correlate with egg size; third: species with larger hatchling sizes (i.e. *C. virgata*) will have the fastest intra-capsular growth rate; and fourth: in species with multiple embryos per capsule, the development fueled by nurse embryos (i.e. *H. scobina*) would be less synchronous within a capsule than in species without them (i.e. *C. maculosa*).

2.3 Material and Methods

2.3.1 Study species and collections

Cominella virgata and *C. maculosa* (Neogastropoda: Buccinidae) are commonly found on moderately sheltered rocky reefs down to the sublitoral fringe in the North Island of New Zealand and the northern part of the South Island, respectively; however, both may occur on soft shores where hard substrata such as dead bivalve shells are available (Morton & Miller 1968). Although both species are scavengers, *C. virgata* has also been reported to feed occasionally on living snails by drilling; however, *C. maculosa* never drills (Stewart & Creese 2004). *Haustrum* (= *Lepsiella) scobina* (Neogastropoda: Muricidae) is abundant on rocky reefs from sheltered to exposed shores throughout New Zealand, but may also occur on soft shores with sufficient hard substrata (Morton

& Miller 1968). The predatory nature of *H. scobina* and its ability to drill through the shell of oysters, little black mussels and barnacle prey is well documented (Stewart & Creese 2004), and has been shown to play a major role in determining the lower limit of distribution for the latter species (reviewed by van der Sman 2007).

In October 2010, groups of egg capsules attached to boulders were collected from actively spawning *C. virgata*, *C. maculosa* and *H. scobina* in the low intertidal zone of Point Halswell (41° 17' S, 174° 49' E), a semi-exposed site located within Wellington Harbour, North Island, New Zealand. All clutches (and boulders) were transported in seawater to the Victoria University Coastal Ecology Laboratory (VUCEL), where they were placed in a large sea table with permanent water flow at a mean (±SD) ambient temperature of 15.4 ± 1.6 °C until hatching. Samples were further used to describe capsule morphology, development and encapsulation traits (see sections 2.3.3 and 2.3.4 below), together with the maternal provisioning on early developmental stages of the three species (see chapters 3 and 4).

2.3.2 Field observations on egg-laying characteristics

In addition to the previous collections, and in order to describe inter-specific patterns of egg-laying (i.e. cluth size) and egg capsule viability (i.e. preyed or infected by bacteria), observations on field-laid clutches of *C. virgata*, *C. maculosa* and *H. scobina* were carried out once a month (i.e. from October to December 2011) at Point Halswell. During each sampling, rocks in a 30m transect line on low-intertidal were randomly overturned to look for groups of egg capsules. Therefore, different groups of capsules were sampled in different months. When found, each group was photographed and the number and viability of egg capsules further evaluated by image analysis (Image J software; http://rsb.info.nih.gov/ij/). Within a group of egg capsules, viability was determined by evaluating number of capsules infected by bacteria (i.e. pink color) or with evident signs of predation. Though, no egg capsules were collected during field surveys.

2.3.3 Egg capsule morphology and intra-capsular development

To characterize the capsule morphology and intra-capsular development of the three study species, 5 - 11 haphazardly selected egg capsules were sampled at weekly intervals until hatch. To ensure that enough capsules were available to consistently describe the developmental pattern over time, only the biggest group of newly deposited egg capsules collected from the field was sampled for each species (i.e. section 2.3.1; ~100 egg capsules). The remaining groups of egg capsules were kept and used in lipid analyses (see Chapters 3 and 4). In all cases, capsules and embryos inside each capsule were examined and measured *in vivo*, no fixatives were used. Estimations of capsule volume were done based on van der Sman (2007). For *C. virgata* capsule volumes were calculated assuming that the capsule approximates a cuboid with a half cylinder top: $V= hwc – wc^2/2 + \pi wc^2/8$ (see Fig. 2.2a, b), whereas for *C. maculosa*, capsule volumes were calculated assuming that the capsule shape approximates an ellipsoid: $V=4/3\pi\ abc$. In both cases h, w and c represented the height, width and depth, respectively (see Fig. 2.2c, d). In *H. scobina*, capsule volumes were calculated assuming the shape approximates a cylinder with a half sphere top: $V=\pi r^2\ (h-r) + 2/3\pi r^3$, where r is the capsule radius and h the height (see Fig. 2.2e, f).

Embryo development, growth, and changes in number of propagules over time were documented at weekly intervals until the emergence of the first hatchlings of each species. All measurements were performed using a dissecting microscope equipped with an ocular micrometer at 10X or 20X (for egg capsules) and 45X (for embryos and hatchlings) magnification. Representative photographs of egg capsules and embryos were also taken at the beginning and at each weekly developmental stage. The relationship between developmental time and embryo size for the three species was obtained by measuring the maximum length (ML), and later shell length (SL), of embryos collected from weekly capsule's measurements (see Moran 1997; Chaparro et al. 2005a, b; Wolf & Young 2012). Embryos' growth (mm d^{-1}) was obtained by averaging the size of embryos obtained in each weekly measurement and estimated as: $GR = (ML_1\text{-}ML_0)\ t^{-1}$, where ML_1: final maximum length (mm), ML_0: initial maximum length (mm) and t: experimental period (days). When multiple embryos were encapsulated in a single capsule (i.e. *C. maculosa* and *H. scobina*), non-parametric Mann-Whitney tests were used to compare the mean number of propagules in newly-laid capsules with the number of propagules at the time of hatching.

2.3.4 Packaging and hatchling traits in *C. maculosa* and *H. scobina*

To estimate the potential relationships among the number of hatchlings emerging per capsule, size at hatching and capsule volume in *C. maculosa and H. scobina*, data on individually dissected capsules of both species obtained from development descriptions (see section 2.3.3) were evaluated using linear regressions. Since the number of embryos encapsulated by female *C. maculosa* did not vary through development, data from all weekly measurements were pooled for evaluating the relationship between number of propagules and capsule volume (i.e. weeks one to ten; n = 57 capsules). However, only weeks nine and ten were pooled for evaluating the relationship between number of embryos per capsule and size at hatching (n = 6 capsules). Because in *H. scobina* the number of encapsulated embryos varied through development, only data from the last two weeks (i.e. 9 and 10; n = 20 capsules) were pooled for evaluating the relationships among the number of hatchlings emerging per capsule, size at hatching and capsule volume.

2.4 Results

2.4.1 Egg-laying characteristics

In the field, the three species were depositing egg capsules at the same time (i.e. first two weeks of October) and exhibited the typical communal egg-laying behavior. Most of the time several conspecific females (e.g. > 10) were observed next to each other or piled on one another depositing egg capsules at the same time in crevices or under boulders (Fig. 2.1a, b, c).

Once the egg-laying finished, the egg capsules of *C. virgata* were always directly attached to the substrata, and, in some cases, a narrow separation among individual clutches was observed (Fig. 2.1a). When individual clutches could be identified, females laid a mean number of 38 ± 2 egg capsules per clutch (± SE; ranging from 3 to 150; n = 155); however, communal clutches consisted of on average 267 ± 35 (± SE; n = 33), and as many as 700 egg capsules. By contrast, *C. maculosa* and *H. scobina* capsules were always deposited in irregular groups, showing no clear separation among

them. Moreover, females of both species laid eggs upon previously deposited clutches, making impossible to distinguish individual clutches (Fig. 2.1b, c). These communal groups consisted of variable numbers of egg capsules, ranging from 35 to 150 (n = 4) in *C. maculosa* and from 4 to ~1000 (n = 12) in *H. scobina*. It was observed that *C. maculosa* also attached their egg capsules upon previously deposited clutches of *C. virgata* and *H. scobina*, and on the shell of conspecifics. The latter was also observed in *H. scobina*.

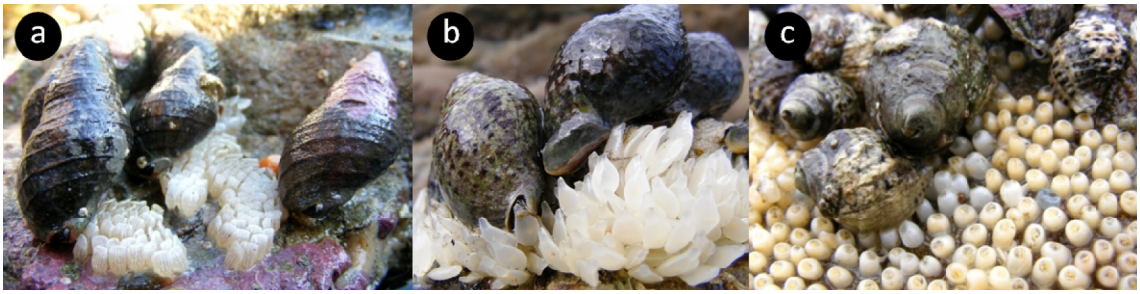

Fig. 2.1 Communal egg-lying behavior in females of the three study species: a) *C. virgata*, b) *C. maculosa* and c) *H. scobina*

2.4.2 Field observations on capsule viability

Intertidal field surveys revealed that egg capsules of *Cominella virgata*, *C. maculosa* and *Haustrum scobina* were mainly damaged due to predation and bacterial infection. When preyed upon, egg capsules were either rasped or drilled, the former being easier to identify due the almost complete destruction of capsule walls, together with the loss of intra-capsular content. Drilled capsules were evidenced by the presence of usually a single hole on their surfaces, which was also accompanied by the loss of intra-capsular fluid. When egg capsules were subject of bacterial contamination, a distinctive pink to purple coloration covered the whole surface of the otherwise withe/yellow capsules, turning the encapsulated embryos, and all the intra-capsular content, in an undistinguishable whitish fluid. None of the egg capsules and embryos affected by these threats was viable.

Egg capsules of the three species were not equally vulnerable to these factors however. By the third week of sampling (i.e. ~3 week-old), egg capsules of *C. virgata* and *C. maculosa* experienced relatively low losses, with approximately 5% and 14% of the total "clutches" or group of egg capsules found (n = ~140 and 7, respectively) exhibiting some of the typical signs of predator-induced damage. In *H. scobina* losses were higher, and all the 19 groups of egg capsules sampled were affected by either: bacterial infection (n = 15), rasping (n = 3) or drilling (n = 1). After approximately 8 weeks in the field (i.e. December; when development was complete), most egg capsules of *C. virgata* and *C. maculosa* were covered by a thin brown film (e.g. benthic diatoms) or small filamentous green algae (possibly *Ulva* sp.). Additionally, 44 % and 20 % of the total "clutches" or groups of egg capsules sampled (n = 93 *C. virgata* and n = 5 *C. maculosa*) showed damage by rasping predators. Once again, vulnerability of *H. scobina* was higher, with around 58 % of the total egg capsules sampled (n = 12) showing clear damage caused by rasping predators (n = 4) and bacterial infection (n = 3). No drillings of the capsule walls were observed during this final survey.

2.4.3 Capsule morphology

Cominella virgata: The capsules (mean ± SE) were rectangular with a flat top and measured 4.08 ± 0.08 mm height (range: 3.6 - 4.4 mm; Fig. 2.2a), 2.73 ± 0.04 mm width (range: 2.5 - 3.0 mm; Fig. 2.2a) and 2.20 ± 0.06 mm depth (range: 1.85 - 2.45 mm; Fig. 2.2b), with an overall volume of 23.09 ± 0.97 µl (range: 16.99 - 27.34 µl). Irregular ridges lined the longitudinal axis of the capsule's surfaces. The top was closed by the union of both capsule walls. Two sides were recognizable because of the inner and outer curvature of the capsule, respectively. An almost indistinguishable flat and short peduncle attached the capsule to the hard substrata (see Fig. 2.2a, b for a frontal and lateral right view of the capsule, respectively).

Cominella maculosa: The measures of the ellipsoid capsules (mean ± SE) were 6.92 ± 0.19 mm height (range: 6.5 - 8.2 mm; Fig. 2.2c), 3.82 ± 0.06 mm width (range: 3.5 - 4.1 mm; Fig. 2.2c) and 2.64 ± 0.02 mm depth (range: 2.60 - 2.70 mm; Fig. 2.2d), with an overall volume of 29.67 ± 0.86 µl (range: 26.47 - 33.93 µl). No distinguishable separate sides were identifiable, and a tough mucus plug of ~1.5 mm length covered the top of the capsules. There were no visible ornamental structures on the smooth surface

of the capsule, which was attached to the substrata by a narrow peduncle of variable size (i.e. 0.9 - 2 mm) (see Fig. 2.2c, d for a frontal and lateral right view of the capsule, respectively).

Haustrum scobina: The measures of the dome-shaped capsules (mean ± SE) were 1.82 ± 0.05 mm radius (range: 1.55 - 2.10 mm; Fig. 2.2e) and 3.60 ± 0.14 mm height (range: 2.75 - 4.20 mm; Fig. 2.2 f), with a mean volume of 18.63 ± 1.66 µl (range: 9.66 - 24.47 µl). The hole on the top of the capsule was covered by a tough and translucent layer. No ornamental structures covered the surface of the capsule, which was directly attached to the hard substrata (see Fig 2.2e, f for a superior and lateral view of the capsule, respectively).

Fig. 2.2 Egg capsules of the three study species: (a - b) frontal and lateral view of *C. virgata*; (c - d): frontal and lateral view of *C. maculosa*; (e - f): superior and lateral view of *H. scobina*. Abbreviations: *h*, height; *w*, width; *c*, depth; *r*, radius. Scale bars: 1 mm.

2.4.4 Intra-capsular development

The intra-capsular development in *C. virgata*, *C. maculosa* and *H. scobina* followed the typical patterns described for other direct developing species of neogastropods. All three species hatched after 10 weeks at a constant temperature (±SD) of 15.4 ± 1.6 °C. Intra-capsular morphology at each developmental stage of the three species is summarized in the Table 2.1, figures 2.3, 2.4, 2.5 and in the descriptions provided below.

Table 2.1 Chronology (e.g. hours and weeks) of intra-capsular development and morphological structures of *C. virgata*, *C. maculosa* and *H. scobina* under laboratory conditions at 15.4 ± 1.6 °C.

Time (h, wk)	*C. virgata*	*C. maculosa*	*H. scobina*
4 h	2 cells	2 cells	2 or 4 cells
48-72 h	Unequal spiral cleavage (4 macromeres, 6 micromeres)	Unequal spiral cleavage (4 macromeres, multiple micromeres)	Not observed
2 wk	Spherical blastula Yolky macromeres vegetal pole Transparent cells animal pole No structures defined	Spherical blastula Yolky macromeres vegetal pole Transparent cells animal pole No structures defined	Morula
3 wk	Late trocophore Head vesicle Ciliated velar lobes Embryonic shell Larval kidneys	Trocophore Incipient velum Embryonic shell Larval kidneys	Late trocophore Desintegration embryos in morula Incipient velum Embryonic shell Larval kidneys
4 wk	Early veliger Embryonic shell growing Soft tissues inside shell Cephalic vesicle Larval kidneys Incipient cilia Foot	Early veliger Embryonic shell growing Soft tissues inside shell Cephalic vesicle Larval kidneys Incipient cilia Prismatic cells	Early veliger Embryonic shell growing Soft tissues inside shell Cephalic vesicle Larval kidneys Incipient cilia
5-6 wk	Late veliger/Pediveliger Shell lacks pigmentation Coiling Head Cephalic tentacles/eyes Bilobed velum Foot Incipient operculum	Veliger Shell lacks pigmentation Coiling Head Larval kidneys Cephalic tentacles/eyes Ciliated velar lobes Foot Incipient operculum	Veliger Shell lacks pigmentation Prismatic cells Larval kidneys Eyes Ciliated velar lobes Foot Incipient operculum
7-8 wk	Early hatchling Pigmentation No velum Enlarged cephalic tentacles/eyes Siphon Foot Operculum	Pediveliger/early hatchling Pigmentation Velum disappearing Prismatic cells Cephalic tentacles/eyes Siphon Foot Operculum	Pediveliger/early hatchling Shell lack pigmentation Coiling Prismatic cells Ciliated velum Larval kidneys Cephalic tentacles/eyes Head Foot Operculum
9-10 wk	Hatchling Pigmentation Siphon Enlarged cephalic tentacles/eyes Foot Operculum Shell with double whorl	Hatchling Pigmentation Siphon Enlarged cephalic tentacles/eyes Foot Operculum Shell with single whorl	Hatchling Pigmentation Prismatic cells Reduced velum Larval kidneys Siphon Enlarged cephalic tentacles/eyes Foot Operculum Shell with single whorl

Cominella virgata: Females consistently encapsulated a single embryo per capsule, which developed without nurse-eggs, presumably relying on albumen. The intra-capsular fluid was translucent and viscose, enabling the embryos to maintain a constant position. Four hours after collection (which occurred immediately after deposition by females), embryos were typically found in a two-cell stage (Fig. 2.3a). After 48 h, the consecutive clevage stages with an unequal spiral cleavage were observed, resulting in four vegetative macromeres and around six small micromeres in the animal pole (Fig. 2.3b). By the second week of development a spherical blastula was already formed, with the yolky macromere in the vegetal pole, and more transparent cells in the animal pole. No organized structures were evident at this stage (day 14; Fig 2.3c). After three weeks, embryos attained the late "trochophore" stage, where the ciliated velar lobes began their growth as projections from the body wall. Early signs of structures such as larval kidneys, head vesicle and a thin embryonic shell were also observed (day 21; Fig 2.3d). By the fourth week the early "veliger" stage was attained. The embryonic shell began to grow, enclosing most of the embryo's soft tissues. The cephalic vesicle, larval kidneys, foot and incipient cilia were also observed in the antero-ventral margin (day 28; Fig 2.3e). In weeks five and six (days 35 and 42, respectively) similar patterns of development were observed, with embryos attaining the late "veliger" and "pediveliger" stages, respectively (the latter comparable to the competent hatchling stage, with reduced velum and more developed foot). Although the embryonic shell still lacked pigmentation, its growth and coiling were evident. Head, cephalic tentacles with basal eyes, bilobed velum, foot and an incipient operculum were also developed. At this stage, embryos actively moved inside the egg capsules (Figs. 2.3f, g). By weeks seven and eight the velum disappeared, and there was pigmentation of the shell, operculum and soft tissues. The siphon, enlarged cephalic tentacles with basal eyes and foot were also observed. During these stages the top of the capsule began to dissolve (days 49 and 56, respectively; Figs. 2.3h, i). By the last two weeks before hatching (days 63 and 70) the intra-capsular fluid was completely absorbed, and just a thin layer (i.e. albumin sack) separated the pre-hatching embryo from the capsule walls. All body structures were developed, i.e. pigmented shell, siphon, cephalic tentacles with basal eyes, foot and operculum (Figs. 2.3j, k). Shell color varied among black, dark-brown, orange, white-brown and white, and was generally characterized by a "double whorl" in the posterior end (see Fig. 2.3k).

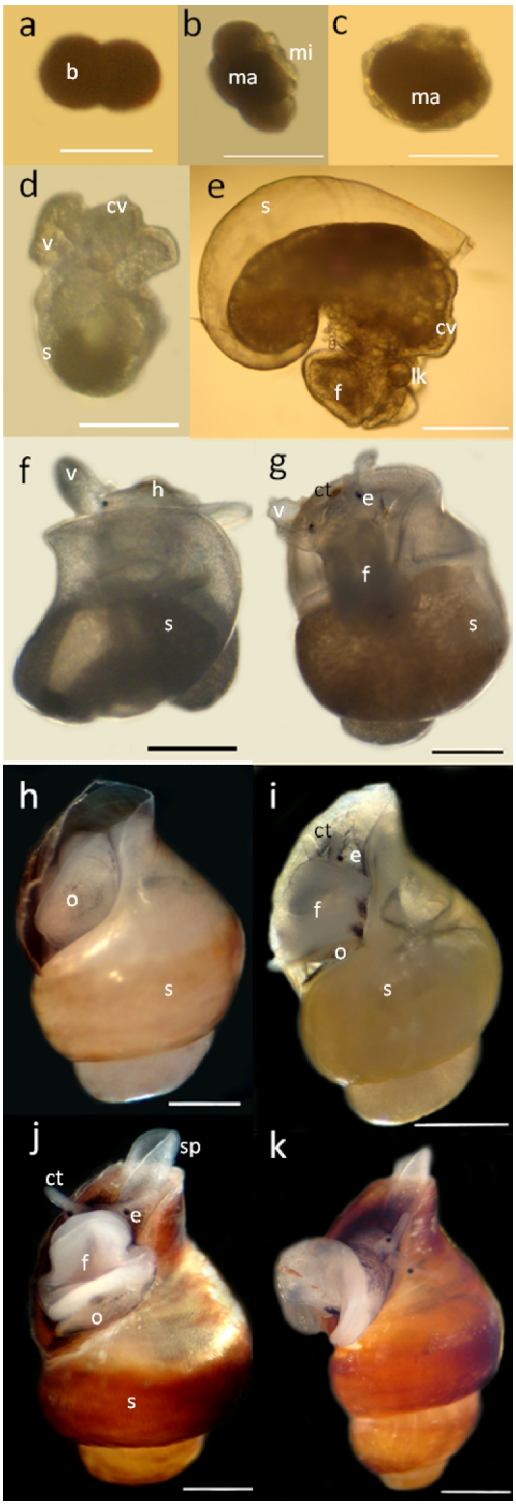

Fig. 2.3 Intra-capsular development of *C. virgata*. Photographs were taken at weekly intervals, where: (a - b) indicate 4 hrs and 48 hrs after collection, respectively; (c - k) indicate weeks two to ten. Scale bars: a - e = 0.25 mm; f - k = 0.5 mm. Abbreviations: b, blastomere; ma, macromeres; mi, micromeres; v, velum; cv, cephalic vesicle; s, shell; lk, larval kidneys; f, foot; e, eye; h, head; ct, cephalic tentacle; o, operculum; sp, siphon.

Cominella maculosa: Females typically encapsulated a mean (± SE) number of 7.7 ± 0.3 embryos per capsule (ranging from 4 to 12). Similar to *C. virgata*, intra-capsular nutrition (i.e. nurse eggs) was not observed, and embryos were surrounded by a jelly-like albumen matrix which became more fluid as development proceeded. After collection, embryos were found at the two-cell stage (four hours; Fig 2.4a). Consecutive clevage stages were further observed, and within 72 h, the unequal spiral cleavage resulted in four big vegetative macromeres and multiple small micromeres in the animal pole (Fig 2.4b). By the second week of development a spherical blastula was observed, with a yolky macromere in the vegetal pole and transparent cells in the animal pole. No evident structures were recognized at this stage (day 15; Fig 2.4c). By week three, embryos reached the "trochophore" stage, where incipient structures such as velum, embryonic shell and larval kidneys seem to be in development (day 21; Fig 2.4d). During week four, the early "veliger" stage was observed and characterized by the development of embryonic soft tissues, including cephalic vesicle, larval kidneys and ciliated velum, which were mostly enclosed within the thin embryonic shell. Large "prismatic" cells were clearly evident in the central portion of the embryo's body (day 28; Fig 2.4e). By weeks five and six, the "veliger" stage was reached (days 35 and 42, respectively), where growing and coiling of the unpigmented shell occurred. At this stage, structures such as larval kidneys and ciliated velum still persisted. The head, cephalic tentacles with basal eyes, foot and insipient operculum were clearly developing. Large "prismatic" cells were still evident in the posterior region of embryos (Figs. 2.4f, g). During the following weeks, i.e. seven and eight, the "pediveliger" and "hatching" stages were observed. The ciliated velum was still present in the former stage (Fig. 2.4h), and was completely absent in the latter one (Fig. 2.4i). Hatching stage snails were completely developed, and had increased pigmentation of the shell and soft tissues. "Prismatic" cells were barely observed. Cephalic tentacles with basal eyes, siphon, foot and operculum were clearly evident. At this stage capsule plugs began to dissolve (days 49 and 56, respectively; Figs. 2.4h, i). During the pre-hatching stages (i.e. weeks nine and ten) development was synchronous among embryos and capsules examined, and body structures were clearly evident, including siphon, enlarged cephalic tentacles with basal eyes, foot and pigmented operculum (days 63 and 70, respectively; Figs. 2.4j, k). Hatchling shell colour was typically dark-brown with small white dots spread through the dorsal and ventral surfaces. Only a "single whorl" was observed at the shell's posterior end (see Fig. 2.4k).

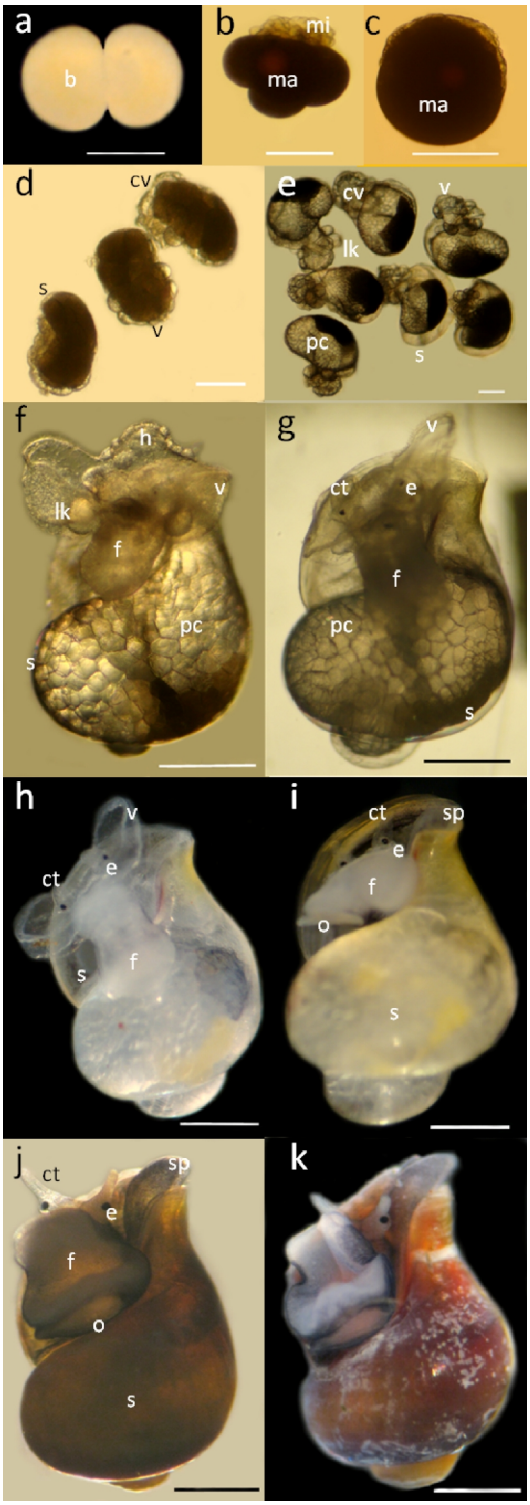

Fig. 2.4 Intra-capsular development of *C. maculosa*. Photographs were taken at weekly intervals, where: (a - b) indicate 4 hrs and 72 hrs after collection, respectively; (c - k) indicate weeks two to ten. Scale bars: a - e = 0.25 mm; f - k = 0.5 mm. Abbreviations: b, blastomere; ma, macromeres; mi, micromeres; v, velum; cv, cephalic vesicle; s, shell; lk, larval kidneys; pc, prismatic cells; f, foot; e, eye; h, head; ct, cephalic tentacle; o, operculum; sp, siphon.

Haustrum scobina: Females encapsulated a mean (± SE) number of 235 ± 17 propagules per capsule (range: 175 - 317). Contrary to *C. virgata* and *C. maculosa*, developing siblings were provisioned with intra-capsular nurse embryos, which could not initially be distinguished. Immediately after collection, the pale-yellow embryos were typically found in two or four cells stages (four hours; Fig. 2.5a). By the second week of development, embryos had entered the "morula" stage (Fig. 2.5b). After three weeks, some embryos in the "morula" stage progressively began to disintegrate due to blastomere separation, whereas the developing siblings reached the late "trochophore" stage. At this stage, an incipient embryonic shell was observed, and the larval kidneys were seen as lateral ectodermic projections. The development of cilia was also observed in the antero-ventral region. Developing siblings were characterized by the consumption of nurse embryos, clearly observed in their stomachs (day 21; Fig. 2.5c). By week four, embryos were in the early "veliger" stage, characterized by evident calcium deposition in the more opaque embryonic shell and a clear development of the cephalic vesicle, larval kidneys and ciliated velum (day 28; Fig. 2.5d). During the following weeks, i.e. five and six, the "veliger" stage was reached (days 35 and 42, respectively), and most morphological structures were developed and enclosed by the thin embryonic shell (i.e. head, larval kidneys, eyes, ciliated velum, foot and incipient operculum). Small "prismatic" cells were just noticeable in the central portion of the embryo's body (Figs. 2.5e, f). Intra-capsular consumption of the few remaining nurse-embryos was still evident (see Fig. 2.5f). By the next two weeks, i.e. seven and eight, the "pediveliger" and "early hatching" stages were observed. The ciliated velum and larval kidneys were also still present in both stages. The unpigmented shell continued growing and began coiling, and head, cephalic tentacles with basal eyes, foot and operculum were clearly seen (Figs. 2.5g, h). During weeks nine and ten, a reduced velum, larval kidneys and small "prismatic" cells still persisted on the remaining embryos. Enlarged cephalic tentacles with basal eyes were observed, and the juvenile shell was completely developed and progressively became pigmented. Development seems to be less synchronous among siblings compared with embryos *C. maculosa*. Hatchling shells were soft and almost translucent under light microscopy, allowing the observation of the consumed nurse-eggs that gave them the typical yellowish pigmentation. A purplish coloration in the antero-ventral surface of the shell was also commonly observed (e.g. days 63 and 70; Figs. 2.5i, j), being a distinctive character of adults *H. scobina*. As in *C. maculosa*, a "single whorl" was observed at the shell's posterior end (Fig. 2.5j).

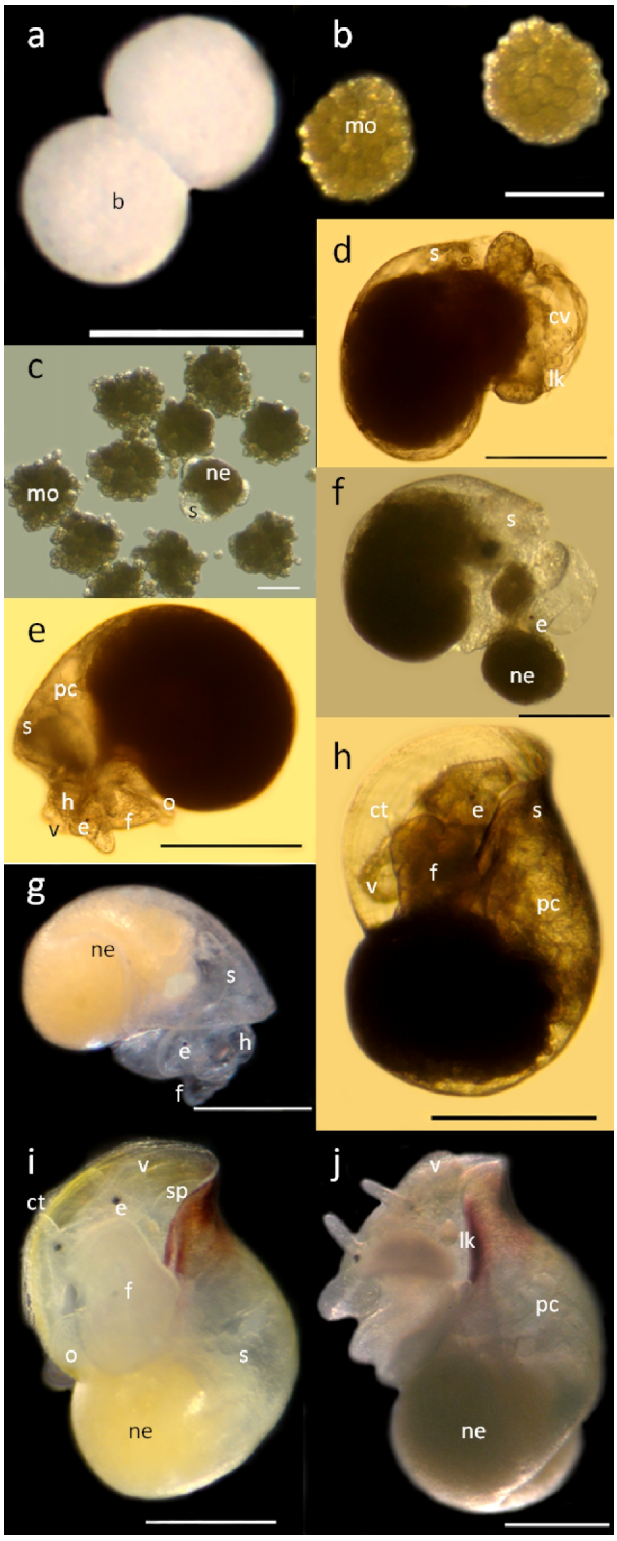

Fig. 2.5 Intra-capsular development of *H. scobina*. Photographs were taken at weekly intervals, where: (a) indicate 4 hrs after collection; (b - j) indicate weeks two to ten. Scale bars: a - c = 0.25 mm; d - j = 0.5 mm. Abbreviations: b, blastomere; mo, morula; v, velum; cv, cephalic vesicle; s, shell; lk, larval kidneys; pc, prismatic cells; f, foot; e, eye; h, head; ct, cephalic tentacle; o, operculum; ne, nurse embryo; sp, siphon.

2.4.5 Embryo size, growth and changes in propagule number

Cominella virgata embryos increased 12.5 times in size during the intra-capsular development, from an initial size of 0.22 ± 0.01 mm (mean ± SE) at two-cell stage, to a final size of 2.75 ± 0.01 mm shell length (SL) after 10 weeks in laboratory conditions (Fig. 2.6a). The maximum growth rates were observed between weeks four and nine, with values ranging between 0.052 and 0.060 mm d^{-1} (except for the week eight: 0.012 mm d^{-1}). During encapsulated period the number of propagules remains unchanged, with just one embryo encapsulated in each capsule.

Cominella maculosa embryos increased in size 6.8 times during the ten weeks of development, from an initial size of 0.24 ± 0.01 mm (mean ± SE) at two-cell stage to a final size of 1.63 ± 0.02 mm SL at hatch (Fig. 2.6b). The maximum growth rates were observed in weeks three, four and five (i.e. 0.073, 0.049 and 0.037 mm d^{-1}, respectively). The initial mean number of propagules encapsulated was 7.7 ± 0.3 (± SE; ranging from 4 to 12), and was not significantly different from the 6.5 ± 0.5 (mean ± SE) juveniles hatched (Mann-Whitney, $U = 28.50$; $p = 0.450$).

Haustrum scobina embryos increased in size 5.4 times, from 0.23 ± 0.001 mm at two-cell stage (mean ± SE) to 1.24 ± 0.02 mm SL in hatchling snails (Fig 2.6c). The maximum growth rate was observed at week four, i.e. 0.053 mm d^{-1}, coinciding with the obvious decrease in propagule number also observed during this time (Fig. 2.6c). The initial number of propagules per capsule was 235 ± 17 (mean ± SE; ranging from 175 to 317), decreasing considerably by the fourth week of development, when embryos had developed cilia in the antero-ventral region and the ingested yellowish nurse eggs were also observed in their stomachs (see Fig. 2.5c, d). By the tenth week, the number of propagule initially encapsulated was significantly different from the 10 ± 1 (mean ± SE) juveniles hatched per capsule (Mann-Whitney, $U = 100.00$; $p < 0.001$; Fig 2.6c).

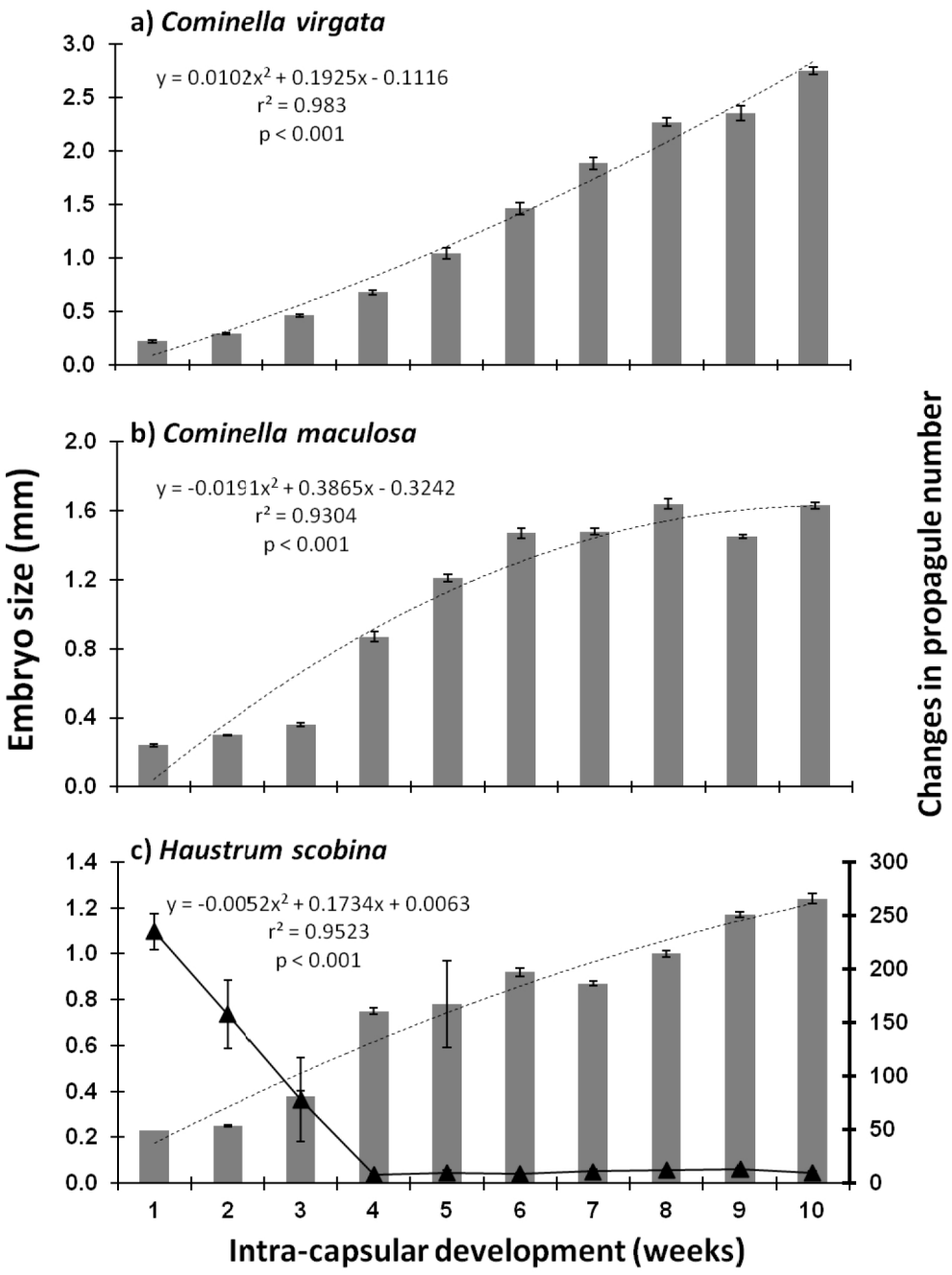

Fig. 2.6 Increase in size (grey bars) and changes in propagule number (continuous line) during the intra-capsular development of *C. virgata*, *C. maculosa* and *H. scobina*. Vertical lines represent standard errors (± SE).

2.4.6. Packaging and hatchling traits in *C. maculosa* and *H. scobina*

The number of *C. maculosa* hatchlings emerging per capsule exhibited a weak but significant relationship with an increase in the capsule volume (y = 0.0561x + 5.7419; R^2 = 0.0856; $F_{1, 55}$ = 5.161; p = 0.027; Fig. 2.7a) and a significant decrease in hatchling size (y = -0.034x + 1.7825; R^2 = 0.191; $F_{1, 60}$ = 14.252; p < 0.001; Fig. 2.7b). The number of *H. scobina* hatchlings emerging from individual capsules was not related to capsule volume (y = -0.1746x + 13.629; R^2 = 0.0667; $F_{1, 18}$ = 1.269; p = 0.275; Fig. 2.7c); nevertheless, and as in *C. maculosa*, it was negatively related to hatchling size (y = -0.0159x + 1.3943; R^2 = 0.4237; $F_{1, 18}$ = 13.253; p = 0.002; Fig. 2.7d).

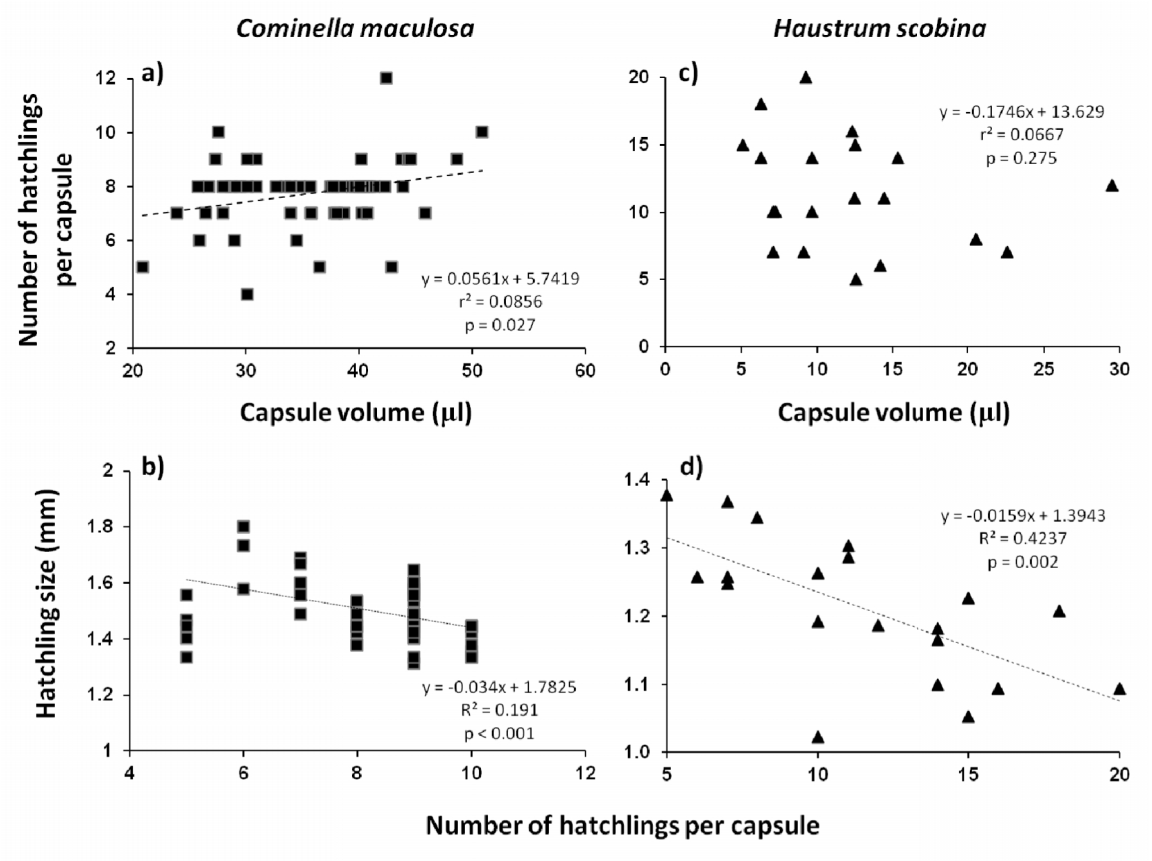

Fig. 2.7 Relationships among number of hatchlings emerging per capsule, capsule volume and hatchling size in (a, b) *C. maculosa* and (c, d) *H. scobina*.

2.5 Discussion

2.5.1 Egg-laying characteristics

Capsule deposition by female *C. virgata*, *C. maculosa* and *H. scobina* occurred over a well defined period during the year, beginning in October and continuing until mid December, when recently deposited egg capsules were still observed. Similar to our findings, van der Sman (2007) and colleagues (van der Sman et al. 2009) observed that females of these three species kept in the laboratory began to lay egg capsules between September and October, continuing for more than 4-5 weeks. As reported in other southern latitude whelks (i.e. 42-43°S; *Odontocymbiola magellanica* and *Trophon geversianus*; Bigatti et al. 2008 and Cumplido et al. 2010, respectively) and in some fresh-water snails (i.e. *Lymnaea stagnalis*; Ter Maat et al. 2012), both temperature and/or photoperiod may have played an important role in regulating oviposition timing. However, since those variables were not evaluated here, further studies should target identification of the environmental triggers of oviposition considering the plastic responses of their reproductive outputs with a wide range of environmental variables (i.e. food availability and water temperature).

Cominella virgata, C. maculosa and H. scobina exhibited communal egg-laying behavior, with several females, i.e. usually more than 10, depositing capsules in such close proximity that made it difficult to recognize which female laid which clutch. Nonetheless, individual clutches of *C. virgata* were, in several cases, identified with some degree of confidence due a narrow separation among them, as also described by van der Sman (2009). Communal egg-laying behavior is a well documented pattern in many other neostropods such as *Buccinum undatum* (Martel et al. 1986; Nasution 2003), *Thais* (*Stramonita*) *chocolata* (Romero et al. 2004), *Rapana venosa* (Saglam & Duzgunes 2007), *Odontocymbiola magellanica* (Bigatti et al. 2008), *Buccinanops cochlidium* (Averbuj & Penchaszadeh 2010), *Trophon geversianus* (Cumplido et al. 2010), among others. This behavior could be associated with a chemical response of females to egg-lying conspecific as a strategy to ensure stable substrata for egg capsule deposition (see Yokoyama & Amaral 2011) or to provide protection against predators owing safety in numbers (reviewed in Dumont et al. 2008).

The substrate selected for egg capsules deposition varied by species. While clutches of *C. virgata* were always deposited on hard substrata (i.e. rocks), female *C. maculosa*

and *H. scobina* were less "choosy", using in some cases clutches of *C. virgata* and *H. scobina* and also the shells of conspecifics in addition to rocks. This may be due to the much denser aggregations of these two species observed during spawning periods (SA Carrasco, pers. obs.; see Fig. 2.1c). Although female whelks typically attach their egg capsules on relatively stable substrata, several other surfaces have also been described, such as the own female shell in *B. cochlidium* (Averbuj & Penchaszadeh 2010), shells of conspecific males in *Solenosteira macrospira* (see Kamel et al. 2010b), the green algae *Ulva lactuca* and *Udotia occidentalis* in *Nassarius vivex* and *Voluta ebrea* (Yokoyama & Amaral 2011 and Matthews-Cascon et al. 2010, respectively), the brown alga *Agarum cribrosum* in *B. undatum* (Martel et al. 1986) and the red algae *Gigartina stellata*, *Polydes rotundus* and *Laurencia pinnatifida* in *N. reticulatus* (reviewed in Chatzinikolaou & Richardson 2010).

2.5.2 Field observations on capsule viability

The egg capsules of the three study species, being sessile in the field, were subject of substantial loss rates in the natural environment. From this study, it is evident that capsules of *H. scobina* were the most vulnerable, both to bacterial contamination (i.e. purple-colored capsules) and two evident kinds of predation (i.e. rasping and drilling). This higher susceptibility observed in egg capsules of *H. scobina* has also been previously documented in laboratory conditions by van der Sman (2007), who showed that while 80% of the *C. maculosa* capsules were viable at hatching, only 23% of *H. scobina* capsules contained hatchlings that completed development.

Similar to *H. scobina*, pink to purple-colored capsules have also been previously described in other gastropods such as *Nucella crassilabrum*, *Plicopurpura pansa*, *Concholepas concholepas* and *Thais haemastoma floridiana* (see Gallardo 1979; Naegel & Gómez del Prado-Rosas 2004). In all cases this coloration seems to be caused by physical stress and bacterial colonization, and results in embryonic death (op. cit), suggesting low protection from physical preassure and/or limited antimicrobial properties in the egg capsules of these species. On the other hand, these findings also suggest that egg coverings of both *Cominella* species have been additionally provisioned with antimicrobial components that will allow for enhanced resistance to an important environmental threat. This sort of provisioning should therefore be considered as an important maternal effect, since as observed here, could realistically play an

ecological role in the prevention of microbial infection and have significant consequences during the early-life history stages of these congeneric whelks as well as in many others marine, fresh-water and terrestrial invertebrates (for examples see Bekendorff et al. 2001; Lim et al. 2007; Peters et al. 2012).

Likewise, predator marks on field-laid capsules of the three study species were evident soon after deposition (i.e. within a month). Based on these observations and in other predation studies, some potential predators of these egg capsules in the field could include rasping predators such as juvenile sea-urchins (e.g. *Evechinus chloroticus*) or chitons (e.g. *Chiton glaucus*), and in the case of *H. scobina*, drilling predators such as the whelks *Buccinulum* sp., and adult *H. scobina*, which are usually found in proximity of the egg capsules. Small crabs present in the study site (e.g. *Heterozius rotundifrons* and *Cyclograpsus lavauxi*) could also be responsible for some of the predation marks observed, as other decapod crustaceans have been previously described as important predators of whelk egg capsules (for examples on predators see Rawlings 1990; Dumont et al. 2008; Roche et al. 2011). Although sea-urchins and chitons are primarily herbivorous and feed on microalgae attached to hard substrate, Dumont et al. (2008) and Roche et al. (2011) reported that these taxa can also remove gastropod capsules and feed on them by taking the intra-capsular fluid, food that will likely represent a rich source of lipids and proteins.

2.5.3 Capsule morphology

Despite being under similar intertidal environmental pressures in the field (e.g. wave action, salinity, temperature and predation), the morphology of the encapsulating structures of *C. virgata, C. maculosa* and *H. scobina* differed considerably in size and shape. These morphologies likely affect the effectiveness against environmental stressors and therefore, have significant implications for embryonic development and survival (Rawlings 1994). These species-specific differences could be directly related with the different loss rates observed in the field, as capsules *H. scobina* presented the smallest volumes compared with those of *C. virgata* and *C. maculosa* (18, 23, 29 µl, respectively) and also were the only ones that lack a peduncle to separate the capsule's body from the substrata, increasing the possibility of predation risk and bacterial contamination. Similarly, the much denser aggregations of egg capsules observed

during spawning events of *H. scobina* may have increased risks by limiting the exchange of respiratory gases (i.e. hypoxia) and elimination of wastes, reducing the chances of encapsulated embryos to complete development and notably increasing mortality (see Fernandes & Podolsky 2011).

In the field, the cuboid egg capsules of *C. virgata* were clearly distinguishable from the spawns of all other co-occurring whelks in both shape and provisioning. The ellipsoid capsules of *C. maculosa* (and its provisioning) were remarkably similar to those of *C. adspersa*, another congener found subtidally in Wellington Harbour, suggesting that these two species could be more closely related than either is to *C. virgata*. Additionally, since *C. maculosa* and *C. adspersa* use different habitats to lay egg capsules (intertidally and subtidally, respectively) and adults are similar in size and shape, it is likely that the environmental pressure on embryos is not the factor mediating capsule morphology, but instead maternal traits and /or phylogenetics constraints. The dome-shaped capsules of *H. scobina* were also very similar to those of the congeneric *H. haustorium*; however, the capsules of the latter are less abundant in the intertidal sites and are almost twice the size of *H. scobina* (i.e. 6 mm diameter and 4 mm height; NE Phillips, unpl. data).

2.5.4 Intra-capsular development

Although differences in capsular traits among the three species were observed, a similar chronology in the developmental stages was recognizable through their intra-capsular life. By the second week of development, *C. virgata* and *C. maculosa* embryos were observed in the later "blastula" stage, whereas in *H. scobina* embryos in the earlier "morula" stage still persisted. During the 3rd and 4th weeks however, embryos of the three species developed to the "trochophore" and early "veliger" stages, respectively. At these early stages, it was evident that only *H. scobina* siblings were provisioned with nurse embryos, which resulted from eggs that stopped their development in the "morula" stage (see Fig. 2.5c). Nonetheless, since the albumen surrounding *C. virgata* and *C. maculosa* embryos declined as development continued, and their body size increased, it is also possible that this capsular fluid provided nourishment for these developing embryos, similarly to the findings in the gastropods *Urosalpinx cinerea* (Rivest 1986), *Buccinum undatum* (Nasution 2003), *Rapana venosa* (Saglam &

Duzgunes 2007), *Voluta ebrea* (Matthews-Cascon et al. 2010) and *Nassarius reticulatus* (Chatzinikolaou & Richardson 2010). As observed in the present work, and regardless of embryo provisioning (e.g. albumen or nurse embryos) and developmental strategy (e.g. direct or indirect), a similar chronology has also been described for other temperate neogastropods, attaining the "trochophore" and "veliger" stages in 4-5 wk and 5-8 wk, respectively, at a temperature between 12-14°C [e.g. *Chorus giganteus* (Leiva et al. 1998); *Thais* (*Stramonita*) *chocolata* (Romero et al. 2004) and *Trophon geversianus* (Cumplido et al. 2011)].

During the second half of intra-capsular development, all juvenile structures became more evident (i.e. weeks 5-10; shell coiled, head vesicle, cephalic tentacles with basal eyes, ciliated velum, foot and incipient operculum; see also van der Sman 2007), and larval characteristics such as velum and embryonic kidneys were less frequently observed. Nonetheless, even after 10 weeks of encapsulation and despite emergent crawling juveniles, some hatchling *H. scobina* still retained some "larval" traits (see table 2.1 and Fig. 2.5j), suggesting that this nurse-embryo-based provisioning could result in asynchrony of development and hatching at different developmental stages and sizes, which will probably depend on the number of embryos enclosed in individual capsules and the number of nurse embryos a developing sibling can consume (see Rivest 1983).

2.5.5 Embryo size, growth and changes in propagule number

Interestingly, although *C. virgata*, *C. maculosa* and *H. scobina* had clear differences in intra-capsular provisioning and number of propagules encapsulated, the egg size after the first cleavage was almost identical (i.e. two-cells stage; 0.22, 0.24 and 0.23 mm, respectively). These findings suggest that some other mechanisms apart from initial egg size (e.g. intra-capsular nutrition, maternal provisioning through lipids or growth trajectories), could be influencing the hatching size of these three species. For example, the present observations suggest that albumen-fed embryos grew faster and thus, increased dramatically in size compared with nurse embryo fed ones. *Cominella virgata* and *C. maculosa* grew at an average rate of 0.040 and 0.022 mm d^{-1}, respectively, with hatchlings 12.5 and 6.8 times larger than the eggs after the first cleavage, while embryos *H. scobina* grew at an average rate of 0.016 mm d^{-1} and were only 5.4 times larger than

the eggs by the time of hatching. To compare these values, the study of Romero et al. (2004) provides examples of how egg size and reproductive strategies correlate with hatchling size within some muricacean snails. In this review, the authors showed that this relationship greatly differed among reproductive strategies and modes of development, with some hatchlings being only 1.6 times larger than eggs in species with planktonic development (e.g. *Concholepas concholepas*), around 3.3 times larger in direct developing species without nurse eggs (e.g. *Urosalpinx cinerea*) or 5.5 times larger in species with direct development and nurse eggs (e.g. *Nucella* spp.). Nevertheless, this relationship seems also highly variable depending on the number of propagules encapsulated (e.g. ranging from 240 to more than 13000).

As in those examples, differences in propagule numbers among the three study species were highly variable, as *C. virgata* was the only species that encapsulated a single embryo per capsule, while *C. maculosa* and *H. scobina* encapsulated multiple ones, with the number of embryos encapsulated being related to the average hatchling size (i.e. C. *virgata* > C. *maculosa* > *H. scobina*). The number of propagules per capsule however, decreased significantly only in the latter species (see Fig. 2.6). Similar to the findings in *H. scobina*, previous studies have also suggested that the consumption of nurse eggs begins early during development (3-4 wk), with embryos ingesting nurse eggs when they attain the trochophore stage in *Nucella crassilabrum* (Gallardo 1979) and *Searlesia dira* (Rivest 1983), and the early-veliger stage in *Trophon geversianus* (Cumplido et al. 2011). Since in the latter study the trochophore stage was not observed, it is also possible than consumption of nurse eggs begin earlier but was not recorded.

During this process, and as described by Gallardo (1979) and Rivest (1983), nurse eggs are swallowed whole, and can be difficult to ingest due the similar size of the embryos. In those species (i.e. *N. crassilabrum* and *S. dira*), micromeres may play an important role by providing a "handle" to incorporate nurse eggs into the stomodeum, and then swallowing the macromeres (Rivest 1983). However, as in *H. scobina* the development is arrested and some embryos seemed to disintegrate at the "morula" stage, it is possible that in this species developing siblings consume the individual blastomeres obtained after such a process. As observed here for *H. scobina* and also by previous authors, the ingestion of nurse eggs/embryos can continue for 1-3 wk once started (around week 3), with the swallowed material remaining intact for until a week or more before feeding ended. This can clearly explain the fact that this nourishing material was

still observed in embryo's guts at advanced stages of development, and even after hatching.

Despite patterns of encapsulation seemed to be relatively consistent, intra-specific differences may also occur. For example, van der Sman (2007) recorded laboratory-laid capsules of *C. virgata* containing multiple hatchlings (i.e. eight with 2, one with 3 and one with 12), and capsules with 2 embryos have also been found in the field (SA Carrasco, pers. obs, although rarely and not in this study). These observations, added to the fact that the co-occurring *C. maculosa* and *C. adspersa* have multiple embryos per capsule, could suggest that the evolutionary step between a capsule with a single large hatchling and one with several smaller hatchlings within the genera *Cominella* is not a large one, and that having multiple hatchlings per capsule is an ancestral trait. Therefore, further studies should aim to closely evaluate taxonomic and phylogenetics relationships within this genus to further clarify this issue.

2.5.6 Packaging and hatchling traits in *C. maculosa* and *H. scobina*

Although both species contained multiple embryos per capsule, their packaging strategies were different. Only *C. maculosa* exhibited a significant increase in hatchling number with an increase in capsule volume. Nonetheless, in *C. maculosa* and *H. scobina* the classical trade-off between offspring size and offspring number occurred (see Bernardo 1996b; Kamel et al. 2010b; Fig. 2.7b, d). As observed here, similar relationships between capsule morphometry and hatchling traits have also been documented in other species of marine snails regardless of their reproductive strategies (i.e. indirect or direct development), with larger capsules usually containing higher numbers of embryos, and/or smaller hatchling sizes being obtained from capsules with higher numbers of encapsulated siblings. Some typical examples are shown in the gastropods *Acanthina spirata* and *Thais emarginata* (Spight 1976b), *Searlesia dira* (Rivest 1983), *Urosalpinx sinerea* (Rivest 1986), *Nucella lapillus* (Pechenik et al. 1984), *Crepidula dilatata* (Chaparro et al. 1999), *Rapana venosa* (Saglam & Duzgunes 2007), *Buccinanops cochlidium* (Averbuj & Penchaszadeh 2010); *Nassarius reticulatus* (Chatzinikolaou & Richardson 2010), *Stramonita haemastoma haemastoma* (Lahbib et al. 2011), among others.

2.5.7 Evolutionary ecology of direct developers

A remarkable aspect in the life-cycle of marine invertebrates in general and benthic mollusks in particular, is the considerable variation in their reproductive and developmental strategies (Gallardo & Perron 1982). It is generally thought that the ancestral condition is the production of pelagic eggs, which develop into planktotrophic trochophore larvae that undergo development into the veliger stage, but in many different evolutionary lines the pelagic larval existence of some benthic mollusks (and other invertebrate species) has been evolutionarily lost, and in some cases repeatedly omitted (see Strathmann 1978; Gallardo & Perron 1982; Pechenik 1999).

In species with such a reproductive strategy (i.e. direct development), an important step in reproductive evolution took place with the acquisition of internal fertilization and deposition of eggs within protective gelatinous envelopes, elaborated egg capsules or brood chambers, where individuals develop to the juvenile stage. Given morphological constraints, Archaeogastropods are free-spawners and cannot produce egg capsules nor undertake internal fertilization, and thus, the evolution of encapsulation patterns is almost entirely restricted to the Meso and Neogastropods mollusks (Gallardo & Perron 1982; Buckland-Nicks et al. 2002).

Interestingly, and as observed in the present study, reproductive and developmental traits may greatly differ among closely related species (i.e. a single or multiple embryos per capsule in *Cominella*). However, it has been generally assumed that gastropods with planktotrophic larval development produce smaller and more numerous eggs, corresponding to the primitive condition compared with the development of lecithotrophy and/or direct development (Gallardo & Perron 1982). Nonetheless, it must also be considered that a shift in developmental mode is not always associated with selection of large yolky eggs (reviewed in Romero et al. 2004), and as also observed in the present study, there are other ways for varying offspring size without necessarily varying egg size.

Comparisons of the developmental traits of these three direct developing species allowed recognizing some common traits among these co-occurring whelks with other gastropod in which analogous reproductive strategies occurred. Some of those patterns include similarities in encapsulation modes, such as a single embryo or multiple embryos per capsule with different sources of provisioning (e.g. albumen or nurse eggs/embryos). Despite evident differences in provisioning and hatching size within

Gastropoda, the encapsulation of a single embryo per capsule seems to be less common compared with the encapsulation of multiple siblings. For example, Collin (2003) showed that from 78 species of Calyptraeid reviewed, only 2 encapsulated a single embryo (i.e. *Crepidula monoxyla* and *C. philippiana*), both of them relying on nurse eggs. Likewise, Romero et al. (2004) found no single embryos in the 49 Muricacean snails reviewed. Even considering just those two studies, it is evident that the encapsulation of a single embryo per capsule will involve fascinating trade-offs in terms of offspring provisioning and performance, and since size and numbers are not the only ways maternal provisioning can vary across species, further chapters will explore those traits under a more integrative morphological and physiological approach.

Chapter 3

Developing a new protocol for evaluating provisioning of lipids in hatchling whelks

3.1 Abstract

The initial provisioning that offspring receive from their mothers is a well studied maternal effect that can influence the evolution of life histories, affecting both maternal and offspring fitness. Although several studies have provided accurate descriptions of the maternal provisioning (i.e. proteins, carbohydrates or lipids) in eggs or embryos of several species with planktotrophic and lecithotrophic larval stages, only a few have managed to provide qualitative and quantitative data on maternally-derived resources for individual offspring. In this chapter, I describe a modified methodology to provide qualitative and quantitative data of lipid classes obtained from individual egg capsules and hatchlings of an intertidal whelk (i.e. *Cominella virgata*) with direct development and crawl-away juveniles. The method uses the Iatroscan thin-layer chromatography-flame ionization detection system (TLC/FID) for lipid extraction and quantification following similar protocols to those previously used in species with planktonic larvae (e.g. echinoderms) with some modifications and additions. Analyses on individual egg capsules and hatchlings of *C. virgata* revealed that all the samples had six different lipid classes commonly described in several marine invertebrate species. Two structural lipids (phospholipids and cholesterol) and four energy-storage lipids (diglyceride, aliphatic hydrocarbon, free fatty acid and triglyceride). The protocol described in this chapter aims to standardize and summarize a methodology that can be applied to study maternal provisioning on an individual basis in any species of direct developing gastropod.

3.2 Introduction

The energetic input that offspring receive from their mothers is one of the most important and well known maternal effects (Marshall et al. 2008). This initial provisioning can influence the evolution of life histories, affecting both maternal and offspring fitness, and can be important in determining offspring size in benthic marine invertebrates (Rivest 1983; Bernardo et al. 1996b; McEdward & Miner 2006). Understanding the quality and quantity of maternal provisioning is an integral part in species' life-history studies, because it mediates offspring development, performance, and the concomitants traits associated with juvenile growth (e.g. age at first reproduction or vulnerability to predators).

Research on the importance of these maternally-derived energy reserves has been conducted in several taxa with a range of different egg sizes and developmental strategies. Numerous comparative and energetic studies have focused on echinoderms (e.g. Sewell 2005; Falkner et al. 2006; Byrne et al. 2008a, b; Prowse et al. 2008; Prowse et al. 2009), mollusks (e.g. Mann & Gallager 1985; Jaeckle & Manahan 1989; Rodriguez et al. 1990; Whyte et al. 1990; Heras et al. 1998; Videla et al. 1998; Labarta et al. 1999; Moran & Manahan 2003; Steer et al. 2004; Pernet et al. 2006; Phillips 2006; Chaparro et al. 2012), crustaceans (Thiyagarajan et al. 2002a, b; Phillips 2006; Guay et al. 2011; Pochelon et al. 2011), fish (Sargent et al. 1999; Hilton et al. 2008; Fuiman et al. 2011) and chondryctians (Pethybridge et al. 2011).

To evaluate physiological condition, these studies have usually characterized and/or quantified protein, lipids and carbohydrates, and it seems that different species use different compounds (or a combination of them) as a primary source of nutrition during different life-stages. Nonetheless, in many of those studies it has also been recognized that lipids are a major metabolic energy reserve, playing important roles during the early life-history stages of most species regardless of contrasting developmental or reproductive patterns (for examples see Mann & Gallager 1985; Jaeckle & Manahan 1989; Sewell 2005; Martínez et al. 2008). Yolk lipids are not only important sources of metabolic energy, they also provide important structural components of biomembranes and hormone precursors for the developing embryos of fish, echinoderms, mollusks, among many others organisms (reviewed in Sargent et al. 1999; Sewell 2005). The quantity, quality and dynamics of those lipid reserves are therefore important to understand. Understanding allocation patterns is fundamental for exploring reproductive

potential, maternal provisioning, embryonic development and condition in a number of marine organisms (see Pethybridge et al. 2011).

The total lipid pool of any given organism consists of many lipid classes, which are not equally metabolically available and participate in different physiological processes. Thus, examining allocation patterns in a suite of distinct lipid classes allows for a better understanding of nutritional requirements during the early-life stages of the species (Pethybridge et al. 2012). Nonetheless, not all analytical techniques tolerate such small scales and limited amounts of samples. Further, traditional methods of lipid quantification [i.e. gravimetric, calorimetric, spectrophometrical or traditional Thin Layer Chromatography (TLC)] are time consuming, require a large number of samples to obtain sufficient lipid material, have low power of detection for the less abundant lipid classes and do not discriminate lipid classes (Moran & McAlister 2009). In this sense, the development of the thin-layer chromatography coupled with flame ionization detection (TLC-FID, Iatroscan) is a major improvement for quantification of lipids, providing shorter analytical times and high sensitivity by quantifying a wide range of compounds in a single analysis. This technique involves the separation of individual lipid classes by chromatography on quartz rods coated externally with silicic acid (Chromarods) and subsequently quantifying the individual zones by passing the rods through flame ionization detection (for details see Fraser et al. 1985; Bergen et al. 2000; Moran & McAlister 2009).

Over recent years this methodology has proven to be extremely successful in the study of the evolution of maternal provisioning in a range of planktotrophic and lecithotrophic echinoderm larvae (Sewell 2005; Falkner et al. 2006; Byrne et al. 2008a, b; Prowse et al. 2008; Prowse et al. 2009). However, no previous attempts have focused on organisms that lack planktonic dispersal stages. In direct-developing species, maternal provisioning to the embryo at the time of spawning is the primary source of nutrition for the offspring through to the beginning of juvenile life (Spight 1976b; Rivest 1983). Therefore, species with crawl-away juveniles are an interesting model system to understand how contrasting reproductive strategies, and thus maternally-derived resources, can have direct consequences in mediating offspring size, provisioning and further juvenile performance in organisms exposed to variable environmental or laboratory experimental conditions (e.g. female's food environments).

Here, based on Sewell (2005), I describe a modified protocol for evaluating maternal provisioning of lipids in a direct developing species using the Iatroscan Mark V^{new}

TLC/FID system. The intertidal whelk *Cominella virgata* was used as a model organism. Consequently, this chapter aims to provide a complete description of the methodology to identify and quantify different lipids classes on a per-individual basis in direct developing species. The applicability of this technique will be exemplified in the following Chapter (four) by providing inter- and intra-specific comparisons among the three study species at small scales of variation (i.e. among siblings; see Chapter 2).

3.3 Materials and methods

3.3.1 Specimen collection

Newly-laid egg capsules of *Cominella virgata* collected in October 2010 (Chapter 2) were used in this Chapter to exemplify the modified methodology for lipid extraction and analysis on a per-individual basis. Immediately after collection, subsets of 3 egg-capsules were taken from a single cluch and individually stored in Eppendorf tubes with seawater at -80 °C. The remaining capsules were maintained in flowing seawater, and after approximately 2.5 months of intra-capsular development (see Chapter 2), a group of 3 new hatchlings (i.e < 1 day old) were collected from the same clutch. Eppendorf tubes were immediately placed in a freezer at -80 °C until further lipid analyses.

3.3.2 Estimation of sample weight

Prior to the lipid extraction, different approaches had to be undertaken in order to estimate the amount of intra-capsular fluid and soft tissue analyzed in egg capsules and hatchlings. Firstly, given that egg capsule walls are primarily composed of proteins, carbohydrates and polysaccharides (see Rawlings 1999; Ojeda & Chaparro 2004), and preliminary tests also indicated that capsule walls did not dissolve during sonication (i.e. first step of the lipid extraction), the complete egg capsules and contents were weighed. Capsule walls were separated from each other, the membrane containing the fluid carefully dissected, sonicated, and then the empty capsule walls (including both sides) dried with towel paper and re-weighed. The amount of intra-capsular fluid to be analyzed (mg) was obtained by subtraction. Secondly, since hatchlings' shells cannot be

recovered after the sonication process, a set of 10 siblings collected from the same clutch were dried and ashed in order to estimate the organic content, and therefore, the flesh weight (FW, mg) for a snail of a particular shell length (SL, mm). The best-fit regression between both factors was further used for estimations of FW in the samples analyzed.

3.3.3 Lipid Extraction and Analyses

Lipid was extracted from egg capsules and hatchlings using a chloroform/methanol extraction previously used with echinoderm larvae (see Sewell 2005), but modified as described below. Sample homogenates were prepared by the addition of 250 µl of ultrapure water to the Eppendorf tubes containing one single capsule (previously disected to expose the fluid) or hatchling and ultrasonication with a Sanyo Soniprep 150 fitted with an exponential probe for 20 to 30 s. To avoid an increase in water temperature, tubes were always maintained in ice during the process. The sonicate (250 µl) was transferred with a drawn Pasteur pipette to a 1 ml glass V-vial (Wheaton) containing 25 µl of the internal standard in a solution of 100 µl chloroform and 250 µl methanol. Ketone was used as internal standard because natural concentrations are low in marine tissues and also because this lipid does not co-elute with any other lipid classes in the development system used (reviewed by Sewell 2005).

Lipid extraction followed the water: chloroform: methanol method (Bligh & Dyer 1959) as described in Sewell (2005). If necessary, the extracts were held in chloroform at -20 ^{o}C before quantification using an Iatroscan Mark V^{new} TLC/FID system and silica gel S-III Chromarods, following the protocols defined by Parrish (1987, 1999). Immediately before lipid extraction, the extract was dried down in a stream of N_2 gas and 10 µl of chloroform added using a Gilson positive displacement pipette. After ensuring that the Chromarods were clean by running a blank scan, the entire lipid extract (10 µl, corresponding to 1 single egg capsule or hatchling) was applied to each Chromarod with a fixed volume Drummond Microdispenser fitted with Drummond Precision Glass Bores (volumetric tolerance of ± 1%). In this case six individual samples were analyzed on each run, and the remaining four Chromarods were used as blanks (unspotted) to test for contamination of the developing solvents.

Lipid classes were separated on the Chromarods using a double development system derived from the triple development of Parrish (1999) but with the second chromatogram resulting from a complete scan (see Fig. 3.1). Development solutions, preparations of the developing tank and timings were as in Parrish (1999) with the exception that the first development [hexane (H): diethyl ether (DEE): formic acid (FA) of 98.95:1:0.05, respectively] had a Partial Scan Mode of PPS27, and the Chromarods were developed in the second development (H: DEE: FA of 79:20:1) for 33 min to ensure that the triglyceride peak was retained in the second chromatogram. Chromarods were run in the Iatroscan with a 30 s scan and settings of 2000 ml min^{-1} O_2 and 160 ml min^{-1} H_2. Chromarods were burnt from the solvent front towards the origin (Fig. 3.1). Data collection was with SES-Chromstar version 4.10 (SES Analysesysteme).

Quantification of the lipid per sample was based on multilevel calibration curves generated for each lipid class on one rack of 10 chromarods. Rods were calibrated with an 8-component composite standard made from highly purified lipid standards (99%) in HPLC-grade chloroform and stored under nitrogen at -20°C (Parrish 1987, 1999; Bergen et al. 2000). The lipid classes were phospholipid (PL: L-α-phosphotidylcholine), free sterol (ST: cholesterol), free fatty acid (FFA: palmitic acid), acetone-mobile phospholipid (AMPL: 1 monopalmytoyl glycerol), triglyceride (TG: tripalmitin), diglyceride (DG: 1,2 dipalmitoyl-rac-glycerol [C16:0]), ketone (KET: 3- hexadecanone) and aliphatic hydrocarbon (HC: nonadecane) using standards as described in Sewell (2005).

Peak areas for the calibration curves were based on the mean of 3 separate Chromarods; R^2 values were > 0.994 for all lipid classes. The concentrations of each lipid class per sample were determined based on the percent recovery of the internal standard and the calibration curve appropriate for each lipid class. Total lipids were calculated by summing the amounts of PL, ST, HC, TG, FFA and DG in each sample; structural lipid by summing PL and ST; and energy lipid by summing HC, TG, FFA and DG.

3.4 Results and Discussion

Six samples of early stages of *Cominella virgata* (n = 3 egg capsules; 9.09 ± 0.33 mg TW [mean ± SE] and n = 3 hatchlings; 2.7 ± 0.06 mm SL [mean ± SE]) were individually analyzed, and all of them showed six lipid classes (Fig. 3.1; Table 3.1), with an approximate mean total lipid content of 17 ± 1 µg capsule^{-1} (± SE) in newly laid egg capsules, and 22 ± 3 µg hatchling^{-1} (± SE) in hatchlings. In both cases, relatively low variability of total lipid composition was observed among samples from specific stages. Around 85 % of the total amount was contributed by the structural lipids phospholipids (PL) and cholesterol (ST), with lesser amounts of the energy-storage lipids diglyceride (DG), aliphatic hydrocarbon (HC), free fatty acid (FFA) and triglyceride (TG) (Table 3.1).

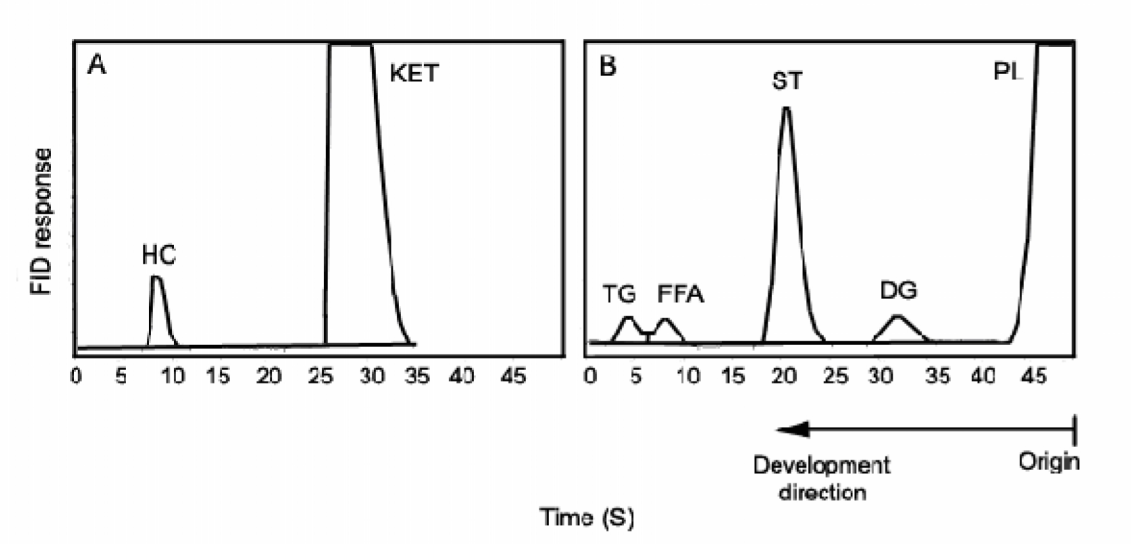

Fig. 3.1 Example of the TLC/FID chromatograms of lipids extracted from a single *C. virgata* hatchling (SL: 2.8 mm) using the Parrish (1999) double development. The two panels are the chromatograms resulting from the 2 scans of the Chromarods: A) Hexane: diethyl ether: formic acid (69 ml; 0.7 ml; 0.035 ml) for 24 min, 5 min conditioned, redeveloped for 19 min and dried 5 min. Partial scan on Iatroscan (PPS27). B) Hexane: diethyl ether: formic acid (55 ml; 14 ml; 0.7 ml) for 33 min and 5 min dried. Iatroscan full scan. *x*- axis is time (s); *y* shows FID response to the same relative scale. As each lipid class has a different FID response, peak areas cannot be directly compared between different lipid classes. HC: aliphatic hydrocarbon; KET: ketone (internal standard); TG: triglyceride; FFA: free fatty acid; ST: cholesterol; DG: diglyceride; PL: phospholipid.

Table 3.1 Lipid class analysis of individual egg capsules and hatchlings *C. virgata* (µg mg^{-1}) using Iatroscan TLC/FID. Morphological traits of egg capsules and hatchlings are also provided.

Sample traits	Egg capsules			Hatchlings		
	Capsule 1	Capsule 2	Capsule 3	Hatchling 1	Hatchling 2	Hatchling 3
Total weight, mg	8.45	9.29	9.53	-	-	-
Fluid weight, mg	5.38	5.18	6.80	-	-	-
Shell length, mm	-	-	-	2.6	2.7	2.8
Flesh weight, mg	-	-	-	0.35	0.37	0.38
Lipid classes (µg mg^{-1})						
Phospholipids (PL)	11.137	8.579	10.967	17.184	11.154	21.545
Cholesterol (ST)	3.795	3.950	4.084	2.954	2.830	2.856
Aliphatic hydrocarbon (AH)	0.739	0.590	0.650	0.584	0.296	0.421
Triglycerids (TG)	0.582	0.583	0.718	0.381	0.196	0.814
Free fatty acids (FFA)	0.444	0.444	0.644	0.845	0.658	0.580
Diglycerids (DG)	0.911	0.781	0.840	0.579	0.564	0.537
TOTAL	17.609	14.927	17.902	22.527	15.699	26.754
Structural	14.932	12.529	15.051	20.139	13.985	24.401
Energetic	2.677	2.398	2.852	2.388	1.714	2.353

The use of the double development of Parrish (1999) and the Iatroscan TLC/FID system is a novel approach for qualitative and quantitative evaluate the offspring provisioning (i.e. lipids) in individual egg capsules and hatchling snails. Using this method I have found that most of the lipid classes indentified in the hatchlings of *C. virgata* are typical of other marine invertebrates and fish larvae [i.e. echinoderms (Sewell 2005; Falkner et al. 2006; Byrne et al. 2008a, b; Prowse et al. 2008; Prowse et al. 2009), mollusks (Heras et al. 1998; Moran & Manahan 2003; Steer et al. 2004; Pernet et al. 2006) and fish (Hilton et al. 2008)], with phospholipids (PL) and cholesterol (ST) as the main structural lipids and aliphatic hydrocarbon (HC), triglyceride (TG), diglyceride (DG) and free fatty acids (FFA) as the main energy-storage lipids. While structural lipids are largely located in cell membranes, the energy-storage lipids are the reserves that fulfill the hatchling's needs immediately after hatch. In a wide range of species it has been recognize that TG is the most important lipid class, since it is the primary endogenous energy reserve fueling the basal metabolism and the construction of the larval body (Gallager et al. 1986; Moran & Manahan 2003; Sewell 2005; Steer et al. 2004; Falkner et al. 2006; Pernet et al. 2006; Byrne et al. 2008a).

Although this methodology has proven to be a successful technique to quantify the amount of different lipid classes in species with relatively small planktonic stages of dispersal (i.e. echinoderm, mollusks, and fish), the use of this method in species with larger hatchlings and hard protective structures can cause some minor problems that will need to be addressed according the species. For example, because the lipids must be extracted directly from the soft tissues, it was not feasible to combust the hatchlings in order to obtain the flesh weight (FW, mg) or the organic material from the same samples. Additionally, because the lipids must be obtained under clean or undisturbed conditions, a proper dissection of hundreds of experimental hatchlings (i.e. 2 mm) for analysing their soft tissues may increase the possibility of sample contamination. Therefore, the best approach in this case was first: to evaluate the organic content in a set of siblings (ideally in a range of sizes) to estimate the relationship FW vs shell length (SL, mm), and second: measure the SL for each of the real samples and then analyze the entire hatchling (even sonicating the shell). Nevertheless, this problem will be more important for species with protective hard structures such as shells, and I assume it could be easily avoided in species with soft bodied early-stages (i.e. cephalopod paralarvae or small crustacean larvae).

In summary, the protocol described here using an Iatroscan TLC/FID based-method allowed the examination of lipids in even the smallest hatchlings of *C. virgata* on an individual basis. Alternative methods for lipid extraction and analysis, such as traditional Thin Layer Chromatography (TLC), require a large number of individuals to be combined to obtain sufficient lipid material, have low power of detection for the less abundant lipid classes, and do not discriminate lipid classes. The method that has been developed here can be applied to study maternal provisioning in any species of direct developing gastropod, being especially useful for comparisons across developmental strategies, over the course of development and stages post-hatch, and for examination of variability at different scales (i.e. within and among females, populations, etc.). Data collected on an individual basis in a variety of direct developers will fill an important knowledge gap in understanding the consequences of variation in maternal provisioning, and will allow energetic comparisons with alternative life-history strategies.

Chapter 4

Maternal provisioning of lipids in three sympatric whelks with contrasting reproductive strategies and offspring size

4.1 Abstract

The early-life stages of marine invertebrates initiate development fueled by maternally-derived resources. Here, using three sympatric whelks: *Cominella virgata* (one embryo per capsule), *C. maculosa* (multiple embryos per capsule) and *Haustrum scobina* (multiple embryos per capsule and nurse embryo consumption), I examined how contrasting reproductive strategies mediate inter- and intra-specific differences in maternal investment (i.e. lipid composition and quantity). A maximum of eight lipid classes were identified in newly laid egg capsules, with a total lipid content (µg egg capsule^{-1} ± SE) of 16.8 ± 0.9 (*C. virgata*), 26.1 ± 0.7 (*C. maculosa*) and 218.7 ± 9.9 (*H. scobina*). The ~10-fold higher lipid provisioning observed in *H. scobina* was mainly attributed to differences in phospholipids (PL) and triglycerides (TG). The total amount of lipid increased during the encapsulated development (wk 1 to 9); however, for one species (*H. scobina*), the content of the energetic lipid free fatty acid (FFA) was depleted after 3 wk. Total lipid content of hatchlings (µg hatchling^{-1} ± SE) was not related to offspring size, as the species with the smallest hatchlings, *H. scobina*, had the highest lipid content (i.e. *C. maculosa*: 6.6 ± 0.4, *C. virgata*: 21.7 ± 3.2 and *H. scobina*: 33.8 ± 8.1). Only capsules and hatchlings of *C. virgata* lacked wax ester (WE) and methyl ester (ME); other species-specific differences in provisioning of both capsules and hatchlings was due to *H. scobina* having greater PL and TG. Inter- and intra-capsular variability in provisioning was higher in *H. scobina*, suggesting less control of allocation for individual embryos. As observed in these whelks, inter-specific variability in hatchling size may not always be a reliable indicator of variability in lipids, and thus, to fully understand the relationships among offspring size, condition and performance, different reproductive strategies and species' life-history stages need to be considered.

4.2 Introduction

The early-life stages of marine invertebrates initiate development fueled by maternally-derived resources stored in eggs (e.g. yolk; see Moran & Manahan 2003; Sewell 2005; Hilton et al. 2008; Byrne et al. 2008a; Prowse et al. 2008) or additionally provided to the embryo in the form of intra-capsular food (e.g. albumen-like fluid, nurse eggs or sibling consumption; see Chaparro et al. 1999; Collin 2003; Cubillos et al. 2007; Martínez et al. 2008; da Costa et al. 2011; Chaparro et al. 2012). Despite different patterns of provisioning and developmental modes, the initial per-offspring maternal investment in terms of offspring size or quality affects offspring performance (see Marshall et al. 2008; Byrne et al. 2008b). Since it is generally recognized that larger offspring often perform better than small peers (see Chapters 5 and 6 for examples), initial differences in size and provisioning can have far-reaching consequences that may persist into the juvenile or even to the adult stages, thereby affecting the performance and phenotype of individuals (Phillips 2004; Marshall et al. 2008; Marshall & Morgan 2011).

Although such studies have highlighted the key role of initial offspring size for several species, variability in provisioning on a per individual basis or among siblings has been rarely examined. Much research is therefore still necessary in order to understand the physiological bases that underlie the production of: *i*) offspring of variable phenotypes as an adaptive maternal strategy to face unpredictable environments (or "bet-hedging") and *ii*) strategies in which mothers may adjust, or have more control over the mean phenotype of their offspring according to changes in the maternal environment (reviewed in Crean & Marshall 2009).

In species with planktotrophic larvae, the amount of resources with which a mother provisions an egg represents the entire parental investment and the offspring develop independently in the water column where growth and survival depend on larval feeding (McEdward & Miner 2006). In contrast, in direct developers the influence of maternal provisioning can extend further into the life of the offspring. Since there is no planktonic larval feeding stage, maternal provisioning to the embryo at the time of spawning is the primary source of nutrition for the offspring through to the beginning of juvenile life (Spight 1976b; Rivest 1983) and thus, the relationship between maternal provisioning and performance is likely to be more direct than in species with planktotrophic larval stages (Marshall et al. 2008).

In many phylogenetically diverse species of planktotrophic marine invertebrates, it has long been recognized that lipid reserves are the major source of nutrition during early stages and are rapidly depleted by prefeeding embryos and larvae (see Sewell 2005; Byrne et al. 2008b). In planktotrophic and lecithotrophic mollusks, the greatest reliance on stored energy reserves is during the beginning and end of the development process when the feeding larvae lack a functional mechanism for particulate feeding (i.e. embryogenesis and metamorphosis; for examples see: Jaeckle & Manahan 1989; Rodriguez et al. 1990; Whyte et al. 1987; Whyte et al. 1990; Heras et al. 1998; Videla et al. 1998; Labarta et al. 1999; Moran & Manahan 2003). Similarly, research in echinoderm larvae suggests that lipid reserves account for a significant amount of the total energy available in eggs, with the energetic lipid triglyceride (TG) being depleted during prefeeding development, while structural lipids (i.e. phospholipids, PL) remain relatively stable as these nutrients are used to construct the larval body (Sewell 2005; Byrne et al. 2008b). Even though there are numerous studies regarding maternal provisioning in marine invertebrates with planktonic and lecithotrophic dispersal stages (op. cit.), there is an important knowledge gap regarding the maternally-derived resources and their dynamics during development of non-dispersive, direct developer species.

Since even closely related species may differ in their reproductive strategies [i.e. planktotrophy vs lecithotrophy (see Falkner et al. 2006; Prowse et al. 2008) and planktonic vs direct development (see Collin 2003)], there are important species-specific differences in maternal investment. For example, studies on the evolution of several species of echinoderms with larval stages suggest that the energetic lipids in the small eggs of echinoderms with feeding larvae are primarily TG (a class of short-term storage lipid), whereas DAGE (diacylglycerol ether) dominates the large eggs of echinoderms with non-feeding larvae (Prowse et al. 2009). Therefore, species with different developmental strategies may have different needs for particular lipid classes, and they will not be equally available as they play different biochemical and energetic roles during embryogenesis and larval development (Moran & McAlister 2009; Pethybridge et al. 2011). Studying allocation patterns of different energetic resources may therefore provide an interesting approach to evaluate how differential provisioning among species could be attributed to their differences in ecology, physiological adaptations, phylogenetics and/or evolution of contrasting life-history modes.

Physiological signatures of different lipid classes and quantities should therefore be expected to also occur in other invertebrates with contrasting reproductive modes, and thus be especially evident in species for which maternal provisioning is the primary source of nutrition for the offspring through the beginning of juvenile life (i.e. direct developers). In this sense, the New Zealand gastropod fauna is large and diverse (over 2000 marine species) including around 70 direct developers. Within these species, there are different reproductive strategies, ranging from multiple embryos per capsule (e.g. *Cominella maculosa*), to multiple embryos per capsule with intra-capsular nurse embryo consumption (e.g. *Haustrum scobina*) or just a single embryo per capsule (e.g. *Cominella virgata*) (see Chapter 2). These contrasting reproductive strategies, and thus modes of provisioning, make whelks an interesting group in which to examine how initial energy reserves (i.e. yolk, energy lipids) are provided by the mother to the embryos according to variable packaging strategies, or more interestingly, how these reserves may vary depending on the initial hatchling size.

In this study I used as model organisms the co-occurring intertidal whelks *Cominella virgata*, *C. maculosa* and *Haustrum scobina* to examine inter- and intra-specific differences in hatchling provisioning in species with direct development. There were four major aims: first, to identify and compare the type and quantity of lipids present in newly laid egg capsules of the three species; second, to examine the dynamics of the lipid classes during encapsulated development; third, to compare the maternally-derived lipid resources of newly hatched snails, and fourth, to evaluate the lipid provisioning in siblings of the species that encapsulate multiple embryos per capsule (i.e. *C. maculosa* and *H. scobina*). I hypothesized that: a) the amount of resources in newly laid egg capsules will be related to the number of embryos encapsulated rather than capsule size, b) intra-capsular provisioning will vary over time as embryos grow and use the lipids in the intra-capsular fluid, c) energetic provisioning per hatchling will be a function of hatchling size and d) higher variability is expected in the species with less “control” in hatchling provisioning (i.e. *H. scobina*, with nurse embryo feeding).

4.3 Materials and methods

4.3.1 Study species, collection and samples selection

Newly laid egg capsules of the three study species were collected at the same time, from the same site, and following the same methodology previously described in Chapter 2.

Once in the laboratory, a large group of capsules from each species (i.e. > 50 egg capsules) was selected to evaluate lipid content over time. Within four hours after collection, an initial set of three randomly selected capsules per species were sampled and classified as week 1. Thereafter, a similar set of samples (n = 3) was taken fortnightly (i.e. weeks, 3, 5, 7, 9) during the intra-capsular development and at hatch (i.e. week 10) for all species. In all cases, egg capsules and hatchlings were stored individually in Eppendorf tubes and kept at -80 °C until lipid analysis.

4.3.2 Lipid analyses

Lipid extraction and analyses followed the methodology previously described in Chapter 3. Nonetheless, given species-specific differences in capsule and hatchling traits, slightly different approaches were additionally considered for each species and described below.

For analyzing the lipid provisioning and dynamics of lipid classes in the capsular content (fluid and embryos) over time, egg capsules of each of the three species were dissected. For *C. virgata*, egg capsules' walls were first separated from each other and then the layer surrounding the fluid (and embryo) transversally dissected to expose the content to the sonication probe. Because in capsules from *C. maculosa* there were not distinguishable sides to separate, a lateral incision was made along one of the capsule walls, allowing the fluid (and embryos) to be released once in contact with the sonication probe. For *H. scobina*, a transversal incision in the capsules' base allowed the fluid and embryos to be sonicated. As stated in Chapter 3, the amount of fluid analyzed in each capsule (mg) was obtained by subtraction of the capsules' weight before (entire capsule) and after (capsule walls) sonication. Given the species-specific differences in investment (e.g. number of propagules encapsulated) and the sensitivity of the Iatroscan system, different amounts of the extracted lipid were analyzed for each

species. As also previously explained for *C. virgata* (see Chapter 3), the entire lipid extract (10 µl, corresponding to a single egg capsule) was applied to each Chromarod. Nonetheless, in order to get adequate measurements of the lipid content per egg capsule, only 5 µl and 1 µl (of the 10 µl extracted) were spotted on each Chromarod for *C. maculosa* and *H. scobina*, respectively. The final lipid amount obtained was then scaled to 10 µl for appropriate inter-specific comparisons.

Quantification of the entire capsular content (fluid and embryos) was carried out until week 7 in all species. By week 9 (i.e. pre-hatching stage; n = 3 capsules per species), the lipid content of *C. virgata* was separately evaluated in the single hatchling and the remaining intra-capsular fluid of each capsule. Similarly, as many embryos as possible from *C. maculosa* and *H. scobina* (usually from 2 to 6) were extracted from the three capsules analyzed at this stage. In both species, the remaining fluid in the capsule (entire lipid extract 10 µl) and its corresponding hatchlings were individually analyzed. For these pre-hatchling stages, lipids were extracted following the same methodology as in newly hatchlings snails (10 wks) described below. Lipid content in the remaining capsular fluid and in pre-hatchling snails was combined (and scaled to the total amount of hatchlings per capsule if necessary) in order to obtain the per-capsular lipid content at this week.

Lipid provisioning in hatchlings was analyzed similarly for each species. As stated in Chapter 3, a preliminary set of about 15 siblings per species was collected and their organic content evaluated in order to determine the relationship between flesh weight (FW, mg) and shell length (SL, mm) of each individual. Different equations were fitted for each species as follows: *C. virgata* ($y = 0.146\ e^{0.341x}$; n = 18), *C. maculosa* ($y = 1.8636x^2 - 5.7214\ x + 4.6447$; n = 17) and *H. scobina* ($y = 0.2776\ \ln(x) + 0.1753$; n = 15). For all the three species, the individual lipid content was analyzed in the entire lipid extract (10 µl) corresponding to a single hatchling.

In order to account for differences in capsule and hatchling size and allow for comparisons among species, the total amount of individual lipid content at both stages (µg ind^{-1}) was standardized to weight (i.e. fluid in egg capsules and of soft tissue in hatchlings) and expressed as µg mg^{-1}.

For egg capsules and hatchlings, lipid classes were separated on the Chromarods using a double development system derived from the triple development of Parrish (1999) and with the same modifications described in Chapter 3. In this case, rods were calibrated with an 11-component composite standard made from highly purified lipid

standards (99%) in HPLC-grade chloroform and stored under nitrogen at -20°C (Parrish 1987, 1999; Bergen et al. 2000). The lipid classes were phospholipid (PL: L-α-phosphotidylcholine), free sterol (ST: cholesterol), free fatty acid (FFA: palmitic acid), acetone-mobile phospholipid (AMPL: 1-monopalmytoyl glycerol), triglyceride (TG: tripalmitin), diglyceride (DG: 1,2 dipalmitoyl-rac-glycerol [C16:0]), ketone (KET: 3-hexadecanone), aliphatic hydrocarbon (HC: nonadecane), wax ester (WE: lauric acid myristyl ester), methyl ester (ME: methyl palmitate) and cetyl alcohol (FALC: 1-hexadecanol) using standards as described in Sewell (2005).

4.3.3 Data analyses

To investigate differences in the lipid class composition among eggs, during the intra-capsular development and among hatchlings of *C. virgata*, *C. maculosa* and *H. scobina*, multivariate analyses were conducted using Primer v 6.0 (Plymouth Routines In Multivariate Ecological Research) .

Differences in the lipid class composition in newly laid egg capsules and hatchlings of the three species were analyzed separately but with an identical statistical design using permutational analyses of variance (PERMANOVA; Anderson 2001, 2008) based on the Bray-Curtis dissimilarity index of untransformed amounts of lipid classes (µg mg^{-1}) and unrestricted permutation of raw data with 999 permutations. Only the factor species (3 levels, fixed) was included in these analyses. When significant effects were detected by PERMANOVA using Monte Carlo p-values ($p_{(MC)} < 0.05$), differences among species were identified by comparing all the possible combinations (i.e. post-hoc pairwise comparisons). Similarity percentage analyses (SIMPER) were further performed to evaluate the contribution of each lipid class to the observed dissimilarities.

Dynamics of lipid classes during the intra-capsular development of the three species was evaluated with PERMANOVA based on the Bray-Curtis dissimilarity index of untransformed amounts of each lipid class (µg mg^{-1}) using unrestricted permutation of raw data and 999 permutations. In the PERMANOVA design, the fixed factors were species (i.e. *C. virgata*, *C. maculosa* and *H. scobina*) and time (i.e. weeks 1, 3, 5, 7, 9). When significant effects were detected by PERMANOVA ($p_{(MC)} < 0.05$), differences among species, time, and their corresponding interactions, were identified by comparing all the possible combinations with post-hoc pairwise comparisons. The contribution of

individual lipid classes to Bray-Curtis similarities and dissimilarities was then examined with SIMPER analysis.

For evaluating the per-offspring investment in the multi-encapsulated siblings *C. maculosa* and *H. scobina*, a nested PERMANOVA (using Bray-Curtis, 999 unrestricted permutations of the raw data set) of untransformed amounts of each lipid class (µg mg^{-1}) was used with the factors species (2 levels, fixed) and capsules nested within species (6 levels, random). When significant effects were detected by PERMANOVA ($p_{(MC)} < 0.05$), post-hoc pairwise comparisons were used to explore significant factor effects. SIMPER analysis further allowed quantifying the contribution of individual lipid classes to Bray-Curtis similarities and dissimilarities. Discriminant plotting methods were used to help in the interpretation of PERMANOVA results (nMDS plot). Coefficients of variation (CVs) were also used to explore differences in lipid provisioning among siblings within individual capsules per species.

4.4 Results

4.4.1 Maternal provisioning of egg capsules

A maximum of eight lipid classes were identified in newly laid egg capsules of the whelk species examined. *H. scobina* had the greatest total lipid content (µg egg $capsule^{-1}$ ± SE), with 13-fold and 8.4-fold more than *C. virgata* and *C. maculosa*, respectively (see Table 4.1).

Table **4.1** Lipid class analysis of newly laid egg capsules *C. virgata*, *C. maculosa* and *H. scobina* (µg mg^{-1}) using Iatroscan TLC/FID. Morphological traits of egg capsules are also provided. Data are mean ± SE (n = 3 in all cases).

Egg capsule traits	*C. virgata*			*C. maculosa*			*H. scobina*		
Embryos per capsule	1			7.7 ± 0.2			235 ± 17		
Height (mm)	4.42 ± 0.08			8.70 ± 0.30			3.27 ± 0.23		
Wide (mm)	2.55 ± 0.12			4.67 ± 0.09			-		
Depth (mm)	2.08 ± 0.03			2.90 ± 0.06			-		
Radius (mm)	-			-			1.78 ± 0.06		
Volume (µL)	22.25 ± 0.91			50.04 ± 2.93			15.03 ± 2.53		
Total weight (mg)	9.09 ± 0.33			39.61 ± 0.99			18.39 ± 0.71		
Fluid weight (mg)	5.79 ± 0.51			33.56 ± 0.79			16.08 ± 0.76		
Lipid classes	µg mg^{-1}	µg egg $cap.^{-1}$	% per egg cap.	µg mg^{-1}	µg egg $cap.^{-1}$	% per egg cap.	µg mg^{-1}	µg egg $cap.^{-1}$	% per egg cap.
Phospholipids (PL)	1.8 ± 0.1	10.2 ± 0.8	60.7 ± 1.7	0.1 ± 0.01	4.2 ± 0.3	16.2 ± 1.3	7.1 ± 0.4	113.5 ± 6.1	51.9 ± 1.6
Cholesterol (ST)	0.7 ± 0.05	3.9 ± 0.1	23.6 ± 1.5	0.03 ± 0.03	1.1 ± 0.1	4.2 ± 0.3	0.3 ± 0.1	5.1 ± 0.9	2.3 ± 0.3
Aliphatic hydrocarbon (AH)	0.1 ± 0.01	0.7 ± 0.04	3.9 ± 0.2	0.02 ± 0.002	0.8 ± 0.1	3.0 ± 0.4	0.03 ± 0.006	0.5 ± 0.1	0.2 ± 0.03
Triglycerids (TG)	0.1 ± 0.002	0.6 ± 0.04	3.7 ± 0.2	0.5 ± 0.03	16.1 ± 0.6	61.7 ± 1.0	5.7 ± 0.4	91.1 ± 3.9	41.7 ± 0.9
Free fatty acids (FFA)	0.01 ± 0.004	0.5 ± 0.07	3.0 ± 0.3	0.01 ± 0.0002	0.5 ± 0.01	1.9 ± 0.1	0.1 ± 0.05	1.2 ± 0.7	0.6 ± 0.06
Diglycerids (DG)	0.1 ± 0.01	0.8 ± 0.04	5.0 ± 0.2	0.01 ± 0.002	0.3 ± 0.1	1.3 ± 0.3	0.1 ± 0.05	1.7 ± 0.7	0.8 ± 0.3
Wax ester (WE)	n.d	n.d	n.d	0.03 ± 0.004	1.1 ± 0.2	4.3 ± 0.5	0.1 ± 0.02	2.2 ± 0.2	1.0 ± 0.09
Methyl ester (ME)	n.d	n.d	n.d	0.06 ± 0.006	1.9 ± 0.2	7.5 ± 0.8	0.2 ± 0.03	3.4 ± 0.4	1.5 ± 0.1
TOTAL	2.9 ± 0.2	16.8 ± 0.9	100	0.8 ± 0.03	26.1 ± 0.7	100	13.7 ± 0.9	218.7 ± 9.9	100
Structural	2.5 ± 0.2	14.2 ± 0.8	84.3 ± 0.3	0.1 ± 0.01	5.3 ± 0.3	20.4 ± 1.1	7.4 ± 0.4	118.6 ± 6.8	54.2 ± 1.5
Energetic	0.5 ± 0.02	2.6 ± 0.1	15.7 ± 0.3	0.6 ± 0.03	20.7 ± 0.8	79.6 ± 1.1	6.3 ± 0.6	100.1± 5.0	45.8 ± 1.5

The structural lipids phospholipid (PL) and cholesterol (ST), and the energetic lipids aliphatic hydrocarbon (AH), triglycerides (TG), diglycerides (DG) and free fatty acids (FFA) occurred in all three species. Only egg capsules of *C. maculosa* and *H. scobina* were provisioned with the energetic lipids wax ester (WE) and methyl ester (ME) (Table 4.1). Energetic provisioning to egg capsules varied among species, but in most cases was dominated by TG, ranging from 3.7% to 61.7% as a proportion of the total lipid content. Structural lipids were dominated by PL in all species, and this lipid class ranged from 16.2% to 60.7% as proportion of the total lipids (Table 4.1).

When differences in capsule size were removed by standardizing to the amount of fluid analyzed ($\mu g\, mg^{-1} \pm SE$), the total maternal investment in lipid varied significantly among egg capsules of the three species (i.e. *C. maculosa*: 0.8 ± 0.03 µg; *C. virgata*: 2.9 ± 0.2 µg and *H. scobina*: 13.7 ± 0.9 µg; PERMANOVA, pseudo-$F_{2, 8}$ = 305.87, p = 0.001; Table 4.1), all of them significantly different from each other (pairwise comparisons, $p < 0.001$ in all cases). Further, SIMPER analysis also showed high average dissimilarities between species [i.e. *C. virgata* vs *C. maculosa* (82.95%), *C. virgata* vs *H. scobina* (70.95%), and *C. maculosa* vs *H. scobina* (89.15%)]. Between *Cominella* species, 91.6% of those differences were explained by amounts of PL> ST> TG and DG, whereas 93% of the differences between both *Cominella* species and *Haustrum* were explained only by amounts of PL and TG (see Table 4.1; see appendix A).

4.4.2 Dynamics of lipid classes during intra-capsular development

Embryos of *C. virgata*, *C. maculosa* and *H. scobina* developed at a similar rate, attaining the pre-hatching stage in around 9 weeks (see Chapter 2). During the encapsulated development of all three species, the amounts of lipid in each class was relatively stable during the first 5 weeks, only increasing by weeks 7 and 9 when capsule fluid and embryos were separately analyzed. Nonetheless, for one species (*H. scobina*), the content of the energetic lipid FFA was completely depleted after 3 weeks of development (Fig. 4.1).

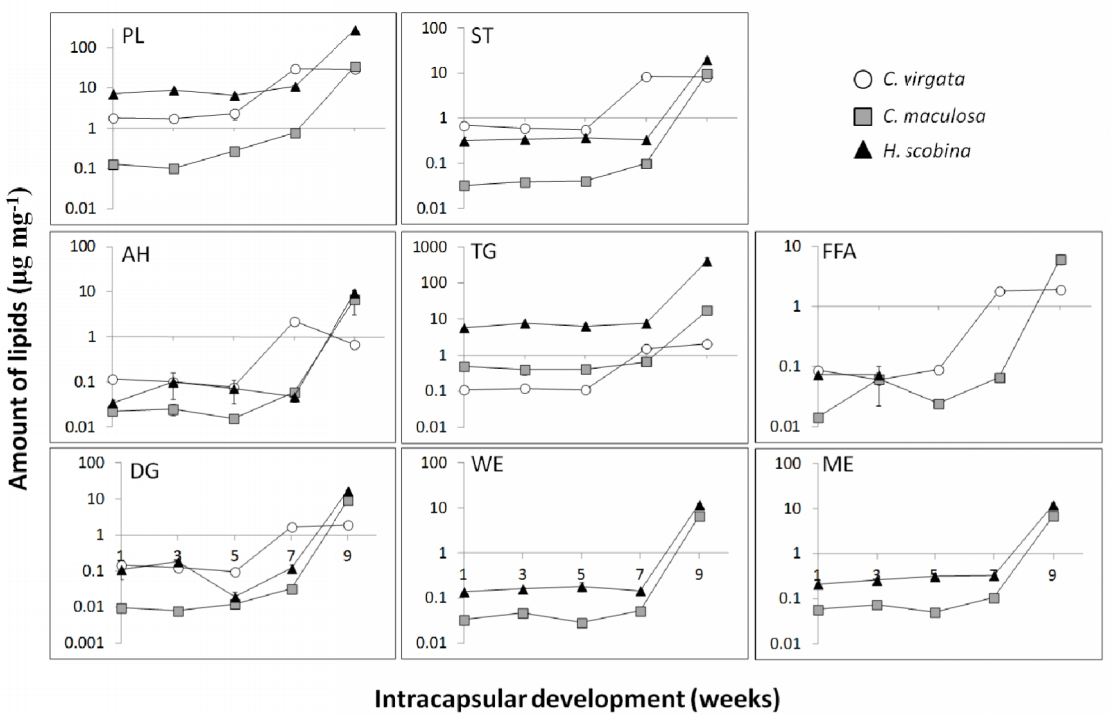

Fig. 4.1 Changes in amount of each lipid class during nine weeks of encapsulated development of three whelk species. Top row shows the two structural lipids phospholipid (PL) and cholesterol (ST); central and bottom rows shows the energetic lipids aliphatic hydrocarbon (AH), triglyceride (TG), free fatty acid (FFA), diglyceride (DG), wax ester (WE) and methyl ester (ME). Y-axis is shown in a logarithmic scale for a better visualization of the standardized lipid class amounts (µg mg^{-1}). For each week and species, data are mean ± SE of 3 egg capsules. In most cases SE bars are not evident as they are smaller than the symbols.

Analyses of total lipid composition revealed that in addition to the significant main effects observed for species and weeks (PERMANOVA, p = 0.001 in both cases), there were significant interactive effects between both factors (PERMANOVA, interaction species x weeks: pseudo-$F_{8, 29}$ = 42.31, p = 0.001). Overall, the lipid composition of egg capsules for the three species (µg mg^{-1} ± SE) was not consistent overtime, and almost all lipid classes significantly increased during development (Fig. 4.1). When lipid composition for the three species was analyzed across all weeks, SIMPER analyses revealed that 91.8% of the average dissimilarity between *C. virgata* and *C. maculosa* (75.19%), was due to differences in the lipid classes PL > ST > TG > DG and AH. The 92.8% of the dissimilarities between *C. virgata* and *H. scobina* (71.12%) were explained by differing amounts of TG >PL and ST, whereas 94.6% of the average

dissimilarity between *C. maculosa* and *H. scobina* (86.38%) where explained due differences in PL and TG (see appendix B). When examining weeks across all species, SIMPER analyses also showed that weeks 1, 3 and 5 were similar to each other (i.e. average dissimilarity: 16.74%). Differences increased significantly when these initial weeks were compared with week 7 (47.68%) and 9 (93.67%) (see appendix C).

4.4.3 Maternal provisioning to hatchlings

As in egg capsules, the same eight lipid classes were detected in hatchlings, varying from six in *C. virgata*, seven in *H. scobina* and eight in *C. maculosa*. The total lipid content in hatchlings (µg hatchling^{-1} ± SE) differed among species, with *H. scobina* having 5.1-fold and 1.6-fold more lipids than *C. maculosa* and *C. virgata*, respectively (Table 4.2). The two structural lipids PL and ST were present in all hatchlings, but only three (AH, TG, DG) of the six energetic lipids were detected in all the three species. WE and ME were only detected in the multiple encapsulated hatchlings *C. maculosa* and *H. scobina*. The latter species was also the only one that lost the energetic lipid class FFA during intra-capsular development (see Table 4.2 and Fig. 4.1). The energetic lipids varied among hatchlings of the three species, but in almost all cases were dominated by TG, ranging from 2% in *C. virgata*, 12.9 % in *C. maculosa* and 38.9 % in *H. scobina* as a percentage of total lipids. Structural lipids were dominated by PL in all species, ranging from 40.1% (*C. maculosa*), 49.3% (*H. scobina*), and 76% (*C. virgata*) as proportion of the total lipids (Table 4.2). In terms of per-hatchling investment, *C. virgata* provisioned their hatchlings with ~9-fold more structural lipids than energetic ones, whereas in *C. maculosa* and *H. scobina* the proportions of both lipid groups were almost the same (1:1) (Table 4.2).

Table 4.2 Lipid class analysis in hatchlings of *C. virgata*, *C. maculosa* and *H. scobina* using Iatroscan TLC/FID. Morphological traits of hatchlings are also provided. Data are mean ± SE (n = 3 in all cases)

Hatchling traits	*C. virgata*			*C. maculosa*			*H. scobina*		
Shell length (mm)	2.7 ± 0.06			1.69 ± 0.03			1.28 ± 0.04		
Flesh weight (mg)	0.37 ± 0.01			0.33 ± 0.02			0.24 ± 0.01		
Lipid classes	µg mg^{-1}	µg $hatchling^{-1}$	% per hatchling	µg mg^{-1}	µg $hatchling^{-1}$	% per hatchling	µg mg^{-1}	µg $hatchling^{-1}$	% per hatchling
Phospholipids (PL)	45.2 ± 7.8	16.3 ± 3.0	76.0 ± 2.7	7.6 ± 1.3	2.7 ± 0.4	40.1 ± 3.9	59.8 ± 20.5	17.6 ± 6.3	49.3 ± 5.9
Cholesterol (ST)	7.9 ± 0.2	2.9 ± 0.04	13.9 ± 2.2	1.8 ± 0.1	0.6 ± 0.03	9.9 ± 0.4	2.5 ± 0.3	0.7 ± 0.1	2.3 ± 0.2
Aliphatic hydrocarbon (AH)	1.2 ± 0.2	0.4 ± 0.08	2.0 ± 0.3	1.9 ± 0.6	0.6 ± 0.2	9.6 ± 2.6	3.3 ± 2.4	0.9 ± 0.6	3.9 ± 3.3
Triglycerids (TG)	1.2 ± 0.5	0.5 ± 0.2	2.0 ± 0.5	2.3 ± 0.6	0.8 ± 0.2	12.9 ± 3.8	43.8 ± 7.4	12.8 ± 2.5	38.9 ± 3.6
Free fatty acids (FFA)	1.9 ± 0.2	0.7 ± 0.1	3.4 ± 0.6	1.7 ± 0.2	0.6 ± 0.1	9.1 ± 1.5	n.d	n.d	n.d
Diglycerids (DG)	1.5 ± 0.06	0.6 ± 0.01	2.7 ± 0.5	1.2 ± 0.1	0.4 ± 0.04	6.7 ± 0.4	2.6 ± 0.6	0.7 ± 0.2	2.6 ± 1.0
Wax ester (WE)	n.d	n.d	n.d	1.1 ± 0.03	0.4 ± 0.01	5.9 ± 0.4	1.5 ± 0.1	0.4 ± 0.05	1.4 ± 0.2
Methyl ester (ME)	n.d	n.d	n.d	1.1 ± 0.05	0.4 ± 0.01	5.8 ± 0.5	1.7 ± 0.2	0.5 ± 0.01	1.6 ± 0.2
TOTAL	59.0 ± 8.2	21.7 ± 3.2	100	18.8 ± 1.4	6.6 ± 0.4	100	115.3 ± 25.8	33.8 ± 8.1	100
Structural	53.1 ± 7.8	19.5 ± 3.0	89.9 ± 0.7	9.5 ± 1.4	3.3 ± 0.4	50 ± 3.6	62.3 ± 20.8	18.3 ± 6.4	51.6 ± 5.6
Energetic	5.9 ± 0.6	2.1 ± 0.2	10.1 ± 0.7	9.3 ± 0.3	3.3 ± 0.05	50 ± 3.6	52.9 ± 5.1	15.4 ± 1.9	48.4 ± 5.6

When lipid content was standardized to amount of soft tissue analyzed (µg mg^{-1} ± SE), maternal investment in hatchlings' lipid composition varied significantly among the three species (i.e. *C. maculosa*: 18.8 ± 1.4 µg; *C. virgata*: 59 ± 8.2 µg and *H. scobina*: 115.3 ± 25.8 µg; PERMANOVA, $F_{2, 8}$ = 18.91, p = 0.001), all of them significantly different from each other (pairwise comparisons, p < 0.015 in all cases). SIMPER analysis also showed that the average dissimilarities between species were lower compared with the values observed in egg capsules [i.e. *C. virgata* vs *C. maculosa* (61.94%), *C. virgata* vs *H. scobina* (46.83%), and *C. maculosa* vs *H. scobina* (73.66%)]. Between hatchlings of both *Cominella* species, 92.1% of those differences were explained by amounts of PL > ST and TG. Differences between *Cominella* species and *Haustrum* were also similarly explained in 90% due to PL > TG and ST (see Table 4.2; see appendix D).

4.4.4 Per-offspring investment in siblings *C. maculosa* and *H. scobina*

After nine weeks of development the egg capsules, and thus encapsulated siblings of *C. maculosa* and *H. scobina*, differed in maternal provisioning (µg mg^{-1} ± SE). Lipid composition varied significantly between species (i.e. *C. maculosa*: 17.64 ± 0.97 µg vs *H. scobina*: 73.25 ± 12.33 µg; PERMANOVA, pseudo-$F_{1, 20}$ = 20.61, p = 0.001) and among capsules within species (PERMANOVA, pseudo-$F_{4, 20}$ = 6.74, p = 0.002). Multivariate analysis of the inter- and intra-specific lipid provisioning among siblings from different capsules is shown in the MDS plot (Fig. 4.2).

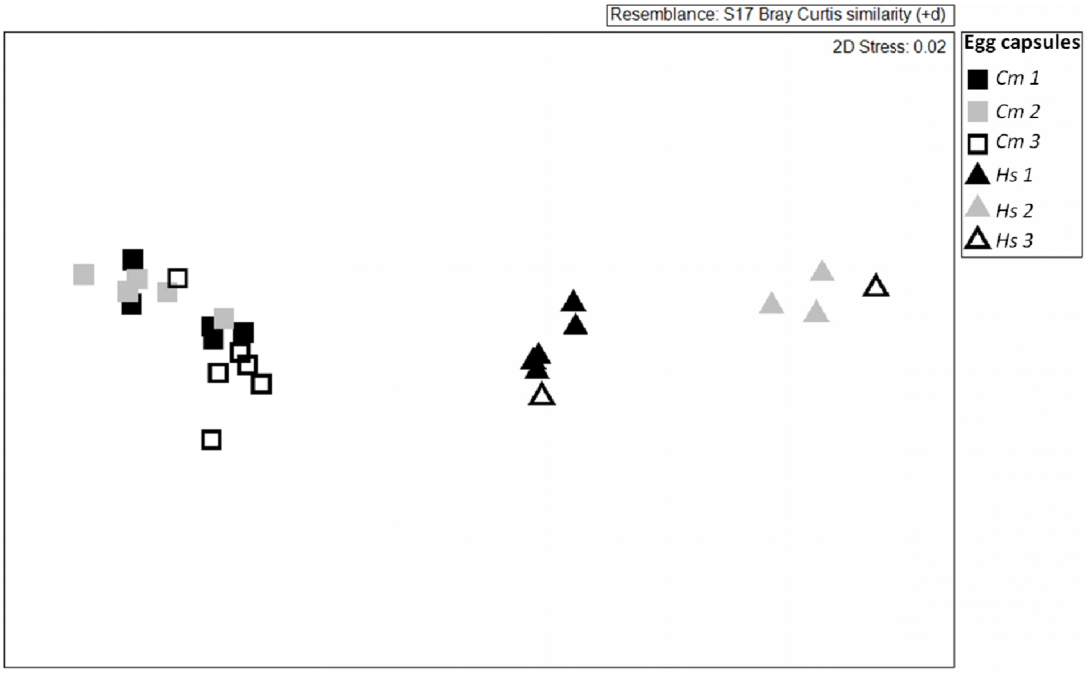

Fig. 4.2 MDS plot of lipid composition for siblings within individual capsules of *C. maculosa* (squares) and *H. scobina* (triangles). Different colors indicate different egg capsules. Stress value for the two-dimensional plot = 0.02. Axes have no scale.

Within species, the lipid composition among capsules and hatchlings *C. maculosa* had a low average dissimilarity of around 17.40% (75% explained by the differences in the lipid classes PL> TG> DG and AH), whereas for *H. scobina*, those among-capsule dissimilarities in lipid provisioning were 31.78%, differences explained in a 93% only by the amounts of PL and TG. Inter-specific differences in lipid composition were higher (average dissimilarities around 65%), and explained in a 92% by differences in the amount of TG > PL > DG and FFA. The range of provisioning in siblings within capsules (µg mg^{-1}) for the two most important structural (PL) and energy (TG) lipid classes, together with the CVs among individual capsules are presented in Table 4.3.

Table 4.3 Range of provisioning (µg mg^{-1}) and coefficient of variation (CV) for the most important lipid classes, PL and TG, in siblings *C. maculosa* and *H. scobina* within three individual capsules per species.

	C. maculosa			*H. scobina*		
PL	1	2	3	1	2	3
Range of provisioning among siblings within capsule (µg mg^{-1})	4.49 - 7.85	4.65 - 7.00	5.09 - 8.30	13.59 - 22.90	34.93 - 49.67	11.99 - 63.13
CVs siblings within capsule (%)	22.3	17.8	16.1	27.0	19.9	96.3
TG	1	2	3	1	2	3
Range of provisioning among siblings within capsule (µg mg^{-1})	2.22 - 4.10	2.03 - 3-90	0.44 - 5.20	23.89 - 25.77	57.15 - 69.08	29.24 - 72.04
CVs siblings within capsule (%)	24.1	25.4	45.3	3.2	9.6	59.8

4.5 Discussion

4.5.1 Maternal provisioning to egg capsules

Unlike many studies in marine invertebrate species with planktonic development, in which egg size has shown not to always be a good predictor of provisioning (McEdward & Morgan 2001; Moran & McAlister 2009); maternal investment for egg capsules in species with direct development is a reliable indicator of provisioning, since no additional resources are provided to the embryos after spawning (see Marshall et al. 2008). Some studies have previously explored the role of maternal provisioning, hatchling size, and organic content in marine snails (e.g. Spight 1976b; Gosselin 1997; Moran & Emlet 2001; Gosselin & Rehak 2007; Lloyd & Gosselin 2007; van der Sman et al. 2009), but only a few have provided detailed information about investment or energy-storage reserves in offspring (see Chaparro et al. 1999; Marínez et al. 2008; Chaparro et al. 2012).

Most of the lipids identified in the egg capsules of these three direct developers (i.e. *Cominella virgata*, *C. maculosa* and *Hautrum scobina*) are common in the early life stages of vertebrates and invertebrates, including several species of echinoderms (reviewed by Prowse et al. 2009), fresh water and marine mollusks (Heras et al. 1998; Moran & Manahan 2003; Steer et al. 2004; Pernet et al. 2006) and fish (Hilton et al. 2008). As in those studies, phospholipids (PL) and cholesterol (ST) were the main structural lipid classes, and aliphatic hydrocarbon (HC), triglyceride (TG), diglyceride (DG), free fatty acids (FFA), wax ester (WE) and methyl ester (ME) the lipids classes used for energy-storage.

Egg capsules of the three whelk species were not equally provisioned. Lipids in *C. virgata* capsules were mainly dominated by PL, attaining values as high as 60% of the total lipid composition and suggesting a higher structural investment for offspring of this species, a trait that will further be reflected in their large hatching size. On the other hand, energetic lipids classes presented remarkable species-specific differences, and in contrast to the structural PL, TG dominated the egg capsules of the species that encapsulated multiple embryos per capsule, *C. maculosa* (61.7%) and *H. scobina* (41.7%), suggesting a differential provisioning for offspring of these species and possible trade-offs between offspring size and energetic reserves. Despite occurring in lesser amounts, DG and FFA were present in egg capsules of the three species;

however, these lipid classes were more abundant in *C. virgata*, providing in total ~8% of the total lipid content. In contrast, WE and ME were only present in the egg capsules of *C. maculosa* and *H. scobina*, accounting for ~12% and 2.5% of the total lipid content, respectively. The functional role of these lipid classes will be discussed further below. Inter-specific comparisons of standardized values (µg mg^{-1}) also clarified that despite differences in the lipid classes and quantities among egg capsules of the three species, those differences were mainly attributable to the two lipid classes PL (structure) and TG (energy).

4.5.2 Dynamics of lipid classes during intra-capsular development

Per-offspring maternal investment is an integral part of life-history theory (McEdward & Miner 2006), yet empirical data on how maternal provisioning is used during development and metamorphosis of offspring are still scarce (Byrne et al. 2008a). Studies on provisioning dynamics carried out in the last decade have clearly demonstrated that many species continuously use their endogenous reserves (and especially TG) to fuel the developmental process [i.e. abalone (Moran & Manahan 2003), sea urchins (Sewell 2005; Byrne et al. 2008a; Byrne et al. 2008b), sea stars (Prowse et al. 2008) and fish (Hilton et al. 2008)], with this lipid class providing the growing larvae part of the necessary energy to face planktonic life. Unlike this pattern, the egg capsules of the three species of whelks examined in the present study showed a notable increase in the amount of almost all lipid classes during encapsulated development (weeks 1 to 9; except for FFA in *H scobina*). Since the highest increases occurred only during the last two weeks of development, these results could possibly be attributable to the different approach used to analyze the samples in these weeks (i.e. separating the shelled embryos from the remaining fluid and then summing both values to obtain a final measurement). Nonetheless, and regardless of the method, significant increases in lipid resources have also been documented during the last weeks of the encapsulated period of other fresh water and marine gastropods such as *Pomacea canaliculata* (Heras et al. 1998), *Chorus giganteus* (Martínez et al. 2008), *Crepipatella dilatata* and *C. fecunda* (Chaparro et al. 2012), suggesting that this increase in provisioning is not only an artefact of the methodology. Further studies in this system using the same technique should therefore explore changes over time by carefully

comparing different methodologies of lipid extraction (i.e. whole capsular content vs embryo extraction), to evaluate the magnitude and timing at which these changes in provisioning occurre.

Interestingly, of the three species and of all lipid classes detected, only FFA was completely depleted after three weeks of development in *H. scobina*, suggesting an important role of this lipid class during the development of these small multi encapsulated embryos. The use of FFA has also been previously highlighted in other invertebrate and vertebrate species with planktonic development. Sewell (2005) suggested that FFA may be an important lipid class fueling the metamorphic and perimetamorphic period in fed larvae of the sea urchin *Evechinus chloroticus*. In the same way, an almost complete depletion in the levels of FFA were observed in eggs of the kingfish *Seriola lalandi* by the time of hatching, suggesting an active use of this energetic lipid class for the synthesis of proteins (reviewed in Hilton et al. 2008).

Although during the encapsulated development of *H. scobina* most of the intra-capsular nurse embryos were consumed by developing siblings in these same weeks (see Chapter 2), it is not clear whether the depletion of FFA is related with this consumption of nurse embryos. Other sources of provisioning (e.g. proteins and carbohydrates) were not directly evaluated in this study, but the utilization of those compounds, especially proteins, has been clearly identified in embryos of several gastropod species (reviewed in Chaparro et al. 2012). Further studies should evaluate if depletion of FFA observed during development of these gastropods could be associated with a synthesis of proteins similar to larval fish.

4.5.3 Maternal provisioning for hatchlings

As also identified in egg capsules, the lipid classes of hatchling whelks differed among species, varying from six in *C. virgata*, seven in *H. scobina* and eight in *C. maculosa*. The main differences in lipid classes corresponded to the absence of WE and ME in *C. virgata* and the total depletion of FFA in *H. scobina*. Interestingly, the presence of both WE and ME has also been independently detected in eggs and planktonic stages of several species, including abalone (Moran & Manahan 2003), squid (Steer et al. 2004), sea stars (Prowse et al. 2008), fish (Hilton et al. 2008) and corals (Dodds et al. 2009). In the case of WE, this lipid class appears to play important roles in buoyancy and long-

term energy storage in polar and deep sea zooplankton (reviewed by Prowse et al. 2009). It is particularly important for shallow-water coral polyps in times of low food input, and indicates a variable food supply (Dodds et al. 2009). The presence of WE and ME in hatchling *C. maculosa* and *H. scobina* could therefore suggest a similarity between these smaller multi-encapsulated embryos with other planktonic larval stages of dispersal. It has generally been recognized that the latter stage is the ancestral condition for many other invertebrate species, with the so-called aplanktonic or direct development being derived (e.g. as observed in vestigial larval organs in many aplanktonic species; reviewed by Pechenik 1999; also see development of *H. scobina* in Chapter 2).

TG was the energetic lipid class that accounted for major differences among hatchlings of the three species. However, among species there was an inverse relationship between hatchling size and the amount of TG as a proportion of the total lipid content (i.e. *C. virgata*: 2%, *C. maculosa*: 12.9 % and *H. scobina*: 38.9%). This suggests an important trade-off between hatchling size and energy content and a key role of nurse embryo provisioning during the intra-capsular development of the smaller of *H. scobina*. Energetic benefits of nurse egg feeding have also been highlighted in other gastropods with encapsulated embryos, showing that the energy content of nurse eggs can even exceed by 30% the energy of the developing siblings (Chaparro et al. 2012). Differences in maternally-derived energetic resources were evident among species, as large *C. virgata* hatchlings had ~9-fold more structural lipids than energetic ones (reflected in their hatchling sizes: ~3mm SL); whereas in the smaller *C. maculosa* and *H. scobina* the proportions of both lipid groups were almost the same (1:1). These findings suggests that smaller hatchlings *C. maculosa* and *H. scobina* could withstand a prolonged period without food and not necessarily start foraging directly after hatching, using microhabitats (instead of shell size) as a refuge from predators. In contrast, larger *C. virgata* hatchlings would probably need food soon after hatching; however, their larger and thicker shells can act as an effective predator deterrent, at least for some of the most common shell crushing predators (i.e. crabs; see Chapter 6 for examples).

Preliminary lipid extraction on juvenile *C. virgata* (i.e. 8 wk and 1 yr old) showed that 8 wk old laboratory-raised snails still maintained the same six lipid classes as hatchlings after being fed on a high protein mussel diet (i.e. *Perna canaliculus*). However, all 1yr field-collected snails showed just five classes of lipids, with no detectable TG present (SA Carrasco, unpubl. data). Although this finding could suggest

that, as in many other marine invertebrates, TG is being utilized as the primary source of nutrition during early juvenile development and growth, further research still needs to evaluate how these lipid reserves may be fueling later life stages of this direct developing species.

4.5.4 Per-offspring investment in siblings of *C. maculosa* and *H. scobina*

Several studies of invertebrate reproduction in general, and gastropod mollusks in particular, have highlighted the importance of different maternal investment strategies in mediating specific traits such as hatchling size. Females can produce variable offspring phenotypes as a bet-hedging strategy to face unpredictable environments, or produce progeny with more similar characteristics by "adjusting" the mean phenotype of their offspring according to changes in their own environment (see Crean & Marshall 2009). In direct developing mollusks, several studies have shown that specific characteristics such as packaging strategies and intra-capsular mechanisms of embryo provisioning will play key roles in determining hatchling size. Those studies have suggested that species that produce eggs which all complete development will generate hatchlings of a relatively uniform size (see Rivest 1983). By contrast, in species that produce other intra-capsular resources (i.e. nurse eggs), embryos have to compete for the limited food (i.e. oophagy, adelphophagy or cannibalism) and therefore, hatching size will vary owing the variable consumption of resources (Spight 1976b; Rivest 1983; Collin 2003; Chaparro et al. 2012).

In the present study, the contrasting provisioning strategies observed in *C. maculosa* (albumen-like fluid) and *H. scobina* (nurse embryos) clearly evidenced different maternal investment (lipid quantity) and patterns of embryo provisioning. The egg capsules and siblings of *C. maculosa* had a similar intra-specific composition of lipids compared with the greater differences observed among egg capsules and siblings of *H. scobina*. This suggests that female *C. maculosa* may more effectively control the allocation of resources among individual embryos within a capsule (i.e. intra-capsular fluid) compared with the nurse-embryo-based strategy of females *H. scobina*. Likewise, these different sources of provisioning also lead to a differential consumption of resources during development, with *H. scobina* embryos exhibiting higher variability in size and developmental asynchrony by the time of hatching (i.e. some hatchlings still

retaining larval traits; see Chapter 2), suggesting that with this strategy, females may increase variation in offspring phenotypes, and as a consequence, bet hedge in a higher degree compared with other albumin-fed hatchlings such as the ones of *C. maculosa* or *C. virgata*. As observed in newly-laid capsules of these two species, inter- and intra-specific differences in provisioning were attributed to the variable amounts of TG and PL, enhancing the role of these two lipid classes as the main energetic and structural components, respectively.

Overall, this inter-specific comparative approach allowed for a better understanding of how co-occurring species of direct developing whelks that have contrasting reproductive strategies mediate hatchling provisioning. As observed in this study, and also previously suggested for other marine invertebrates, even relatively closely related species may differ in the allocation of resources to their progeny. Provisioning in the congeneric *C. virgata* and *C. maculosa* significantly varied in the type (i.e. WE and ME) and quantity (i.e. PL and TG) of lipid, highlighting the fact that not all lipid classes will be equally available for all species as they may be utilized in different physiological or ecological processes. As observed in these three direct developers, inter-specific variability in hatching size may not always be a reliable indicator of variability in energetic reserves, as smaller hatchlings of *H. scobina* had ~7-fold more energetic resources than larger hatchlings of *C. virgata*. Furthermore, since the observed trade-offs in hatchling size, number and energetic provisioning will interact with many other ecological traits such as resistance to starvation, foraging activity, vulnerability to predators, etc., it is evident that offspring size and provisioning are factors that cannot be independently considered in these direct developers species, and characteristics that may seem disadvantageous at one specific life-history stage, may have advantageous consequences for another.

Chapter 5

Offspring size and maternal environments mediate the early juvenile performance of two congeneric whelks[2]

5.1 Abstract

Offspring size variation can have pervasive ecological and evolutionary implications for both offspring and mother, affecting an organism's performance throughout its life. Here, using two marine intertidal gastropods *Cominella virgata* and *Cominella maculosa* as model organisms, I examined how different maternal environments and contrasting hatchling size influence juvenile performance. The average size of field-collected hatchlings greatly differed between species and at different scales of variation (i.e. among sites). Species-specific differences in hatching size were reflected in juvenile performance; overall, the species with larger hatchlings, *C. virgata* (~ 3 mm), exhibited faster growth rates and higher survival compared with the smaller *C. maculosa* (~1.5 mm). Subjection to a desiccation treatment did not affect the performance of fed juveniles; however, large hatchlings had higher growth rates than small conspecifics for both species. Starved hatchlings of both species performed more poorly than fed ones; however, species-specific and size differences were less significant on evaluated traits, suggesting a non size-related allocation of resources, and similar resource utilization in starvation conditions (i.e. within species). As has been described for many taxa, large offspring often perform better than small conspecifics; however, because this performance is likely to be context-dependent, understanding the importance of the different scales of variation is crucial for determining when variation in size is an advantage or a disadvantage in terms of an organism's performance.

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[2] Carrasco SA, Phillips NE, Pérez-Matus A (2012) Offspring size and maternal environments mediate the early juvenile performance of two congeneric whelks. Marine Ecology Progress Series 459: 73-83.

5.2 Introduction

Offspring size is a key trait for most organisms, influencing an individual's subsequent performance, and having direct consequences in fitness for both offspring and mother. For a range of taxa across a variety of habitats, individuals that start juvenile life with a large size often perform better than smaller conspecifics [e.g. gastropods (Spight 1976a, Gosselin 1997, Moran & Emlet 2001), mussels (Phillips 2002), barnacles (Thiyagarajan et al. 2003), ascidians (Marshall et al. 2003, 2006), beetles (Fox 2000, Clark et al. 2011), isopods (Tsai & Dai 2001), spiders (Walker 2003); fish (Green & McCormick 2005, Fisher et al. 2007) and birds (Krist 2011)]; therefore, intraspecific variation in offspring size is of fundamental ecological and evolutionary importance (Marshall & Keough 2008a). Offspring size often varies spatially (i.e. among and within populations, clutches or siblings) and temporally (Bernardo 1996b). Several explanations have been proposed for these scales of variation, but they are often attributed to either non-adaptive stochastic variation in provisioning or to a maternal bet-hedging strategy in unpredictable environments (see Kamel et al. 2010b).

The ability of mothers to anticipate the environment their offspring will experience may therefore mediate variation in offspring size. When mothers are able to predict their offspring's environment, the expectation is that plasticity in mean offspring size will be favored ("anticipatory" maternal effects, Marshall & Uller 2007). Conversely, when mothers are unable to predict the environment, the expected outcome is that increases in the variation of offspring size will ensure that some offspring will be optimal for the environment (Crean & Marshall 2009). Regardless of the strategy, the division of finite reproductive resources should ultimately result in an optimal equilibrium between the offspring fitness and the maximization of the parental fitness, and therefore, simple models have typically suggested that as the relationship between progeny size and progeny fitness change, the optimal size of progeny is also expected to change as environmental conditions vary (Fox 2000; Walker 2003). Examples provided by Allen et al. (2008) for marine invertebrates, suggest that harsh environmental conditions are predicted to have a steeper offspring size/fitness relationship than more benign conditions. Hence, an evolutionary response would result in larger offspring sizes being more beneficial in adverse conditions (e.g. highly competitive environments, high predation pressure or high wave exposure), whereas offspring size would be less important in more favorable environments.

Spight (1976a) suggested predation as a component of environmental severity for early juvenile benthic invertebrates. Food limitation, especially in the beginning of benthic life, has also been described as a critical factor that can influence growth and survival of juveniles (Gallardo et al. 2004a; Moran & Manahan 2004). For juvenile intertidal organisms, however, vulnerability also includes other physical factors that do not occur in most terrestrial and fresh water habitats (e.g. wave exposure, desiccation, temperature fluctuations), which often result in mortality and affect the distributional limits of the species (Gosselin & Chia 1995a, b; Helmuth et al. 2002).

Several studies on invertebrates with larval stages have shown that larger sizes are often correlated with better performance in terms of feeding rate, duration of planktonic period, settlement rate, growth rate and survival (Phillips 2002; Marshall et al. 2003; Marshall & Keough 2004; Marshall & Keough 2009). By contrast, in direct developers which lack a planktonic larval stage, maternal provisioning to the embryos at the time of spawning is the primary source of nutrition until offspring enter juvenile life (Spight 1976a; Rivest 1983). This characteristic makes direct developing invertebrates an interesting model system to evaluate how maternal environments and initial maternal-derived resources might affect offspring traits (e.g. size, growth rate, overall survival), and to determine the scales of variation at which these maternal effects are evident and comparable within or among populations. To date, few studies have examined this system; however, they have found consistent relationships between: hatchling size and performance (e.g. larger hatchlings showing better growth and survival than small conspecifics; Moran & Emlet 2001), hatchling size and maternal habitat (e.g. smaller hatchling sizes from more protected habitats; Lloyd & Gosselin 2007), and maternal resources and offspring performance (e.g. higher growth from juveniles obtained from mothers in better food conditions; van der Sman et al. 2009).

Since maternal food resources can ultimately limit the energy available for allocation, variation in food availability can be an important factor influencing the amount of energy available for reproduction (Du 2006). One coastal region where resources vary between two neighbouring locations is at the southern end of the North Island of New Zealand, first described by Morton & Miller (1968), i.e. the semi-enclosed and nutrient-rich Wellington Harbor vs. the exposed and low productivity Wellington south coast. Compared to the nearby south coast, Wellington Harbour has denser and more species rich intertidal communities with greater recruitment rates of fish and invertebrates (Phillips & Hutchison 2008; Shima & Swearer 2009; Demello &

Phillips 2011), higher standing stocks of phytoplankton and concentration of particulates (Gardner 2000; Helson et al. 2007), higher quality and faster growing larval reef fish (Shima & Swearer 2009, 2010; Swearer & Shima 2010) and higher growth rates for several species of whelks (NE Phillips, unpubl. data).

Here, I used sympatric direct-developing whelks with different reproductive strategies *Cominella virgata* (single large hatchling per capsule) and *C. maculosa* (multiple small hatchlings per capsule) as models to examine how maternal environments influence the size and performance of juveniles. There were two major aims: (1) to examine variation in hatchling size within and among different *C. virgata* and *C. maculosa* populations, and (2) to determine whether initial hatchling size mediates the performance of juveniles when exposed to different stressors (i.e. desiccation and starvation). I hypothesized that: first, hatchlings from different maternal environments will perform differently, i.e. hatchlings from productive maternal environments (i.e. Wellington Harbour) will perform better compared with hatchlings from less productive maternal conditions (i.e. the south coast), and second, the performance of hatchlings from the same maternal environment will be a function of size, with large individuals performing better than small conspecifics.

5.3 Materials and methods

5.3.1 Study species and collections

Cominella virgata and *C. maculosa* (Neogastropoda: Buccinidae) are common scavenging whelks found in pools, under stones, or in moist depressions in the low intertidal zone down to the sublittoral fringe, and subtidally, on rocky shores throughout New Zealand (Morton & Miller 1968). Females of both species attach morphologically different egg capsules to the rock's surfaces. *Cominella virgata* encapsulates a single embryo per capsule (~3 mm hatching size); while *C. maculosa* deposits ~8 embryos inside each capsule (~1.5 mm hatching size) but all usually develop. Intra-capsular nutrition (e.g. nurse eggs) is absent in both species, and embryos rely entirely on the initial maternal provisioning at the time of spawning. The egg laying period begins in mid-September, and the development period lasts ~90 days (van der Sman 2007).

In order to evaluate hatchling size and performance of *C. virgata* and *C. maculosa* in response to contrasting maternal environments, groups of egg capsules (> 20 capsules per group), in which hatching was imminent (i.e. fully developed snails were clearly visible inside the capsule), were collected from four sites around the Wellington region of New Zealand (41° 17' S, 174° 50' E). Two sites within Wellington Harbour: Point Howard (PH) and Point Halswell (PHS), and two sites on the Wellington south coast: Moa Point (MP) and Te Raekaihau Point (TR). At each of the four field sites twenty groups of egg capsules of *C. virgata* and twelve of *C. maculosa* were collected.

All groups of egg capsules were collected during low tides in December 2008 (*C. virgata*) and December 2009 (*C. maculosa*) from intertidal pools, in crevices or on the undersides of boulders. In order to ensure that each of them represented the reproductive output of at least one female, egg capsules were collected from different rocks within each sampling site. For both species, groups of egg capsules were carefully detached from the rocks using a scalpel and then transported in seawater to the laboratory, where each of them was placed in a 500-ml jar fitted with mesh sides (500-µm mesh) that allowed for water flow. Jars were half submerged in a large shallow tank supplied with constantly flowing fresh seawater at ambient temperature until hatching (16.4 ± 0.5°C in 2008 and 15.7 ± 0.7°C in 2009). Experiments were conducted at National Institute of Water and Atmospheric Research (NIWA) and at Victoria University Coastal Ecology Laboratory (VUCEL).

5.3.2 Hatchling size

To quantify variation in hatchling size among the different *C. virgata* and *C. maculosa* populations, 20 haphazardly selected hatchlings from a subset of five of the groups of egg capsules collected per site (n = 100 hatchlings per site; except *C. maculosa* from TR, n = 99) were measured from the apex of the shell to the end of siphonal notch (shell length [SL], mm) using a dissecting microscope equipped with an ocular micrometer at 20 X and 40 X magnification, respectively. Differences in hatchling size evaluated in this study were: between locations (Harbour and South Coast) and among sites (PH, PHS, MP, TR). Due to difficulties in accurately identifying individual clutches in the field, this source of variability was not included in the model. Nested mixed-model ANOVAs were used for each species to examine whether hatchling size varied by

location (fixed factor) with sites nested within locations (random factor). When the mixed models failed to find significant differences between locations, but substantially variation among sites, differences among sites were subsequently examined with one-way ANOVAs.

5.3.3 Size-dependent performance as a function of the maternal environment and experimental conditions

The performance of different-sized juveniles was evaluated with manipulative experiments under two separate conditions: desiccation and starvation (*C. virgata*: December 2008 to February 2009 and *C. maculosa*: December 2009 to February 2010). Hatchlings (48 h after hatch) from 4 sites (PH, PHS, MP and TR) were placed in 500-ml jars fitted with 500-µm mesh sides. The jars were half submerged in a large shallow tank supplied with constantly flowing fresh seawater at ambient temperature. For each site and species, snails above the mean length (e.g. average size of 100 individuals) were considered large (L) and below the mean were considered small (S). Within each size class, hatchlings were haphazardly assigned to each container.

For both species, the desiccation experiments were conducted using 200 hatchlings per site: 100 in control conditions (i.e. always submerged in seawater) and 100 in a desiccation treatment. For each treatment, 5 replicate containers of each size class (L and S) were used with 10 individual hatchlings in each. In this region adults of these species probably experience tidal emersion 3-5 times a week, for 1 to 3 hours per low tide, although duration and amplitude of low tides varies considerably depending on tidal cycle, wind swell and other factors. Further, juveniles of these species are almost exclusively found at the sublittoral fringe, and in moist, shaded habitats such as in crevices and under boulders (NE Phillips, pers. obs), and thus likely are more limited in duration of aerial exposure at low tide compared with adults. Therefore, the desiccation treatment for hatchlings was consistently simulated for 1.5 hrs during mid-day 3 times per week by removing containers from the seawater and placing them in a dry tray. Food was provided once a week using the adductor muscle ($1cm^2$) of the mussel *Perna canaliculus* (after van der Sman et al. 2009). In the starvation treatment, for both species (i.e. second experiment; no food provided), 60 hatchlings per site were used; with 30 in each size class (L and S) divided into 3 replicate containers with 10 snails in each. Given the limited number of hatchlings available for this experiment, the performance

of large and small food-deprived individuals from each site was contrasted with those in control conditions from the first experiment (i.e. fed and submerged in water).

In both experiments (e.g. desiccation and starvation), shell lengths (SL, mm) were measured weekly using a dissecting microscope at 20 X and 40 X magnification (for *C. virgata* and *C. maculosa*, respectively), allowing us to estimate the daily growth rate (GR, mm d^{-1}). Due to the logistics of handling and tagging so many individuals, I was unable to follow individual hatchlings during the experiments, therefore growth rates were calculated based on a per jar average (after van der Sman et al. 2009).

To compare the variation in GR among sites, treatments, size classes and their interactions, either a three way ANOVA (e.g. desiccation experiment) or a two way ANOVA (e.g. starvation experiment) were used, followed by a Tukey's HSD post-hoc test when appropriate. Homogeneity of variance was tested using the Cochran's C test, and data did not require transformation to meet ANOVA assumptions.

In both experiments, hatchling survival was evaluated weekly, and dead individuals removed from the jars, until survival in any of the replicate jars decreased to 2 individuals. To determine if survival under experimental conditions was significantly different to hatchlings under control conditions, a generalized linear model was used (GLM; Crawley 2007) because the response variable was based upon counts (e.g. number of hatchlings) which had unequal variances and non-normally distributed errors. Two separate analyses were conducted, one consisting of orthogonal contrast between survival of large and small individuals from each site against the hatchlings in control conditions, and another analysis comparing survival of hatchlings under desiccation and food-deprived conditions from the four study sites against hatchlings in control conditions. For these GLMs a binomial error distribution and a logit link function (Crawley 2007) was specified. Data were not overdispersed (i.e. desiccation experiment: *C. virgata*, residual deviance = 29.9 over 555 df and *C. maculosa*, residual deviance = 135.4 over 288 df; starvation experiment: *C. virgata*: residual deviance = 23.8 over 112 df and *C. maculosa*: residual deviance = 29.9 over 43 df). Statistical analyses were performed using R 2.12 and JMP 9 software packages.

5.4 Results

5.4.1 Hatchling size

Consistent with other reports, *C. virgata* hatchlings (mean SL: 2.67 ± 0.03 mm [± SE]), were almost twice as large as *C. maculosa* hatchlings (mean SL: 1.51 ± 0.02 mm [± SE]; Fig. 5.1).

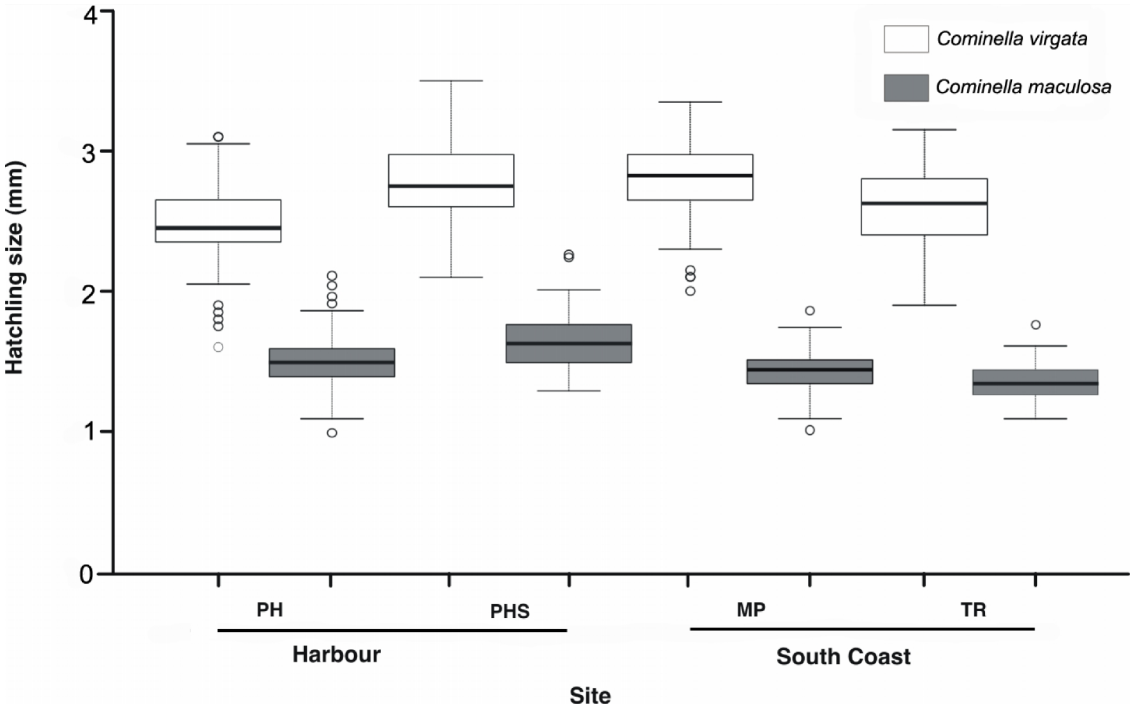


Fig. 5.1 Variation in hatchling size of *C. virgata* and *C. maculosa* from two locations in the Wellington region, New Zealand. Site abbreviations are: PH: Point Howard, PHS: Point Halswell, MP: Moa Point and TR: Te Raekaihau Point. The central line in each box represents the median, and the upper and lower edges of the boxes the 75th and 25th percentiles, respectively. Horizontal lines represent the range without outliers and the circles indicate the outliers.

Cominella virgata hatchling size did not differ between the two locations ($F_{1, 2}$ = 0.140, p = 0.744), with hatchlings from the South Coast and Harbour exhibiting similar SL [e.g. 2.70 ± 0.05 vs. 2.63 ± 0.05 (mean ± SE), respectively]. The mixed-model ANOVA with sites as random factor further revealed that a large proportion of the variance in hatchling size (32 %) occurred among sites, with the residual 68 % variance in hatchling size occurring within or among groups of egg capsules (Fig. 5.1).

Hatchling size of *C. maculosa* also showed no differences between the two locations ($F_{1, 2}$ = 5.564, p = 0.142), with similar SL obtained from the Harbour and South Coast (e.g. 1.60 ± 0.2 vs. 1.42 ± 0.14 [mean ± SE], respectively). Similar to *C. virgata*, 16 % of the variance in hatchling size occurred among sites, with the residual variance (84 %) occurring within or among groups of egg capsules (Fig. 5.1).

Subsequent one-way ANOVA revealed that hatchling size significantly varied among sites for both species (*C. virgata:* $F_{3, 396}$ = 34.419, p < 0.0001; *C. maculosa*: $F_{3, 396}$ = 51.892, p < 0.0001). For *C. virgata*, hatchlings from the south coast site MP and the Harbour site PHS were significantly larger than those from TR, and hatchlings from the Harbour site PH were smallest (post-hoc Tukey tests, p < 0.05). For *C. maculosa*, hatchling size was significantly different among all sites with PHS>PH>MP>TR.

Since for both species the variability in hatchling size was not significant between locations, but did vary by sites, only those differences were further evaluated in the manipulative experiments. Locations (i.e. Harbour and South Coast) were removed from the models in the following performance analyses.

5.4.2 Size-dependent performance of juveniles in desiccation conditions

Cominella virgata hatchlings grew in control and desiccation treatments over the eight weeks of the experiment, from an initial mean size of 2.62 ± 0.02 mm SL to a final mean size of 4.3 ± 0.07 mm SL (± SE). Growth rates (GR, mm d^{-1}) did not significantly differ between treatments (e.g. control vs. desiccation: $F_{1, 544}$ = 0.11, p = 0.738); however, there was a significant effect of initial size class ($F_{1, 544}$ = 10.87, p = 0.001), with large hatchlings growing at a faster rate compared with small conspecifics (Fig. 5.2a, b). The four sites also showed significant differences in GR ($F_{3, 544}$ = 4.13, p = 0.006); hatchlings from PH grew faster than those from PHS and TR (post-hoc Tukey

tests, p = 0.047 and p = 0.004, respectively; Fig. 5.2a, b). There were no significant interactive effects between the factors (p > 0.05 for all interactions).

Cominella maculosa hatchlings in control and desiccation treatments also grew over the five weeks of experiments, increasing from an initial mean size of 1.52 ± 0.06 mm SL to a final size of 1.81 ± 0.11 mm SL (± SE). Growth rates were not affected by treatment (i.e. control and desiccation: $F_{1, 248} = 0.11$, p = 0.74,) or site ($F_{3, 248} = 1.78$, p = 0.15; Fig. 5.2c, d); however, the two size classes showed significant differences in GR ($F_{1, 248} = 6.57$, p = 0.01), with large hatchlings growing faster than small ones (Fig. 5.2c, d).

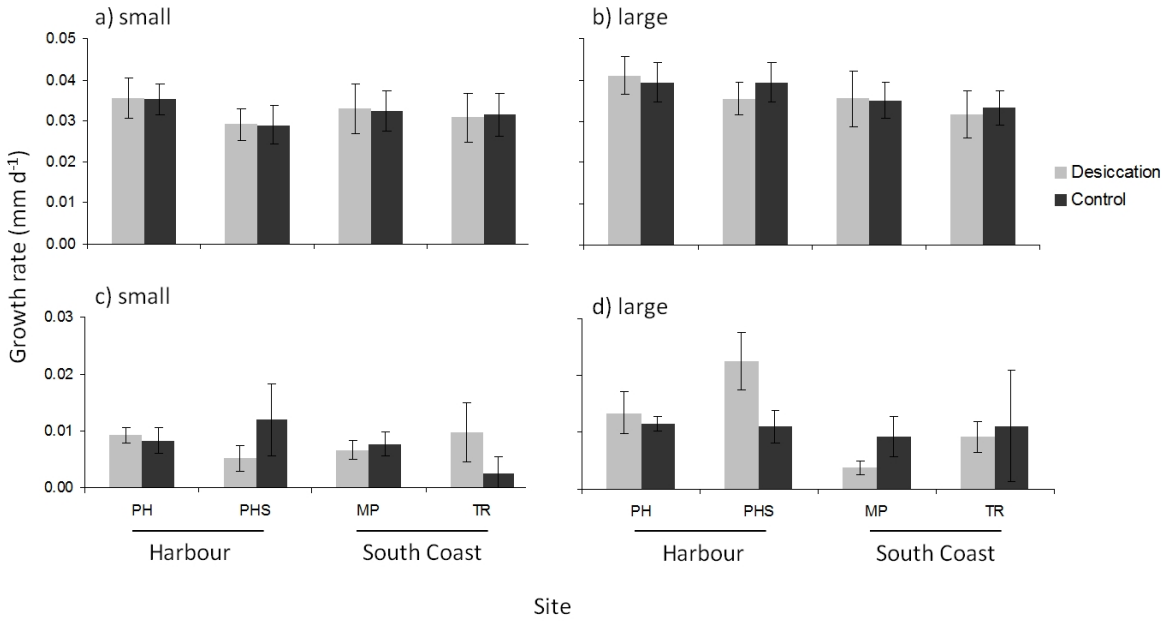


Fig. 5.2 Growth rates (±SE) of fed hatchlings of: (a, b) *C. virgata* and (c, d) *C. maculosa*, from four sites around the Wellington region, New Zealand (see Fig. 5.1. for site abbreviations). Light-grey bars represent the desiccation treatment and dark-grey bars the control conditions.

Hatchling survival (%) of *C. virgata* decreased through the 8 wk experimental period to a final value of 61 ± 3.4 % (mean ± SE). The desiccation treatment was not significant ($z = -0.188$, $p = 0.85$), and neither was hatchling size class ($z = 0.54$, $p = 0.59$). For each site, survival of large individuals exposed to the desiccation treatment did not differ significantly from those in the control conditions (PH: $z = -0.35$, $p = 0.71$; PHS: $z = -0.44$, $p = 0.66$; MP: $z = -0.47$, $p = 0.63$; TR: $z = -0.98$, $p = 0.31$; Fig. 5.3a). Similarly, survival of small individuals from each site did not differ between desiccation treatment and control conditions either (PH: $z = -1.45$, $p = 0.13$; PHS: $z = -0.35$, $p = 0.71$; MP: $z = -0.87$, $p = 0.38$; TR: $z = -0.75$, $p = 0.45$; Fig. 5.3b). Survival of *C. virgata* from different sites was similar. Relative to MP, hatchling survival was similar among the other three study sites: TR ($z = -0.14$, $p = 0.89$), PHS ($z = -0.27$, $p = 0.78$), and PH ($z = 0.16$, $p = 0.87$). There were no significant interactive effects among the factors included in the analysis ($p > 0.05$ for all interactions; Fig. 5.3a, b).

Hatchling survival (%) of *C. maculosa* also decreased through the experimental period (5 wk) to a final value of 8.5 ± 1.5 % (mean ± SE). For each site, survival of large individuals did not differ by control or desiccation treatment (PH: $z = -1.18$, $p = 0.235$; PHS: $z = 1.37$, $p = 0.19$; MP: $z = -0.86$, $p = 0.38$; TR: $z = -0.31$, $p = 0.75$; Fig. 5.4a). Similarly, survival of small *C. maculosa* did not differ between treatments (PH: $z = 0.31$, $p = 0.75$; PHS: $z = 0.01$, $p = 1$; MP: $z = -0.01$, $p = 1$; TR: $z = -0.55$, $p = 0.57$; Fig. 5.4b). However, significant differences were detected between size classes ($z = 2.98$, $p = 0.0029$), where large hatchlings had higher survival compared with small ones (Fig. 5.4a, b). Hatchling survival from different sites was also significantly different. Relative to MP, survival of *C. maculosa* was lower from TR ($z = 2.97$, $p < 0.003$) and PHS ($z = 2.66$, $p = 0.008$), but was similar to PH ($z = -0.036$, $p = 0.97$). There were no significant interactive effects among the factors included in the analysis ($p > 0.05$ for all interactions; Fig. 5.4a, b).

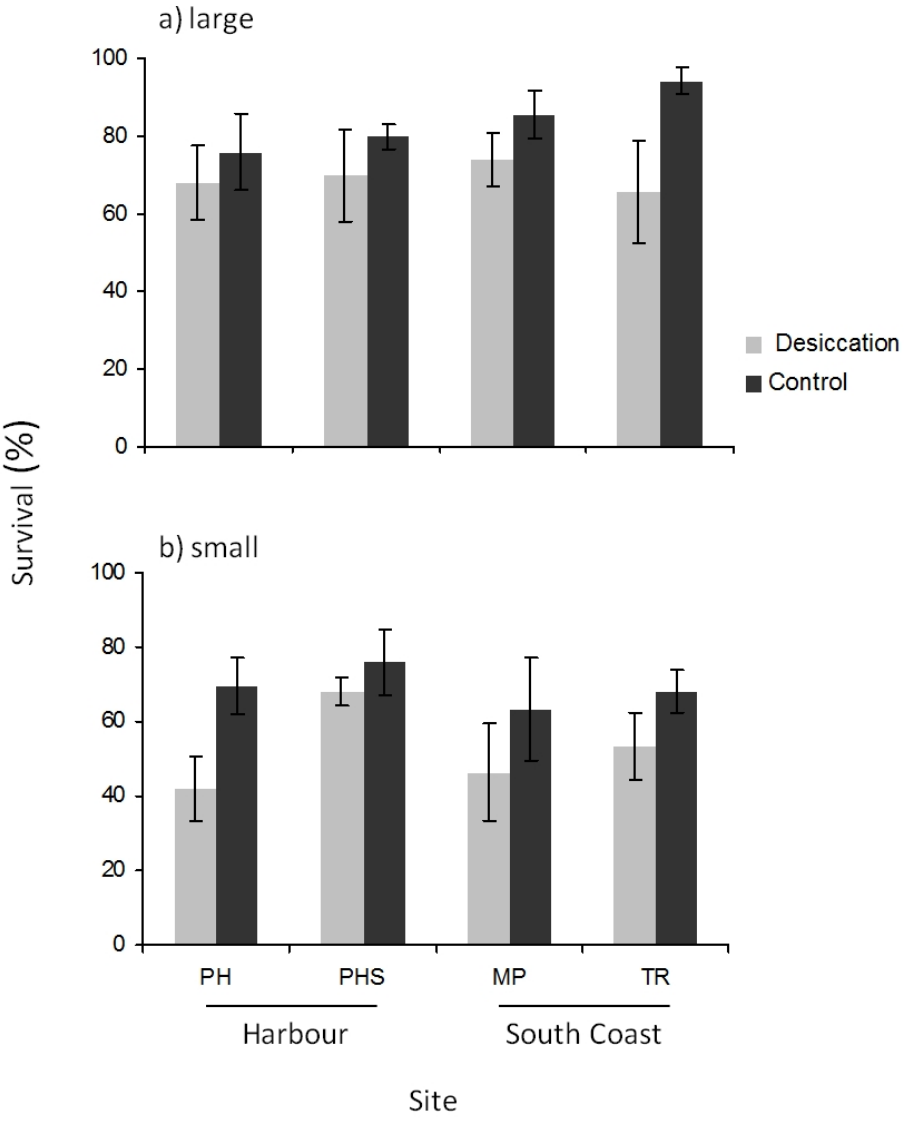


Fig. 5.3 Survival (±SE) of fed hatchlings of *C. virgata* from two initial size classes: (a) large and (b) small, and four sites around the Wellington region, New Zealand (see Fig. 5.1. for site abbreviations). Light-grey bars represent the desiccation treatment and dark-grey bars the control conditions.

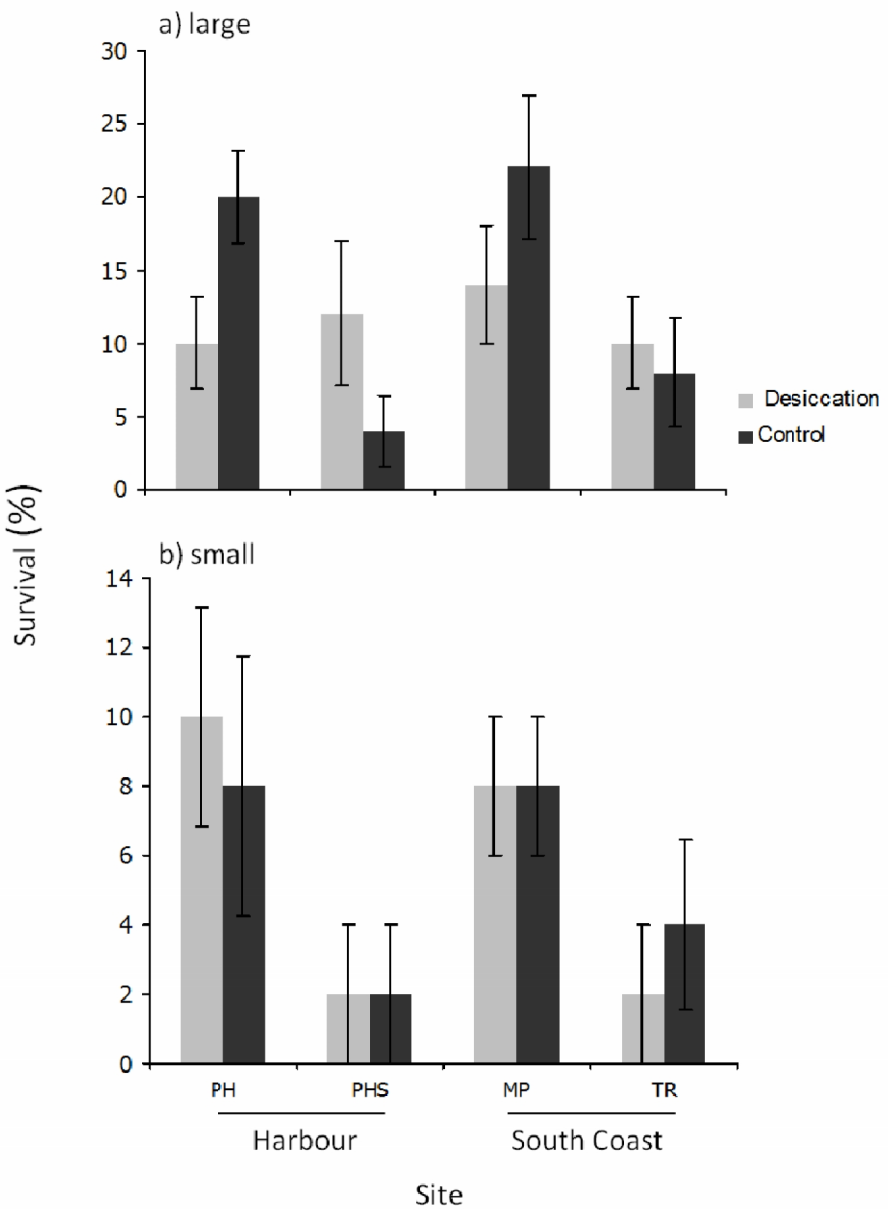


Fig. 5.4 Survival (±SE) of fed hatchlings of *C. maculosa* from two initial size classes (a) large and (b) small, and four sites four sites around the Wellington region, New Zealand (see Fig. 5.1. for site abbreviations). Light-grey bars represent the desiccation treatment and dark-grey bars the control conditions.

5.4.3 Size-dependent performance of juveniles in starvation conditions

In food-deprived conditions, *C. virgata* hatchlings increased from 2.60 ± 0.05 mm SL to 2.91 ± 0.05 mm SL (mean ± SE) over the six weeks of the experiment, which contrasted with the size attained by snails with a constant supply of food over this same time period (3.6 ± 0.06; 6th week; experiment 1). The two size classes showed significant differences in GR ($F_{1,\ 112}$ = 4.93, p = 0.028), but in contrast to the desiccation experiment, small hatchlings grew faster compared with large conspecifics (Fig. 5.5a). Once again, there were significant differences among sites in GR ($F_{3,\ 112}$ = 3.84, p = 0.011), with higher GR from MP compared with PHS and TR (post hoc Tukey tests, p = 0.015 and p = 0.003, respectively). No interactive effects were observed between site and size (p > 0.05; Fig. 5.5a).

Cominella maculosa hatchlings held in food-deprived conditions increased from an initial size of 1.52 ± 0.06 mm SL to a final size of 1.61 ± 0.06 mm SL (mean ± SE) during the 3 weeks of the experiment. This was similar to the size attained by peers in a constant supply of food over this same period (1.65 ± 0.06 mm [mean ± SE]; 3rd week; experiment 1). There were significant interactive effects between sites and hatchling size class ($F_{3,\ 80}$ = 3.45, p = 0.02), with large hatchlings from MP growing at a lower rate compared with large individuals from TR and small ones from PH, PHS and MP (post hoc Tukey tests, p < 0.018 in all cases; Fig. 5.5b).

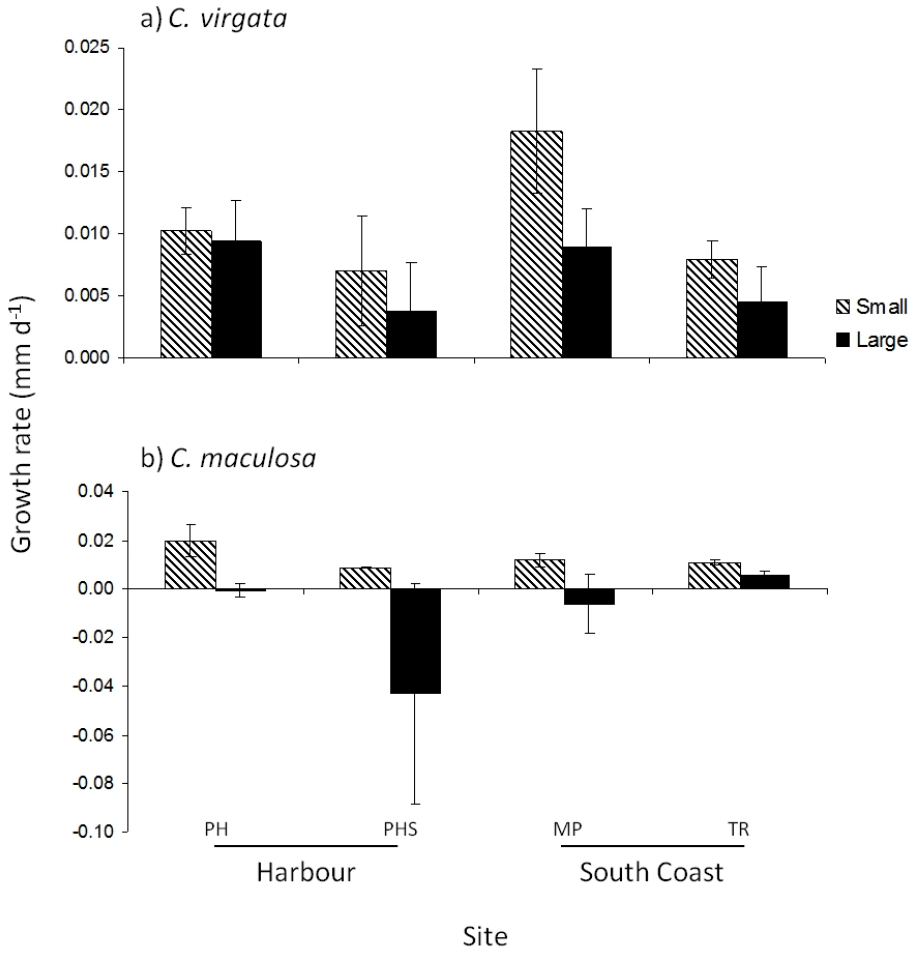


Fig. 5.5 Growth rates (±SE) of food-deprived hatchlings: (a, b) *C. virgata* and (c, d) *C. maculosa*, from four sites around the Wellington region, New Zealand (see Fig. 5.1. for site abbreviations). Light-grey bars represent the desiccation treatment and dark-grey bars the control conditions.

Survival of food-deprived *C. virgata* hatchlings decreased 45 % compared to fed hatchlings over the same 6 wks period. Relative to control conditions, the survival of large and small *C. virgata* from one of the sites (PHS) under food-deprived conditions was not different (small: $z = 0.87$, $p = 0.38$; large: $z = -1.35$, $p = 0.174$). However, the survival of large individuals under food-deprived conditions was significantly reduced compared to hatchlings in control conditions for the other three sites (PH: $z = 2.37$, $p = 0.017$; MP: $z = 2.04$, $p = 0.04$; TR: $z = 2.61$, $p = 0.009$; Fig. 5.6a). Similarly, survival of small *C. virgata* under food-deprived conditions was significantly lower relative to control conditions at these sites (PH: $z = 4.76$, $p = 0.006$; MP: $z = 1.95$, $p = 0.05$; TR: $z = 2.57$, $p = 0.01$; Fig. 5.6b). The survival of food-deprived individuals did not change among sites or between large and small individuals ($z = - 0.045$, $p = 0.96$). No interactive effects were observed between the factors ($p > 0.05$; Fig. 5.6a, b).

By contrast, survival of *C. maculosa* hatchlings in food-deprived conditions was 26 % higher when compared to snails in control conditions after the first three week period. Relative to food-deprived conditions, survival was significantly different only for hatchlings from TR, where it was lower in control conditions for both large and small size classes (large: $z = -2.33$, $p = 0.01$; small: $z = -2.05$, $p = 0.04$; Fig. 5.7a, b). There were no differences in survival between small and large size classes under food-deprived conditions ($z = 0.83$, $p = 0.40$). Relative to MP, the survival of juveniles did not vary among the other three study sites: TR ($z = 0.33$, $p = 0.74$), PHS ($z = 1.53$, $p = 0.13$) and PH ($z = 1.59$, $p = 0.11$). No interactive effects were observed between the factors ($p > 0.05$; Fig. 5.7a, b).

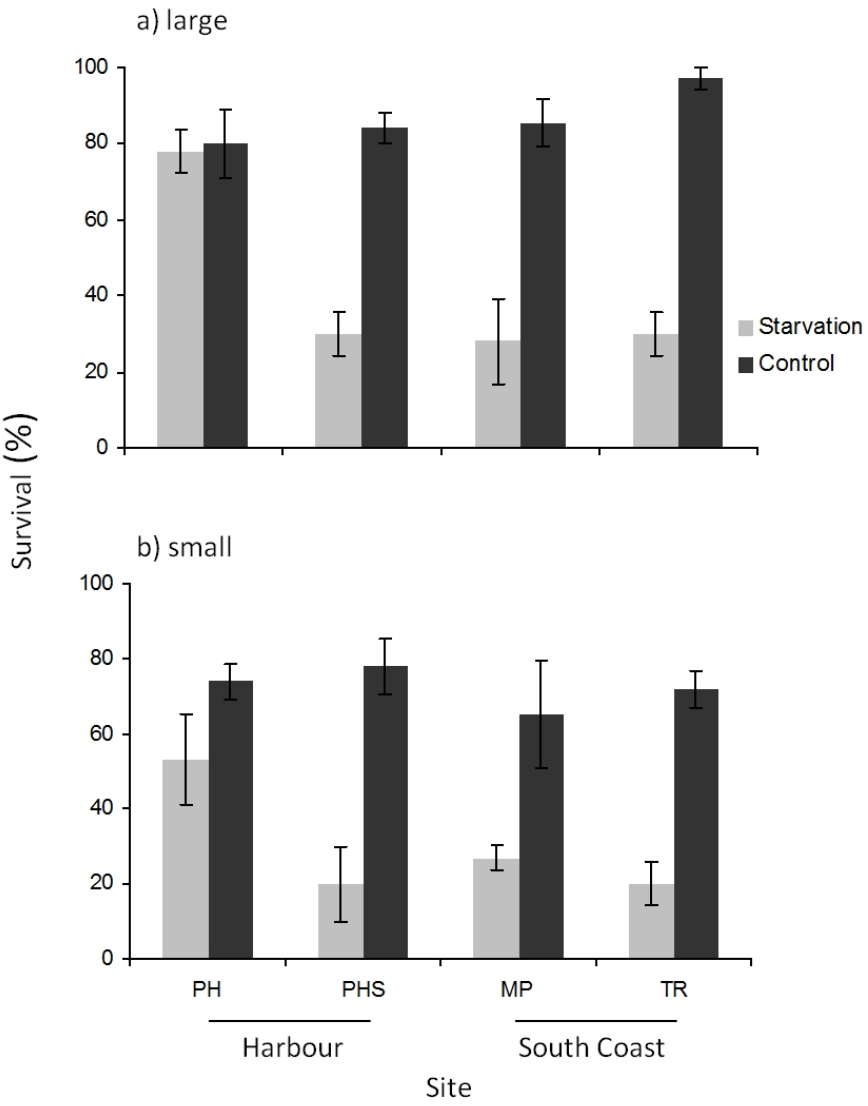


Fig. 5.6 Survival (±SE) of food-deprived hatchlings of *C. virgata* from two initial size classes: (a) large and (b) small, and four sites around the Wellington region, New Zealand (see Fig 5.1. for site abbreviations). Light-grey bars represent the starvation treatment and dark-grey bars the control conditions.

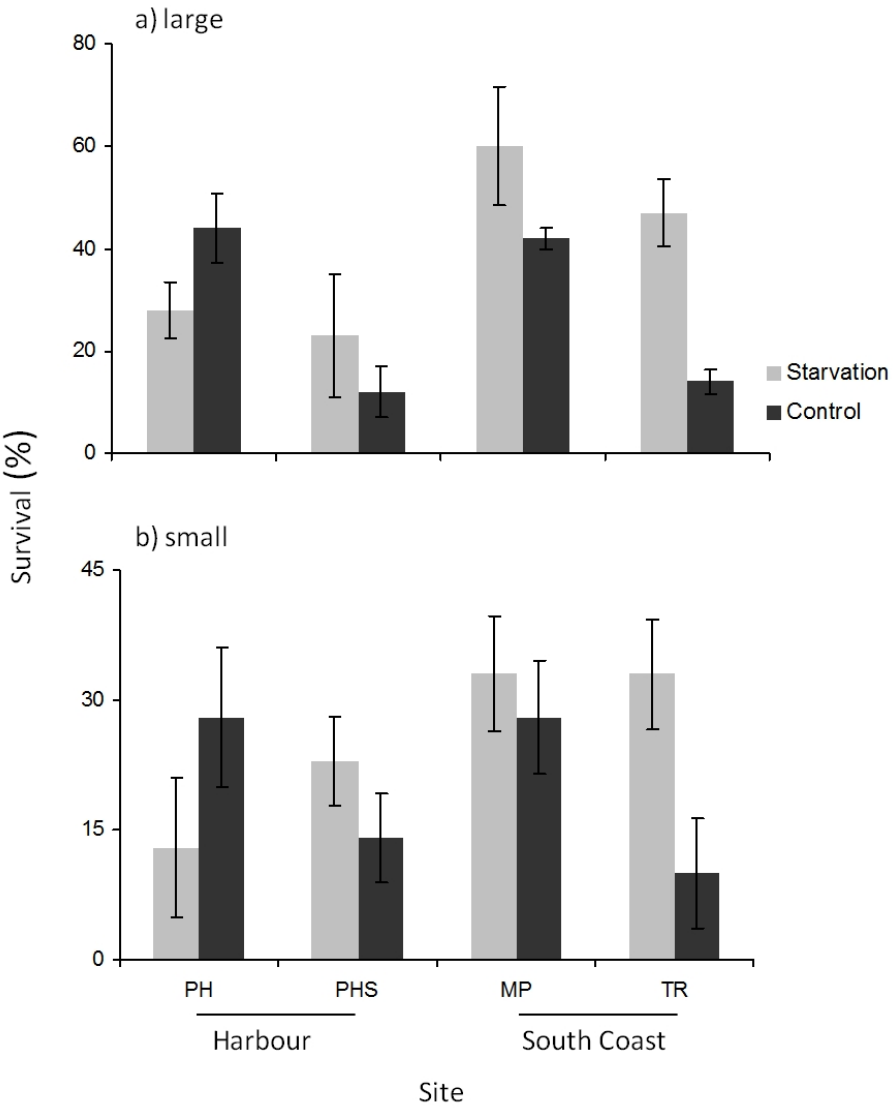


Fig. 5.7 Survival (±SE) of food-deprived hatchlings of *C. maculosa* from two initial size classes: (a) large and (b) small, and four sites around the Wellington region, New Zealand (see Fig. 5.1. for site abbreviations). Light-grey bars represent the starvation treatment and dark-grey bars the control conditions.

5.5 Discussion

5.5.1 Hatchling size

In both species of whelks hatchling size was variable, and the most important source of variation was at the smallest scale explicitly identified, among sites, with substantial variation also occurring within and among females. Contrary to the expectations, and despite the large differences in conditions and available resources between Harbour and South Coast, the two locations did not influence the hatchling size of either species. As in other taxonomic groups, offspring size variation can be dramatically affected by different maternal environments; nonetheless, this trait will also be dependent upon the level of observation. At a population level, offspring size can vary considerably within and among individual females (Bernardo 1996b). However, among populations, environmental factors appear to play a greater role in determining offspring size, and the overall quality of the habitat into which offspring emerge may act as a selective force on offspring size (see Johnston & Leggett 2002; Gosselin & Rehak 2007; Shima & Swearer 2009).

Studies spanning the last twelve years have demonstrated that dramatic differences can be observed between both locations examined, with the Wellington Harbour having the greater densities and recruitment rates of invertebrates and fish (Phillips & Hutchison 2008; Shima & Swearer 2009; Demello & Phillips 2011), higher stocks of phytoplankton and particulate concentration (Gardner 2000; Helson et al. 2007), higher quality and faster growing larval fish (Shima & Swearer 2009, 2010; Swearer & Shima 2010) and higher whelk growth rates (NE Phillips, unpubl. data), all suggesting a more productive environment in Wellington Harbour. Nonetheless, our study in hatchling whelks suggests that at least for these two direct-developing species, smaller scale site-to-site and individual female attributes are more important than these larger scale differences between locations in mediating offspring traits, contrary to what has been found in species with planktonic larvae in this system (Shima & Swearer 2009, 2010; Swearer & Shima 2010).

5.5.2 Size-dependent performance of juveniles in desiccation conditions

When the performance of *C. virgata* and *C. maculosa* hatchlings from different maternal habitats were compared in equivalent laboratory conditions (i.e. food *ad libitum*, control and desiccation treatments), variation in initial size of hatchlings among the four sites was not reflected in hatchling performance (e.g. growth rates, survival), suggesting that factors mediating hatchling size are not necessarily consistent with those acting on hatchling performance. Further, there was no effect of the desiccation treatment. It is possible that the level of stress caused by this treatment was relatively benign compared to desiccation conditions in the field, although observations suggest that hatchlings and juveniles are rarely found above the sublittoral fringe, or in habitats where they are likely to be endure considerable aerial exposure at low tide. Perhaps more than desiccation itself, air temperature is a more important stressor for hatchlings at low tide. For example, Moran & Emlet (2001) found that sun exposure affected hatchling survival in intertidal *Nucella ostrina*, and further that large hatchlings had higher survival in the shaded (i.e. less severe) environment than smaller hatchlings, with no size advantage in the sun-exposed environment.

By contrast, when sizes of hatchlings (i.e. large and small) were compared within each site, the expected response was observed, and in both species large snails performed better than small conspecifics in terms of growth rates and survival (the latter only for *C. maculosa*). Although few studies have examined the relationship between maternal environments, offspring size and offspring performance in marine snails, some of the few published data describe similar responses. *Nucella ostrina* hatchling size can be influenced by environmental factors such as exposure to wave action, resulting in smaller sizes from less wave-exposed sites (Gosselin & Rehak 2007); whereas Moran & Emlet (2001) reported a size-dependent relationship in survival and growth of juvenile *N. ostrina* outplanted in the field (~20 days), with large hatchlings showing higher overall recovery and growth rates than small siblings. Further, in a comparative study among prosobranch gastropods, Spight (1976a) suggested that large hatchlings will have higher survivorship than small ones because they can: tolerate better physical stress (e.g. dehydration), are susceptible to fewer predators, can travel further to find food or shelter, and have a larger food supply (e.g. larger prey can be taken), although maternal environments were not explicitly examined in that study.

Although the results for *C. virgata* and *C. maculosa* cannot be directly compared due to the experiments occurring in different years, it is interesting to point out that the larger *C. virgata* hatchlings performed better than the smaller *C. maculosa* hatchlings, with at least ~3-fold faster growth rates and ~7-fold higher survival. Similar levels of mortality in laboratory were also recorded by van der Sman (2007) for hatchlings of both species, suggesting that regardless the sampling years and maternal habitats, the small size of *C. maculosa* hatchlings could result in an increased vulnerability, especially during the first two weeks of the crawl-away dispersal period. This higher mortality however, could be traded-off with the higher fecundity of *C. maculosa*, which can encapsulate an average of 8-fold higher number of hatchling per capsule compared with the single embryo per capsule produced by *C. virgata*.

5.5.3 Size-dependent performance of juveniles in starvation conditions

Not unexpectedly, starved hatchlings of both species performed more poorly than fully-fed conspecifics, attaining smaller shell lengths, slower growth rates and lower survival (e.g. except for starved *C. maculosa* compared with fed peers from one site). Interestingly, different maternal habitats and variation in initial size for both species had no effect in the overall survival of starved hatchlings; however, intra-specific differences in growth rates seems to be highly influenced by those factors individually (e.g. *C. virgata*), or acting in positive interactions (e.g. *C. maculosa*).

Although inter-specific comparisons of hatchling traits cannot be directly evaluated, the larger species (*C. virgata*) had overall higher growth and survival when starved compared to the smaller *C. maculosa*, which could be a direct consequence of a higher maternal investment in larger individuals, especially in terms of the total lipid content that have been recorded in hatchlings of the former species (see Chapter 4). In this sense, it has long been recognized that neutral lipids, particularly triglycerides (TG), are an important energy source that fuels the early larval development in planktotrophic echinoids and the embryogenesis and metamorphosis in mollusks (reviewed by Sewell et al. 2005). The possibility of size-dependent allocation of energy reserves in hatchlings, and consequences for performance, is the subject of on-going studies.

As in the present experiments, other studies in whelks have also shown that food limitation during early life stages reduces growth rates and survival of individuals,

compared with fully-fed conspecifics [e.g. *Hemifusus tuba* (Morton1986), *Chorus giganteus* (Gallardo et al. 2004b) and *C. virgata* (van der Sman et al. 2009)]. It is interesting to note that although the starvation experiments finished after 6 wk and 3 wk (*C. virgata* and *C. maculosa*, respectively) due the low numbers of individuals in some of the replicate containers, the remaining juveniles were kept in the same laboratory conditions, and were able to survive unfed for 18 wk (*C. virgata*) and 8 wk (*C. maculosa*). These observations demonstrate the potential for hatchling whelks to withstand prolonged periods of time without food, although as is the case for many marine invertebrates, there is the possibility that these species may uptake dissolved organic matter (DOM) which can be particularly important for juveniles (Morton 1986).

Overall, examining performance of newly hatched direct-developing snails under different conditions allowed to understand the importance of considering different scales of variation when the size-performance relationship is evaluated. Because different scales may evaluate completely different contexts in the life-history of the species (Marshall & Uller 2007), maternal and offspring characteristics should ideally both be considered. Variation at the site level was important for both size and performance of hatchlings, particularly growth rates, however these responses were not necessarily consistent among sites, and sometimes hatchling size interacted with sites, resulting in complex patterns. Further, size may be important in mediating performance in response to some stressors, but not others, or to multiple stressors in different ways. Thus the causes and consequences of offspring size variation can be complex, even for direct-developing whelks which develop and hatch in the same environment as their mothers. Further studies should evaluate the mechanisms underlying the variable performance exhibited by juveniles and how it may be related to maternal history or environmental conditions.

Chapter 6

Differential vulnerability to predation in two sympatric whelks is mediated by juvenile traits[3]

6.1 Abstract

In many taxa, initial differences in offspring size play an important role in mediating subsequent performance; however, the consequences of interspecific variation in size for the performance of co-occurring taxa have been rarely examined. I used the whelks *Cominella virgata* and *C. maculosa*, which co-occur on rocky shores throughout their life cycles, to examine the vulnerability of early life-stages to native predators under controlled laboratory conditions. Among all the predators evaluated (the cushion sea star *Patiriella* spp., the olive rockfish *Acanthoclinus fuscus*, the oyster borer snail *Haustrum scobina*, the smooth shore crab *Cyclograpsus lavauxi*, and the pebble crab *Heterozius rotundifrons*), hatchlings of both species (*C. virgata*: ~3 mm shell length [SL] and *C. maculosa*: ~1.5 mm SL) were especially vulnerable to the smooth shore crab *C. lavauxi*, the only potential predator in which mortality was greater than in the control treatment. Small shore crabs (~8 mm carapace width [CW]) were unable to eat hatchlings of either whelk species, whereas medium and large shore crabs (~12 and ~18 mm CW, respectively) consumed hatchlings of both prey species. Hatchlings of *C. virgata* were less vulnerable to predation by medium crabs than large ones, and those of *C. maculosa* were equally vulnerable to both sizes of crabs. In hatchlings of both prey species, shell length and shell thickness increased over time. Two months after hatching, only individuals of *C. virgata* had reached a size refuge from predation. The results show that interspecific vulnerability to predators can be mitigated by larger sizes and thicker shells at hatching; nonetheless, results also suggest that other species-specific factors, such as juvenile growth rate, may also play key roles in determining the vulnerability of hatchling and juvenile snails to shell-crushing predators.

[3] Carrasco SA, Phillips NE (2012) Differential vulnerability to predation in two sympatric whelks is mediated by juvenile traits. Invertebrate Biology 131(3): 187-196

6.2 Introduction

A central observation underlying most studies of life-history is the considerable variability in offspring size observed in all multicellular organisms (Bernardo 1996b). Such variation is particularly evident in the vast range of eggs and offspring sizes exhibited across marine invertebrates at a variety of levels (e.g. among species, populations within species, females within populations, clutches within mothers and individuals within clutches). This variation can have strong effects on offspring performance (Marshall & Keough 2008a; Kamel et al. 2010b). Several studies across a variety of taxa and habitats have shown that larger offspring often perform better than smaller conspecifics in terms of growth rate, probability of survival, reproductive success, outcome of competition and vulnerability to predation [e.g. gastropods (Spight 1976a; Gosselin 1997; Moran & Emlet 2001; Chapter 5), mussels (Phillips 2002), barnacles (Thiyagarajan et al. 2003), ascidians (Marshall et al. 2003, 2006), beetles (Fox 2000), isopods (Tsai & Dai 2001), spiders (Walker et al. 2003)]; thus, differences in initial size are of major ecological and evolutionary importance (Marshall & Keough 2008a).

Offspring size has the potential to mediate population dynamics, as initial size *per se* may modify traits of adult individuals within a given population (reviewed by Marshall & Keough 2008b). For example, in the bryozoan *Watersipora subtorquata* Marshall & Keough (2008b) showed that larger offspring exhibited better survival and faster growth compared with smaller peers. Similarly, Spight (1976a) found that larger intertidal snail hatchlings often performed better than small conspecifics because they could better tolerate physical stress (e.g. desiccation), could travel further to find food or shelter, had a larger food supply (because larger prey could be taken), or were susceptible to fewer predators. Although data from a variety of studies demonstrates these sorts of size-mediated effects within species, comparisons among species with similar life-histories but contrasting levels of per-offspring maternal investment are less common. The early life stages of many taxa co-occur in benthic habitats, and are therefore subject to similar potential stressors or threats. If there are size-dependent or species-specific responses, those traits may influence the subsequent community structure of early recruits.

In terrestrial and aquatic environments, predation can be an important process in determining prey population size and overall community structure; however, due to its rapid and decisive nature, predation is notoriously difficult to study (Holmes &

McCormick 2010). For benthic marine invertebrates, it has been suggested that predation during early life stages is the main source of mortality, often exceeding 90% (Gosselin & Qian 1997). Decapod crustaceans are often identified as a major source of predation; their potentially large impact results from their generally high abundances, broad distributions (e.g. intertidal and subtidal habitats), high motility, ability to crush protective structures and their metabolic requirements to process large amounts of food (see Gosselin & Qian 1997).

Initial differences in offspring size may be one of the key drivers determining the probability of surviving encounters with predators; such as decapod crustaceans; therefore, these differences may play an important role in determining which individuals will be able to reach larger sizes or develop more resistant protective structures (e.g. shells, carapaces or tegument) (Spight 1976a; Gosselin & Qian 1997). Because vulnerability generally scales inversely with body size, rapid growth during the early juvenile period is also considered an important strategy to reduce the likelihood of mortality by minimizing the time spent in the smallest and most vulnerable size classes (Spight 1976a; Palmer 1990; Gosselin & Chia 1995a; Gosselin 1997; Gosselin & Rehak 2007).

From a prey organism's perspective, larger size at any given life-history stage should generally result in a survival advantage through lower predation, increased competitive ability and reduced susceptibility to starvation (i.e. "bigger-is-better" hypothesis; see Marshall & Keough 2008a). Some recent studies have identified exceptions to this pattern, however, showing for example that in some cases, small marine invertebrate larvae are less vulnerable to visual predators than are large larvae (Allen 2008; Vaughn 2010). From the perspective of a predator, a variety of traits (e.g. morphological attributes, nutritional composition, life stage and sex) in addition to size may mediate prey selection to maximize rate of energy intake (Lawton & Hughes 1985; Jensen et al. 2012). The characteristics of the targeted prey are contingent on the selective preferences of each predator, and so to fully understand the influence of body size on the outcome of predatory encounters, the relative size of predators and prey need to be considered (Lawton & Hughes 1985; Holmes & McCormick 2010).

In this study, I evaluated vulnerability of young post-hatching stages to potential predators in two congeneric marine snails that co-occur in rocky shore habitats, but have different hatching sizes: the red-mouthed whelk *Cominella virgata* (~3 mm shell length [SL] at hatching) and the spotted whelk *C. maculosa* (~1.5 mm SL at hatching). The

specific goals of this study were to (1) identify some of the possible predators of hatchlings and early juveniles of these two whelk species, (2) evaluate potential differences between these two species in susceptibility to predators, (3) determine how predator size affects vulnerability to predation of these two species, and (4) determine if juveniles experience a shift in susceptibility to predators thorough ontogeny. I hypothesized that initial differences in hatchling size would mediate vulnerability to predation, with smaller hatchlings of *C. maculosa* exhibiting higher mortalities when exposed to predators than would the larger *C. virgata*; larger predators would consume higher numbers of juveniles of both species than would smaller predators; and finally, that the vulnerability of juvenile whelks to predators would decrease as they reached an invulnerable size through growth, with the species with larger hatchlings (*C. virgata*) becoming less vulnerable to predation earlier than the species with smaller hatchlings (*C. maculosa*).

6.3 Materials and methods

6.3.1 Study species and collections

Cominella virgata and *C. maculosa* are common scavenging whelks found on the rocky intertidal shores of the North Island of New Zealand and the northern part of the South Island (Morton & Miller 1968). Females of both species engage in communal egg-laying and attach morphologically distinguishable egg capsules to rocky substrata underneath boulders or in crevices. Females of *Cominella virgata* encapsulate a single embryo (hatchling size ~3 mm) in each cuboid, flat-topped capsule, whereas females of *C. maculosa* encapsulate ~8 embryos in each ellipsoid capsule (hatchling size ~1.5 mm). Nurse eggs are absent, and development to hatching takes ~90 d, over the period from mid-October to mid-December (van der Sman 2007).

Groups of egg capsules of *C. virgata* and *C. maculosa* in which hatching was imminent (i.e. fully developed snails were clearly visible inside the capsule) were collected during December 2010 from Point Halswell (41°17’S, 174°49’E), a semi-exposed rocky shore site in Wellington Harbour, New Zealand. Capsules (either attached to small boulders, or in some cases carefully detached from the substrate with a

scalpel) were collected and transported in seawater to the Victoria University Coastal Ecology Laboratory. Boulders with attached capsules were placed directly in a large sea table, and dettached capsules were placed in 500-ml jars fitted with 500-µm mesh sides in a sea table with constant water flow at ambient temperature (16.2 ± 1.13°C) until hatching.

6.3.2 Whelk vulnerability to potential predators

I identified co-occurring potential predators of whelk hatchlings and juveniles and estimated their abundance along a 30 m transect line placed parallel to the coastline in the mid-intertidal zone at the study site. Sampling took place within 0.25 m^{-2} quadrats (n = 15) placed at 2 m intervals along the 30 m transect. Rocks were overturned to search for predators.

Predators were brought to the laboratory, and their hunger levels standardized by starvation for 72 h prior to feeding trials. Five species were selected as potential predators because of their high abundance at the study site: the cushion sea star *Patiriella* spp., the olive rockfish *Acanthoclinus fuscus*, the oyster borer snail *Haustrum scobina*, the smooth shore crab *Cyclograpsus lavauxi* and the pebble crab *Heterozius rotundifrons*. As the latter species also shows strong sexual dimorphism, with an enlarged right claw in males, members of both sexes were tested separately. Males and females of the crab *C. lavauxi* were also sampled in the field; however, only males were used in laboratory experiments, as there were no evident differences in claw morphology between sexes, and because we wanted to avoid disturbing females, many of which were brooding. Although other species of potential predators were also present at this intertidal site (e.g. hermit crabs and other muricid snails), they were not used in experiments because of their low abundance.

Predators were measured to the nearest 0.1 mm using calipers. Sizes of sea stars were measured from the tip of the longest arm to the opposite armpit (total length, TL). Fish size was determined by measuring the distance from snout to caudal fork (standard fork length, SF). Snails were measured from the apex to the shell to the anterior tip of the aperture (shell length, SL). Crabs size was measured at the widest part of the carapace (carapace width, CW).

In the laboratory, each potential predator was tested for predatory behavior. Five individuals of each predator, each held in a separate cage, were each offered ten individuals of *C. virgata*, and five individual predators were each offered ten individuals of *C. maculosa*. Hatchling sizes for each whelk were on average (mean ± SE) 1.74 ± 0.02 mm SL for *C. maculosa*, and 3.07 ± 0.05 mm SLfor *C. virgata* (n = 50 hatchlings in each case). Each predator was placed in a plastic cage (9.5 x 9.5 x 7.5 cm) fitted with 500-µm mesh sides to allow water exchange. The cages provided sufficient space for predators and prey to move about, and were half submerged in a large shallow tank supplied with constantly flowing fresh seawater at ambient temperature (16.5 ± 0.71°C). To each container, ten hachling whelks of the appropriate species were added. Predators and prey were held in the cages for 24 h, after which time were scored as dead or alive; mortality was expressed as percentage of hatchlings that died in 24h. Differences in consumption among predators and hatchling species (i.e. fixed factors) and their corresponding interactions were analyzed using a two-way ANOVA. When appropriate, significant differences were further examined using Tukey's HSD multiple comparison test. Assumptions of normality and homocedasticity were tested with Kolmogorov-Smirnov and Levene tests, respectively. To improve normality, percentage data were arc-sin transformed (Underwood 1997).

6.3.3 Effects of predator size and prey species on predation

To evaluate whether predation on *C. maculosa* and *C. virgata* hatchlings was mediated by predator size, I measured predation on new (. < 1 day old) hatchlings of both species when exposed for 24 h to predators. Of the predators I examined, *C. lavauxi* preyed the most heavily on hatchling snails (see Table 6.1); therefore, I used this species as predator in the experiments. Three different size classes of crabs were selected for this experiment: small (6-10 mm CW), medium (11-13 mm CW) and large (17-20 mm CW). For each crab size-class I used eight crabs: four were offered *C. maculosa*, and four *C. virgata*. All crabs were starved for 72 h and then individually placed in plastic containers (9.5 x 9.5 x 7.5 cm) fitted with 500-µm mesh sides. To each container, ten hatchling whelks of the appropriate species were added. For both species of whelks, a haphazardly selected mix of hatchlings from at least six groups of egg capsules was used.

At the end of the experiment, the hatchlings were scored as eaten or not eaten. Differences in consumption among crab size-class, hatchling species (i.e. fixed factors), and their corresponding interactions were analyzed using a two-way ANOVA. Significant differences were further examined using Tukey's HSD multiple comparison test. Assumptions of normality and homocedasticity were tested with Kolmogorov-Smirnov and Levene tests, respectively. Count data were square-root transformed to improve normality (Underwood 1997).

6.3.4 Vulnerability to predation over ontogeny

To determine whether *C. maculosa* and *C. virgata* experienced shifts in vulnerability to predators with growth, I evaluated predation on juveniles of both species by members of the two larger size classes of the smooth shore crab (*C. lavauxi*) utilized in the above experiment. Predation was evaluated in members of both species of snails at three different ages post-hatching, between January and March 2011: 1 day post-hatching (when snail shell lengths were on average 1.74 ± 0.02 mm and 3.07 ± 0.05 mm for *C. maculosa* and *C. virgata*, respectively, with n = 50 in both cases); 1 month post-hatching (2.20 ± 0.02 mm and 4.08 ± 0.03 mm; n = 80), and 2 months post-hatching (2.60 ± 0.05 mm and 4.81 ± 0.04 mm; n = 80). Hatchlings were reared in the laboratory in a large shallow tank supplied with constantly flowing fresh seawater at ambient temperature (16.5 ± 0.71°C), and fed *ad libitum* with chopped pieces of the green mussel *Perna canaliculus*. For each test at each age, a completely new set of crabs was collected from the field and starved for 72 h before being used in experiments. Crabs were placed individually in plastic containers (9.5 x 9.5 x 7.5 cm) fitted with 500-µm mesh sides, and ten snails were added to each cage. For each crab size-class (e.g. medium and large) I used eight crabs: four were offered *C. maculosa*, and four *C. virgata*. At the end of each 24 h experimental period, juveniles were scored as eaten or not eaten. Differences in consumption were analyzed using a three-way ANOVA using the fixed factors of crab size, prey species, age, and all possible interactions. When appropriate, significant differences were examined using Tukey's HSD multiple comparison tests. Assumptions of normality and homocedasticity were evaluated with Kolmogorov-Smirnov and Levene tests, respectively. To improve normality, count data for juvenile survival were square-root transformed (Underwood 1997).

In addition, I evaluated the relationship between shell length and shell thickness on 11 new hatchlings of both species of whelks. To generate fragments in the laboratory, hatchlings' shells were fractured with a dissecting needle near the posterior end of the shell (the apex). Measurements were obtained from at least five different locations (some more posterior, some more anterior) and those values averaged for each individual hatchling. Thickness measurements in the subsequent juvenile stages of both species (i.e. 1 and 2 mo post-hatching) were also estimated, using shell fragments recovered from the experimental containers. In all cases, thickness measurements were performed using a dissecting microscope equipped with an ocular micrometer, at 45X magnification. Between-species differences were evaluated using ANCOVA, with shell length as the covariate, species the fixed factor and thickness as the response variable.

6.4 Results

6.4.1 Whelk vulnerability to potential predators

The five potential predator species evaluated were common at the field site surveyed. The population density (mean ± SE) of the cushion sea star *Patiriella* spp. was the highest (26.5 ± 7.5 m^{-2}; size 19.5 ± 0.8 mm TL), followed by females and males of the pebble crab *Heterozius rotundifrons* (10.2 ± 3.8 m^{-2}; 16.4 ± 0.7 mm CW and 9.5 ± 3.2 m^{-2}; 14.7 ± 0.6 mm CW, respectively), the oyster borer snail *Haustrum scobina* (9.1 ± 3.5 m^{-2}; 16.1 ± 0.6 mm SL), the olive rockfish *Acanthoclinus fuscus* (5.5 ± 2.4 m^{-2}; 35.8 ± 4.3 mm SF), and males of the smooth shore crab *Cyclograpsus lavauxi* (4.7 ± 3.9 m^{-2}; 12.6 ± 0.9 mm CW).

Over the 24 h feeding trials, at least one hatchling of each whelk species died in each treatment (including controls, in the absence of predators). Overall, hatchlings of *C. maculosa* were significantly more vulnerable than those of *C. virgata* (two-way ANOVA, $F_{1,\ 126} = 18.08$, $p < 0.001$; Table 6.1). Hatchling mortality was also significantly affected by the species of predator evaluated (two-way ANOVA, $F_{6,\ 126} = 12.61$, $p < 0.001$; Table 6.1). *Cyclograpsus lavauxi* was the only potential predator that led to higher whelk mortality than in the control (no predator) treatment (Tukey post

hoc tests, all $p < 0.001$; Table 6.1). No interactive effects were observed between hatchling prey and predator species.

Table 6.1 Potential predators tested to determine the consumption upon the two species of hatchling whelks offered as prey (*C. maculosa*: 1.74 ± 0.02 and *C. virgata*: 3.07 ± 0.05 [mean ± SE]; n = 50 in both cases). Predator size is shown as mean (± SE) of 10 individuals per species. Abbreviations in the table are as follows: TL = total length measured from the tip of the longest arm to the opposite armpit; SF = standard fork length measured from the snout to the caudal fork; SL = shell length measured from the apex to the shell to the anterior tip of the aperture; CW = carapace width measured at the widest part of the carapace. Asterisks indicate significant differences in hatchling mortality among predators and between preys using Tukey's HSD test ($p < 0.05$).

Potential predator	**Predator size (mm; mean ± SE)**	**Hatchling mortality (%) over 24h (mean ± SE)**	
		C. maculosa	***C. virgata***
Control	No predator	2.3 ± 0.51	2.3 ± 1.22
Equinodermata			
Asteroidea			
Patiriella spp.	31.8 ± 1.0 TL	3.7 ± 1.26	0.7 ± 0.67
Chordata			
Perciformes			
Aconthoclinus fuscus	51.4 ± 2.2 SF	2.7 ± 1.71	0.7 ± 0.44
Mollusca			
Gastropoda			
Haustrum scobina	20 ± 0.4 SL	4.3 ± 1.58	1 ± 1.51
Arthropoda			
Crustacea			
Heterozius rotundifrons (♀)	22.7 ± 0.5 CW	11.3 ± 4.04	1.3 ± 1.74
Heterozius rotundifrons (♂)	20 ± 0.4 CW	7.7 ± 1.65	4.3 ± 3.26
Cyclograpsus lavauxi (♂)	15.4 ± 0.3 CW	24 ± 3.97	20 ± 5.02

6.4.2 Effects of predator size and prey species on predation

During the 24 h experimental period, the mean mortality of hatchling *C. virgata* and *C. maculosa* was significantly affected by the interaction between predator size and prey species (two-way ANOVA, predator size x prey species, $F_{2, 18} = 4.04$, p = 0.036; Fig. 6.1; Table 6.2). This interaction arose because large smooth shore crabs consumed three times more hatchlings of *C. virgata* than did medium conspecifics, whereas medium and large crabs consumed similar numbers of *C. maculosa* (Tukey post hoc tests, $p < 0.05$ in both cases; Fig. 6.1; Table 6.2). Small smooth shore crabs did not consume any of the prey offered (Fig. 6.1).

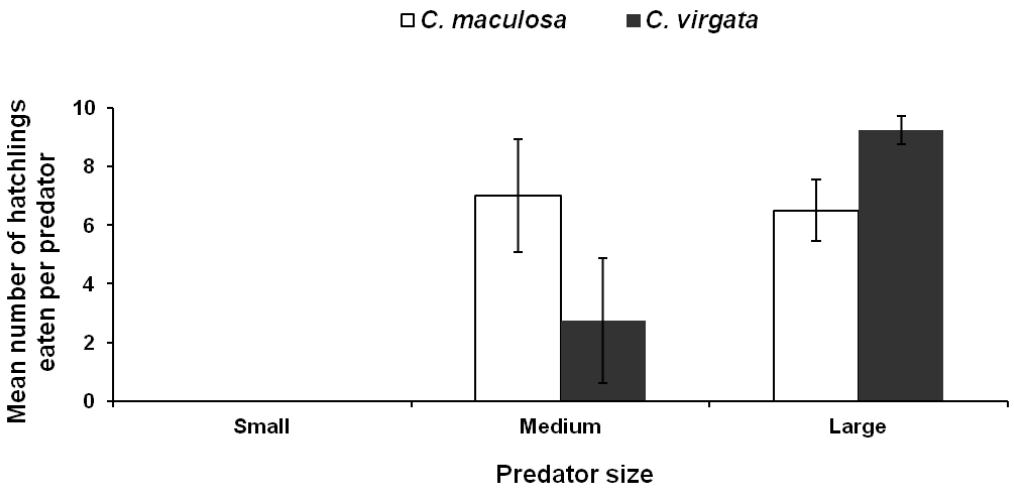


Fig.6.1 Mean mortality of *C. maculosa* (white bars) and *C. virgata* (dark-grey bars) hatchlings when exposed to 24 h predation by differently-sized smooth shore crab *Cyclograpsus lavauxi* in a density of one predator and ten hatchlings per replicate container (± SE). Three different size classes of crabs were selected: small (6-10 mm CW), medium (11-13 mm CW) and large (17-20 mm CW).

Table 6.2 Results of two-way ANOVA comparing mortality of hatchlings *C. virgata* and *C. maculosa* when exposed for 24 h to smooth shore crabs, *Cyclograpsus lavauxi*, of three different size classes: small (6-10 mm carapace width [CW], medium (11-13 mm CW) and large (17-20 mm CW).

Source	df	F	p
Crab Size	2	26.59	0.000
Prey species	1	0.688	0.418
Crab Size x Prey species	2	4.041	0.036
Error	18		

6.4.3 Vulnerability to predation over ontogeny

The smooth shore crab *C. lavauxi* consumed substantial numbers of both prey species during the 24 h experimental trials, with the mean mortality of hatchlings significantly affected by the interaction among crab size, prey species and time (three-way ANOVA, crab size x prey species x time, $F_{2, 36} = 10.62$, $p < 0.001$; Fig. 6.2; Table 6.3). This interaction arose because the vulnerability of both prey species was not consistent over time for both sizes of predators evaluated. The first day after hatching, large smooth shore crabs consumed more hatchlings of *C. maculosa* than did medium crabs, whereas hatchlings of *C. virgata* were consumed at equal rates by predators of both size classes (Fig. 6.2). One month after hatching, medium-sized crabs consumed approximately 5-fold fewer juveniles of *C. virgata* (compared with day one hatchlings: Tukey *post hoc* test, $p < 0.05$; Fig. 6.2). By the second month, large crabs also consumed approximately 4-fold fewer juveniles of *C. virgata* compare with the previous month (Tukey post hoc test, $p < 0.05$), and medium-sized crabs were completely unable to crush the shells of 2-month-old juveniles of *C. virgata* (which, at this point, averaged 4.81 ± 0.39 mm SL (Fig. 6.2; Table 6.3).

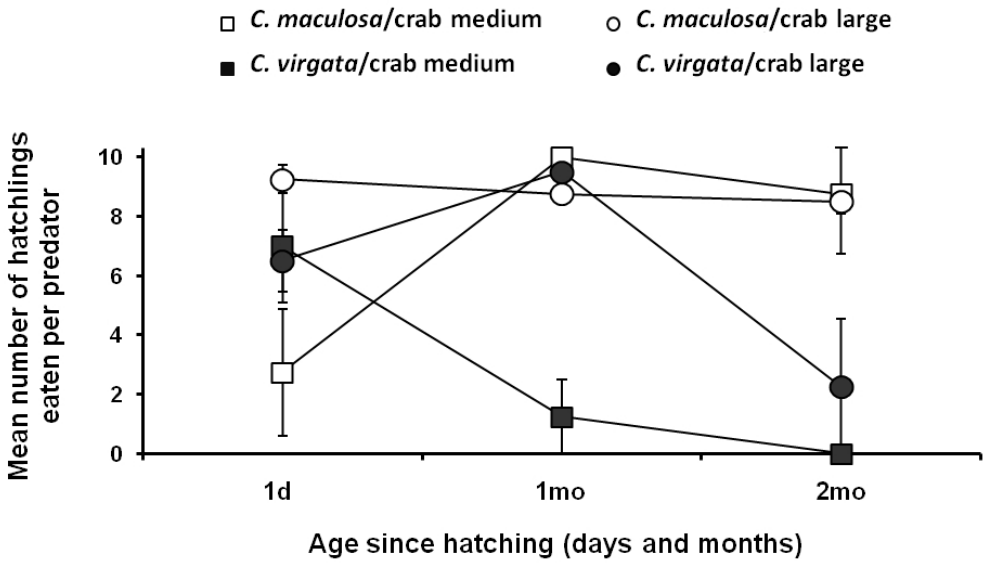


Fig. 6.2 Mean numbers (± SE) of juvenile *C. maculosa* (open symbols) and *C. virgata* (filled symbols) consumed at different ontogenetic stages when exposed to smooth shore crabs (*Cyclograpsus lavauxi*) of different sizes. Each replicate container contained one predator and ten hatchlings.

Table 6.3 Results of a three-way ANOVA comparing mortality of hatchlings *C. virgata* and *C. maculosa* at three different ages since hatching (1d, 1 month, and 2 months), when exposed to smooth shore crabs, *Cyclograpsus lavauxi*, of medium (11-13 mm carapace width [CW]) or large (17-20 mm CW) sizes for 24 h.

Source	**df**	**F**	**p**
Crab Size	1	14.119	0.001
Prey species	1	26.414	0.000
Time	2	4.68	0.016
Crab Size x Prey species	1	1.47	0.233
Crab Size x Time	2	1.308	0.283
Prey species x Time	2	14.452	0.000
Crab Size x Prey species x Time	2	10.624	0.000
Error	36		

There was no significant relationship between shell size and shell thickness in hatchlings of *C. maculosa* (y = 0.0275x - 0.0127, R^2 = 0.2427, p = 0.140; n = 11; Fig. 6.3a), but shell thickness was positively related to shell size in hatchlings of *C. virgata* (y = 0.0452x - 0.069, R^2 = 0.6732, p = 0.002; n = 11; Fig. 6.3b).

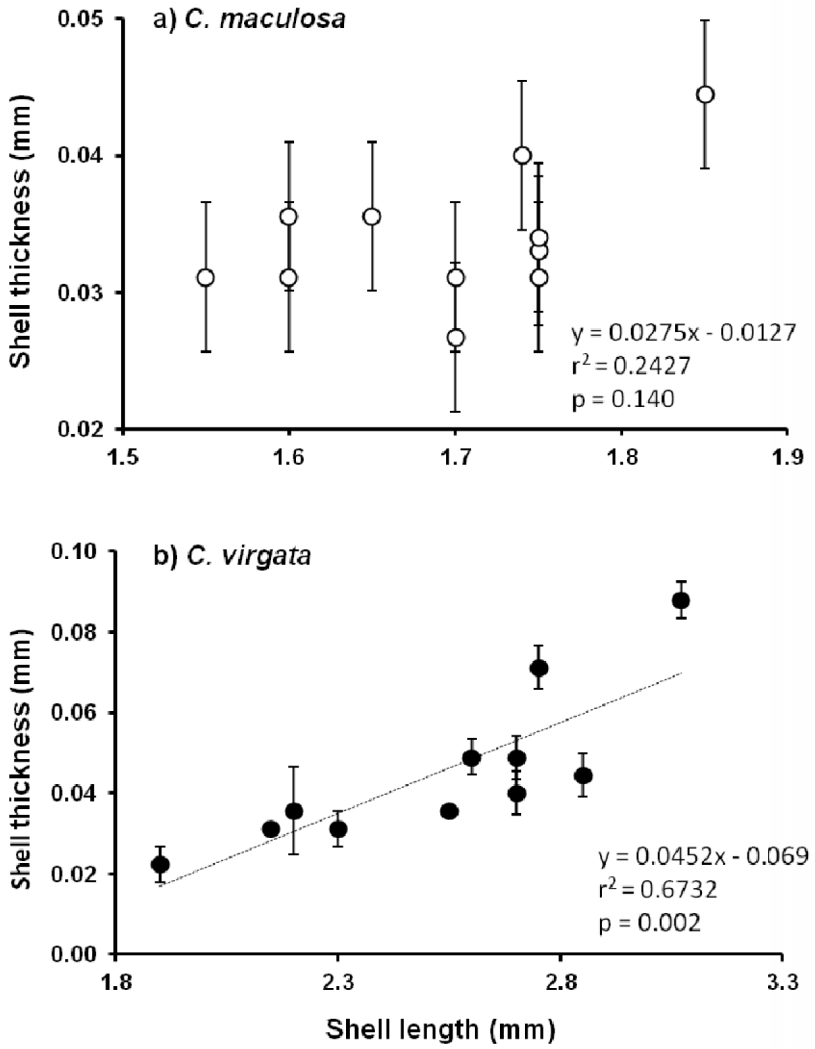


Fig. 6.3 Relationship between shell length (mm) and shell thickness (mm) at hatching for the whelks (a) *C. maculosa* (n = 11) and (b) *C. virgata* (n = 11). Points represent means of multiple measurements per individual; error bars represent standard errors (± SE). The dashed line in the plot for *C. virgata* represents the significant linear regression.

Shell thickness increased significantly over two months of growth in juveniles of both species of whelks, from mean values of 0.04 mm to 0.07 mm in *C. maculosa* ($y = 0.0002x + 0.0355$, $R^2 = 0.2542$, $p < 0.001$; Fig. 6.4a), and from 0.09 mm to 0.11 mm in *C. virgata* ($y = 0.0002x + 0.0813$, $R^2 = 0.1436$, $p < 0.001$; Fig. 6.4b). For a given shell length at a particular age since hatching, shell thickness was significantly greater for *C. virgata* (ANCOVA, $F_{1, 363} = 496.18$, $p < 0.001$); even two months after hatching, shells of juvenile *C. virgata* were 37% thicker than those of *C. maculosa* (see Fig 6.4a, b).

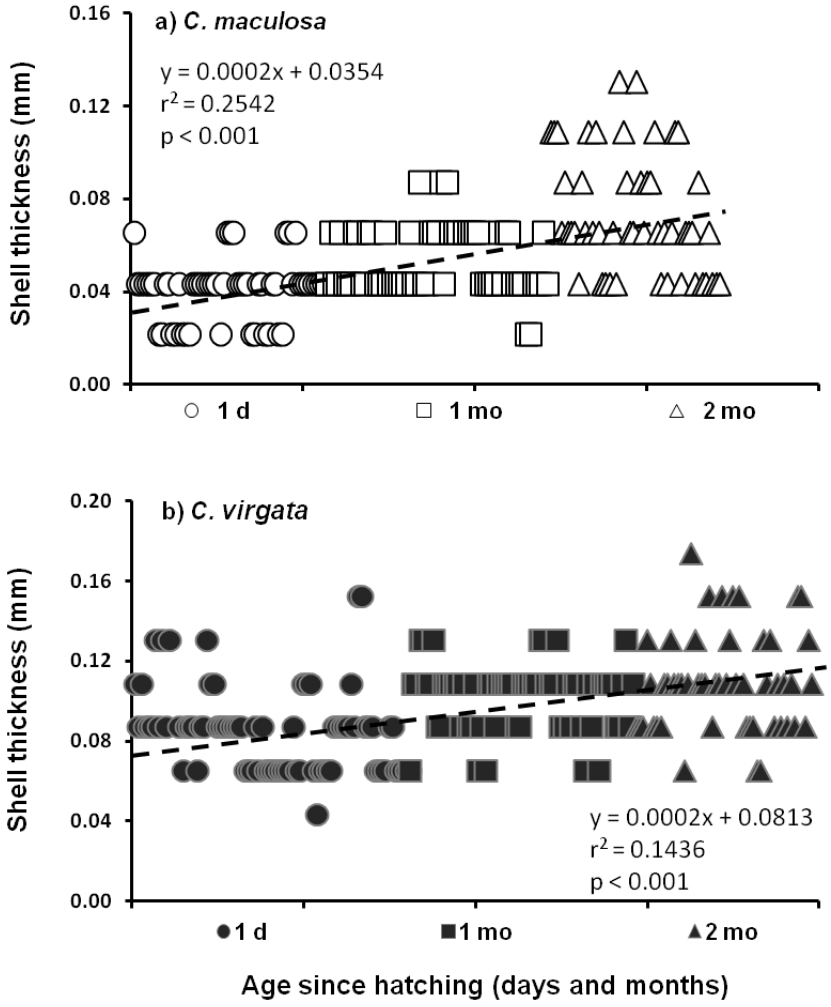


Fig. 6.4 Changes in shell thickness (mm) over the two months after hatching in the whelks (a) *C. maculosa* and (b) *C. virgata.* Dashed lines represent the significant linear regressions.

6.5 Discussion

6.5.1 Whelk vulnerability to potential predators

Hatchlings of *Cominella maculosa* and *C. virgata* were not equally vulnerable to all the potential predators evaluated. As hypothesized, overall more hatchlings *C. maculosa* were consumed compared with the larger hatchlings of *C. virgata*, suggesting that differences in hatchling size could have major ecological consequences for juvenile survival. The predatory responses varied among the potential predators evaluated, from no visible signs of predation by some of the most abundant potential predators (e.g. the sea star *Patiriella* spp., the rockfish *Acanthoclinus fuscus* and the oyster borer snail *Haustrum scobina*) to near-complete predation by the relatively less abundant smooth shore crab *Cyclograpsus lavauxi*. Although I did not exhaustively evaluate all possible predators, others were rare (e.g. muricid snails, hermit crabs, other grapsids). It is plausible that decapod crustaceans are a major threat during the early life stages of these whelks, as has been reported for the gastropods *Nucella lapillus*, *Littorina littorea*, *Haliotis kamtschatkana*, *Crepidula fornicata* (Lawton & Hughes 1985; Griffiths & Gosselin 2008; Pechenik et al. 2010, respectively), and in the bivalves *Mytilus edulis*, *Ischadium recurvum* and *Crassostrea virginica* (Smallegange & van der Meer 2003; Aronhime & Brown 2009; Kulp et al. 2011; Rindone & Eggleston 2011, respectively).

In the present experiments, crabs were not offered alternative prey, so it is not possible to assess the degree to which hatchling snails are selected as prey in the field; nevertheless, their importance as predators on small juvenile invertebrates is likely high due to their broad vertical distributions (intertidal and subtidal), their ability to handle and crush protective structures (e.g. shells), their need to obtain large quantities of food, and their high mobility (Yamada & Boulding 1996; Gosselin & Qian 1997). Even though the smooth shore crab *C. lavauxi* was was the only potential predator that exhibited significant levels of predation in this experiment, previous observations have shown that the pebble crab *H. rotundifrons* could also consume hatchlings of *C. virgata* in experimental trials (SA Carrasco, unpubl. data). Some of these differences in consumption observed, herein, could therefore be due to interactive effects of laboratory conditions, their contrasting life styles, and possibly metabolic rates of these intertidal crabs. Grapsids, such as *C. lavauxi*, are usually found under boulders from the middle-

to-upper intertidal zone, and exhibit a modest aerobic scope, foraging in air and running when exposed at low tide. The pebble crab *H. rotundifrons*, however, is a slow-moving crab that occupies the mid-to-lower intertidal zone. At low tide, pebble crabs remain partially buried in sand, with movements subject to sex ratio-dependent factors and a "freezing" response when disturbed (Taylor & Leelapiyanart 2001; Hazlett et al. 2005).

6.5.2 Effects of predator size and prey species on predation

As has previously been demonstrated in many predator-prey systems (e.g. Lawton & Hughes 1985; Palmer 1990; Yamada & Boulding 1996; Griffiths & Gosselin 2008; Aronhime & Brown 2009; Holmes & McCormick 2010; Kulp et al. 2011), the success of the attacks observed in these experiments was contingent on the relative sizes of predators and prey. Small mooth shore crabs were unable to successfully crush and eat any of the hatchling snails offered as food;however, as the sizes of the predators tested increased, the success of the attacks also increased significantly. The small hatchlings of *C. maculosa* were consumed in similar numbers by medium and large crabs. The significantly fewer hatchlings *C. virgata* consumed by medium crabs compared with large ones suggests that hatchlings of this species were not equally vulnerable to all sizes of crabs, and that shell morphology (i.e. size and thickness) could be acting as an effective deterrent for small and medium predators. Although such differences in performance between closely related species with similar life-histories but different per-offspring investment have not been well studied, these findings are consistent with previous results showing within-species size-dependent performance in a broad range of marine and terrestrial invertebrates (e.g. Spight 1976a; Gosselin 1997; Moran 1999; Fox 2000; Moran & Emlet 2001; Tsai & Dai 2001; Phillips 2002; Phillips & Gaines 2002; Marshall et al. 2003; Thiyagarajan et al. 2003; Walker 2003).

A recent study of differently sized juveniles of *C. virgata* and *C. maculosa* kept in laboratory conditions also showed similar within- and between-specific differences in performance, with larger hatchlings of both species being, in most cases, less vulnerable to the stressors evaluated (desiccation and starvation) compared with smaller hatchlings (see Chapter 5). Together, these findings suggest that inter- and intra-specific differences in per-offspring investment will not only mediate the performance of these

juvenile whelks when faced with predators (as shown here), but also will affect growth rates and survival under different physical stressors.

Interestingly, the large numbers of hatchlings of *C. virgata* consumed by large shore crabs support preliminary observations on prey-selectivity, where adults of *C. lavauxi* preferred *C. virgata* to *C. maculosa* when hatchlings of both prey species were offered together, suggesting a possible relationship between size and nutritional reward (SA Carrasco, unpubl. data; see Chapter 4), and also highlighting the fact that ideally both predators and prey traits should be considered when size-dependent interactions are evaluated.

6.5.3 Vulnerability to predators over ontogeny

The susceptibility to predators observed in these experiments varied depending on prey species and the predator size. For one species (*C. virgata*), risk of predation showed a sharp decrease with increasing body size through ontogeny, similar to findings in the dog whelk *N. lapillus* (Lawton & Hughes 1985), the abalone *H. kamtschatkana* (Griffiths & Gosselin 2008) and in the slippershell snails *Crepidula plana* and *C. convexa* (Pechenik et al. 2010). Differences in predation as a function of prey size have also been explored in other predatory crabs (e.g. *Callinectes sapidus*), and have suggested that some species of crabs may select smaller prey to avoid damaging their claws (Aronhime & Brown 2009).

In this study, early juveniles of *C. maculosa* and *C. virgata* (on average 1.7 and 3.1 mm SL, respectively) were both highly vulnerable to predation during the first day after hatching, when their shell thicknesses were 0.04 and 0.09 mm, respectively. However, after one month of growth, when juvenile snails reached (on average) shell lengths of 2.2 and 4.1 mm and shell thickness of 0.05 and 0.10 mm (for *C. maculosa* and *C. virgata*, respectively), notable differences in predation were observed. Large crabs ate almost all the juveniles provided regardless of the species, consuming around 7-fold more *C. virgata* compared to medium-sized crabs. By the second month after hatching, medium-sized crabs were no longer able to crush and eat any of the juvenile *C. virgata* offered, and large crabs decreased their consumption by ~4-fold compared with the previous month. These findings suggest that juvenile of *C. virgata* became less vulnerable to shell-crushing predatory crabs overtime, attaining a size refuge from

predation by smooth shore crabs when the shell length reached ~4.8 mm and shell thickness 0.11 mm. For *C. maculosa*, the risk of predation remained, in almost all cases, unchanged through early ontogeny, from a shell length of 1.7 mm and shell thickness of 0.04 mm one day after hatching to a shell length of 2.6 mm and shell thickness of 0.07 mm two months since hatching. Recent studies on juvenile performance for both species showed that growth rates of *C. maculosa* (~0.02 mm d^{-1}) were nearly half those of *C. virgata* (~0.04 mm d^{-1}) during the first month after hatching in laboratory conditions (Chapter 5), and therefore, it is not surprising that juvenile of *C. maculosa* might require at least two more months to eventually reach the size refuge of ~4.8 mm SL and ~0.11 mm shell thickness observed in the less vulnerable 2-month-old juveniles of *C. virgata*.

The species-specific relationship between shell thickness and size observed in hatchlings of *C. virgata* and *C. maculosa* may indicate that it is not size alone that determines their vulnerability to shell crushing predators. Large hatchlings of *C. virgata* have much thicker shells than those of smaller conspecifics, whereas the range of shell thickness for hatchlings of *C. maculosa* was much smaller, and larger hatchlings did not have significantly thicker shells than those of smaller conspecifics. Thus, hatchling size may not affect risk in this latter species: all hatchlings of *C. maculosa* may be equally vulnerable to shell crushing predators (for examples, see Spight 1976a; Lawton & Hughes 1985; Pechenik et al. 2010). Given that the two species of whelks co-occur and have the same reproductive season, species differences in vulnerability to predators at this early post-hatching stage could have downstream consequences at the community level. Greater juvenile vulnerability to predators of *C. maculosa* could influence the population density of adults (in fact, adults of *C. maculosa* are much less abundant than those of *C. virgata* at the study site, SA Carrasco & NE Phillips unpubl. data). Such effects may vary spatially over sites with different suites or densities of predators.

Overall, this study highlights the importance of initial offspring size in mediating predation in ecologically similar and closely related co-occurring species. These results show that vulnerability to predators can be mitigated by larger sizes at hatching. However, the results also suggest that, in addition to size, shell thickness and juvenile growth rates may also play key roles in determining the vulnerability of hatchling and juvenile snails when exposed to shell crushing predators. To fully understand the species-specific risk of predation for these juvenile whelks, further studies should evaluate the role of juvenile behavior (i.e. refuge seeking) and/or chemical defenses that could mediate their vulnerability to predators over time.

Chapter 7

General Discussion

7.1 Reproductive strategies, embryo packaging and offspring size

Understanding the way in which mothers provision their progeny and how those maternal effects have further consequences for offspring phenotype and performance is a complex and variable process, that can depend on several factors such as species, reproductive strategies, maternal habitat, female size and female condition, among many others. Although for over 20 years these maternal effects have been the subject of several studies in vertebrates (e.g. birds and fish), as well as in many marine and terrestrial invertebrate species (e.g. gastropods, mussels, barnacles, ascidians, isopods, spiders, beetles) (for examples see Bernardo 1996a; Marshall et al. 2008; Chapters 5 and 6), there are still many questions that remain to be answered, particularly in the changing marine environment. Although over the years most studies have considered maternal effects on different life history stages, only a few have simultaneously evaluated the causes and consequences of inter- and intra-specific variation in per-offspring provisioning in terms of both maternal investment and juvenile performance. The study that I have conducted here using as model organisms sympatric direct developing whelks allowed for a better understanding of how species with contrasting reproductive strategies provision their progeny and how that maternal provisioning is linked with offspring size and further, translated into juvenile performance, within and among species.

As observed in these co-occurring whelks, and also in several other invertebrates, even closely related species may considerably differ in their allocation patterns (e.g. Collin 2003), and thus, have many different ways in which offspring will interact with their environment. For example, despite being under the same environmental pressures in the field (e.g. wave action, salinity, temperature and predation), the packaging strategies and encapsulating structures observed in the three study species, *Cominella virgata*, *C. maculosa* and *Haustrum scobina*, varied considerably in size and shape,

resulting in differential effectiveness against some of the most common environmental stressors such as predation and bacterial infection. Even in those early life-history stages, species-specific patterns of encapsulation had significant implications for further embryonic development and survival, especially for hatchlings of *H. scobina*, species that despite encapsulating the highest number of embryos per capsule, was also the most vulnerable to environmental threats (see Chapter 2).

Differences in packaging and provisioning were also reflected in juvenile traits, and as expected, the species that encapsulates a single embryo per capsule (*C. virgata*) exhibited considerably larger hatching sizes than the species that encapsulate multiple ones, without (*C. maculosa*) or with nurse embryo consumption (*H. scobina*). Despite such inter-specific differences, a similar chronology in the developmental features was recognizable through their intra-capsular life. All three species attained the trochophore and early veliger stages by the third and fourth weeks, and the hatching stage after 10 weeks, with coiled and pigmented shells, cephalic tentacles with basal eyes, siphon, foot and operculum. As recorded in several other species of gastropod mollusks that encapsulate their embryos, the classical trade-offs between offspring size and number were also evident in this system, with a higher number of embryos, and/or, smaller hatchling sizes, being usually obtained from capsules with a higher number of siblings (see Spight 1976b; Rivest 1983, 1986; Pechenik et al. 1984; Chaparro et al. 1999; Saglam & Duzgunes 2007; Averbuj & Penchaszadeh 2010; Chatzinikolaou & Richardson 2010; Lahbib et al. 2011).

Although a vast number of studies have evaluated morphological consequences of different packaging strategies and intra-capsular food provisioning on offspring traits, studies regarding the physiological and energetic basis underlying those different ways of maternal investment still remain scarce for gastropod mollusks (see Heras et al. 1998; Martínez et al. 2008; Chaparro et al. 2012). For example, an interesting pattern identified during intra-capsular development of *H. scobina*, was the progressive decrease in the number of nurse eggs coupled with the total depletion of one of the energetic compounds during the fourth week of development, a pattern previously undescribed for this or other species with a nurse-embryo-based strategy. Much information is therefore still necessary to understand the different ways in which direct developing species provision their embryos to face the crawl-away juvenile period, and how those contrasting reproductive strategies could parallel different evolutionary and physiological pathways in the life-history of these species.

7.2 Maternal provisioning and offspring size

The maternally-derived resources that offspring receive from their mothers can strongly influence the evolution of life histories, being an important determinant of offspring size and quality in benthic marine invertebrates (Rivest 1983; Bernardo et al. 1996b; McEdward & Miner 2006). Nonetheless, it has also been recognized that estimations of offspring size are not a reliable indicator of offspring condition for all species (i.e. egg size in echinoids; McEdward & Morgan 2001; Moran & McAlister 2009). In direct developers it also cannot be considered as the total maternal investment, since together with eggs, females also include a range of other nourishing resources to be consumed by embryos during the encapsulated development, including albumen, unfertilized eggs and embryos or even viable siblings (Spight 1976b; Rivest 1983; Collin 2003). Provisioning for a newly laid egg capsule and later for individual hatchlings however, could be considered a reliable indicator of total maternal investment, since none of those early stages are influenced by other external sources of nutrition.

Despite contrasting reproductive strategies, most of the lipids identified in the egg capsules and hatchlings of *C. virgata, C. macuolosa* and *H. scobina* were common in the early-life stages of other invertebrate and vertebrate species, including echinoderms (Prowse et al. 2009), fresh-water and marine mollusks (Heras et al. 1998; Moran & Manahan 2003; Steer et al. 2004; Pernet et al. 2006) and fish (Hilton et al. 2008). Phospholipids (PL) and cholesterol (ST) were the main structural lipid classes, whereas aliphatic hydrocarbon (HC), triglyceride (TG), diglyceride (DG), free fatty acids (FFA), wax ester (WE) and methyl ester (ME) were the lipids classes used for energy-storage.

By comparing those lipid signatures among species, differences in maternally-derived energy resources were evident among them, as *C. virgata* was the only one that lacks WE and ME, and *H. scobina* was the only one that "lost" one lipid class (FFA) during intra-capsular development. Interestingly, the presence of WE and ME have both been independently recorded in early stages of species with planktonic development [i.e. abalone (Moran & Manahan 2003), squid (Steer et al. 2004), sea-stars (Prowse et al. 2008), fish (Hilton et al. 2008) and corals (Dodds et al. 2009)], and in some cases, WE has also been described to play important roles in buoyancy and long-term energy storage in polar and deep sea zooplankton (see Prowse et al. 2009). The presence of these lipid classes in the smaller hatchings of *C. maculosa* and *H.* scobina suggest similar traits between these multi-encapsulated embryos with other planktonic stages of

dispersal, which has been recognized as the ancestral condition for many other marine invertebrate species (see Strathmann 1978; Pechenik 1999; Chapter 2; Chapter 4).

Based on the maternally-derived resources observed among siblings of *C. maculosa* and *H. scobina*, this study also suggests that the reproductive strategy of female *C. maculosa* (i.e. intra-capsular fluid) may be more efficient in controlling allocation of resources for individual siblings compared with the nurse-egg-based strategy of female *H. scobina*. Therefore, females of the latter species would be able to bet-hedge to a higher extent than female *C. maculosa* or *C. virgata*, assumption that could also be reflected in the higher variability in size and developmental asynchrony observed by the time of hatching in *H. scobina*, with some hatchlings still retaining a few larval traits (i.e. velum and larval kidneys; see Chapter 2).

Likewise, the energetic signatures observed among the three species (i.e. especially TG) evidenced important trade-offs between hatchling size and energy content, since the species with the smaller hatchlings, *C. maculosa* and *H. scobina*, provisioned their embryos with higher amounts of TG than the species with the larger hatchlings, *C. virgata*. These findings suggests that the former two species could withstand prolonged period without food in the field and not necessarily start foraging directly after hatching, using intertidal microhabitats (instead of shell size) as a refuge from predators. In contrast, given their lower energy reserves, hatchlings of *C. virgata* would probably need food soon after hatching; however, their larger sizes and thicker shells, could act as an effective predator deterrent for at least some of the most common shell crushing predators in these variable intertidal environments.

7.3 Offspring size mediating early juvenile performance

Across a variety of taxa and habitats, it has been well recognized that initial differences in offspring size will play key roles in mediating the performance of organisms, with direct consequences for the fitness of both offspring and mother (Bernardo 1996b; Marshall & Keough 2008a; Kamel et al. 2010b). Although intra-specific variation in offspring size is of fundamental ecological and evolutionary importance, the consequences of inter-specific variation for co-occurring taxa have been rarely examined.

When hatching size was evaluated in field conditions for the congeneric *C. virgata* and *C. maculosa*, these observations suggested that at least for these two direct-developing species, smaller scale site-to-site and individual female attributes were more important than larger scale differences between locations in mediating offspring traits. These results contrasted with observations in species with planktonic larvae in this same system (i.e. Wellington Harbor vs. South Coast; Shima & Swearer 2009, 2010; Swearer & Shima 2010), but also reinforced the fact that, for both species, the overall quality of the habitat into which offspring emerged was the selective force mediating offspring size (Johnston & Leggett 2002; Gosselin & Rehak 2007; Shima & Swearer 2009).

As variation in offspring size differs across the scales examined (i.e. inter- and intra-specific or habitat related), identifying the consequences of such variability was the key to identify the ecological role of hatchling size between these direct developing whelks. When the intra-specific performance of *C. virgata* and *C. maculosa* hatchlings from different maternal habitats were compared in equivalent laboratory conditions, variation in initial size was not reflected in hatchling performance (e.g. growth, survival), suggesting that factors mediating hatchling size in the field (i.e. among sites) were not consistent with those acting on hatchling performance. Contrastingly, when hatchling sizes (i.e. large and small) were compared within each site, the expected response was observed, and in both species, large snails performed better than small conspecifics; agreeing with similar findings previously described in other marine gastropods by Spight (1976a) and Moran & Emlet (2001). Unlike these patterns, when the performance of hatchlings was compared in unfed conditions, different maternal habitats or variation in initial size had no effect in the overall survival of starved hatchlings, suggesting an "optimal" allocation and similar utilization of resources during unfavourable conditions. As in this study, Moran & Emlet (2001) also suggested that the advantage of larger hatchling sizes in juvenile *Nucella ostrina* was decreased under more severe environmental conditions, and that during periods of high heat stress, mortality was largely random with respect to size (see Chapter 5).

In inter-specific comparisons, the expected responses were observed, with larger *C. virgata* hatchlings performing better than the smaller *C. maculosa* when fed or starved, exhibiting faster growth rates and higher survival, and suggesting that these differences in performance could be a direct consequence of the higher per-offspring maternal investment observed in the former species (see Chapter 4). Although smaller hatchlings of *C. maculosa* had more TG than larger *C. virgata*, huge differences in the total lipid

composition were observed among species, suggesting that the total provisioning of lipids (sum of all classes), as well as the concomitant advantages of initial larger sizes at hatch, could also play key roles in determining those differences their overall fitness. Further inter-specific studies should ideally evaluate similar questions on provisioning and performance under more realistic field conditions.

Under a predator-prey context, these two species exhibited similar responses to the ones observed in the performance experiment. In all different scenarios evaluated, larger *C. virgata* always had a size advantage compared with the smaller *C. maculosa*, suggesting that initial differences in hatchling size would have major ecological consequences for later juvenile stages of these two co-occurring whelks. Similarly to findings in other predator-prey systems, the success of the attacks was contingent on the relative sizes of both predators and prey (e.g. Lawton & Hughes 1985; Palmer 1990; Yamada & Boulding 1996; Griffiths & Gosselin 2008; Aronhime & Brown 2009; Holmes & McCormick 2010; Kulp et al. 2011), with smaller *C. maculosa* being consumed by almost all predators irrespective of the initial hatchling size, whereas larger *C. virgata* were consumed in higher numbers only by larger predators, suggesting that shell morphology in this species (i.e. size and thickness) could be an effective deterrent for some predators in the field.

Likewise, although the susceptibility to predators of both hatchlings whelks varied depending on the prey species and the predator size, only in *C. virgata* the risk of predation decrease with increasing body size over ontogeny, similar to the findings in other gastropod species such as whelks (Lawton & Hughes 1985), abalone (Griffiths & Gosselin 2008) and slippershells (Pechenik et al. 2010). These findings suggest that the faster growth rates observed in juvenile *C. virgata* also allowed this species to attain an earlier size refuge after two months post-hatch, contrasting with the findings in *C. maculosa*, in which the risk of predation remained unchanged over the vulnerable post-hatching crawl-away period (see Chapter 6).

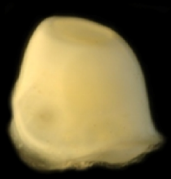
7.4 Concluding remarks and future directions

The research conducted during this thesis aimed to incorporate different approaches in the study of the early life-history stages of direct-developing whelks with contrasting reproductive strategies, including: (a) descriptions of different patterns of encapsulation

and food provisioning, (b) exploration of variation in maternal investment of lipid resources and (c) evaluating the performance of juveniles with variable traits during the crawl-away juvenile period. This integrative approach allowed for a better understanding that offspring size does not necessarily reflect energetic content, as for example, the smaller hatchlings of *H. scobina* and *C. maculosa* presented more triglycerides (i.e. the most important energy lipid class) than the larger hatchlings of *C. virgata*. As the two former species also encapsulated multiple embryos per capsule, it was clear that trade-offs between offspring size and offspring energy resources occurred. Results suggest that although larger hatchlings of *C. virgata* had less energy stored, they could start feeding soon after hatching, being less vulnerable as they were more protected by their larger and thicker shells, faster growth rates, and higher survival in the presence of food. On the other hand, although hatchlings of *C. maculosa* and *H. scobina* escaped from the capsules in higher numbers and with more energetic resources (i.e. TG), their initial life will be associated with smaller and thinner shells, slower growth rates, lower survival, and higher vulnerabilities when facing crushing predators.

These findings suggest that when defining offspring size, provisioning and performance relationships, many context-dependent scenarios are likely to arise. Therefore, integrative studies considering different species (e.g. contrasting reproductive strategies), life-history stages (e.g. hatchlings and juveniles), scales of variations (e.g. sites and locations), habitats (e.g. protected vs exposed), and conditions (e.g. fed vs starved), are needed in order to better understand how these complex relationships arises and how they could affect an individual's life-history. Further studies still need explore in more detail the links between maternal condition and offspring size, condition and/or performance. Similar ecological and/or physiological approaches across species with different reproductive strategies, and particularly those that may share habitat during part or their complete life history, would inform our understanding of ecological consequences. Further, such comparative data generated across related species that differ in maternal provisioning to their offspring, coupled with a resolution of phylogenetic relationships among those taxa, would allow us to evaluate evolutionary trends in allocation patterns.

7.5 Schematic summary

Offspring size, provisioning and performance as a function of maternal investment in direct developing whelks			
	Cominella virgata	*Cominella maculosa*	*Haustrum scobina*
Egg capsule morphology			
Egg capsule size	4.08 ± 0.08 mm height 2.73 ± 0.04 mm width 2.20 ± 0.06 mm depth 23.09 ± 0.97 µl volume	6.92 ± 0.19 mm height 3.82 ± 0.06 mm width 2.64 ± 0.02 mm depth 29.67 ± 0.08 µl volume	1.82 ± 0.05 mm radius 3.60 ± 0.14 mm height 18.63 ± 1.66 µl volume
Egg capsule lipids	PL, ST (84.3%) AH, TG, FFA, DG (15.7%)	PL, ST (20.4%) AH, TG, FFA, DG, WE, ME (79.6%)	PL, ST (54.2%) AH, TG, FFA, DG, WE, ME (45.8%)
Development time	10 wk	10 wk	10 wk
Hatchling morphology			
Hatchling traits	2.7 ± 0.06 mm shell length 0.37 ± 0.01 mg flesh weight	1.69 ± 0.03 mm shell length 0.33 ± 0.03 mg flesh weight	1.28 ± 0.04 mm shell length 0.24 ± 0.01 mg flesh weight
Hatchling lipids	PL, ST (89.9%) AH, TG, FFA, DG (10.1%)	PL, ST (50%) AH, TG, FFA, DG, WE, ME (50%)	PL, ST (51.6%) AH, TG, FFA, DG, WE, ME (48.4%)
Hatchling growth (mm d^{-1})	Fed Large: ✓✓✓ Small: ✓✓ Starved Large: ✓ Small: ✓✓	Fed Large: ✓✓✓ Small: ✓✓ Starved Large: ✓ Small: ✓✓	Not evaluated
Hatchling survival (%)	Fed Large: ✓✓✓ Small: ✓✓✓ Starved Large: ✓✓ Small: ✓✓	Fed Large: ✓✓✓ Small: ✓✓ Starved Large: ✓✓ Small: ✓✓	Not evaluated
Vulnerability to predators	✓	✓✓✓	Not evaluated
Size refuge	4.8 mm shell length (2 months)	Not attained (2 months)	Not evaluated

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Appendix A

SIMPER analyses of lipid classes in newly laid egg capsules of *C. virgata*, *C. maculosa* and *H. scobina*

Groups: *C. virgata* & *C. maculosa*

Average dissimilarity = 82.95

Variable	Mean *Cv*	Mean *Cm*	Average dissimilarity	SD	% contribution	Cumulative %
PL	1.78	0.13	44.48	15.36	53.62	53.62
ST	0.69	0.03	17.74	10.43	21.38	75
TG	0.11	0.48	10.07	8.04	12.14	87.14
DG	0.15	0.01	3.72	13.07	4.48	91.62

Groups: *C. virgata* & *H. scobina*

Average dissimilarity = 70.95

Variable	Mean *Cv*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
TG	0.11	5.7	33.62	18.95	47.39	47.39
PL	1.78	7.07	31.88	13.41	44.93	92.32

Groups: *C. maculosa* & *H. scobina*

Average dissimilarity = 89.15

Variable	Mean *Cm*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
PL	0.13	7.07	48.19	23.74	54.05	54.05
TG	0.48	5.7	36.05	20.05	40.44	94.5

Appendix B

SIMPER analyses of lipid classes in egg capsules during intra-capsular development of *C. virgata, C. maculosa* and *H. scobina* analyzed across all weeks.

Groups: *C. virgata* & *C. maculosa*

Average dissimilarity = 75.19

Variable	Mean *Cv*	Mean *Cm*	Average dissimilarity	SD	% contribution	Cumulative %
PL	12.86	8.41	40.96	2.14	54.47	54.47
ST	3.74	2.4	13.37	2.19	17.78	72.25
TG	0.78	4.52	7.91	1.88	10.52	82.77
DG	0.79	2.29	3.73	2.87	4.96	87.73
AH	0.64	1.65	3.02	1.54	4.02	91.75

Groups: *C. virgata* & *H. scobina*

Average dissimilarity = 71.12

Variable	Mean *Cv*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
TG	0.78	63.62	32.19	2.4	45.26	45.26
PL	12.86	46.46	29.9	3.91	42.04	87.3
ST	3.74	3.14	3.9	0.85	5.49	92.79

Groups: *C. maculosa* & *H. scobina*

Average dissimilarity = 86.38

Variable	Mean *Cm*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
PL	8.41	46.46	43.63	5.43	50.51	50.51
TG	4.52	63.62	38.07	7.39	44.07	94.58

Appendix C

SIMPER analyses of lipid classes in egg capsules during intra-capsular development of *C. virgata, C. maculosa* and *H. scobina* across all species.

Groups: 1 & 3

Average dissimilarity = 13.40

Variable	Mean 1	Mean 3	Average dissimilarity	SD	% contribution	Cumulative %
TG	2.1	2.72	5.34	0.76	39.89	39.89
PL	2.99	3.55	4.41	1.36	32.9	72.79
FFA	0.06	0.07	1.34	0.45	9.99	82.77
ST	0.35	0.33	0.79	0.8	5.91	88.69
ME	0.09	0.11	0.44	0.68	3.29	91.97

Groups: 1 & 5

Average dissimilarity = 16.05

Variable	Mean 1	Mean 5	Average dissimilarity	SD	% contribution	Cumulative %
PL	2.99	3.04	9.7	1.59	60.43	60.43
TG	2.1	2.3	3.28	0.96	20.42	80.85
ST	0.35	0.32	1.28	0.61	7.96	88.81
DG	0.09	0.04	0.49	1.19	3.05	91.85

Groups: 3 & 5

Average dissimilarity = 20.77

Variable	Mean 3	Mean 5	Average dissimilarity	SD	% contribution	Cumulative %
PL	3.55	3.04	11.45	1.72	55.16	55.16
TG	2.72	2.3	5.21	0.98	25.11	80.26
FFA	0.07	0.04	1.2	0.47	5.79	86.06
ST	0.33	0.32	0.97	0.59	4.66	90.71

Appendix C Continued

Groups: 1 & 7

Average dissimilarity = 48.59

Variable	Mean 1	Mean 7	Average dissimilarity	SD	% contribution	Cumulative %
PL	2.99	13.74	30.63	1.53	63.04	63.04
ST	0.35	2.98	6.35	0.88	13.06	76.09
TG	2.1	3.25	5.2	1.84	10.7	86.8
AH	0.06	0.77	2.01	0.95	4.13	90.93

Groups: 3 & 7

Average dissimilarity = 47.07

Variable	Mean 3	Mean 7	Average dissimilarity	SD	% contribution	Cumulative %
PL	3.55	13.74	29.35	1.33	62.36	62.36
ST	0.33	2.98	6.31	0.85	13.4	75.77
TG	2.72	3.25	5.24	0.9	11.13	86.9
AH	0.08	0.77	2.05	0.97	4.36	91.26

Groups: 5 & 7

Average dissimilarity = 47.39

Variable	Mean 5	Mean 7	Average dissimilarity	SD	% contribution	Cumulative %
PL	3.04	13.74	28.72	1.44	60.6	60.6
ST	0.32	2.98	6.27	0.85	13.23	73.83
TG	2.3	3.25	5.96	1.74	12.58	86.41
AH	0.05	0.77	2.13	1.01	4.5	90.91

Appendix C Continued

Groups: 1 & 9

Average dissimilarity = 93.83

Variable	Mean 1	Mean 9	Average dissimilarity	SD	% contribution	Cumulative %
PL	2.99	94.95	43.62	3.81	46.49	46.49
TG	2.1	109.61	20.92	1.1	22.29	68.78
ST	0.35	12.53	10.63	1.93	11.33	80.1
DG	0.09	9.2	5.69	1.68	6.06	86.16
FFA	0.06	3.51	3.84	1.54	4.1	90.26

Groups: 3 & 9

Average dissimilarity = 93.87

Variable	Mean 3	Mean 9	Average dissimilarity	SD	% contribution	Cumulative %
PL	3.55	94.95	43.66	3.73	46.5	46.5
TG	2.72	109.61	20.81	1.1	22.17	68.67
ST	0.33	12.53	10.71	1.91	11.41	80.09
DG	0.1	9.2	5.71	1.69	6.08	86.16
FFA	0.07	3.51	3.86	1.55	4.11	90.27

Appendix C Continued

Groups: 5 & 9

Average dissimilarity = 93.31

Variable	Mean 5	Mean 9	Average dissimilarity	SD	% contribution	Cumulative %
PL	3.04	94.95	43.02	3.92	46.1	46.1
TG	2.3	109.61	20.9	1.1	22.4	68.5
ST	0.32	12.53	10.69	1.9	11.46	79.95
DG	0.04	9.2	5.72	1.7	6.13	86.08
FFA	0.04	3.51	3.83	1.53	4.1	90.18

Groups: 7 & 9

Average dissimilarity = 65.59

Variable	Mean 7	Mean 9	Average dissimilarity	SD	% contribution	Cumulative %
PL	13.74	94.95	25.02	1.99	38.14	38.14
TG	3.25	109.61	19.31	0.96	29.44	67.58
ST	2.98	12.53	5.17	1.31	7.89	75.47
DG	0.61	9.2	4.4	1.03	6.71	82.18
AH	0.77	5.69	3.24	1.03	4.94	87.12
ME	0.15	6.22	3.03	0.94	4.62	91.74

Appendix D

SIMPER analyses of lipid classes in newly hatchlings of *C. virgata*, *C. maculosa* and *H. scobina*.

Groups: *C. virgata* & *C. maculosa*

Average dissimilarity = 61.94

Variable	Mean *Cv*	Mean *Cm*	Average dissimilarity	SD	% contribution	Cumulative %
PL	45.24	7.61	47.35	5.37	76.45	76.45
ST	7.86	1.85	7.94	5.45	12.81	89.26
TG	1.25	2.33	1.76	1.2	2.84	92.1

Groups: *C. virgata* & *H. scobina*

Average dissimilarity = 46.83

Variable	Mean *Cv*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
TG	1.25	43.78	24.44	6.21	52.19	52.19
PL	45.24	59.83	13.5	1.27	28.82	81.01
ST	7.86	2.51	3.26	3.38	6.96	87.97
AH	1.19	3.27	1.84	0.74	3.93	91.9

Groups: *C. maculosa* & *H. scobina*

Average dissimilarity = 73.66

Variable	Mean *Cm*	Mean *Hs*	Average dissimilarity	SD	% contribution	Cumulative %
PL	7.61	59.83	36.13	3.19	49.05	49.05
TG	2.33	43.78	31.15	7.64	42.28	91.34