

Juvenile biology and captive rearing
of the freshwater pearl mussel
Margaritifera margaritifera

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Abstract

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Captive breeding of the freshwater pearl mussel (*Margaritifera margaritifera*) is an important short-term strategy to conserve this critically endangered species. The aim of this thesis was to improve current knowledge of the factors affecting juvenile *M. margaritifera* in a captive setting, and to develop understanding of juvenile anatomy, ontogeny and the ecological requirements of juveniles in captivity. The substrate requirements of newly-excysted juveniles were investigated in an experimental flow-through system (Chapter 3) by analysing differences in survival and growth in two different substrate size clasts (0.25 - 1 mm or 1 - 2 mm), and cleaning regimes (weekly or monthly). Factors potentially affecting juvenile survival and growth were further investigated in Chapter 4. Results indicate that dissolved oxygen and flow were crucial for juveniles in this system. Investigations of juvenile anatomy and ontogeny (Chapter 5) using scanning electron microscopy have greatly improved our knowledge of the timing of key developmental stages, such as the onset of gill reflection. Analyses of gill ciliation suggest the species is capable of retaining very small particles (<2 µm diameter), offering a potential reason for why *M. margaritifera* is so sensitive to turbid and enriched conditions. Improving the efficiency and effectiveness of monitoring juveniles in captivity should be an objective for all rearing programmes. Batch marking of juveniles through immersion in calcein (Chapter 6) was shown to offer a quick and reliable method and has the potential to save rearing programmes time and money whilst improving juvenile monitoring. The findings of these investigations should inform other captive rearing programmes in order to improve juvenile survival. Rearing efforts should focus initially on ensuring sufficient flow and dissolved oxygen for post-excystment juveniles, before tailoring systems to ensure low-stress conditions for transforming juveniles.

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Here's to the next chapter...

Author's declaration

I declare that all of the work presented in this thesis is my own and has not been submitted for consideration for a degree at any other university or institution. Any section of this thesis that has been published has been clearly identified. The copyright of this thesis rests with the author.

Chapter 1

Introduction

1.1. Background and context of freshwater bivalve conservation

Freshwater mussels (Unionida) are among the most endangered invertebrates in the world (Machordom *et al.*, 2003; Primack, 2006) and are disproportionately imperilled compared to other groups (Williams *et al.*, 1993). They are a keystone group in aquatic systems making them a priority for conservation (Primack, 2006) and *Margaritifera margaritifera* (Linnaeus, 1758) has simultaneously been described as fulfilling the role of a keystone, umbrella, indicator and flagship species (Geist, 2010). Freshwater mussels also provide valuable environmental services, with their presence greatly enhancing biodiversity (Killeen *et al.*, 2004; Geist, 2010).

Due to the unprecedented decline of freshwater bivalves, the number of studies focusing on freshwater mussel biology, ecology and propagation began to gather pace at the end of the 19th century (Lopes-Lima *et al.*, 2014). One of the earliest concerted efforts to understand freshwater mussel ecology and propagation took place at Fairport Biological Laboratory, Iowa, USA, between 1908 – 1941 (Pritchard, 2001; Lopes-Lima *et al.*, 2014). Lopes-Lima *et al.* (2014) provide an excellent review of important events in freshwater mussel biology, ecology and conservation research in which they describe the importance of concurrent and subsequent works by various groups and individuals; notably those of Ortmann (1911a; 1911c; 1911b; 1912), Haas (1948), Bauer (1979; 1988; 1998) and Bauer & Wächtler (2001), Barnhart (2003; 2004; 2006), Bogan (1998; 2008) and Bogan & Roe (2008), Neves; Neves & Widlak (1987), Gatenby *et al.* (1996), Beaty & Neves (2004), Hanlon & Neves (2006), Hua *et al.* (2013), and Strayer (1999a; 1999b), Sparks & Strayer (1998) and Newton *et al.* (2008).

These works have gone a long way to describe bivalve biology, ecology and propagation but have mainly focused on unionid species. This is understandable considering that much of this early work was carried out in the USA and unionids make up the majority of the North American freshwater mussel fauna. Conservation of freshwater mussels is particularly difficult as they have complex life histories involving a parasitic stage on a wide range of hosts (usually fish), can occur in complex species assemblages which may have taken decades to establish, and may have very specific water quality requirements or be sensitive to different stressors at different life history stages. The freshwater pearl mussel *M. margaritifera* is classified as critically endangered (Moorkens, 2011a) and declined by over 90 % during the 20th Century (Bauer, 1988). Consequently, *M. margaritifera* has become the subject of increased research interest, particularly in Europe where the majority of remaining populations are found. The

life history and ecology of this species have been extensively studied since the early 1980's, e.g. Bauer (1979); Bauer *et al.* (1980); Valovirta (1980); Young & Williams (1983); Young & Williams (1984a, 1984b); Bauer (1987a, 1987b); Bauer & Vogel (1987); Buddensiek (1989), and more recently the topic of captive breeding (propagation) has become popular as an increasing number of programmes have been initiated to save dwindling populations e.g. Hastie & Young (2003a); Lange (2005); Preston *et al.* (2007); Schmidt & Vandr  (2010); Thomas *et al.* (2010); Gum *et al.* (2011); Moorkens (2011b); Scheder *et al.* (2011); Scriven *et al.* (2011); Eybe *et al.* (2013); Scheder *et al.* (2014); Simon *et al.* (2015); Gui *et al.* (2016); Lavictoire *et al.* (2016). Previous ecological and environmental studies have tended to focus on conditions in the wild and those affecting adult mussels. Whilst some have focused on benthic habitats and the effects on juvenile distribution and survival, e.g. Buddensiek *et al.* (1993); Geist & Auerswald (2007);  sterling *et al.* (2008); Moorkens & Killeen (2014), there is still a paucity of research in this area. Similarly, propagation programmes for *M. margaritifera* are still in their infancy and because juveniles take a long time to grow and become sexually mature, concrete evidence of large-scale captive rearing successes are not widely published. Studies on releases of captive-bred *M. margaritifera* are even more scarce as only a handful of research groups have reached this stage, e.g. Buddensiek (1995); Hruška (1999); Preston *et al.* (2007); Wilson *et al.* (2011); F. Thielen (pers. comm.). It has also become apparent that different rearing methods have variable success rates in different countries and that methods showing success at one location may not necessarily work at others (Thielen *et al.*, 2015). This highlights the need for further investigative work to clarify the primary factors affecting survival of juvenile *M. margaritifera* so that propagation practitioners can refine and standardise rearing systems. The following sections serve to introduce the freshwater pearl mussel and the factors affecting survival in the wild, as well as to summarise current methods of captive breeding and their relative successes. Finally, the thesis objectives and structure is provided.

1.2. The freshwater pearl mussel *Margaritifera margaritifera*

1.2.1 Life history, distribution, habitat requirements and factors causing decline

The freshwater pearl mussel (*Margaritifera margaritifera*) is a large freshwater bivalve occurring in very clean, highly oxygenated, low-calcium, neutral to slightly acidic, fast flowing

rivers and streams (Skinner *et al.*, 2003; Geist & Auerswald, 2007). Within these habitat parameters, the species has a holarctic distribution (Young *et al.*, 2001) and is classified as critically endangered in the UK (Seddon *et al.*, 2014) and throughout Europe (Moorkens, 2011a). The species is long-lived, slow-growing and requires a host fish in order to complete its life cycle (Fig. 1.1). Throughout this thesis, the term ‘adult’ refers to mussels which are sexually mature and ‘juvenile’ to those which are not. Juveniles are thought to become sexually mature at around 12 years old, or when shell length reaches approximately 6.5 - 7 cm (Young & Williams, 1984a). The freshwater pearl mussel is typically dioecious (Skinner *et al.*, 2003) but may become hermaphroditic at low population levels (Bauer, 1987b). Each year, male mussels release sperm between May – July, which are inhaled by female mussels during normal filtering activity. Sperm fertilise eggs which are brooded within the female’s gills for several weeks before mature larvae (glochidia) are released into the water column, typically in a temperature-dependent, synchronised event between July – September (Hastie & Young, 2003b). Glochidia must encyst upon a salmonid fish gill, typically salmon, *Salmo salar* (Linnaeus, 1758), or brown/sea trout, *Salmo trutta* (Linnaeus, 1758) in order to continue development. Once encysted within the fish gill epithelium, glochidia remain there for around 9 - 10 months and grow to around 5 – 6 times their original size (typically from approximately 0.07 – 0.08 mm to 0.4 mm). Between May – July the following year, juvenile

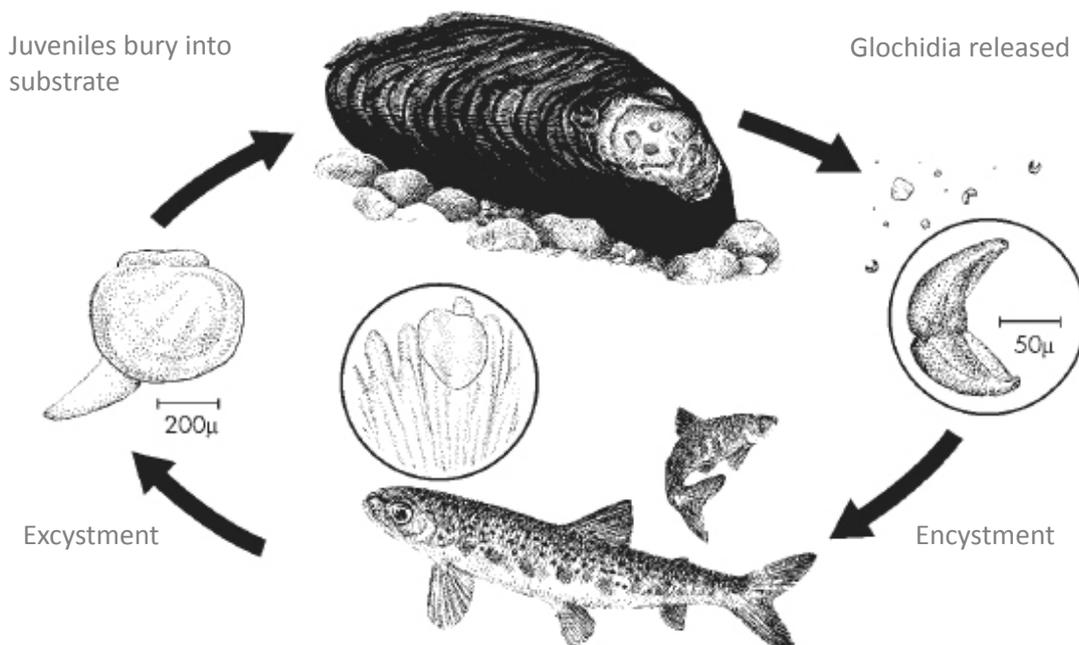


Fig. 1.1: Diagram showing the life cycle of the freshwater pearl mussel (*Margaritifera margaritifera*) showing glochidia release, encystment on a salmonid host and eventual excystment into river gravels. Modified from Skinner *et al.* (2003).

mussels excyst (drop off) the fish gills and must fall into suitable habitat if they are to continue growth and development into adult mussels.

M. margaritifera is a good indicator species as it has very high water quality requirements and only thrives in very clean, oligotrophic rivers (Young, 2005; Geist & Auerswald, 2007; Geist, 2010). The species is declining throughout its range (Young *et al.*, 2001) with > 90 % loss of individuals during the 20th Century (Bauer *et al.*, 1980) due to factors including aggravated siltation, eutrophication, loss/decline of host fish species and illegal pearl fishing (Bauer, 1988; Bogan, 1993; Young *et al.*, 2001). By far the most significant factor leading to population declines across Europe has been degradation of juvenile habitats through siltation and increased nutrient inputs (Moorkens & Killeen, 2014). As is the case throughout its range, English pearl mussel populations have seen significant declines and population extinctions since the early 20th Century (Fig. 1.2).

Juveniles are particularly vulnerable to sub-optimal habitat conditions as they inhabit substrate interstices for the first few years of their post-parasitic life. Recruiting populations are found in areas where the river bed consists of stable, well-mixed coarse substrates with low proportions of fine material (Boycott & Howell, 1898; Brim Box & Mossa, 1999; Hastie *et al.*, 2000; Morales *et al.*, 2004; Altmüller & Dettmer, 2006; Geist & Auerswald, 2007; Moorkens & Killeen, 2014) so that there is good exchange between the water column and interstitial layer

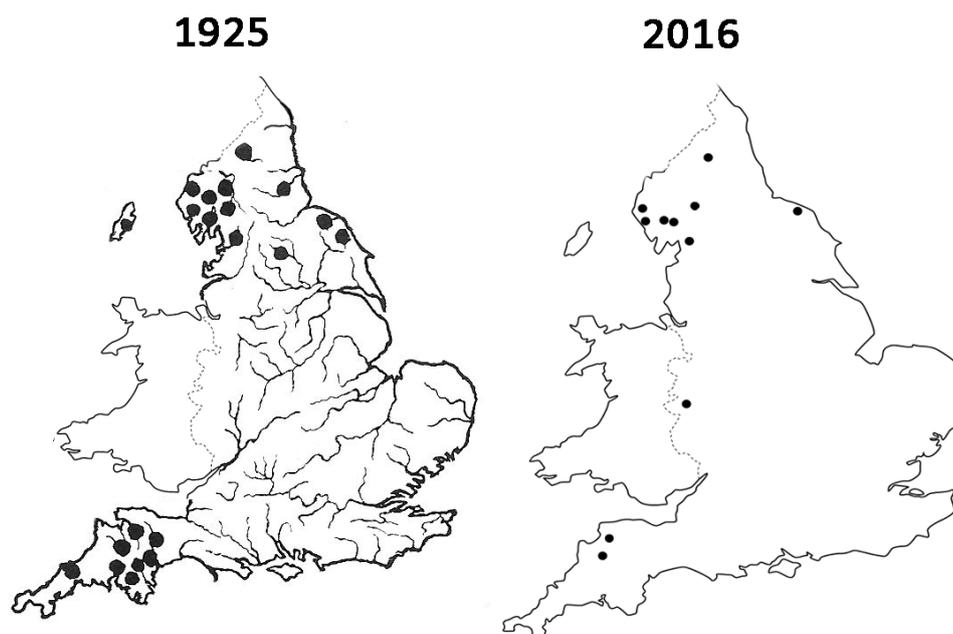


Fig. 1.2: Map of England showing location of known English freshwater pearl mussel populations reported by Jackson (1925) and those present in 2016. N.B. Size of dots not indicative of population size.

(Buddensiek *et al.*, 1993; Geist & Auerswald, 2007). This provides juveniles with sufficient food and oxygen (Brady, 2000). Where this is not the case, low dissolved oxygen (DO) concentrations may cause mortality due to asphyxiation but may also cause behaviours which make juveniles more susceptible to being washed away, e.g. moving to the surface in search of higher DO conditions (Sparks & Strayer, 1998; Moorkens, 2011b). Such behavioural changes can also have energetic consequences as juveniles spend time searching for suitable habitat conditions rather than foraging.

1.2.2 Taxonomy

It is believed that the Margaritiferidae radiated from Asia (Bauer, 2001; Smith *et al.*, 2001) and members of the family are known from the North American continent, Europe (including northern-most Africa), the Middle East and much of south and east Asia (Smith *et al.*, 2001). Taxonomic studies of freshwater mussels foster lively debate and the relationships between factions of the Unionida are being regularly revised e.g. Smith *et al.* (2001); Carter *et al.* (2011). Many scholars believe the Margaritiferidae to be a primitive group within the Unionida (Ortmann, 1911c; Hannibal, 1912; Graf & Ó Foighil, 2000; Bauer, 2001; Smith *et al.*, 2001), but this stance has been challenged by some e.g. Heard & Guckert (1970); Hoeh *et al.* (2001). More recent molecular studies tend to place the Margaritiferidae as a sister taxa of the Unionidae, but as new evidence comes to light this position may change (Graf & Cummings, 2007).

The main features which differentiate the Margaritiferidae from other families were outlined in the early 20th Century by Ortmann (1911b):

- Incomplete diaphragm
- Incomplete fusion of mantle margins resulting in failure to form separate siphons
- Lack of complete septa in the gills (and subsequent lack of water tubes)
- All four demibranchs marsupial
- Small size of hookless glochidia

Matrices of morphological characters (Graf & Ó Foighil, 2000), particularly brooding characters (Cannuel & Beninger, 2006) have been analysed with genetic data to produce evolutionary trees for the Unionida e.g. Carter *et al.* (2011). However, at present there is insufficient agreement between researchers to achieve high phylogenetic resolution for the Order (Graf, 2013). To construct robust phylogenetic hypotheses, consideration of the 'whole picture' is needed. This

requires combining information from all pertinent sources in the areas of genetics, anatomy, morphology, behaviour and paleontology in order to build robust hypotheses on phylogeny e.g. Bogan & Roe (2008). Different scholars have based their hypotheses on a range of character features. For example the early work of Ortmann (1911c) and later Heard & Guckert (1970), based their taxonomic revisions primarily on soft-part anatomy and reproductive habits, and ignored conchological (shell) features, believing them to be too variable within species (Ortmann, 1911c; Smith, 1976). Walker *et al.* (2001) based their suggestions upon the differences in larvae (glochidia and lasidia), Beninger & Dufour (2000) consider the abfrontal gill surface and mucocyte distribution, Atkins (1938), Owen (1978) and Beninger *et al.* (1994) discuss the usefulness of laterofrontal cirri in phylogenetic studies whereas Smith (1986) tried (unsuccessfully) to separate Margaritiferids by considering stomach anatomy.

Characters for which there is open debate about their supposed ancestral or derived states are outlined in Table 1.1 with references arguing their individual cases. New investigations into juvenile anatomy and development could provide an important missing link between traditional biological and modern genetic studies and inform taxonomic debates.

1.3. Captive rearing strategies

Captive rearing is seen as a last resort strategy when habitat improvements are unlikely to deliver significant benefits to mussel populations within an appropriate timescale or where population numbers are so low that reproduction in the wild is not feasible (Neves, 2004). The aim of reintroduction/augmentation of propagated juveniles is to enhance long term population survival, to re-establish a keystone species and to establish viable populations in the wild within the species' former range (IUCN, 1998; Lyons *et al.*, 2005). Some populations of *M. margaritifera* require immediate attention if they are to persist and some countries have therefore undertaken captive rearing programmes as a short term strategy until catchment pressures can be alleviated. Different captive rearing strategies have been employed in different countries with some success. The methods used depend upon a range of factors including available resource (money, time, expertise and facilities), population-specific conservation priorities, and local environmental conditions.

A major benefit of mussel culture is that environmental parameters can be controlled in order to optimise habitat and ultimately increase juvenile survival. Regardless of the already

Table 1.1: Defining character state descriptions for *M. margaritifera* and references which argue their ancestral or derived origins showing there is still debate about the origins of *M. margaritifera*.

Character state	Ancestral	Derived
Tetrageny – use of all 4 demibranchs to brood glochidia	Ortmann (1911b; 1911c); Heard & Guckert (1970); Hoeh <i>et al.</i> (2001)	Hoeh <i>et al.</i> (1998); Graf & Foighil (2000)
Endobranchy – Use of only the inner pair of demibranchs for brooding	Hoeh <i>et al.</i> (1998)	Heard & Guckert (1970); Hoeh <i>et al.</i> (2001)
Ectobranchy – Use of only the outer pair of demibranchs for brooding	Graf & Foighil (2000)	Heard & Guckert (1970); Hoeh <i>et al.</i> (1998); Hoeh <i>et al.</i> (2001)
Incomplete diaphragm	Ortmann (1911b; 1911c)	Graf & Foighil (2000); Hoeh <i>et al.</i> (1998)
Interlamellar junctions (rather than complete septa)	Ortmann (1911b; 1911c)	Hoeh <i>et al.</i> (1998); Graf & Foighil (2000)
Bradytictia - Long term brooding over winter before glochidial release the following spring		Ortmann (1911b, c); Graf & Foighil (2000)
Tachytictia - Short term brooding during spring before glochidia released in summer	Ortmann (1911b)	
Lack of supra-anal aperture		Graf & Foighil (2000)

substantial research activity into captive rearing of *M. margaritifera*, relatively little empirical information is available regarding parameters critical to juvenile survival. In order of least to most resource-intensive, these methods include:

1. Bankside encystment of resident salmonids for immediate release. This strategy is only successful if employed where habitat conditions are sufficient to support juvenile mussels (Altmüller & Dettmer, 2006).
2. Allowing juveniles to excyst directly into gravels within raceways before placing out into rivers as older juveniles (Preston *et al.*, 2007; Moorkens, 2011b). A recent modification of this method has been proposed by Moorkens (2015), whereby juveniles excyst into clean gravels which are immediately transported to mussel rivers and introduced into suitable habitat patches.
3. Collection of juveniles and culture during the first growth period up to a size of > 1 mm length before transfer into “Buddensiek cages” or boxes either in raceways or rivers e.g. Buddensiek (1995); Schmidt & Vandr  (2010); Lange & Selheim (2011).

4. Collection of juveniles and subsequent transfer into holding trays/baskets, (Hastie & Young, 2003a; Taylor, 2007; Lange & Selheim, 2011; Scriven *et al.*, 2011; Sweeting & Lavictoire, 2013) or incubators (Hruška, 1999; Lange, 2005; Eybe *et al.*, 2013) for growth and release as older juveniles.

Gum *et al.*, (2011) provide a useful review of *M. margaritifera* captive rearing programmes, their methods and rearing successes in Europe. Studies from the USA on unionid species employ a number of additional methods for species with less specific and/or demanding habitat requirements and include transfer of larger juveniles to different systems more appropriate to their larger size and more advanced stage of development (Barnhart, 2015).

The type of captive rearing activities undertaken depends upon the specific set of threats within a mussel population or area. For example, where population numbers are not yet critically low but sustainable recruitment is not taking place, catchment restoration coupled with bankside encystment and release of encysted fish may be the most appropriate option e.g. Altmüller & Dettmer (2006). In contrast, in catchments with more complex environmental issues which will take several years to address, or where pearl mussels are sparsely distributed, aggregating adult mussels in captivity such as the FBA's Freshwater Pearl Mussel Ark (Chapter 2) may be the only way to safeguard remaining individuals whilst improving the probability of successful fertilisation between male and female mussels (Downing *et al.*, 1993). This strategy also decreases the probability of mussels becoming hermaphroditic and potentially impacting population genetic diversity. Similarly, where populations consist of slightly higher numbers, captive rearing programmes need to consider the implications of broodstock selection and using different batches of mussels for broodstock to reduce the potential consequences of inbreeding depression and loss of genetic diversity (Neves, 2004; Jones *et al.*, 2006; Geist, 2010) as well as maximising reproduction if females display different levels of fecundity (Hanlon, 2000; Mummert, 2001).

Overall rearing success appears to depend upon survival within the first few months post-excystment (Gum *et al.*, 2011) with mortality within the first few weeks being particularly high (Gatenby *et al.*, 1996; Gatenby *et al.*, 1997; O'Beirn *et al.*, 1998; Jones *et al.*, 2005). Survival may be linked to pre-winter size (Buddensiek, 1995; Denic *et al.*, 2015) with some researchers suggesting juveniles should be reared to a minimum size of 1 mm before the onset of the first winter (Lange, 2005; Lange & Selheim, 2011; Eybe *et al.*, 2013). Whilst this stage is

clearly vital, steady mortality of juveniles appears to occur until juveniles are approximately 3 years old, after which mortality is generally lower (pers. obs.). This may be due to other important ontogenic stages such as transformation from pedal to filter feeding (see Chapter 5) or changing habitat requirements with age.

Captive rearing practices for *M. margaritifera* have typically favoured the more resource-intensive methods (numbers 2, 3 and 4 above) due to the specific habitat requirements of the species. Some programmes favour the use of flow-through systems where untreated river/lake water is used with no supplemental feeding e.g. Preston *et al.* (2007); Moorkens (2011b); Lavictoire *et al.* (2016), whilst others use recirculating or static systems which are supplemented with artificial diets e.g. Lange (2005); Lange & Selheim (2011); Capoulade (2012); Eybe *et al.*, (2013). Only a small number of these programmes have reared juveniles to an age where release of free-living individuals has been trialled e.g. Wilson (2010); F. Thielen (pers. comm.), and it is too early to tell if these juveniles will survive to sexual maturity and contribute to the next generation.

1.4. Thesis outline and objectives

The aim of this thesis is to investigate factors affecting juvenile mussel survival at the Freshwater Biological Association's Freshwater Pearl Mussel Ark project (see Chapter 2) in order to tailor rearing techniques and ultimately improve survival. As mentioned in section 1.1, despite over 30 years of attempts to refine rearing methods for *M. margaritifera*, there is still a lack of information on juvenile ecology, ontogeny and captive rearing in general. Examples of unanswered questions include:

- Juvenile mussel ecology:
 - What biotic and abiotic parameters affect juvenile mussels and how do they affect survival?
 - What parameters are juveniles particularly sensitive to?
 - What is categorised as 'good' juvenile mussel habitat and do habitat requirements change with age/size?
 - Juvenile mussel ontogeny:
 - What developmental stages do juveniles go through post-excystment from the host?
 - How do these stages affect behaviour and the ability of juveniles to survive?
 - How may ontogenic factors affect captive rearing practices?
-

- Captive rearing:
 - How can conditions in captivity be improved to maximise survival whilst bearing in mind financial and practical constraints?
 - What are the main advantages/disadvantages of different captive rearing methods and can they be tailored to juveniles at different ages/developmental stages to increase survival?
 - How robust are captive-bred juveniles and once they are sexually mature, do they contribute to subsequent generations?

Investigations in this thesis will focus on three main topics, which are outlined below and in the thesis structure diagram (Fig. 1.3):

1. **What are the key environmental factors affecting juvenile survival and growth in captivity?** This work will assess the suitability of a flow-through captive rearing system for newly-excysted juveniles and will investigate the importance of parameters such as substrate size, cleaning regime, interstitial dissolved oxygen, interstitial ammonia concentration and interstitial flow on juvenile growth and survival. These experiments seek to investigate which parameters are most important to juvenile survival so that captive rearing programmes may be tailored to juvenile requirements in order to improve overall survival rates – Chapters 3 & 4.
2. **What ontogenic stages do juvenile mussels undergo in their early post-parasitic life and how might these affect feeding behaviour, the switch from pedal to filter feeding, and survival?** This work seeks to describe biological development of juvenile mussels between the ages of 1 – 44 months old, compare feeding behaviour at different developmental stages, and investigate the period when juveniles are thought to switch from pedal to filter feeding. This work is the first known attempt to describe the development of juvenile *M. margaritifera* at different ages in order to better understand the potential factors limiting survival – Chapter 5.
3. **Is fluorescence marking with calcein a feasible method to improve monitoring of immature stages of *M. margaritifera* in captivity?** This work will consider if batch-marking of juvenile mussels using the fluorophore calcein is a useful tool to improve monitoring in captive rearing programmes. This work has the potential to enhance efficiencies within captive rearing programmes making them more time- and cost-effective – Chapter 6.



Fig. 1.3: Diagram showing the structure of this thesis outlining facilities and pilot studies (Chapter 2), experimental chapters investigating substrate parameters (Chapters 3 & 4), juvenile biological development (Chapter 5), a method for batch-marking juvenile mussels (Chapter 6) and a final discussion chapter putting these findings in to context of the current knowledge of freshwater pearl mussel captive rearing and conservation.

Experimental work took place at the captive rearing programme run by the Freshwater Biological Association which is described in more detail in Chapter 2 and a synopsis of findings and the wider implications of this work are covered in the final discussion (Chapter 7) along with suggested research priorities going forward.

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Chapter 2

Freshwater Pearl Mussel Ark project and pilot studies

2.1. Introduction

This chapter describes the captive rearing facilities and the Freshwater Pearl Mussel Ark project at the Freshwater Biological Association (FBA) and also describes two pilot studies undertaken to inform the investigations carried out in Chapters 3 and 6 of this thesis.

2.1.1 Freshwater Pearl Mussel Ark project and the FBA captive rearing facility

In 2007 the FBA commenced a captive rearing programme entitled the Freshwater Pearl Mussel Ark project ('Ark project' or 'Ark') for priority populations of the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) in England. In 2009, after two years of limited success rearing juveniles, work on this thesis commenced to investigate ways of improving captive rearing techniques for the species and increase survival for populations held at the FBA. At the time of writing, the project is ongoing and juvenile mussels are reared using captive adult mussels from each population as broodstock. All rearing activities take place at the FBA's Windermere site. The Ark project's aims are to:

1. Maintain in captivity adult mussels from all priority English populations in captivity to provide a genetic 'Ark' to safeguard against local population extinction.
2. Rear juvenile mussels using captive adult mussels as broodstock.
3. Reintroduce juvenile mussels into suitable habitat in rivers from which the parent mussels originated.

Up until 2014, nine populations were housed at the FBA and breeding activities attempted with all present populations in all years. In 2014, surviving individuals from two populations were returned to their native rivers due to poor survival and breeding activity at the FBA. During 2015 all individuals from a further population died, leaving six remaining populations at the FBA at time of writing.

The FBA Ark uses water from Windermere, a mesotrophic lake, which is filtered to 30 μm (adult mussels and fish) or 20 μm (juvenile mussels). The rearing system encompasses all life cycle stages of *M. margaritifera* from glochidia encystment on salmonid hosts through to excystment (drop-off) and on to medium-term (up to 10 years) juvenile maintenance and monitoring (Fig. 2.1). Adult mussels are kept within gravels in stainless steel cages in 1.6 m diameter fish tanks (<https://www.youtube.com/watch?v=qPhFJUva2vc>). Around the time of

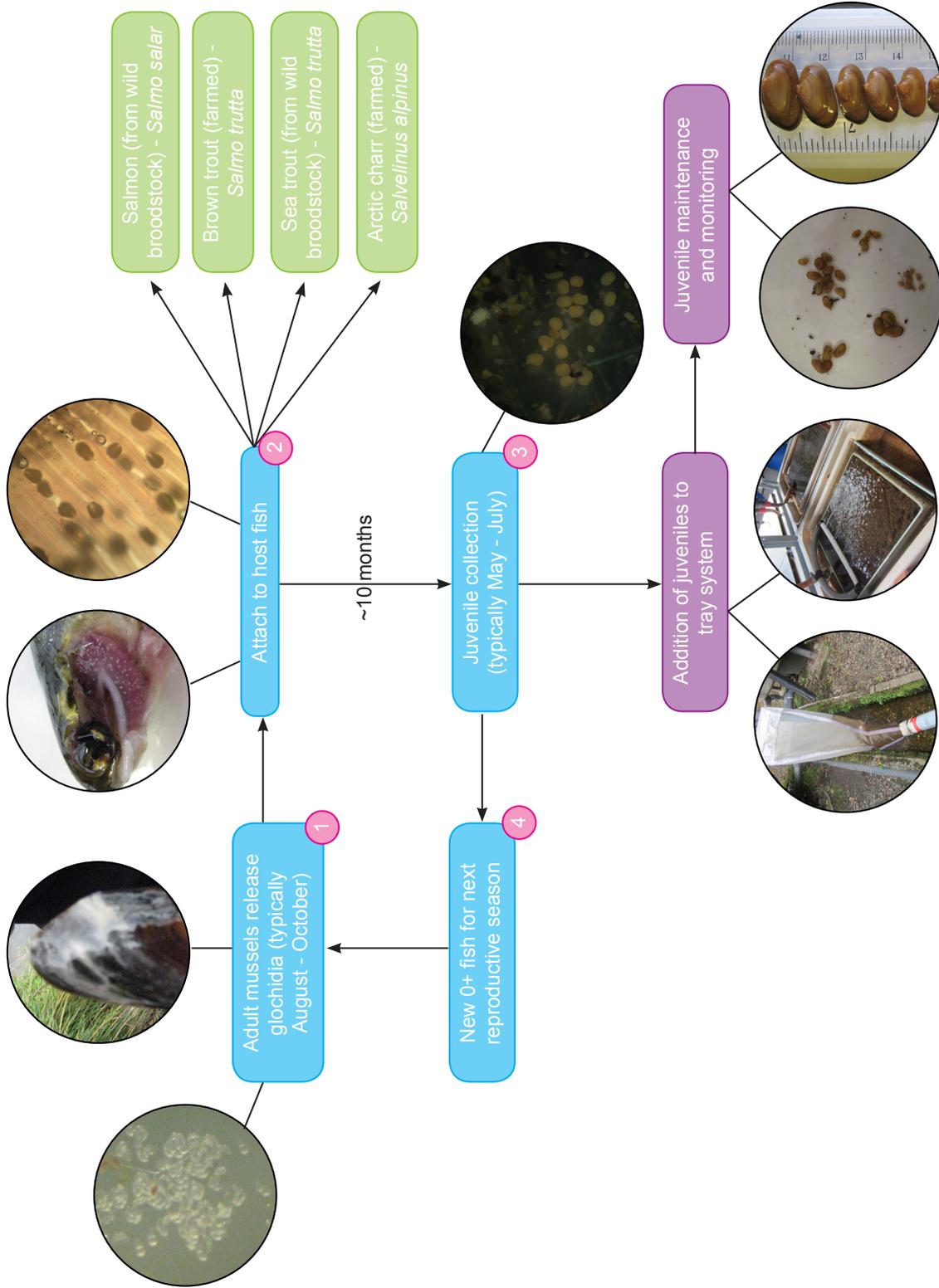


Fig. 2.1: Diagram of the captive rearing system at the FBA Freshwater Pearl Mussel Ark. Adult mussels are kept at the FBA for population security purposes and for use as broodstock. Depending upon the population, salmon, brown/sea trout or Arctic charr are encysted with glochidia. Juveniles are then collected and transferred to trays to continue growth.

glochidia release, host fish can be kept either in the same tank as mussels or in an adjoining tank. In some populations more than one host fish has been suitable for glochidial development. In these cases, two or more tanks can be connected so that the effluent from the tank containing mussels and one fish species can run directly into the adjoining tank meaning that several different host fish species may be used. At the Ark, adult mussels release glochidia typically between mid-August to mid-October each year (Lavictoire *et al.*, 2014). This is later than observed in lotic environments due to the temperature regime of the lake being different from rivers with respect to different temporal patterns; Windermere warms up slower in spring/summer and cools down later in autumn/winter with extremes of temperature minimised. Work in previous years at the Ark has established suitable host fish species for each mussel population so that in subsequent years, only the most suitable host is provided. The most suitable hosts have primarily been salmon (*Salmo salar*) or brown/sea trout (*Salmo trutta*), but trials on Arctic charr (*Salvelinus alpinus*) have also had some success in some populations (Miles & Sweeting, 2011). Glochidia of *M. margaritifera* over-winter on fish and typically begin excysting around mid-May in the year following encystment (temperature dependent). At this time, plankton nets with a removable plastic bottle at the terminal end are used to collect excysting juveniles on a daily basis. From these bottles, juveniles are transferred into trays (Fig. 2.1) containing substrate measuring 1 - 2 mm. Substrate in this system and for all investigations in this thesis was obtained from the western shore of Windermere (Cumbria). Substrate is left to air-dry before being sieved into different size clasts with a Fritsch Analysette sieve shaker. Trays containing juveniles are cleaned every two weeks and regular monitoring of survival and growth takes place twice per year.

2.2. Pilot studies

Mussels from the River Ehen (Cumbria) lineage were used for all experimental studies detailed in this thesis. These mussels provide the Ark project with a 'control' population against which to compare the performance of other mussel populations in captivity. The river Ehen population is the largest and healthiest pearl mussel population in England and juveniles from this population provide opportunities for research in order to refine captive rearing techniques. Two pilot studies were performed to refine methods for further investigations described in

Chapters 3 and 6. The pilots are described in the following sections, and a brief outline of results is provided. Pilot studies sought to investigate:

- The effect of substrate size, depth and cleaning regime on growth and survival of juvenile mussels (to inform Chapter 3), and;
- The success and usefulness of using the fluorophore calcein to mark juvenile mussels in captivity (to inform Chapter 6).

2.2.1 The effect of substrate size, depth and cleaning regime on growth and survival of juvenile mussels

2.2.1.1 Method

This experiment sought to investigate the effect of substrate size, substrate depth and cleaning regime on growth and survival of newly-excysted juveniles. In spring 2011, five aquaria (600 x 297 x 300 mm) were set up as shown in Fig. 2.2 and supplied with water filtered to 20 µm. Plastic styrene sheets were cut to fit inside each aquarium (600 x 297 mm) and holes were cut to comfortably house an effluent water pipe and 12 square *Artemia* sieves (Hobby, Germany). *Artemia* sieves consist of plastic sides and a plastic mesh bottom and were used to house substrate and juveniles. The styrene sheet was fixed to aquaria sides with EVO-STIK Wet Grab sealant (Bostick, UK). This product had been used previously in culture trays with no detrimental effects observed on juvenile survival. Gasket tape (10 x 10 mm) was stuck around the top lip of the sieves, which were then inserted into the holes so the gasket tape formed a loose seal with the styrene sheet in order to encourage water to flow through the substrate. Water was provided via a spray bar and flowed from the top chamber (above styrene sheet) into the bottom chamber (below styrene sheet), creating a down-welling, flow-through system.

The parameters investigated were:

- **Substrate size:** small (S1: 0.3 - 0.5 or S2: 0.18 - 0.5 mm); medium (M: 0.5 - 1 mm); large (L: 1 - 2 mm); mixed (MIX1: 0.3 - 2 or MIX2: 0.18 - 2 mm).
 - **Substrate depth:** 0 cm(enough substrate to just cover the mesh); 1 cm; 3 cm.
 - **Cleaning regime:** 2 months; 6 months; undisturbed (sampled at end of experiment at 10 months).
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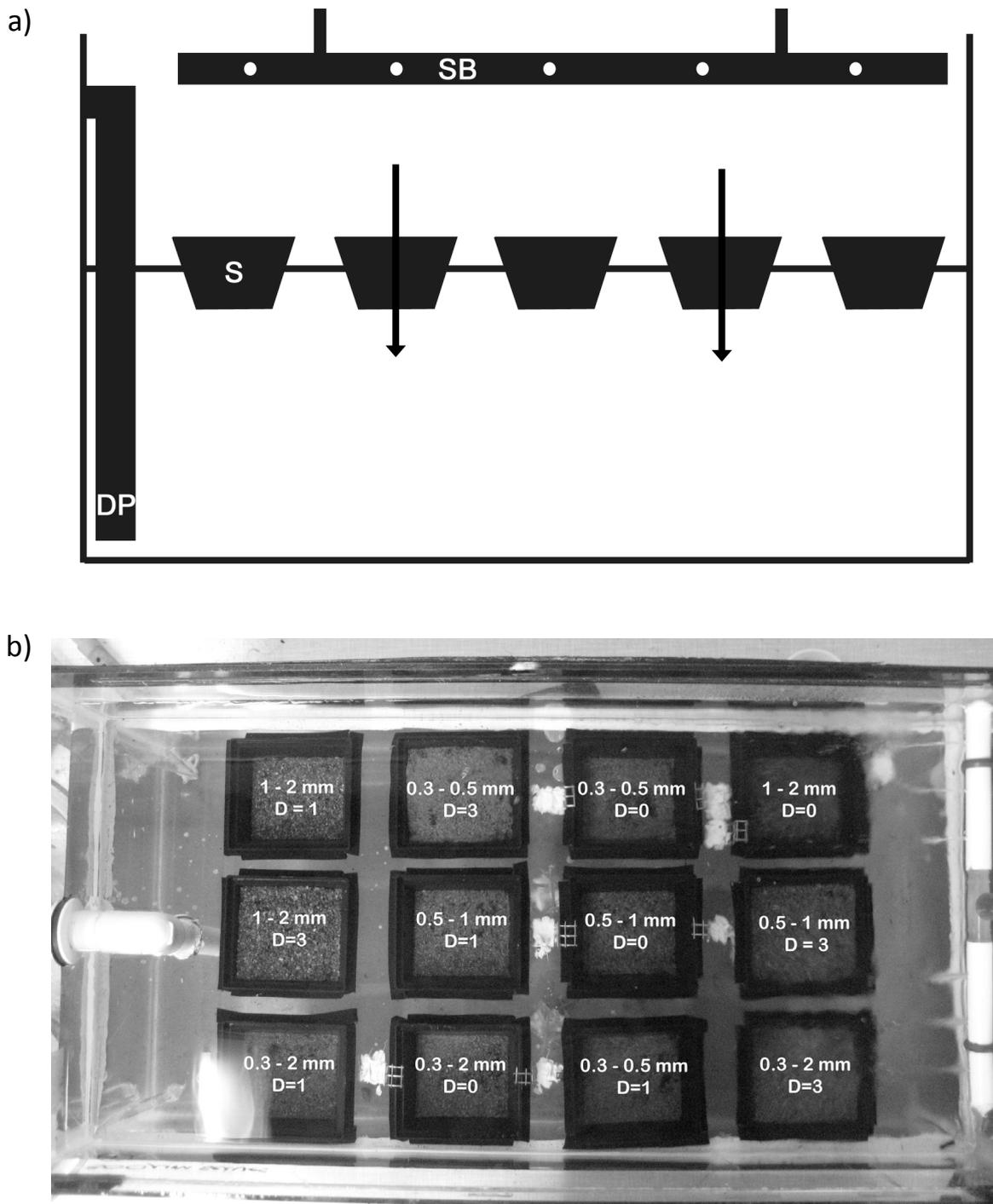


Fig. 2.2: Diagrammatic representation (a) and photograph (b) of substrate pilot experimental set-up in an aquarium; (a) Water enters at the top of the system via a spray bar (SB) and flows through sieves (S) containing substrate and juveniles (arrows show direction of flow). Water exits from the bottom chamber via the downpipe (DP); (b) Sieves were filled with different substrate clasts (0.3 - 0.5, 0.5 - 1, 1 - 2 & 0.3 - 2 mm) and to different depths (D = 0, 1 & 3 cm).

Aquarium 1 was set up as outlined in Fig. 2.2 and sieves with a mesh size of 0.3 mm were used to hold gravels and juveniles. Sieves in this aquarium were cleaned and sampled every two months. Aquaria 2 and 3 were set up with the same treatments as aquarium 1 but sieves in aquarium 2 were cleaned and sampled at six & ten months and in aquarium 3 at ten months. Sieve position was randomly selected and different in each aquarium.

Aquaria 4 and 5 tested the S2 and MIX2 substrate treatments and so required sieves with a mesh size of 0.18 mm, again with the same treatments described above but with the 'small' treatment containing substrate measuring 0.18 - 0.5 mm. Aquarium 5 contained the same treatments as in aquarium 4 with an additional three sieves containing an extra replicate each for the small (0.18 - 0.5 mm) substrate size at 0, 1 & 3 cm depth (15 sieves in total). Aquarium 4 was cleaned and sampled every two months and aquarium 5 cleaned and sampled at six and ten months.

Substrate was exposed to running water for at least five days prior to the experiment beginning in order for a biofilm to begin to establish on the sediment as food for juveniles. One hundred active juveniles were added to each sieve (6,300 individuals total), of which 30 from each sieve were measured (length and height; Fig. 2.3).

At two-month intervals, all sieves from aquaria 1 and 4 were checked exhaustively for juveniles. This was done by washing small amounts of substrate into a petri dish and checking for juveniles under a low-power microscope. This process was repeated until all substrate within the sieve had been checked. The number of live and dead individuals was recorded.

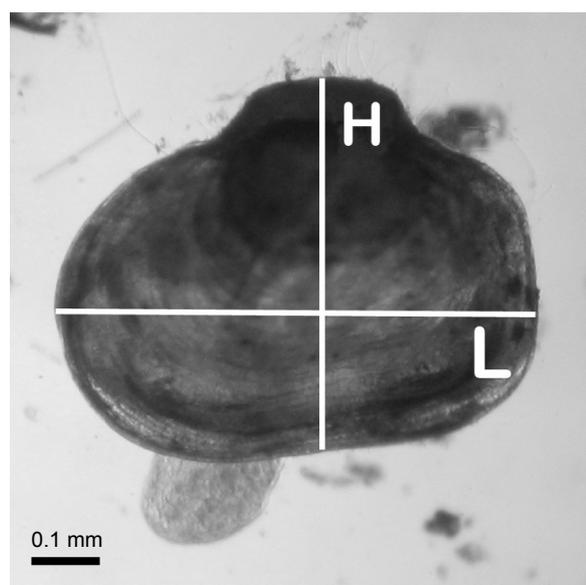


Fig. 2.3: Light micrograph of juvenile mussel showing length (L) and height (H) measurements taken for each individual.

Any discrepancy between the number of juveniles found (alive plus dead) and the original number in the sieve was recorded as 'unaccounted'. The length and height of 30 live individuals was recorded. Where fewer than 30 individuals remained, all individuals were measured. The same process of checking was carried out on aquaria 2 and 5 after six and ten months and on aquaria 3 after ten months. Dead individuals were measured, removed and were assigned to a 'degradation class' (Fig. 2.4) to indicate how degraded shells were when they were found. This was measured using a 4-point scale:

1. Recently dead individual. Shell still has all of its calcium present. Soft tissue may still be present inside the valves.
2. Less than $\frac{1}{4}$ of the shell dissolved.
3. More than $\frac{1}{4}$ of the shell dissolved. Some parts of the shell may be transparent.
4. No calcium present. Shell appears skeletal and transparent.

2.2.1.2 Data analysis

Throughout this thesis the statistical software SPSS (versions 19 - 22, IBM) was used for analysis unless otherwise specified. Data were checked for normality (Shapiro-Wilk test) and one-way Analysis of Variance (ANOVA) with *post hoc* Tukey's HSD tests were used to analyse survival differences between treatments on the same sampling occasion where data were normal. Where data were not normal, Kruskal–Wallis tests were employed. Unless otherwise stated

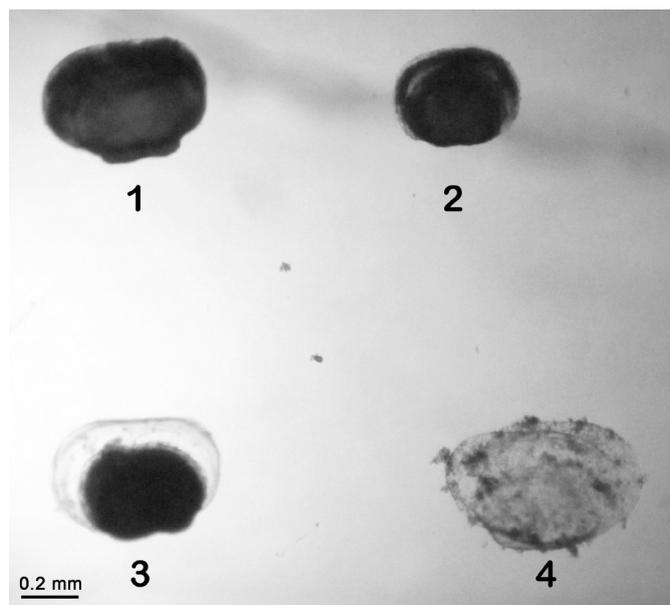


Fig. 2.4: Light micrograph of dead freshwater pearl mussel shells showing degradation classes; 1 = recently dead, no degradation; 2 = less than $\frac{1}{4}$ dissolved; 3 = more than $\frac{1}{4}$ dissolved; 4 = No calcium present, shell transparent.

standard deviation is given after mean values. Length and height are highly correlated in *M. margaritifera* (see Chapter 3) so only length was used for analysis in both pilot studies.

2.2.1.3 Results

Original length of juveniles were significantly different between sieves from aquaria 1 and four ($F_{(23,696)} = 2.825, P < 0.001$) meaning that size could not be used as an accurate indicator of differences in juvenile performance over the course of the experiment. Size data can however provide a useful general benchmark for how much juveniles can grow within ten months post-excystment under the conditions described. Considering all juveniles measured, juveniles grew from a mean initial length of 0.45 mm (± 0.04) on approximately 22 June 2011 to 0.63 mm (± 0.06) on approximately 2 May 2012 (Fig. 2.5).

None of the treatments provided conditions suitable for adequate juvenile survival to ten months old. A summary of survival in aquaria 1 and 4 is provided in Table 2.1. Of the 6,300 individuals added to the system initially only ten individuals were found after ten months across all treatments and aquaria (0.16 %). Survival was recorded every two months in aquaria 1 and 4, providing opportunities for further analysis. Considering only aquarium 1 and ignoring any effect of substrate depth, there was a significant difference in survival between substrate size clasts in August 2011 ($F_{(3,8)} = 8.891, P = 0.006$) and October 2011 ($F_{(3,8)} = 5.623, P = 0.023$) but not in December 2011 ($F_{(3,8)} = 2.613, P = 0.123$), February 2012 ($\chi^2_{(3)} = 5.50, P = 0.139$) or April 2012 ($\chi^2_{(3)} = 4.156, P = 0.245$). For August and October 2011, *post hoc* tests showed that juveniles in the 1 - 2 mm substrate treatments displayed higher survival compared with all other treatments. In aquarium 4, survival was not significantly different in any of the size classes in any month; August 2011 ($F_{(3,8)} = 0.749, P = 0.553$), October 2011 ($F_{(3,8)} = 3.031, P = 0.093$), December 2011 ($\chi^2_{(3)} = 5.144, P = 0.162$), February 2012 ($\chi^2_{(3)} = 1.745, P = 0.627$) or April 2012 ($\chi^2_{(3)} = 2.750, P = 0.432$).

When substrate size is ignored, there was no significant difference in survival at 0, 1 and 3 cm depth in aquaria 1 or 4 in any month ($P > 0.05$) but sampling did take much longer for

Table 2.1: Total survival in aquaria one and four on all sample occasions. Total survival has been rounded to whole individuals except where survival < 1 %.

	June 2011	Aug. 2011	Oct. 2011	Dec. 2011	Feb. 2012	April 2012
Total survival	2,400	1,072	629	157	32	9
Total survival (%)	100	45	26	7	1	0.38

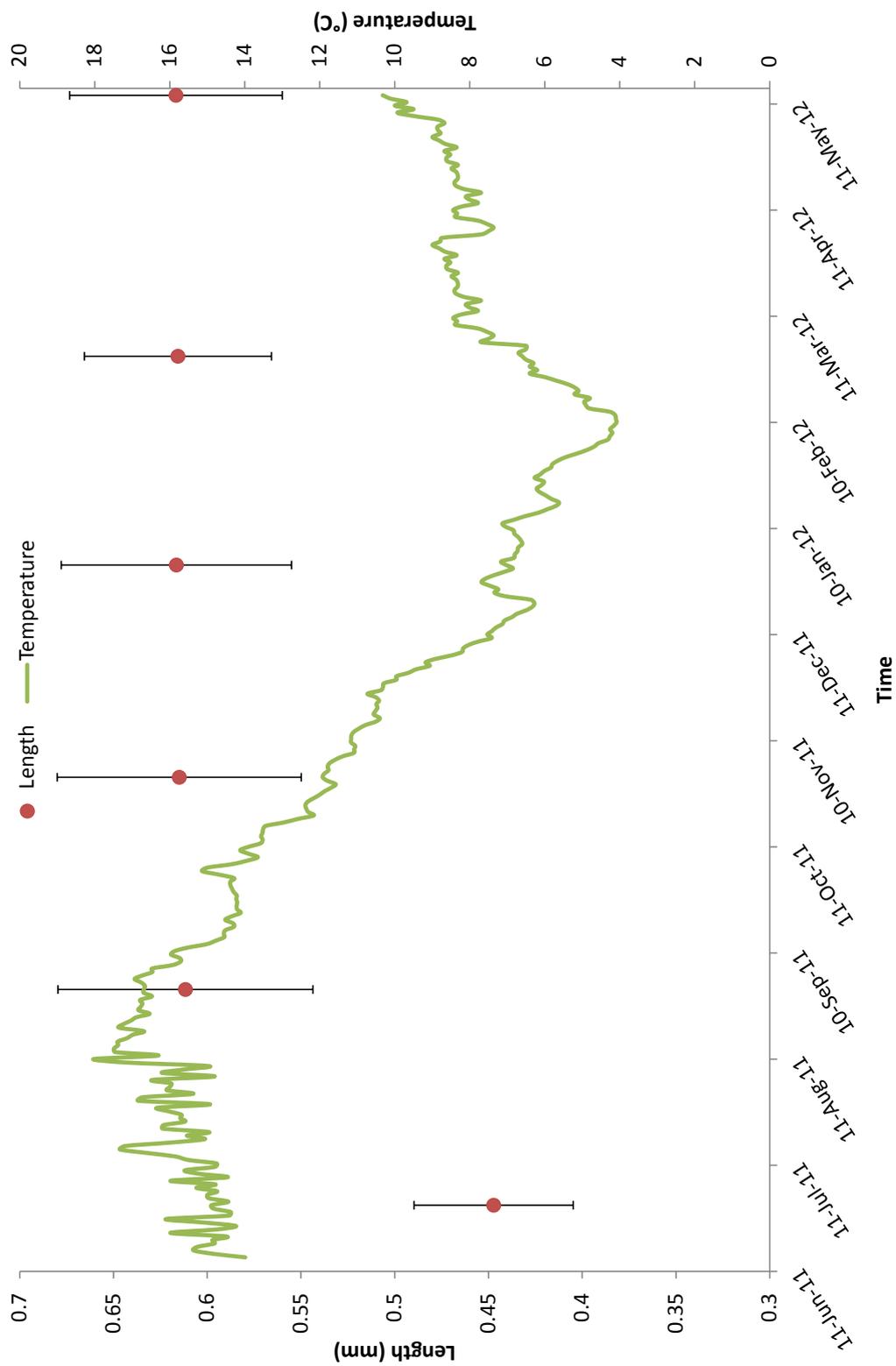


Fig. 2.5: Time series graph showing mean length of juvenile freshwater pearl mussels (SD error bars) over the 10 month experimental period. Mean daily temperature is provided to show the relationship between growth and temperature (cessation of growth as temperature decreases).

deeper treatments and those with smaller substrate clasts due to the volume of substrate (up to 8 hours per sieve for the smallest substrate sizes when $D = 3$ cm).

At the six month check in December 2011, there was no significant difference in survival between treatments cleaned every two (aquarium 1) or six (aquarium 2) months ($\chi^2_{(1)} = 0.145$, $P = 0.704$). Similarly at the ten month check (May 2012), there was no significant difference in survival between these two aquaria ($\chi^2_{(1)} = 1.338$, $P = 0.247$).

At the two month check the percentage of dead individuals assigned to degradation classes 1, 2, 3 and 4 was 46, 18, 22 and 13 % respectively. Whilst it is not possible to know exactly when these individuals died, the majority (46 %) died close enough to the 2 month sample point for the shells to degrade only slightly. Thirteen percent of individuals were assigned to degradation class 4 (shell contains no calcium) showing that it can take less than two months for shells of dead juveniles of this size to dissolve completely in this system.

2.2.1.4 Discussion

This pilot investigated factors thought to be important for juvenile survival such as substrate size, depth and cleaning regime as well as considering logistical aspects of experimental design such as sieve mesh size. As a pilot, replicate numbers were low and therefore statistical power was weak but some valuable insights were gained which informed experimental design for the final study (Chapter 3).

Care was taken to ensure healthy and active juveniles were selected for this study as juveniles excysting at the beginning of the drop-off period can be under-developed and often suffer high mortality (Jones & Neves, 2002; Schmidt & Vandr e, 2010, Eybe *et al.*, 2015). Active juveniles were taken from the middle portion of the excystment period to ensure this potential bias was not observed. Substrate depth did not appear to affect survival in this experimental system and therefore does not warrant further investigation. Similar findings have been reported for *Villosa iris* by Beck (2001) and Beaty & Neves (2004). In addition, deeper treatments took longer to clean and check (up to 8 hours), a problem also observed by Hastie & Young (2003) in some of the systems investigated in Scotland. This is an important consideration for captive rearing programmes which have finite resources.

Substrate size did appear to have an effect on juvenile size and survival in the early months of the experiment and larger substrates were easier and quicker to clean. Whilst

cleaning regime showed no differences in survival after 6 months (December 2011) or 10 months (April 2012), mortality rates were high across all treatments posing concerns that cleaning every two months was not sufficient to provide suitable scope for survival. This is likely due to build-up of organic matter blocking interstitial spaces. As a result, substrate size and the frequency of cleaning were the two parameters chosen to be investigated in further detail (Chapter 3).

2.2.2 Investigating the suitability of calcein immersion as a marking technique for juvenile mussels

2.2.2.1 Method

This pilot study was carried out between 14 September – 10 October 2010 to test the efficacy of calcein (Fluorescein di-(methyliminodiacetic acid) sodium salt; Fisher Scientific Ref. No. F/1250/44) for marking juvenile *M. margaritifera* and the effects of calcein concentration and immersion duration on growth and survival. The calcein concentrations and immersion duration treatments investigated were 0 (control), 30, 60 and 120 mg/L and 3, 6, 12 and 24 days respectively.

Four hundred and eighty juvenile mussels were selected at random from the Ehen juveniles excysting in 2010. Individuals were split into four groups of 120 individuals and allocated to a treatment (0, 30, 60, 120 mg/L). As in the substrate pilot, *Artemia* sieves were used to hold juvenile mussels (mesh size 0.18 mm). Approximately 1 cm of substrate (50 g dry weight) measuring 0.25 – 1 mm was added to each sieve. The substrate was exposed to running water for two days prior to the start of the experiment so a biofilm could begin to establish as food for the juvenile mussels. Length and height (Fig. 2.3) of 30 individuals from each treatment was recorded.

Calcein stock solutions (1 L) were prepared in a dark room by dissolving powdered calcein, in lake water filtered to 20 µm. Calcium concentration was taken on 3 August 2010 as part of Environment Agency routine monitoring in Windermere south basin and was recorded as 5.99 mg/L (© Environment Agency and database right). On 16 September 2010, calcein stock solutions plus a blank for the control (filtered lake water) were added to four aerated treatment tanks containing 19 L of filtered lake water (Fig. 2.6). pH was measured during calcein addition to each aquarium using a Troll 9500 multi-parameter sonde (In-Situ, USA). Readings



Fig. 2.6: Photograph of tanks containing (left to right) 30, 60, 120 and 0 (control) mg/L calcein solutions. Sieves containing substrate and juveniles were suspended in aquaria with aeration provided throughout the experiment.

were taken from tanks before stock solutions were added, after addition, and on days one, four and ten. Where pH showed a marked drop after addition of the calcein stock solution, sodium bicarbonate was used to buffer solutions and increase pH (Wilson *et al.*, 1987; Frenkel *et al.*, 2002; Mohler, 2003; Thébault *et al.*, 2006). Sieves containing juveniles were placed into the tanks. Tanks were situated in a dark room and were covered with a surround to block out any external light.

On day three, the contents of the each sieve were emptied in turn into a beige-coloured metal tray and examined. The beige tray contrasted with the darker brown colour of juveniles and thus allowed them to be picked out by eye. The first 30 individuals encountered (alive or dead) were removed from the container. Where 30 individuals could not be located, the maximum number found was removed. The number of live and dead juveniles was recorded. Ten live juveniles from each treatment were placed individually onto a cavity slide with enough water to cover the mussel and examined under a Leitz Diaplan compound fluorescence microscope fitted with a calcein-specific filter cube (445 – 495 nm excitation filter; and a 510 – 570 nm emission filter). Illumination was with a mercury short arc lamp. Photographs of juveniles were taken with a Canon EOS 350D which was set to manual focus (ISO = 100, file type: Canon RAW). Previous tests investigating potential suitable shutter speeds for photographing juveniles informed the decision to further test shutter speeds of 0.6, 1 and 2 seconds with control and treatment individuals. Once photographed, juveniles were transferred to new sieves with fresh substrate (0.25 - 1 mm) and were placed into a large holding aquarium with aerated,

static water. This process was repeated on days six, 12 and 24, when final measurements of remaining individuals were taken in addition to recording survival. After 24 days, all sieves from the holding tank were placed onto the bottom of a fish egg tray and supplied with flowing water. Additional checks for survival and growth were made in January 2011 and January 2012.

Fluorescence intensity was quantified from photographs using Adobe Photoshop (version 12.0.4), as described by Frenkel *et al.* (2002) and Mohler & Kehler (2007). RAW files were batch-processed and convert into high-quality JPEG files. Photoshop was used to select the green (fluorescent) pixels in each image. Mean values for green luminosity was recorded and these values were used to compare the level of fluorescence in each picture.

2.2.2.2 Data analysis

Due to this study being a pilot, there was only one replicate of each treatment so robust statistical analysis of survival of juveniles between treatments was not possible. Size and luminosity data (taken from photographs) are however suitable for statistical analysis. Data were tested for normality (Shapiro-Wilk test) before ANOVA or Kruskal-Wallis tests were carried out. *Post hoc* Tukey's HSD tests were carried out where significant differences were observed during ANOVA's. 2-way ANOVA tests were carried out to assess the interaction between immersion time and calcein concentration. Unless otherwise stated, standard deviation is given after mean values.

2.2.2.3 Results

During this pilot, temperature ranged from 14.7 – 15.3 °C and so growth of juveniles (and therefore sequestration of calcein) should have occurred. On day ten, an error was made when adding sodium bicarbonate to the 120 mg/L treatment tank which increased the pH to an average of 7.77 (max 7.94). Whilst this did not appear to be acutely or chronically toxic, it is possible that it could have affected feeding activity and therefore uptake of calcein after day ten in this treatment.

Table 2.2 shows the highest total survival was found in the 30 mg/L calcein treatment (73 %) and the lowest was found in the 60 mg/L treatment and the control (58 % each). On day 24 in the 120 mg/L treatment only a small number of live juveniles and a large number of dead

Table 2.2: Number of live and dead individuals found in the control (0 mg/L calcein) and treatment (30, 60 or 120 mg/L calcein) sieves after 3, 6, 12 and 24 days.

Day	Control		30 mg/L		60 mg/L		120 mg/L	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
3	28	2	30	0	30	0	29	1
6	22	1	29	0	21	1	27	3
12	12	1	19	0	11	1	12	3
24	8	1	10	1	8	1	3	7
Total number	70	5	88	1	70	3	71	14

individuals were found compared to the other treatments. Total mortality was highest in the 120 mg/L treatment also.

Juveniles were found to have different mean starting lengths between the different treatments ($F_{(3,116)} = 4.005, P = 0.009$). *Post hoc* analysis found individuals in the 120 mg/L treatment were significantly larger than in the 30 or 60 mg/L treatments (Fig. 2.7), but were not larger than the control ($P < 0.05$). The technique used for sampling juveniles was found to be biased with larger individuals being removed first because they were easier to see. Due to this, mean length of juveniles on day 24 was generally smaller than original measurements (Table 2.3). As a result of this the effect of calcein on growth of juveniles between day 0 and 24 could not be analysed.

No auto-fluorescence was observed in unmarked juveniles at shutter speeds of 0.6, 1 and 2 seconds. A shutter speed of 1 second was found to be adequate for analysis of calcein-labelled individuals. It is almost impossible to discern any difference in mark intensity by eye by simply comparing photographs of treatment individuals (Fig. 2.8). There was no significant interaction between immersion time and calcein concentration on mark intensity ($F_{(6,109)} = 1.07, P = 0.385$) but when considering the effect of immersion time on mark intensity separately, there was a significant difference between treatments ($F_{(3,151)} = 3.644, P = 0.014$) with individuals exposed for 24 days displaying brighter marks (mean luminosity = 73.55 ± 33.08) compared to those exposed for only 3 days (mean luminosity = 49.08 ± 29.70 ; $P = 0.015$; Fig. 2.9). All other comparisons were not significantly different from each other ($P > 0.05$). A significant difference was also found between calcein concentration and mark intensity ($F_{(3,151)} = 207.90, P < 0.001$). Individuals in each of the calcein treatments had significantly brighter marks compared with the control ($P < 0.001$) and individuals in the 120 mg/L treatment also had significantly brighter marks (mean luminosity = 86.60 ± 18.09) compared with the 30 mg/L

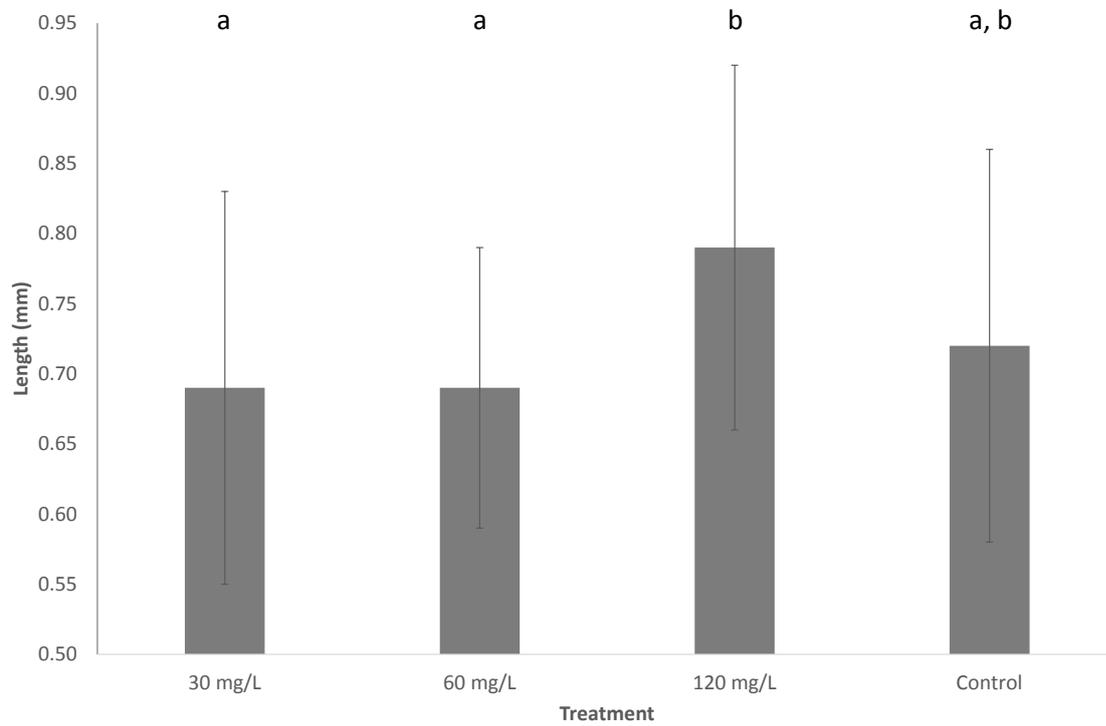


Fig. 2.7: Bar chart showing starting lengths of juvenile freshwater pearl mussels in the different calcein treatments (30, 60, & 120 mg/L) and the control (0 mg/L calcein). Letters above bars indicate statistically homogeneous groups ($P = 0.05$).

Table 2.3: Mean length (mm, \pm SD) of juvenile freshwater pearl mussels at the start (day 0) and end (day 24) of the investigation. Measurements of the 120 mg/L individuals at the end of the experiment were not taken in error.

	Control	30 mg/L	60 mg/L	120 mg/L
Day 0	0.72 \pm 0.14	0.69 \pm 0.14	0.69 \pm 0.10	0.79 \pm 0.13
Day 24	0.65 \pm 0.07	0.69 \pm 0.11	0.67 \pm 0.08	-

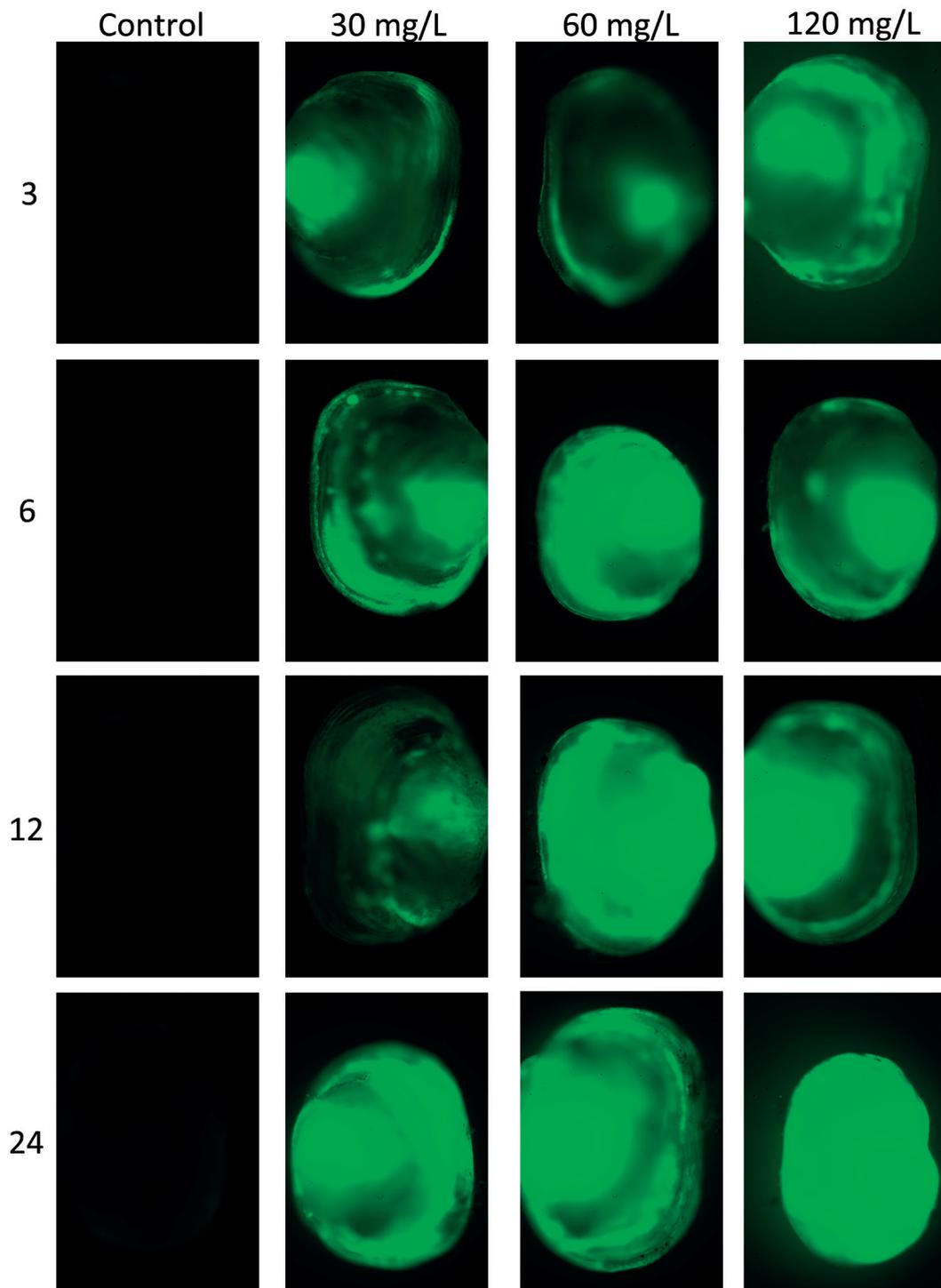


Fig. 2.8: Example fluorescence micrographs of juvenile freshwater pearl mussels in the control (0 mg/L calcein) and 30, 60 & 120 mg/L calcein treatments (L - R) and those immersed for 3, 6, 12 & 24 days (top to bottom). No fluorescence was observed in control individuals but it is impossible to discern by eye any difference between different calcein treatments or immersion periods.

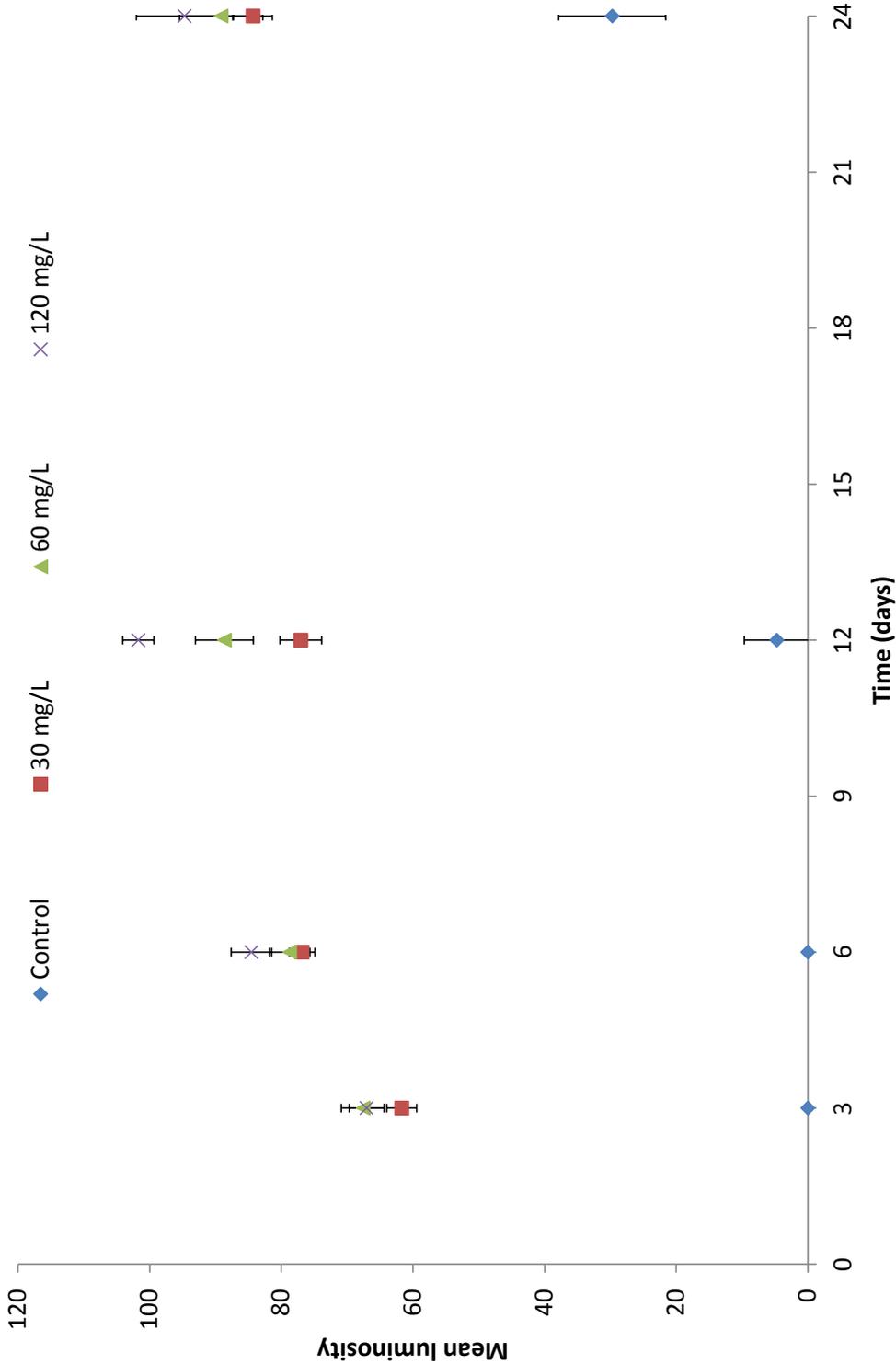


Fig. 2.9: Scatter plot showing mean luminosity in the control (0 mg/L calcein), 30, 60 and 120 mg/L calcein treatments over 3, 6, 12 and 24 days (\pm SD bars). Individuals exposed for 24 days had significantly higher luminosity values compared to those exposed for only 3 days ($F_{(3,151)} = 3.644, P = 0.014$). Individuals in the 120 mg/L calcein treatment had significantly brighter marks compared to those in the 30 mg/L calcein treatment ($F_{(3,151)} = 207.90; P < 0.0001$).

treatment (mean luminosity = 74.92 ± 11.43 ; $P < 0.009$). There was no significant difference in luminosity between the 60 and 120 mg/L treatments ($P = 0.544$; Fig. 2.9).

2.2.2.4 Discussion

Detectable marks from calcein-labelled calcium were visible after three days of immersion in calcein. Marks were significantly brighter after 24 days compared to three days but were not significantly brighter compared to those at six and 12 days. This finding concurs with previous studies that increased immersion time produces brighter marks (Day *et al.*, 1995). This suggests that 3 days immersion provides sufficient marks for juvenile *M. margaritifera*. Live juveniles were observed in all treatments after 24 days immersion. Small numbers of live individuals were found approximately 120 days after the end of the experiment in all treatments exposed for 3 days, and in the 30 mg/L treatment exposed for 24 days. Extended immersion durations of up to 24 days were tested in this pilot which, to the author's knowledge, is longer than any other study seeking to mark mussels in this manner. Other studies have found that immersion times of between 12 and 48 hours produce good quality marks in bivalves with faster growth rates than *M. margaritifera* (Day *et al.*, 1995; Crocker, 1998; Eads & Layzer, 2002; Moran & Marko, 2005; Linard *et al.*, 2011). Immersion durations of only four hours can produce successful results in the brown mussel, *Perna perna* (Kaehler & McQuaid, 1999).

Luminosity was significantly lower in the 30 mg/L treatment compared to the 120 mg/L treatment but there was no significant difference between any other treatments. This suggests that concentrations of at least 60 mg/L will provide clear, bright marks in *M. margaritifera*. This finding concurs with previous studies on other bivalves (Day *et al.*, 1995; Crocker, 1998; Eads & Layzer, 2002; Linard *et al.*, 2011). Whilst it is almost impossible to discern any difference in mark intensity by eye, analysis using the luminosity method previously described by Frenkel *et al.* (2002) and Mohler & Kehler (2007) provides a way to quantify this. Findings from this pilot suggest a suitable marking regime for *M. margaritifera* may be exposing juveniles to concentrations of at least 60 mg/L calcein for at least three days. This hypothesis will be further tested in Chapter 6.

2.3. Conclusions

This chapter describes the location where most of the investigations in this thesis took place, and puts the experimental work in to context of what the FBA's Freshwater Pearl Mussel Ark project is trying to achieve. This captive rearing programme provides an important opportunity to research potential factors affecting juvenile survival, an area of study which is impossible in the wild due to current poor recruitment levels and the small size of juvenile mussels.

Whilst both pilot studies used low replicate numbers meaning that statistical power was weak, some conclusions can be drawn. The experiment considering substrate size, depth and cleaning regime found that survival was higher in larger substrates. This warrants further investigation with a smaller number of treatments and more replicates. Substrate depth does not appear to affect survival but does make a substantial difference to sampling effort with smaller, deeper substrate treatments taking over eight hours to check compared with larger, shallower substrate treatments taking < 1.5 hours. Cleaning every two months does not appear to be sufficient to ensure satisfactory juvenile survival so increasing the cleaning frequency will be considered in the full study.

Marking with a fluorophore has the potential to save rearing programmes time when monitoring very small juveniles. The pilot found that calcein appears to be a suitable fluorophore for batch-marking juvenile *M. margaritifera* and does not appear to be acutely or chronically toxic. Marking juveniles at a concentration of 60 mg/L calcein over 6 days produces adequate marks in this species, and increasing immersion time or calcein concentrations does not appear to increase mark brightness significantly. The effect of calcein on mussel growth needs to be established and a comprehensive marking protocol outlined using evidence with increased statistical power. Further investigations in Chapter 6 will use more replicates to investigate the effects on growth and survival of a similar range of calcein concentrations as considered in this pilot.

2.4. References

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Chapter 3

Investigating the effects of substrate size and cleaning regime on growth and survival of juvenile freshwater pearl mussels *Margaritifera margaritifera*

3.1. Introduction

In the wild, juvenile mussels excysting from host fish must fall into habitat suitable to support continued development. Juveniles are particularly vulnerable to factors which decrease substrate and water quality, for example, aggravated erosion, nutrient enrichment and pollution incidents. In captive rearing programmes parameters affecting juvenile survival can be controlled and manipulated in order to achieve higher survival rates than may be possible in the wild. Research in the early 2000's into optimising captive rearing conditions for freshwater mussels (mainly by North American and some European practitioners) has led to near perfection of these techniques (Lopes-Lima *et al.*, 2014). Rearing of particularly sensitive species such as the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758) however has proven slightly more problematic and has required significant and ongoing investigation. Several different methods of rearing *M. margaritifera* have been trialled in Europe (Gum *et al.*, 2011) including propagating juveniles in trays or baskets (Hastie & Young, 2003; Taylor, 2007; Lange & Selheim, 2011; Scriven *et al.*, 2011; Eybe *et al.*, 2013; Lavictoire *et al.*, 2014), suspending cages containing juveniles in raceways or rivers (Buddensiek, 1995; Schmidt & Vandr e, 2010; Scheder *et al.*, 2014), allowing juveniles to excyst directly into raceways (Preston *et al.*, 2007; Moorkens, 2011) and holding juveniles in static systems in incubators (Lange & Selheim, 2011; Eybe *et al.*, 2013). Additionally, wild populations may be augmented via bankside encystment and immediate release of encysted salmonids (Altm uller & Dettmer, 2006).

When designing captive rearing systems it is important to consider maintenance requirements in addition to maximising juvenile survival. Some early attempts at captive rearing of *M. margaritifera* were over-laborious due to small juvenile size and holding containers being disproportionately large (Hastie & Young, 2003). This led to poor monitoring and low survival. Subsequent captive rearing efforts have sought to use relatively simple systems which minimise handling e.g. Buddensiek (1995); Barnhart (2006); Preston *et al.* (2007); Lange & Selheim (2011); Eybe *et al.* (2013), but these also have their drawbacks.

Post-excystment there is often an initial period (approximately 4 - 8 weeks) of high mortality before survival rates stabilise (Gatenby *et al.*, 1996; Gatenby *et al.*, 1997; O'Beirn *et al.*, 1998; Beaty, 1999; Rogers, 1999; Hanlon, 2000; Jones *et al.*, 2005). It is important to understand the reasons behind high-mortality periods in order to reduce stress or tailor

environmental conditions for juveniles and maximise the number reared, especially for populations which are close to extinction.

3.1.1 Culture conditions affecting juvenile survival

Factors thought to affect the growth and survival of newly-excysted juveniles include substrate size (Beaty & Neves, 2004; Liberty *et al.*, 2007; Hua *et al.*, 2013) and depth (Yeager *et al.*, 1994; Beaty & Neves, 2004; Jones *et al.*, 2005), maintenance (cleaning) regime (O’Beirn *et al.*, 1998; Liberty *et al.*, 2007), diet (Gatenby *et al.*, 1997; Lange, 2005; Kovitvadhii *et al.*, 2006; Schmidt & Vandr , 2010; Eybe *et al.*, 2013), mussel density and the presence of potentially harmful ions, such as ammonium (Eybe *et al.*, 2013).

3.1.1.1 Substrate

Substrate characteristics are the most important habitat parameters and are most likely to be the limiting factor in pearl mussel rivers (Hastie *et al.*, 2000; Weber, 2005; Geist & Auerswald, 2007; Tarr, 2008). In the wild, adult and juvenile pearl mussels can be found in the same habitat patches (Hastie *et al.*, 2000) but utilise different microhabitats (Geist & Auerswald, 2007); lack of suitable substrate impedes juvenile growth and survival (O’Beirn *et al.*, 1998). A functional pearl mussel population requires substrates which are stable, have a low proportion of fine sediments and organic matter, are well oxygenated and which are not too compact so as to inhibit burrowing (Bauer *et al.*, 1980; Lewis & Riebel, 1984; Hruška, 1992; Geist & Auerswald, 2007; Englund *et al.*, 2008). It is the author’s belief that, where possible, these conditions should be adhered to in captivity in order to provide the best scope for survival and growth and to ‘prime’ juveniles for similar conditions in their native catchments upon reintroduction.

Previous studies on the requirements of unionids use species which are relatively fast-growing compared to *M. margaritifera*, but these studies provide useful benchmarks for comparison. Higher juvenile survival rates are typically observed in coarser substrates (Brady, 2000; Liberty *et al.*, 2007), especially if substrate is compacted (Rogers, 1999) but there are exceptions to this (Beaty & Neves, 2004; Hua *et al.*, 2013). Where compaction is not a problem, Rogers (1999) found that finer substrate sizes produced better survival rates for *Lampsilis fasciola*. Finer substrates also appear to provide better opportunities for growth (Rogers, 1999; Beaty & Neves, 2004; Liberty *et al.*, 2007). This may be due to higher food

availability (Wahlström, 2006; Liberty *et al.*, 2007). Contradictions to this have been reported by Brady (2000) who observed higher growth in larger substrate sizes for *Lampsilis cardium* and Hudson & Isom (1984) found the addition of silt increased survival in *Anodonta (=Utterbackia) imbecillis*. These contradicting findings suggest that substrate size preferences are likely to be specific to species or perhaps rearing systems.

The effect of substrate depth appears to be of limited importance. Beaty & Neves (2004) found depth had no effect on survival or growth in *Villosa iris* and the pilot study for this experiment found the same for *M. margaritifera* (see Chapter 2). In captivity, juveniles tend to be found within the top layer of the substrate, usually within the first centimetre (Yeager *et al.*, 1994; Beaty & Neves, 2004). Some studies attempting to rear *M. margaritifera* have had success without any substrate (Lange & Selheim, 2011; Eybe *et al.*, 2013) but studies on other species have found that the presence of substrate improved survival (Jones *et al.*, 2005). The long-term implications (if any) of rearing juveniles in conditions which do not in some way mimic conditions in the wild, such as no substrate or constant, elevated temperatures, are as yet unknown.

3.1.1.2 Culture density

The density of juveniles in culture may have an effect on juvenile survival and growth. Eybe *et al.* (2013) reported no difference in survival of *M. margaritifera* juveniles in densities of 200 - 400 per 500 ml of water but a significant difference in size was found with higher growth rates at lower densities. Beaty (1999) found no affect of juvenile density on growth or survival of *V. iris* and Barnhart (2006) reported good survival in small containers approximately 6 cm in diameter each supporting 2000 individuals. Density-dependent survival and growth is likely to differ between species and also between culture systems depending upon a variety of factors, for example, flow, temperature and mode of feeding (pedal or filter feeding).

3.1.1.3 Disturbance

In bivalve culture, routine maintenance and monitoring are required which may disturb juveniles. Some studies have found that cleaning substrate or simply sampling can positively affect growth and survival (O'Beirn *et al.*, 1998; Hanlon, 2000; Zimmerman, 2003) but other studies have observed the opposite, with increased mortality through damage, accidental loss

(Beaty, 1999) and handling stress (O'Beirn *et al.*, 1998). Zimmerman (2003) found that cleaning every two months or when siltation became a problem had a positive effect on growth and survival in *Epioblasma capsaeformis* and *L. fasciola* but Liberty *et al.* (2007) found cleaning more frequently had a detrimental effect on growth and survival in *V. iris*. O'Beirn *et al.* (1998) reported mixed results with *L. fasciola* showing improved survival but slower growth in treatments sampled more frequently.

3.1.1.4 Other factors

Juvenile survival during the first winter post-excystment may be positively correlated with size in *M. margaritifera* (Buddensiek, 1995; Lange, 2005; Lange & Selheim, 2011). Buddensiek (1995) found 100% mortality in juveniles measuring <0.7 mm in length at the onset of the first winter and Lange & Selheim (2011) found juveniles grown to >1 mm in captivity generally survive the first winter. However Moorkens (2011) found the mean length of captive juvenile *Margaritifera (margaritifera) durrovensis* after the first growth season to be 0.65 mm. These contradictions indicate the relationship between size and survival over the first winter is not a simple one. Under a near-natural temperature regime in captivity, individuals from different English pearl mussel populations display different growth rates (Sweeting & Lavictoire, 2013). Therefore growth and eventual maximum size may also be population-specific. If shell size is an indication of biological development or of specific individuals having more nutritional reserves than others, it stands to reason that size-dependent over-winter survival should be observed.

Timing of glochidial excystment from host fishes may also be an important factor affecting growth and survival. Juveniles excysting earlier in the season have more time to grow before the onset of winter and therefore may have an advantage over juveniles which excyst later (Buddensiek, 1995; Beaty & Neves, 2004). However, juveniles which excyst at the beginning of the drop-off period are often under-developed, not very active and can suffer high mortality (Jones & Neves, 2002; Schmidt & Vandré, 2010). Captive rearing programmes should ensure that rearing efforts are focussed on healthy individuals to maximise output.

3.1.2 Objective of this study

This investigation sought to test a new experimental culture system employing some features from previous studies e.g. Barnhart (2006), for *M. margaritifera* and to identify the most

appropriate substrate size class and cleaning/maintenance regime. These initial investigations informed additional work to be undertaken (Chapter 4) to better understand the conditions limiting juvenile growth and survival within substrates.

3.2. Methods

3.2.1 Experimental design

Experimental design was based on the system described in Chapter 2, Section 2.2.1.1 with some key improvements. In this study, the effects on growth and survival of juveniles was tested in four treatments. Two substrate size classes (0.25 - 1 mm and 1 - 2 mm) and two cleaning regimes (weekly and monthly) were investigated in the following treatments:

- 0.25 - 1 mm substrate cleaned weekly;
- 0.25 - 1 mm substrate cleaned monthly;
- 1 - 2 mm substrate cleaned weekly;
- 1 - 2 mm substrate cleaned monthly.

There were nine replicates of each treatment (36 sieves total). As in the pilot study the system was a down-welling flow-through system supplying filtered lake water to juveniles at a rate of approximately 67 ml s^{-1} (4 L/min). A single aquarium (995 mm x 357 mm x 510 mm) was set up as outlined in the pilot with a spray bar, effluent pipe and a styrene sheet with 36 square holes cut out created upper and lower chambers in the aquarium (Fig. 3.1). *Artemia* sieves (Hobby, Germany) with a mesh size of 0.9 mm were adhered to the styrene sheet with EVO- STIK

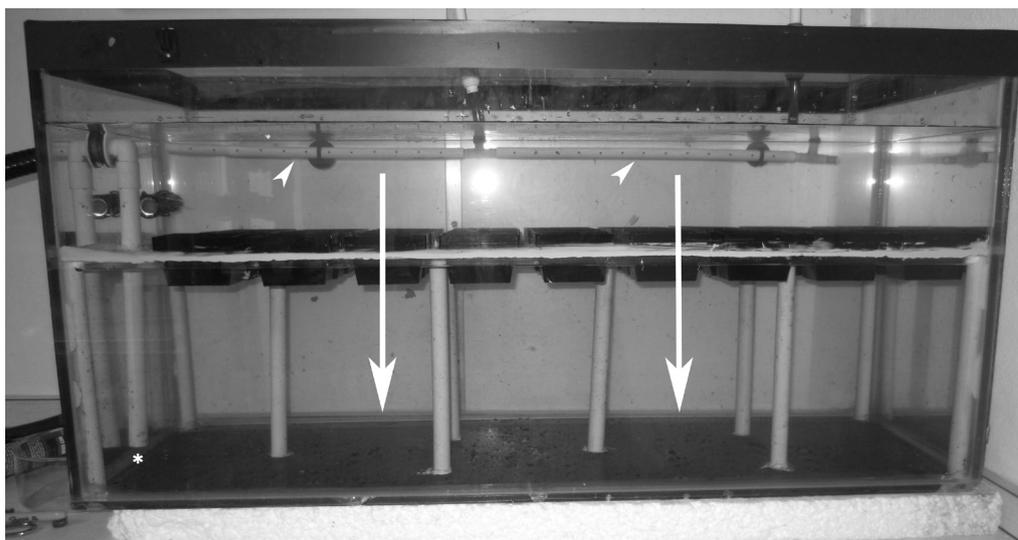


Fig. 3.1: Photograph of rearing system used for the substrate experiment. Water enters at the top of the system via a spray bar (arrow heads) and flows through sieves holding substrate and juvenile freshwater pearl mussels (arrows showing direction of flow). Water exits from the bottom of the aquarium (*).

Wet Grab sealant (Bostick, UK) to create a fixed support for experimental sieves (mesh size 0.18 mm) to clip in to. Experimental sieves were therefore removable for monitoring, cleaning and sampling purposes. This design ensured that the sole pathway for water from the top chamber into the bottom was through the substrate.

Each experimental sieve contained one of the treatment substrate mixes to a depth of approximately 1 cm (50 g dry weight). Substrate was collected from the western shore of Windermere and left to air-dry before being sieved to the appropriate size. Substrate was exposed to flowing lake water for a minimum of 21 days prior to the start of the experiment to facilitate biofilm development. A suitable biofilm may develop after one week (Gum *et al.*, 2011) but a stable biofilm should be formed after 21 days (Battin *et al.*, 2003; Romani, 2009). One hundred newly-excysted active juveniles were added to each experimental sieve (total 3,600 individuals) between 26 June and 3 July 2012. Thirty individuals from each sieve were randomly selected and their length and height measured (to the nearest 50 μm) before addition to sieves. The position of experimental sieves within the aquarium was assigned on a random basis so that each column within the aquarium had one of each of the four treatments (Fig. 3.2).

Sieves in treatments cleaned weekly (18 sieves in total) were removed from the aquarium once per week (7 days ± 1.87) and the substrate was gently emptied into a glass container with filtered lake water. The substrate was elutriated by swilling it gently in the container to suspend organic particles and the elutriate poured through a 0.18 mm mesh sieve to retain any juveniles. Sieves were inspected under a low power microscope (x 10) and any juveniles replaced into the experimental sieve along with the substrate. The sieve was then returned to the aquarium. The process above was repeated on a monthly basis (30 days ± 7.71) for the treatments cleaned monthly.

All sieves were sampled exhaustively approximately every two months during the first year on 51, 112, 167, 247, 308 and 362 days post excystment with a final check done on day 758 days post excystment. On these occasions the number of live and dead juveniles was recorded. Where available, thirty live individuals from each sieve were chosen at random and measured. All dead juveniles were measured and removed. Sampling in this manner constituted a cleaning event as organic matter was removed during the sampling process.

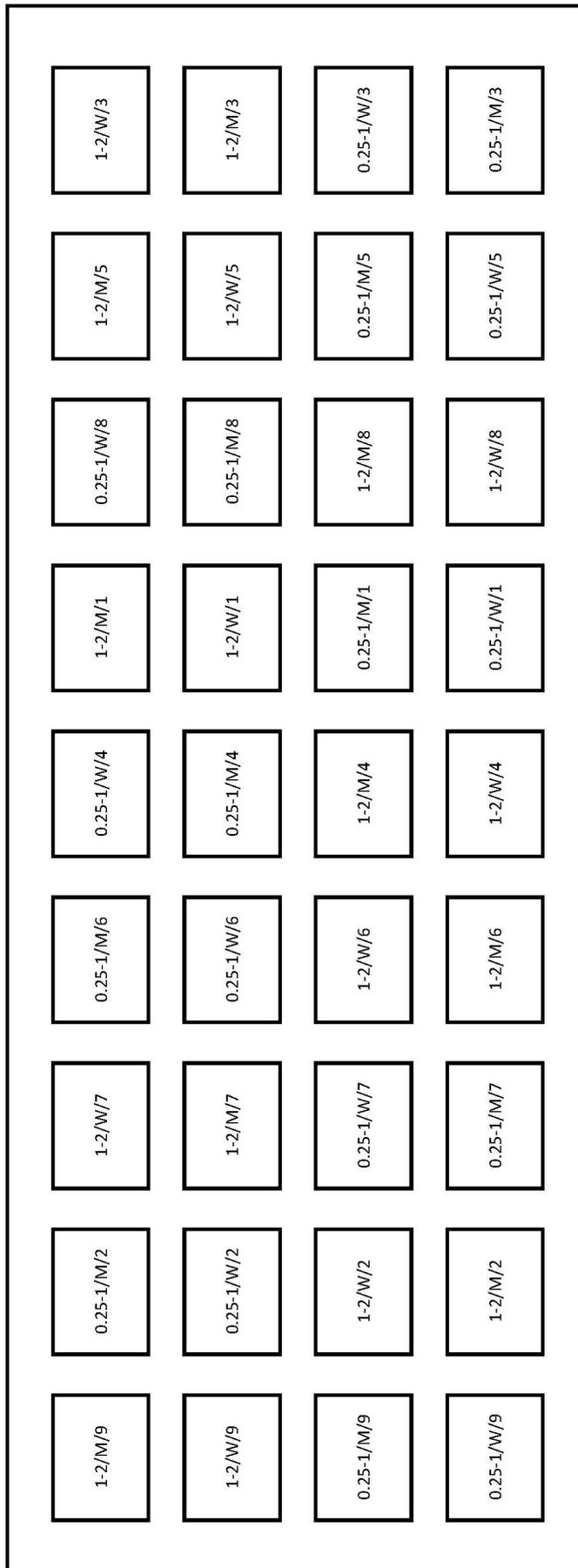


Fig. 3.2: Diagrammatic representation of the layout of experimental sieves. One hundred juvenile freshwater pearl mussels plus one of the substrate types of was placed in to each sieve. Sieves were placed randomly in the aquarium but in a way which ensured that only one of each treatment type occurred in each column. For every sieve, substrate size (either 0.25 - 1 mm or 1 - 2 mm) is followed by cleaning regime (W = weekly; M = monthly) which is followed by replicate number (1 - 9), e.g. 1-2/M/9 is the 9th replicate of the 1 - 2 mm substrate treatment cleaned monthly.

Sixteen days after the experiment commenced, the surface of the styrene sheet was siphoned and 11 juveniles were found outside sieves, indicating some escapement. Similar observations have been made in studies on unionids (Hanlon, 2000; Liberty *et al.*, 2007). As a result, 0.3 mm mesh sieves were placed on top of experimental sieves so juveniles could not escape. A small number of escaped mussels continued to be found (presumably circulating in water currents) until 31 October 2012, when numbers of juveniles outside of sieves dropped to zero. An additional 4 juveniles (2 alive, 2 dead) were found in the aquarium during a spot check on 05/04/2013. All escaped juveniles were removed from the system after each check. This experiment ran between June 2012 – July 2014. Temperature was logged every hour throughout the experiment using a Hobo temperature logger (U22-001, Onset, USA).

3.2.2 Data analysis

Central Limit Theorem (Elliott, 1993) was applied to assume normality where appropriate. When $n < 30$, normality was tested using a Shapiro-Wilk test and if samples were found to be normally distributed, parametric statistical tests were used. Unless otherwise stated, \pm SD values are provided after mean values. The presence of outliers in data was tested using methods outlined in Hoaglin *et al.* (1986) and Hoaglin & Iglewicz (1987).

One-way Analysis of Variance (ANOVA) with *post hoc* Tukey's HSD tests were used to assess the significance of survival, survival rates and size between treatments on the same sampling occasion when data were normal. Where data were not normal Kruskal-Wallis tests were employed with *post hoc* Mann Whitney U tests. Two-way ANOVA's were used to investigate the interaction of substrate size and cleaning regime and their effects on both survival and size in treatments on day 362. Repeated Measures ANOVA's with pairwise comparisons were used to test survival between 0 – 362 days to see if survival changed at specific times (e.g. seasonal variation). For Repeated Measures ANOVA's a Greenhouse-Geisser correction was applied if the assumption of sphericity was not met. Student's *t*-tests were used to compare length pre- and post-winter to help establish if juveniles displayed size-dependent over-winter survival within treatments. Spearman's rank correlation coefficient tests were used to test the significance of correlations between shell length and height, shell growth and temperature, and mean survival and shell length. Juvenile length and survival across rows and columns in the aquarium were tested in June 2013 to rule out any bias due to sieve positioning (lateral and

top-to-bottom positioning within the aquarium). There was no significant difference in survival between different columns ($F_{(8,27)} = 0.196, P = 0.989$) or rows ($F_{(3,32)} = 0.025, P = 0.994$) indicating that any significant results between treatments were not due to sieve positioning. The same was true for mean juvenile length in June 2013; there were no significant differences between columns ($F_{(8,27)} = 0.097, P = 0.999$) or rows ($F_{(3,32)} = 0.163, P = 0.920$).

Initial size in June 2012 was also tested to ensure juveniles in each sieve had the same starting length. Starting lengths of individuals in all 36 sieves were not significantly different ($F_{(35,1044)} = 1.35, P = 0.083$) at the beginning of the experiment (mean length = 0.40 ± 0.02 mm).

3.3. Results

3.3.1 Survival

In this investigation survival was comparable to, and in most cases greater than, previous studies of similar duration on other species of freshwater mussel (Fig. 3.3a), and specifically *M. margaritifera* (Fig. 3.3b). Higher survival was observed in the 1 - 2 mm monthly treatment with an average of over 55 % survival after 362 days and 23 % after 758 days (Table 3.1). The poorest survival was in the 0.25 - 1 mm weekly treatment, averaging 14 % and 7 % survival after 362 and 758 days respectively, with the other two treatments showing intermediate survival (Table 3.2 & Fig. 3.4).

Table 3.1: Survival (total number, mean and range) of juvenile freshwater pearl mussels in each treatment after 362 and 758 days.

Treatment	Total no. survived	362 days		Total no. survived	758 days	
		Mean survival (%)	Survival range (%)		Mean survival (%)	Survival range (%)
0.25 - 1 mm weekly	123	14	8-19	67	7	8-12
0.25 - 1 mm monthly	253	28	19-40	61	7	4-15
1 - 2 mm weekly	333	37	18-47	184	20	7-30
1 - 2 mm monthly	498	55	45-63	206	23	15-29

Table 3.2: Mean survival (\pm SD) of juvenile freshwater pearl mussels at each sampling point in order of highest to lowest survival. Survival was significantly different between all treatments (Tukey's HSD tests; $P < 0.05$) within each sampling point except where indicated on 51 ($*P = 0.93$), 112 ($^{\wedge}P = 0.97$) and 758 days post-excystment ($^{\%}P = 0.734$ and $^{\S}P = 0.992$). N.B. Results have been rounded to whole juveniles.

Treatment	Days post excystment						
	51	112	167	247	308	362	758
1 - 2 mm monthly	84 \pm 4	75 \pm 4	71 \pm 4	68 \pm 3	65 \pm 3	55 \pm 6	23 \pm 5 $^{\%}$
1 - 2 mm weekly	76 \pm 6 *	51 \pm 5 $^{\wedge}$	50 \pm 6	48 \pm 6	46 \pm 6	37 \pm 9	20 \pm 8 $^{\%}$
0.25 - 1 mm monthly	75 \pm 4 *	51 \pm 8 $^{\wedge}$	43 \pm 8	40 \pm 8	33 \pm 7	28 \pm 6	7 \pm 3 §
0.25 - 1 mm weekly	40 \pm 7	18 \pm 4	16 \pm 3	15 \pm 3	13 \pm 3	14 \pm 3	7 \pm 3 §

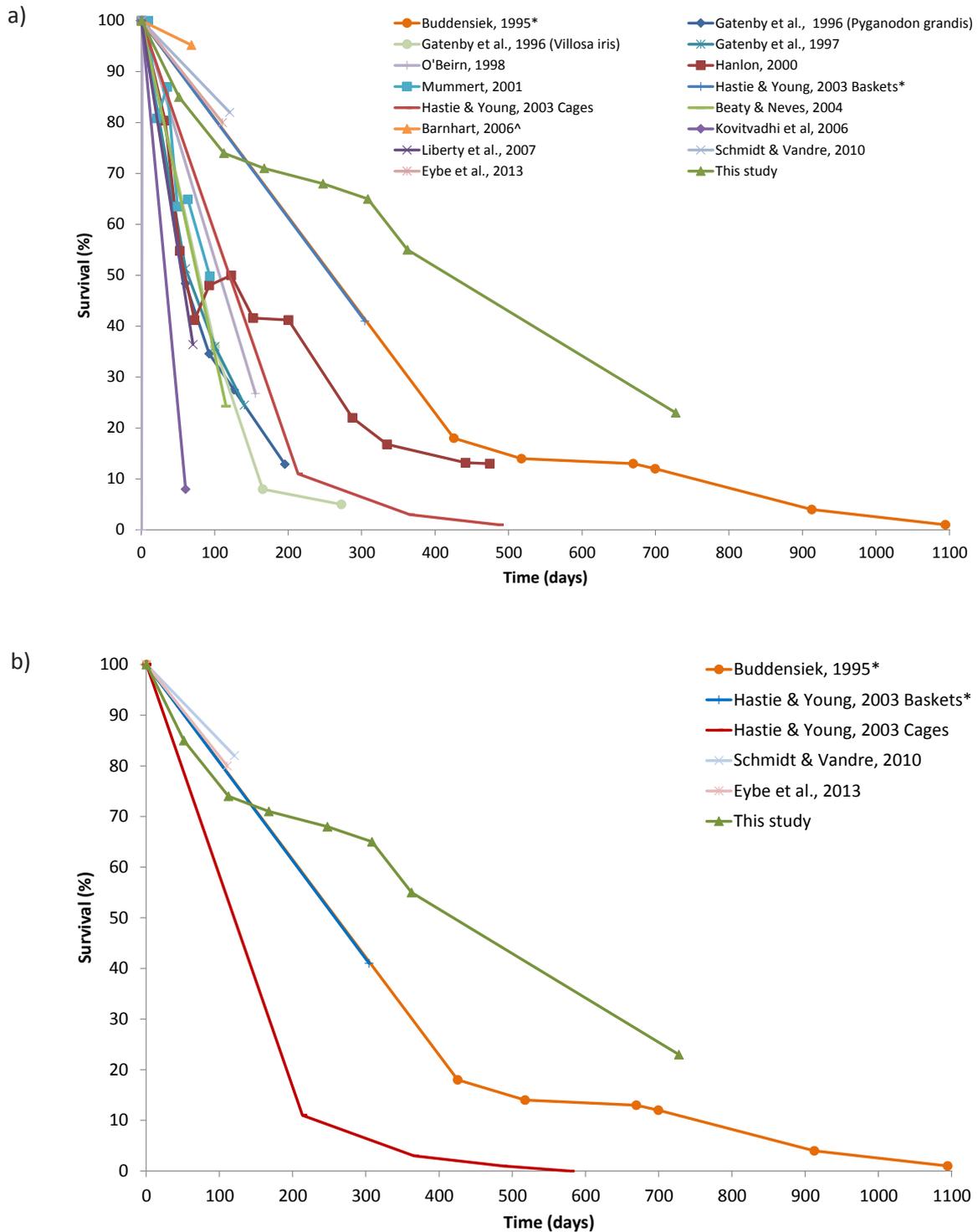


Fig. 3.3: Time series graphs showing mean survival in (a) other published bivalve captive rearing studies and (b) more specifically on *M. margaritifera*. 'Buddensiek (1995)' figures taken from survival graphs; 'Hastie & Young (2003) Baskets' estimated a sample of known volume and multiplied up to indicate likely survival in total volume. *Indicates figures based upon estimates; ^Indicates only one treatment selected for illustrative purposes; this study investigated survival of 8 different species over different timescales. N.B. For all studies where several treatments are reported, only the best survival results are reported here for comparison.

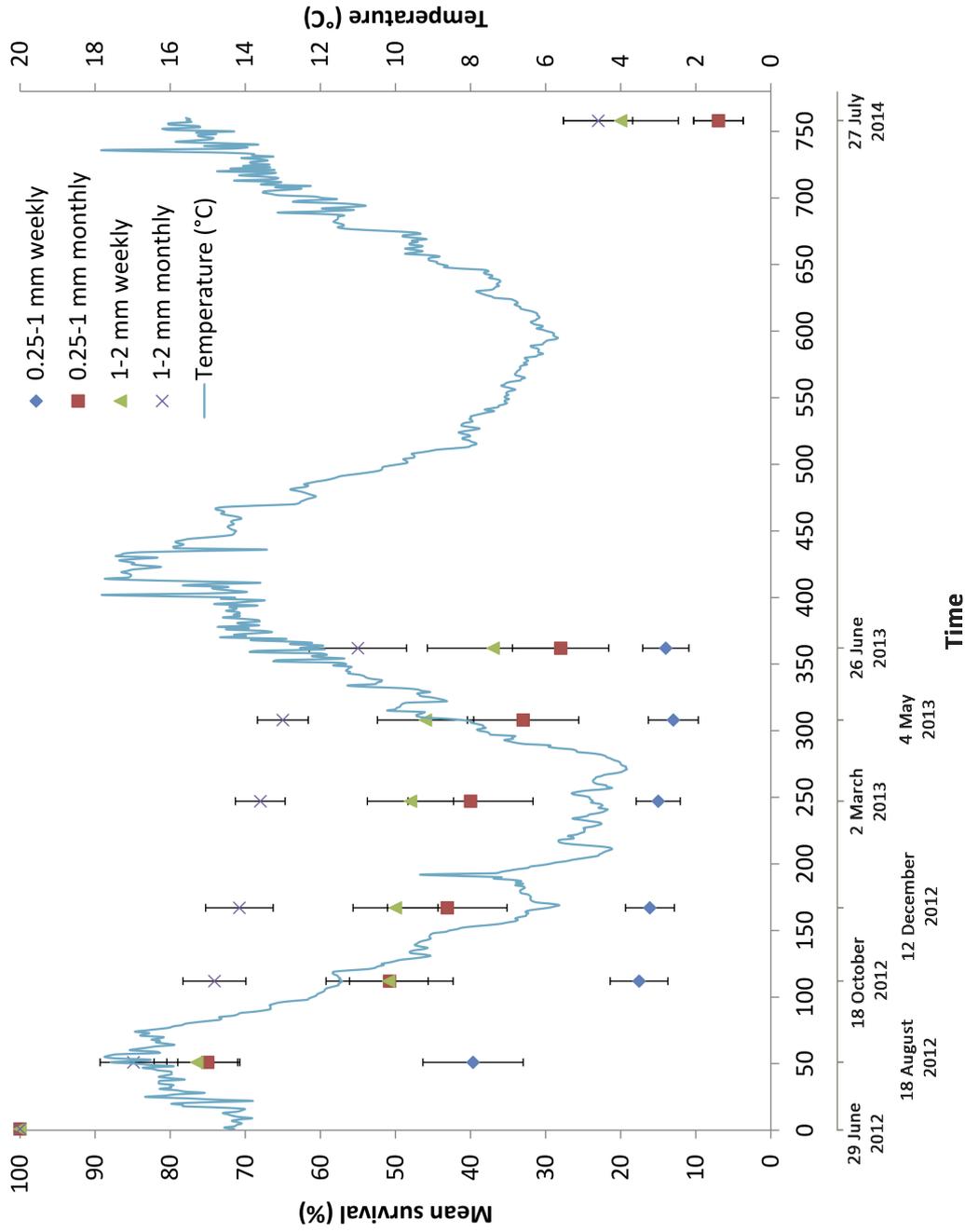


Fig. 3.4: Time series graph showing mean survival of juvenile freshwater pearl mussels in different treatment with SD bars. The x-axis is provided both in days since experiment commenced and detailing sample dates (months) to show how survival relates to time of year. N.B. The final data points for 0.25 - 1 mm treatments overlap precisely. Temperature is also provided for reference.

Juvenile survival between treatments was significantly different on all sampling occasions ($P < 0.001$); 51 days ($F_{(3,32)} = 128.30$), 112 days ($F_{(3,32)} = 148.285$), 167 days ($F_{(3,32)} = 145.296$), 247 days ($F_{(3,32)} = 140.117$), 308 days ($F_{(3,32)} = 145.350$), 362 days ($F_{(3,32)} = 64.670$) and 758 days ($F_{(3,32)} = 25.400$). The same pattern of survival was observed on all sampling occasions; 1 - 2 mm monthly > 1 - 2 mm weekly > 0.25 - 1 mm monthly > 0.25 - 1 mm weekly. *Post hoc* tests indicate survival was the same where indicated in Table 3.2.

There was no significant interaction between substrate size and cleaning regime affecting survival ($F_{(1,32)} = 0.805$, $P = 0.376$) although when considering these two factors separately, survival was significantly higher in 1 - 2 mm substrate ($F_{(1,32)} = 136.022$, $P < 0.001$) and in treatments cleaned monthly ($F_{(1,32)} = 57.178$, $P < 0.001$). Likewise, on day 758 there was no significant interaction between the effects of substrate size and cleaning regime on survival ($F_{(1,31)} = 1.572$, $P = 0.219$). When considering these two parameters separately survival was significantly higher in 1 - 2 mm substrate ($F_{(1,31)} = 83.381$, $P < 0.001$) but there was no significant difference in survival between cleaning regimes ($F_{(1,31)} = 0.052$, $P = 0.822$).

3.3.1.1 Survival rate

Survival rates (i.e. difference between survival at one sampling point and the next as a percentage) were considered to examine the different mortality rates between treatments taking into account the high initial mortality observed in the 0.25 - 1 mm weekly treatment. Survival rates between the treatments were significantly different on days 51 ($F_{(3,32)} = 128.303$; $P < 0.001$), 112 ($F_{(3,32)} = 41.388$; $P < 0.001$), 167 ($F_{(3,32)} = 9.743$; $P < 0.001$), 247 ($F_{(3,32)} = 6.553$; $P = 0.001$), 308 ($H_{(3)} = 15.179$; $P = 0.002$) and 758 ($F_{(3,32)} = 9.404$; $P < 0.001$) but not different on day 247 ($F_{(3,32)} = 0.619$; $P = 0.608$).

To investigate the relationship between juvenile size and survival rate, mean shell length was plotted against mean survival rate. There appears to be no relationship for either of the 1 - 2 mm treatments (Fig. 3.5) but a slight positive relationship is observed in the 0.25 - 1 mm treatments, which is particularly strong in the treatment cleaned weekly. There was no significant correlation in the 0.25 - 1 mm monthly treatment ($r_{s(4)} = 0.515$, $P = 0.296$) but there was a significant correlation in the 0.25 - 1 mm weekly treatment ($r_{s(4)} = 0.947$, $P < 0.01$). In this treatment larger individuals had significantly higher survival rates compared to smaller individuals.

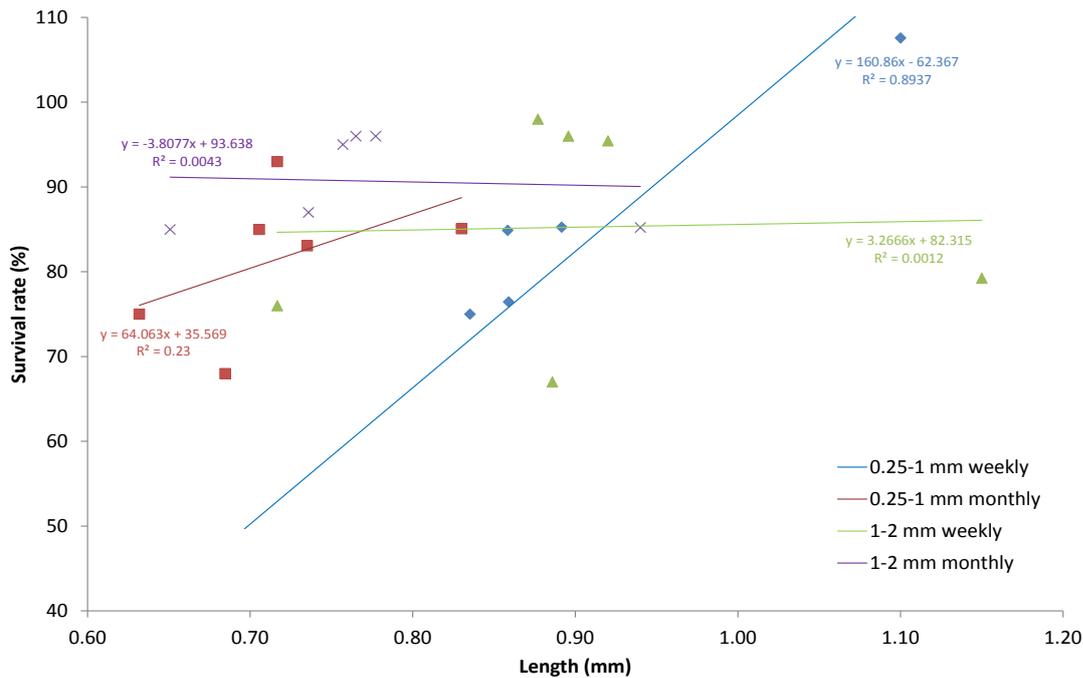


Fig. 3.5: Scatter plot showing mean survival rate (%) against mean shell length (mm) of juvenile freshwater pearl mussels for each treatment. Only the 0.25 - 1 mm weekly treatment shows a significant correlation ($P < 0.01$) between survival rate (%) and length (mm).

3.3.1.2 Survival over time

Survival differences within treatments over the first 362 days of the experiment were tested with Repeated Measures ANOVAs to see if survival changed at specific times of year. Table 3.3 provides a summary of results. Mean survival was different over the course of the experiment for all treatments ($P < 0.001$); 0.25 - 1 mm weekly ($F_{(2,021, 16,171)} = 1147.196$), 0.25 - 1 mm monthly ($F_{(6, 48)} = 315.484$), 1 - 2 mm weekly ($F_{(2,126, 17,008)} = 324.543$) and 1 - 2 mm monthly ($F_{(2,030, 16,243)} = 167.912$). *Post hoc* tests revealed that survival was the same only in the 1 - 2 mm weekly treatment between days 112 & 167 (October & December 2012; $P = 0.086$) and in the 0.25 - 1 mm weekly treatment between days 167 & 247 (December 2012 & February 2013;

Table 3.3: Mean survival (\pm SD) of juvenile freshwater pearl mussels in all treatments at each sampling point (days) in order of highest to lowest survival. Survival was significantly different within all treatments (i.e. between time points) using Repeated Measures ANOVA; $P < 0.001$, except where indicated by the same symbols: % (1 - 2 mm weekly) and *, \$, ^ (0.25 - 1 mm weekly). N.B. Results have been rounded to whole juveniles.

Treatment	Days						
	51	112	167	247	308	362	758
1 - 2 mm monthly	84 \pm 4	75 \pm 4	71 \pm 4	68 \pm 3	65 \pm 3	55 \pm 6	23 \pm 5
1 - 2 mm weekly	76 \pm 6	51 \pm 5%	50 \pm 6%	48 \pm 6	46 \pm 6	37 \pm 9	20 \pm 8
0.25 - 1 mm monthly	75 \pm 4	51 \pm 8	43 \pm 8	40 \pm 8	33 \pm 7	28 \pm 6	7 \pm 3
0.25 - 1 mm weekly	40 \pm 7	18 \pm 4	16 \pm 3*	15 \pm 3*\$	13 \pm 3^	14 \pm 3^s	7 \pm 3

$P = 0.122$), 247 & 362 (February & June 2013; $P = 0.128$) and 308 & 362 (April & June 2013; $P = 0.288$), indicating that survival stabilised over winter in the 0.25 - 1 mm weekly treatment but not in any of the others.

3.3.1.3 Over-winter survival

To establish if juveniles display size-dependent over-winter survival, t -tests were carried out comparing juvenile size pre- and post-winter in October 2012 (day 112) & April 2013 (day 308). As shown in Fig. 3.6, juveniles were significantly larger post-winter ($P < 0.001$) in all treatments; 0.25 - 1 mm weekly ($t_{(272)} = -4.377$), 0.25 - 1 mm monthly ($t_{(522)} = -5.239$), 1 - 2 mm weekly ($t_{(538)} = -3.717$), 1 - 2 mm monthly ($t_{(530)} = -5.027$). Considering Fig. 3.6, there were more juveniles in larger size classes in April 2013 compared to October 2012. This implies that, rather than there being high mortality of smaller juveniles over winter, individuals have grown, pushing them into larger size classes.

3.3.2 Size

Length and height were found to be highly correlated in all treatments ($P < 0.001$; Fig. 3.7) therefore only length was used for analysis in this study.

Length of juveniles between treatments was analysed for each sampling occasion (Table 3.4). Juvenile length was significantly different between all treatments ($P < 0.001$) on days 51 ($F_{(3,1076)} = 77.295$), 112 ($F_{(3,964)} = 195.723$), 167 ($F_{(3,951)} = 158.522$), 247 ($F_{(3,941)} = 175.247$), 308 ($F_{(3,906)} = 162.465$), 362 ($F_{(3,883)} = 167.377$) and 758 ($F_{(3,514)} = 70.576$). The same pattern was observed throughout the experiment; 1 - 2 mm weekly > 0.25 - 1 mm weekly > 1 - 2 mm monthly > 0.25 - 1 mm monthly (Fig. 3.8). Fig. 3.9 shows length histograms for each treatment during the first year.

Table 3.4: Mean length \pm SD (mm) of juvenile freshwater pearl mussels in all treatments at each sampling point (days) in order of largest to smallest. Size was significantly different between all treatments (Tukey's HSD tests; $P < 0.05$) within each sampling point except where indicated on days 167 (* $P = 0.31$) and 308 (^ $P = 0.17$).

Treatment	Days						
	51	112	167	247	308	362	758
1 - 2 mm weekly	0.72 \pm 0.07	0.89 \pm 0.11	0.88 \pm 0.10*	0.90 \pm 0.09	0.92 \pm 0.10^	1.14 \pm 0.17	2.82 \pm 0.54
0.25 - 1 mm weekly	0.70 \pm 0.07	0.84 \pm 0.11	0.86 \pm 0.11*	0.87 \pm 0.10	0.90 \pm 0.11^	1.10 \pm 0.17	2.60 \pm 0.62
1 - 2 mm monthly	0.65 \pm 0.08	0.74 \pm 0.10	0.76 \pm 0.10	0.77 \pm 0.10	0.78 \pm 0.11	0.94 \pm 0.18	2.16 \pm 0.59
0.25 - 1 mm monthly	0.63 \pm 0.07	0.69 \pm 0.10	0.71 \pm 0.10	0.72 \pm 0.10	0.74 \pm 0.11	0.83 \pm 0.16	1.81 \pm 0.49

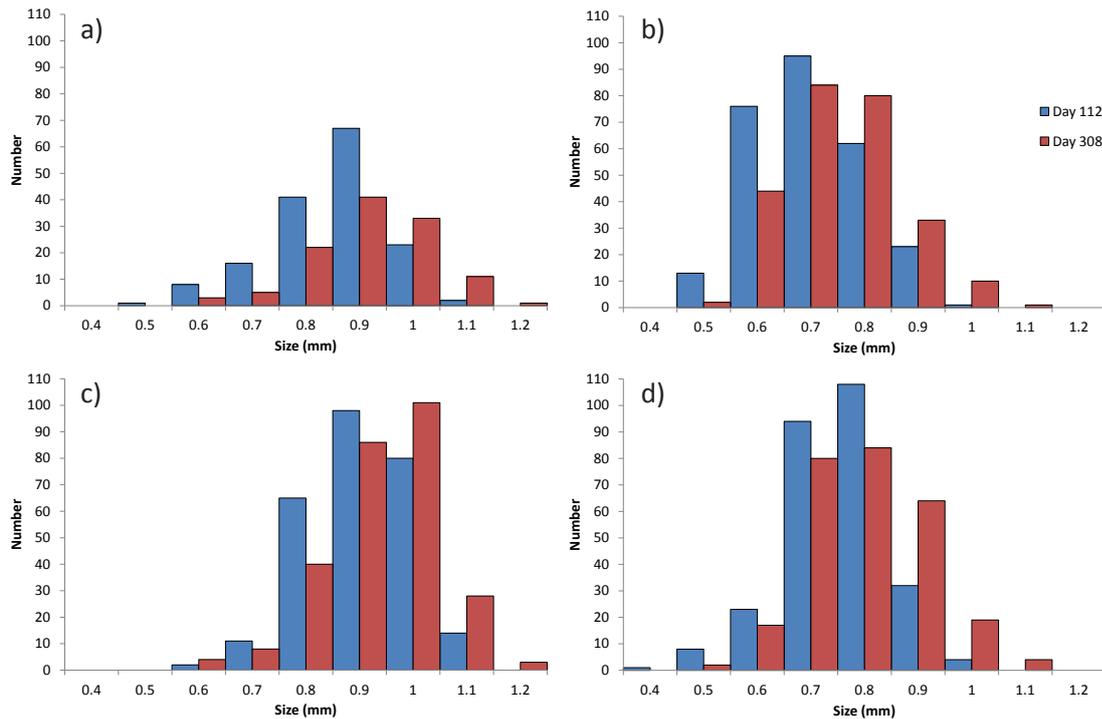


Fig. 3.6: Histograms showing length (mm) of juvenile freshwater pearl mussels before (day 112) and after winter (day 308) for treatments; a) 0.25 - 1 mm weekly; b) 0.25 - 1 mm monthly; c) 1 - 2 mm weekly; d) 1 - 2 mm monthly.

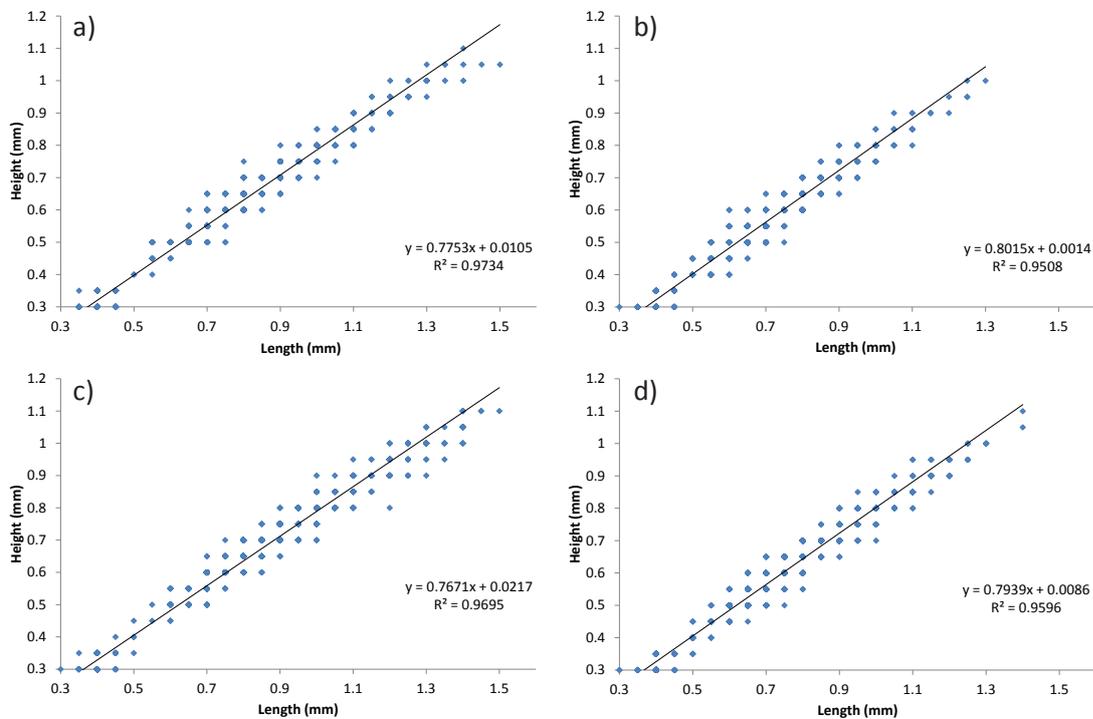


Fig. 3.7: Scatter plots showing length and height (mm) of juvenile freshwater pearl mussels in the different treatments; a) 0.25 - 1 mm weekly; b) 0.25 - 1 mm monthly; c) 1 - 2 mm weekly; d) 1 - 2 mm monthly. Length and height were highly correlated in all treatments ($P < 0.001$).

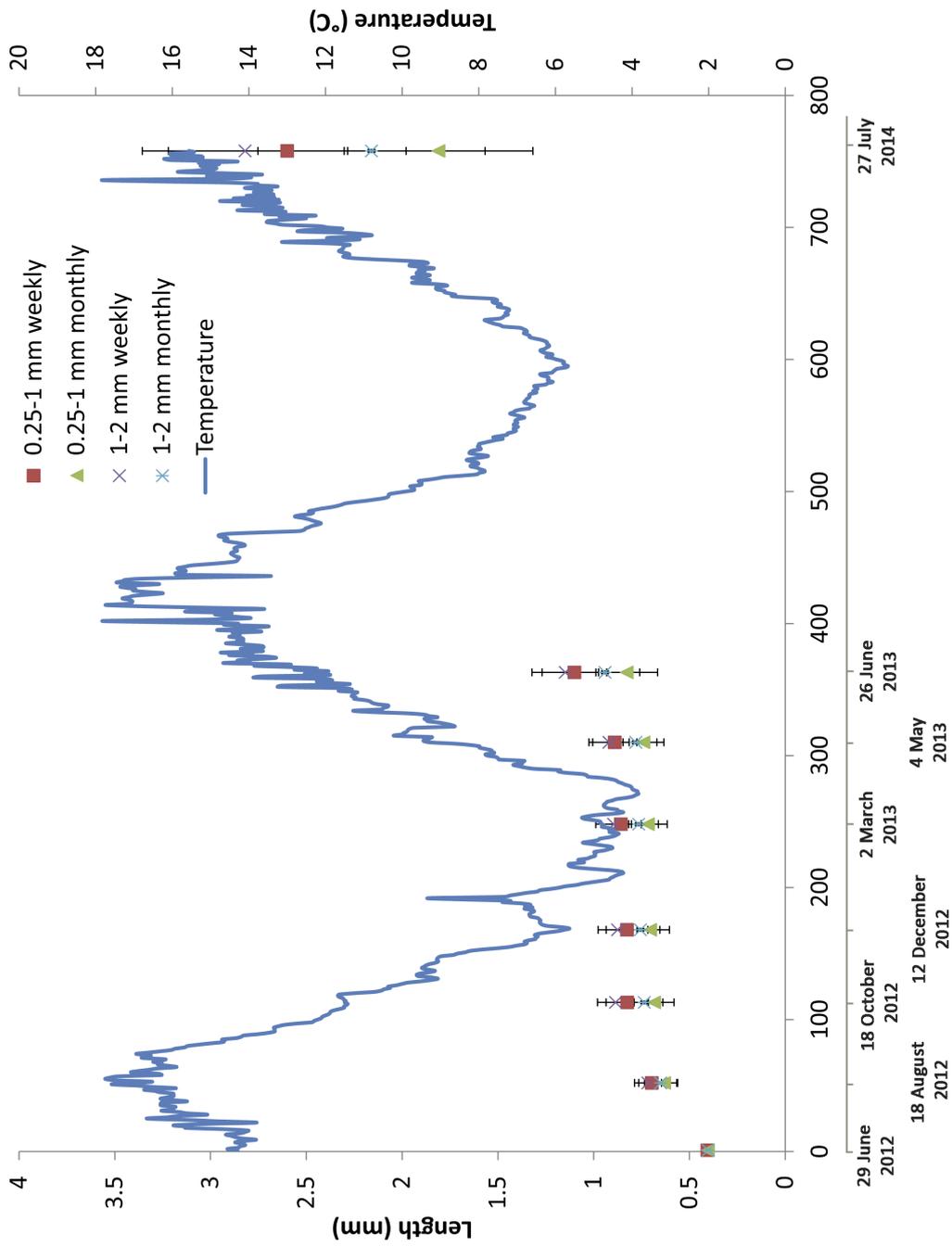


Fig. 3.8: Time series graph showing length (mm) of juvenile freshwater pearl mussels over the experiment duration in all treatments (\pm SD bars). Temperature ($^{\circ}$ C) is provided for reference to show near cessation of growth over winter. The x-axis is provided both in days since experiment commenced and per month to show how growth relates to time of year.

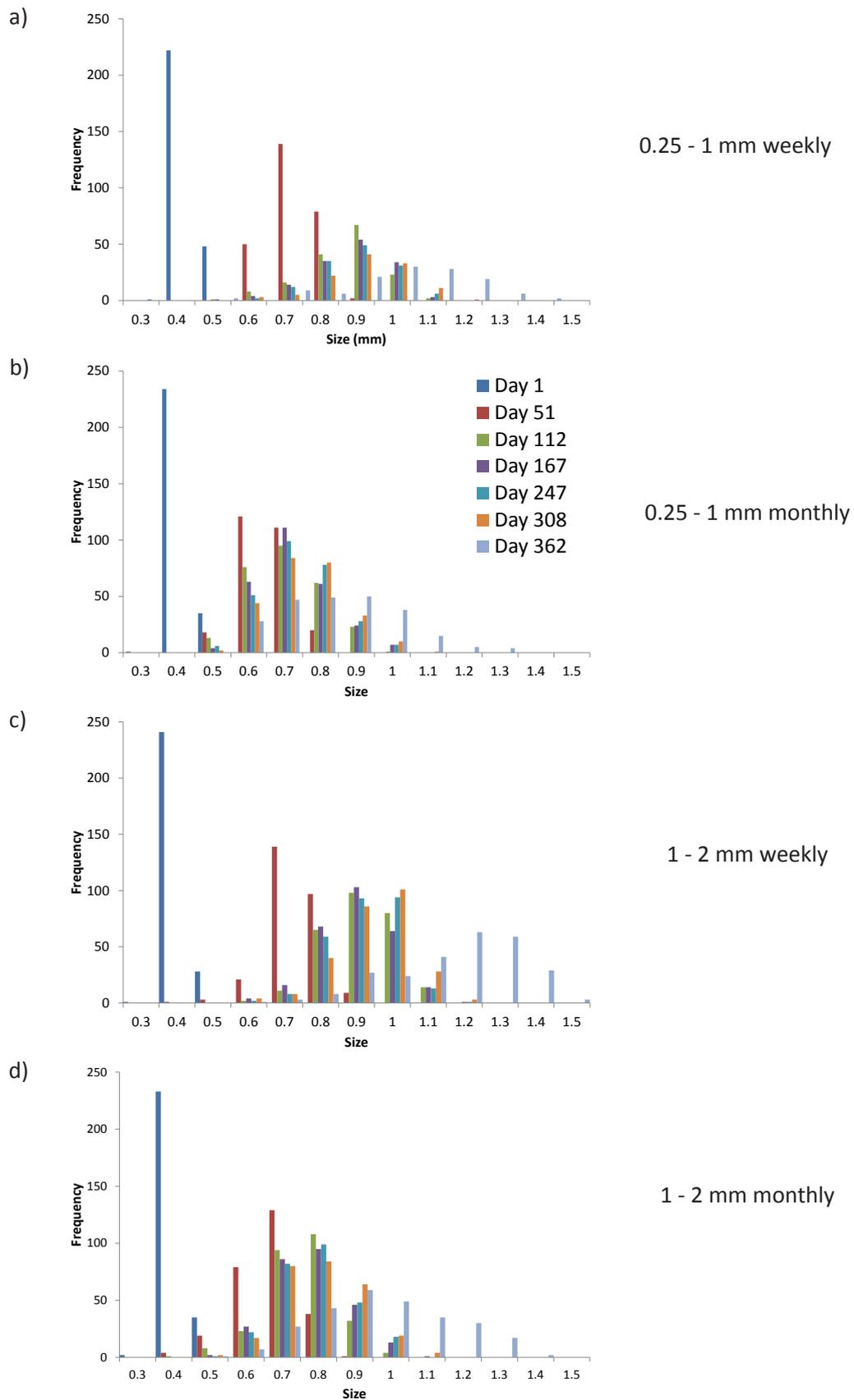


Fig. 3.9: Histograms showing frequency distribution of shell length (mm) of juvenile freshwater pearl mussels on each sampling occasion for each of the different treatments; a) 0.25 - 1 mm weekly; b) 0.25 - 1 mm monthly; c) 1 - 2 mm weekly; d) 1 - 2 mm monthly.

A 2-way ANOVA carried out on length data after 362 days found a significant interaction between substrate size and cleaning regime ($F_{(1,883)} = 7.414$, $P = 0.007$). Analysis of simple main effects found juveniles were significantly larger in the 1 - 2 mm substrate when cleaned both weekly ($F_{(1,883)} = 6.336$; $P = 0.012$) and monthly ($F_{(1,883)} = 54.916$; $P < 0.001$) and the individuals cleaned weekly were significantly larger in both the 0.25 - 1 mm ($F_{(1,883)} = 204.378$; $P < 0.001$) and 1 - 2 mm substrates ($F_{(1,883)} = 191.658$; $P < 0.001$). Another 2-way ANOVA was carried out for juveniles at 758 days but no significant interaction was found ($F_{(1,514)} = 1.358$; $P = 0.244$), although when considering these two factors separately, juveniles were significantly larger in 1 - 2 mm substrate ($F_{(1,514)} = 23.731$, $P < 0.001$) and those treatments cleaned weekly ($F_{(1,514)} = 157.734$, $P < 0.001$).

3.3.2.1 Growth per day and the effect of temperature

Shell growth is positively correlated with temperature ($r_{s(214)} = 0.76$, $P < 0.001$). Near cessation of growth was observed below approximately 10 °C (Fig. 3.10). A summary of temperature data recorded at hourly intervals throughout the experiment is provided in Table 3.5.

Individuals in sieves cleaned weekly, regardless of substrate size, showed superior growth compared to those cleaned monthly (Table 3.4 & Fig. 3.8). Mean daily growth rates (Fig. 3.11) during the warmest period (June - August 2012) were almost 11 times higher than during the coolest period (March - May 2013). Growth decreased with temperature over winter but did not stop completely (Table 3.6) before increasing again between April and June 2013.

Table 3.5: Summary of water temperature data within rearing system for the different sampling intervals during 2012 - 2013. This shows summer maximum temperatures between June - August 2012, temperature dropping over winter to a minimum between March - May 2013 before temperatures rise again between May - June 2013.

Sampling interval	Temperature (°C)		
	Mean ±SD	Max	Min
29 June - 18 August 2012	15.4 ±1.0	17.6	13.8
19 August - 18 October 2012	14.7 ±2.0	17.7	11.4
19 October - 12 December 2012	9.0 ±1.7	11.7	5.9
13 December 2012 - 2 March 2013	5.6 ±1.1	9.3	4.2
3 March - 2 May 2013	5.5 ±1.5	8.6	3.8
3 May - 26 June 2013	10.7 ±1.3	13.9	8.6

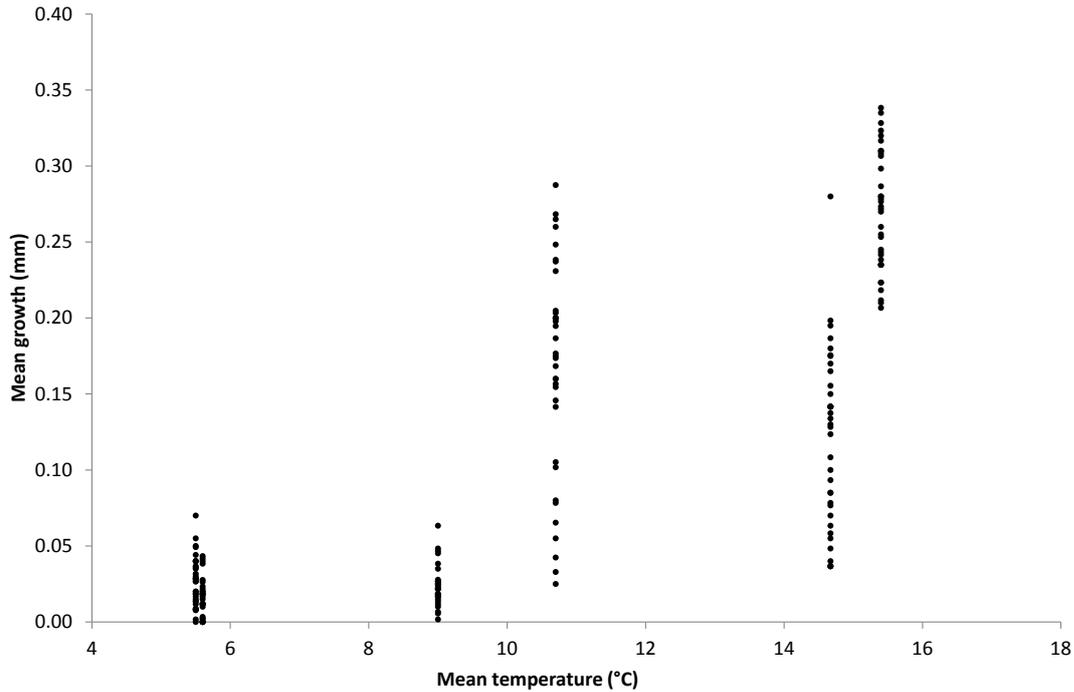


Fig. 3.10: Scatter plot showing mean shell growth (mm) of juvenile freshwater pearl mussels in individual sieves between sampling occasions against mean water temperature from June 2012 - June 2013. This shows higher growth when temperature exceeds approximately 10 °C and near-cessation of growth below this.

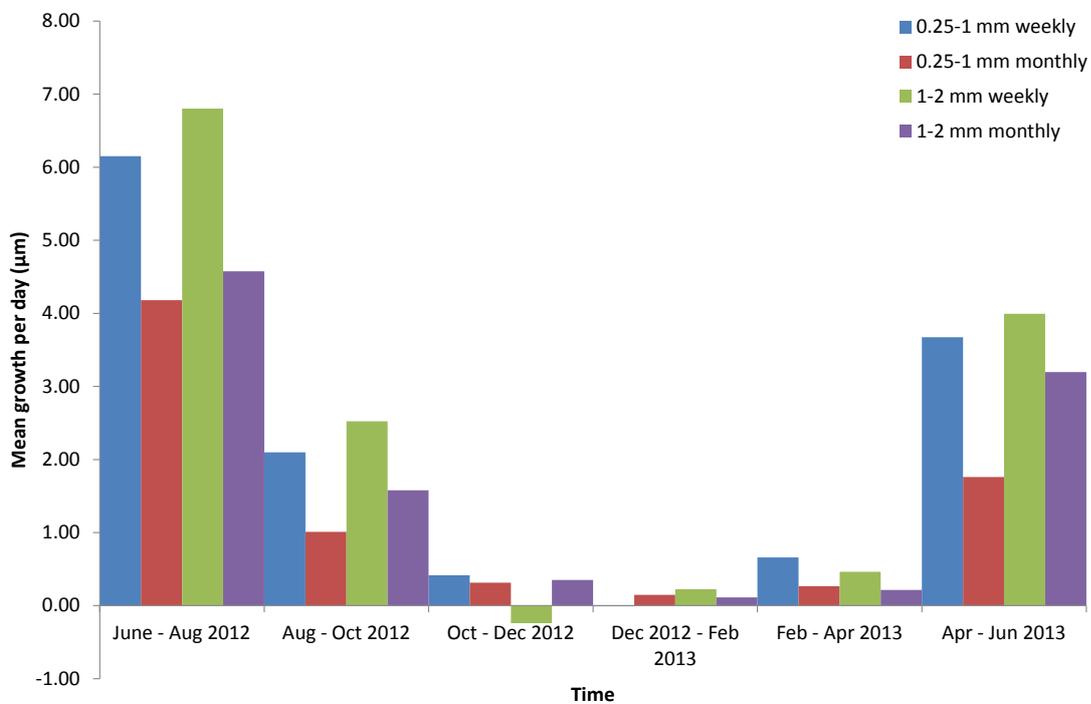


Fig. 3.11: Bar chart showing mean daily shell growth (μm) of juvenile freshwater pearl mussels in the different treatments over the first year of the experiment. June - August 2012 had the highest mean water temperature (Table 3.5) leading to the highest growth (shell length) per day.

Table 3.6: Mean daily growth in shell length (μm) for juvenile freshwater pearl mussels for the different sampling intervals in each treatment. N.B. Negative values in the 0.25 - 1 mm weekly treatment between December 2012 and February 2013 and in the 1 - 2 mm weekly treatment between October and December 2012 are due to sampling error.

Treatment	June – Aug 2012	Aug – Oct 2012	Oct – Dec 2012	Dec 2012 – Mar 2013	Mar – May 2013	May – Jun 2013
0.25 - 1 mm weekly	6.15	2.10	0.42	-0.01	0.66	3.67
0.25 - 1 mm monthly	4.18	1.01	0.31	0.15	0.27	1.76
1 - 2 mm weekly	6.80	2.52	-0.24	0.22	0.46	4.00
1 - 2 mm monthly	4.58	1.58	0.35	0.11	0.21	3.20

3.3.3 Escaped mussels

A total of 88 juveniles were found outside of sieves between 19 July 2012 and 5 April 2013. This equates to around 2.5 % of experimental individuals. The majority (84 individuals) were found during the first four months of the experiment and the number decreased markedly once the 0.3 mm sieves were placed on top of experimental sieves. Lengths of escaped juveniles are consistent with experimental individuals (Fig. 3.12).

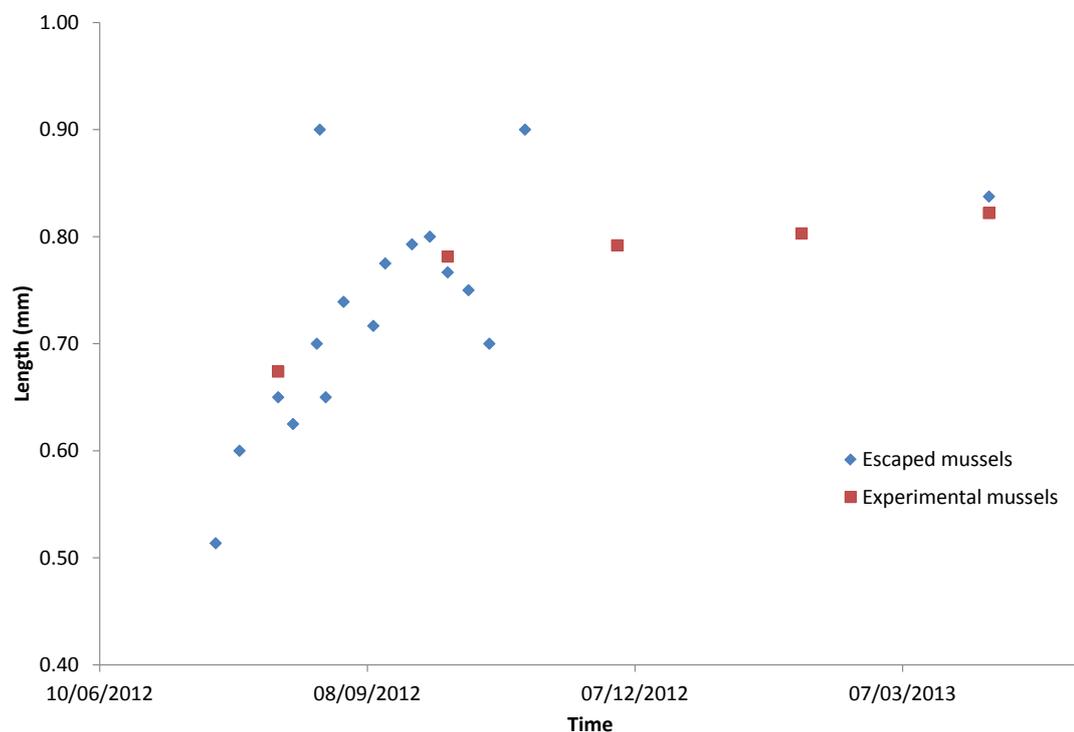


Fig. 3.12: Scatter plot showing mean shell length (mm) of escaped juvenile freshwater pearl mussels compared to the overall mean shell length (mm) of juveniles found within sieves on normal sampling occasions.

3.4. Discussion

The objective of this study was to test the effectiveness of this culture system for rearing newly-excysted juvenile mussels and to examine the effects of different substrate sizes and cleaning regimes on growth and survival of the freshwater pearl mussel *Margaritifera margaritifera*. This is a semi-natural rearing technique employing salient features from previous studies e.g. Hastie & Young (2003); Lange (2005); Barnhart (2006); Preston *et al.* (2007); Gum *et al.* (2011) to create a low-maintenance system which does not require supplemental feeding. This culture system proved successful in rearing juveniles to 25 months old and, if scaled up, could rear up to 2000 juveniles per year to a size of > 1 mm using 1 - 2 mm substrate cleaned monthly at a density of 100 juveniles per sieve. This equates to a maintenance effort of approximately three hours per month. This system may also safeguard against disease or fungal infection because juveniles are kept in separate sieves. If fungal infection was detected in a particular sieve then this could be removed to safeguard individuals in other sieves. Eybe *et al.* (2013) found fungal infection could be problematic and could spread rapidly, particularly when density is high.

The proportion of mussels escaping experimental containers was minimal (total 2.5%) and in future this can be prevented by placing covers over containers from the beginning. Other studies have reported juvenile mortality in culture associated with predation by other invertebrates (Henley *et al.*, 2001; Barnhart, 2003; Barnhart, 2004; Jones *et al.*, 2005) but there was no evidence of predation in this system. Before the addition of juveniles, substrate was exposed to lake water to allow biofilm development. Whilst not a topic of focus in this study, other researchers have found the presence of a biofilm increases growth and survival during the earliest juvenile stages compared to a sterile surface (Ó Foighil *et al.*, 1990, Gum *et al.*, 2011) and the use of substrate exposed for at least 21 days as per Battin *et al.* (2003) appears to be adequate for *M. margaritifera* juveniles.

Juveniles were reared to an age of 758 days with the most successful treatment achieving over 55 % mean survival by day 362 and 23 % by day 758. No other captive rearing programme has published this level of survival over a similar time scale (Fig. 3.3). However, published studies tend to lack specific information on periodic growth and survival over extended time periods. For example Schmidt & Vandr  (2010) reported mean survival of 82 % over approximately 120 days but no subsequent data, whereas Buddensiek (1995) details much lower survival but over a longer period e.g. 17% survival at 476 days and 2% survival at 1081 days. Hruška (1999)

detailed the rearing of 30,000 individuals to over 3 years old but does not provide information on original numbers of juveniles or survival rates. For a long-lived, slow growing species such as *M. margaritifera*, long term studies with periodic monitoring and standardised reporting are required to fully understand factors affecting growth and survival of juveniles in captivity.

In this investigation growth was comparable with other published studies on *M. margaritifera* e.g. Hruška (1999); Scheder *et al.* (2011); Scriven *et al.* (2011); Eybe *et al.* (2013). Juveniles achieved 170 - 220 % growth during the first season (equating to lengths of 0.68 – 0.89 mm) which compares favourably with Hruška (1999) who reported 250 % growth and Eybe *et al.* (2013) reporting 150 - 200 % growth (0.96 – 1.13 mm). It is not possible to use growth as an indicator of rearing success when comparing different rearing systems due to confounding issues such as differences in culture conditions, temperature regimes, handling and different growth rates displayed by different mussel lineages (pers. obs.). For example Eybe *et al.* (2013) exposed juveniles to a constant temperature of between 17 - 18 °C at all times to extend the growth period, whereas the mean temperature for the same period during this study was 15.0 ± 1.7 °C. Equally Scheder *et al.* (2014) kept juveniles at 18 °C and achieved mean daily growth rates of up to 9.3 µm; higher than this study's maximum of 6.80 µm. It is however encouraging that juvenile growth in this study is within the limits of other studies on this species.

This study found that juveniles of the same age can display a large size range after a relatively short time period. This has also been observed in other populations at the Freshwater Pearl Mussel Ark (Sweeting & Lavictoire, 2013) and concurs with findings from studies on other species e.g. Hudson & Isom (1984); Beaty & Neves (2004); Barnhart (2006); Schmidt & Vandr  (2010). By day 758, some individuals were four times the length of others and the standard deviations within the treatments were larger on this sampling occasion compared to all previous ones. It is thought that juveniles switch from pedal to filter feeding around 18 months old (E. Moorkens, pers. comm.) so this may indicate that differences in growth rates may be observed once filter feeding commences. This topic is explored further in Chapter 5. It is unlikely that the observed differences in growth rates arise from differences in excystment date as the maximum difference in this study was only 8 days. Some have suggested that different growth rates are governed by genetics or may even be due to the vitality of individual glochidia before leaving the female mussel (Loosanoff & Davis, 1963).

Whatever the reason, this pattern has been shown to continue as juveniles get older with some individuals reaching over 9 times the length of conspecifics by five years old (Sweeting & Lavictoire, 2013).

3.4.1 Effect of substrate size

Significantly higher survival was observed in larger substrates (1 - 2 mm) averaging between 37 - 55 % after 362 days compared with 14 - 28 % in the smaller (0.25 - 1 mm) substrate treatments (Table 3.1). These findings concur with previous studies on unionids e.g. Rogers (1999); Brady (2000); Liberty *et al.* (2007). By day 758, survival in the 1 - 2 mm monthly treatment appears to drop closer to other treatments although it is still significantly higher (Fig. 3.4). This may be because juveniles are outgrowing this system and the 0.3 mm sieve designed to retain juveniles may be causing some mortality due to squashing (pers. obs.). Removal of the top sieve could be beneficial after 12 months but escapement may still occur. On day 758 juveniles were observed clinging to the top edge of the 0.3 mm sieve approximately 30 mm above the substrate, proving that they are still very mobile at this age and capable of climbing out of containers. An alternative option to ensure juvenile containment within sieves would be to place a piece of netting over the top of the sieve, secured with an elastic band.

When considering juvenile length, a significant interaction between substrate size and cleaning regime was found after 362 days whereby juveniles were larger in 1 - 2 mm substrate and substrate which was cleaned weekly. This interaction was not present after 758 days. One hypothesis for why this interaction was lost between these two sampling points is that pedal feeding juveniles (day 362) may be particularly vulnerable to sub-optimal substrate conditions which may impede feeding and thus affect size. It is anticipated that there are higher flow rates through 1 - 2 mm substrates due to larger interstitial spaces which should mean higher levels of available dissolved oxygen or food, thus leading to larger juvenile size. Likewise, it is anticipated that weekly cleaning should increase water flow through sieves, providing a more suitable foraging environment for juveniles and perhaps increasing food delivery. Therefore it is thought that the interaction of substrate size and cleaning regime had a significant effect on the growth of pedal-feeding juveniles (362 days old) but this interaction disappeared when juveniles were filter feeding (758 days) because these juveniles are more capable of pumping water from the water column rather than being dependent solely upon the food available

within the substrate. For survival, there was no interaction between substrate size and cleaning regime on either of days 362 or 758.

Culture conditions which allow water to flow through interstitial spaces but do not allow too much fine organic or particulate matter to infiltrate can supply juveniles with suitable habitat conditions with good levels of oxygen and food (Liberty *et al.*, 2007). The potential benefits of using larger substrate sizes for *M. margaritifera* in culture are improved oxygen and food supply and also the more efficient removal of potentially toxic ions such as ammonium or nitrite, found to be a limiting factor for juvenile survival by Eybe *et al.* (2013). The superior growth and survival found in larger substrates in this study indicates that substrates measuring 1 - 2 mm in diameter allow sufficient flow-through but provide suitable substrates for juveniles to move through. The ability of mussels to survive in different substrate clasts appears to be species specific, or at least relates to the habitat niche of the species in question. Survival may also be affected by the facility or culture system employed. For example, Hua *et al.* (2013) recorded better growth and survival for *Villosa iris* in substrates < 0.2 mm in a recirculating system at the Freshwater Mollusk Conservation Centre in Virginia, USA, but Liberty *et al.* (2007) found superior survival in substrates between 0.50 - 0.85 mm in a flow-through system at the Aquatic Wildlife Conservation Centre, also in Virginia.

Individuals in the 0.25 - 1 mm weekly treatment were the only ones to show size-dependent survival over the first 362 days of the experiment. This may be because this treatment provided the least suitable conditions for *M. margaritifera* so only the largest (and presumably healthiest) individuals could survive. It appears that the combination of small substrate size and weekly cleaning does not provide conditions under which *M. margaritifera* juveniles can thrive.

3.4.2 Effect of cleaning regime

Cleaning substrate on a weekly basis detrimentally affects survival compared to monthly cleaning (Table 3.2 & Fig. 3.4). Differences in survival rates (i.e. the proportion of juveniles which survived between one sampling point and the next) between treatments were significant which indicates that observed differences in survival were not artefacts of high initial mortality in the 0.25 - 1 mm weekly treatment after approximately 112 days. Similar results have been reported documenting poorer survival and growth in substrates cleaned more

frequently in studies on other mussel species (O'Beirn *et al.*, 1998; Hanlon, 2000; Zimmerman, 2003; Liberty *et al.*, 2007) due to stress or accidental damage/loss during sampling.

Whilst survival was compromised in treatments cleaned more frequently, growth was found to be significantly higher. This may be because cleaner substrates allow juveniles to forage for more or better quality food and there is less build-up of potentially toxic waste products such as ammonia. Superior growth in treatments cleaned more frequently contradicts findings of studies on other mussel species e.g. Hanlon (2000) and Liberty *et al.* (2007). It appears that most captive-bred species require enough cleaning to remove fine particles in order that they do not affect normal behaviour (e.g. foraging) but too much cleaning may cause stress and damage/loss, leading to lower growth rates and higher mortality. Whilst this finding is interesting, higher growth rates should not be sought at the expense of survival in captive rearing programmes.

3.4.3 Effect of temperature

This investigation confirms the findings of other studies showing juvenile growth is negligible when temperatures are below approximately 10 °C (Ziuganov *et al.*, 1994; Buddensiek, 1995; Hruška, 1999; Scheder *et al.*, 2014). Mean daily temperature dropped below 10 °C between early November 2012 and late May 2013, approximately half of the first year post-excystment. The temperature regime of Windermere is different from lotic systems because the lake is slower to heat up in summer and cool down in autumn (see Fig. 3.4 for temperature profile). This may provide an extended growth period for juveniles before the onset of winter. During the first growth season there were 127 days where temperatures were above 10 °C, equating to 1845 degree days. Whilst a higher rate of daily growth occurred at higher temperatures (Table 3.6), it is important to note for this experimental system that the growth season may be extended as long as water temperature remains above 10 °C. Some rearing programmes have adopted the strategy of holding juveniles at artificially high temperatures to promote growth to > 1 mm in early juvenile life (Lange, 2005; Eybe *et al.*, 2013, Scheder *et al.*, 2014). The long-term effects of this are unknown. This strategy could lead to selection for individuals better suited to surviving at higher temperatures or it could lead to delayed mortality once individuals are introduced into a natural temperature regime. Wild individuals growing at the southern extreme of the natural range of *M. margaritifera* grow more quickly than those in northern

populations due to increased metabolism but these individuals also die younger (Bauer, 1992; Hruška, 1992). Due to these concerns, a fluctuating temperature regime encompassing temperature ranges observed in native pearl mussel rivers was used in this study but the effects of selection or delayed mortality until later juvenile life of stable and/or elevated temperatures in early juvenile life should be investigated further.

Across all treatments mortality was highest during the first growth season, after which it was relatively low and stable over winter before increasing slightly at the beginning of the second growth season (Fig. 3.4). This implies that juvenile survival is relatively stable when water temperature (and therefore metabolic rate) is low but increases with rising water temperature. At higher temperatures it is anticipated that certain processes will increase such as metabolic rate (leading to higher DO consumption and increased production of waste materials) and primary production (with subsequent breakdown of organic and waste materials), thus decreasing sediment quality for juveniles and increasing mortality. Some of these factors are investigated and discussed further in Chapter 4.

Regardless of the hypothesis posed above, the observed pattern of survival in this study was not expected because it was assumed that mortality would increase over winter for those juveniles lacking sufficient nutritional reserves (Lange & Selheim, 2011; Denic *et al.*, 2015). Buddensiek (1995) found high mortality during the first few months post-excystment (June – December) but also found size-dependent over-winter mortality of smaller mussels < 0.7 mm; a result which has not been replicated in this study. This investigation found some individuals in the treatment showing the slowest growth (0.25 - 1 mm monthly) had still not attained 0.7 mm by 362 days. Whilst growth rates decreased substantially between 112 and 308 days in line with decreasing water temperature, a small amount of growth was still observed (approximately 0.3 $\mu\text{m}/\text{day}$) which matches almost exactly with the 0.31 $\mu\text{m}/\text{day}$ Scheder *et al.* (2014) observed over late autumn and winter. This minimal growth was distinguished from mortality of smaller individuals by considering the number of individuals in the different size classes pre- and post-winter (Fig. 3.6). There are more juveniles in larger size classes in April 2013 compared to October 2012 and not simply fewer juveniles in the smaller size classes (which would indicate mortality in the smaller individuals).

3.4.4 Conclusions and further research

This work has informed breeding practices at the FBA's Freshwater Pearl Mussel Ark. A rearing protocol using 1 - 2 mm substrate cleaned every 2 weeks in modified fish egg trays described in Sweeting & Miles (2009) was introduced for all new juvenile cohorts collected from spring 2013. Some success in this modified system allows tentative optimism that aspects of this investigation can be used to scale-up propagation of threatened populations at the FBA. This system facilitated rapid sampling and cleaning which is an important consideration when designing culture systems in order to maximise efficiency.

It is thought that juveniles undergo transformation from pedal to filter feeding at approximately 18 months old (~548 days - E. Moorkens pers. comm.) and observations made at 758 days indicated that most individuals were filtering. This system is therefore suitable for both pedal and filter feeding juveniles although as mentioned previously it may be beneficial to remove the 0.3 mm sieve after 12 months and replace it with an alternative mesh cover to avoid crushing juveniles.

Whilst this study has demonstrated significant differences in survival and growth in different substrate sizes and cleaning regimes a number of questions remain:

- 1. What habitat parameters are responsible for these differences?** This investigation sought to establish if there were differences between substrate size clasts and cleaning regimes but more information is required on why these differences were observed. Chapter 4 seeks to explore this by investigating parameters such as interstitial dissolved oxygen concentration, ammonia concentration, interstitial space and flow characteristics in this system.
- 2. Does juvenile density affect survival and growth within this system?** This question is important to refine the system and optimise the number of juveniles reared. Eybe *et al.* (2013) found better juvenile growth at densities of < 300 juveniles per 500 ml water whilst Barnhart (2006) achieved good survival for several North American mussel species at densities of 2000 individuals in small cups. Similarly Beaty (1999) found no density dependent effects for *Villosa iris*.
- 3. Is this system suitable for juveniles beyond 758 days?** Investigating this would demonstrate suitability of the system beyond 758 days and would also clarify when

survival stabilises. Sweeting & Lavictoire (2013) observed > 90 % year-on-year survival in some populations at the FBA after reaching 3 - 4 years old.

- 4. Can juveniles 'choose' their habitat?** Choice or preference for different types of substrate is likely to be species-specific (Huehner, 1987; Downing *et al.*, 2000; Lara & Parada, 2009) and may change with age (or size). The current study did not provide a choice to juveniles so this aspect is worthy of further study. Larger substrate sizes (> 2 mm) could also be considered to see if survival could be increased in the current system.

Findings of this chapter have been published in the following paper which can be found in the supplementary material at the end of this thesis:

Lavictoire, L., Moorkens, E., Ramsey, A.D., Sinclair, W. and Sweeting, R.A. (2016) Effects of substrate size and cleaning regime on growth and survival of captive-bred juvenile freshwater pearl mussels, *Margaritifera margaritifera* (Linnaeus, 1758). *Hydrobiologia*. **766**: 89-102. <http://dx.doi.org/10.1007/s10750-015-2445-4>.

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Chapter 4

Interstitial factors affecting growth and survival of juvenile freshwater pearl mussels
Margaritifera margaritifera

4.1. Introduction

In the wild, juvenile mussels are dependent upon the substrate conditions within the habitat patch into which they fall upon excystment. Juveniles are more vulnerable to poor interstitial habitat conditions as they live within the gravels for several years post-excystment (Skinner *et al.*, 2003) and are much more likely to be washed away if poor habitat conditions force them to rise to the surface in search of better conditions (Moorkens, 2011).

Physical habitat structure influences the organisms residing within substrates and factors such as habitat type, flow and biological activity act over different spatial scales, causing variations at the level of individuals and populations. In a natural river setting, hyporheic exchange delivers water rich in dissolved oxygen (DO) to the shallow hyporheic zone and removes the build-up of waste products and toxic chemicals (Buss *et al.*, 2009; Tonina & Buffington, 2009). Flow also affects the structure and function of biofilm communities (Battin *et al.*, 2003) which has the potential to be an important food source in early juvenile life.

In captive or laboratory settings, parameters such as flow (rate and direction), fine particle inputs, supplementary diet, substrate characteristics and disturbance can be controlled and monitored to achieve improved survival and/or growth of cultured species. Whilst many culture facilities adopt methods which are semi-natural e.g. Buddensiek (1995), Hruška (1999), Mummert (2001), Beaty & Neves (2004), Preston *et al.* (2007), Lavictoire *et al.* (2014), others have adopted methods which promote accelerated growth in systems which do not require water flow or sediment at all e.g. Lange (2005), Eybe *et al.* (2013). This brings into question which parameters truly limit juvenile growth and survival in captivity and where captive rearing programmes should focus efforts to optimise survival.

4.1.1 Interstitial conditions affecting juvenile mussels

Many studies have been carried out to characterise pearl mussel habitat but relatively few have focused on the interstitial habitats which juveniles inhabit and are dependent upon (Scheder *et al.*, 2015). Water flow through stream substrates is complex and dynamic often varying over very small spatial scales (cm) and is affected by a wide range of factors such as substrate particle size, discharge and channel slope (Geist & Auerswald, 2007; Quinlan *et al.*, 2014a). Such factors are outside of the scope of this study but it is important to note which

habitat parameters in the wild predict presence or absence of juvenile mussels in order to tailor rearing systems and investigate why these factors are good predictors.

Margaritifera margaritifera (Linnaeus, 1758) requires pristine habitat conditions with high DO concentrations. In 1898, (Boycott & Bowell) described *M. margaritifera* inhabiting rivers with “...more or less a sandy bottom...”, “...found in abundance at the head of rapids where the bottom consists of fairly large stones, the interstices filled with sand and fine gravel...” and “...sheltering behind larger stones...”. These early descriptions of pearl mussel habitat have since been echoed by many researchers, all of whom agree that the species prefers mixed substrate of coarse sand and smaller stones stabilised by larger cobbles and boulders (Boycott, 1933; Vannote & Minshall, 1982; Purser, 1985; Neves & Widlak, 1987; Ziuganov *et al.*, 1994; Gittings *et al.*, 1998; Beasley & Roberts, 1999; Hastie *et al.*, 2000; Moorkens, 2000; Skinner *et al.*, 2003; Morales *et al.*, 2004; Geist & Auerswald, 2007; Moorkens & Killeen, 2014; Quinlan *et al.*, 2014a). Neither grain-size nor compaction should limit free-flowing water exchange with the interstitial (Buddensiek *et al.*, 1993; Geist & Auerswald, 2007) and flow should be high enough to discourage sedimentation (Hastie *et al.*, 2003; Morales *et al.*, 2004).

The most significant factor leading to population declines across Europe has been habitat degradation through siltation and increased nutrient inputs (Buddensiek *et al.*, 1993; Moorkens & Killeen, 2014; Santos *et al.*, 2015). Interstitial spaces can become clogged by fine particles (Buddensiek, 1995; Brim Box & Mossa, 1999; Geist & Auerswald, 2007) and anoxic through a combination of factors including direct smothering, decreasing water column-interstitial water exchange, and decomposition of organic matter (Buddensiek *et al.*, 1993; Patzner & Müller, 2001; Tarr, 2008; Buss *et al.*, 2009) although the exact cause of juvenile mussel mortality has not yet been identified (Quinlan *et al.*, 2014a). Juvenile mussels are intolerant of anoxia (Dimock & Wright, 1993). Therefore when interstitial conditions become sub-optimal they may seek higher oxygen levels by rising to the substrate surface (Moorkens, 2011) where the exchange with the water column is higher. This puts juveniles at risk of being washed away during high flows, indirectly leading to mortality (Sparks & Strayer, 1998). Many factors affect DO and concentrations can be highly variable even within dense mussel beds (Quinlan *et al.*, 2014b). In addition to flow rate and the breakdown of organic matter, nitrification can also affect DO. Waste products (mainly ammonia) from juvenile mussels and other interstitial invertebrates are oxidized to nitrite and again oxidized to nitrate under aerobic conditions,

decreasing available oxygen. In addition, un-ionized ammonia and nitrite can be toxic to aquatic organisms (Patzner & Müller, 2001) so poor flow through substrates (and thus failure to remove nitrogenous waste products) may also contribute to mortality.

In a controlled environment such as captive rearing systems, much of this habitat complexity can be removed or simplified and it is possible to measure the effects of individual factors on juvenile survival and growth.

4.1.2 Objective of this study

A number of studies have investigated nutrient concentrations from the water column in pearl mussel rivers e.g. Bauer (1988); Buddensiek *et al.* (1993); Moorkens (2006a); Geist & Auerswald (2007) but the suggested targets/limits are for the water column and not the interstitial layer where juveniles reside.

The findings reported in Chapter 3 and published by Lavictoire *et al.* (2016) outline a lack of basic knowledge of the physical, chemical and biological requirements of juvenile mussels and why these are important for survival. The current chapter seeks to quantify some of the parameters which may contribute to the differences in survival and growth observed in the previous chapter. This will provide additional information on the habitat requirements of newly-excysted juvenile mussels and how these parameters ultimately affect survival.

In Chapters 2 & 3, an aquarium system was described which uses a hydrostatic head to create a down-welling system through sieves containing juvenile mussels. Superior survival rates were observed in coarser substrates and higher growth was observed in treatments cleaned more frequently (Lavictoire *et al.*, 2016). Substrate size and interstitial clogging affects interstitial flow, DO concentrations and the removal of potentially harmful ions. It is therefore probable that differences in these parameters are the cause of these significant differences in growth and survival in treatments with different substrate sizes (0.25 - 1 mm & 1 - 2 mm) and cleaning regimes (weekly & monthly). This chapter considers the habitat parameters below, as well as the growth and survival of newly-excysted juvenile mussels over a two month period in summer 2015 immediately following excystment. In the experiment carried out in 2012/13 (Chapter 3), this was the period of highest mortality for newly-excysted juveniles and is likely to have the most challenging conditions with lower DO concentrations and higher productivity due to higher (summer) temperatures.

Parameters to be considered are:

1. Effective pore space
2. Flow rate
3. Interstitial DO concentration
4. Interstitial ammonia concentration
5. Total phosphorus (TP) concentration from organic matter within the interstices
6. Dry weight of organic and inorganic material adhered to substrate (biofilm biomass).

4.2. Methods

4.2.1 Experimental set-up

An aquarium (620 x 310 x 310 mm) was set up in the same way as described in Chapter 3 but with only three replicates of each treatment (total 12 sieves). New substrate was sieved to the two required sizes (0.25 - 1 mm and 1 - 2 mm) and heated in a muffle furnace (Carbolite 301) to 550 °C for 4 hours to ash any organic matter. Forty grams (± 0.01 g) of substrate was added to each experimental sieve. Substrate was exposed to running lake water for at least 21 days before addition of juveniles to allow the establishment of a stable biofilm. Substrate was cleaned as described in Chapter 3 before the addition of juveniles. One hundred juveniles were added to each sieve (30 measured to obtain initial size as per Chapter 3) between 18 - 23 July 2015 and a 0.3 mm sieve placed on top to stop juveniles escaping. The mesh of each 0.3 mm sieve had a hole through which 6 mm aquarium tubing was inserted so the tip rested approximately half way in to the substrate for water samples to be extracted. Tubing had 0.2 mm mesh over the end within the substrate to stop the removal of juvenile mussels when water samples were taken. Three sieves also had a second hole so DO loggers could be inserted to the same depth (Fig. 4.1). Sieves in the weekly treatments were cleaned weekly following the cleaning method described in Chapter 3 and the process repeated on a monthly basis for the monthly treatments. Where DO loggers were present in sieves which needed to be cleaned, loggers were downloaded and removed from sieves before cleaning.

A multi-parameter sonde (Troll 9500, In-Situ, USA) was suspended in the water column in the top chamber of the aquarium and this logged conductivity, DO, pH, redox potential, temperature and turbidity every 15 minutes for the duration of the experiment. DO loggers were introduced to the system at the beginning of week 5 (day 31; 17 August 2015). Table 4.1

outlines the treatments for which DO was logged, when, and for how long they were deployed. For the treatments cleaned weekly the PreSens DO dipping probe was sited in a 0.25 – 1 mm sieve for a single week, followed by a single week in a 1 – 2 mm sieve followed by a two week period in the same 0.25 – 1 mm sieve (different to the sieve in week 5). At experiment termination, the 0.25 – 1 and 1 – 2 mm monthly sieves were cleaned on 13 September 2015 and the probes reinserted until the following day while sampling of the other sieves took place. This was to ascertain if cleaning increased DO concentration in the monthly treatments following

Table 4.1: Summary of types of dissolved oxygen and multi-parameter loggers used, parameters measured, position in aquarium and duration of deployment.

Make/model of logger	Position	Duration of deployment	Parameters
In-Situ, Troll 9500	Water column	Whole experiment	Conductivity, DO, pH, redox potential, temperature, turbidity
PreSens DO dipping optode with Fibox 4 logger	0.25 – 1 mm weekly	Week 5	DO, temperature
	1 - 2 mm weekly	Week 6	
	0.25 – 1 mm weekly	Weeks 7 & 8	
Hobo U26-001	0.25 - 1 mm monthly	Weeks 5 - 8	DO, temperature
Hobo U26-001	1 - 2 mm monthly	Weeks 5 - 8	DO, temperature

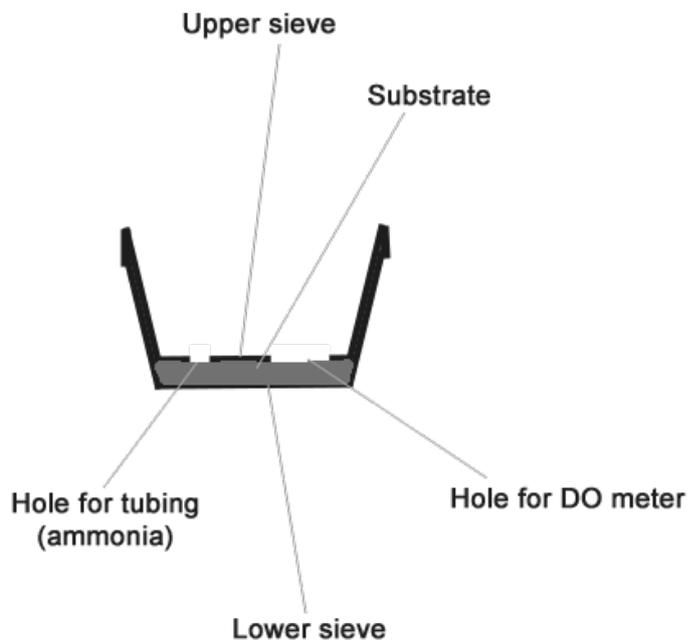


Fig. 4.1: Diagram showing sieve set-up in the rearing system. Upper and lower sieves sandwich the substrate. Holes are present in the mesh of the upper sieves for taking DO measurements and ammonia samples.

a cleaning event. Optodes/sensors were calibrated following manufacturer's instructions and measured to within an average of 0.32 mg/L and 0.08 °C of each other.

Water samples for ammonia analysis were taken before cleaning on day 29 (see section 4.2.2.1 below). On day 57 (14 September 2015) the experiment was terminated and all sieves were checked for juvenile survival. Size (length and height) of 30 individuals was measured before they were removed to the Ehen 2015 tray in the tray system described in Chapter 2. When checking substrate for juveniles on day 57, care was taken to retain all organic material from each sieve for analysis of TP (see section 4.2.2.2 below). Effective pore space, flow rate through substrate and organic content of biofilm was analysed as described in section 4.2.3 below.

4.2.2 Chemical analyses

4.2.2.1 Ammonia

On day 29 (17 August 2015) before sieves were cleaned, a 2 ml water sample was extracted from each sieve and three samples taken from random spots in the water column. Initially, 1 ml was siphoned out and discarded (water present in the tube) before the sample was taken.

Ammonia-free water was prepared in advance using Amberlite IR 120 resin (Na⁺ form, Aldrich Chemistry). Three hundred and sixty ml of 1N HCl solution was passed through the resin at a rate of 7 ml/min until all Na⁺ ions on the resin had been replaced by H⁺ ions. The resin was flushed three times the bed volume with de-ionised water and the conductivity measured to ensure all acid had been flushed from the resin. De-ionised water was then passed through the resin to obtain ammonia-free water.

The phenate method for determination of ammonia concentration was used (Greenberg, 1985). Standards of ammonium chloride were prepared to concentrations of 0.00625, 0.0125 and 0.025 µg/ml. A blank (ammonia-free water) was also used to check for potential sample contamination. The blank and standard solutions were used to create a calibration curve before analysis of samples using a Double-beam Cary 60 UV-VIS spectrophotometer (Agilent Technologies, USA) at 630 nm.

4.2.2.2 Total phosphorus

The amount of total phosphorus (TP) from organic matter washed from within the substrate interstices was analysed at the end of the experiment to give the amount of TP present after one week (treatments cleaned weekly) and one month (treatments cleaned monthly). After experiment termination, the contents of each sieve were emptied into a pyrex dish and elutriated in the same way as for juvenile cleaning to collect organic matter. Initially, attempts were made to filter two samples through Whatman GF/C filters but the pores became too clogged. All other samples were poured into a measuring flask, allowed to settle and the supernatant discarded. The organic material and excess water were then added to crucibles and dried at 105 °C overnight. In the two samples where filtration had been attempted the filter papers and the remaining organic matter and water were treated in the same way. The dry samples were weighed before being heated to 550 °C for 4 hours. The burned weight of each sample was recorded once crucibles had cooled to room temperature. Empty crucibles were also weighed.

To each ash sample, 5 ml of 5N hydrochloric acid was added and left to stand for 2 hours to make P available for analysis. Samples were filtered through Whatman No.1 filters and made up to 100 ml with distilled water. A 1 ml sub-sample was taken from each and neutralized by adding 1 drop phenolphthalein solution and concentrated sodium hydroxide dropwise until the solution turned bright pink. These sub-samples were then made up to 20 ml with distilled water. Mixed reagent was prepared by combining 5N hydrochloric acid with solutions of sodium molybdate, ascorbic acid and antimony potassium tartrate in the ratio 5:2:2:1. Phosphate standard solutions were prepared with potassium dihydrogen orthophosphate to concentrations of 0 (blank), 0.02, 0.05, 0.10, 0.15, 0.20, 0.40 and 1.00 µg/mL. To the 20 ml samples and standard solutions, 5 ml mixed reagent was added and solutions left for at least 15 minutes for a blue colour to form and stabilise. In an acidified solution, orthophosphate reacts with acidified molybdate and antimony potassium tartrate to form molybdophosphoric acid which is reduced by ascorbic acid to the intensely blue coloured molybdenum blue. The blank and standard solutions were used to create a calibration curve before analysis of samples via spectrophotometry at 880 nm. Results are therefore reported as TP (filtered) but are not TP dissolved in interstitial water; they are the TP bound in organic material trapped within substrate interstices.

4.2.3 Flow and organic content analysis

Sieves were removed carefully from the test aquarium and placed over a bucket on a mesh surface. One litre of water was carefully poured into the sieve making sure that substrate and organic matter were not disturbed. The time taken for 1 L of water to pass through the sieve was recorded. Each sieve was examined to record the number of live juveniles remaining and the length and height of 30 randomly selected individuals. This process was repeated for all sieves. During juvenile processing, care was taken to collect all organic material from within the sieves for analysis of TP.

The effective pore space in each sieve's substrate was measured after juveniles were removed and substrate had been elutriated to remove organic matter. Substrate was placed into a measuring cylinder and water added until the meniscus rested on the substrate surface. Water was then drained into another measuring cylinder and the volume recorded. The drying and burning procedure described in section 4.2.2.2 was then carried out to measure the weight of biofilm on substrate. Loss On Ignition (LOI) was calculated using the following equation:

$$\text{Loss on ignition} = ((DS - AS) / (DS - DC)) \times 100$$

Where:

DS = weight (g) of the crucible containing the sample dried at 105 °C over night.

AS = weight (g) of the crucible containing the ignited sample.

DC = weight (g) of the empty crucible.

4.2.4 Data analysis

As mentioned in Chapter 3, juvenile length and height are highly correlated and so only length was used for analysis here. Juvenile length was not significantly different between sieves ($F_{(11,348)} = 1.366, P = 0.187$) at the beginning of the experiment (mean length = 0.45 ± 0.05 mm).

Central Limit Theorem (Elliott, 1993) was applied to assume normality where appropriate. ANOVA's with *post hoc* Tukey's HSD tests were used for data relating to juvenile survival, juvenile length, weight of biofilm, weight of interstitial organic matter, total LOI, total phosphorus in the substrate and ammonia. Student's *t*-tests were used to compare interstitial space in the two different substrate sizes. Unless otherwise stated, standard deviations are provided after means.

To ensure there was no temporal autocorrelation bias within DO data, water column data were analysed using the autocorrelation and partial autocorrelation function in the statistical package R (version 3.2.2). This analysis indicated an appropriate interval of 11 data points. Every 11th data point was therefore extracted and used for analysis. Spikes in data relating to cleaning events (section 4.3.3) were removed for data analysis purposes but are explained and discussed in detail in the following sections. Paired *t*-tests were performed to explore differences in DO concentrations between the water column and treatments. For these tests a Bonferroni correction for multiple tests was applied so that $\alpha = 0.006$ ($n = 9$). Plots of DO data before and after cleaning in the 0.25 - 1 mm weekly treatments were visually inspected to observe any effect of cleaning on DO concentration.

Juvenile survival during the first two months post excystment, temperature profiles and information on when juveniles were collected during the excystment period were also analysed between 2012 and 2015 to see if any differences could be found.

4.3. Results

The Troll 9500 sonde measured water quality parameters every 15 minutes during its deployment. Analysis and summary of the period 23 August - 15 September 2015 are provided (Table 4.2 & Fig. 4.2) to compliment DO data. All parameters measured by the sonde were within the ecologically acceptable range for *Margaritifera margaritifera* throughout the experiment and are within

Table 4.2: Mean (\pm SD) values for water quality parameters measured by the Troll 9500 sonde and from spot samples taken by the Environment Agency on 17 August 2015.

Parameter	Mean (\pm SD)
TROLL 9500 SONDE	
Temperature ($^{\circ}$ C)	16.62 \pm 0.39
Turbidity (FNU)	4.21 \pm 3.68
Redox potential (Volts)	0.53 \pm 0.02
pH	7.28 \pm 0.09
Dissolved oxygen (mg/L)	8.57 \pm 0.25
Dissolved oxygen (% saturation)	88.42 \pm 2.96
Conductivity (μ S/cm)	56.06 \pm 2.13
ENVIRONMENT AGENCY DATA	
Phosphorus-P (mg/L)	0.02
Nitrogen-N (mg/L)	0.71
Filtered orthophosphate (mg/L)	0.002
Nitrogen-oxidised filtered (mg/L)	0.18
Ammonia filtered (mg/L)	0.002

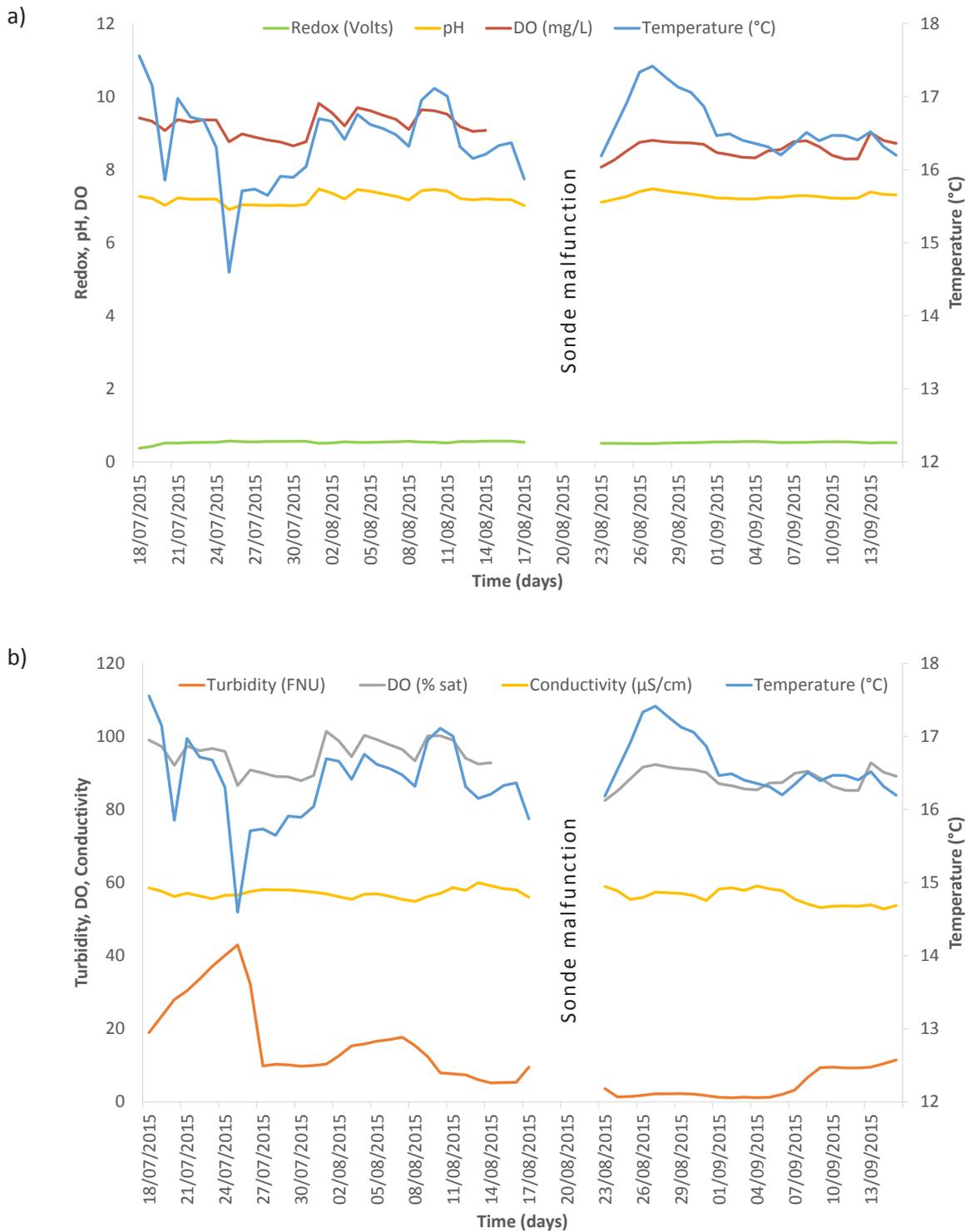


Fig. 4.2: Time series graphs showing mean daily values for water quality parameters measured in the aquarium throughout the experimental period. (a) Redox (Volts), pH & DO (mg/L); (b) Turbidity, (FNU), conductivity ($\mu\text{S}/\text{cm}$) and DO (% saturation). Daily mean temperature also provided on both graphs for reference. Data is missing between 17 - 23 August 2015 due to sonde malfunction.

the expected range for the time of year. Sonde malfunction between 17 - 23 August meant that data are missing for this period. Additional data were also provided by the Environment Agency (© Environment Agency and database right) in Table 4.2 for Windermere South Basin (grid ref: SD 38230 91552). Turbidity appeared to increase towards the end of the period but this was thought to be due to fouling on the sensor rather than an actual increase in suspended material.

4.3.1 Survival

Survival differed significantly between treatments ($F_{(3,8)} = 4.713$; $P = 0.035$) with highest survival in the 1 - 2 mm monthly treatment and the lowest survival in the 0.25 - 1 mm monthly treatment (Table 4.3). Despite this significant result, *post hoc* tests could find no significant difference between any two treatments, although the difference between the 0.25 - 1 mm monthly treatment was almost significantly different ($P = 0.053$) from the 1 - 2 mm monthly treatment. This may be because the number of replicates was low meaning that the *post hoc* tests did not have sufficient power to detect differences. The pattern of survival observed after 2 months in 2015 differs from 2012 results, with the 0.25 - 1 mm weekly treatment showing higher survival than the 0.25 - 1 mm monthly treatment in 2015 although since these differences in 2015 are not significant this is of limited importance (Table 4.3). In both 2012 and 2015, highest mean survival was observed in the 1 - 2 mm monthly treatment.

Table 4.3: Mean survival (\pm SD) of juvenile freshwater pearl mussels after 2 months in the 2012 substrate experiment (Chapter 3) and the current (2015) experiment. Superscript letters within columns indicate statistically homogeneous subsets.

Treatment	2012	2015
0.25 - 1 mm weekly	40 \pm 7 ^a	72 \pm 2 ^a
0.25 - 1 mm monthly	75 \pm 4 ^b	68 \pm 4 ^a
1 - 2 mm weekly	76 \pm 6 ^b	80 \pm 4 ^a
1 - 2 mm monthly	85 \pm 4 ^c	81 \pm 8 ^a

4.3.2 Size

Juvenile length was not significantly different ($F_{(3,356)} = 0.744$; $P = 0.526$) between treatments in September 2015. This is in contrast to findings in 2012 where juvenile size was significantly different after two months (Table 4.4).

The mean starting length of juveniles used in the pilot substrate experiment in 2011, the full experiment in 2012 and the current experiment in 2015 were significantly different ($F_{(2,3327)} = 461.487$, $P < 0.001$) with *post hoc* tests finding significant differences between all years. Juveniles in 2012 were the smallest ($0.40 \text{ mm} \pm 0.02$) followed by those in 2011 (0.44 ± 0.02) and the largest juveniles were collected in 2015 ($0.45 \text{ mm} \pm 0.05$). When considering temperature profiles (Fig. 4.3), excystment patterns (Fig. 4.4) and additional data such as degree days during the encystment and collection periods (Table 4.5) the effect of temperature (degree days) on juvenile growth does not appear to be a simple one. Juveniles collected in 2011 and 2012 had a similar number of degree days between encystment and excystment and total number of days encysted, yet the 2011 individuals were significantly larger than those in 2012. Juveniles in 2011 were collected during the peak excystment period (Fig. 4.4) whilst juveniles in 2012 were collected during the main excystment period but just before the 4-day peak. In comparison, the 2015 juveniles were collected at the end of the excystment period after the peak and had a larger number of degree days compared to the other two study periods. Individuals in 2015 were exposed to higher mean temperatures (and thus higher number of degree days) compared to 2012 individuals (Table 4.6) and the pattern of them being larger than the 2012 individuals thus continued when sampled after two months.

Table 4.4: Mean length (mm) of juvenile freshwater pearl mussels after 2 months in the 2012 substrate experiment and the current (2015) experiment (\pm SD). Superscript letters within columns indicate statistically homogeneous subsets.

Treatment	2012	2015
0.25 - 1 mm weekly	0.70 \pm 0.07 ^a	0.82 \pm 0.10 ^a
0.25 - 1 mm monthly	0.63 \pm 0.07 ^b	0.83 \pm 0.08 ^a
1 - 2 mm weekly	0.72 \pm 0.07 ^c	0.84 \pm 0.10 ^a
1 - 2 mm monthly	0.65 \pm 0.08 ^d	0.81 \pm 0.09 ^a

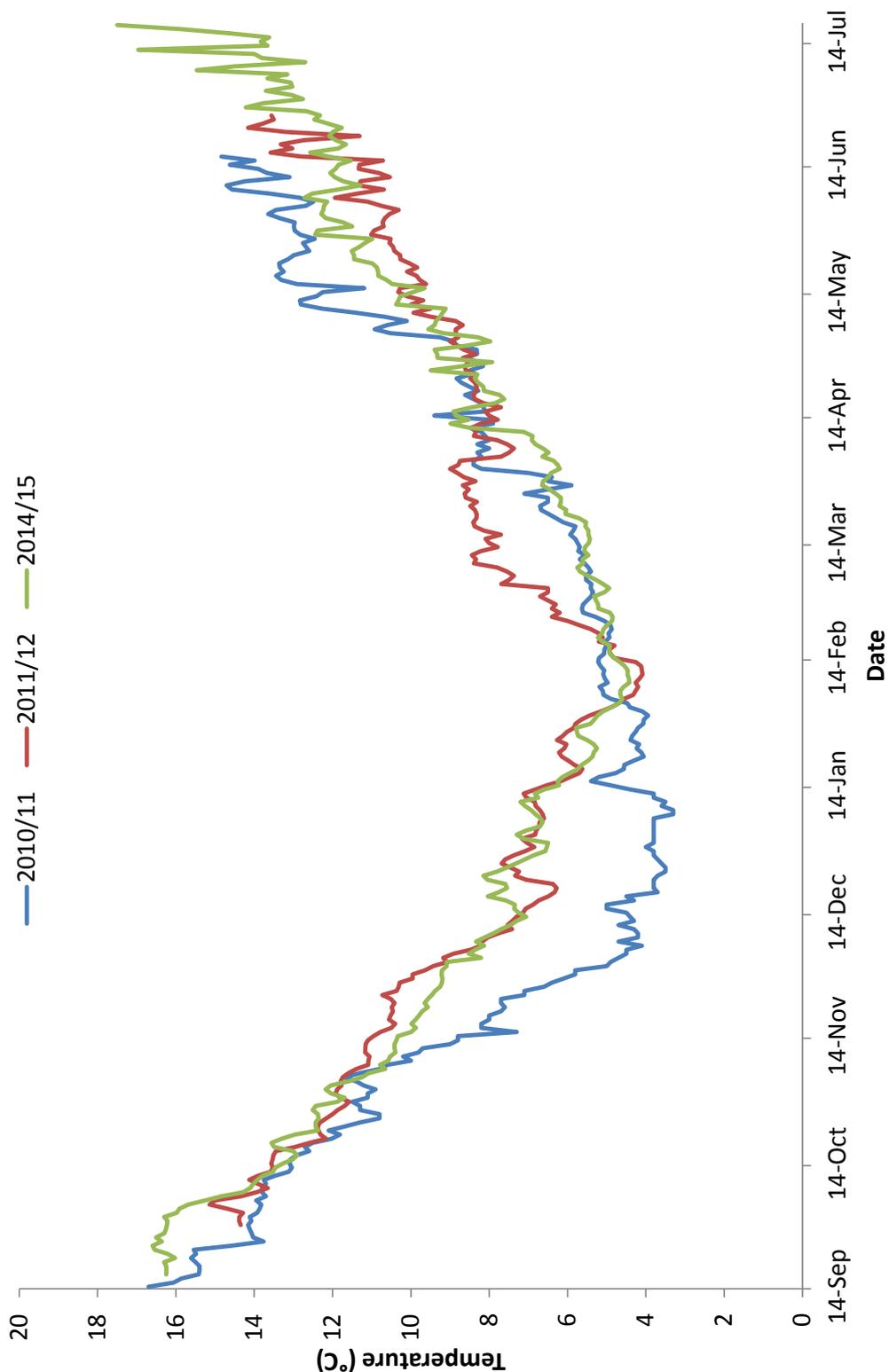


Fig. 4.3: Time series graph showing temperature profiles for the encystment periods of freshwater pearl mussel glochidia in 2010/11, 2011/2012 and 2014/15 from date encystment was first observed to date collection of juveniles commenced.

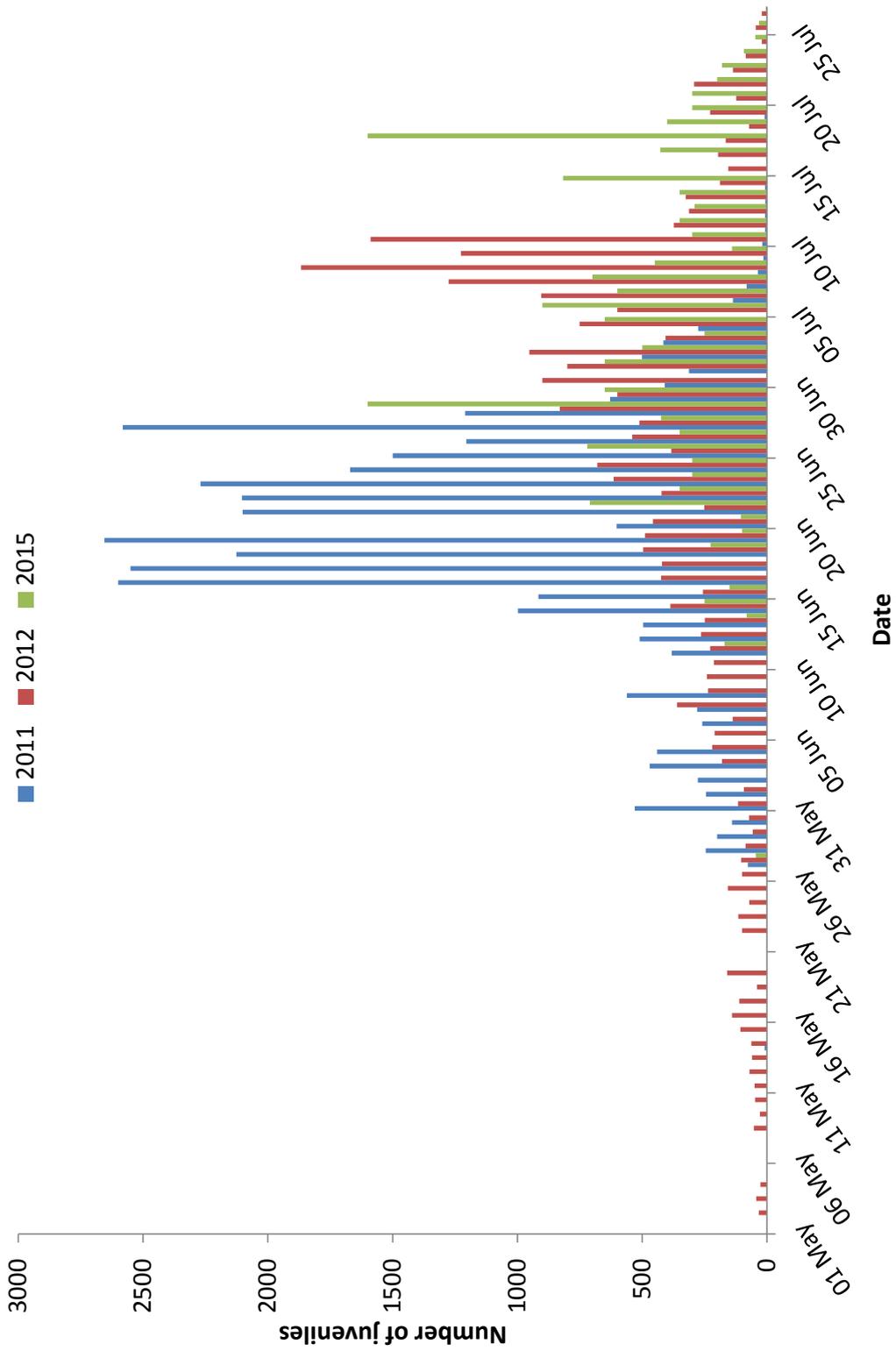


Fig. 4.4: Bar chart showing number of juvenile freshwater pearl mussels collected from the mussel population used in all experiments in this thesis by the Freshwater Pearl Mussel Ark project in 2011, 2012 and 2015. Note the extended drop-off period, particularly in 2012 (starting in early May) and the different pattern of excystment between years.

Table 4.5: Summary information on juvenile freshwater pearl mussel encystment and excystment during the three years juveniles have been used for experimentation during this thesis. Numbers in brackets in first row are the number of individuals measured (30 % of total).

	2010/11 (pilot)	2011/12 (Chapter 3)	2014/15 (current study)
Total number (measured)	6300 (1890)	3600 (1080)	1200 (360)
Size at collection (mm)	0.44 (± 0.02)	0.40 (± 0.02)	0.45 (± 0.05)
No. degree days (encystment - excystment)	1896	1845	2206
No. degree days (encystment - collection)	2335	2452	2877
No. days encysted	277	271	305
Drop-off period	14 May - 20 July	1 May - 26 July	27 May - 26 July
Collection period	16 - 28 June During peak excystment	26 June - 3 July During main excystment but just before the 4-day peak	18 - 23 July End of excystment after peak
No. of collection days	11	9	6

Table 4.6: Summary of temperature data between when juvenile freshwater pearl mussels were added to the aquarium system and next sampled in the 2012 and 2015 experiments. This data shows that higher mean temperatures in 2015 contributed to larger juveniles in 2015 compared to 2012.

	2012	2015
Number of days between sampling	52	57
Mean temperature ($^{\circ}\text{C}$)	15.44 (± 0.99)	16.31 (± 0.74)
Degree days ($^{\circ}\text{C}$)	803	930
Max. temperature ($^{\circ}\text{C}$)	17.58	17.52
Min. temperature ($^{\circ}\text{C}$)	13.80	13.74

4.3.3 Dissolved oxygen

Sonde malfunction meant that the first week of DO data from the water column was not usable. In addition, in the 0.25 – 1 mm monthly treatment DO concentration did not regain the pre-cleaning level after the first cleaning event at approximately 11 am on 23 August 2015 and there was no apparent reason for a step-change in DO concentration during the middle of the second week. Therefore data between 10:00 on 23 August and 12:30 on 25 August 2015 were excluded from analysis for this treatment. In the 0.25 - 1 mm weekly treatment a step-change in DO concentration was also observed at approximately 11:15 on 20 August 2015 which also cannot be explained. Data from this first week are therefore also excluded from analysis.

Fig. 4.5 and Fig. 4.6 show DO for all treatments in mg/L and % saturation respectively. Severe low spikes in DO were recorded in all treatments when sieves were removed for cleaning; as low as 0.07 mg/L (<1 % saturation) in the 0.25 – 1 mm weekly treatment on 23 August 2015 (see spikes on 23 & 30 August and 6 September - Fig. 4.5 & Fig. 4.6). These spikes are thought to be due to a lack of flow through substrates. When any sieve is removed from the system for cleaning, a hole between the top and bottom chambers is created through which water preferentially flowed because it follows the path of least resistance. When the sieve being cleaned was replaced, flow was restored through substrate interstices. These low DO spikes were not recorded in water column data because flow to this sensor was not affected by sieve removal.

Water column DO concentrations were consistently and significantly higher than any of the interstitial measurements (Fig. 4.5 and Table 4.7). Water column DO remained consistently high, never dropping below 7.94 mg/L (81 % saturation) and displayed a synchronous pattern with water temperature. Statistical comparisons of DO concentration between treatments are provided in Table 4.8 with significant differences highlighted in bold.

Table 4.7: Summary of dissolved oxygen data logged from the different probes (Table 4.1) in mg/L and % saturation (\pm SD). DO spikes identified as anomalies from cleaning events have been removed from analysis. N.B. Range includes low spike data in brackets.

Treatment	DO (mg/L)		DO (% saturation)	
	Mean (\pm SD)	Range (spike)	Mean (\pm SD)	Range (spike)
Water column	8.55 (\pm 0.25)	7.94 - 9.17 (N/A)	88 (\pm 3)	81 - 95 (N/A)
0.25 - 1 mm weekly	7.98 (\pm 0.44)	6.90 - 8.81 (1.54)	82 (\pm 5)	70 - 100 (16)
0.25 - 1 mm monthly	6.78 (\pm 1.27)	2.04 - 8.88 (0.07)	70 (\pm 14)	21 - 93 (1)
1 - 2 mm weekly	8.26 (\pm 0.19)	7.75 - 8.76 (5.12)	86 (\pm 2)	81 - 92 (53)
1 - 2 mm monthly	8.24 (\pm 0.44)	7.13 - 9.57 (2.67)	85 (\pm 5)	73 - 100 (27)

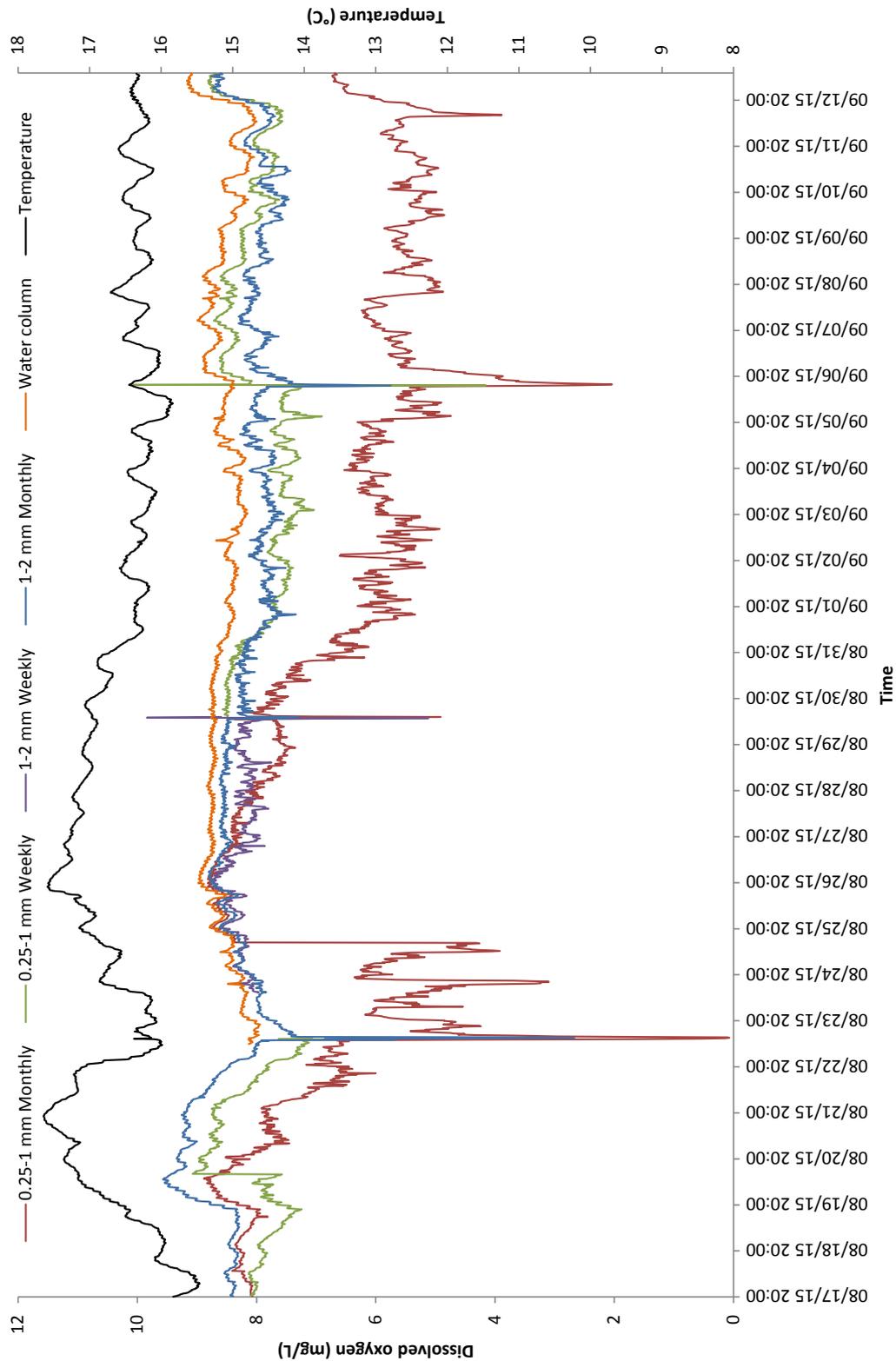


Fig. 4.5: Time series graph showing dissolved oxygen (mg/L) in all treatments and the water column. Temperature (°C) is also provided for reference.

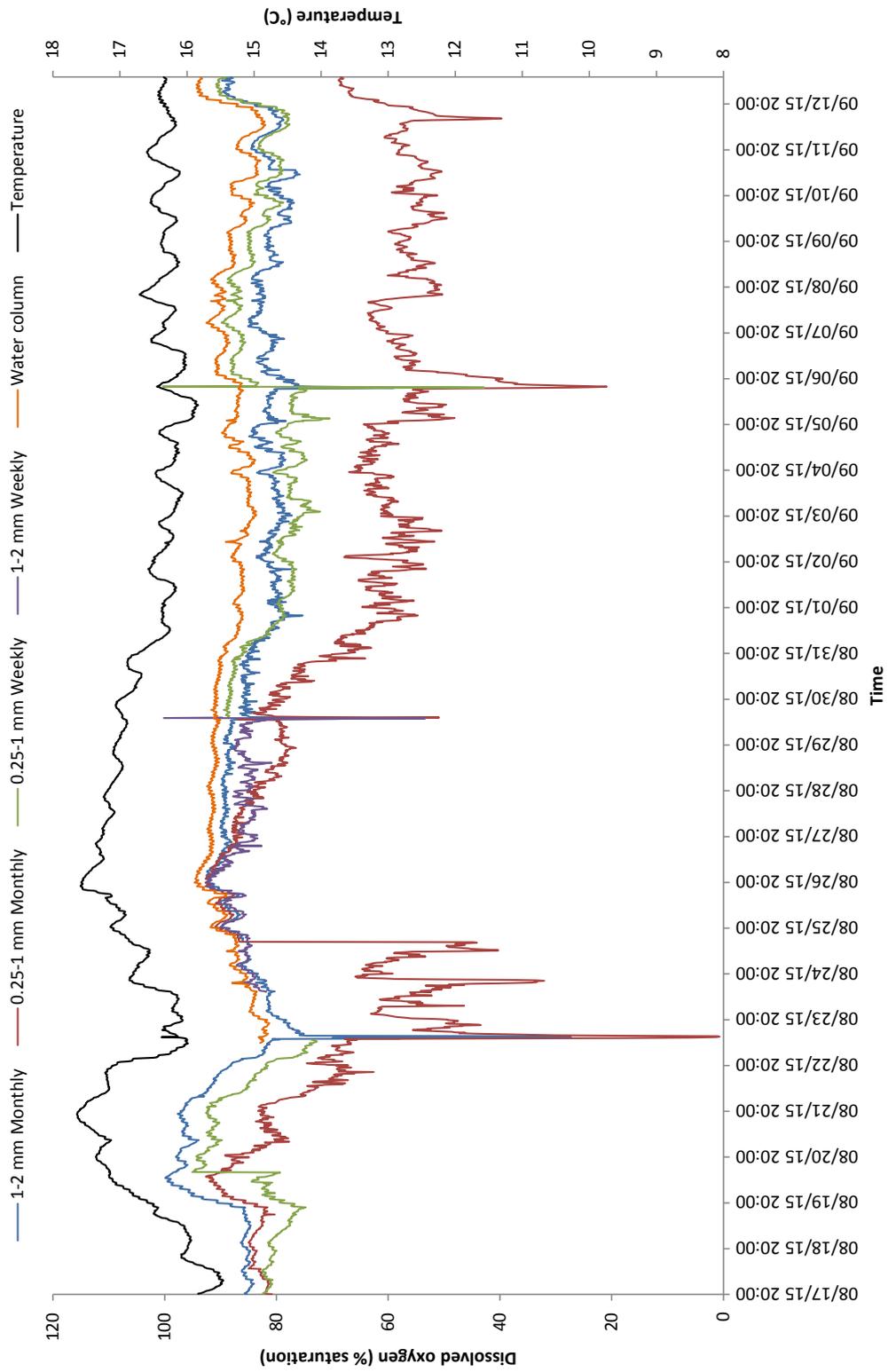


Fig. 4.6: Time series graph showing dissolved oxygen (% saturation) in all treatments and the water column. Temperature (°C) is also provided.

Table 4.8: Results of paired *t*-tests carried out to investigate differences between dissolved oxygen concentrations (mg/L) over time. N.B. $\alpha = 0.006$ due to Bonferroni correction for multiple tests ($n = 9$). Significant differences are highlighted in bold.

Test	<i>P</i>	Mean ⁽¹⁾	Mean ⁽²⁾	<i>t</i>	<i>n</i>
Water column ⁽¹⁾ + 1 – 2 mm monthly ⁽²⁾	<0.001	8.55	8.10	28.399	183
Water column ⁽¹⁾ + 0.25 – 1 mm monthly ⁽²⁾	<0.001	8.58	6.47	23.590	166
Water column ⁽¹⁾ + 0.25 – 1 mm weekly ⁽²⁾	<0.001	8.52	7.98	21.420	123
Water column ⁽¹⁾ + 1 – 2 mm weekly ⁽²⁾	<0.001	8.67	8.25	14.671	52
1 – 2 mm monthly ⁽¹⁾ + 0.25 – 1 mm monthly ⁽²⁾	<0.001	8.25	6.78	22.166	214
1 – 2 mm monthly ⁽¹⁾ + 0.25 – 1 mm weekly ⁽²⁾	0.489	7.96	7.98	-0.694	123
1 – 2 mm monthly ⁽¹⁾ + 1 – 2 mm weekly ⁽²⁾	<0.001	8.47	8.25	7.578	52
0.25 – 1 mm weekly ⁽¹⁾ + 0.25 – 1 mm monthly ⁽²⁾	<0.001	7.98	5.86	30.396	123
0.25 – 1 mm monthly ⁽¹⁾ + 1 – 2 mm weekly ⁽²⁾	0.450	8.23	8.27	-0.762	43

DO concentration in the one week the 1 – 2 mm weekly treatment was monitored showed that DO remained high and did not decrease substantially over the course of the week. Whilst the 1 – 2 weekly and monthly treatments did show a significant difference, the average DO concentrations were both high (Table 4.8). When considering the treatments cleaned monthly, DO concentrations were significantly higher in the 1 – 2 mm treatment (Fig. 4.7 & Table 4.8) with the gap between the two increasing over time. The 1 – 2 mm treatment followed the same fluctuations observed in the water column and low spikes in DO were smaller in magnitude compared to the 0.25 – 1 mm treatment. DO concentration in the 0.25 - 1 mm monthly treatment began to drop around 28 August 2015 and generally fluctuated around 5.0 – 6.5 mg/L (51 – 66 % saturation; min & max values = 3.89 - 6.77 mg/L; 40 – 69% saturation) from approximately 1 September 2015 to experiment termination on 14 September 2015. This compares with concentrations generally around 8.0 mg/L (82 % saturation; min & max values = 7.13 – 8.76 mg/L; 73 – 89 % saturation) in the 1 - 2 mm monthly substrate over the same period. The only instances when any treatment had a higher DO concentration than the 1 – 2 mm monthly treatment was when the 0.25 – 1 mm weekly treatment had been cleaned (Fig. 4.6).

After cleaning events on 30 August and 6 September 2015, DO concentrations in the 0.25 – 1 mm weekly treatment fell below the 1 - 2 mm monthly treatment after approximately three and five days respectively indicating that when interstitial flow is limited, cleaning gravels can provide a temporary increase in DO to juveniles, although this increase lasts only a few days in this system.

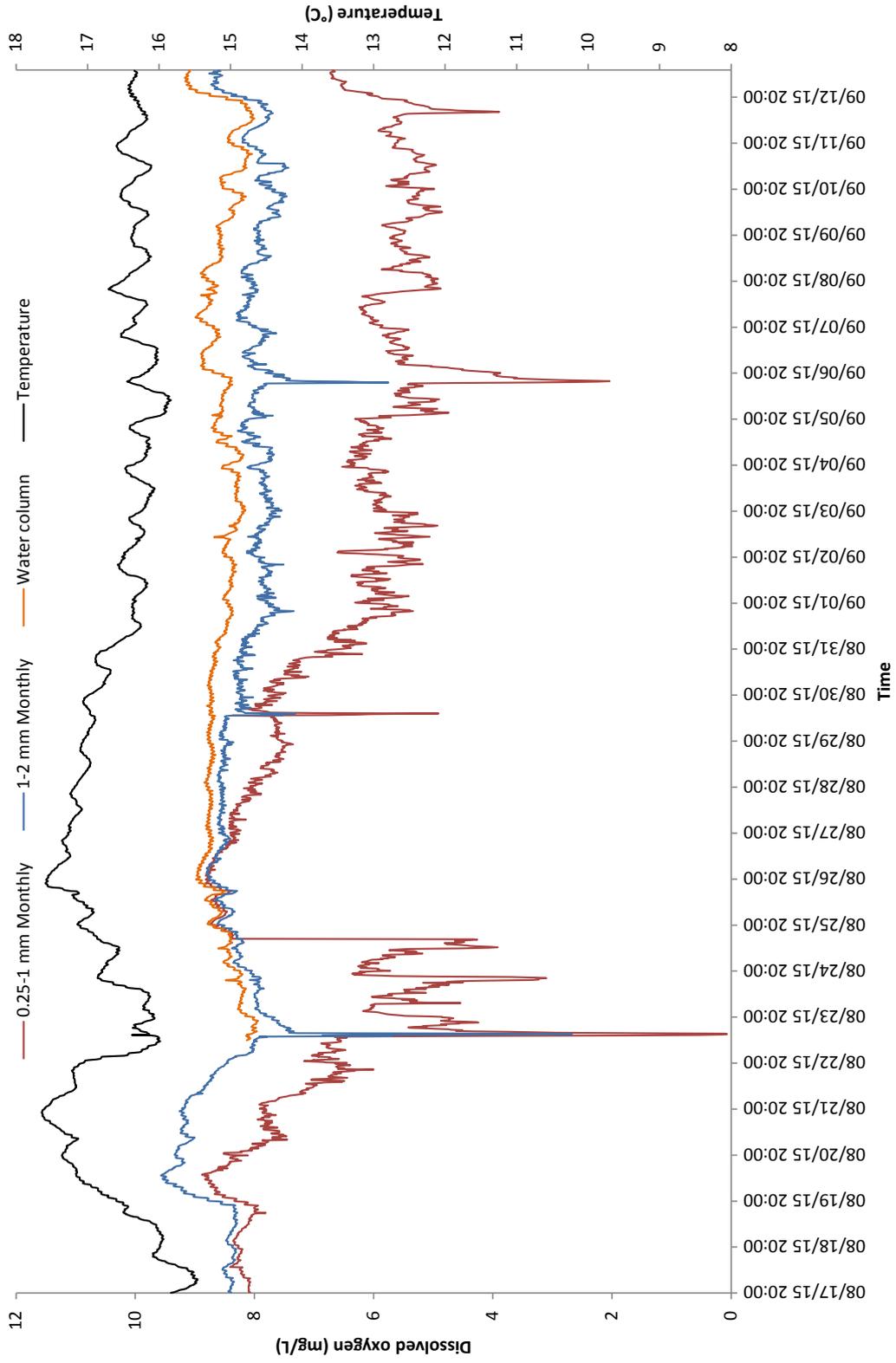


Fig. 4.7: Time series graph showing dissolved oxygen (mg/L) in only the 0.25 - 1 mm monthly & 1 - 2 mm monthly treatments for clarity compared to the water column. Temperature (°C) is also provided. The 0.25 - mm monthly treatment is much more variable and generally lower than the 1 - 2 mm monthly treatment.

When the experiment was terminated, the 0.25 – 1 mm and 1 – 2 mm monthly sieves were cleaned on 13 September 2015 and the probes reinserted to test the effect of cleaning on DO concentration in the monthly treatments (Fig. 4.8). In the 1 – 2 mm treatment DO concentration increased slightly after cleaning from around 8.5 mg/L before cleaning to 8.8 mg/L after cleaning. The increase in the 0.25 – 1 mm treatment was more substantial however, increasing from around 6.5 to 8.5 mg/L. The same pattern was observed in the 0.25 – 1 mm weekly treatment after the cleaning event on 06 September 2015 when DO increased from approximately 7.3 mg/L pre-cleaning to 8.1 mg/L post-cleaning (Fig. 4.9). As Fig. 4.8 shows, DO concentration in the 0.25 – 1 mm monthly treatment was more affected by sieve removal from the system than the 1 – 2 mm monthly treatment, leading to spikes of reduced DO concentrations when sieves were absent from the system (denoted by asterisks).

Non-significant differences between the 0.25 – 1 mm monthly and 1 – 2 mm weekly treatments (Table 4.8) were a reflection of when the measurements were taken within the month. Comparisons were made before DO began to decrease substantially in the 0.25 – 1 mm weekly treatment (Fig. 4.5). The other non-significant comparison was between the 1 – 2 mm monthly and 0.25 – 1 mm weekly treatments. In the 0.25 – 1 mm weekly treatment the DO concentration dropped below the 1 – 2 mm monthly treatment towards the end of the week but the overall difference in DO values was not significantly different.

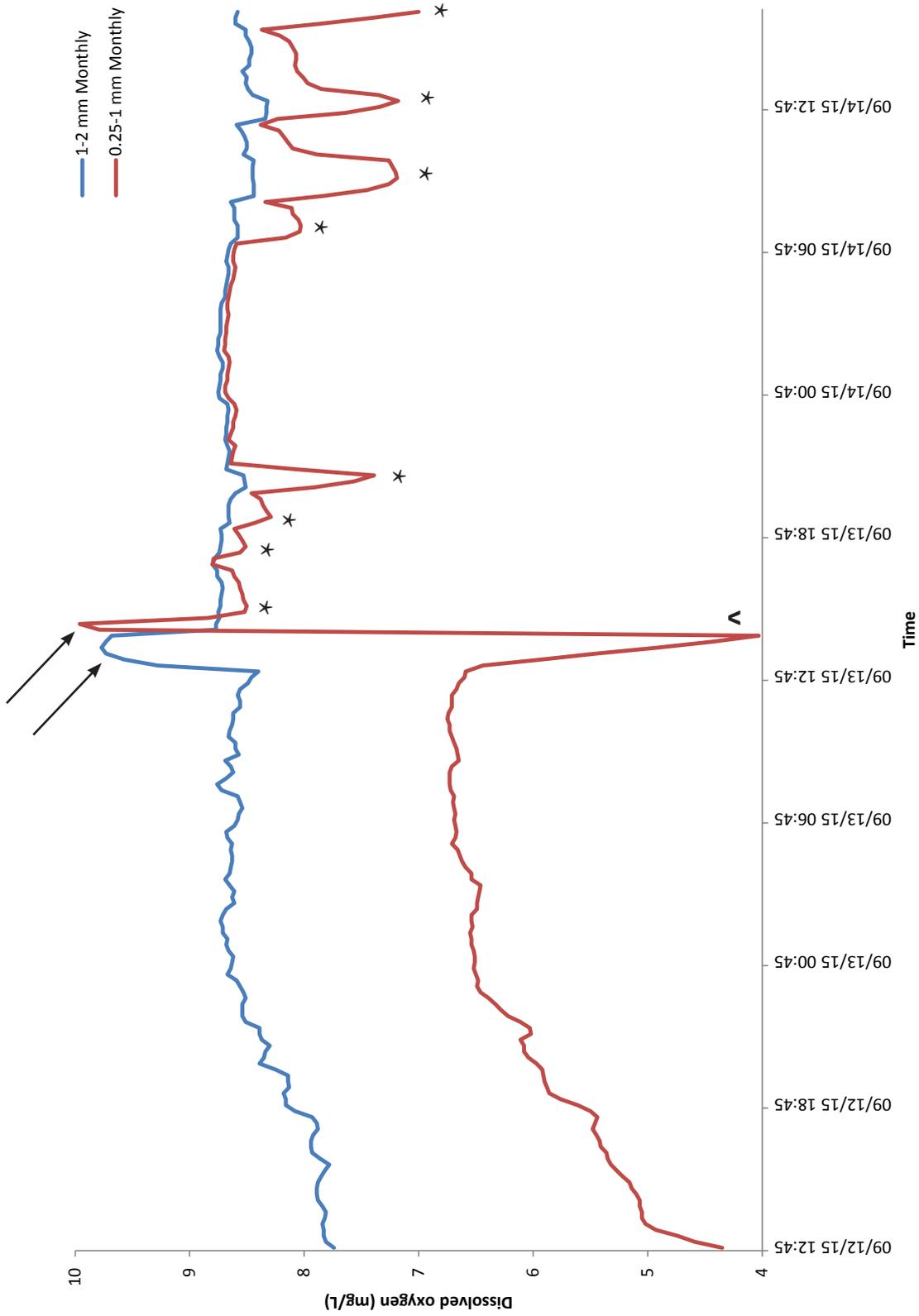


Fig. 4.8: Time series graph showing dissolved oxygen (mg/L) in the 0.25 - 1 mm monthly & 1 - 2 mm monthly treatments 24 hours before and 24 hours after cleaning. Sensors were exposed to air during cleaning (arrows); low DO spike (^) is due to 1 - 2 mm monthly sieve being removed from aquarium; spikes denoted by (*) were when other sieves were removed for sampling showing that the 0.25 - 1 mm substrate is affected more by removal of flow (and therefore DO) when sieves are removed from the system.

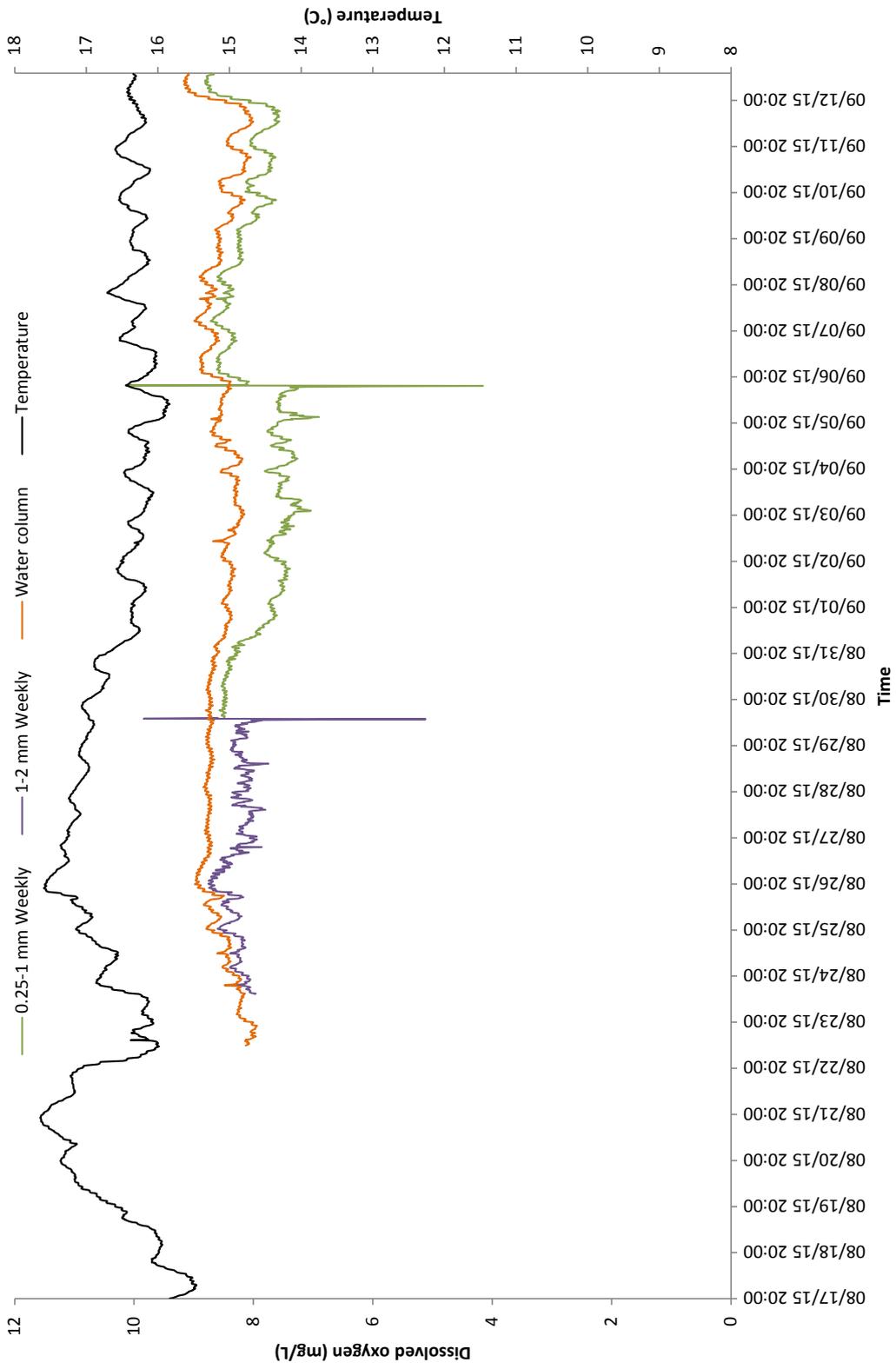


Fig. 4.9: Time series graph showing dissolved oxygen (mg/L) in only the 0.25 - 1 mm weekly & 1 - 2 mm weekly treatments for clarity compared to the water column. Temperature (°C) is also provided. Dissolved oxygen in the 1 - 2 mm weekly treatment shows a similar pattern to in the water column but is consistently lower. Dissolved oxygen in the 0.25 - 1 mm weekly treatment improves after cleaning on the 6 September.

4.3.4 Interstitial space and flow

Comparison of interstitial space in the two substrate sizes showed that 0.25 - 1 mm substrates had significantly less space ($t_{(10)} = -4.725$, $P = 0.001$; 2.6 ± 0.61 ml) compared with 1 - 2 mm substrates (4.27 ± 0.25 ml).

Flow through undisturbed (dirty) sieves exposed to the different cleaning regimes was significantly slower through the 0.25 - 1 mm monthly treatment compared with all other treatments ($F_{(3,8)} = 8.834$, $P = 0.006$) with the slowest sieve taking almost 43 minutes to clear 1 L of water. All 0.25 - 1 mm monthly sieves had to be lightly agitated periodically to encourage water flow as it often stopped completely indicating that there was minimal to no flow through these sieves within the system. Fig. 4.10 shows that flow through substrates once cleaned was significantly faster through the 1 - 2 mm substrates (28 ± 5 seconds; $F_{(3,8)} = 18.802$, $P = 0.001$) compared to the 0.25 - 1 mm substrate (44 ± 4 seconds). Comparing each treatment before and after cleaning found flow was significantly faster post-cleaning in all treatments (Table 4.9).

Table 4.9: Mean flow rate of water through dirty (before) and cleaned (after) substrate. Flow rates are provided in SI units (mL s^{-1}) and the more widely reported litres per minute (L/min). All flow rates are reported \pm SD. Comparisons between flow rate (mL s^{-1}) before and after cleaning found significant differences in all treatments; Student's t values and P provided.

Treatment	Flow rate before (mL s^{-1})	Flow rate after (mL s^{-1})	Flow rate before (L/min)	Flow rate after (L/min)	t	P
0.25 – 1 mm weekly	3.50 (\pm 0.93)	23.61 (\pm 2.87)	0.21 (\pm 0.05)	1.42 (\pm 0.17)	-11.54	<0.001
0.25 – 1 mm monthly	0.61 (\pm 0.35)	22.11 (\pm 0.75)	0.04 (\pm 0.02)	1.33 (\pm 0.04)	-44.95	<0.001
1 – 2 mm weekly	3.72 (\pm 3.29)	32.22 (\pm 3.95)	0.22 (\pm 0.20)	1.93 (\pm 0.24)	-9.60	0.001
1 – 2 mm monthly	3.78 (\pm 2.50)	40.33 (\pm 4.32)	0.23 (\pm 0.15)	2.42 (\pm 0.26)	-12.67	<0.001

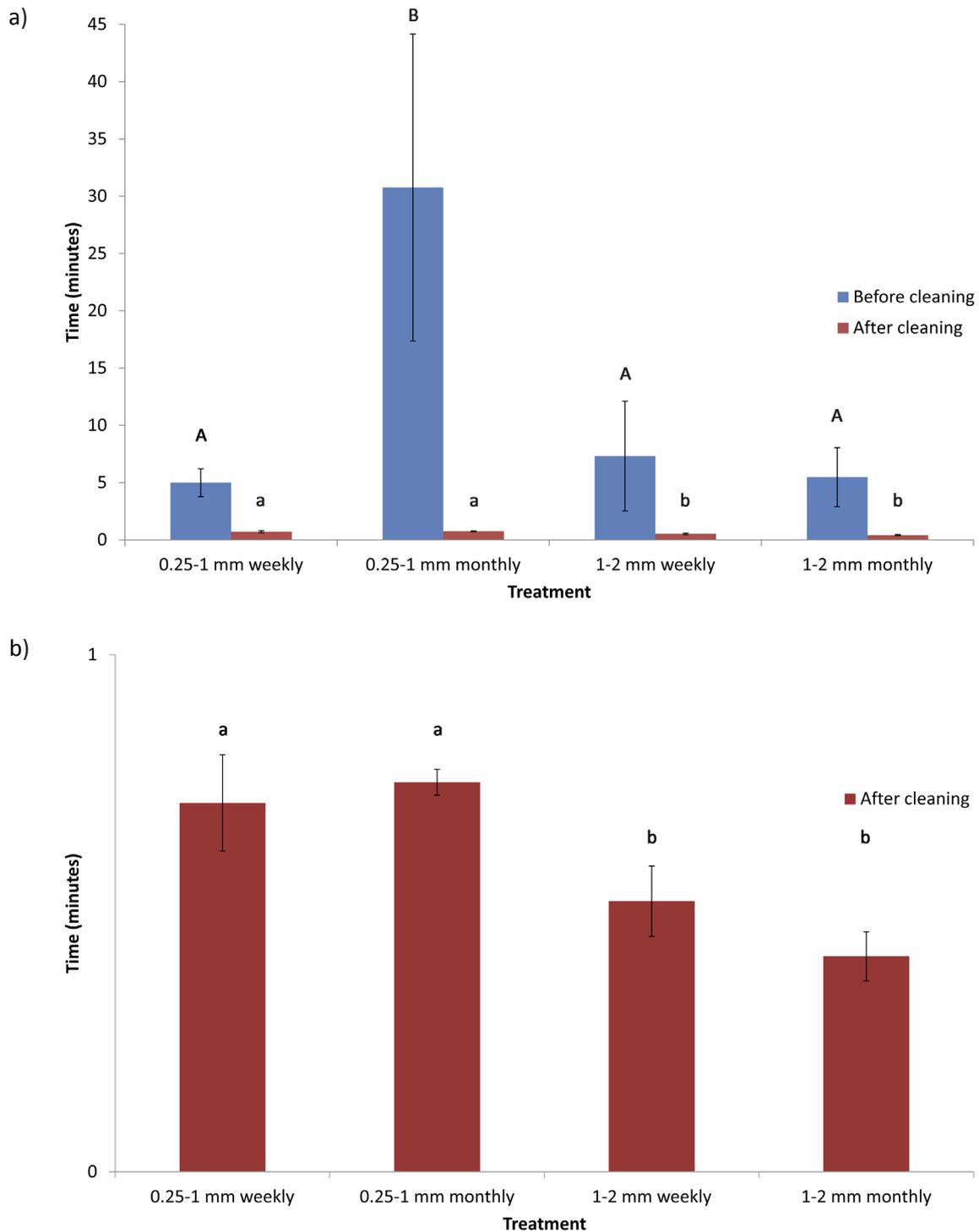


Fig. 4.10: Bar graphs showing average time (minutes \pm SD bars) for 1 L of water to flow through substrates in the different treatments before and after cleaning (a) and after cleaning enlarged to show detail (b). Letters over bars denote significant differences (note capital letters used for before cleaning and lower case used for after cleaning in (a)).

4.3.5 Substrate organic content and ion concentrations

The weight of biofilm on the substrate of different treatments was not significantly different ($F_{(3, 8)} = 2.756$; $P = 0.112$). Whilst the weight of organic matter from within the interstices at experiment termination appeared higher in treatments cleaned monthly (Fig. 4.11), there was no significant difference between any of the treatments ($F_{(3, 8)} = 3.432$, $P = 0.072$). When combining the weight of all organic matter in the samples (biofilm and loose organic matter) the LOI ranged between 0.47 - 0.76 % (mean = 0.59 ± 0.08 %). There was no significant difference in the LOI results between different treatments ($F_{(3, 8)} = 0.542$; $P = 0.667$).

Inorganic TP concentrations taken from burned organic matter in the interstices were not significantly different between treatments (Table 4.10; $F_{(3, 8)} = 2.119$, $P = 0.176$). The values in Table 4.2 show that whilst orthophosphate concentrations in Windermere would not indicate cause for concern during the spot sample on 17 August 2015 (0.002 mg/L), a large amount of organic material was found within substrate interstices in the captive rearing system indicating that available P is being taken up by phytoplankton.

Ammonia samples taken before cleaning on day 29 (17 August 2015) showed no significant difference between treatments or the water column ($F_{(4, 14)} = 1.384$; $P = 0.307$).

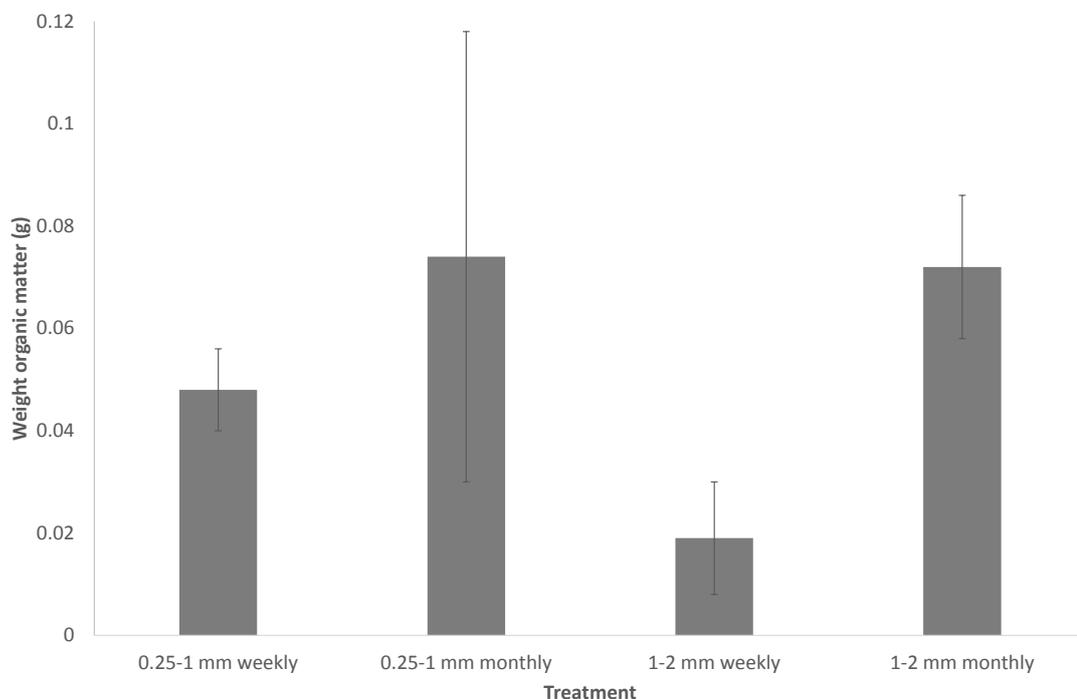


Fig. 4.11: Bar graph showing the mean dry weight (\pm SD bars) of organic matter collected from substrates from the four treatments at experiment termination. The weight of organic matter was lower in the treatments cleaned weekly, particularly in 1 - 2 mm substrate, but this difference was not statistically significant ($P = 0.072$).

Table 4.10: Mean (\pm SD) concentrations (mg/L) of ammonia (taken on day 29) and total phosphorus (TP; taken on day 60) from the four different treatments.

Treatment	Ammonia (mg/L)	TP (mg/L)
Water column	0.014 (\pm 0.007)	N/A
0.25 - 1 mm weekly	0.008 (\pm 0.009)	1012.33 (\pm 307)
0.25 - 1 mm monthly	0.019 (\pm 0.018)	998.00 (\pm 610)
1 - 2 mm weekly	0.026 (\pm 0.008)	462.93 (\pm 330)
1 - 2 mm monthly	0.012 (\pm 0.008)	1245.33 (\pm 397)

4.4. Discussion

The aim of this investigation was to build upon the findings in Chapter 3 and to investigate a range of parameters potentially affecting survival and growth of juvenile mussels in different substrate sizes (0.25 – 1 and 1 – 2 mm) and cleaning regimes (weekly and monthly). Analysing the effects of various environmental parameters thought to be important to juvenile pearl mussel survival will help refine captive rearing methods and reduce the high mortality rates often observed in the first few weeks of juvenile life post-excystment. Dissolved oxygen, substrate size (and resulting interstitial space) and flow appear to be the limiting factors in this system with higher survival being observed in treatments with larger substrates which allow higher flow rates and DO concentrations, leading to significantly higher juvenile survival after two months.

The period of highest mortality in the 2012 experiment (Chapter 3) was during the first four months post-excystment. This period has high summer temperatures which are particularly pronounced at this site because the water source is a large lake which has a more stable temperature regime with a high temperature lag into the autumn months. The decision was therefore taken to investigate the response (survival and growth) of juveniles to various environmental parameters during a two month period from 20 July – 14 September 2015; a period when environmental conditions are likely to be least suitable (Geist & Auerswald, 2007).

4.4.1 Effects on growth and survival – current investigation and comparisons over several years

In this investigation, survival was highest in treatments with coarser substrate cleaned monthly, mirroring results reported previously (Chapter 3). The most notable difference between the 2012 and 2015 investigations was that survival in the least suitable treatment (0.25 - 1 mm weekly) was markedly higher in 2015 (72 ± 2 %) compared with 2012 (40 ± 7 %). This may

be because 2015 juveniles were significantly larger upon excystment potentially leading to improved survival.

Growth was not significantly different between treatments in this study, contradicting findings in Chapter 3 which showed juveniles were significantly larger in treatments cleaned weekly. Size is a difficult parameter with which to assess breeding success, particularly when it has been shown to vary significantly within age cohorts in several unionoid species (Beatty & Neves, 2004; Barnhart, 2006; Schmidt & Vandré, 2010). Buddensiek (1995) found size-dependent survival in pearl mussel streams whereas Lavictoire *et al.* (2016) did not find this in captivity. Table 4.5 shows that for the three years that juveniles were collected for experimentation, the timing of collection within the drop-off period and the number of degree days between the first sighting of glochidial encystment and juvenile collection differs. These factors may partly explain why the 2015 individuals were significantly larger than both the 2011 or 2012 individuals upon excystment. In addition, the higher mean temperature and number of degree days during the first 2 months post-excystment in 2015 compared with 2012 is likely to have contributed also.

Degree days have been used previously to try and describe when certain aspects of the life cycle may occur (Hruška, 1992; Bauer, 1994; Hastie, 1999; Thomas *et al.*, 2010) but they are only a crude tool because they usually describe the cumulative temperature for phenomena observed at the level of populations rather than individuals e.g. cumulative temperature between first observation of glochidia encystment to first observation of juvenile excystment. Whilst glochidial release can be highly synchronous in the wild (Young & Williams, 1984; Hastie, 1999; Hastie & Young, 2003), in captivity glochidial releases have been observed up to 6 weeks apart (FBA, unpublished data) indicating variability within populations even when maintained under the same environmental conditions. This could give rise to the observed extended period over which juveniles excyst in captivity (Lavictoire *et al.*, 2014). The number of degree days between glochidial encystment and the start of juvenile excystment at the FBA (ranging from 1845 - 2206) is comparable to previous reports of between 1600 and 2619 degree days (Hruška, 1992; Bauer, 1994; Thomas *et al.*, 2010). The temperature required for the excystment period to commence, however, has not been as high as the 15 °C Hruška (1992) reported. Mean daily temperatures on the days when juvenile excystment has commenced at the FBA range from 8.82 - 12.25 °C in the various study years indicating that excystment

may commence upon attainment of sufficient size or when sufficient degree days have been accumulated regardless of ambient water temperature. Eybe *et al.* (2015) found that juveniles excysting at the beginning of the drop-off period did not grow as quickly or survive as well as juveniles excysting later. Further work is required on this topic to investigate the biological reasons for these differences and why breeding success may differ between years. This information is valuable to captive rearing programmes as it could help focus resources on juveniles which are more likely to survive, or focus on years when recruitment is likely to be particularly good. Selecting the fittest juveniles for captive rearing raises important questions about whether this constitutes genetic selection and whether this may affect genetic diversity and heterozygosity in wild populations when these juveniles are eventually reintroduced.

4.4.2 Environmental parameters affecting juvenile growth and survival

4.4.2.1 Organic content, flow and total phosphorus & ammonia concentrations

The amount of organic matter in the interstices at experiment termination showed no significant difference between treatments, although those cleaned weekly had less organic matter compared to monthly treatments and the 0.25 - 1 mm substrates trapped more compared with their 1 - 2 mm counterparts (Fig. 4.11). Few data exist describing the amount of organic matter juveniles can withstand within the interstices. The percentage of organic matter (LOI) within the interstices and as biofilm adhered to the substrate was similar to that reported by Tarr (2008), who found 0.5 – 1 % in substrates supporting juveniles in Scottish pearl mussel rivers. Tarr (2008) however analysed only fractions < 0.5 mm compared to this study which did not discriminate on size. Therefore figures reported by Tarr (2008) may be an under-estimate of the true organic matter content. Regardless of this, the organic matter percentage of total substrate weight in this study was low and does not appear to be a problem in the context of concentrations found in this study. The amount of interstitial space in sieves did not allow for a sample of suitable volume to be taken to analyse interstitial soluble reactive phosphorus concentration. Little work has been done on determining toxic concentrations of phosphorus for adult mussels or juveniles but measurements taken by the Environment Agency (Table 4.2) do not indicate levels which are likely to be toxic to juveniles in the water column. The spot sample in August 2015 indicated that concentrations are within the range of values reported previously for European pearl mussel rivers (Bauer, 1988; Buddensiek *et al.*, 1993;

Moorkens, 2006b). Total phosphorus concentrations were not significantly different between treatments which was expected since the organic matter results were not significantly different. TP concentrations reported here highlight the effects of elevated P availability as it is transferred into unavailable P during phytoplankton growth, underlining the problem of nutrient loading in Windermere (Reynolds & Irish, 2000).

The system described is a downwelling one with 0.3 mm sieves placed on top of substrate in order to retain juveniles. This may impede the size of organic matter entering the substrate but would not stop organic matter growing within the substrate if conditions were suitable. Equally, the 0.18 mm sieve within which substrate and juveniles are held would stop organic matter > 0.18 mm passing through the bottom of the sieve, theoretically providing the opportunity for organic matter to accumulate over time. Data on flow through the pre- and post-cleaning substrates indicates that whilst the amount of organic matter within sieves did not differ significantly, treatments with smaller substrates and those cleaned monthly did accumulate more matter (Fig. 4.11). In addition, smaller pore size provided more resistance to flow so that the combination of small substrate size and monthly cleaning meant this treatment had significantly lower flow rates. High water column-interstitial exchange is a predictor of functional pearl mussel habitats and the presence of juvenile mussels (Buddensiek *et al.*, 1993; Geist & Auerswald, 2007). Captive rearing systems should strive therefore to emulate these conditions. In this system increased flow rates could be achieved by increasing sieve mesh size as juveniles grow.

Ammonia concentrations in this investigation are lower than acutely or chronically toxic concentrations reported for North American unionids (Augsburger *et al.*, 2003; Mummert *et al.*, 2003; Wang *et al.*, 2007; Wang *et al.*, 2008). The potential toxic effects of ammonia are not thought to be a source of mortality in this system at the FBA. It is assumed that the flow-through nature of the system prevents the accumulation of high ammonia concentrations and, whilst higher than the 0.01 mg/L concentration suggested by Moorkens (2006a), the treatments do not differ significantly from the ammonia concentration in the water column and do not appear to have negative effects on juvenile survival in this system. The presence of biofilm on the substrate and oxic conditions may help oxidise ammonia into less harmful ions. Eybe *et al.* (2013) reported lower ammonium and nitrite concentrations in treatments containing detritus and suggested that nitrifying bacteria found in detritus were the cause of this. The total mass of

biofilm was found to be the same across all treatments. If biofilm is a food source for juveniles, substrate size and cleaning frequency do not affect the biomass available, and therefore would not affect survival or growth in this system. The species composition of biofilm and its nutritive value was outside of the scope of this study but further studies would be beneficial to assess its potential as a food source for *M. margaritifera*.

4.4.2.2 Dissolved Oxygen

The results of DO analyses in this system echo findings of Quinlan *et al.* (2014b) who found that surface water DO concentrations were generally higher than in the hyporheic zone in an English pearl mussel river. In smaller substrates (both cleaning regimes), there was a general trend of declining DO over time. This trend was not observed in the 1 - 2 mm sieves, even those cleaned monthly, with DO concentrations remaining consistently high and following the same fluctuations as water column readings.

DO concentration in the 0.25 - 1 mm monthly treatment began to decrease after approximately 1.5 weeks and remained low for the rest of the sampling period. DO fluctuations in the 0.25 - 1 mm gravel were also higher than in the 1 - 2 mm substrate. Both low and fluctuating DO could cause stress to juveniles and may alter their behaviour, making them seek out more suitable conditions. The results reported here are similar to those of Quinlan *et al.* (2014b) who found that DO concentrations measured in different locations in a pearl mussel river varied substantially and that the site with the lowest DO concentrations also had the highest variability. The effects of low DO spikes (created by interrupting flow when removing sieves for cleaning) are not known, but the same phenomenon must have occurred during the 2012 experiment for which there was relatively good survival after 25 months (14 % across all treatments). These results outline the importance of good interstitial flow in providing oxygen to juveniles and the unknown effects of routine activities such as cleaning in culture systems. To stop spikes in this system in future, a 'blank' sieve containing substrate should be placed in holes created when sieves are cleaned to minimise this effect and reduce stress on juveniles.

In the 0.25 - 1 mm weekly treatment DO decreased over the course of the 7 days between cleaning events but increased post-cleaning. As observed in the monthly treatments DO decreases in smaller substrates over time. Decreases in DO may be observed in as little as two days but this is likely to depend upon the amount of organic matter entering the system.

As Quinlan *et al.* (2014a) point out, the direct cause of detrimental effects of fine sediment on hyporheic organisms (including salmonid eggs and juvenile mussels) are unknown but may be due to physical smothering effects, lowering of water exchange between the water column and the interstitial, or because of the oxygen-limiting effects of organic matter. Too much fine material entering the substrate could also lead to reduced feeding activity causing stress and starvation (see discussion in Chapter 5). In streams, DO can vary over small spatial scales (both vertically and horizontally) depending upon water surface velocity, substrate size and permeability of the stream bed (Quinlan *et al.*, 2014a). All three parameters can be controlled in the system outlined in this thesis (surface velocity = flow rate) so equal flow and DO concentrations should be present in all sieves where the substrate size and cleaning regime is the same.

Whilst all treatments provided significantly lower DO concentrations compared to the water column, the 1 – 2 mm monthly treatment provided the highest and most stable DO profile of all treatments. An average of 8 mg/L (82 % saturation) appears to be suitable for juvenile *M. margaritifera*. The 1 – 2 mm weekly treatment also provided high DO conditions, however this treatment required four times more maintenance compared with the 1 - 2 mm monthly treatment.

4.4.3 Conclusions and significance for captive rearing programmes

This study confirms previous findings that coarser, uncompacted substrates lacking fine sediments and organic material provide better interstitial environments for a wide range of biota, including juvenile mussels (Wood & Armitage, 1997; Brim Box & Mossa, 1999; Geist & Auerswald, 2007; Liberty *et al.*, 2007; Quinlan *et al.*, 2014a; Lavictoire *et al.*, 2016). Juveniles are often the limiting stage in populations which are displaying a lack of recruitment regardless of apparently sufficient adult mussel and host fish densities (Österling *et al.*, 2008). In this system 1 – 2 mm substrate cleaned monthly provided the highest juvenile survival with the highest DO concentrations due to higher flow rates through larger substrate pores. All other parameters (ammonia, TP concentrations of organic matter within substrates, and the amount of organic material) did not significantly affect juvenile survival in this flow-through system. The 1 – 2 mm monthly treatment was also one of the least resource-intensive treatments, with cleaning occurring only once per month and taking between 3 – 5 minutes per sieve to clean.

Sampling and monitoring of newly-excysted juveniles was also easier and quicker in larger substrate because newly-excysted juveniles are in a different size bracket compared to the substrate, making them easier to find. Another important practical requirement in this system is temporarily replacing sieves which have been removed for cleaning with a blank sieve (just substrate) to maintain flow through other sieves. These practical considerations are important for captive rearing programmes which need to maximise the number and quality of juveniles reared, but which have finite resources.

This study has outlined several areas requiring additional investigation including:

- **Dissolved oxygen:** The body of evidence is growing that interstitial DO is one of the most critical parameters affecting juvenile mussel survival. To date, no work has been carried out on the dissolved oxygen limits for juvenile *M. margaritifera* or how different patterns of DO concentrations (e.g. low DO spikes) affect behaviour and survival. This makes it difficult to assess the reasons for poor juvenile survival in captive rearing programmes and impossible to provide guidelines for practitioners considering potential suitable donor sites for juvenile augmentation in to wild populations. As previously described, coarse but stable substrates provide suitable hyporheic exchange and DO concentrations of around 8 mg/L (> 80 % saturation) in this study provided suitable conditions for high juvenile survival during summer conditions at the FBA Pearl Mussel Ark.
- **Timing of excystment:** Comparison of juvenile size and survival in this study with previous years (2011 and 2012), and analysis of timing of excystment has corroborated findings by Eybe *et al.* (2015) that juveniles excysting at different times within the drop-off period may display differences in growth and survival rates. This topic, and the link of glochidial development and degree days, warrants further investigation in order for captive rearing programmes to determine which environmental conditions produce the highest quality juveniles in the most cost- and labour-effective manner.
- **Different substrate mixes:** Substrate mixes of 0.25 – 1 mm and 1 – 2 mm were used in this study and in Chapter 3 but use of larger substrate clasts have not been considered. Coarser (1 – 2 mm) substrates appear to allow sufficient flow, even when only being cleaned monthly, but consideration of larger substrate clasts within the same rearing system is warranted. Additionally, switching the 0.18 and 0.3 mm sieves for larger mesh sizes once juveniles are large enough may improve flow further and decrease the build

up of organic matter. The hypotenuse of the mesh size is the largest gap through which juveniles could fall or escape, but measurements from the 2012 experiment suggest that the number of juveniles lost to this by replacing the 0.18 mm with 0.3 mm sieves would be minimal after 1 year.

- **Biofilm and diet:** The role and importance of biofilm for juvenile mussels has not been sufficiently described. Eybe *et al.* (2013) suggested that detritus could act to reduce ammonia concentrations in static systems and biofilm could have a similar role to play in flowing systems. The natural diet of *M. margaritifera* has not been studied and it is not known if food preferences or dietary requirements change with age and/or size. Phytoplankton species abundance and availability is season-specific. European captive rearing programmes have had success rearing *M. margaritifera* in systems with 'natural' food sources as well as in systems where supplemental feeding is provided (e.g. cultured algae/shellfish diets) without specific knowledge of dietary requirements. However, tailored diets for juvenile mussels during different seasons and developmental stages have the potential to improve juvenile condition, thus increasing survival.

There is still much we do not know about the biological requirements of juvenile *M. margaritifera* but the experiments outlined here (and in Chapters 2 and 3) provide some preliminary information about how simple factors such as substrate size and cleaning regime can have major impacts by influencing environmental conditions and therefore juvenile growth and survival. Ensuring high interstitial DO should be a primary consideration for captive rearing programmes and future research should focus on topics such as how diet and timing of excystment affects the quality of juveniles, their growth, and ultimately their suitability for restocking wild populations.

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Chapter 5

Investigations into the transformation from pedal to filter feeding in the freshwater pearl mussel *Margaritifera margaritifera*

5.1. Introduction

The amount of research into the ecology, captive rearing techniques and pressures facing *Margaritifera margaritifera* (Linnaeus, 1758) in the wild has increased markedly over the last three decades. Despite this, relatively little work is being undertaken on the fundamental biology of the species, particularly the vulnerable early-life stages (glochidia and juveniles). A better understanding of early life ontogeny is particularly important for practitioners undertaking captive rearing activities in order to improve breeding protocols and increase survival. Juveniles may have different requirements depending upon their mode of feeding (Henley *et al.*, 2001) and mortality may increase when developmental changes occur (Fitt *et al.*, 1984; Beninger *et al.*, 1994; Cannuel & Beninger, 2006) due to inability to meet energetic demands during morphogenesis (Veniot *et al.*, 2003). For example, poor understanding of dietary requirements is the main reason for stalling efforts to culture the commercially important New Zealand green-lipped mussel, *Perna canaliculus* (Gui *et al.*, 2016).

Margaritifera margaritifera undergoes several substantial ontogenic stages before adulthood which are undoubtedly sources of mortality:

- 1. Glochidia development and metamorphosis:** Initially, glochidia must encyst within the gills of a host fish where they grow to over five times their original size before metamorphosing into juveniles. This process includes the development of all major internal organs and two adductor muscles instead of the single adductor glochidia possess (Lasee, 1991; Nezhlin *et al.*, 1994; Ziuganov *et al.*, 1994; Wächtler *et al.*, 2001; Araujo *et al.*, 2002).
- 2. Early juvenile development:** Upon excystment, juveniles pedal feed which involves probing substrate with their foot and directing food particles into the pedal gape on water currents generated by cilia (<https://www.youtube.com/watch?v=nHtE4rtkF9A>). This stage also likely includes final development of digestive organs as juveniles begin to derive nutrients from algae and bacteria instead of from the fish host (Lasee, 1991). Very little is known about the feeding behaviour and dietary requirements of juvenile mussels once they have become established in river gravels (Ziuganov *et al.*, 1994).
- 3. Transformation:** At some point during early juvenile life, a second metamorphosis occurs. Substantial development of the gills enables them to be used as a highly efficient feeding organ, and the primary mode of feeding switches from pedal feeding to siphonal

filter feeding. From this point forward, this second metamorphosis will be termed 'transformation' in this chapter.

For the majority of freshwater mussels, including *M. margaritifera*, very little is known about this transformation stage. For example, it is unknown whether it is age- or size-dependent, the timing of transformation, what the main morphological changes are, or how these changes affect feeding behaviour. It is also not clear if habitat and/or dietary requirements or preferences change during or after transformation, or whether the stress caused by the process itself is a cause of mortality. Only a small number of studies have investigated the biological development of glochidia and juvenile freshwater mussels over time using scanning or transmission electron microscopy (SEM & TEM) e.g. Hudson & Isom (1984); Lasee (1991); Pekkarinen & Valovirta (1996); Kovitvadhi *et al.* (2001); Araujo *et al.* (2002); Fishera & Dimock (2002); Neumann & Kappes (2003); Kovitvadhi *et al.* (2007); Trump (2010). Even fewer have looked specifically at early life stages of *M. margaritifera*; Le Pennec & Jüngbluth (1983) considered ligament formation in juvenile *M. margaritifera* while Nezlin *et al.* (1994) studied the ultrastructure of glochidia. Additionally, Schartum *et al.* (2016) considered gill development in juvenile *M. margaritifera* using histology so this may be useful when considering findings from this investigation.

Recently there has been renewed interest in the field of particle capture and transport in adult bivalves but a paucity of research on juvenile mussels and their development is apparent, particularly in freshwater species. There are some notable single-species exceptions, e.g. Kovitvadhi *et al.* (2007), but research is preferentially carried out on commercially important species (Ó Foighil *et al.*, 1990; Beninger *et al.*, 1994; Veniot *et al.*, 2003; Cannuel & Beninger, 2006; Cannuel *et al.*, 2009; Gui *et al.*, 2016). This lack of research is possibly due to the small size of juveniles and their scarcity in the wild but the recent proliferation of freshwater mussel captive rearing programmes can now supply experimental animals for these types of studies. In addition, a more comprehensive understanding of mussel biology and development, particularly of juvenile stages, also informs the ongoing development of our taxonomic understanding (see Chapter 1).

The transition from pedal to filter feeding has been demonstrated to be a gradual one in unionids and individuals may employ both methods simultaneously for some time before the switch to filter feeding is complete (Gatenby *et al.*, 1997). There is no reference or definition

which states what processes or biological features must be satisfied in order for an organism to be officially classified as filter feeding. Yonge (1947) suggests that gill filaments are not functional until the food groove and associated cilia develop but other questions remain unanswered. For example, does filter feeding become possible once the gills and the labial palps grow close enough to touch, as suggested by Veniot *et al.* (2003) and Trump (2010)? How does the development of different types of cilia on gill filaments affect filter feeding ability? What are the functions of pedal cilia once water transport is achieved using primarily gill cilia? These are all fundamental questions which are yet to be answered.

5.1.1 Anatomy and juvenile ontogenesis in bivalves

An outline of mussel anatomy, development and feeding behaviour is provided below as a benchmark against which *M. margaritifera* development was measured during this investigation. A glossary of all anatomical terms and abbreviations used in this chapter is provided in section “5.6.1 Appendix 1: Glossary and abbreviations” on page 187. Killeen *et al.* (2004) outline the basic general anatomy of adult freshwater mussels and McMahon & Bogan (2001) provide a useful illustration of the main features (Fig. 5.1). Figure 5.2 shows the positioning of the left and right inner demibranchs in relation to the foot and a cross-section through the gills is provided in Fig. 5.3.

Bivalve gills are unique and vital organs which serve three main purposes: respiration, feeding and as a brooding organ for glochidia in sexually mature females (Neumann & Kappes, 2003). In adults, the primary pumping mechanism is driven by the lateral cilia on gill filaments, although cilia around the inhalant siphon are also used. Cilia on the gills filter suspended particles and direct them to the ventral gill surface where they travel anteriorly along the oral groove towards the labial palps for sorting. Once sorted, particles are either directed towards the mouth to be ingested, or to mantle ciliary tracts to be rejected as pseudofaeces. Juvenile mussels with under-developed gills use cilia located on the foot, mantle and gill to pump water and suspended particles into the mantle cavity (Kovitvadhi *et al.*, 2006). Juveniles also use their muscular foot to move through gravel interstices. Cilia on the foot may dislodge particles from substrate surfaces and direct them into the mantle cavity via self-generated water currents. Little is known about the process of particle sorting for ingestion or egestion as pseudofaeces.

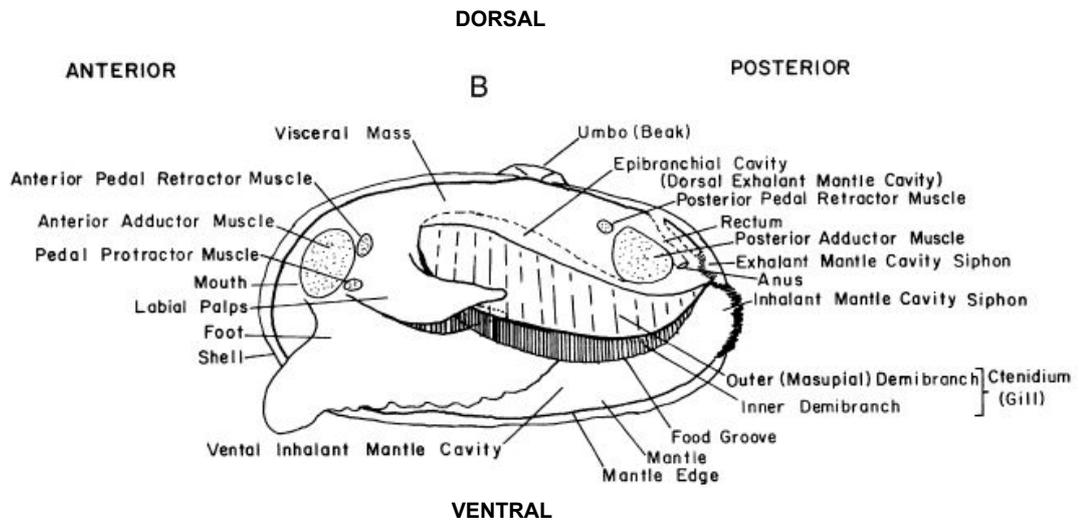


Fig. 5.1: Diagram of the general anatomy of the soft tissues of an unionoid freshwater mussel from Fig. 2b, p. 333 of McMahon & Bogan (2001).

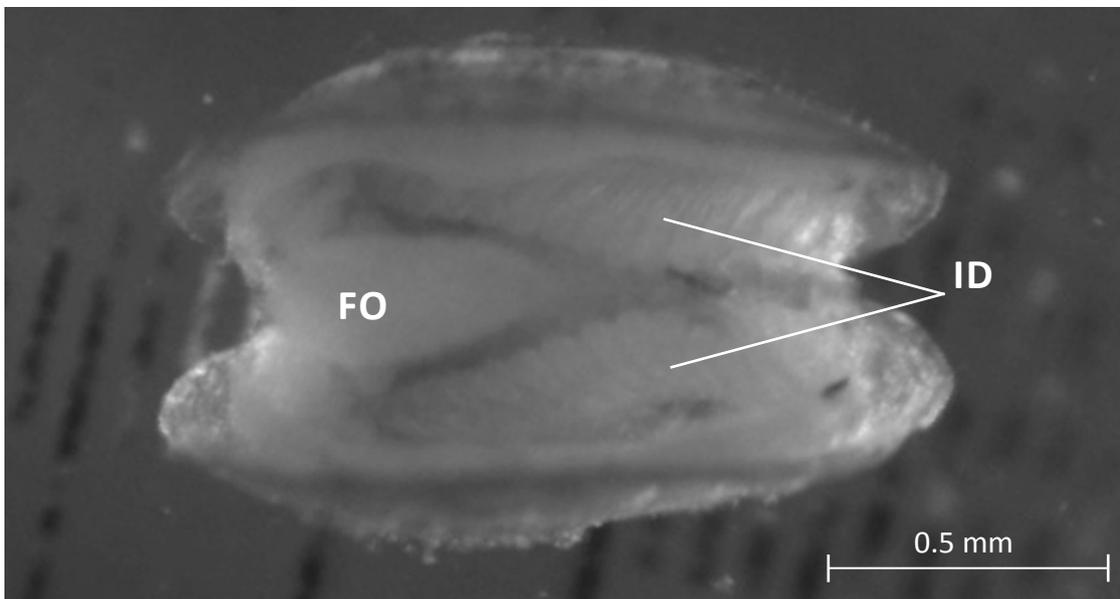


Fig. 5.2: Light micrograph of a 14 month old juvenile *M. margaritifera* showing the foot (FO) and left and right inner demibranchs (ID).

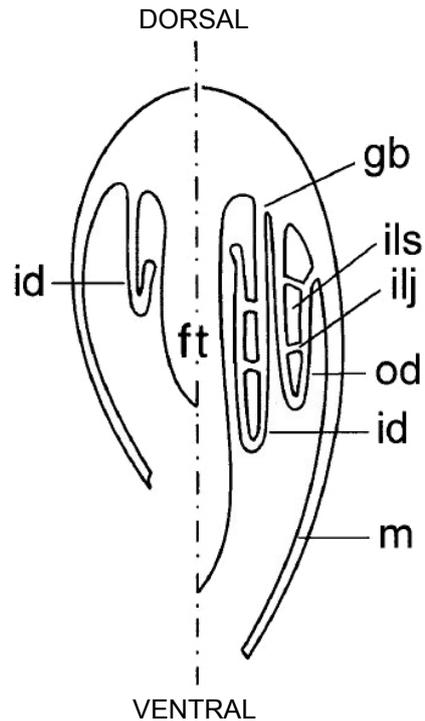


Fig. 5.3: Diagram of a dorso-ventral section through a eulamellibranch mussel showing the foot (ft), gill base (gb), inner demibranch (id), interlamellar junction (ilj), interlamellar space (ils), mantle (m) and outer demibranch (od). From Neumann & Kappes (2003).

This study focuses primarily on the structure and function of juvenile mussel gills, but will also consider important additional features such as the foot, labial palps, siphons, mouth and mantle.

5.1.1.1 Gills

Broadly, there are two major gill types in bivalves: protobranch and lamellibranch, although pseudolamellibranchs also exist. Protobranchs have reduced simple plate-like gills whereas lamellibranchs have enlarged ciliated gills consisting of filaments which are connected via ciliary (filibranch) or tissue (eulamellibranch) connections (Barnes, 2006). Pseudolamellibranchs possess tissue connections which are not as extensive as those in eulamellibranchs (Saleuddin & Wilbur, 1983). In addition to gill type, species are also defined by the type of gill filaments they possess. Individuals with heterorhabdic gills have principal and ordinary filament types and particle selection can occur on the gills. Individuals displaying the homorhabdic condition possess only ordinary filaments with particles being transported to the labial palps for sorting (Saleuddin & Wilbur, 1983; Barnes, 2006). *M. margaritifera* is a eulamellibranch mussel displaying the homorhabdic condition. Homorhabdic filibranch gills are considered to be

plesiomorphic since both the heterorhabdic filibranchs and the pseudolamellibranchs display a homorhabdic state in the early stages of gill development (Cannuel *et al.*, 2009).

Some aspects of gill development, morphology and function are very well documented (Morton, 1983; Jørgensen, 1990; McMahon & Bogan, 2001), however some important aspects have not been adequately researched such as ciliation, creation of junctions, and type of filament growth (Cannuel *et al.*, 2009). *M. margaritifera* is tetragenous (using all four demibranchs for brooding) which is widely thought to be a primitive trait (see Table 1.1, Chapter 1). Other primitive gill features which *M. margaritifera* displays are the lack of complete septa (and therefore the absence of water tubes) and the incomplete fusion of mantle margins resulting in a failure to form separate siphons (Ortmann, 1911b; Heard & Guckert, 1970).

Growth of bivalve gills is from the posterior end of the budding zone through proliferation of new gill filaments (Neumann & Kappes, 2003; Trump, 2010). Gills undergo continuous terminal growth correlated with body size (Neumann & Kappes, 2003; Veniot *et al.*, 2003; Trump, 2010). This continuous growth implies that biological development is linked to size rather than age. It is therefore feasible that the phenomenon of large size ranges within age cohorts reported previously e.g. Beaty & Neves (2004); Barnhart (2006); Schmidt & Vandr  (2010), could mean that individuals are at different stages developmentally. Mature filaments are made up of two limbs. Moving ventrally from the gill axis (called the gill base in Fig. 5.3), the descending limb terminates at the ventral bend where the oral groove may be present at the most ventral point. From here, the ascending limb runs dorsalward where it contacts the mantle cavity again via connective tissue, or is free but joined to the terminus of the other ascending limbs on adjacent filaments via the fused dorsal bend. Different types and stages of gill filament development have been described such as:

1. The presence of unreflected but curved filaments forming a temporary gill basket in scallops (Beninger *et al.*, 1994; Veniot *et al.*, 2003; Cannuel *et al.*, 2009). This condition usually progresses to one of the stages below.
 2. Ventral elongation of the descending limb before dorsalward reflection for growth of the ascending limb (Neumann & Kappes, 2003; Veniot *et al.*, 2003; Trump, 2010).
 3. Cavitation extension where the ascending and descending limbs grow simultaneously e.g. Ansell (1962); Cannuel & Beninger (2006).
-

The descending and ascending limbs of the lamellae are connected by interlamellar junctions. As well as being attached at the budding zone and the oral groove, adjacent filaments are connected to each other by interfilamentary junctions. In eulamellibranchs, interfilamentary junctions may develop as ciliary connections initially before tissue junctions form (Cannuel & Beninger, 2006; Cannuel *et al.*, 2009; Trump, 2010). The spaces delimited by adjacent filaments and interfilamentary junctions (i.e. the gaps between filaments) are known as gill pores or ostia (Tankersley & Dimock, 1992; Cannuel & Beninger, 2006; Cannuel *et al.*, 2009). True ostia may develop on thin sheets of tissue filling the gill pores depending upon the species (Ortmann, 1911c; Kovitvadhi *et al.*, 2007).

In newly-excysted juveniles, only a few gill filaments (< 5) are present which consist of only the descending limb of the inner demibranch (Trump, 2010). Several groups have shown that rather than the filaments bending back upon themselves and growing in a dorsalward direction as the term 'reflection' indicates, descending limbs continue to grow ventrally and ascending limbs grow in a dorsalward direction, originating from the ventral bend (Ansell, 1962; Neumann & Kappes, 2003; Cannuel *et al.*, 2009). Development of the inner demibranchs usually precedes that of the outer demibranchs and the outer demibranch lamellae may develop simultaneously or sequentially; see Table 1 of Cannuel *et al.* (2009). Additionally, attainment of a certain body size may be required for the onset of outer demibranch growth (Ansell, 1962; Neumann & Kappes, 2003).

Each filament is ciliated with groups of cilia or cirri responsible for different functions. Lateral cilia are responsible for water movement through the gills from the infrabranchial into the suprabranchial cavity. Laterofrontal cirri are responsible for particle capture although debate remains as to whether they act as bats/sieves to mechanically remove particles, or whether they create localised currents which direct particles onto the frontal cilia e.g. Owen & McCrae (1976); Jørgensen *et al.* (1984); Jørgensen, (1996); Riisgård *et al.* (1996); Silverman *et al.* (1996); Riisgård *et al.* (2014). A video taken through a low-power microscope of laterofrontal cirri of a 6 month old *M. margaritifera* juvenile can be viewed here https://www.youtube.com/watch?v=_rwi98T7B4w. The frontal cilia transport particles ventrally towards the oral groove. From here, particles are directed anteriorly towards the labial palps and mouth. At an ultrastructural level, important structures such as laterofrontal cirri have been observed from

the time of excystment in some bivalve species (Lasee, 1991; Trump, 2010) but not in others (Beninger *et al.*, 1994; Kovitvadhi *et al.*, 2007).

Development of the filaments and of cilia and cirri with different functions may be a useful tool to estimate the onset and efficiency of filter feeding. Large interfilamentary spaces which would not facilitate effective particle capture have been observed in immature gills with spaces closing as development continues (Beninger *et al.*, 1994; Veniot *et al.*, 2003). Indeed, if laterofrontal cirri are to be an effective sieve for particles, they will be most efficient if the tips on adjacent filaments overlap slightly (Gui *et al.*, 2016). The complexity of laterofrontal cirri can vary widely between species from simple cilia to those consisting of fused plates (Silverman, 1995). Large, compound laterofrontal cirri are widespread throughout the Bivalvia (Riisgård & Larsen, 2001) although there are exceptions such as *Bathypecten vulcani*, a peri-hydrothermal vent bivalve which does not possess laterofrontal cirri (Beninger *et al.*, 2003).

Whilst the form of gills is largely the same across the majority of bivalve species, cilia and/or cirri specialisations allow the exploitation of different niches. The number of cilia per laterofrontal cirrus can vary between species (Silverman, 1995). Lentic unionids have been found to have smaller laterofrontal cirri and fewer cilia per cirral plate compared with lotic species (Silverman *et al.*, 1997). Because of these specialisations, Silverman *et al.* (1997) also found that lotic species could clear laboratory-raised *Escherichia coli* at a higher rate compared to lentic species. This provides evidence that complex cirri aid capture of smaller particles.

5.1.1.2 Labial palps

The labial palps are paired triangular flaps located either side of the mouth (Morton, 1983) which sort particles into items which are either ingested or expelled as pseudofaeces. In *Utterbackia imbecillis*, Trump (2010) found that development of the labial palps began as a fold of tissue anterior to the foot 3 days post excystment and cilia were present around the mouth. The most anterior gill filament contacted the growing labial palps at around 113 days old and by 130 days old the labial palps surrounded the heavily ciliated mouth and had begun to take on a ridge and groove morphology typically observed in adult labial palps. An important consideration for the development requirements of juvenile mussels is the size of food particles in relation to the mouth and oesophagus; particles larger than these structures

are unlikely to be ingested (Lasee, 1991; Beck & Neves, 2003) and this may limit juveniles' ability to exploit particular algal species.

5.1.1.3 Siphons

In contrast to the Unionidae, *M. margaritifera* does not have a complete diaphragm and does not form a supra-anal opening (Ortmann, 1911c; Heard & Guckert, 1970; Graf & Ó Foighil, 2000). Descriptions of siphon development in juvenile freshwater mussels are rare but the presence of a pigmented and plicated inhalant siphon may be a sign of filter feeding as the ridges are tactile and used as a loose sieve for particle selection before water enters the infrabranchial chamber (Cummings & Graf, 2009).

5.1.1.4 Mantle

In *Utterbackia imbecillis*, Trump (2010) found that the mantle cavity of newly excysted juveniles was lined with cilia. Ciliary tufts developed on the anterior medial surface by 44 days old with cilia appearing in the posterior portion near the developing siphons by day 62. Thicker, compound cilia were present by 130 days old. Mantle ciliation is important for pseudofaeces rejection as captured particles are sorted by the labial palps before being transported from the anterior to the posterior region for ejection from the inhalant siphon or along the region of the ventral valve opening. This is done via particle rejection tracts which extend most of the length of the animal (Beninger *et al.*, 1999). The authors describe different types of cilia covering the mantle surface, with longer compound cilia responsible for carrying pseudofaeces being elevated above simple cilia. Simple cilia also may be present sporadically across the rest of the mantle (Beninger *et al.*, 1999). The presence of acid mucopolysaccharide-secreting mucocytes in this region helps to bind the pseudofaeces so that particles may be carried against the prevailing current in the mantle cavity (Beninger *et al.*, 1999).

5.1.1.5 Foot

In adults the function of the foot is mainly to anchor the individual within the substrate to avoid being washed away. In pedal feeding juveniles, the function of the foot is to move through substrate in order to find food particles which are directed into the pedal gape by water currents created by pedal cilia (<https://www.youtube.com/watch?v=nHtE4rtkF9A>). In

Utterbackia imbecillis, Trump (2010) found that 3-day-old juveniles had a ring of cilia on the distal portion of the foot but cilia were absent elsewhere. Tufts of cilia in the proximal region only formed later (113 days old). This incomplete pattern of ciliation indicates that particle transport into the pedal gape is unlikely to be via a train of particles travelling up the foot on cilia as suggested by Lasee (1991).

5.1.1.6 *Byssus threads*

In some species, the presence of byssus threads may indicate a switch from pedal to filter feeding (Hanlon, 2000). Due to the lack of investigation into the juvenile life stage, the presence of byssus threads in juvenile mussels is poorly documented, although Ziuganov *et al.* (1994) observed them in juvenile *M. margaritifera* < 20 mm in length. The author has observed byssus threads in *M. margaritifera* from juveniles up to 25.5 mm (FBA, unpublished data) and they are common in wild juveniles that are still buried (E. Moorkens, pers. comm.).

5.1.2 Feeding behaviours of juvenile freshwater mussels

Acquiring food through an anterior aperture (as opposed to the posterior inhalant siphon) is a trait common to most juvenile and small bivalves (Reid *et al.*, 1992) and one which the author has observed first-hand in *M. margaritifera*. There are many examples of species employing some type of pedal feeding behaviour immediately after excystment and before filter feeding commences, most likely due to the undeveloped gills and their inability to efficiently pump water. The term 'pedal feeding' has been used to describe several slightly different behaviours in a range of species, leading to confusion. For example, the movement of particles into the pedal gape on water currents generated by cilia has been referred to as 'interstitial pedal feeding', 'interstitial suspension feeding', 'locomotory pedal feeding' (when the individual is mobile) and 'pedal-mantle-gill feeding' (Reid *et al.*, 1992; Yeager *et al.*, 1994; Gatenby *et al.*, 1996; Gatenby *et al.*, 1997; Hanlon, 2000; Kovitvadhi *et al.*, 2006). The terms 'pedal-sweep feeding' and 'pedal-probe feeding' have been used for when particles adhered to pedal cilia are transferred directly in to the pedal gape or even to directly on to the labial palps (Reid *et al.*, 1992; Yeager *et al.*, 1994; Gatenby *et al.*, 1996; Gatenby *et al.*, 1997). Pedal feeding itself could be described as a type of filter feeding, as cilia on the foot and mantle entrain particles in water currents depending upon factors such as particle size and shape. It is clear

that the mechanisms and behaviours exhibited during this early feeding stage are still not fully understood and differ between species. Investigations in this area will help clarify the terminology used for describing feeding behaviours in juveniles before gill development facilitates true siphonal filter feeding. In this thesis, the term 'pedal feeding' is used to describe the movement of particles into the pedal gape on water currents generated by cilia on the foot, mantle or gill filaments, before the onset of siphonal filter feeding. This is typified by juveniles actively moving through substrate and particles entering the pedal gape predominantly from areas other than the posterior siphonal region.

Descriptions of juvenile feeding behaviour specifically in *M. margaritifera* are scarce. Buddensiek (1995) describes juveniles probing and retracting their foot into the mantle cavity and particles being drawn into the mantle cavity around the anterior and ventral edges. Lange & Selheim (2011) demonstrate the activity of juvenile mussels during feeding through documenting trails left in fine particulate organic matter.

Some species are reported to display both deposit and filter feeding behaviours into adulthood, e.g. *Corbicula* spp. (Reid *et al.*, 1992), but reports of deposit feeding in adult mussels are uncommon and Raikow & Hamilton (2001) believe that the widely-held perception that adult mussels feed exclusively by filtering from the water column is not supported by empirical evidence. In fact, several studies have postulated that adult freshwater mussels may derive a significant proportion of their diet from the substrate, and this is obtained by deposit feeding (Hornbach *et al.*, 1984; Way, 1989; Raikow & Hamilton, 2001, Nichols *et al.*, 2005). Whilst the assumption has always been that *M. margaritifera* adults are strictly filter feeders, this has not been backed up by experimental data. It is therefore important to fully understand the feeding mechanisms of juvenile *M. margaritifera* pre- and post-transformation to establish if they exclusively filter feed or use a combination of feeding methods. These findings could have important implications for both captive rearing practices and river restoration activities in pearl mussel catchments.

5.1.3 Objective of this study

The purpose of this study was to describe the feeding behaviour of juvenile *M. margaritifera* at different ages and feeding stages and to investigate the biological development of feeding apparatus and other pertinent structures. The aim is to provide evidence for the timing of

switching from pedal to filter feeding (transformation). This in turn could assist with the improvement of captive rearing practices where benefits may be derived from employing different rearing strategies at different developmental stages. The stages to be considered are:

1. Post-metamorphosis (excystment); 1 - 8 months old;
2. 10 - 20 month old juveniles (around the suspected age of transformation), and;
3. Post-transformation (> 3 years old).

These investigations will consider feeding behaviour through observation of video recordings and biological development using light and scanning electron microscopy (SEM).

5.2. Methods

5.2.1 Comparing foraging and feeding behaviour

5.2.1.1 Experimental procedure

On 10 January 2015 three live individuals which excysted in 2014 (approximately 6 months old) were examined under a low-power light microscope (x10 to x200 magnification) to help inform the methodology for this investigation. Experimental work took place over ten months between May 2015 – March 2016. The feeding behaviour of juveniles was assessed at different ages and sizes by taking video recordings of individuals which had been observed filter feeding and had thus undergone transformation (excysted summer 2012), those which were expected to undergo transformation within the sampling period (excysted summer 2014) and newly excysted juveniles (excysted July 2015). Throughout this chapter size refers to shell length (mm).

In May 2015, 10 month old juveniles from the river Ehen population were sampled by disturbing sediment in the tray system described in Chapter 2 and siphoning water through a 0.3 mm sieve. Juveniles were emptied into a petri dish and individuals encompassing a range of sizes were chosen for further investigation. The presence and activity of juveniles was recorded using a light microscope (x 100) with additional side lighting from a cold light source. Juveniles were placed into a glass petri dish containing filtered lake water. Locomotory behaviour was recorded for 3 minutes using a camera (Moticam 2000, Motic, China) mounted on a trinocular microscope (Meiji, UK). Individuals were then prepared for further ultrastructural investigation via scanning electron microscopy (SEM). The same procedure was carried out on all occasions for all cohorts. On 9 September 2015 an adult mussel from the FBA Ark (length = 99 mm)

was found to have recently died and the opportunity was taken to dissect some of the gill tissue for SEM investigation. Small square sections were taken from the mid-gill on the outer demibranch.

5.2.1.2 Analysis of video recordings

Video files were assessed to document behaviour over a three minute period starting from one minute after the mussels were placed into the petri dish. Individuals therefore had one minute to recover from handling before recordings began. The total time spent active was converted into proportion of time active (%) for data analysis and different types of movements recorded:

- Pedal foot movement - the foot is extended away from the shell before the tip (or less frequently the entire region from the tip to the 'heel') is anchored and used to pull the individual in the direction of the foot tip;
- Pedal sweep - the foot is swept in a single motion along an arc following the curvature of the shell, usually in an anterior to posterior direction, and;
- Shell flipping - the juvenile starts from resting on one valve and performs a pedal foot movement whereby it rotates upon its ventral axis, and which results in the individual coming to rest on the opposite valve at the end of the manoeuvre.

5.2.2 Ultrastructural analysis with scanning electron microscopy

After behavioural observations, each individual was transferred into an eppendorf tube and immersed in 1 mg/mL MS-222 (tricaine methanesulfonate) as per Galbraith *et al.* (2009) to relax the valves and expose soft tissues within the shell. Juveniles were processed with either both valves intact (to observe gill and siphonal connections between left and right sides), valves were teased open to better observe feeding structures, or one valve was removed completely (to observe shell-facing structures and gain a better overall view of gill filaments). Juveniles were fixed in 2 % glutaraldehyde in 0.1 M Sorenson's Phosphate Buffer (SPB) over night. They were then washed in SPB (x 2) before being dehydrated through 25%, 50%, 75% and 2 x 100% ethanol washes. Samples were immersed in Hexamethyldisilazane (HMDS) for 2 x 30 minutes in place of critical point drying before being mounted onto SEM stubs and sputter coated with gold. A Leo 1450VP scanning electron microscope (Zeiss, Germany) at the University of Derby was used to view all samples. Slightly different methodologies were undertaken on different

sampling occasions to try and streamline the work process. Some of these variations led to poor quality samples. For example, some samples were treated up to the HMDS step before being mailed to the University of Derby for mounting and sputter coating whereas others were mounted on stubs before being mailed for sputter coating. The consequences of these different methodologies are discussed in the results. Adult mussel tissue was dissected and stored in 2 % glutaraldehyde in 0.1 M SPB for 3 weeks before the alcohol dehydration step was undertaken. All other steps were the same as described above.

5.2.3 Data analysis

All measurements of features on micrographs were taken with ImageJ (version 1.48; National Institutes of Health, Maryland, USA). As a measure of filtering efficiency at different ages, the distance between filaments (interfilamentary space) and distance between laterofrontal cirrus couplets were measured from micrographs. Where pictures contained interfilamentary junctions (ciliary or tissue), measurements were taken in the vicinity of the junction because, at this point, filament distance is less variable. Due to potential tissue shrinkage during sample preparation (Silverman *et al.* 1995; Beninger *et al.* 1999), all measurements reported should be considered minimum measurements as no attempt was made to quantify tissue shrinkage for this work.

All data were checked for normality using Shapiro-Wilk tests for normality before performing parametric tests. ANOVA's (with *post hoc* Tukey's HSD tests) were performed to test the difference in the size of interfilamentary space for individuals at different ages, to test the number of cilia per laterofrontal cirrus, and to test the proportion of time active between individuals of different ages. Unless otherwise specified, numbers given after means are standard deviation. The number of inner demibranch filaments was plotted against mussel length and age, and regression lines plotted. Linear regression analysis was performed for interfilamentary space against shell length and also for the number of inner demibranch 'vs' outer demibranch filaments, where the outer demibranch was present.

5.3. Results

The following descriptions and micrographs outline the most noteworthy features of juveniles at different ages observed using SEM. Table 5.1 provides information about the ranges of shell length (mm) and range of inner demibranch filaments observed for the different age cohorts.

Table 5.1: Summary information about the ranges of shell length (mm) of juvenile freshwater pearl mussels and number of inner demibranch (ID) filaments for the different age cohorts (months) described.

Age (months)	Shell length range (mm)	Number of ID filaments
1	0.49 - 0.66	5 - 6
2	0.58 - 0.83	Poor sample prep. prevented accurate counts
3	0.65 - 0.81	6 - 7
4	0.75 - 0.94	6 - 11
8	0.95 - 1.00	7 - 9
13	0.81 - 1.37	6 - 14
14	0.96 - 1.28	9 - 13
15	1.00 - 1.30	7 - 12
16	1.13 - 1.56	9 - 17
20	1.20 - 1.44	12 - 16
34	2.66 - 5.90	28 - 62
44	3.10 - 8.90	34 - 94
Adult	99	Not counted

Where possible, qualitative descriptions have been matched with micrographs from the correct age cohort. On some occasions however it was not possible to obtain images of adequate quality to illustrate structures within the correct age cohort and so electron micrographs from other cohorts are used for illustrative purposes. The age of individuals is provided in the top right corner of each micrograph.

5.3.1 1 month old

Shortly after excystment, gill filaments of the inner demibranch were observed as long finger-like projections (Fig. 5.4). Adjacent filaments were not connected other than by the gill axis. The distance between adjacent filaments was large. Frontal cilia, lateral cilia and laterofrontal cirri (illustrated in Fig. 5.5 of a 34 month old individual) were observed on filaments. Laterofrontal cirri had a similar structure to those reported previously in other species (Owen, 1974; Silverman *et al.*, 1996; Gui *et al.*, 2016). Cirral plates were orientated perpendicular to the filament and each laterofrontal cirrus consisted of two parallel rows of cilia which were shortest towards the frontal surface and became progressively longer towards the lateral surface (illustrated in Fig. 5.6 of a 34 month old individual). At this stage, the large interfilamentary distance meant that laterofrontal cirri on adjacent filaments were not able to span the gap between adjacent filaments. They are therefore unlikely to form an effective sieve at this stage.

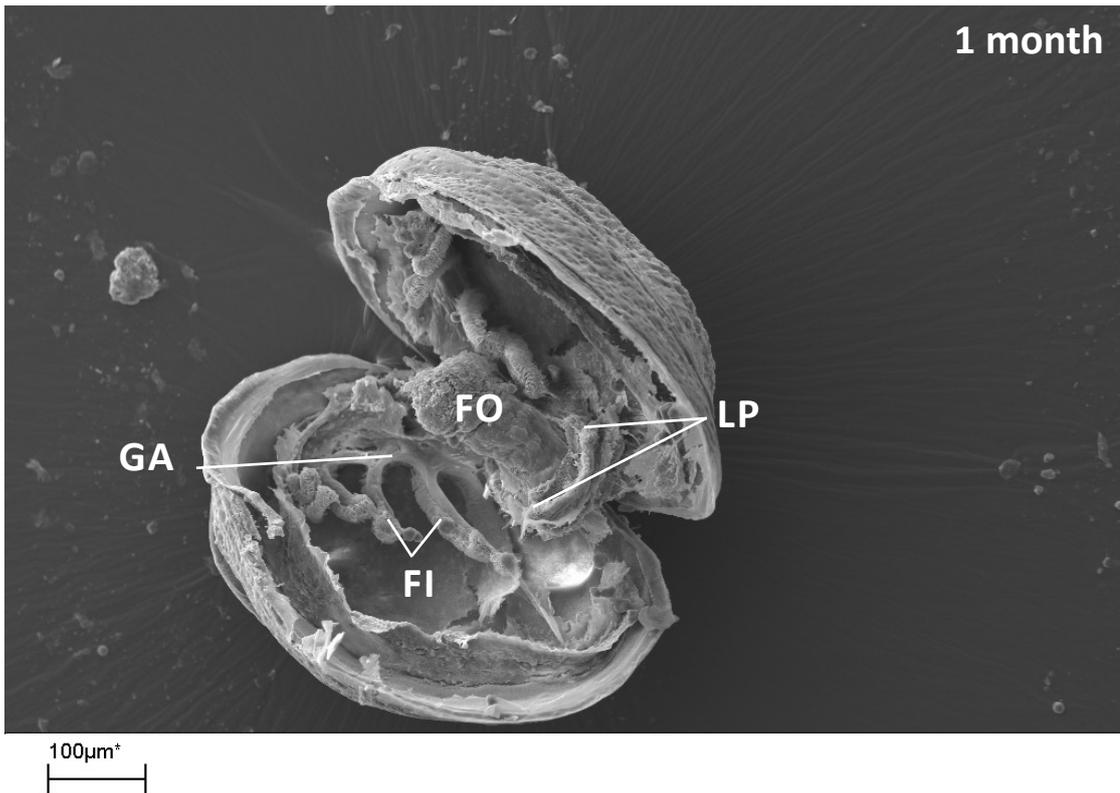


Fig. 5.4: Scanning electron micrograph of a 1 month old juvenile; foot (FO), unreflected filaments (FI), gill axis (GA), left and right labial palps (LP).

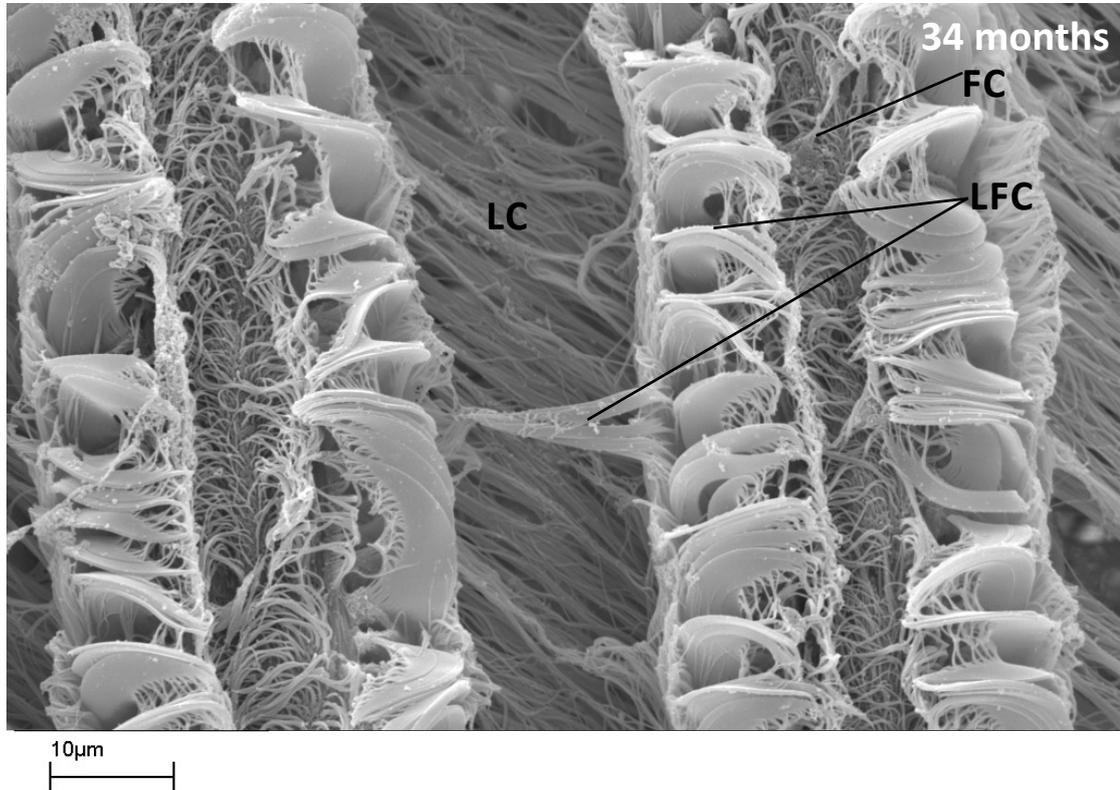


Fig. 5.5: Scanning electron micrograph of the frontal view of two gill filaments. The three different types of cilia/cirri; frontal cilia (FC), lateral cilia (LC) and laterofrontal cirri (LFC). In developed individuals like this 34 month old, the LFC are capable of reaching over half way across the interfilamentary space (as shown here).

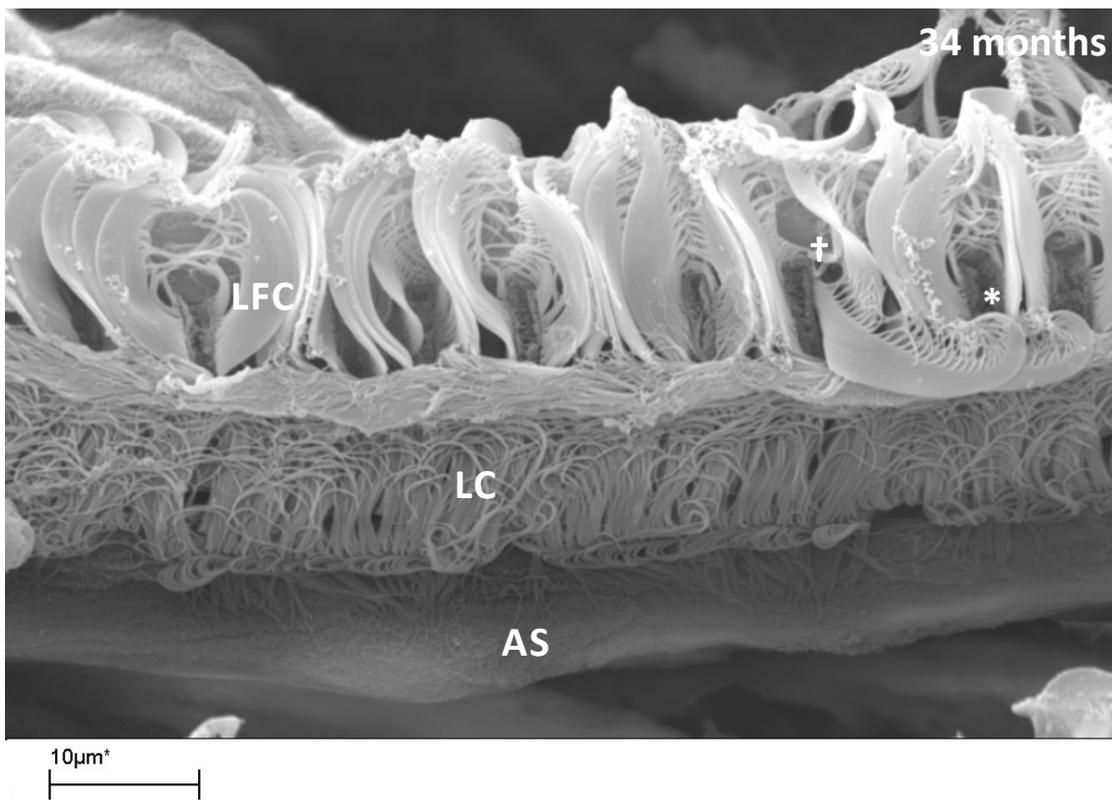


Fig. 5.6: Scanning electron micrograph showing the lateral view of a gill filament. Lateral cilia (LC), laterofrontal cirri (LFC) and the abfrontal surface (AS) are marked. Cilia making up the LFC are longer towards the lateral surface (*) and shorter towards the frontal surface (+).

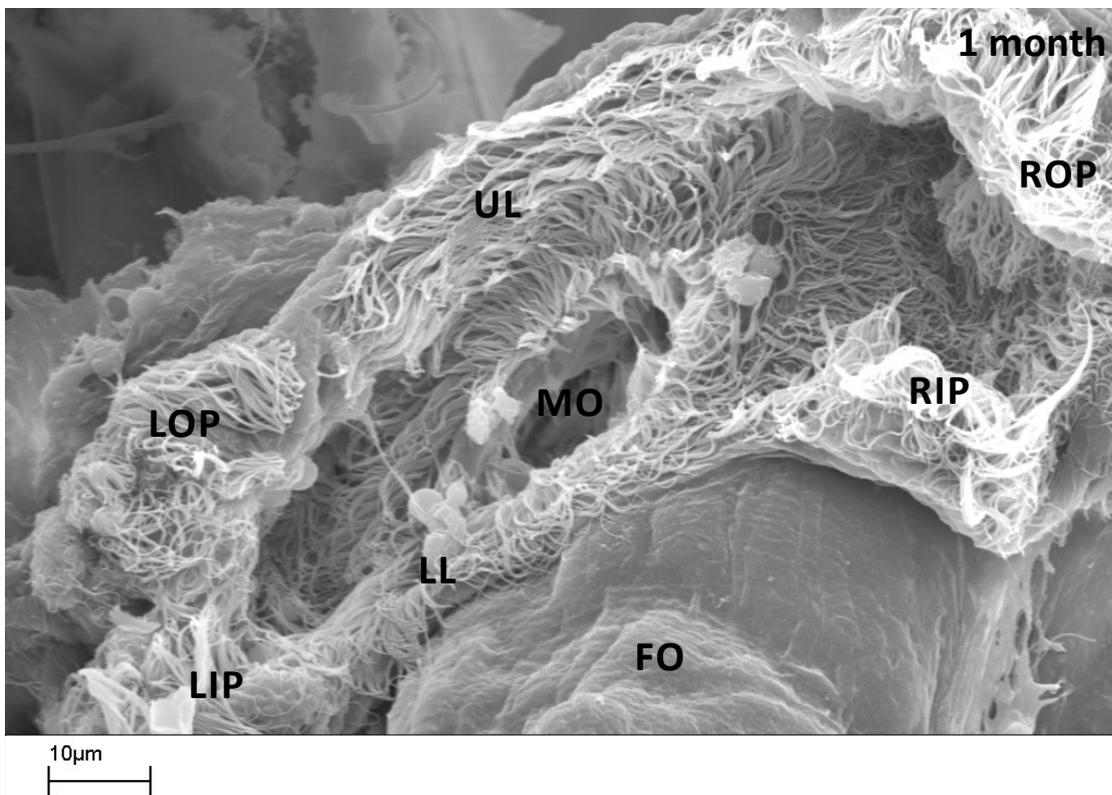


Fig. 5.7: Scanning electron micrograph of the labial palp primordia, lips and mouth (MO); foot (FO), left inner palp (LIP), left outer palp (LOP), lower lip (LL), right inner palp (RIP), right outer palp (ROP), upper lip (UL).

Filaments were observed to follow the curvature of the shell (Fig. 5.4) but because valves were separated during sample preparation it was not possible to tell if juveniles formed a gill basket, as described by Beninger *et al.* (1994). For the same reason it was also impossible to ascertain if the labial palp primordia had made contact with any of the gill filaments at this stage. Labial palp primordia, lips and the area around the mouth were heavily ciliated (Fig. 5.7).

5.3.2 2 & 3 months old

Experimentation of suitable sample preparation techniques meant that all 2 & 3 month old samples were either too damaged or did not show enough detail to ensure meaningful data were collected (see section 5.3.16). However, the mouth and labial palp primordia were observed in several 3 month old specimens and both were heavily ciliated, as observed in the 1 month old individuals. Gill filaments were still unreflected at this stage.

5.3.3 4 months old

Filaments were joined at the distal tips by tissue connections (illustrated in Fig. 5.8 of a 14 month old individual) which were covered in long simple cilia. Larger individuals had tissue connections between most filaments whereas smaller individuals had connections between only some filaments. Connections usually started developing between filaments at the posterior end (nearest the budding zone).

Simple cilia were visible around the mantle margin, with more dense aggregations near the posterior end. Cilia around the mantle margin were organised into rows (Fig. 5.9). On the outer-most margin was a band of short cirri composed of 6 - 10 individual cilia. Slightly dorsal to this ran two bands of long cilia. In addition to this were sparse aggregations of long cilia distributed over the visible part of the mantle and occasional instances of long cilia in two parallel rows (Fig. 5.10).

5.3.4 8 months old

Specimens were slightly damaged leading to a potential under-estimation of the number of gill filaments present. Filaments remained unreflected and showed the same arrangement as described for 4 month old individuals. The labial palps were again observed but the morphology

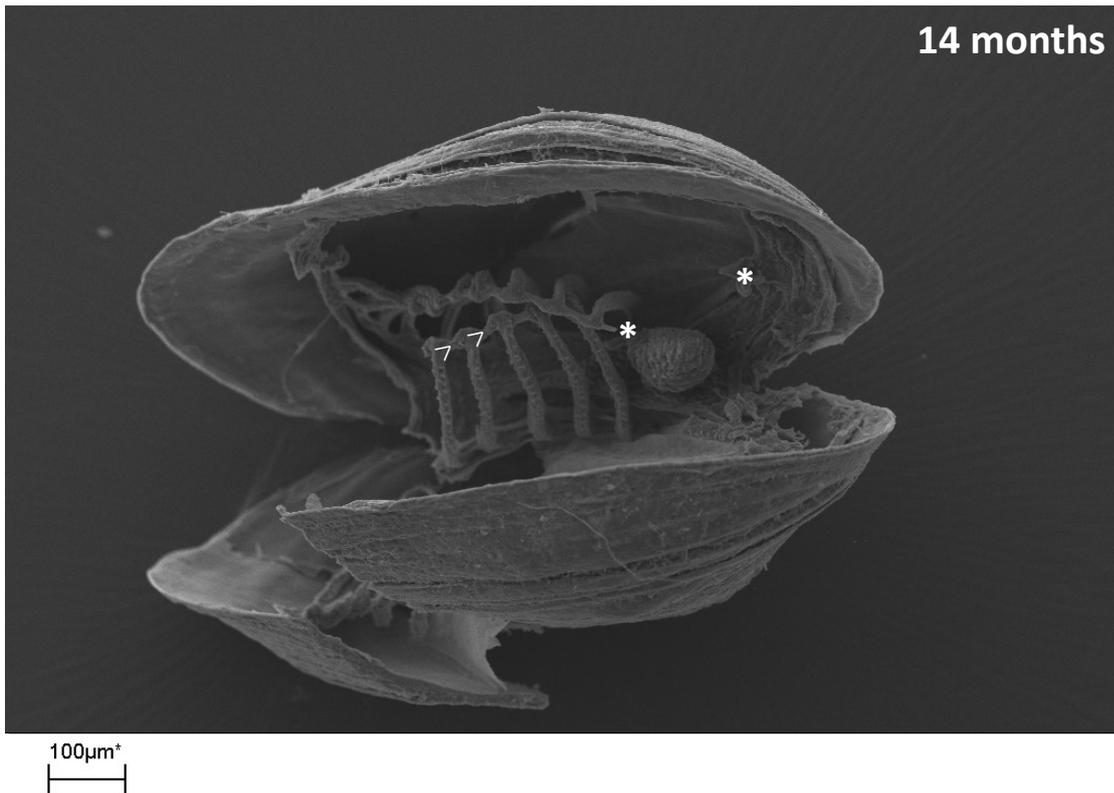


Fig. 5.8: Scanning electron micrograph of the distal tips of filaments which are joined to each other by thin tissue connections (arrow heads). Points of connection between the anterior-most filament and the mantle are indicated (*).

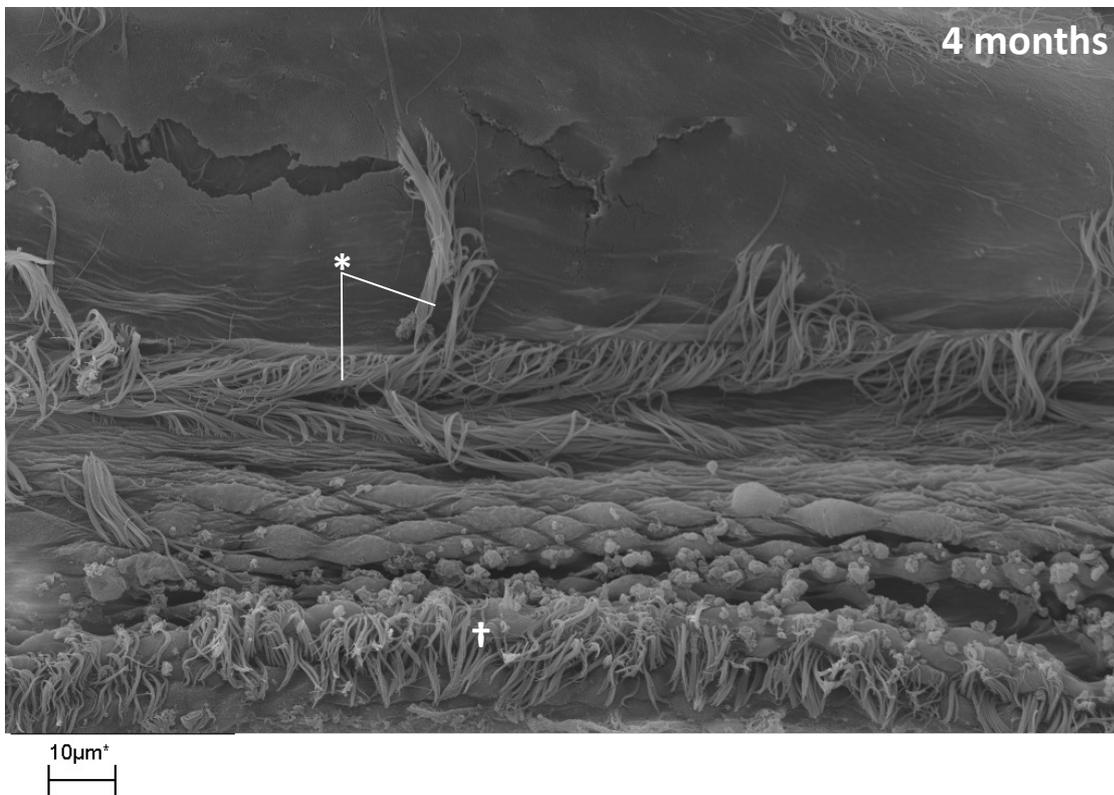


Fig. 5.9: Scanning electron micrograph of long simple cilia (*) and short cirral tracts (†) near the mantle margin. Ventral shell margin is to the bottom of the image.

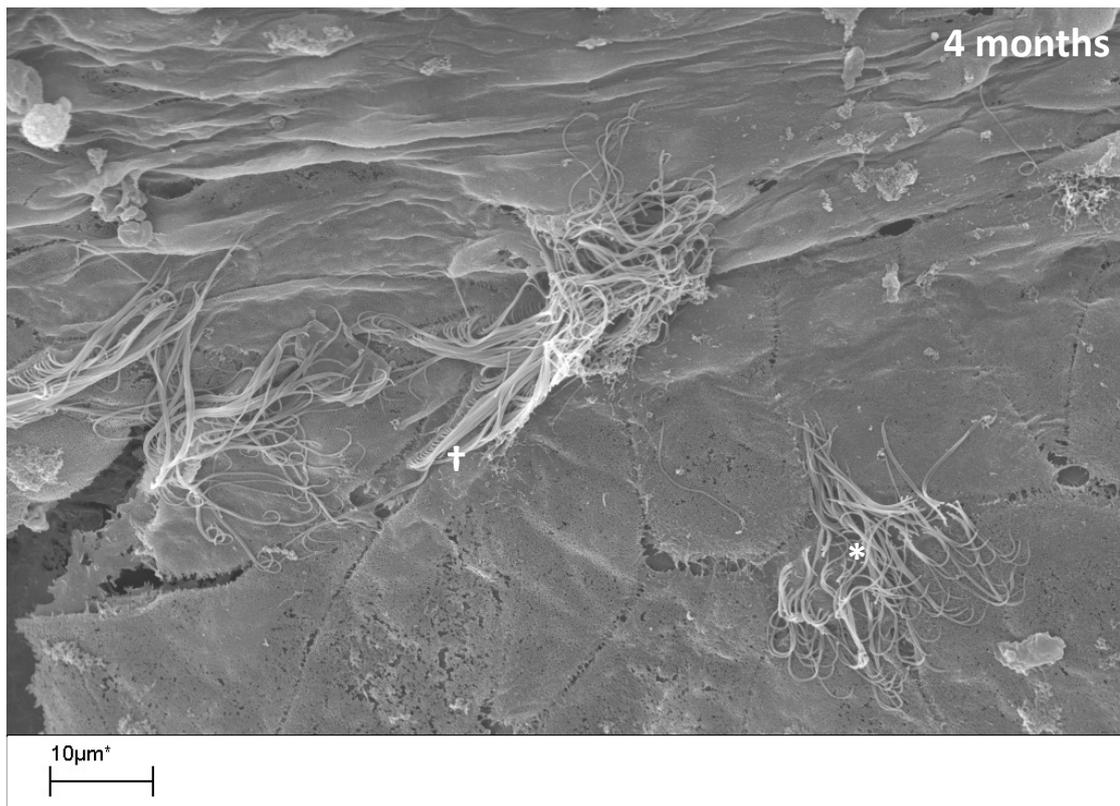


Fig. 5.10: Scanning electron micrograph of patches of long simple cilia observed over the whole visible part of the mantle either in sparse patches (*) or arranged in two parallel rows (†).

did not differ from previous observations. Sparse aggregations of cilia covered the mantle surface with denser ciliation around the posterior mantle margin as previously described.

5.3.5 13 months old

In the largest individual (1.37 mm, 14 filaments), the first instance of gill reflection was observed. The most distal portion of filaments had a bulbous appearance and were still connected by thin tissue connections, but the terminal end of the ascending limb had a continuous, thicker piece of tissue joining all filaments which projected dorsally (illustrated in Fig. 5.11 of a 16 month old individual). This structure is known as the fused dorsal bend and had a sparse covering of short simple cilia. Reflection was observed concurrently on both the right and left inner demibranch. The ascending limbs on the middle filaments were longer than on the most anterior and posterior filaments, indicating that reflection may begin in the middle portion of the demibranch and radiate anteriorly and posteriorly (Fig. 5.11). Ciliation on the ascending limb was the same as on the descending limb with all types of cilia/cirri present (Fig. 5.11) although laterofrontal cirri were smaller and consisted of fewer cilia per cirrus (see section 5.3.14.3 on page 159). Labial palp morphology did not differ to previous observations.

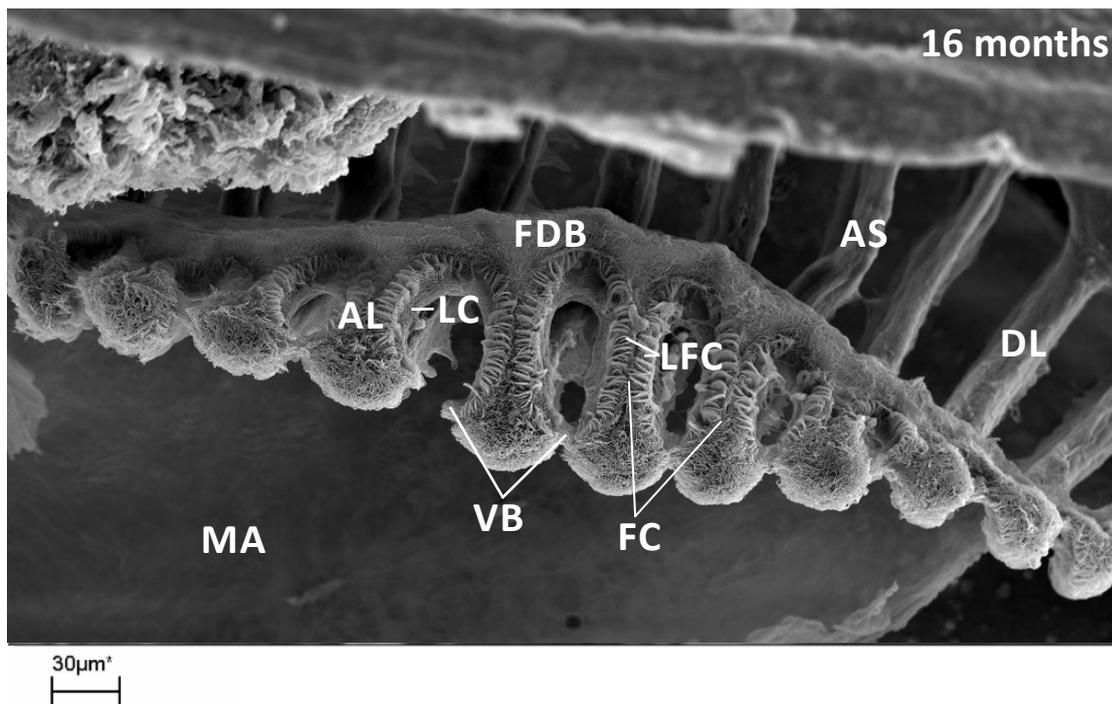


Fig. 5.11: Scanning electron micrograph showing gill reflection of the inner demibranch. Thin tissue connections join filaments at the ventral bend (VB) and the thicker fused dorsal bend (FDB) joins the terminal ends of the ascending arms. Even in the early stages, reflected filaments develop all three cilia types almost immediately; lateral cilia (LC), laterofrontal cirri (LFC) and frontal cilia (FC). Ciliation progresses with ascending limb (AL) growth. The ascending limb is longer on medial filaments compared to those at either anterior or posterior ends (to the left and right of the figure). Other features of note are the abfrontal surface (AS), descending limb (DL) and mantle (MA).

5.3.6 14 months old

Reflection was not observed in smaller individuals in this age class but filaments had started to reflect in the largest individual (1.28 mm, 13 filaments). Unreflected filaments were joined to each other by thin tissue connections at the distal tips as described previously, but the most anterior and posterior filaments were also connected to the mantle both anteriorly and posteriorly (as outlined previously in Fig. 5.8). There was no sign of oral groove development at this stage.

5.3.7 15 months old

Samples from this age class were either too damaged or not preserved adequately due to specimen preparation trials discussed in section 5.3.16, but followed the same general pattern of development as observed in the 14 month old specimens.

5.3.8 16 months old

Of the five individuals checked, gill reflection was observed in three. This was the first time that reflection was obvious under a light microscope before specimen preparation for SEM; subsequent micrographs confirmed the condition. As described in the 13 month old individual, filaments were observed reflecting from the medial filaments, followed by filaments in more anterior and posterior positions (Fig. 5.11). Development of laterofrontal cirri on the ascending limb was more advanced in medial filaments compared with those at the anterior and posterior ends, and again laterofrontal cirri on the ascending limb consisted of fewer cilia per cirrus compared with those on the descending limb (13 - 14 on ascending limb 'vs' 43 on descending limb).

Connections between filaments at the ventral bend were observed beginning as ciliary connections before tissue connections formed. Tissue connections were again covered in simple cilia and those connecting medial filaments were more developed than at either the anterior or posterior ends (Fig. 5.11). No oral groove was present at this stage but developing tissue connections at this most distal point is likely a precursor to oral groove development.

The smallest individual (1.13 mm) did not have reflected filaments but thin tissue connections joined the distal tips and reflection appeared imminent. From the individuals in this age cohort, filament reflection was observed in individuals measuring just over 1.13 mm up to 1.22 mm (largest individual in age class), and when the inner demibranch consisted of more than nine filaments.

Dense patches of short cirri were present around the mantle margin, particularly around the posterior region where siphons will develop at a later stage.

5.3.9 20 months old

The length of the two 20 month old individuals observed overlapped with those of 16 month olds, as did the numbers of inner demibranch filaments. Gill reflection was observed in both individuals and new filaments were budding already reflected (i.e. via cavitation extension). Development of the labial palps was progressing with the first observation of folds developing in this organ. A ciliary connection was also observed for the first time between the labial palp and the penultimate anterior filament. This may have been present in younger specimens but due to the way in which samples were prepared the connection may have been broken previously. Again, no oral groove was observed.

On the ascending limb, laterofrontal cirri development appeared to start from near the fused dorsal bend and progress towards the ventral bend because the bases of cirral plates were observed near the ventral bend but they lacked cilia (Fig. 5.12). However, this could have been due to specimen damage so this observation warrants further investigation.

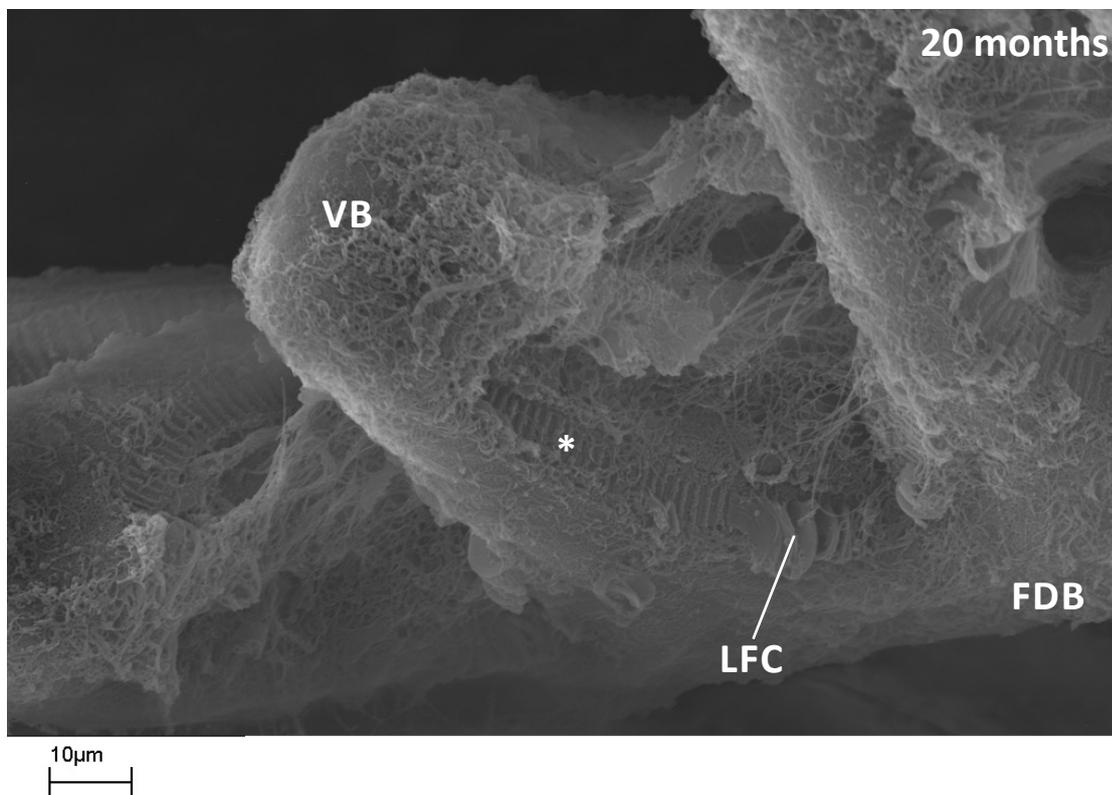


Fig. 5.12: Scanning electron micrograph showing the ascending limb of the inner demibranch with bases (*) of the laterofrontal cirri (LFC) present, but cilia absent towards the ventral bend (VB). Cilia are present near the fused dorsal bend (FDB).

5.3.10 34 months old

The budding zone was observed in detail for the first time in 34 month old individuals. The whole region was densely covered in simple cilia. Gill buds were distinguished from 'true' filaments by the absence of laterofrontal cirri. Each individual typically had 3 - 5 buds before true filaments were observed (Fig. 5.13) and the budding zone was free within the mantle cavity (i.e. not connected to the mantle). All individuals had reflected inner demibranch filaments and new filaments were budding already reflected via cavitation extension. The most anterior gill filament was attached to the visceral mass along its entire length and only consisted of a descending limb. Laterofrontal cirri could reach over half way across interfilamentary spaces (as illustrated in Fig. 5.5) meaning that they were capable of forming an effective sieve for particle capture. In all individuals, the fused dorsal bend of the inner demibranch was not connected to the visceral mass i.e. the same condition as present in younger individuals (Fig. 5.14). As in younger juveniles, the abfrontal surface of filaments had only a very sparse coverage of cilia which did not appear to be organised into tracts (Fig. 5.15).

Interfilamentary junctions between adjacent filaments were observed for the first time. These began as ciliary junctions between posterior (recently budded) filaments, giving way to tissue junctions between more anterior filaments. Between approximately filaments 1 - 11 there were no interfilamentary junctions. After this, ciliary interfilamentary junctions were present until approximately the 14 - 15th filament, after which they became tissue (Fig. 5.16). In addition to interfilamentary junctions in a posterior-anterior direction, additional interfilamentary junctions were present along the dorsal-ventral axis. These may be created as filaments elongate. In a single individual (length = 5.8 mm) the distance interfilamentary junctions were observed from the gill axis was measured. The maximum length of inner demibranch filaments was 1.59 mm and tissue interfilamentary junctions were present 0.90 mm and 1.17 mm down the filament from the gill axis and a final ciliary interfilamentary junction (Fig. 5.17) was present 1.53 mm from the gill axis (most ventrally).

Formation of both the outer demibranch (Fig. 5.18) and the oral groove on the inner demibranch (Fig. 5.19) were observed for the first time in 34 month old individuals. Development of the oral groove began on filaments which were approximately 3 - 13 true filaments away from the last gill bud. The outer demibranch had started to form in all but the smallest individual (< 3.2 mm) but no oral groove was observed on the outer demibranch

in any individual (Fig. 5.19). Outer demibranch filaments appear to proliferate via cavitation extension only, and are of a similar length to each other with the budding zone appearing to give rise to several filaments at once (Fig. 5.18).

The labial palps were observed in several individuals. At this stage they consisted of two pairs of flattened plates which are highly ciliated on the inner surfaces but devoid of cilia on exterior surfaces (Fig. 5.18).

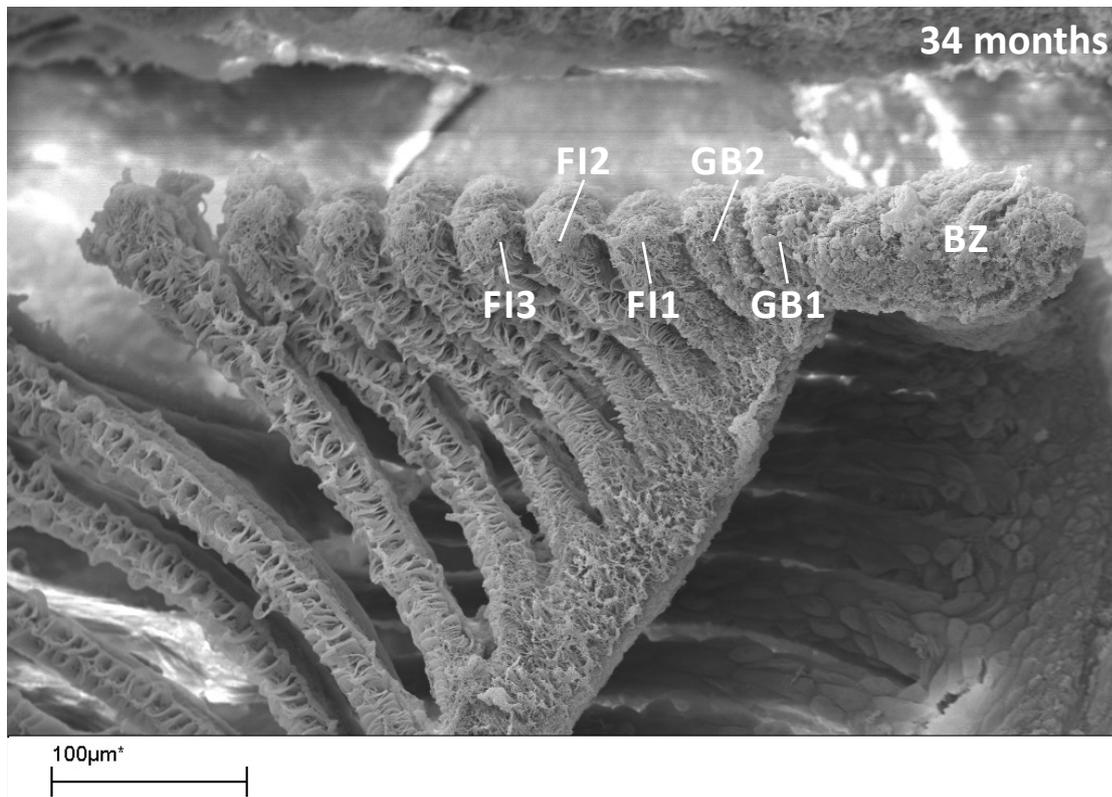


Fig. 5.13: Scanning electron micrograph of the budding zone (BZ) with two gill buds (GB) and three true filaments (FI) numbered.

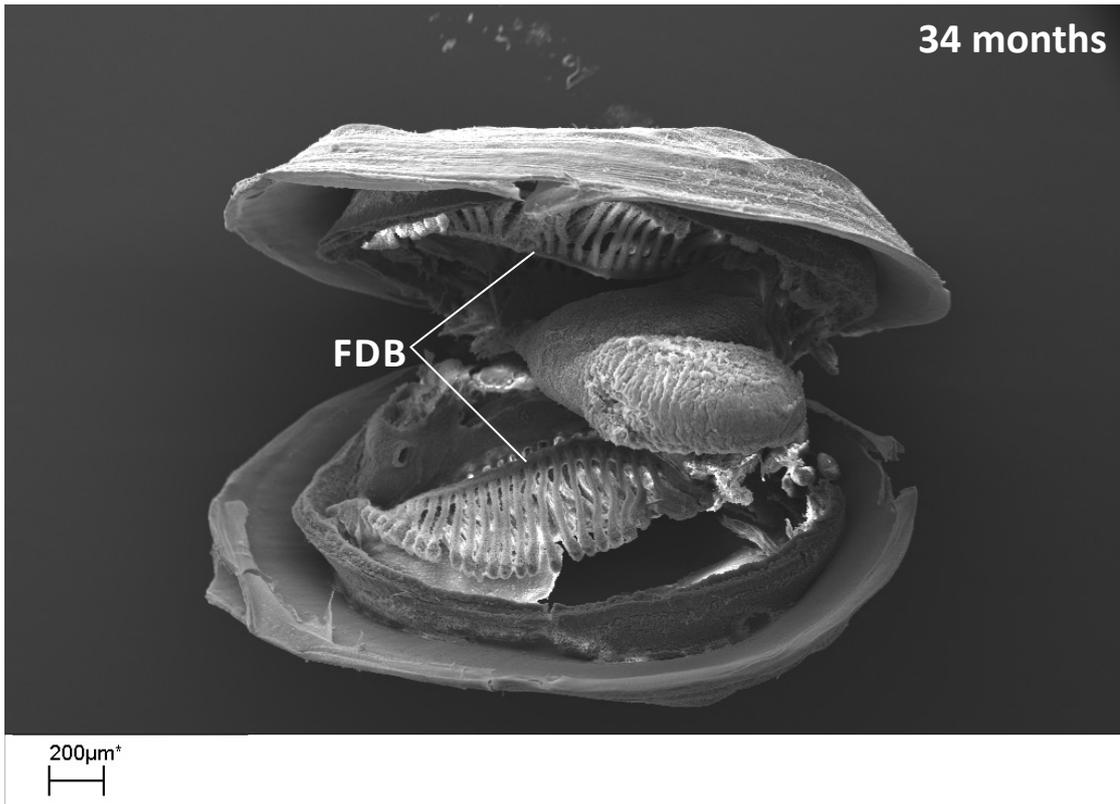


Fig. 5.14: Scanning electron micrograph of the fused dorsal bend (FDB) which is not attached to the foot or visceral mass.

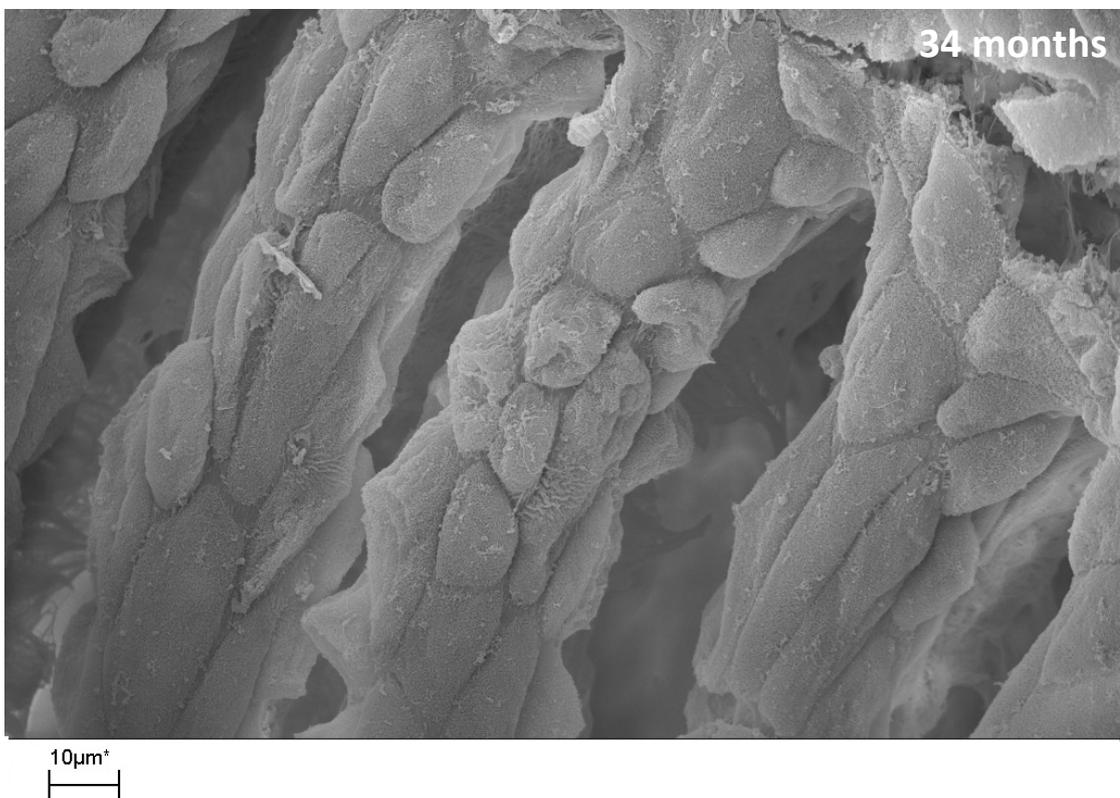


Fig. 5.15: Scanning electron micrograph of the abfrontal surface of filaments which is sparsely covered in cilia which were not organised into tracts.

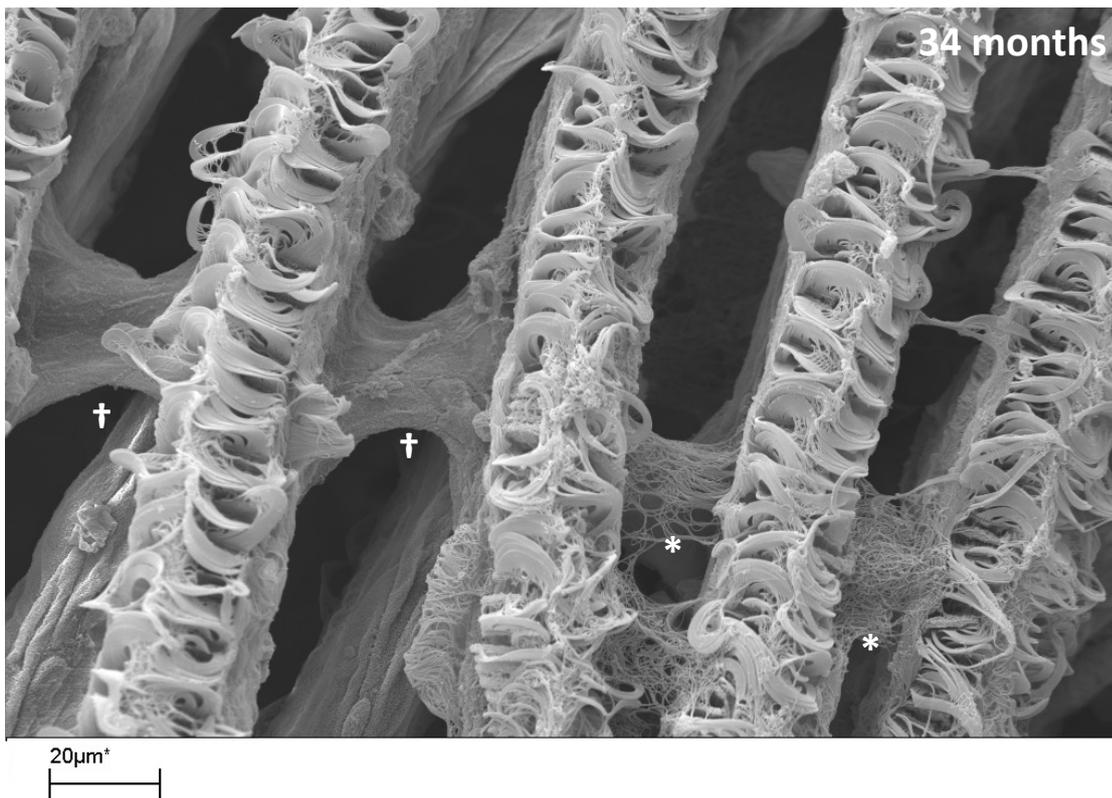


Fig. 5.16: Scanning electron micrograph of ciliary and tissue interfilamentary junctions between filaments. No interfilamentary junctions were present between the first 1 - 11 filaments. Ciliary interfilamentary junctions (*) were present between approximately filaments 11 - 14 after which tissue interfilamentary junctions were present (+).

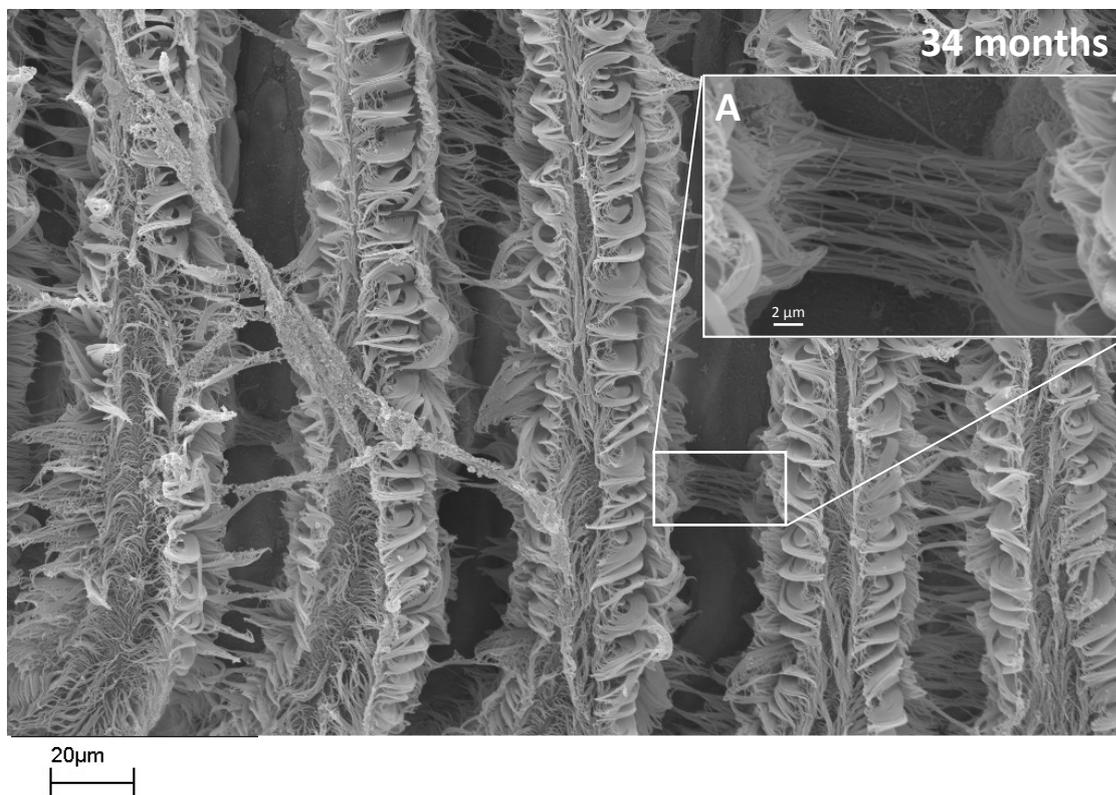


Fig. 5.17: Scanning electron micrograph showing ciliary interfilamentary junctions present at the most ventral (distal) region of lamellae. Tissue interfilamentary junctions were present between filaments in dorsal and medial regions.

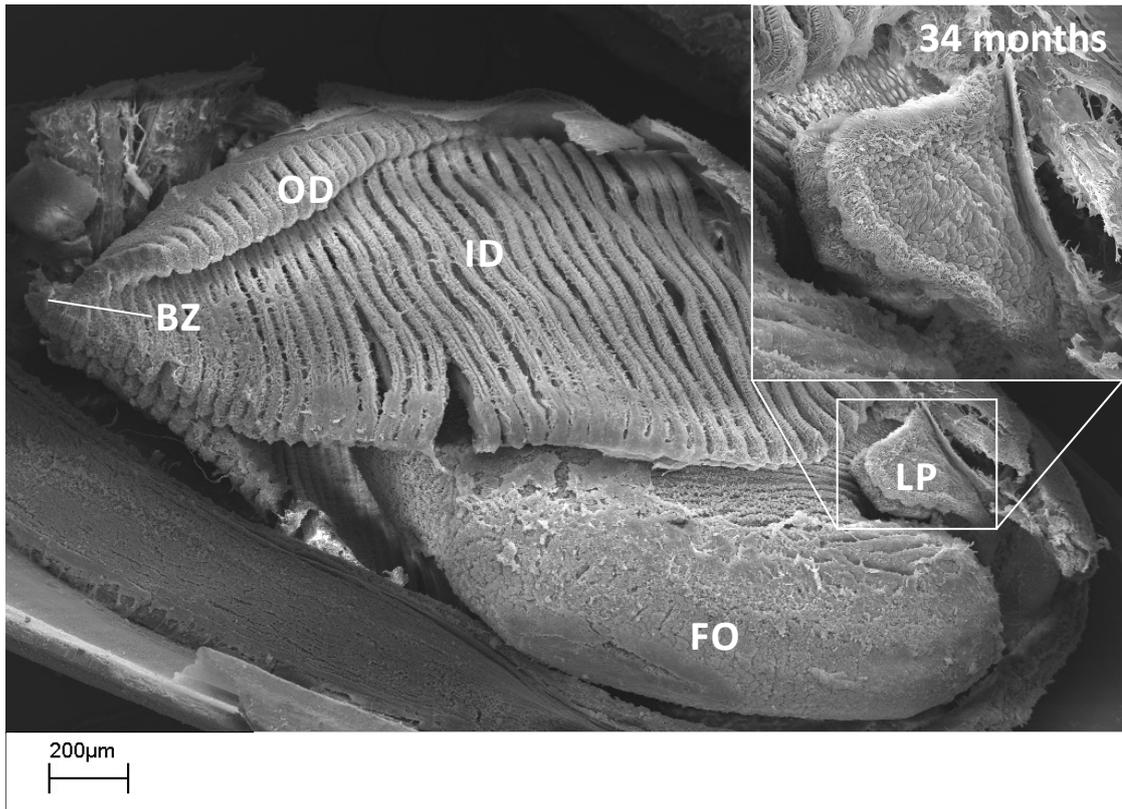


Fig. 5.18: Scanning electron micrograph showing the right inner demibranch (ID), outer demibranch (OD) and labial palps (LP). Outer demibranch development occurs via cavitation extension with several filaments developing at once leading to filaments uniform in length. FO = foot; BZ = budding zone. Inset: Labial palps are highly ciliated on the inner surface but devoid of cilia externally.

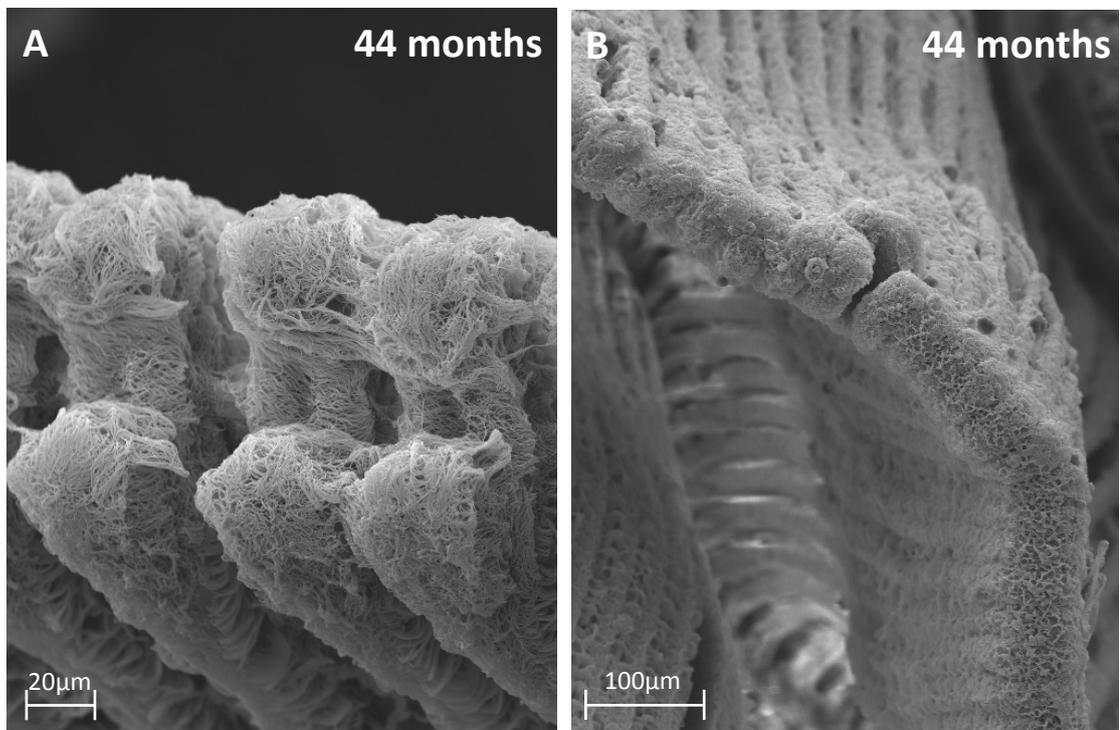


Fig. 5.19: Scanning electron micrograph showing the oral groove (A) developed on the inner demibranch some time between 20 - 34 months old but was not observed on the outer demibranch (B).

5.3.11 44 months old

The outer demibranch was present in the three largest out of the four individuals observed. These data suggest that the outer demibranch begins to form in individuals which are > 3.1 mm long. The budding zone was not attached to the mantle and was again highly ciliated with only simple cilia on developing gill buds. In all individuals, new buds on both the inner and outer demibranchs were already reflected (as in those at 34 months) and a ciliary connection joined the different parts of the budding zone which gave rise to the left and right budding zones (Fig. 5.20). Laterofrontal cirri on newly-budded filaments consisted of fewer cilia per cirral plate (i.e. were not as wide) as laterofrontal cirri on older filaments. The laterofrontal cirri on more developed filaments were capable of reaching over half way across the interfilamentary space making them an effective sieve for food particles.

Adjacent filaments at the anterior end of the inner demibranch were joined at the ventral bend but it was difficult to see if these connections were tissue or ciliary. If they were tissue as has been observed in younger individuals, cilia may still play an important role in ensuring these connections are strong (Fig. 5.21). The inner demibranch of the largest individual had six rows of tissue interfilamentary junctions in a dorsal-ventral direction; no ciliary interfilamentary junctions were observed. Tissue interfilamentary junctions were first observed between filaments 9 and 10 with no interfilamentary junctions before this. As filaments elongated, additional interfilamentary junctions were added in a dorsal-ventral direction.

The oral groove was observed on the inner demibranch in all specimens but never on the outer demibranch. The ventral bend on the outer demibranch was flattened and was covered in simple cilia, but no invagination into an oral groove was observed in any specimen. Invagination on inner demibranch filaments for the oral groove was observed after approximately 2 - 8 true filaments.

The labial palps were more developed than previously observed and had a 'corrugated' morphology (Fig. 5.22). They were heavily ciliated on internal surfaces with both simple cilia and more complex cirri present. These structures are likely to play different roles in particle sorting and processing. The outer (mantle-facing) surface of the lips was ciliated in a similar manner to the rest of the mantle (see below).

The mantle surface was covered in rows of simple cilia (Fig. 5.23) with ciliation becoming more dense close to the inhalant siphon. The inhalant siphon tissue was plicated (Fig. 5.24)

and ciliation extended to the shell-facing side of the mantle. Detailed observation of ciliation around the mantle margin was not possible in these individuals due to the unattached portion of the mantle folding down.

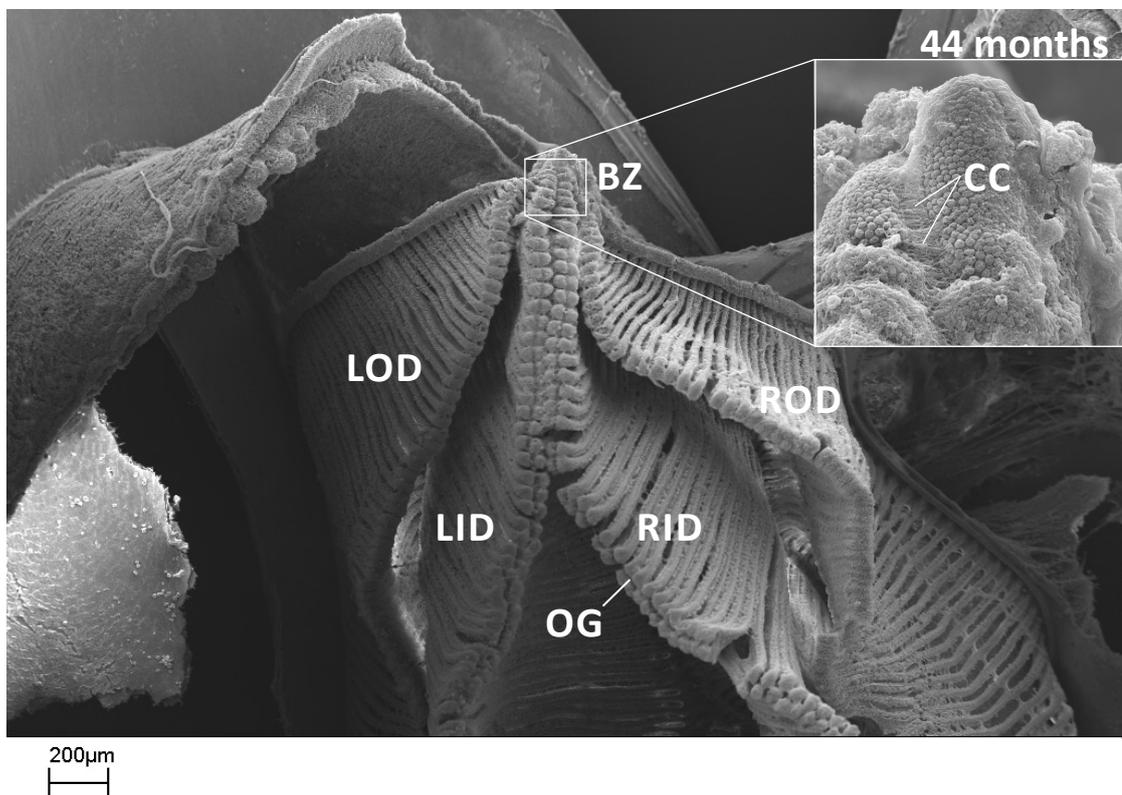


Fig. 5.20: Scanning electron micrograph of the budding zone (BZ) and left inner (LID), left outer (LOD), right inner (RID) and right outer demibranchs (ROD) of 44 month old specimen. Inset box shows ciliary connection (CC) between left and right BZ. OG = oral groove.

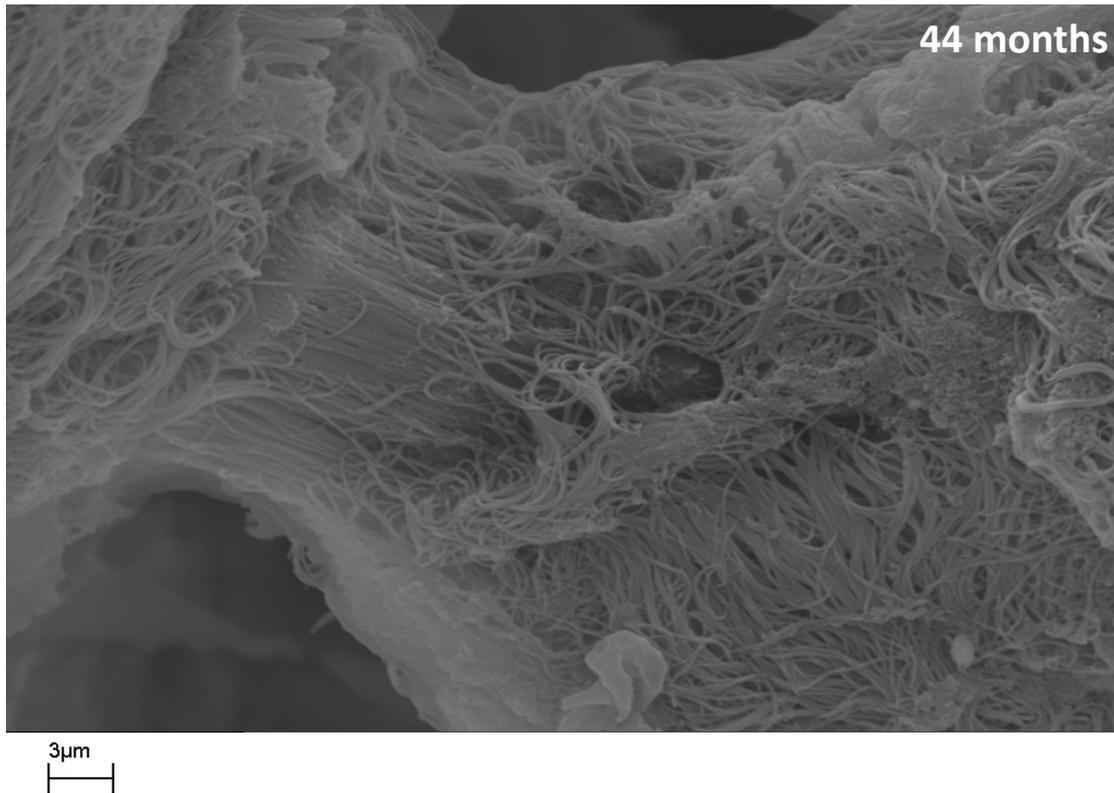


Fig. 5.21: Scanning electron micrograph of the ventral bend connection in 44 month old individual. It is difficult to tell from micrographs if these are ciliary or tissue connections.

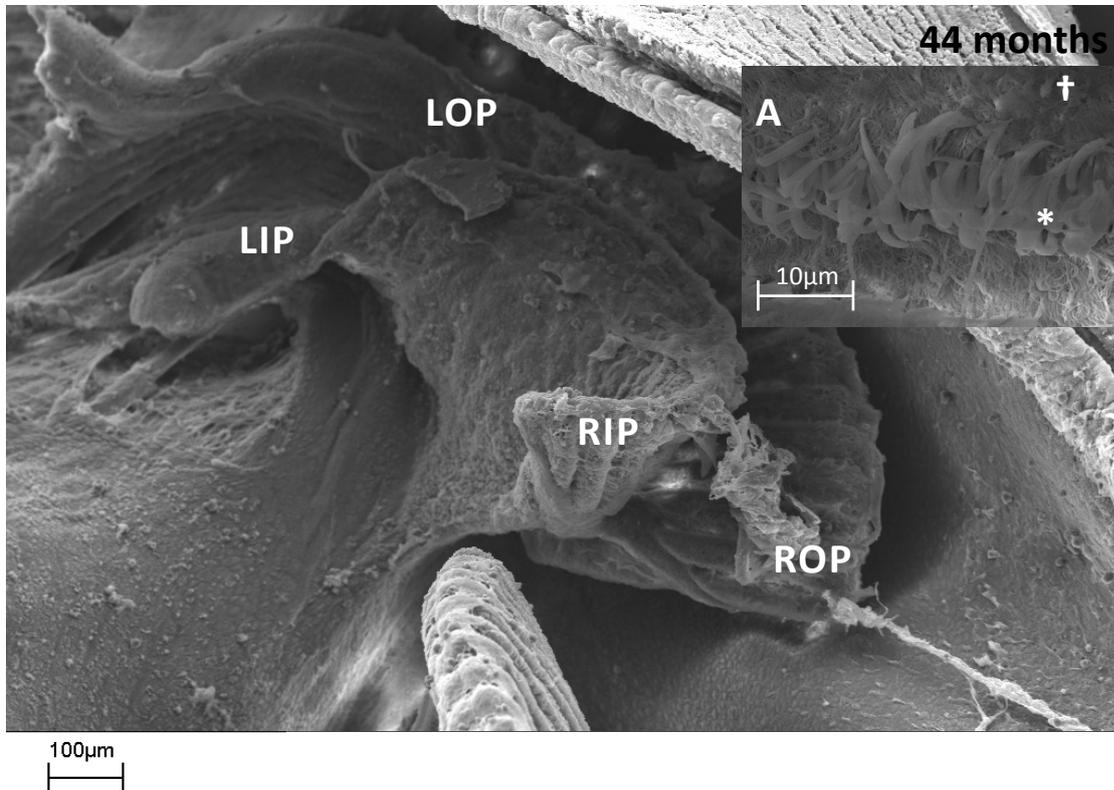


Fig. 5.22: Scanning-electron micrograph of the right inner (RIP), right outer (ROP) and left inner (LIP) and left outer (LOP) labial palps which are plicated internally but still retain their flattened appearance on the outer surface. Inset (A): Internally labial palps are heavily ciliated with simple cilia (+) and more complex cirri (*).

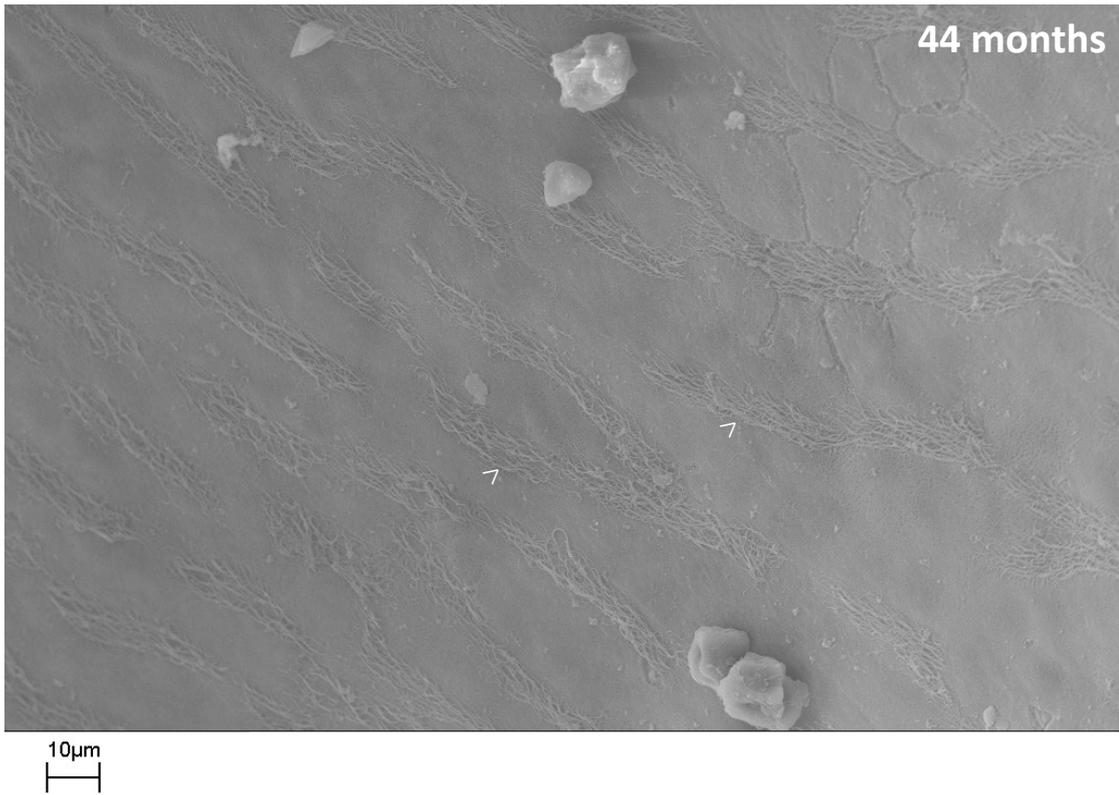


Fig. 5.23: Scanning electron micrograph of the mantle surface covered in rows of cilia (arrow heads).



Fig. 5.24: Scanning electron micrograph of plication of the inhalent siphon (IS).

5.3.12 Adult (> 50 years)

Adult gills were observed to have the same overall structure as the oldest juveniles in this study with some notable exceptions. Firstly, adults possessed a thin layer of tissue attached to the abfrontal surface of filaments which bore ostia. Ostia were arranged in approximate transverse rows (Fig. 5.25). Ostia were oval-shaped and were not uniform in size. A sample ($n = 70$) was measured and averaged (mean) $110 \mu\text{m} (\pm 47)$ long and $40 \mu\text{m} (\pm 16)$ wide. The abfrontal surface of this tissue was sparsely ciliated with some arrangement into tracts (Fig. 5.26). There did not appear to be any pattern to these tracts; some ostia had ciliary clumps or tracts around them while others did not. Sparse cilia were also still present on the abfrontal surface of filaments (Fig. 5.27). The filaments in adult gills appeared closer in proximity to each other compared to juvenile mussels (see section 5.3.14.2 for more information).

The second significant observation was the presence of thick tissue projections (interlamellar junctions) joining the descending and ascending lamellae (Fig. 5.25), projecting out of the tissue layer on the abfrontal surface. The presence of interlamellar junctions was not noted in juvenile mussels, although these specimens were prepared with a focus on keeping them intact so observations of interlamellar junctions would have been difficult if not impossible if they were present.

Finally, the sublateral surface of filaments (the lateral surface between the lateral cilia and the abfrontal surface) was wider in adults compared to juveniles, indicating the same form as Cannuel *et al.* (2009) observed in *Mytilus edulis*.

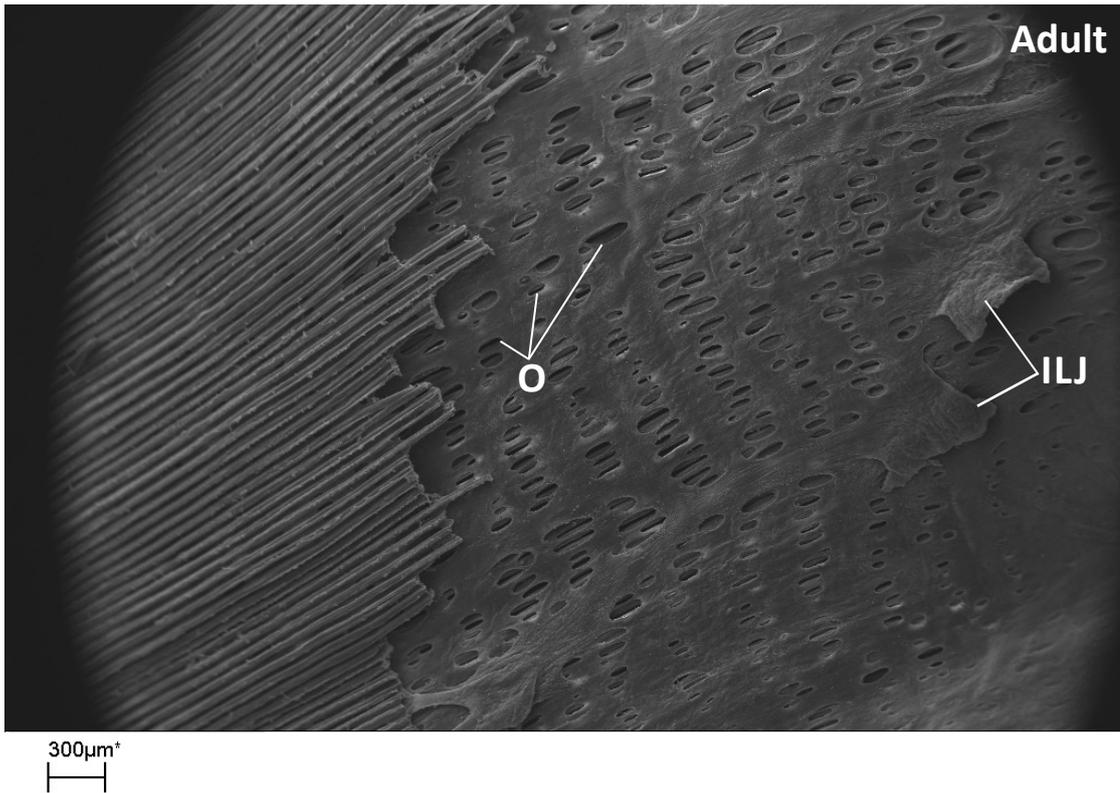


Fig. 5.25: Scanning electron micrograph showing tissue covering the abfrontal surface of filaments. Ostia (O) are variable in size and arranged in transverse rows. Interlamellar junctions (ILJ) were also present.

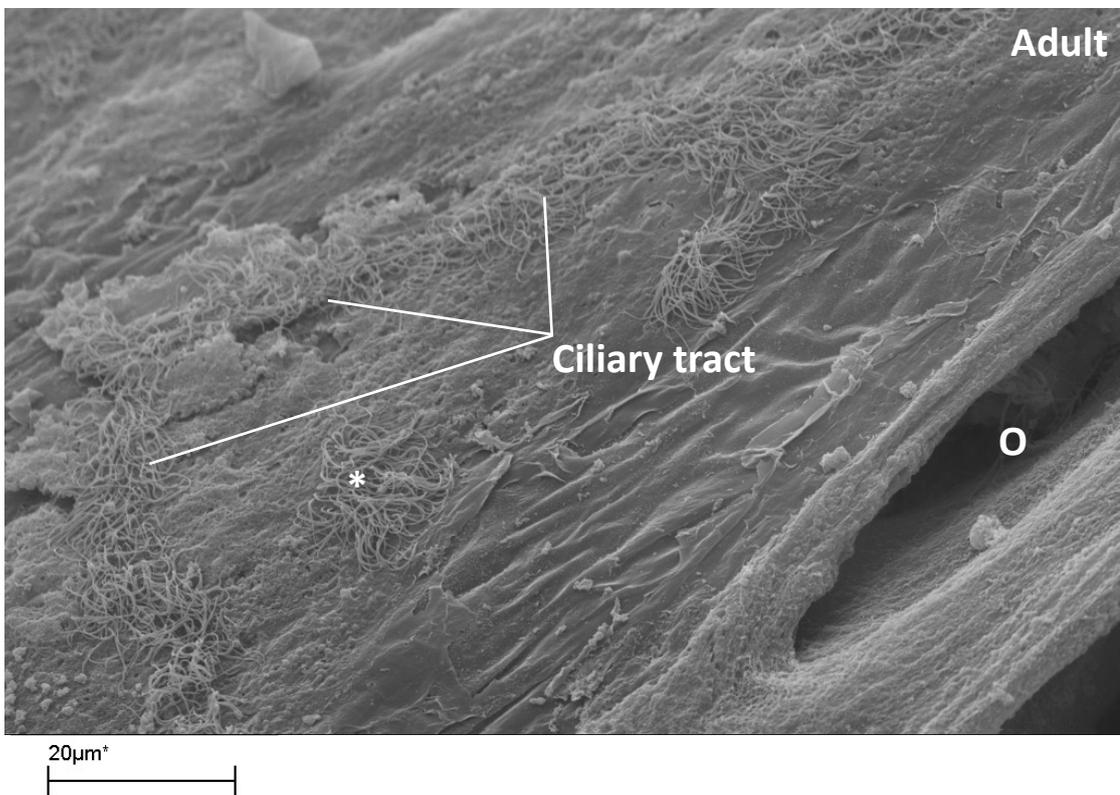


Fig. 5.26: Scanning electron micrograph showing ciliary tracts and clumps (*) on the abfrontal tissue surface in between the ostia (O) but no pattern of arrangement was obvious.

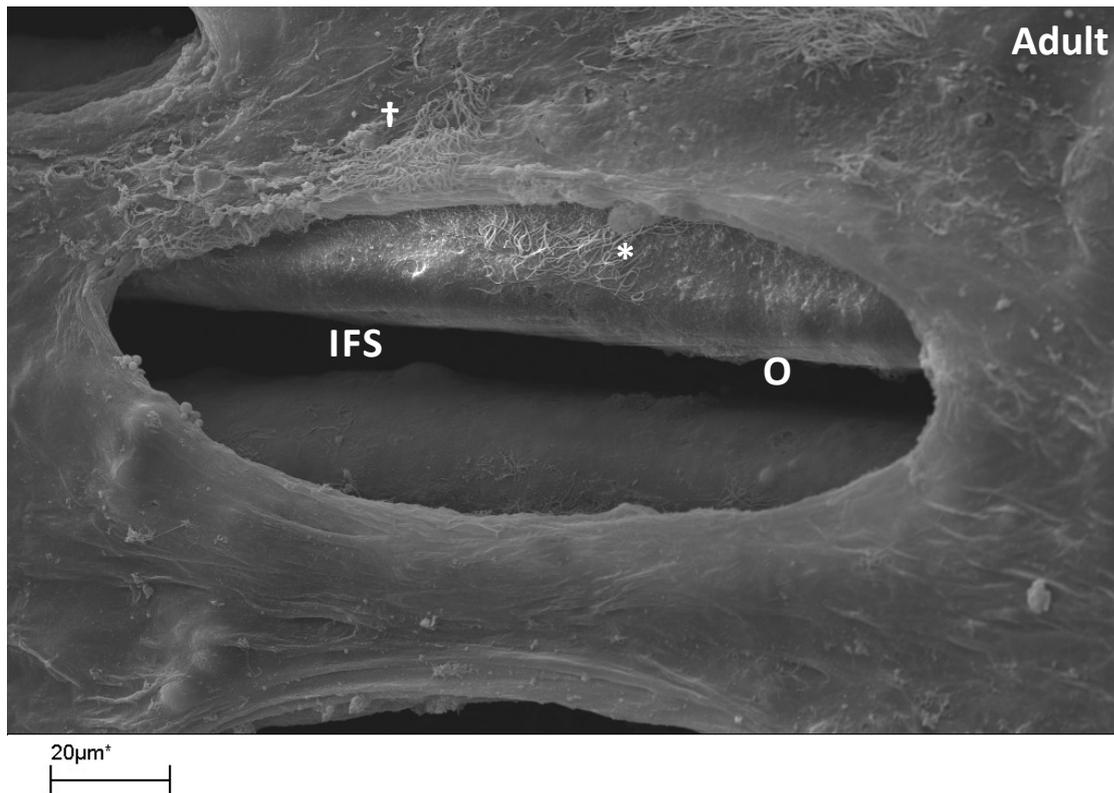


Fig. 5.27: Scanning electron micrograph showing cilia presence on the abfrontal surface of gill filaments (*) as well as on the tissue attached to the abfrontal surface of filaments (†). O = Ostium; IFS = Interfilamentary space.

5.3.13 Other observations

5.3.13.1 Foot (all ages)

Foot form and ciliation was consistent throughout all age classes observed. The foot appears to have two distinct regions (Fig. 5.28). The distal region has a dense covering of simple cilia (Fig. 5.29) whilst the proximal region above the 'heel' is only very sparsely covered in patches of cilia (inset Fig. 5.29).

The largest 44 month old juvenile (8.9 mm), had a fine byssus thread attached to a single grain of substrate when taken for processing. A thin hole was observed along the distal tip of the foot in this individual through which the byssus likely originated (Fig. 5.30). Byssus threads in juveniles have been observed at the Ark project in juveniles from 2.5 years old measuring from 2.8 mm long (FBA, unpublished data).

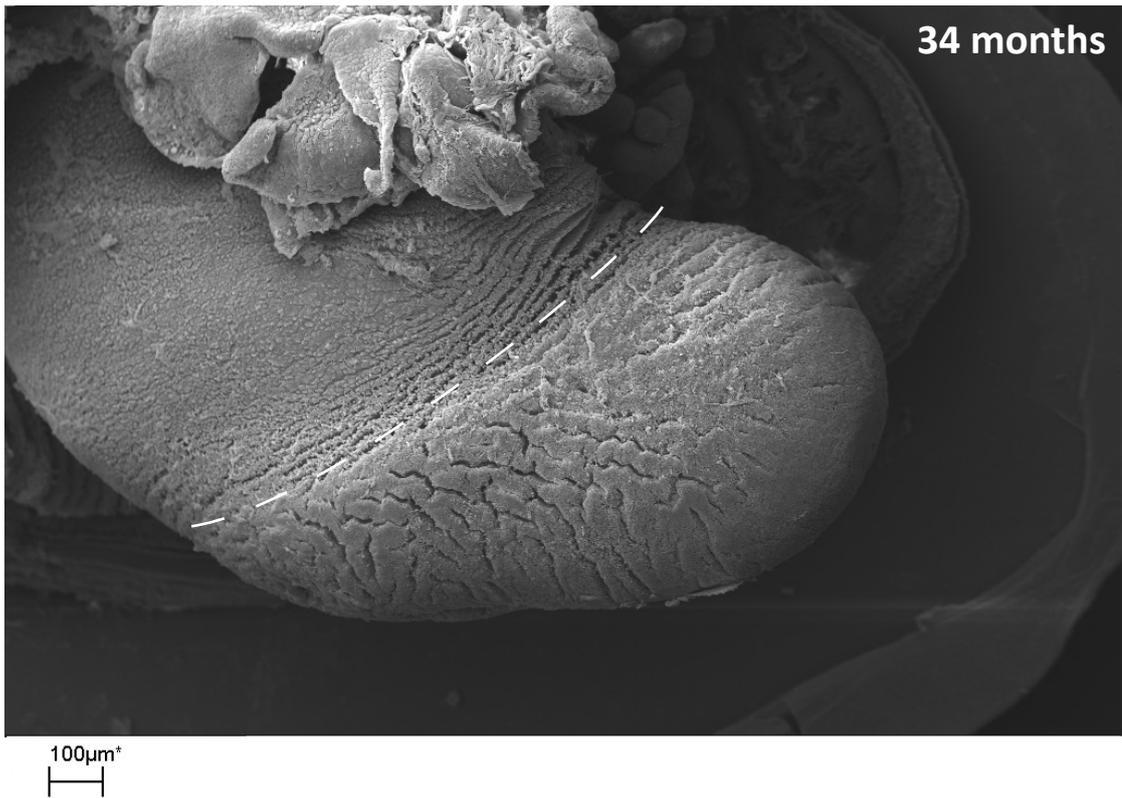


Fig. 5.28: Scanning electron micrograph showing the two distinct regions present on the foot. The most distal part, below and right of the 'heel' (dashed line) is heavily ciliated. The more proximal region, above and left of the 'heel', bears only sparse ciliation.

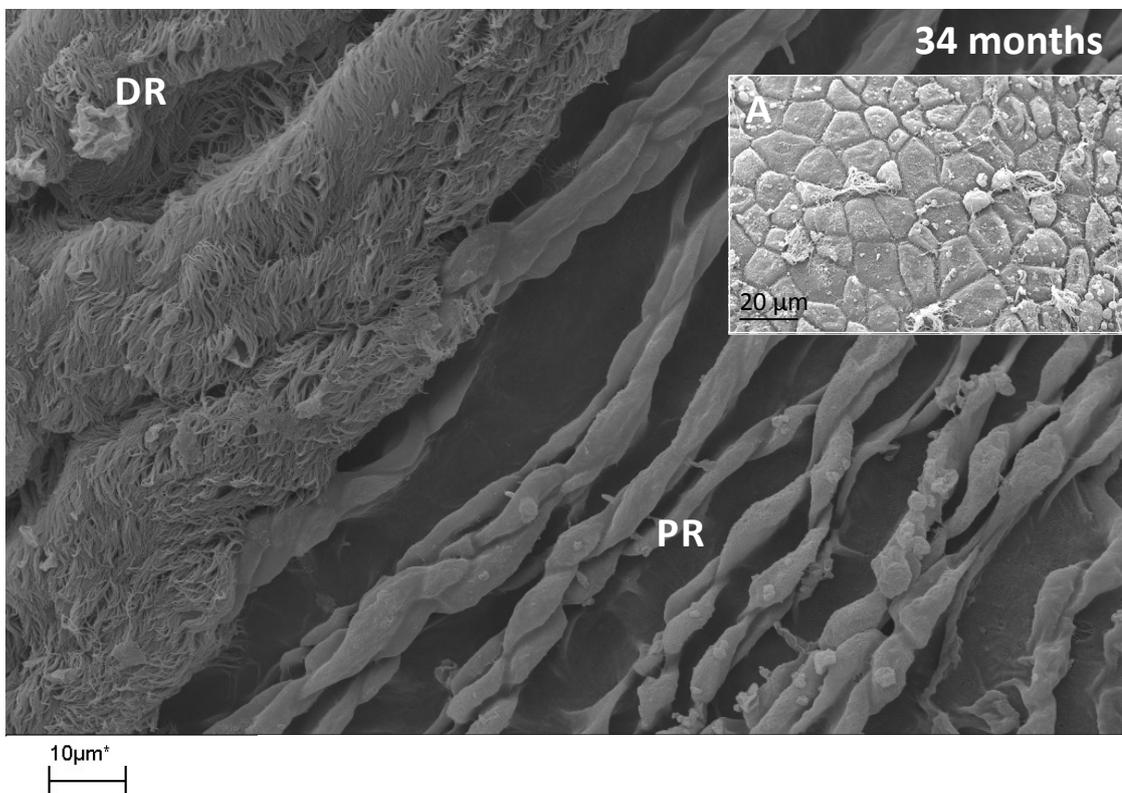


Fig. 5.29: Scanning electron micrograph showing the distal region (DR) and proximal region (PR) of the foot. Inset (A) shows sparse ciliation covering parts of proximal region.

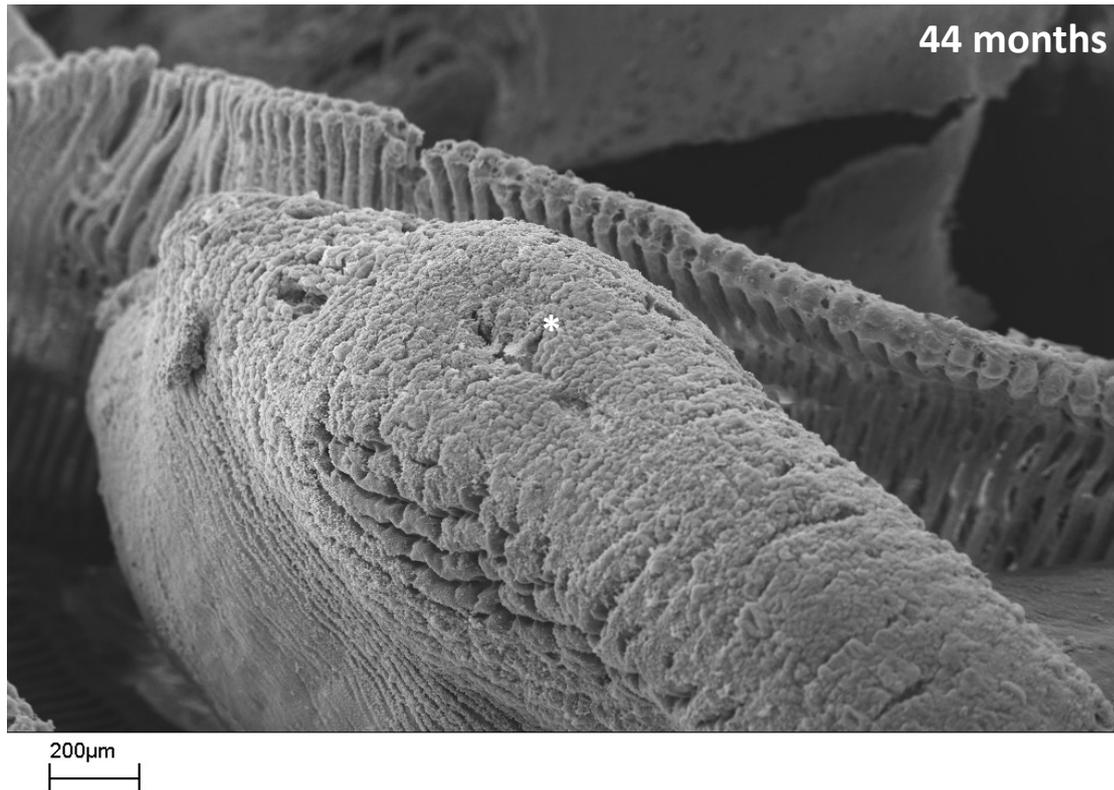


Fig. 5.30: Scanning electron micrograph showing the thin hole (*) along the most distal tip of the foot where the byssus thread likely originated.

5.3.13.2 Shell (all ages)

Whilst investigation of shell structure did not form a major part of this study, some useful observations were made. In one 14 month old individual there appeared to be 2 parts of the shell laying down calcium products (Fig. 5.31). In some 16 and 34 month old individuals balls of calcium carbonate were seen on the shell-facing part of the mantle which may be for sequestration into the nacreous layer (Fig. 5.32).

5.3.13.3 Style

During preparation of 4 month old juveniles, the rotating style was observed through the transparent cells in one individual (0.95 mm) - https://www.youtube.com/watch?v=Pvr_s89NCh4&list=PLBCF8F795289C1093&index=12. The style helps to break down food particles through mechanical means (rotation of the crystalline rod) and by enzymes present in the style sac.

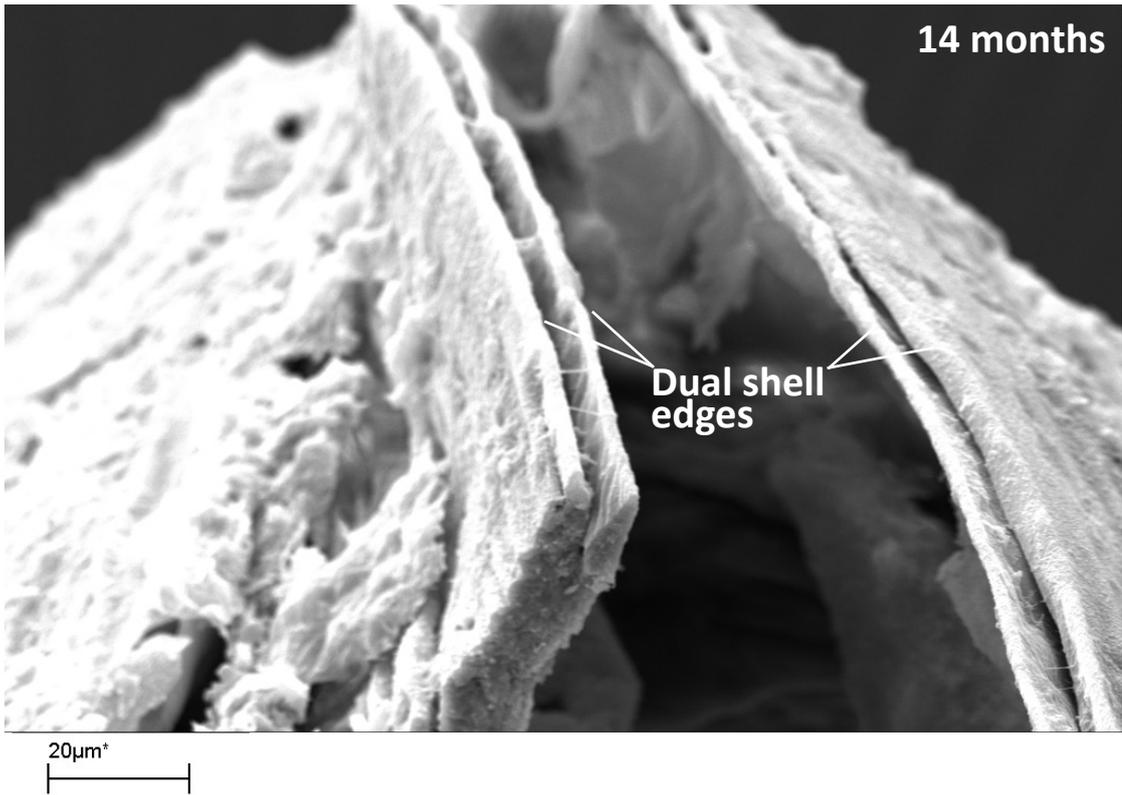


Fig. 5.31: Scanning electron micrograph showing the two shell 'edges' observed which appeared to be laying down material during new shell growth.

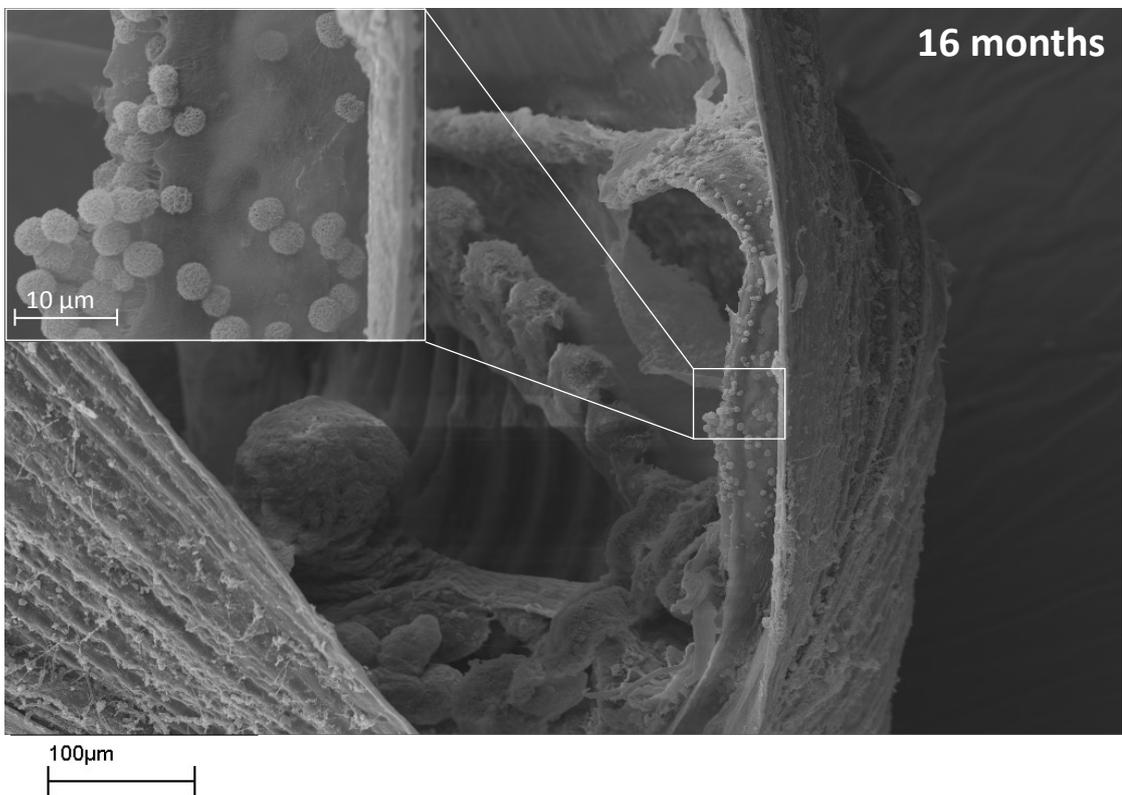


Fig. 5.32: Scanning electron micrograph showing aragonite balls on the shell-facing part of the mantle, most likely for sequestration into the nacreous layer.

5.3.14 Gill morphology

5.3.14.1 Number of filaments as a function of age/size

The number of filaments on the inner demibranch ($n = 47$) was counted and plotted against age (months) and shell length (mm). Shell length was correlated with the number of inner demibranch filaments ($F_{(1,45)} = 3520.585$; $P < 0.001$; $R^2 = 0.99$) and this relationship was stronger than the correlation of age with the number of inner demibranch filaments ($R^2 = 0.84$). Length and age are covariates so both showed a strong relationship with the number of inner demibranch filaments (Fig. 5.33). The number of inner demibranch filaments was also able to predict the number of outer demibranch filaments (Fig. 5.34; $F_{(1,4)} = 483.909$, $P < 0.001$, $R^2 = 0.99$).

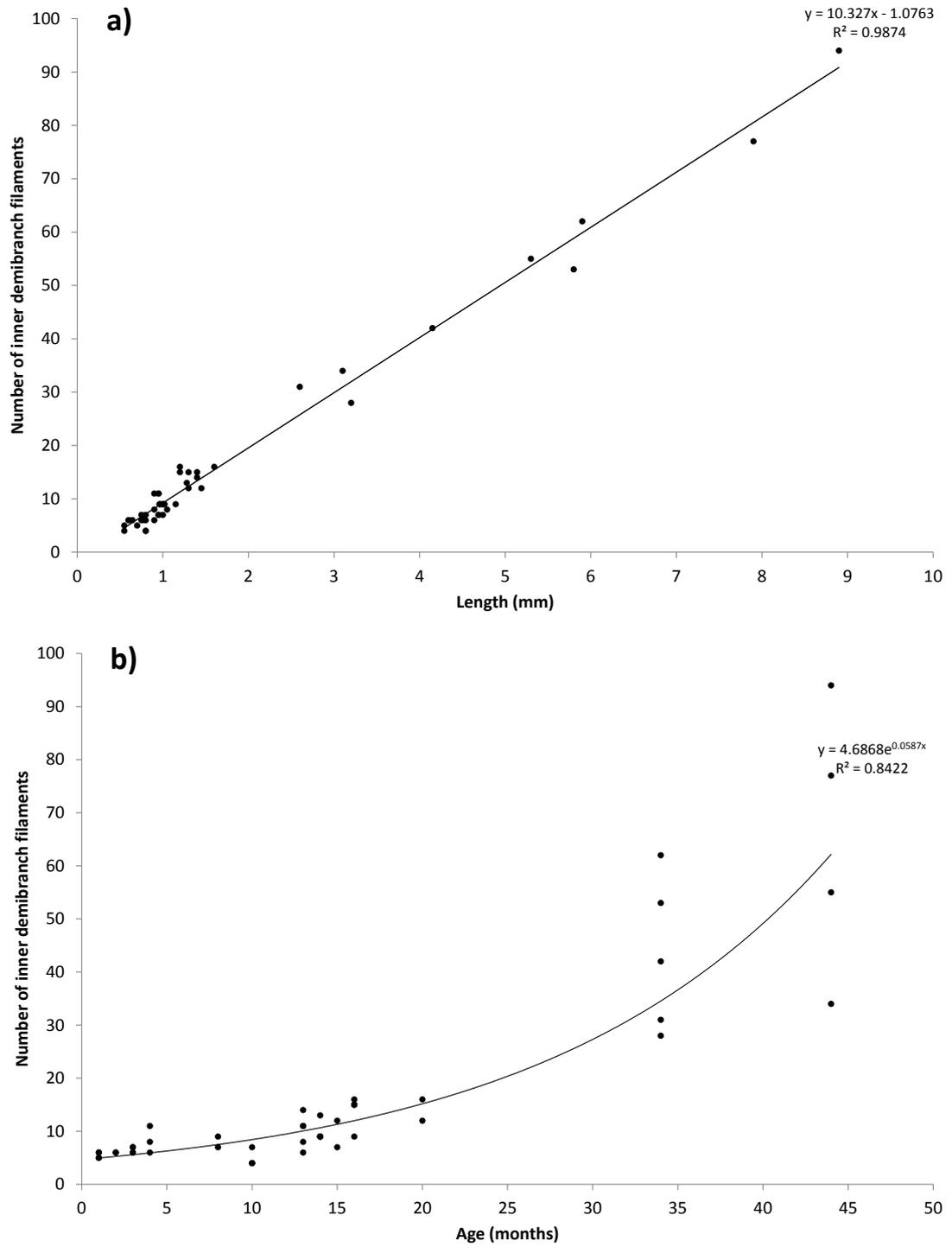


Fig. 5.33: Scatter plots showing the number of inner demibranch filaments against shell length (a) and age (b) in juvenile freshwater pearl mussels. The relationship between shell length and the number of inner demibranch filaments ($R^2 = 0.99$) is stronger than the relationship between age and the number of inner demibranch filaments ($R^2 = 0.84$).

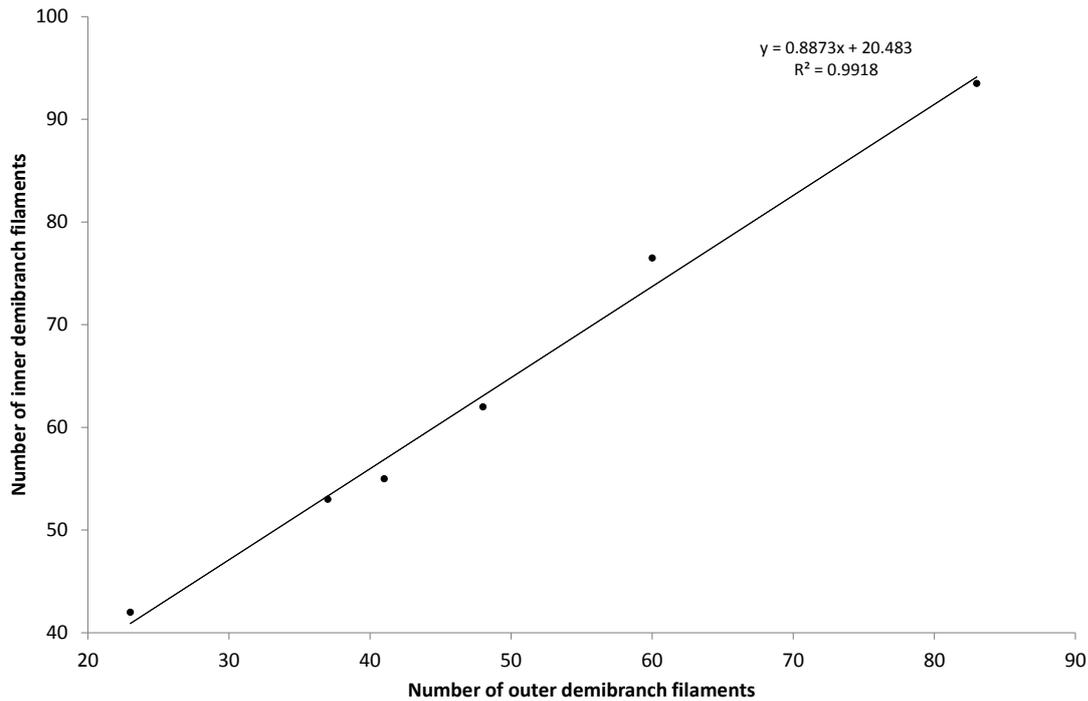


Fig. 5.34: Scatter plot of the number of inner 'vs' outer demibranch filaments in juvenile freshwater pearl mussels. The number of inner demibranch filaments is correlated with the number of outer demibranch filaments and accounted for 99 % of the explained variability in number of outer demibranch filaments.

5.3.14.2 Interfilamentary space

There was a significant difference between interfilamentary space (Fig. 5.35) at different ages ($F_{(5,80)} = 15.487$, $P < 0.001$) and shell lengths ($F_{(8,70)} = 8.761$, $P < 0.001$). Interfilamentary space was generally wider in younger and smaller individuals although this was not always the case (Table 5.2 & Fig. 5.36). When differences with age are examined, *post hoc* tests found interfilamentary space was significantly narrower in 34 month old juveniles and adults compared with the other age cohorts ($P < 0.05$). The distance between filaments joined by interfilamentary junctions was set by the junction width whereas filaments with no junction could be a variable distance apart, hence the larger SD values in individuals < 16 months old (Table 5.2). The interfilamentary space between filaments joined by ciliary interfilamentary junctions was narrower ($14 \mu\text{m} \pm 6$) compared to filaments joined by tissue interfilamentary junctions ($23 \mu\text{m} \pm 9$) probably because ciliary connections are less rigid (Fig. 5.16). The length and width of ostia are strongly associated with each other (Fig. 5.37; $R^2 = 0.64$).

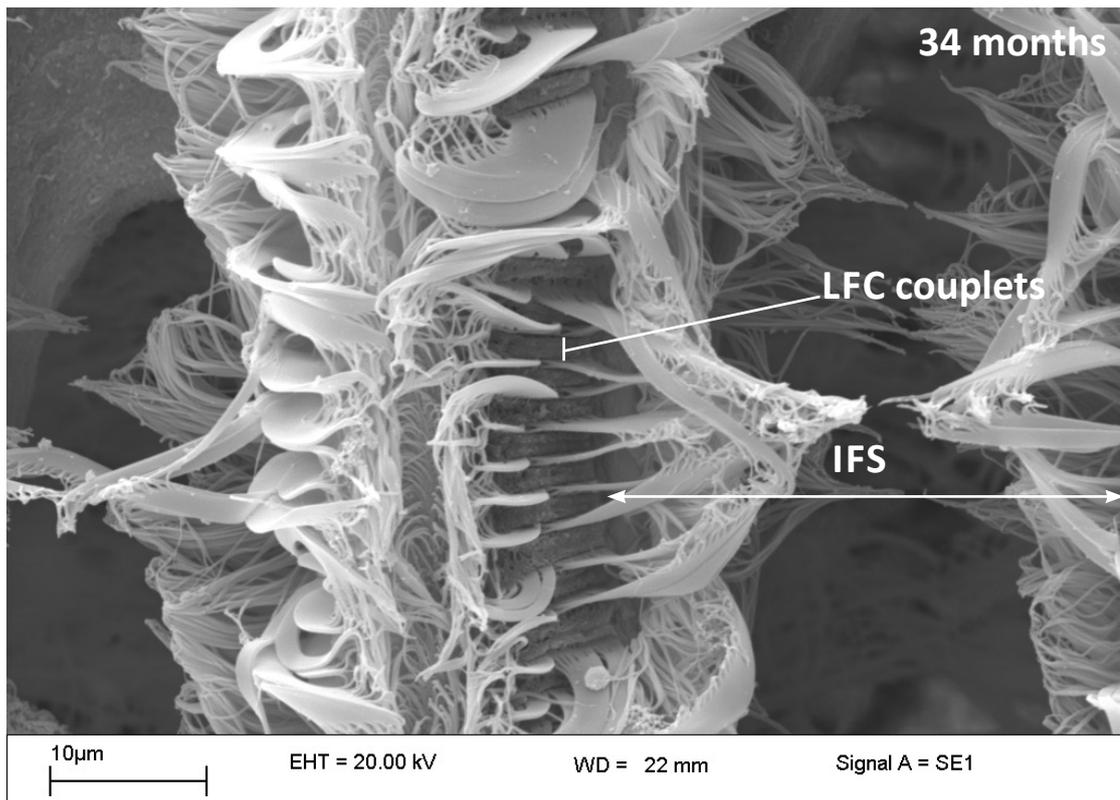


Fig. 5.35: Scanning electron micrograph showing interfilamentary space (IFS) and the space between laterofrontal cirri couplets (LFC) which were measured using ImageJ.

Table 5.2: Mean interfilamentary space of individual freshwater pearl mussel specimens (\pm SD). The number of measurements taken from each individual is also provided (n).

Age (months)	Shell length (mm)	Interfilamentary space (μm)	n
4	0.75	30 (\pm 7)	3
4	0.9	33 (\pm 17)	3
14	0.97	38 (\pm 10)	4
16	1.15	40 (\pm 12)	8
16	1.2	24 (\pm 6)	7
34	3.2	23 (\pm 9)	10
34	5.8	21 (\pm 7)	12
44	7.9	37 (\pm 3)	14
44	8.9	36 (\pm 6)	18
Adult (> 50 years)	99	14 (\pm 3)	7

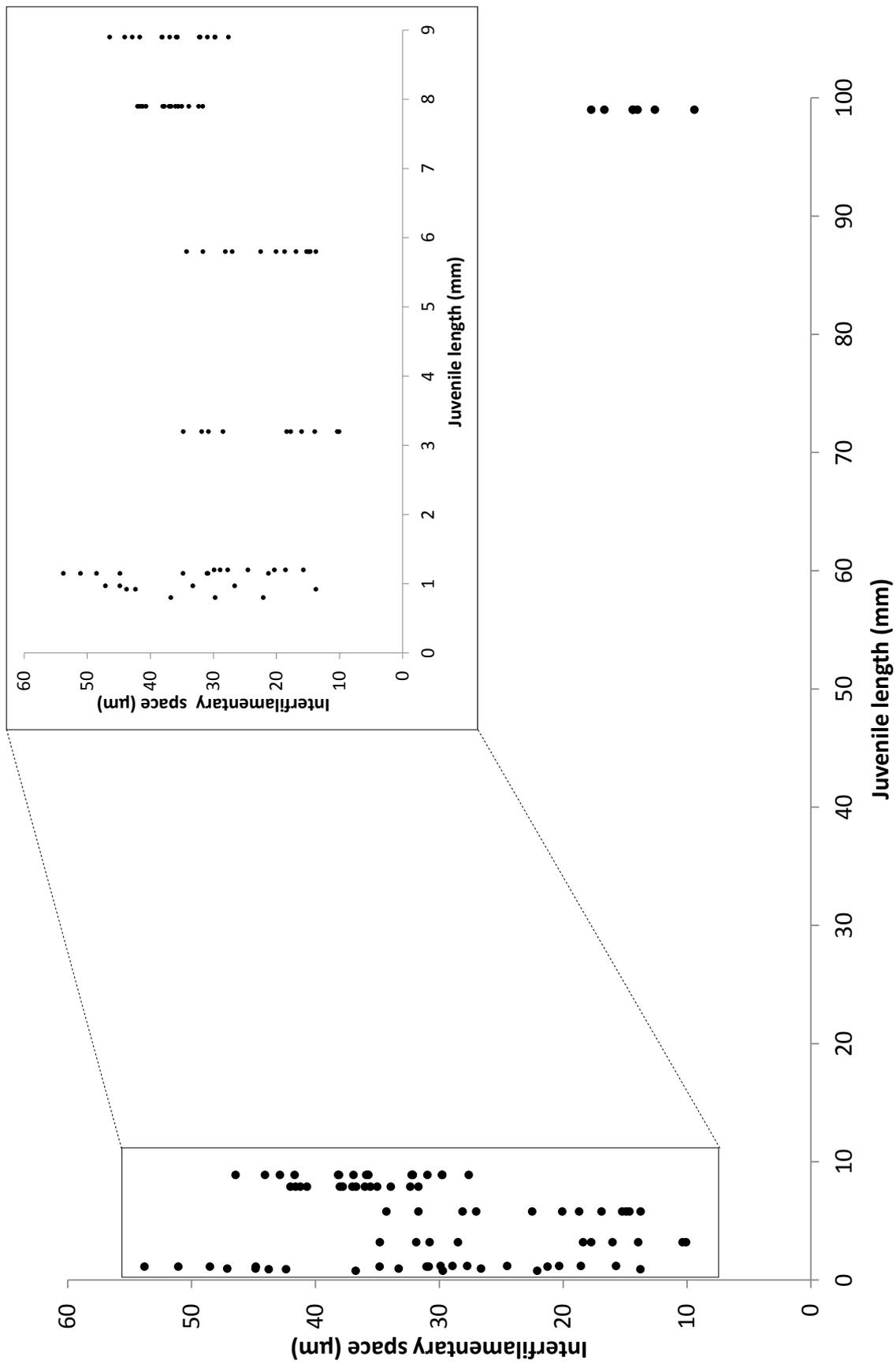


Fig. 5.36: Scatter plot showing interfilamentary space against shell length of juvenile freshwater pearl mussels. Interfilamentary space is more variable in younger juveniles (inset) and less variable and generally narrower in adults.

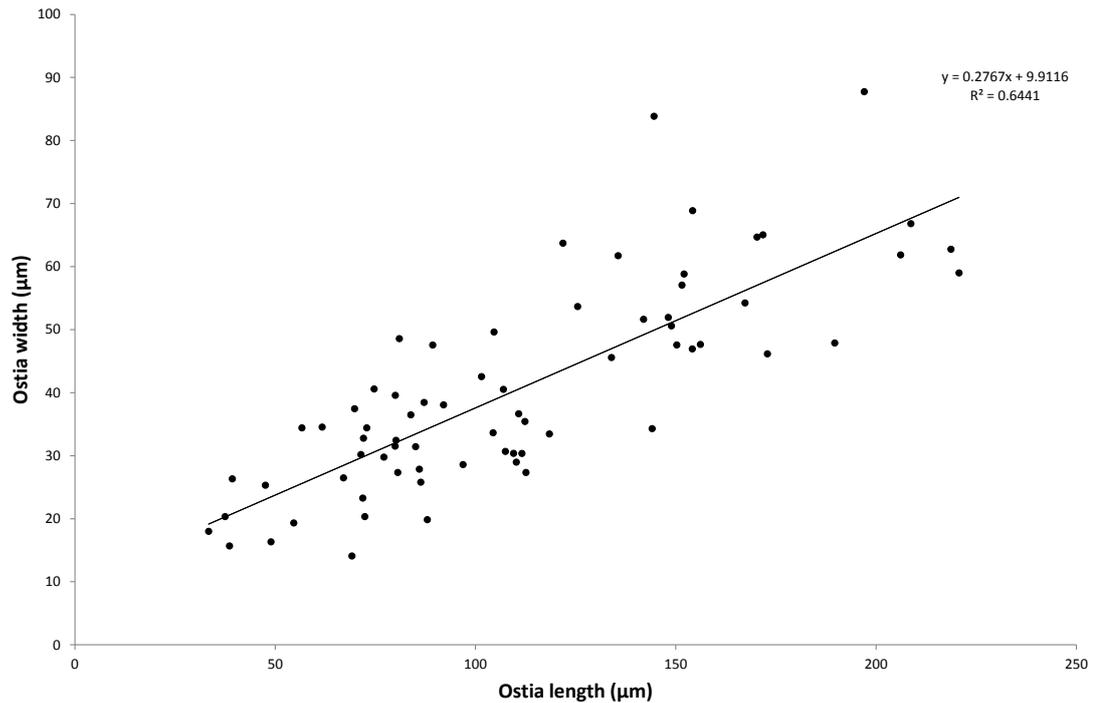


Fig. 5.37: Scatter plot showing ostia length against width. There is a strong association between ostia length and width.

5.3.14.3 Laterofrontal cirri

The number of cilia per laterofrontal cirrus depends upon the stage of cirral development. It has already been noted in 16 month old individuals that there were fewer cilia per laterofrontal cirrus on the developing ascending limb compared with the descending limb. Relatively few laterofrontal cirri were in the correct position for photographing but Table 5.3 shows that on developed laterofrontal cirri (i.e. only those on the descending limb of reflecting filaments), there is little difference in the number of cilia per laterofrontal cirrus between different age classes. Laterofrontal cirri couplets were spaced an average of $1.54 \mu\text{m}$ (± 0.40) apart (Fig. 5.35; $n = 21$ from three individuals at 16 and 34 months old).

Table 5.3: Mean (\pm SD) number of cilia per laterofrontal cirrus (LFC) in 4, 16 and 34 month old individuals. Counts were made when position of the laterofrontal cirrus allowed sufficient scope for counting individual cilia. The number of individuals and the number of laterofrontal cirri considered to obtain the mean number of cilia per LFC is also provided.

Age (months)	Number of individuals	Number of LFC	No. of cilia per LFC
4	5	6	42 (± 2)
16	1	3	43 (± 3)
34	1	2	39 (± 9)

5.3.15 Feeding behaviour

The proportion of time spent active had no relationship with age or length (Fig. 5.38; $R^2 = 0.12$). Thirty four and 44 month old specimens were less active than younger juveniles with the majority of time spent resting on one or other of their valves with siphons protruding and them actively filtering. When movements were made they indicated attempts to bury and were observed to be slower compared to smaller juveniles; no 'foraging' type behaviour was observed in larger juveniles. Younger juveniles made more pedal foot movements, foot sweeps and shell flipping movements associated with 'foraging' activity (Fig. 5.39) but some individuals from around 10 months old did show behaviours which appeared to be attempts to bury. Fewer foraging-type movements were observed in 14 month old juveniles which coincides with the timing of when gill reflection begins to occur. When testing the proportion of time active, 1 and 2 month old juveniles were significantly more active than all other age classes except 8, 10 and 20 month old individuals ($F_{(10,46)} = 5.116, P < 0.001$) although these comparisons do not take into account the type of movements being made (e.g. foraging, burying).

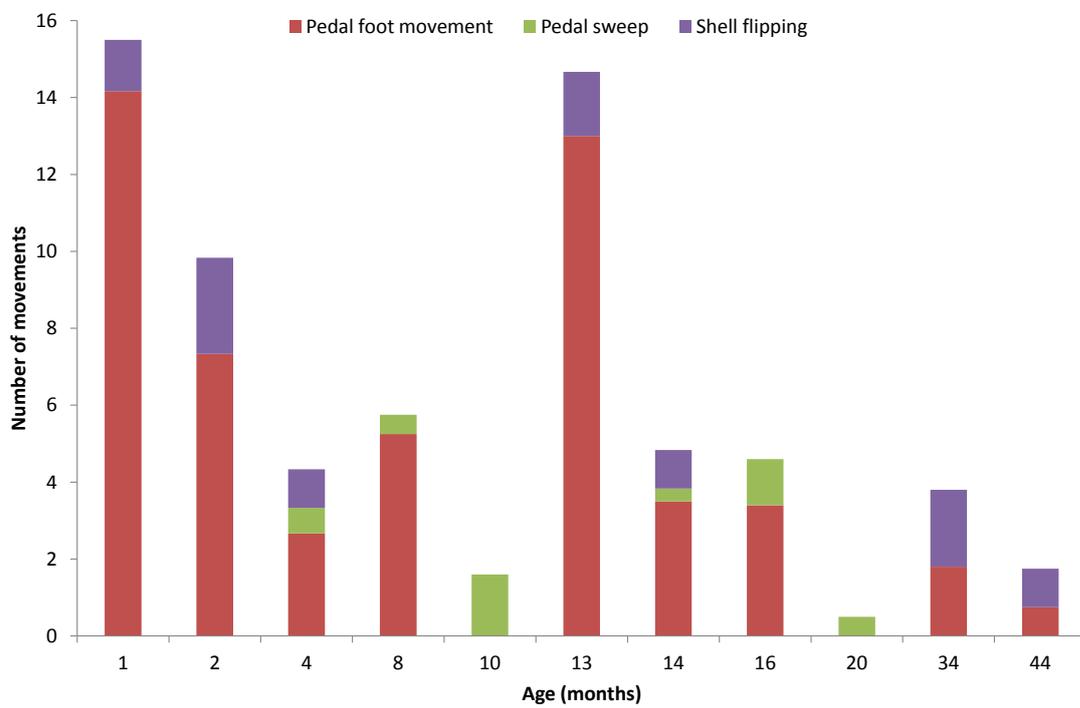


Fig. 5.39: Stacked bar chart showing the mean number of pedal foot movements, pedal sweeps and shell flipping movements of juvenile freshwater pearl mussels arranged by age class.

5.3.16 Findings on procedure and advised methodology

Slightly different methodologies were used to try and streamline the work flow because specimen preparation was carried out in Cumbria and SEM at the University of Derby. These variations and their consequences are discussed in Table 5.4. SEM provides an invaluable tool for studying the ultrastructure of juvenile mussels, but the way in which specimens are prepared can affect the overall quality and positioning of structures as well as the connections between structures. One of the main problems encountered with the method described was that the mussel valves had to be open wide enough to allow sufficient exposure of the soft tissues to reagents. When not opened sufficiently, specimen quality was poor. Opening specimens too widely however may have affected the connections between structures, leading to an under-representation of these in the observed specimens.

Table 5.4: Procedural deviations from the suggested best method for preparing *M. margaritifera* specimens for scanning electron microscopy and their consequences for specimen quality.

Procedure deviation	Consequence
Individuals not teased apart enough after treatment with MS-222 in order to try and preserve any cross connections/structures which may bridge left and right valves.	Exposure to reagents is impeded leading to poor preservation of structures and loss of fine detail. In addition, sputter coating is less effective leading to poor specimen quality.
Delay in sputter coating after the HMDS step.	Specimens had the opportunity to partially rehydrate leading to poor preservation of structures and loss of fine detail.
After fixing, valves and soft tissues were either separated completely or valves were just teased apart to expose internal organs.	Separation of valves completely could lead to damaged and unusable specimens but leaving valves attached could obscure some structures.

5.4. Discussion

The objectives of this investigation were to describe the feeding behaviour and ontogeny of juvenile freshwater pearl mussels (*Margaritifera margaritifera*) in order to inform captive rearing practices at the FBA's Pearl Mussel Ark. This work also intended to provide evidence for the timing of the switch from pedal to filter feeding (transformation) and to put any potential implications of this morphogenesis into context for captive rearing programmes.

The nature of bivalve filter feeding is under regular review and certain topics remain the subject of ongoing discussion, e.g. the role of mucus versus water currents in feeding and particle retention and sorting (Ansell, 1962; Ward *et al.*, 1993; Jørgensen, 1996; Ward *et al.*, 1998; Beninger *et al.*, 2003; Ward & Shumway, 2004). Over the last 30 - 40 years, research in the field has focused on freshwater mussel ecology, habitat preferences and reasons for decline. The early works of only a handful of early researchers such as Lefevre & Curtis (1910; 1912), Ortmann (1911a; 1911c; 1911b) and Atkins (1936; 1937a) sought to describe the basic anatomy of a wide variety of freshwater mussels but relatively little attention has since been paid to this topic, leading to a lack of understanding about how juvenile biology and development may affect survival in captivity or in the wild. In addition, this lack of information and knowledge is holding back advances in systematics of freshwater mussels (Graf & Cummings, 2006), and morphological data are still required to complement genetic information to form robust phylogenetic hypotheses (Bogan & Roe, 2008). It is important to address these knowledge gaps in order to be able to make evidence-based decisions about captive rearing practices and river restoration/catchment enhancement activities for juvenile augmentation.

There is still no single description of what biological features or behaviours an individual must attain before it is considered to be 'filter feeding'. From the anatomical and behavioural observations during this work, the author considers pedal feeding to be movement of particles in to the pedal gape on water currents generated by cilia on the foot, mantle and gills, as described by Kovitvadhi *et al.* (2006). This in itself is a type of filter feeding which is different to siphonal filter feeding, described below. Pedal feeding is typified by high activity levels as individuals move through the substrate to maintain supply of particles. In contrast, juveniles are more sedentary when performing siphonal filter feeding, and can be observed in the more typical position with the most posterior and dorsal areas pointing upwards and the foot being used as an anchor.

It is suggested that, to be classed as filter feeding, individuals should:

- Use cilia on the ctenidia as the primary pump creating water currents.
- Have the ability to efficiently capture particles, i.e. small interfilamentary space (Gui *et al.*, 2016).
- Have a functional oral groove or the ability to transmit particles to the labial palps (Yonge, 1947).
- Have contact between anterior gill filaments and the labial palps (Trump, 2010).

In this investigation 20 month old individuals (1.45 mm long) had not yet developed an oral groove but labial palps were becoming plicated and filaments were beginning to contact the labial palps. By 34 months old (2.66 - 5.9 mm long) the oral groove had developed and juveniles were obviously filtering. It is therefore assumed that individuals began filter feeding between these two time points.

This study is the first of its kind to describe the early ontogeny of juvenile *M. margaritifera* using SEM and has made several important findings which may help explain why the species is so sensitive to sub-optimal habitat conditions. Firstly, due to the slow-growing nature of *M. margaritifera*, the onset of transformation occurs much later than reported in any other bivalve species to date (Table 5.5). Gill reflection was observed to commence at approximately 13 months old when length > 1.2 mm and the number of inner demibranch filaments was > 9. The extended period over which *M. margaritifera* pedal feeds may make them vulnerable to sub-optimal substrate conditions because pedal feeding involves high activity levels compared with filter feeding. In addition, juveniles are smaller for longer making them anatomically unable to exploit larger food particles due to the size of their mouth/oesophagus, thus limiting their dietary range. Secondly, observations of the complex structure of laterofrontal cirri in *M. margaritifera* and measurements of inter-cirral distance and the number of cilia per laterofrontal cirrus supports the previous observation that the species is capable of filtering very small particles (Baker & Levinton, 2003). Previous findings on studies from *M. margaritifera* and other species implicate sub-optimal substrate conditions to be the main cause of juvenile mortality (Aldridge *et al.*, 1987; Geist & Auerswald, 2007; Österling *et al.*, 2008; Lavictoire *et al.*, 2016) and findings from the current study offer an explanation as to why this is so. The ability to filter very small particles makes juvenile *M. margaritifera* particularly sensitive

Table 5.5: Age, size and number of inner demibranch filaments present at time of gill reflection in previous studies on freshwater and marine bivalves.

Species	Age at onset of inner demibranch reflection	Size at onset of inner demibranch reflection (mm)	Number of true inner demibranch filaments	Reference
Freshwater pearl mussel (<i>Margaritifera margaritifera</i>)	13 - 16 months	~1.2	> 9	This study
Atlantic deep sea scallop (<i>Placopecten magallanicus</i>)	-	0.95 - 1.1	> 9	Veniot <i>et al.</i> (2003)
Paper pondshell (<i>Utterbackia imbecillis</i>)	113 days	1.3	~15	Trump (2010)
<i>Anodonta</i> sp.	-	-	~18	Kaestner (1967)
Great scallop (<i>Pecten maximus</i>)	~42 days	0.9 - 1.0	~20	Beninger <i>et al.</i> (1994)
Pacific oyster (<i>Crassostrea gigas</i>)	Cavitation extension from outset	0.33	1	Cannuel & Beninger (2006)
<i>Hyriopsis (Limnoscapha) myersiana</i>	30 days	-	10	Kovitvadhi <i>et al.</i> (2007)
*Blue mussel (<i>Mytilus edulis</i>)	-	< 1	15 - 16	Cannuel <i>et al.</i> (2009)

* Filaments already reflected at this stage so the size and no. of filaments at reflection is an over-estimate.

to the presence of unsuitable particles, including organic particles too large to consume, or inorganic particles with no nutritive value. Sub-optimal habitat conditions are likely to cause juveniles to close more frequently or for longer periods to avoid taking in unsuitable particles which may clog the gills (Ellis, 1936; Aldridge *et al.*, 1987; Jørgensen, 1990). Taking in particles during these unsuitable periods has an energetic cost via the over-production of mucus and pseudofaeces. Thirdly, even the oldest/largest specimens observed during this study do not yet bear the adult condition of having true ostia meaning that their filtering capability may not be as efficient as it would be in fully developed individuals. It is also a useful indicator of when female mussels may be capable of reproduction because without this tissue, glochidia cannot be brooded within the gills. The information presented in this chapter is vital to captive rearing programmes and helps inform rearing and reintroduction practices in order to improve juvenile survival.

5.4.1 Ontogenic stages

Mussels at different ages have displayed overlapping ranges of shell length and the number of inner demibranch filaments (Table 5.1) indicating that shell length is a better predictor of development compared to age. This is confirmed by regressions of the number of inner

demibranch filaments against both shell length and age (Fig. 5.33). Different numbers of developmental stages have been identified for different mussel species (Beninger *et al.*, 1994; Veniot *et al.*, 2003; Cannuel & Beninger, 2006) and Schartum *et al.* (2016) recently suggested 3 stages for *M. margaritifera*; the 'I', 'V' and 'W' stages based upon single, unreflected filaments (I stage), reflected inner demibranch filaments (V stage) and fully developed, tetragenous ctenidia (W stage). These stages were based upon data from individuals between 1 - 29 months old (1 - 3 mm long). Here, a wider age range was considered and four stages of gill development are proposed for *M. margaritifera* based upon current data (Table 5.6).

Stage 1 lasts for approximately the first 13 months post-excystment or when juveniles measure < 1.2 mm. Juveniles display approximately the same morphological features with new inner demibranch filaments being added with increasing length. Tissue connections form

Table 5.6: Description of the four stages of gill development in juvenile freshwater pearl mussels in the age classes considered as part of this study. The age (months) at which individuals begin to display particular structures/developments is approximate and no attempt has been made to postulate when development of certain structures begins if they were not directly observed during this study. The number of inner demibranch (ID) and outer demibranch (OD) filaments are the number observed during this study and may differ depending upon population or other parameters.

Stage	Age (months)	Length (mm)	Description	No. of ID filaments	No. of OD filaments
1	0 - 14	0.40 - 1.17	Proliferation of unreflected filaments with the gradual formation of connections between adjacent filaments at the ventral bend in individuals ~4 months old (~0.75 mm). Labial palp primordia simple, flat and unfolded but heavily ciliated. No oral groove on inner demibranch.	5 - 9	0
2	13 - 20	1.17 - 1.44	Filaments commence reflection starting with the medial filaments when ind. ~1.2 mm in length. Ascending limb joined at fused dorsal bend which has covering of simple cilia. Labial palps becoming larger and starting to take on folded morphology. No oral groove on inner demibranch. No outer demibranch development.	9 - 17	0
3	34 - 44	2.66 - 8.90	Reflected filaments on inner demibranch with new filaments developing via cavitation extension. Budding zone obvious giving rise to 3 - 5 buds before true filaments develop. Oral groove develops after 2 - 13 true filaments on the inner demibranch. Outer demibranch proliferation via cavitation extension in individuals ~3 mm long. First sighting of ciliary and tissue interfilamentary junctions on inner demibranch.	28 - 94	0 - 83
4	Older juvenile - adult	??	Tissue with semi-circular ostia forms on abfrontal surface of demibranchs.	??	??

between adjacent filaments at the most distal extremity after approximately 4 months when length > 0.75 mm. At this stage the labial palps are simple, flattened flaps which are heavily ciliated, as is the inner surface of the lips and the area around the mouth.

M. margaritifera appears to begin the transformation stage (Stage 2) between 13 - 16 months old when measuring ~1.2 mm in length and when the number of inner demibranch filaments was > 9. To the author's knowledge this is the most delayed example of gill reflection in a freshwater bivalve in terms of age. However, both the size of the individual and developmental stages before reflection are similar to previous reports in other bivalves (Table 5.5). The terminal ends of filaments making up the ascending limb are joined at the fused dorsal bend, which is covered in simple cilia, similar in form to the frontal cilia. Tissue at the ventral bend becomes thicker and more densely covered in cilia in more developed individuals, possibly as a precursor to oral groove development (Trump, 2010). Labial palps become larger and begin to take on a folded appearance. The outer demibranch is still not present at this stage.

During Stage 3, inner demibranch filaments proliferate already reflected (cavitation extension), and the budding zone is clearly visible for the first time. Outer demibranch filaments also bud via cavitation extension in individuals > 3.1 mm. The oral groove is present on inner demibranch filaments only after approximately the 2 – 13th true filament, with invagination of the oral groove increasing in more anterior filaments. Interfilamentary junctions on the inner demibranch are observed for the first time, beginning as ciliary junctions but quickly giving way to tissue junctions. At this stage no ostia are present within the gill pores and so it is suggested that this development occurs in Stage 4. Tissue connected to the abfrontal surface with ostia was present in a single adult specimen but further investigation is required to clarify when the onset of this condition occurs. Once Stage 4 is reached, juveniles can be considered to have fully developed gills capable of brooding, in addition to their respiratory and feeding roles.

5.4.2 Gill development

One of the reasons for considering development in very young juveniles was to try to better understand the early mortality event within the first few weeks post-excystment described by several authors e.g. Ó Foighil *et al.*, (1990), Lasee (1991), Gatenby *et al.* (1997), O'Beirn *et al.* (1998), Jones *et al.* (2005) & Lavictoire *et al.* (2016). There is no significant change in gill form

or function during the first four months post-excystment so it is unlikely that gill development causes this early mortality event in *M. margaritifera*. However, mortality could be caused by the inability of mussels to meet energetic demands during intense morphogenesis of other organs during this period (Lasee, 1991; Veniot *et al.*, 2003). This area warrants further investigation.

Gill development follows patterns previously outlined by a number of researchers (see Table 5.5 for examples). In the youngest individuals there was no evidence of right and left inner demibranch filaments interdigitating to form a gill basket as described in previous studies (Beninger *et al.*, 1994; Veniot *et al.*, 2003), but this may be due to the way samples were prepared in this study which may have disrupted this feature, if present. Wide interfilamentary spaces, no connections between adjacent filaments, absence of the oral groove and the small size and under-developed nature of labial palp primordia makes it unlikely that particle capture and transport could be carried out by any but the most anterior gill filaments at this stage. The mode of particle collection and transport to the labial palps for sorting and ingestion during the first few months post-excystment could not be established in this study.

Whilst there were significant differences between interfilamentary distance and both shell length and age, there was no distinct pattern although 34 month old and adult filaments were significantly closer together compared with all other age classes. This pattern has been observed before (Gui *et al.*, 2016) but the shape of the gills varies depending upon whether active pumping is occurring (Tankersley, 1996) so it is possible that interfilamentary spaces may be variable between individuals and may differ due to the way in which samples are prepared. Once the tissue bearing ostia develops (connected to the abfrontal filament surface in *M. margaritifera*) it is likely that the interfilamentary space will be more fixed.

The most distal points of filaments begin to form junctions at around 4 months old when measuring > 0.75 mm, marking the start of more complex gill development observed during Stage 2 (Table 5.6). Medial filaments are the first to undergo reflection followed by the anterior and posterior filaments. Once reflection has reached the budding zone, proliferation of new filaments is via cavitation extension as reported in previous studies (Ansell, 1962; Cannuel *et al.*, 2009). Outer demibranch filaments proliferate via cavitation extension with no unreflected stage. The R^2 value for the relationship between number of filaments and shell length found in this study is very similar to those reported by Cannuel & Beninger (2006) for *C. gigas* when

specimens had V-shaped ($R^2 = 0.93$) and W-shaped gills ($R^2 = 0.98$), providing evidence that certain traits are homologous between all bivalves.

More observations are required to see how the fused dorsal bend of both the inner and outer demibranchs interact with the mantle surface/visceral mass in older individuals as specimens in this study were not in the correct position to properly observe these features. Cannuel *et al.* (2009) describe cilia on the fused dorsal bend of *M. edulis* (filibranch) matching with cilia on the mantle surface so that the two may interlock, holding the fused dorsal bend against the mantle thus aiding the shunt mechanism. Ansell (1962) also describes ascending filaments connecting with the mantle via ciliary connections in the eulamellibranch *Venus striatula* (= *Chamelea gallina*). Ansell (1962) also described in *V. striatula* that the most anterior filament was attached to the visceral mass along its entire length and only consisted of a descending limb; the same feature was observed in one of the largest individuals of *M. margaritifera* in this study (5.8 mm length). It is not known what function (if any) this filament has but it appears that the overall importance of this feature is minimal as the overall size of the individual increases (Ansell, 1962).

The purpose of the oral groove is to move particles from the frontal tracts of filaments in an anterior direction towards the labial palps and mouth for sorting and ingestion. In *M. margaritifera* the developing oral groove begins as a connection between the distal tips of filaments as early as 4 months old (0.75 mm long), with these tissue junctions becoming thicker as development continues. The oral groove was not present in 20 month old juveniles but was well developed on the inner demibranch of all 34 month old specimens (developing between 1.45 – 2.60 mm long). This is earlier than described by Schartum *et al.* (2016), who observed onset of oral groove development in individuals measuring 3 - 6 mm long. In the current study, specimens were not observed between 20 - 34 months old so the exact mechanism of oral groove development can only be inferred from observations at these two points. It is postulated that the tissue connections thicken bringing adjacent filaments closer together, the distal tips flatten and then invagination begins. The oral groove region was not sampled in the adult specimen but on juvenile *M. margaritifera* the oral groove remains open, unlike the deep, enclosed oral groove of *M. edulis* described by Cannuel *et al.* (2009). In more developed individuals, the oral groove develops after approximately the 2 - 13th filament, similar to the 8 - 10th filament observed in *M. edulis* (Cannuel *et al.*, 2009). An oral groove was never observed

on the outer demibranch in any specimen. Particles captured on the outer demibranch may be conveyed on to the frontal surface of the inner demibranch before continuing ventralwards to the oral groove for onward transport to the labial palps (Atkins, 1937b; Tankersley, 1996). Cannuel *et al.* (2009) is the only reference the author is aware of referring to an oral groove developing on the outer demibranch. Both Cannuel *et al.* (2009) and Tankersley (1996) report that particles are incorporated into a mucus thread at the oral groove but this topic is still being actively discussed in the literature. Ansell (1962) observed anterior movement of particles via ciliary transport before the oral groove was developed in *C. gallina* suggesting that whilst transport is possible, the oral groove may make the process more effective.

In *M. margaritifera* the budding zone is not attached to the mantle and instead projects into the mantle cavity. This condition has also been described for *Anadara*, *Nucula* and *Dreissena* spp. but budding zones of *Unio* spp. and *Mya arenaria* were attached to the mantle (Neumann & Kappes, 2003). Development of the budding zone in *M. margaritifera* requires further investigation because smaller/younger samples in this study were usually not of suitable quality to observe the budding zone properly. Specimens which were 20 months old and > 1.15 mm in length had an early budding zone visible but it was not of the same form as observed in 34 month old individuals. The early budding zone consisted of 1 - 2 gill buds and looked like it may be attached to the mantle. In more developed individuals ciliary connections were observed between the left and right budding zone regions (Fig. 5.20) which were not observed previously in individuals with valves fully separated. This outlines the value of observing individuals prepared in different ways i.e. with valves intact or with one valve removed.

Interfilamentary junctions were first observed in 34 month old specimens (smallest individual 2.66 mm long) but their development likely begins in slightly smaller and younger individuals and is likely borne out of a requirement to stabilise elongating filaments. Initially, a single transverse row of interfilamentary junctions develops with additional transverse rows being added as filaments elongate. Shorter, more recently budded filaments (filaments 0 - 11) had no interfilamentary junctions with ciliary junctions forming between filaments 11 - 14 which then gave way to tissue junctions from approximately the 15th filament. Ciliary junctions also appear to develop before tissue junctions as a new transverse row of interfilamentary junctions is added ventrally. This pattern of ciliary and subsequently tissue junctions confirms

the findings of previous studies on other eulamellibranchs (Cannuel & Beninger, 2006; Cannuel *et al.*, 2009; Trump, 2010). No ciliary junctions were observed in 44 month old specimens and it may be that only tissue connections form after attainment of a certain size. Where several rows of interfilamentary junctions were present they appeared to be spaced approximately evenly along the dorso-ventral axis. Addition of interfilamentary junctions along the dorso-ventral axis suggests that the site of elongation i.e. ventral growth of filaments, may be from the ventral portion of the filament rather than from the gill axis. It is unclear where growth of the ascending lamella originates from although the position of interfilamentary junctions on the ascending lamella roughly matches with the positioning of interfilamentary junctions on the descending lamella along the dorso-ventral axis. Initial growth of the ascending limb upon reflection has been shown to be from the ventral bend region (Ansell, 1962; Neumann & Kappes, 2003; Cannuel *et al.*, 2009) but elongation may be from the terminal end of the ascending limb i.e. near the fused dorsal bend. This area requires further investigation.

The outer demibranch was first observed in 34 month old individuals at around 3.2 mm long and proliferation was via cavitation extension (Ansell, 1962; Cannuel *et al.*, 2009; Schartum *et al.*, 2016). This is later than reported by Schartum *et al.* (2016) who observed development at 4.5 mm in *M. margaritifera* and Neumann & Kappes (2003) observed first development at 4.9 mm in *Unio pictorum*. The onset of outer demibranch development may be population or species specific. Presence of the outer demibranch increases gill surface area for respiration and feeding although the mechanism of particle transfer from the outer demibranch to the labial palps and mouth is as yet unknown for *M. margaritifera*.

The ostia described here are unlike anything previously reported in the primary literature. Normally ostia are delimited by interfilamentary junctions, otherwise known as gill pores (Tankersley & Dimock, 1992; Cannuel & Beninger, 2006; Cannuel *et al.*, 2009) or develop on thin sheets of tissue filling the gill pores (Ortmann, 1911c; Kovitvadhi *et al.*, 2007). However in adult *M. margaritifera* ostia were observed on tissue connected to the abfrontal surface of filaments. The size of ostia in *M. margaritifera* ranged from 33 - 221 μm in length which compares favourably with other studies on marine bivalves (Cannuel & Beninger, 2006; Cannuel *et al.*, 2009). Gill pores delimited by the interfilamentary junctions are very large in 44 month old juveniles and there is still no sign of the abfrontal tissue at this stage meaning that even the oldest juveniles observed in this study do not display the adult condition. Whilst

there is no doubt these individuals are filter feeding, it is perhaps less efficient compared to adult mussels. It also highlights that these individuals are not sexually mature because eggs and glochidia would not be retained in the gills without the presence of the abfrontal surface tissue. This area requires further investigation. Development of the plicated inhalent siphon was complete before the gills were fully formed, as reported for *Hyriopsis myersiana* by Kovitvadhi *et al.* (2007). In captivity it has been observed that mortality is negligible in juveniles older than 3 - 4 years old (FBA, unpublished data) so it would appear that the addition of this tissue to the abfrontal surface and ostia development is not a major contributor to juvenile mortality. To understand the timing and development of this feature, further consideration of specimens older than 44 months and > 8.9 mm length is required.

The adult specimen of *M. margaritifera* displayed the same wide sublateral surface observed in *M. edulis* (Cannuel *et al.*, 2009). This affords a larger respiratory area and if the widening of filaments continues with development, as suggested by Cannuel *et al.* (2009), this may provide larger, more developed individuals with an opportunity to increase metabolism and grow faster than their smaller, less developed counterparts.

5.4.2.1 Gill ciliation

A substantial body of research exists on the topic of gill ciliation, particularly the role of laterofrontal cirri in particle capture. This study shows that the full suite of ciliation is present in even the youngest individuals observed in this study. Ciliation develops shortly after budding, similar to previous reports in other freshwater (Kovitvadhi *et al.*, 2007; Trump, 2010) and marine bivalves (Cannuel & Beninger, 2006; Cannuel *et al.*, 2009). The branched structure of laterofrontal cirri in *M. margaritifera* is similar to that previously described in other species (Owen, 1974; Lasee, 1991; Silverman *et al.*, 1995; Silverman *et al.*, 1996; Silverman *et al.*, 1997; Gui *et al.*, 2016) and form an effective 'net' or 'sieve' for particles. This complex type of laterofrontal cirrus is efficient in removing small particles from suspension compared to simple cirri (Riisgård, 1988). The range of particles filtered by a particular species may be an adaptation to algal/bacterial species present (Ward & Shumway, 2004) and therefore the implications of eutrophication on algal communities in pearl mussel rivers is of particular importance. Studies on the diet of *M. margaritifera* are particularly sparse but are critical to understanding the development and ecology of the species.

The number of cilia per laterofrontal cirrus is variable between species (Table 5.7 and references therein) and in *M. margaritifera* did not differ between individuals of different ages/sizes (Table 5.3) and was comparable with the number found in previous studies albeit towards the higher end of the spectrum (Table 5.7). Silverman *et al.* (1997) found that species from lentic habitats had smaller, simpler laterofrontal cirri with fewer cilia per cirrus. They also found that species with larger gill surface areas and more cilia per cirrus could clear more bacteria and at a faster rate. Cirral plates in *M. margaritifera* averaged 1.54 μm apart (± 0.40) which makes them more closely aligned than the majority of inter-cirral distances previously reported, which typically range between 2.0 - 3.5 μm (Owen, 1974; Cannuel *et al.*, 2006; Cannuel *et al.*, 2009; Gui *et al.*, 2016; Schartum *et al.*, 2016), except in the ribbed mussel (*Geukensia demissa*) where the inter-cirral distance was 1.57 μm (Wright *et al.*, 1982). Inter-cirral distance may increase slightly with age (Gui *et al.*, 2016) but this requires further investigation in *M. margaritifera*. The small inter-cirral distance and high number of cilia per laterofrontal cirrus suggests that *M. margaritifera* juveniles may be capable of retaining very small ($< 2 \mu\text{m}$) algal and bacterial cells. Baker & Levinton (2003) found that *M. margaritifera* adults preferentially ingested particles $< 4 \mu\text{m}$ and rejected larger algal species in pseudofaeces. However, *M. margaritifera* could not distinguish more nutritious algae (*Microcystis aeruginosa*) from less nutritious *Typha* pulp, indicating that selection may be by particle size alone (Baker & Levinton, 2003). This has important implications for captive rearing programmes because diet is a significant consideration, particularly when supplementary feeding is being provided. The findings of Baker & Levinton (2003) coupled with the findings in this study of relatively small inter-cirral distance and complex, branching laterofrontal cirri with a high number of cilia per laterofrontal cirrus indicate that *M. margaritifera* likely requires small algae and bacteria as food items. This may be particularly true for juvenile mussels which are likely to have a smaller mouth and may be less able to ingest large particles. If juveniles are not provided with a diet constituting suitably-sized, nutritious particles, or are in environments with a high suspended solids load they are likely to decrease their filtration rate (Jørgensen, 1990), potentially leading to stress, starvation, reduced growth and/or increased mortality. If inter-cirral distance increases with age/size in *M. margaritifera*, as it did in *P. canaliculus* (Gui *et al.*, 2016), particle preference may also change. Inter-cirral distance was not measured in specimens older than

34 months so further investigation is required to inform decisions about juvenile mussel diets in systems which provide supplementary feeding.

Cilia observed on the abfrontal surface of filaments were sparsely distributed and did not appear to be organised, suggesting they are unlikely to serve any functional purpose and may simply be vestigial (Cannuel & Beninger, 2006; Cannuel *et al.*, 2009). Cilia on the abfrontal surface were still present in the adult specimen despite the abfrontal tissue overlying filaments. These cilia may interlock with cilia on the adjacent tissue surface to hold the tissue in place. Again, the sparse ciliation on the abfrontal surface in juveniles does not provide strong evidence to support this hypothesis and further investigation is required with either transmission electron microscopy or histology.

5.4.3 Labial palps and mouth

The inner surface of the labial palp primordia and the area around the mouth were heavily ciliated in 1 month old individuals. Labial palp morphology remained practically unchanged in subsequent specimens until approximately 20 months old (1.45 mm) when evidence of folding into the plicated form was initially observed. Also at 20 months old, a ciliary connection between the penultimate anterior gill filament and the labial palps was observed indicating that particle transport from the gill filaments to the labial palps may have been possible despite the absence of an oral groove at this stage. Development of labial palp morphology is not well described in the literature making comparisons difficult but Trump (2010) reports the onset of 'ridge and groove' morphology in the labial palps from 130 days in *U. imbecillis*. In the current study, the outer surface of the labial palps in 44 month old specimens had the same pattern of ciliation as the rest of the mantle. Cilia and more complex cirri were distinguishable on the inner labial palp surface suggesting that more complex sorting of particles is possible at this age. Development of the labial palps and connection with the gills is another under-studied topic but obviously an important one which is likely to have a significant bearing on when juveniles can sort particles and therefore filter feed efficiently.

5.4.4 Foot ciliation

Foot ciliation was consistent in all age classes in this study. The proximal portion was only sparsely ciliated, whilst the distal portion was heavily ciliated with short simple cilia. These

Table 5.7: Summary information for freshwater (FW) and marine (MA) bivalves on their preferred habitat, laterofrontal cirrus type and number of cilia per laterofrontal cirrus. Some information is not available (NA).

Species	FW/MA	Habitat	Laterofrontal cirrus type	Number of cilia/LFC	Order/Family	Reference
<i>Margaritifera margaritifera</i>	FW	Oligotrophic, fast flowing, highly oxygenated rivers and streams	Complex, branching	32 - 46	Unionida/ Margaritiferidae	This study
<i>Utterbackia imbecillis</i>	FW	Ponds & lakes or mud-bottomed pools	Simple	14 - 15	Unionida/Unionidae	Silverman <i>et al.</i> (1997); Trump (2010)
<i>Corbicula fluminea</i>	FW	Sandy/gravel substrates; also in mud	Complex, branching	32 - 33	Veneroidea/Cyrenidae	Silverman <i>et al.</i> (1995)
<i>Toxolasma texasensis</i>	FW	Slow flow in mud/sand	Complex, branching	12 - 13	Unionida/Unionidae	
<i>Corbicula fluminea</i>	FW	Slow flow in mud/sand/gravel	Complex, branching	32 - 33	Veneroidea/Cyrenidae	Silverman <i>et al.</i> (1995)
<i>Dreissena polymorpha</i>	FW	Slow flow on hard surfaces	Complex, branching	38 - 42	Veneroidea/Dreissenidae	
<i>Hyriopsis (Limnoscapha) myrsiana</i>	FW	Rivers, streams, lakes and reservoirs	Complex, branching	NA	Unionida/Unionidae	Kovitvadhi <i>et al.</i> (2007)
<i>Actinonaias ligamentina</i>	FW	Sand/gravel in a range of flow types	Complex, branching	13 - 23		
<i>Amblema plicata</i>	FW	Mud/sand/gravel in a range of flow types	Complex, branching	6 - 16		
<i>Obliquaria reflexa</i>	FW	Mud/sand/gravel in moderate flow	Asked about structure of these	21 - 26	All Unionida/Unionidae	Galbraith <i>et al.</i> (2009)
<i>Fusconaia flava</i>	FW	Mud/sand/gravel in moderate flow		15 - 21		
<i>Ligumia subrostrata</i>	FW	Mud/sand in slow flow	<i>Ligumia subrostrata</i>	12 - 16		
<i>Elliptio dilatata</i>	FW	Mud to gravel in medium streams & larger	has smaller cirri	30 - 32		
<i>Lampsilis ovata</i>	FW	Coarse sand/gravel in a range of flow types	than all other	26 - 35		
<i>Ptychobranchus fasciolaris</i>	FW	Coarse substrates in riffle/faster flow areas	species although	25 - 32	All Unionida/Unionidae	Silverman <i>et al.</i> (1997)
<i>Fusconaia flava</i>	FW	Mud/sand/gravel in creeks to large rivers	LFC in all species	25 - 30		
<i>Villosa lienosa</i>	FW	Mud/sand in slow currents	were branched	25 - 30		
<i>Cyclonaias tuberculata</i>	FW	Sand/gravel in moderate flow		38 - 42		
<i>Musculium transversum</i>	FW	Slow-flowing or still waters in mud/sand	Complex, branching	NA	Veneroidea/Sphaeriidae	Way <i>et al.</i> (1989)
<i>Polymesoda caroliniana</i>	MA	Estuaries/brackish waters in mud/sand	Complex, branching		Veneroidea/Cyrenidae	

Species	FW/MA	Habitat	Laterofrontal cirrus type	Number of cilia/LFC	Order/Family	Reference
<i>Crassostrea gigas</i>	MA	Oyster - tidal areas	Simple compound cilia made up of 5-6 cilia. Not present until ~2.7 mm long.	NA	Ostreoidea/Ostreidae	Cannuel (2006)
<i>Pecten maximus</i>	MA	Tidal areas	Not present in observed juveniles (up to 4 mm long) but present in adults	NA	Ostreoidea/Pectinidae	Beninger et al. (1994)
<i>Placopecten magellanicus</i>	MA	Tidal areas	Not present in observed juveniles (up to 7.5 mm long). Adults have short laterofrontal cirri	NA	Ostreoidea/Pectinidae	Veniot et al. (2003)
<i>Mytilus edulis</i>	MA	Rocky shore	Complex, branching	18 - 26 (22 - 26)	Mytiloidea/Mytilidae	Silverman (1999) (Owen, 1974)
<i>Perna canaliculus</i>	MA	Intertidal/tidal zone	Complex, branching	20 - 28	Mytiloidea/Mytilidae	Gui et al (2016)
<i>Nucula sulcata</i>	MA	Mud and sandy substrates	Complex, branching	20	Nuculida/Nuculidae	Owen & McCrae (1976)
<i>Perna perna</i>	MA	Rocky shores	Complex, branching	18 - 23	Mytiloidea/Mytilidae	George & Gregory (2000)
<i>Geukensia demissa</i>	MA	Inter-tidal zone/salt marshes	Complex, branching	22 - 26	Mytiloidea/Mytilidae	Ward & Shumway (2004)
<i>Crassostrea virginica</i>	MA	Tidal areas	Complex, branching	6 - 11	Ostreoidea/Ostreidae	
<i>Argopecten irradians</i>	MA	Tidal areas	None	Simple cilia	Ostreoidea/ Pectinidae	

short cilia have previously been observed directing particles into the pedal gape on water currents (<https://www.youtube.com/watch?v=nHtE4rtkF9A>). This pattern of ciliation differs to that observed Kovitvadi *et al.* (2007) who report the whole foot being ciliated in *Hyriopsis myersiana*, but supports the findings of numerous other studies e.g. Lasee (1991), Passos *et al.* (2005), Trump (2010). These findings support the hypothesis that pedal cilia, along with mantle and gill cilia, have a function in directing particles into the pedal gape on water currents, but particles are not bound in mucus and transported in to the pedal gape on pedal ciliary tracts.

5.4.5 Mantle ciliation

Whilst mantle ciliation was not a major consideration in this study, some general observations were made. At all stages of development, mantle ciliation was denser around the posterior region where the siphons would develop, but ciliation was also present around the majority of the mantle margin. Around the margin, short, compound cirri were present nearest the margin and longer, simple cilia were present slightly dorsal to this. Longer cilia may have a role in pseudofaeces transport as reported by Beninger *et al.* (1999) and the shorter cirri may have a role in creating water currents into the infrabranchial cavity but direct observation in live specimens is required to confirm these hypotheses. Ciliation away from the margins appeared to be unorganised and patchily distributed in younger individuals becoming more organised into tracts by 44 months old. It is likely that these cilia are involved in maintaining water currents through the infrabranchial cavity. By 44 months old, siphons were well-developed and the inhalant siphon had the characteristic plicated form and was highly ciliated.

5.4.6 Behaviour

Smaller and younger individuals at earlier developmental stages were more active than older, more developed individuals. This is most likely due to their ineffective filtering ability and the requirement for them to move through the substrate in search of food due to their under-developed gill pump. Older/larger individuals had more developed gills and mantle cilia, presumably facilitating the movement of water through the mantle cavity without the need to move through the substrate. This is an important consideration for captive rearing programmes using systems with flowing water. Smaller individuals are more susceptible to being washed out of substrate due to their small size and active behaviour. They require

interstitial conditions which have a high exchange with the water column for dissolved oxygen and food delivery but also require stable substrates so that juveniles are not washed out. In contrast, larger individuals with more mature, reflected gills are not as active because they have a more efficient pump and they can orientate themselves within the gravels to take advantage of flow direction to aid water passage through their inhalant siphon. They are also heavier and therefore less likely to be washed out. These findings highlight that captive rearing programmes should consider juvenile behaviour as well as anatomical development when designing rearing systems and have a flexible approach to switching systems as juveniles grow and develop in order to maximise growth and survival.

The switch from active pedal feeding behaviour to the more sedentary filter feeding behaviour occurred from around 14 months old; around the age that gill reflection begins. This is much later than has been reported in faster-growing species such as *Villosa iris* (Gatenby *et al.*, 1997) and *Lampsilis fasciola* (Hanlon, 2000). Scallops in particular seem capable of rapid gill development with the heterorhabdic stage reached by 2 months old (Beninger *et al.*, 1994). Future studies should consider that biological development is more closely correlated with size rather than age (Veniot *et al.*, 2003) so studies which quote development in relation to age alone are of limited value. In addition, growth is temperature-dependent so temperature data are useful to provide context to results. Activity may be linked to temperature with higher temperatures increasing metabolism. No consideration was given to water temperature during this study and so this topic requires further investigation. Temperature data for the period covered during this study can be requested from the author.

5.4.7 Procedure and suggested methodology for SEM work on juvenile mussels

Variable specimen quality was observed in some individuals due to differences in the way juveniles were prepared for SEM (Table 5.4). This led to the loss of some data, but outlined the importance of following a successful procedure for specimen preparation. Critical point drying was not used during these investigations but may offer a suitable alternative for the dehydration step, despite reports of this method leading to increased specimen damage (Trump, 2010). The very thin shells of newly-excysted and very young juveniles were very fragile after the HMDS step which led to increased specimen damage so investigation into

the potential benefits of using critical point drying as an alternative for the dehydration step should be considered.

As a result of these findings, the methodology outlined in Appendix 2 on page 189 is advised for preparation of juvenile *M. margaritifera* for SEM. The procedure was also successful on adult mussel gill tissue. All reagents should be prepared fresh at the time of use and steps should be carried out as soon as possible after one another to ensure the best results. If samples and reagents need to be stored for short periods of time then this should be done in the fridge until use. Once specimens have been sputter coated they can be transported or stored with no structural or tissue degeneration.

5.4.8 Conclusions and implications for captive rearing programmes

This study is the first to comprehensively describe the main anatomical developments in *M. margaritifera* juveniles aged 1 - 44 months old and goes some way to explaining behavioural changes associated with mode of feeding. These investigations considered juveniles from only one population of *M. margaritifera* and only a single adult specimen which was sampled opportunistically when it was found dead at the FBA Ark. It is not known whether the rate of development is population specific therefore sampling of additional populations should be undertaken to confirm the timings of key developments reported in this work.

Gill reflection begins to occur around the middle/end of the second growth season (13 - 16 months old and when length > 1.20 mm) but it is likely that attainment of true filter feeding using fully developed ctenidia does not occur until much later. At termination of the 2012 substrate experiment (Chapter 3) larger individuals were observed to be filter feeding. This agrees with observations of gill reflection and labial palp development by 20 months old and subsequent oral groove development by 34 months old during this study. It is likely that cilia on the foot and mantle are still heavily utilised to move particles in to the pedal gape in juveniles < 20 months old despite the amount of 'active' time decreasing with age. The heavily ciliated nature of the mantle margin around the siphons also implies these cilia play an important role in directing water in to the infrabranchial cavity before it is directed over the ctenidia by lateral cilia. Additional behavioural observations are required to fully understand these processes.

The following conclusions can be drawn about how juvenile ontogeny may affect survival in captivity:

- 1. Mortality during the first few months post-excystment is not related to transformation.**
The high mortality observed within the first 4 - 8 weeks post-excystment does not correlate with substantial ontogenic changes in gill morphology. Mortality during this period may be due to juveniles not being able to find enough food of suitable particle size or poor capture efficiency of suitable particles and the high level of activity required by juveniles in order to feed. Additional environmental risk factors during this period are discussed in Chapter 4 and in the conclusions and discussion chapter (Chapter 7).
- 2. Juveniles may be particularly sensitive to stress factors from around 13 months old (> 1.20 mm) when gill reflection begins and labial palp development increases.**
Additional energy may be required to undergo these significant morphological changes and the timing of reflection suggests that juveniles may have to store additional nutritional reserves over the second growth season to meet this increased demand. Therefore, stress factors should be kept to a minimum during the second growth season and second winter. This area warrants further investigation.
- 3. 44 month old juveniles (up to 8.9 mm) do not display the adult gill condition.** Additional observations are required on older juveniles to see when the abfrontal surface tissue and ostia appear. Juveniles at the FBA Ark have displayed high survival (> 97 %) between the ages of 4 - 8 years (FBA, unpublished data) but presence of the abfrontal surface tissue and ostia cannot be confirmed in these individuals. Further investigation is required.
- 4. *Margaritifera margaritifera* may require/prefer small food particles and gill structure may limit their ecological niche to oligotrophic streams.** Previous findings from Baker & Levinton (2003) on adult *M. margaritifera* and the findings here of high numbers of cilia per laterofrontal cirrus and small inter-cirral distance implies that the species is capable of filtering very small particles (< 2 µm) but not necessarily able to sort them by nutritional value. *M. margaritifera* requires oligotrophic conditions so may have to expend large amounts of energy clearing unsuitable particles where there is eutrophication/aggravated erosion. This hypothesis is supported by the findings of ecological studies on the loss of juvenile function with increased catchment intensification and loss of oligotrophic conditions (Moorkens, 2010; Österling *et al.*, 2010).

These observations and initial measurements have set the benchmark for further ontogenic studies on *M. margaritifera* and other freshwater species. The majority of studies on bivalve

gill ontogeny have been carried out on marine species but the recent increase in captive rearing programmes for freshwater mussels, particularly in Europe, has led to a requirement for additional information about the factors affecting juvenile growth and survival in captivity. Studies into ontogeny should complement those on general ecology and environmental factors affecting juvenile growth and survival in order to build a comprehensive management plan for threatened species.

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5.6. Appendices

5.6.1 Appendix 1: Glossary and abbreviations

Description of terms and abbreviations used in this chapter.

Term	Description	Abbr.
Abfrontal surface	The inward-facing surface of lamellae which are largely devoid of cilia.	AS
Ascending limb	Limb of the filament which ascends (extends dorsally) away from the gill axis.	AL
Budding zone	Zone from which new filaments proliferate at the posterior end.	BZ
Ciliary connection	A connection between features comprised of cilia. Thought to be a more primitive version of tissue connections.	CC
Ctenidium (gill)	Mussels have two ctenidia (gills) each made up of the inner and outer demibranchs. The demibranchs themselves are made up of filaments.	-
Descending limb	Limb of the filament attached to the gill axis which descends (extends ventrally) away from the gill axis.	DL
Filament	A single process of the gill which collectively make up a demibranch. A filament may consist of just a descending limb or both a descending and ascending limb.	FI
Foot	Muscular appendage used to move, anchor in sediment and create water currents into the mantle cavity.	FO
Frontal cilia	Simple cilia covering the frontal surface of filaments which direct particles ventrally towards the oral groove.	FC
Fused dorsal bend	In reflected demibranchs, the fused tissue at the terminal end of ascending filaments which is growing dorsally.	FDB
Gill axis	The supporting axis which attaches the ctenidia to the visceral mass. As well as attaching ctenidia to the body wall the gill axis contains is vascularised, providing filaments with haemolymph for gas exchange.	GA
Infrabranchial (= mantle/pallial) cavity	The cavity into which water is pumped through the inhalant siphon.	-
Inner demibranch	The V-shaped structure consisting of the ascending and descending lamellae which develop first and are closest to the foot.	ID
Interfilamentary junction	A ciliary or tissue junction (connection) between adjacent filaments.	IFJ
Interfilamentary space	The spaces between adjacent filaments on the same lamella.	IFS
Interlamellar junction	A tissue junction joining the ascending and descending limbs of a filament. Also known as a septum.	ILJ
Labial palps	Particle-sorting organ anterior of the foot where particles are directed from the oral groove for sorting before ingestion or rejection as pseudofaeces.	LP
Lamella	General term for the transverse filaments making up either the ascending or descending limbs of a demibranch.	-

Term	Description	Abbr.
Lateral cilia	Cilia covering the lateral (side) portion of filaments. Primary function is creation of water current.	LC
Laterofrontal cirri	Cirri covering the laterofrontal surface of filaments consisting of linear plates of long, simple cilia. Primary function is to capture particles and direct them on to the frontal cilia.	LFC
Mantle	Tissue covering the inner surface of the shell which secretes calcium for shell formation. Attached along the pallial line.	MA
Mouth	Particles are passed from the labial palps to the mouth for ingestion via ciliary mechanisms.	MO
Oral (=food) groove	Particles are directed by frontal cilia ventrally along filaments towards the oral groove where they continue towards the labial palps and mouth.	OG
Ostia	Holes through which water passes from the infrabranhial into the suprabranhial cavity.	O
Outer demibranch	The V-shaped structure consisting of the ascending and descending lamellae which are furthest from the foot. Develop after the inner demibranch has reflected.	OD
Shell	Hard, external skeleton protecting inner tissues.	-
Suprabranhial cavity	The cavity into which water flows from the infrabranhial cavity (through the ostia) and from where water can exit via the exhalent siphon.	-
Tissue connection	A connection between features comprised of tissue.	-
Ventral bend	The site where ascending and descending limbs are connected at the ventral-most tip. On the inner demibranch the oral groove develops here.	VB

5.6.2 Appendix 2: Suggested protocol for specimen preparation

1. Measure individuals (length) and place them into individual eppendorf tubes containing 1 mg/mL MS-222 (enough to cover individuals). Leave for approximately 0.5 - 1 hour until valves are gaping. N.B. Individuals should not be left in MS-222 for too long as soft tissues and delicate structures may degrade.
2. Depending upon study requirements, individuals can be prepared in either of the following ways:
 - a. If the individual is to be kept intact the valves should be teased open wide enough to ensure soft tissues receive adequate exposure to reagents and so that structures are readily visible.
 - b. The valves can be separated entirely to better observe structures with minimal obstruction. This should be done carefully with fine mounted needles and reverse forceps. Larger individuals could be separated by using a sharp scalpel to divide soft tissues in the left and right valves with minimal damage.
3. Specimens should be placed into fresh eppendorf tubes containing enough 2% glutaraldehyde in 0.1 M Sorenson's phosphate buffer (SPB) to cover individuals. Leave over night.
4. Transfer individuals to fresh eppendorf tubes containing SPB for 15 minutes. Repeat this step (2 x SPB rinses).
5. Perform graded dehydration in ethanol; 25 % (30 mins), 50 % (30 mins), 75 % (30 mins), 100 % (1 hour), repeat with 2nd 100 % ethanol wash (1 hour).
6. Transfer to fresh eppendorf tubes with enough Hexamethyldisilazane (HMDS) to cover specimens, for 30 mins. HMDS is highly volatile so replace lids on eppendorf tubes. Repeat this step (2 x HMDS washes). N.B. Whilst not considered for this study, critical point drying provides an alternative method for this HMDS step (Trump, 2010).
7. Pipette juveniles on to filter paper and leave to air dry under a fume hood. Transfer onto carbon stickers mounted on SEM stubs.
8. Gold coat specimens with a sputter coater.

This method provided clear and consistent results of fine structures when followed for all age classes investigated as part of this study. N.B. Appropriate health and safety and COSHH assessments should be carried out prior to any work as this method includes the use of

potentially damaging reagents, particularly glutaraldehyde and HDMS. A suitable fume cupboard is essential when using these reagents.

Chapter 6

Fluorescence marking of immature stages of the freshwater pearl mussel *Margaritifera margaritifera*

6.1. Introduction

Propagating *Margaritifera margaritifera* (Linnaeus, 1758) is labour-intensive, mainly due to the different requirements at the various life cycle stages and their small size and slow growth rate. Due to their small size, juvenile mussels are difficult to observe which can make maintenance and monitoring activities time consuming and difficult in captive settings. Marking juveniles to make them more visible is potentially one way of making rearing activities less laborious. Many methods of marking organisms are available to biologists, including internal and external tags and labels (reviewed by Nielsen, 1992). Due to their small size, marking juvenile mussels using traditional numbered tags or etching the shell is not an option (Eads & Layzer, 2002) while glues or paints may be toxic to small, thin-shelled animals (Moran, 2000). An alternative technique is to mark juvenile mussels with a fluorophore (or fluorochrome, i.e. a fluorescent compound). Fluorophores are chemical compounds which fluoresce upon light excitation. There are several advantages of using fluorophores including the ability to batch-mark large numbers of mussels, provision of a relatively long-lasting mark, and suitability for use on mussels of any size.

In fluorescence microscopy, light passes through an excitation filter which selectively allows the passage of a narrow band of wavelengths corresponding to the absorption maximum of the fluorophore (Rost, 1991). When this light hits the labelled specimen it is absorbed and the electrons are promoted to a higher energy state. This process is known as excitation. Excess energy is emitted from the fluorophore as light of a longer wavelength (lower energy) which passes through the microscope objective. A barrier filter allows light from a specific range to pass through (the emission range of the fluorophore) whilst blocking light of wavelengths outside this range. Light is detected against a dark background as it passes through the ocular lens (Fig. 6.1). Fluorescence fundamentals (2015) provides a good overview of the fundamentals of fluorescence microscopy.

6.1.1 Use of fluorophores in ecological studies

Marking molluscs with fluorescent compounds has a variety of applications such as monitoring augmentation efforts (Eads & Layzer, 2002), tracking dispersal patterns (Moran & Marko, 2005), linking growth patterns with environmental conditions (Ambrose *et al.* 2012) and monitoring shell growth rates (Thébault *et al.*, 2006; Peharda *et al.*, 2007; Trigg, 2009; Cáceres-Puig *et*

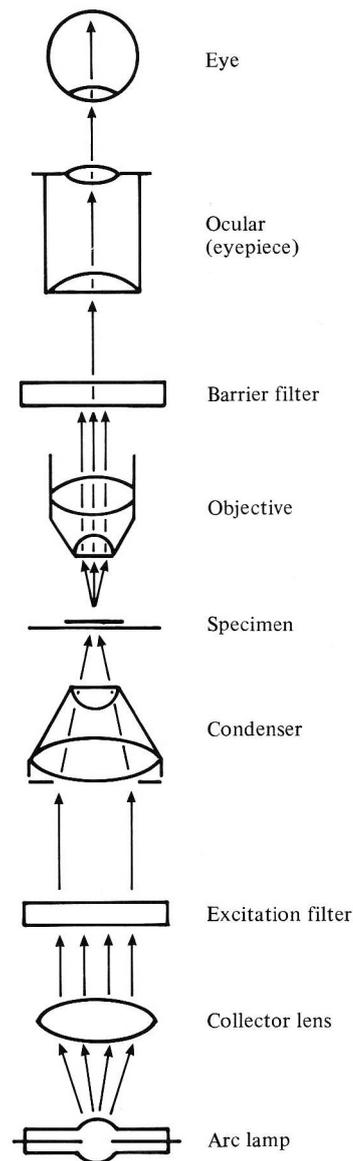


Fig. 6.1: Diagram of processes involved in observing fluorescence in a marked specimen (from Rost, 1991). Light of a narrow band of wavelengths excites the fluorophore on a marked specimen which then emits light of a different wavelength. This light is filtered before reaching the objective and the viewer.

al., 2011). Marking with fluorophores has also been suggested as a way of distinguishing individuals from protected sites to combat poaching (Bolton & Dey, 1979). Here a new use of marking juvenile mussels as an aid to monitoring in a captive situation was investigated.

In this study fluorophores which bind to calcium are required as *M. margaritifera* sequesters calcium into its valves along the growth edge of the shell. Previously, exposure of molluscs to fluorophores has been through immersion, injection or, in one case, through the process of osmotic induction (U.S. Fish and Wildlife Service, 2002). Whilst injection of fluorophores is a useful tool for adult mussels or larger juveniles it is not practical for newly

excysted juveniles due to their small size and is therefore not considered here. Immersion and osmotic induction techniques are considered in more detail below.

6.1.1.1 Immersion

Bolton & Dey (1979) were among the first to describe (and patent) a method for batch marking molluscs with tetracycline via immersion. This method has since been modified and adapted to test different types of fluorophores on a wide range of species. Fluorophores reported for use in marking molluscs include oxytetracycline (OTC) (Day *et al.*, 1995; Eads & Layzer, 2002), calcein (Day *et al.*, 1995; Eads & Layzer, 2002; Riascos *et al.*, 2007; Linard *et al.*, 2011), tetracycline (Dey & Bolton, 1978; Bolton & Dey, 1979; Day *et al.*, 1995), strontium chloride (Riascos *et al.*, 2007), alizarin red and xylene orange (Day *et al.*, 1995). Most marking experiments on molluscs have concentrated on marine molluscs although there are some examples of marking fresh water species e.g. Eads & Layzer (2002).

6.1.1.2 Osmotic induction

Osmotic induction increases the rate of fluorophore uptake by creating an osmotic gradient between the target animal and the immersion medium (Alcobendas *et al.*, 1991). Individuals to be marked are given a brief salt bath to create the osmotic gradient which facilitates faster uptake of fluorophores when immersed in fluorophore solutions. The osmotic induction method has been used to mark fish scales, fins and otoliths (Alcobendas *et al.*, 1991; Mohler, 2003; Negus & Tureson, 2004; Stubbing & Moss, 2007; Honeyfield *et al.*, 2008) but the author knows of only one pilot study involving the osmotic induction method to mark juvenile freshwater bivalves (U.S. Fish and Wildlife Service, 2002). In this pilot on the freshwater mussel *Elliptio complanata*, survival was better in osmotic induction trials compared to static immersion trials (48% and 28% respectively) and there was no difference in growth rates of juveniles between the treatments. The effects of salt on *M. margaritifera* juveniles are unknown and so osmotic induction was not considered for marking juvenile mussels here. However, use of the osmotic induction method to mark encysted glochidia has not been tested. Purser (1985) argued that colonisation of rivers by *M. margaritifera* after the last ice age could have been by fish encysted with glochidia from one river 'straying' into other rivers where juveniles excysted and founded new populations. He hypothesised that short-term exposure to salt

water may not have a detrimental effect on encysted glochidia. Glochidia of *M. margaritifera* grow to over five times their original size whilst encysted in the fish host (Karna & Millemann, 1978; Sweeting & Miles, 2009), so there may be scope for fluorophore uptake by glochidia from fish which are treated after a salt bath. Glochidia of *M. margaritifera* which are encysted in salmonids go through a rapid growth phase before the onset of colder winter temperatures, when their growth slows down or stops. Growth then resumes when temperatures increase, culminating in glochidial drop-off in late spring/early summer. Glochidia obtain nutrients from their host and therefore may also take up fluorophore-labelled calcium.

6.1.2 Suitability of calcein immersion for *M. margaritifera*

The fluorophore chosen for these investigations was calcein ((Fluorescein di-(methyliminodiacetic acid) sodium salt). Calcein (Fig. 6.2) has been used to mark a wide range of aquatic species including ascidians (Lambert & Lambert, 1996), echinoderms (Stewart, 1996), fish (Alcobendas *et al.*, 1991; Mohler, 2003; Negus & Tureson, 2004; Honeyfield *et al.*, 2006; Stubbing & Moss, 2007) and foraminifera (Bernhard *et al.*, 2004). It has been used to mark both marine and freshwater bivalves and investigations have shown it has low toxicity, is easy to work with, produces clear and consistent marks and causes fewer growth and mortality issues for molluscs compared with other fluorophores (Day *et al.*, 1995). Eads & Layzer (2002) recommend calcein as a fluorophore but report that marking success may vary between mussel species.

Calcein forms non-covalent interactions with dissolved calcium via the alanine rings (top of Fig. 6.2) providing a reference mark that is visible under ultraviolet (UV) light (Day *et al.*,

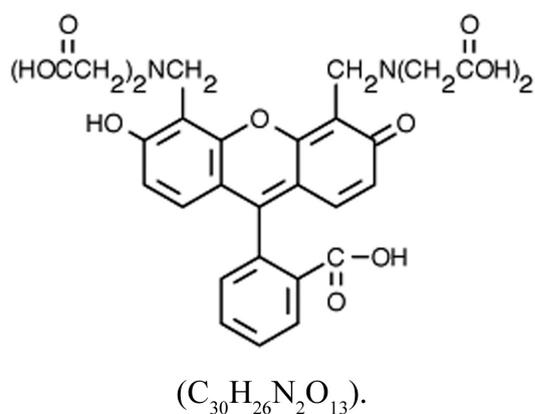


Fig. 6.2: Molecular structure of calcein ($C_{30}H_{26}N_2O_{13}$). Non-covalent interactions with calcium are formed via the alanine rings at the top of this figure.

1995; Smith *et al.*, 2010). Calcein marks have been shown to deteriorate over time (Frenkel *et al.*, 2002) with exposure to light (Stubbing & Moss, 2007). Even so, studies have shown that marks can persist for up to nine months in the brown mussel, *Perna perna* (Kaehler & McQuaid, 1999), 19 months in brown trout, *Salmo trutta* (Stubbing & Moss, 2007) and 3 years in Atlantic salmon, *S. salar* (Mohler, 2003). Marks have the potential to persist for several years in freshwater molluscs due to their burrowing behaviour cutting out light to the marked part of the shell (Eads & Layzer, 2002). In a species which inhabits neutral to slightly acidic waters such as *M. margaritifera*, marks may be lost over time as the umbo is eroded or obscured as the thick periostracum develops (see section 6.1.3 below).

There is evidence to suggest that mussels undergoing rapid growth display more intense marking (Day *et al.*, 1995), presumably because they are sequestering more fluorophore-bound calcium. Marking of species which have distinct growth periods, such as *M. margaritifera* which grows at temperatures exceeding 10 °C (Hruška, 1999; Lavictoire *et al.*, 2016), should only take place when individuals are going through a growth phase (Eads & Layzer, 2002). Some studies have found adverse effects on growth and survival in some species when juveniles are younger than two months old e.g. Eads & Layzer (2002) for *Lampsilis cardium* and *Actinonaias pectorosa*. However, Moran & Marko (2005) found no detrimental effect of calcein on growth and survival in three-day-old veligers of *Argopecten irradians concentricus* and *Mytilus trossulus*.

Marking experiments on a variety of bivalves testing different concentrations of calcein and immersion times have found that increasing calcein concentration and immersion time increases mark intensity (Day *et al.*, 1995; Crocker, 1998; Eads & Layzer, 2002; Linard *et al.*, 2011). Single immersions have been reported most often and produce lower mortality rates compared with multiple immersions over a short period of time (Eads & Layzer, 2002).

6.1.3 Shell structure

Many texts describe shell formation and layering. The following description has been taken from Lowenstam & Weiner (1989), Simkiss & Wilbur (1989) and Killeen *et al.* (2004). In bivalves, the mantle is responsible for shell formation and the valves consist of three layers (Fig. 6.3). The inner nacreous layer contains a proteinaceous conchiolin matrix containing horizontal crystals of aragonite. The middle prismatic layer is composed of vertical prisms of calcite separated by thin layers of conchiolin and the outer periostracum is formed entirely of

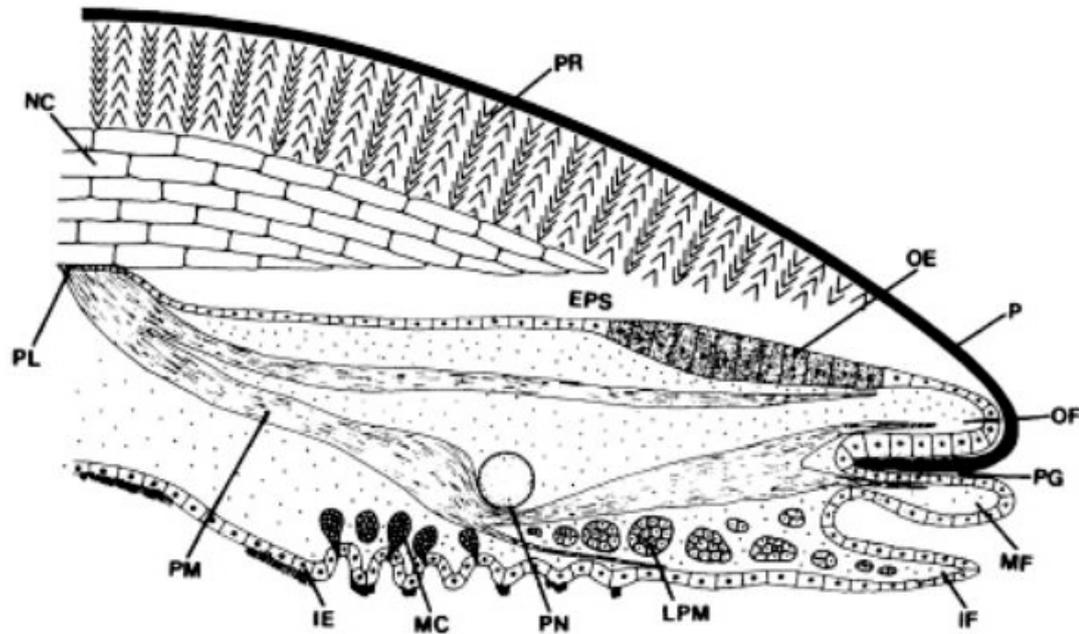


Fig. 6.3: Diagram of a section through the mantle edge and shell (From Simkiss & Wilbur, 1989; p. 232): EPS, extrapallial space; IE, inner epithelium; IF, inner fold; LPM, longitudinal pallial muscle; MC, mucous cell; MF, middle fold; NC, nacreous layer; OE, outer epithelium; OF, outer fold; P, periostracum; PG, periostracal groove; PL, pallial line; PM, pallial muscle; PN, pallial nerve; PR, prismatic layer.

conchiolin and contains no calcium. Therefore, calcein-labelled calcium should be sequestered into the nacreous and prismatic layers but not the periostracum. In long-lived species like *M. margaritifera*, the periostracum is very thick and many conchiolin layers are laid down to prevent dissolution (Bauer & Wächtler, 2001). Indeed, according to Bauer & Wächtler (2001), *M. margaritifera* allocates 30 % of its organic matter to its valves so that it may survive in acid waters. Very young mussels lack a thick periostracum so fluorescence around the shell margin should be obvious.

6.1.4 Objective of this study

These investigations consider several questions surrounding marking of different life cycle stages with calcein:

1. Is calcein immersion a suitable method for marking juvenile *M. margaritifera* and, if so, how do different concentrations affect mark intensity?
2. How do different concentrations of calcein affect growth and survival of juvenile mussels?
3. Does embedding juveniles in resin and freeze-fracturing them allow details of calcein sequestration to be observed under a fluorescence microscope?

4. Can osmotic induction be used as a method of batch-marking glochidia encysted within fish gills?

If either immersion of juveniles or osmotic induction of fish to mark glochidia is successful for *M. margaritifera*, these could represent relatively inexpensive and simple methods for batch-marking early life-stages of *M. margaritifera* to improve monitoring in captive settings.

6.2. Methods

6.2.1 Marking of juveniles via immersion

The methodology for this experiment was largely the same as for the pilot (Chapter 2) with some key changes. Three tanks were prepared containing calcein solutions of 0 (control), 60 and 120 mg/L using water from Windermere to dilute calcein stock solutions. Twelve *Artemia* sieves (four replicates of each of the three treatments) were prepared with 17 g (dry weight) of substrate measuring 1 – 2 mm which had been exposed to running water previously for at least eight days for a biofilm to develop. Thirty juvenile mussels were measured (length and height) and added to each sieve. Sieves were placed into each experimental tank containing calcein solutions of 0, 60 and 120 mg/L on 7 August 2012. Aeration was provided to each tank with a pump and air stones. Dissolved oxygen, pH and conductivity were measured at three points during the experiment: immediately prior to and during addition of calcein, after 24 hours and after 72 hours (end of the experiment). Aquaria were kept in the dark to avoid the potential loss of marks from sunlight (Honeyfield *et al.*, 2008)

After 72 hours sieves from the control treatment were removed to a bucket of clean water and the sieves from the two calcein treatments were transferred to the no-calcein aquarium to stop the uptake of calcein. Each of the 12 sieves were checked for juveniles by washing a small amount of substrate into a petri dish and observing it under a low-power microscope. Juveniles were removed into a separate dish containing lake water and once all of the substrate had been checked, the number of live and dead individuals was recorded. Five live juveniles from each sieve were randomly selected and photographed under the fluorescence microscope (camera: Canon 350D; shutter speed: 1 sec.; ISO: 100; File type: Canon RAW). The intensity of fluorescence in each photograph was measured using the same Photoshop technique described in section 2.2.2.1 (Chapter 2). For each sieve, substrate was replaced with

substrate which had not been exposed to calcein and all live juveniles were placed into the flow-through aquarium system described in Chapter 2.

All sieves were subsequently checked for surviving juveniles in September, October and November 2012 and finally in April 2013. Length and height measurements were recorded on each of these occasions. On 13 October 2012 attempts were made to view juveniles with an SE Mark Detection Device (Mohler, 2004) to see if this device was suitable for searching for fluorescent mussels in larger containers. Briefly, the SE mark detection device is a small hand-held torch which shines UV light on to specimens. The specimens are viewed through a filter cube attached to the device which selectively allows the passage of light of a specified wavelength.

6.2.2 Embedding

On 13 November 2012, five individuals were taken from one sieve from each treatment for fixing in Quetol 651 (EMS Catalogue No. 14640), a water-miscible resin. For this, two mixes were created:

Mix 1

- 8 ml Quetol
- 16 ml Nonenyl Succinic Anhydride (NSA)
- 0.5 ml DMP-30

Mix 2

- 11 ml Quetol
- 14 ml Methyl-5-Norbornene-2,3-Dicarboxylic Anhydride (NMA)
- 0.5 ml DMP-30

The two mixes were combined in a ratio of 85:15 of Mix 1:Mix 2. This mixture was pipetted into circular moulds 11 mm in diameter and live juveniles added to the liquid resin. Moulds were put into an oven and heated to 60 °C for 24 hours before being removed and placed into a freezer at -80 °C until required. On 23 January 2013, the resin capsules were fractured in the vicinity of the juvenile mussels using a sharp blade and hammer. Individuals were observed again under the fluorescence microscope.

6.2.3 Use of the osmotic induction method to test calcein uptake by glochidia

The method used here was similar to the methods described by Mohler (2003) and Stubbing & Moss (2007) to mark fish. Atlantic salmon fry (*Salmo salar*) were challenged with glochidia

in late-summer 2012 at the FBA Pearl Mussel Ark. On 21 May 2013, sixty fish were randomly selected, checked for glochidial encystment by eye and split into two batches of 30. The treatment batch was placed into a net and immersed in a 2.5 % salt bath (125 g of NaCl in 5 L lake water) for 3.5 minutes. Fish were removed from the salt bath and placed momentarily into a fresh water bath to remove excess salt. The net was blotted onto a towel to remove excess water and fish were immersed in an aerated 0.5 % calcein bath (7.75 g calcein in 1.55 L lake water) buffered to pH 7 with ammonium acetate for 3.5 minutes. Fish were removed and dipped into fresh water to remove any excess calcein and then placed into an aquarium supplied with aerated lake water filtered to 20 μm . The control batch was treated in the same way but instead of a calcein bath following the salt bath, fish were immersed in 1.55 L of lake water with no calcein. Fish were provided with a natural light regime for 24 hours. After 24 hours, 10 fish from each of the treatment and control batches were killed by immersing them in an MS-222 solution (tricaine methanesulfonate). Once dead, fish were rinsed with lake water, measured (fork length) and weighed. Ten glochidia from each fish were dissected from within cysts in the gills and placed into marked vials with a little fresh water. Scale scrapings from each fish were mounted on slides to see if fluorescence could be detected.

The help of a colleague was required to ensure a 'blind' review of mark presence or absence in glochidia, similar to the method of Eads & Layzer (2002). The colleague selected a glochidium at random from the vials containing either treatment or control glochidia, measured the juvenile length and height and placed it on a cavity slide with a cover slip before passing it to the author. The author observed the glochidium under the Leitz Diaplan fluorescence microscope described in Chapter 2, and indicated if fluorescence could be detected or not. Presence or absence of a calcein mark was recorded by the colleague and the data analysed to establish how often a mark was correctly identified in glochidia from treated fish.

6.2.4 Data analysis

Survival and luminosity data followed normal distributions (Shapiro-Wilk tests) so ANOVA (with *post hoc* Tukey's HSD tests) and *t*-tests respectively were carried out on these data. Growth data did not follow a normal distribution so Kruskal-Wallis tests with *post hoc* Mann-Whitney U tests were employed to analyse these data. Pairwise comparisons (Mann-Whitney U) were made when testing for differences in initial length between treatments and a Bonferroni

correction for multiple comparisons was applied ($\alpha = 0.0167$). Unless otherwise stated, standard deviation is provided after mean values.

For the osmotic induction investigation fish condition factor was calculated using equation 1 to compare glochidial load between fish:

$$CF = (L/W)^3 \times 100 \quad (1)$$

where CF = condition factor; L = length; W = weight.

6.3. Results

6.3.1 Marking of juveniles via immersion

The abiotic parameters measured remained within the expected range for *M. margaritifera* throughout the experiment in all aquaria; DO (10.94 mg/L \pm 0.13), conductivity (72.16 μ S/cm \pm 8.56) and pH (6.92 \pm 0.26). Temperature was stable throughout the experiment (17.78 \pm 0.26 $^{\circ}$ C) and remained above 10 $^{\circ}$ C so juveniles should have been growing throughout and therefore taking up calcein-labelled calcium.

In October 2012 the SE-Mark detection device was used to see if it was possible to distinguish fluorescent juveniles from gravel. No marks were detectable from juveniles in the control group or from surviving individuals from the 2010 pilot study but marks were detectable in treatment juveniles from this investigation (Fig. 6.4).

A higher incidence of fungal infection was observed in juveniles in the 60 mg/L treatment. Fungal infection was suspected as the cause of death in 12% of juveniles in the 60 mg/L treatment sieves compared to 2 and 0% respectively in the control and 120 mg/L treatments after 3 days.

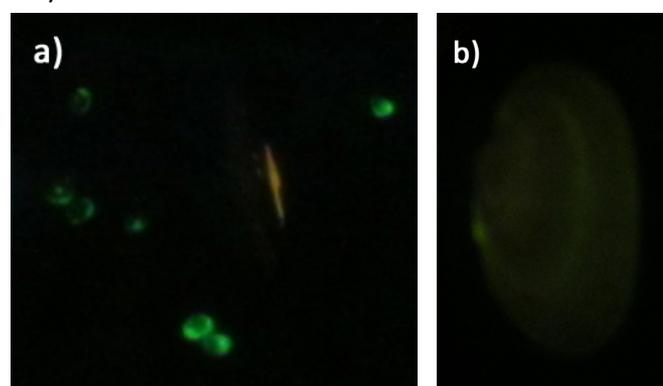


Fig. 6.4: Light micrographs of marked juvenile freshwater pearl mussels observed using the SE mark detection device (not same scale); a) Fluorescent juveniles 2 months post-exposure; b) No observable fluorescence from juvenile marked in 2010 approximately 2 years and 2 months post-exposure.

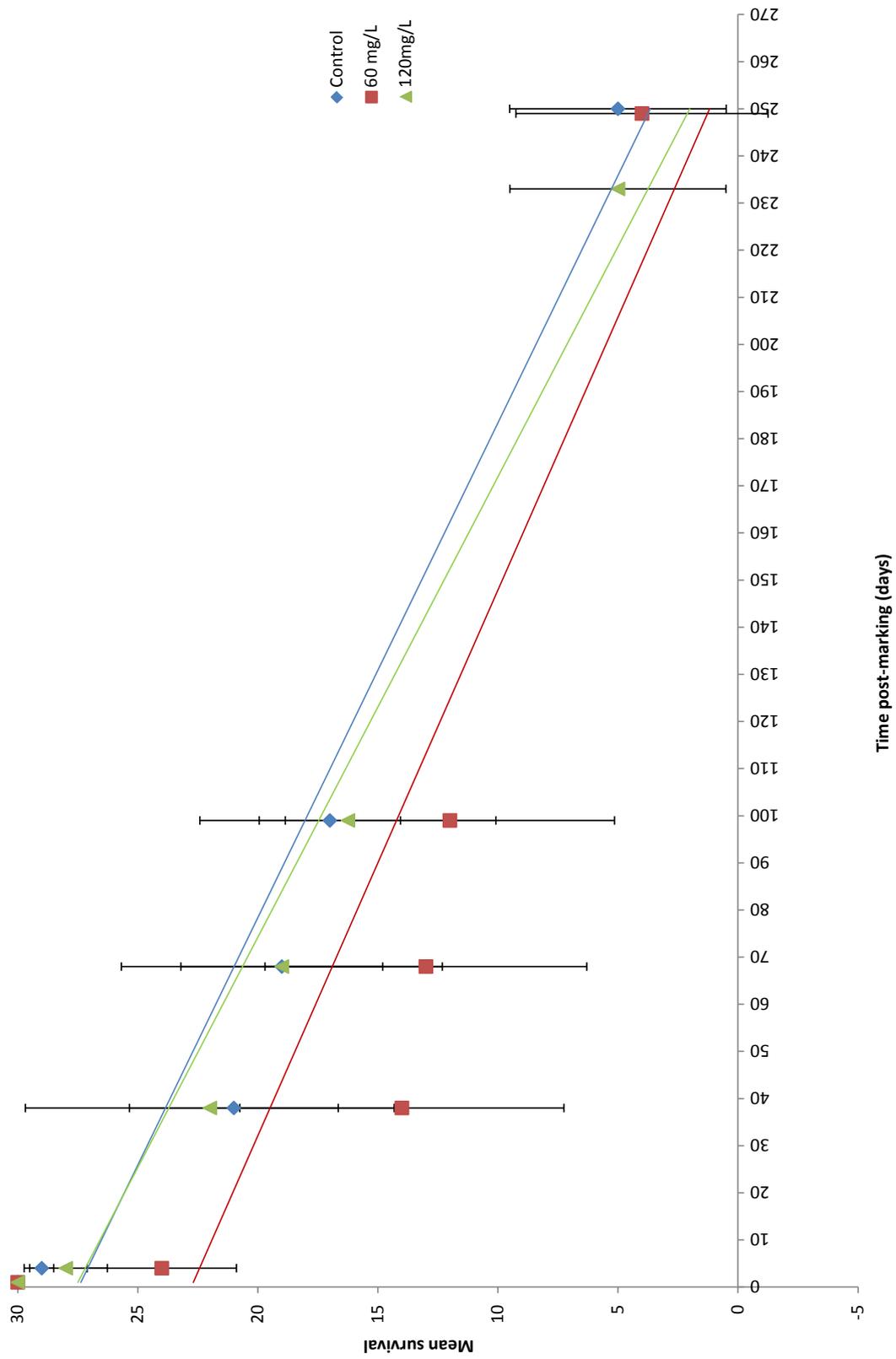


Fig. 6.5: Scatter plot of mean survival (\pm SD bars) of juvenile freshwater pearl mussels at each of the sampling points (38, 68, 99 and approximately 241 days).

Survival was assessed after 3, 38, 68, 99 and approximately 241 days. On day 3 a significant difference in survival was observed between the control (28.75 ± 0.50) and the 60 mg/L treatment (23.5 ± 3.11) ($F_{(2,9)} = 6.987, P = 0.015$) but on all other sampling occasions no significant difference in survival was found between any treatments or the control ($P > 0.05$). Low numbers of surviving juveniles were observed after approximately 241 days in the control (15 %), 60 mg/L treatment (14 %) and 120 mg/L treatment (18 %) (Fig. 6.5). It is thought this relatively low survival rate is due to the post-marking system juveniles were maintained in rather than any effect of marking. The system used to hold individuals post-marking in this experiment did not have the benefit of the refined system outlined in Chapters 3 and 4 meaning that flow and dissolved oxygen concentrations were likely to be sub-optimal.

Analysis of initial length found that juveniles in the different treatments were not the same size at the beginning of the experiment ($\chi^2_{(2)} = 55.542, P < 0.001$), meaning that direct size comparisons between treatments throughout the experiment were not possible.

Some growth was observed in juveniles from all treatments over the 241 days (Fig. 6.6) but because of the reasons stated above, simple length data could not be used to compare the effect of calcein concentration on growth. Instead, growth rates were calculated using equation 2, and 95 % confidence limits between treatments were compared.

$$\frac{\text{length (t2)} - \text{length (t1)}}{\text{length (t2)}} \times 100 \quad (2)$$

As shown in Fig. 6.7 it appears that the growth rate increased between days 99 and 241 (November 2012 - March 2013). However, due to temperatures being $< 10 \text{ }^\circ\text{C}$ during this period (Fig. 6.6) juvenile growth should have been negligible. Using data presented in Chapter 3, the estimated maximum juvenile growth for this entire period was $20 \text{ }\mu\text{m}$. Therefore, it is thought this apparent increase in growth rate was actually an artefact of mortality of smaller juveniles. If no growth during this period is presumed, losses from each juvenile size class give us an indication of how the size distribution for each treatment changed over the period. Fig. 6.8 shows the percentage mortality between these sample points by size class. From this data we can see that a higher proportion of juveniles in smaller size classes died in the control and 60 mg/L treatment and highest mortalities in the 120 mg/L treatment were from across the size distribution.

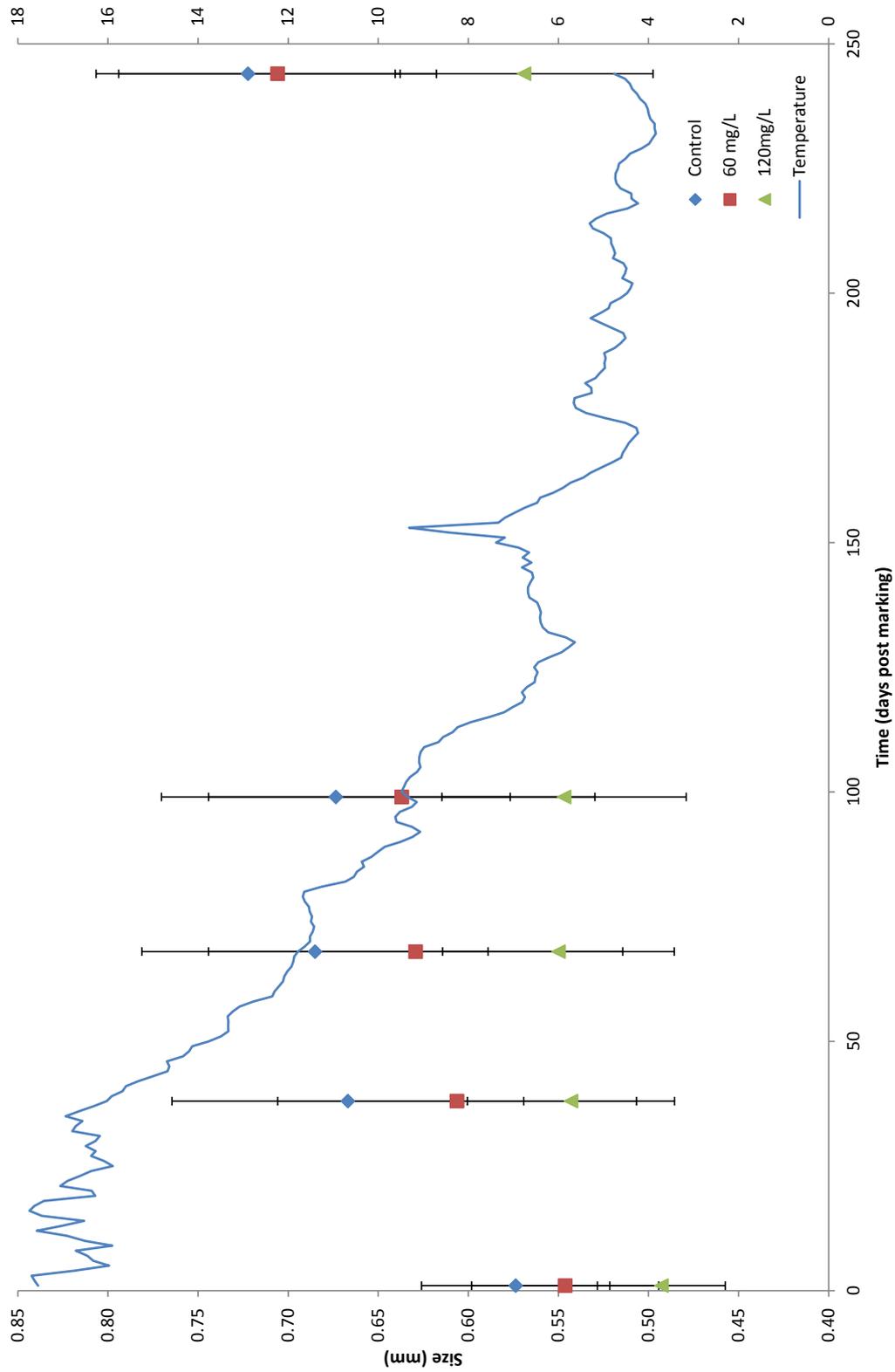


Fig. 6.6: Scatter plot of mean shell length (mm) of juvenile freshwater pearl mussels in each treatment (\pm SD bars) over the course of the experiment. Mean daily temperature is provided for reference.

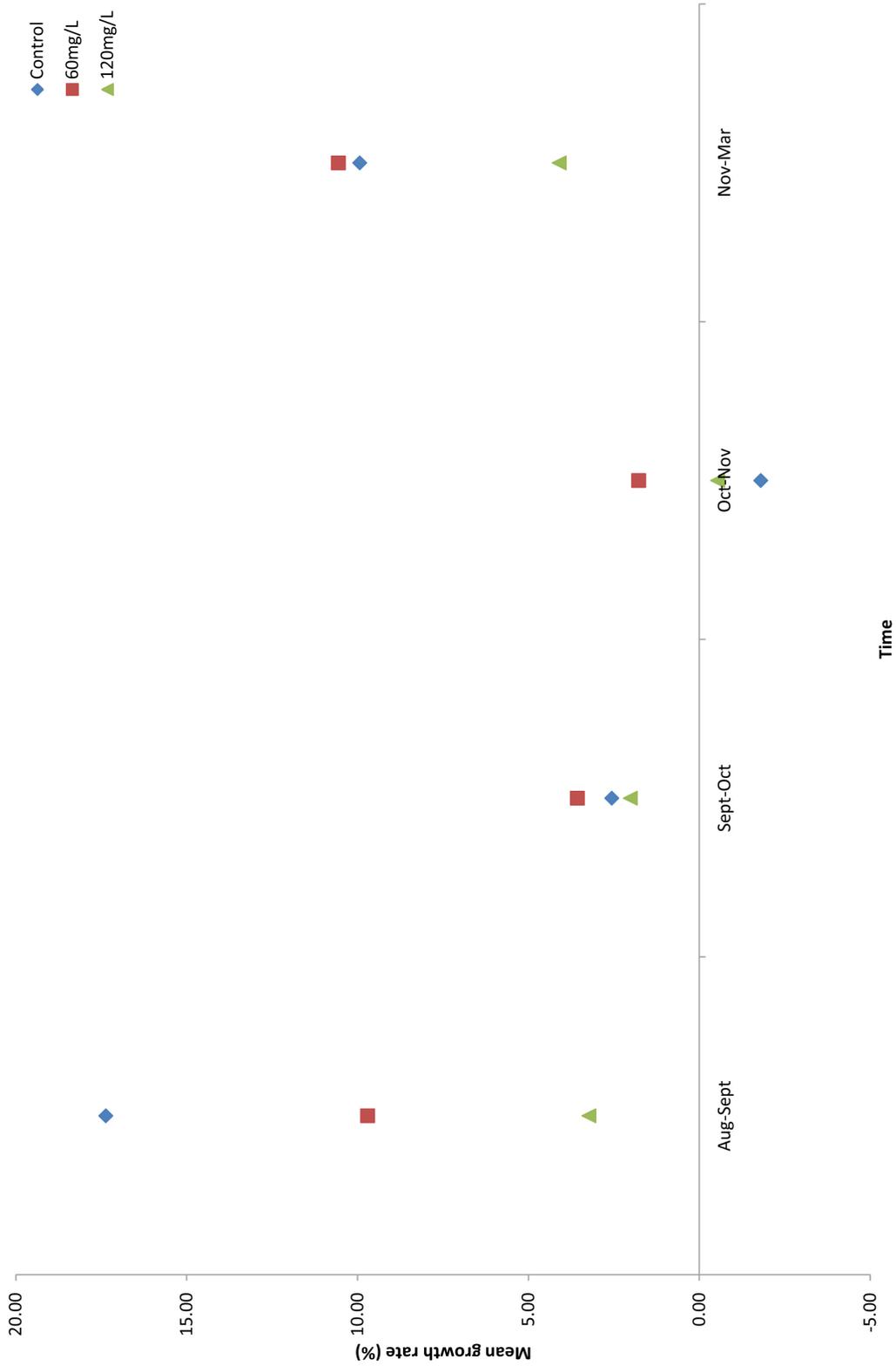


Fig. 6.7: Scatter plot showing growth rates (%) of juvenile freshwater pearl mussels between sampling points for all treatments throughout the experiment.

Considering summary statistics for the different treatments on day 241 (Table 6.1), 95% confidence intervals for the means overlap for the control ($0.72 \text{ mm} \pm 0.02$) and 60 mg/L treatment ($0.71 \text{ mm} \pm 0.02$) indicating individuals are the same size after 241 days of growth. Growth does however appear to be affected in the 120 mg/L treatment with individuals only attaining a mean length of $0.57 \text{ mm} (\pm 0.01 \text{ mm})$ after 241 days. Fluorescence intensity (luminosity) was detected in both calcein treatments but was not observed in the control. Mean luminosity was lower in the 60 mg/L treatment (57.2 ± 1.4) compared to the 120 mg/L treatment (60.5 ± 1.6) (Fig. 6.9), but this difference was not significant ($t_{(35)} = -1.588$; $P = 0.12$).

6.3.2 Embedding

Fragments of embedded juveniles were examined under the fluorescence microscope but no fluorescence could be detected. The Quetol resin was green in colour under the fluorescence microscope but fluorescent juveniles should have fluoresced brighter than the resin. It is thought that a different method of preparing the slides such as described by Eads & Layzer (2002) using a low-speed precision saw to take a thin section before examining under the microscope would have made it easier to see marks but this equipment was not available for this experiment.

6.3.3 Use of the osmotic induction method to test calcein uptake by glochidia

No mortality was observed in either the control or treatment fish after the osmotic induction procedure. Fish scales taken from individuals exposed to calcein fluoresced brightly compared to scales taken from control individuals (Fig. 6.10). Information on each fish is provided in section 6.6. Appendix 3). Out of a possible 100 glochidia, the number of specimens of satisfactory quality for analysis in the calcein treatment and control was 84 and 94 individuals respectively.

Examples of individuals of unsuitable quality were glochidia which had remnants of gill tissue surrounding them, or individuals which were squashed during the handling processes. The number of samples correctly identified as treatment and control were 39 (46 %) and 58 (62 %) respectively. Of these individuals for the treatment and control, 16 % and 21 % respectively were correctly identified with a high level of confidence. There was no significant

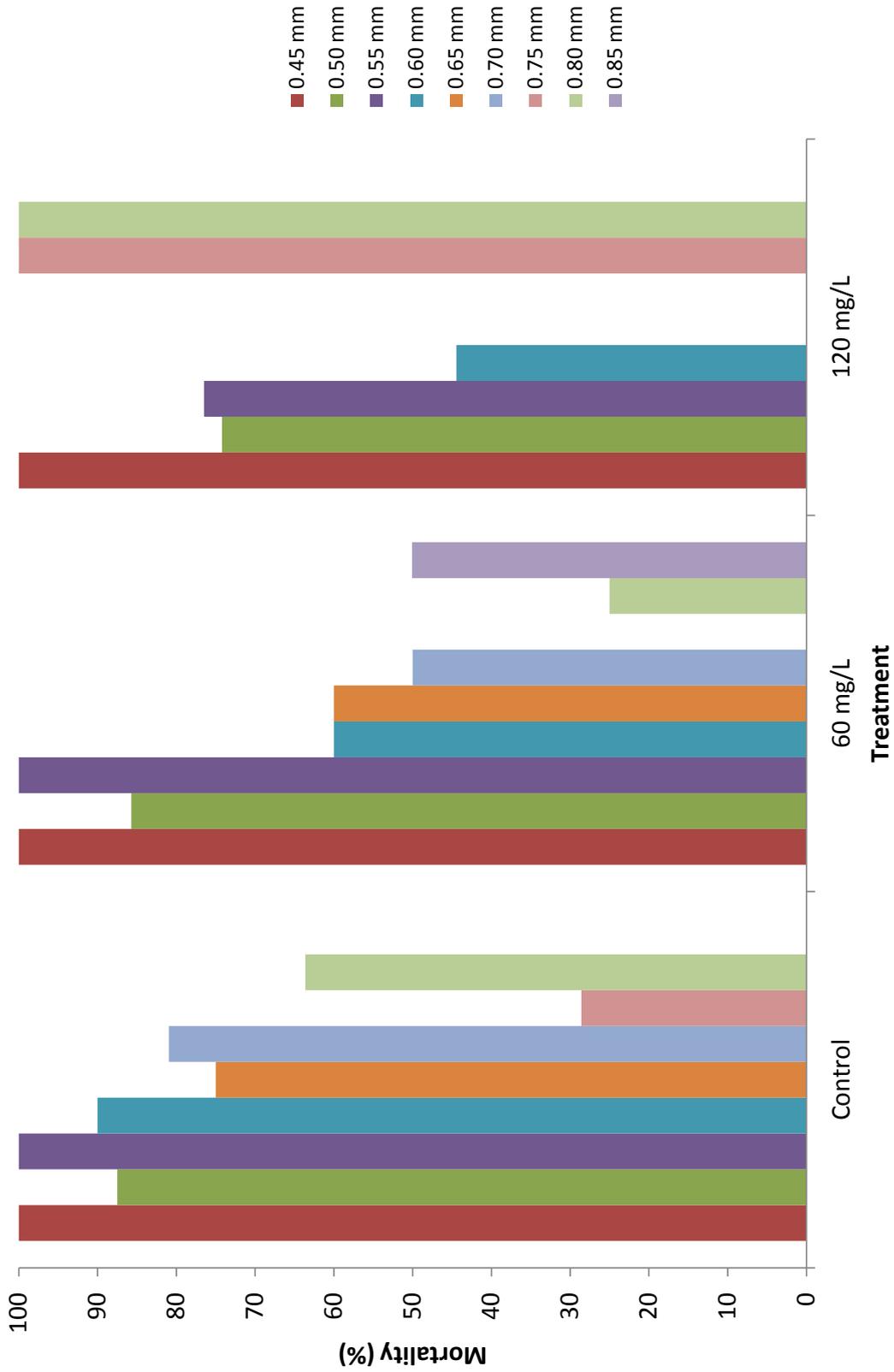


Fig. 6.8: Bar chart showing juvenile freshwater pearl mussel percentage mortality in the different treatments (0, 60 and 120 mg/L calcein solutions) between 99 & 241 days arranged by size class. A higher proportion of smaller juveniles died in the control and the 60 mg/L treatment whereas mortality in the 120 mg/L treatment was more evenly distributed across the size classes.

Table 6.1: Summary shell length (mm) statistics for juvenile freshwater pearl mussels in all treatments (0, 60 or 120 mg/L calcein solutions) on all sampling occasions (1, 38, 68, 99 and 241 days).

	Control					60mg/L					120mg/L				
	1	38	68	99	241	1	38	68	99	241	1	38	68	99	241
Mean length (mm)	0.57	0.67	0.69	0.67	0.72	0.55	0.61	0.63	0.64	0.71	0.52	0.54	0.55	0.55	0.57
SD	0.05	0.10	0.10	0.10	0.08	0.05	0.10	0.11	0.11	0.09	0.04	0.06	0.06	0.07	0.07
SE	0.00	0.01	0.01	0.01	0.02	0.00	0.01	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.02
95% CI	0.01	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01
Max	0.70	0.85	0.85	0.85	0.85	0.70	0.85	0.85	0.85	0.85	0.60	0.75	0.80	0.80	0.70
Min	0.50	0.50	0.50	0.45	0.50	0.50	0.45	0.45	0.45	0.50	0.50	0.45	0.45	0.45	0.50
N	120	83	74	68	18	120	56	53	46	17	120	86	76	64	21

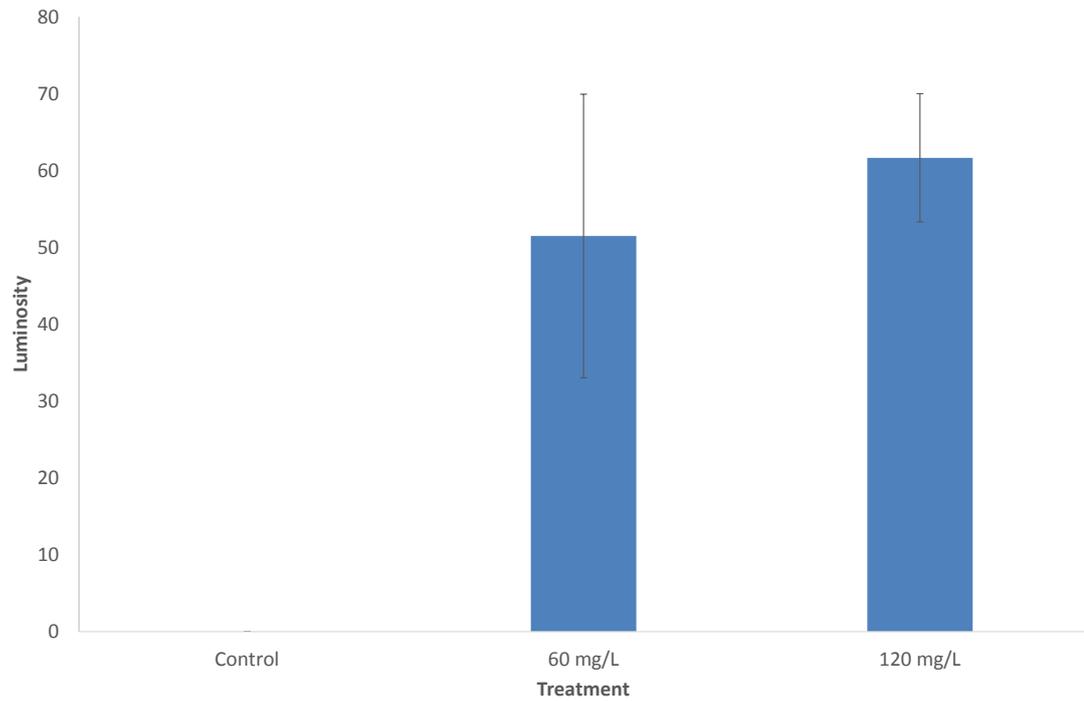


Fig. 6.9: Bar chart of mean luminosity of juvenile freshwater pearl mussels in each treatment (0, 60 or 120 mg/L calcein solutions) after three days immersion. Whilst luminosity in the 60 mg/L treatment was lower than in the 120 mg/L treatment, this difference was not statistically significant ($t_{(35)} = -1.588$; $P = 0.12$). No fluorescence was observed in control individuals.

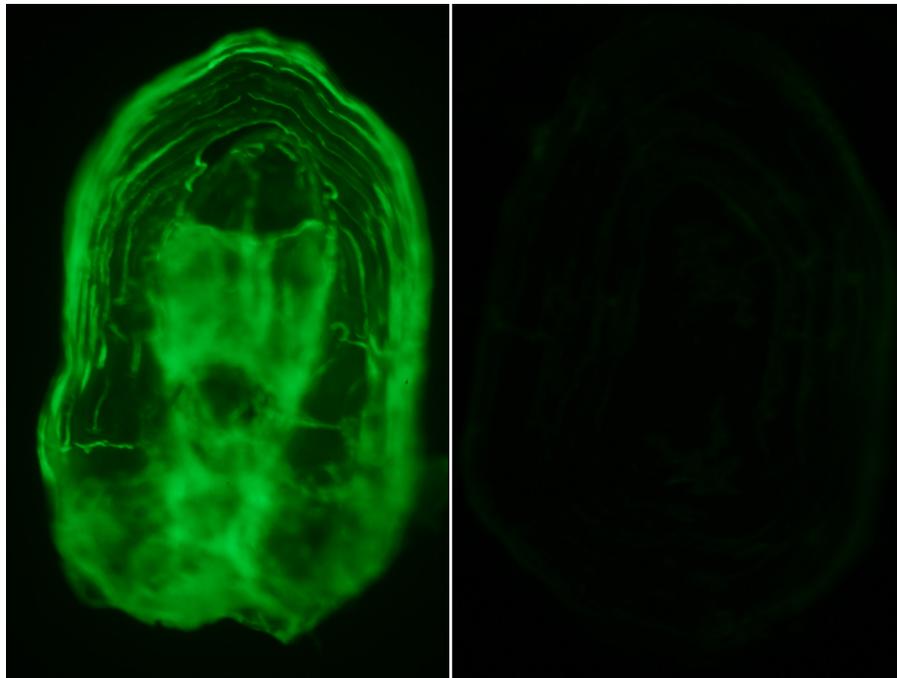


Fig. 6.10: Fluorescence micrograph of salmon fish scale from calcein treatment (left) showing successful marking, and a scale from a control individual (right) showing no fluorescence.

difference in the proportion of individuals correctly identified in the treatment and control ($t_{(18)} = 1.143$, $P = 0.268$). Analysis of fish condition factor found that control fish had a higher condition factor compared with treatment fish ($t_{(18)} = 2.205$, $P = 0.041$) but that the number of glochidia per fish did not differ between control and treatment fish ($t_{(18)} = -1.366$, $P = 0.189$).

Table 6.2 shows that marks (or lack of marks in the control) were more easily identified on some fish compared to others. For example, 80 % of glochidia were correctly identified as controls for fish C1 but only 22 % for fish C7. Similarly for treatment glochidia, 67 % were correctly identified in fish T5 but only 22 % for fish T4. For some specimens it was difficult to identify the presence or absence of marks indicating that this method may need refining before applying to a larger scale study.

Table 6.2: Table showing the number and proportion of freshwater pearl mussel glochidia correctly identified as being fluorescently marked (with calcein) or unmarked from blind review of mark presence/absence. Glochidia were taken from either control (C 1-10) salmon which were not exposed to calcein or from treatment salmon (T 1-10) which were exposed to calcein via osmotic induction.

Fish	N	No. correct	Proportion correct
C1	10	8	0.80
C2	10	6	0.60
C3	10	6	0.60
C4	9	6	0.67
C5	9	8	0.89
C6	10	6	0.60
C7	9	2	0.22
C8	8	4	0.50
C9	10	5	0.50
C10	9	3	0.33
T1	4	0	0.00
T2	10	6	0.60
T3	10	5	0.50
T4	9	2	0.22
T5	9	6	0.67
T6	10	5	0.50
T7	10	3	0.30
T8	9	4	0.44
T9	8	5	0.63
T10	4	3	0.75

6.4. Discussion

The objectives of these investigations were to assess if calcein immersion is a suitable method of batch-marking juvenile freshwater mussels and if so, what effect concentration has on mark intensity, growth and survival. In addition, the method of osmotic induction on encysted fish was tested to see if calcein uptake could be detected in encysted glochidia.

6.4.1 Juvenile marking

Calcein immersion of young (< 4 months old) juveniles provided visible fluorescent marks and appears to have limited acute or chronic effects on survival and growth compared with controls. Fifty six juveniles (16 %) from the 2012 experiment survived for over 241 days before they were added to the mainstream culture trays at the FBA and three juveniles (0.63 %) survived from the pilot study to a minimum of 4 years post-exposure to calcein.

6.4.1.1 Effect of immersion duration

The effect of immersion duration on mark intensity in has been sufficiently covered in Chapter 2 but it should be noted that the slow-growing nature of *M. margaritifera* formed the basis of the decision to keep juveniles immersed for extended time periods compared to other published studies (up to 24 days in the pilot and 3 days in this study). Typically, immersion times of between 12 and 48 hours produce good quality marks in bivalves (Day *et al.*, 1995; Crocker, 1998; Eads & Layzer, 2002; Moran & Marko, 2005; Linard *et al.*, 2011) but immersion durations of as little as four hours have shown good results in some species (Kaehler & McQuaid, 1999). In both the pilot study and the full investigation, detectable marks were observed after 3 days in juvenile *M. margaritifera*. It is difficult to see any difference in mark intensity by simply comparing photographs of individuals (Fig. 2.8 in Chapter 2). However analysis using the Photoshop luminosity method described by Frenkel *et al.* (2002) and Mohler & Kehler (2007) was successful in quantifying fluorescence intensity in juvenile mussels.

6.4.1.2 Effect of calcein concentration

In the pilot study (Chapter 2), luminosity was significantly lower in the 30 mg/L treatment compared to the 120 mg/L treatment but there was no significant difference between any other treatments. This result was replicated in the full experiment with no significant

difference in mark intensity detected between the 60 and 120 mg/L treatments. This agrees with previous studies on other bivalves e.g. Crocker (1998); Eads & Layzer (2002); Linard *et al.* (2011). Day *et al.* (1995) found that increasing calcein concentration above 60 mg/L did not affect mark brightness. This may be because the number of calcium ions in solution is finite and once all calcium ions are bound to calcein, increasing fluorophore concentration will have no additional effect. Marks were not significantly brighter at concentrations of 120 mg/L and it therefore seems likely that this is approaching the maximum useful concentration of calcein in this system.

There was no significant difference in juvenile survival between the control and the two treatments in the 2012 investigation, meaning that immersion in calcein for three days at either 60 or 120 mg/L did not have any measurable negative effect on survival up to approximately 241 days post-exposure. Linard *et al.* (2011) found no mortality in 10 month old pearl oysters (*Pinctada margaritifera*) while Eads & Layzer (2002) found survival depended on the species and age with mortality being low in older juveniles (≥ 4 months old).

Growth rates were retarded in the 120 mg/L treatment compared to the control and the 60 mg/L treatment. This contrasts with the findings of Eads & Layzer (2002), who found no difference in growth between calcein treatments and controls for *Lampsilis cardium* and *Actinonaias pectorosa* of various ages.

6.4.1.3 Suitability of marking for the purpose of monitoring juvenile mussels

Margaritifera margaritifera inhabits slightly acidic rivers where dissolution of the shell may lead to marks being eroded over time. The mark detection rate of calcein in brown trout fry has been shown to decrease over time (Stubbing & Moss, 2007) but this may be due to effects of the fish growing rather than fluorochrome breakdown following exposure to light. In the wild, mussels live buried in gravels so that exposure of marked parts of the shell to light would be minimal, increasing the longevity of the mark compared to species more exposed to light. In captivity however, juveniles are kept in shallow gravels or in some cases, no gravel at all (Lange & Selheim, 2011; Eybe *et al.*, 2013). In these cases, care should be taken to not expose juveniles to direct sunlight.

When observing juveniles under the fluorescence microscope, generally the entire animal fluoresced green but results were variable. For example, in some individuals brighter

marks were observed along the pallial line, in others the gills were brightly marked, while in others the whole animal fluoresced green (Fig. 6.11). It was expected that marks would be bright near the growth edge but it appears that, due to the semi-transparent nature of mussels at this age, the effect of calcein was observed in all soft tissues which were visible through the shell. This may change as juveniles grow and the periostracum becomes thicker but once juveniles are large enough to see with the naked eye, marking for the purpose of improving monitoring becomes redundant. Calcium concentrations are thought to be high along the pallial line where calcium is directed for sequestration along the growth line (Simkiss & Wilbur, 1989). The gills are also sites of higher calcium concentrations and in adult females play a vital role of storing calcium for liberation when glochidia are brooded (Silverman *et al.*, 1987).

The SE Mark Detection Device was successfully used to observe juvenile mussels without a microscope 68 days post-exposure (Fig. 6.4). Marks on individuals from 2010 (27 months old) were not detected. This may have been because subsequent layers of calcium had been sequestered since marking, which obscured the fluorescence. This method of marking is unlikely to be suitable for adult mussels because the periostracum, which does not contain calcium, is relatively thick compared to juvenile mussels and would obscure marks. Handling of mussels and the SE Mark Detection Device had to be undertaken in complete darkness and sampling gravel whilst holding the device was difficult. If this type of device is used in future to observe marked individuals, it is suggested that a modified version is created

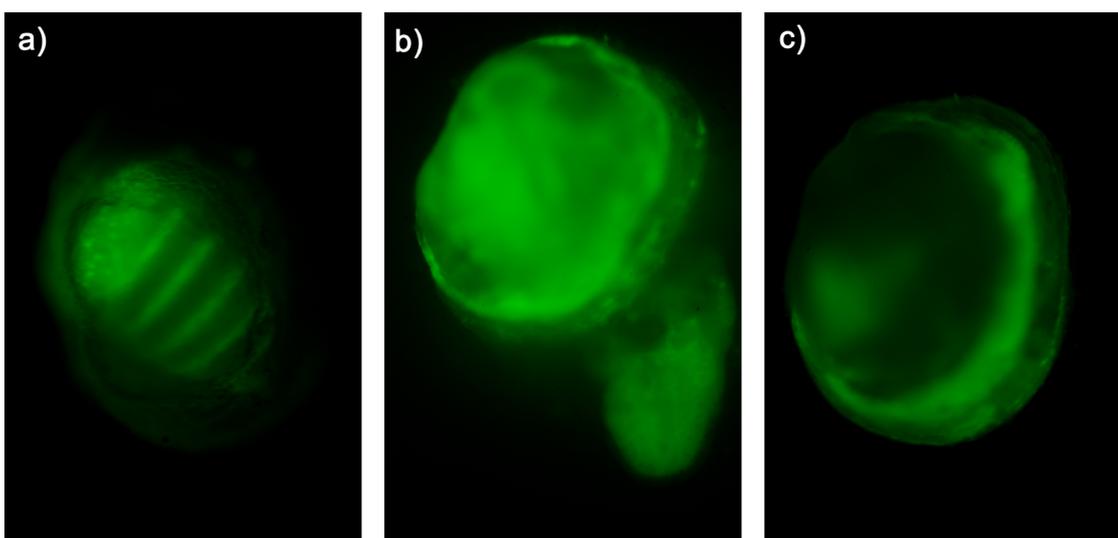


Fig. 6.11: Fluorescence light micrographs of juvenile freshwater pearl mussels showing the variable pattern of fluorescence 99 days post-exposure to calcein; a) Gill filaments fluorescing brightly through shell (60 mg/L treatment); b) More uniform fluorescence over most of shell (3 days post-exposure, 120 mg/L treatment); c) Bright fluorescent band with bright spot on shell (60 mg/L treatment).

which can be attached to the sampler's head (similar to wearing goggles) so that both hands are available for sampling activities. Alternatively, the method described by Moran (2000) using a dissecting microscope and a fibre-optic light, both fitted with suitable filters, could also provide a convenient method for observing juveniles in samples of substrate.

The method employed here of embedding juveniles in a resin and freeze fracturing in order to observe which specific part of the shell was fluorescing was not successful. Well established methods of using a low speed precision saw and smoothing sections should be employed as by Eads & Layzer (2002), but this facility was not available to the author at this time.

6.4.2 Osmotic induction

The method outlined in this study for using osmotic induction as a method for marking glochidia was successful but results were highly variable. The ability to discern fluorescent marks appeared to depend upon the host fish and the author could attribute a high level of confidence to only 16 % of cases in marked individuals. This method requires refinement but is the first known example of attempting to mark this life cycle stage. Osmotic induction has previously been used to mark Atlantic salmon (Mohler, 2003), brown trout (Stubbing & Moss, 2007) and juvenile mussels (U.S. Fish and Wildlife Service, 2002), but searches of the literature found no mention of attempting to mark glochidia encysted within salmonid gills.

Fluorescent marks were correctly identified only 46 % of the time for treatment glochidia. Table 6.2 shows that correct identification was high in some fish but not in others. A larger sample size may determine if there are indeed differences in calcein uptake depending upon the individual fish. As expected, calcein-marked fish scales showed very bright marks compared with scales from control fish but it appears that calcein uptake by glochidia did not occur to a similar degree under these conditions. The exposure time of 3.5 minutes followed by a 24 hour holding period does not appear to be sufficient for glochidia to become marked, at least to a level detectable for samplers to reliably observe. Longer exposure to the calcein bath or holding fish for longer post-exposure (perhaps a week or more) may produce clearer results. Arey (1932) found that glochidia derive nutrients from the 'tissue juices' of fish hosts but the timing of exposure to calcein or holding period may play an important role in uptake success. It is most likely that glochidia will take up calcein when they are going through a growth phase

so the timing of exposure may be important. There is also the question of whether marks may degrade faster in glochidia compared with juveniles due to their relatively low calcium content. Regardless of these considerations, marks were clearer in glochidia originating from some fish compared to others and this emphasises the variability between host fish individuals, which was not addressed in this study.

The effects of calcein exposure to glochidia shortly after release from the female mussel (before encystment) are unknown. Marking glochidia pre-encystment may be a simple way to batch-mark glochidia, removing the need to mark fish or juveniles at a later stage. Going one stage further, however, it may be possible to expose adult female mussels to calcein before brooding when calcium stores are being sequestered as concretions in the gills (Silverman *et al.*, 1987). This may allow female mussels to build up a store of calcein-labelled calcium for transfer to directly to glochidia during brooding. Marking at this stage, however, may produce marks with limited longevity, as glochidia grow over five times their original length on fish and juveniles then grow rapidly through their first growth season as juveniles.

It is clear that further investigations are required to consider marking various life stages of *M. margaritifera* to establish the best protocol for this species. The immersion method was successful in producing lasting marks on juvenile mussels and has potential to be useful for improving monitoring in captive rearing settings. The osmotic induction study presented here utilises a previously-described method but employs it for a new application. Mark intensity was variable between fish and further investigation is required to establish if marking glochidia pre-encystment or marking adult mussels before brooding provides a suitable alternative.

6.4.3 Conclusions

- This work comprises the first known investigations into marking different life cycle stages of *Margaritifera margaritifera* with calcein for the purposes of improved monitoring in a captive rearing setting.
- The recommended marking procedure for young (< 4 month old) juveniles is immersion in calcein at a concentration of 60 mg/L for three days at temperatures of approximately 17 °C. This procedure should produce detectable marks without compromising juvenile growth or survival.

- The first known example of attempting to mark glochidia encysted on host fish is described here. Results were variable depending upon individual host fish and work in future should focus on increasing sample size to determine whether this was an artifact of small sample size or a true effect. Future work could also attempt to mark free-living glochidia (before encystment) or adult female mussels before brooding.

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6.6. Appendix 3

Fish (salmon) fork length, weight, approximate number of attached freshwater pearl mussel glochidia and fish condition factor (CF).

	Code	Fork length (cm)	Weight (g)	Approx. no of glochidia attached	CF
Fish 1 - Control	F1C	7.9	4.90	600	0.99
Fish 2 - Control	F2C	7.1	3.68	300	1.03
Fish 3 - Control	F3C	8.3	6.10	300	1.07
Fish 4 - Control	F4C	7.0	3.37	700	0.98
Fish 5 - Control	F5C	8.0	5.84	400	1.14
Fish 6 - Control	F6C	7.4	4.57	500	1.13
Fish 7 - Control	F7C	6.9	3.45	400	1.05
Fish 8 - Control	F8C	7.3	3.72	600	0.96
Fish 9 - Control	F9C	7.5	4.63	300	1.10
Fish 10 - Control	F10C	7.8	4.73	200	1.00
Fish 1 - Treatment	F1T	8.4	5.40	600	0.91
Fish 2 - Treatment	F2T	7.4	3.54	500	0.87
Fish 3 - Treatment	F3T	7.4	3.63	600	0.90
Fish 4 - Treatment	F4T	7.3	4.00	500	1.03
Fish 5 - Treatment	F5T	7.2	3.80	400	1.02
Fish 6 - Treatment	F6T	7.9	5.10	700	1.03
Fish 7 - Treatment	F7T	7.5	4.16	400	0.99
Fish 8 - Treatment	F8T	6.3	2.22	700	0.89
Fish 9 - Treatment	F9T	8.0	5.19	200	1.01
Fish 10 - Treatment	F10T	6.7	3.30	700	1.10

Chapter 7

Discussion and conclusions

7.1. Introduction and restatement of research questions

The aim of the research presented in this thesis was to investigate factors affecting the growth and survival of juvenile freshwater pearl mussels, *Margaritifera margaritifera* (Linnaeus, 1758), in order to contribute to the conservation of this critically endangered species. The increase in the number of captive rearing programmes for *M. margaritifera* within the last three decades, and the onus to produce sufficient numbers of juveniles to save dwindling populations from extinction, has necessitated a better understanding of the factors affecting juvenile survival in order to maximise captive rearing outputs. This work focused on areas of study which have traditionally been difficult to achieve in the wild due to the small size, slow growth rate and scarcity of juveniles. The findings of this thesis contribute significant information on the importance of various interstitial parameters to juvenile survival and growth in captivity during the first 25 months post-excystment. This work also shows that it is possible to tailor habitat conditions to improve survival rates for unimpacted wild populations (Young & Williams, 1984; Bauer, 2001). Suggestions for improved rearing practices are proposed, including a potentially time-saving method of marking with a fluorochrome to aid monitoring or as a tool for batch identification for research and captive rearing purposes alike. Behavioural and ultrastructural studies using scanning electron microscopy (SEM) during the first 44 months post-excystment are the first known attempt to document ontogeny in *M. margaritifera* and its relation to feeding behaviour and mode during the period juveniles switch from pedal to filter feeding.

Freshwater molluscs (Unionida) are among the most endangered invertebrates in the world (Machordom *et al.*, 2003; Primack, 2006) and are disproportionately imperiled compared to other groups (Williams *et al.*, 1993). They provide valuable environmental services for a wide range of taxa from the bottom to the top of the food chain, and *M. margaritifera* has been described as simultaneously fulfilling the criteria of a keystone, umbrella, indicator and flagship species (Geist, 2010). Attributing these roles to the freshwater pearl mussel demonstrates its importance in aquatic systems and highlights the need for a swift and urgent response to its catastrophic decline during the 20th Century (Bauer, 1988). Regardless of these ecological labels and its recent upgrade on the IUCN Red List of Threatened Species from 'endangered' to 'critically endangered' (Moorkens, 2011), numbers in the wild remain low and many populations are at imminent risk of local extinction.

The number of captive rearing programmes for *M. margaritifera* has increased substantially in Europe over the last 30 years in response to the urgent requirement to maintain the current distribution of the species at both a local and global level. As well as providing the important function of rearing juveniles for population augmentation, rearing programmes also provide unique opportunities to study aspects of the life cycle in controlled environments. For many populations, captive rearing offers a final chance to conserve the genetic integrity of populations from different rivers (Geist & Kuehn, 2005; Jones *et al.*, 2006; Cauwelier *et al.*, 2009; Karlsson *et al.*, 2014), and provides time for ecologists and practitioners to ameliorate the threats to pearl mussel survival in rivers (Atkins, 2012; Freshwater Biological Association, 2013; Horton *et al.*, 2015). Habitat restoration in pearl mussel catchments is difficult and time-consuming due to the size of catchments and the complex range of threats the species faces, such as aggravated sediment and nutrient loading, changes in flow dynamics and habitat structure, loss or displacement of local host fish species and, in some areas, destructive pearl fishing. Even achievement of 'High Ecological Status' as set out in the EU Water Framework Directive does not adequately protect pearl mussel populations as this legislation does not consider the quality of the interstitial environment, which is critical to juvenile mussel survival. The recent introduction of water quality equipment capable of logging some interstitial parameters consistently over extended periods of time (Malcolm *et al.*, 2008; Quinlan *et al.*, 2014), has improved upon traditional methods of spot sampling in pearl mussel rivers e.g. Buddensiek *et al.* (1993); Geist & Auerswald (2007), and has enabled a more complete understanding of the issues facing juvenile mussels in individual catchments. Ecological studies and pearl mussel surveys have long documented the absence of juvenile cohorts in areas where adults survive (Ziuganov *et al.*, 1994; Geist *et al.*, 2010; Moorkens, 2010; Simon *et al.*, 2015) but the frequency and precision of environmental data collection has, thus far, been insufficient to draw definitive conclusions about the specific causes of absences in these areas. More long-term investigations employing high-precision loggers which log at an appropriate frequency are required; every 15 minutes has been adopted by some pearl mussel practitioners and was thus adopted for DO measurements in Chapter 4 of this thesis (Moorkens, E.A. & Killeen, I.J. pers. comm.). Linking data on water column and interstitial water quality is also important and provides information on how the surrounding catchment and the river function together.

Information about juvenile mussel biology and ontogeny are particularly sparse in the primary literature, with only a handful of studies conducted on freshwater species (Hudson & Isom, 1984; Kovitvadhi *et al.*, 2001; Neumann & Kappes, 2003; Kovitvadhi *et al.*, 2007), and hardly any specifically on *M. margaritifera* (Le Pennec & Jungbluth, 1983; Nezlin *et al.*, 1994). There is a paucity of information on how juveniles develop and how sub-optimal habitat conditions may affect their survival. The recent proliferation of captive rearing programmes has made research into this under-studied topic possible, and the findings are important to inform rearing programmes and tailor rearing practices to age/size-specific requirements. For example, juveniles may have changing dietary requirements or habitat preferences depending on their developmental stages (Henley *et al.*, 2001) which need to be understood in order to tailor captive rearing programmes and improve success.

The original research questions posed at the beginning of this work focused on three main areas, summarised here and explored in more detail in the following sections:

- 1. What are the key environmental factors affecting juvenile survival and growth in captivity?** This work focused on designing a successful flow-through system and investigating the importance of environmental parameters in order to better understand the factors limiting juvenile survival and growth in captivity.
 - 2. What ontogenic stages do juvenile mussels undergo in their early post-parasitic life and how might these affect feeding behaviour, the switch from pedal to filter feeding and survival?** This work is the first attempt to document the biological development of juvenile *M. margaritifera* in the early post-parasitic stage and beyond, up to 44 months old. Feeding behaviour was also compared at different developmental stages and the biological and behavioural changes which take place during the period that juveniles switch from pedal to filter feeding were investigated.
 - 3. Is fluorescence marking with calcein a feasible method to improve monitoring of immature stages of *M. margaritifera* in captivity?** This research considered the feasibility and usefulness of marking newly-excysted juveniles with the fluorophore calcein. The effects of different calcein concentrations and immersion durations on growth and survival were considered and a suggested marking protocol outlined for use with *M. margaritifera*. Also considered was the first known attempt in any mussel species of marking encysted glochidia via osmotic induction of host fish.
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7.2. Key findings and significance for captive breeding programmes

Here the salient findings from this thesis are explored and put into context for captive rearing of *M. margaritifera*. First, the biological development of juvenile mussels is considered and how this may affect survival and growth. Secondly, a successful flow-through system for rearing juveniles is outlined and the effects of interstitial substrate conditions on juvenile mussels within this system are described. Finally, a method of batch-marking juvenile mussels with a fluorophore is described and its usefulness for captive rearing programmes explored.

7.2.1 Effect of interstitial substrate conditions on growth and survival of juvenile mussels

This body of work describes a successful and low maintenance captive rearing system for *M. margaritifera* capable of rearing substantial numbers of juveniles up to 25 months old before they are transferred to systems designed for larger mussels (Lavictoire *et al.*, 2016). Larger substrates (1 - 2 mm) cleaned monthly provided the best survival rate and the lowest maintenance effort of all treatments investigated. Utilisation of the system described in Chapter 3, employing 1 - 2 mm substrate and cleaning monthly could achieve survival of at least 820 juveniles to 25 months old (23 % survival). As has been shown in numerous studies on other unionoids, mortality is high during the first few weeks post-excystment (Gatenby *et al.*, 1997; O'Beirn *et al.*, 1998; Jones *et al.*, 2005; Gum *et al.*, 2011) leading to low overall survival during the first year post-excystment (34 % across all treatments in this study). Survival improved slightly during the second year (43 % of surviving juveniles from year 1 across all treatments) and was highest in the third year and beyond (78 % survival between 25 – 45 months old in the tray system described in Chapter 2). This suggests that different systems can be utilised at different ages for *M. margaritifera*, as is the practise in captive rearing programmes on North American freshwater mussel species (Barnhart, 2015). These investigations have improved captive rearing success at the FBA's Freshwater Pearl Mussel Ark and subsequent counts of juveniles from the 2012 cohort confirm that this system is still the most successful medium-term (~4 years) rearing effort for *M. margaritifera* published to date (Fig. 7.1).

Additional investigations were carried out to consider parameters thought to affect juvenile survival and growth. These parameters were substrate interstitial space (volume),

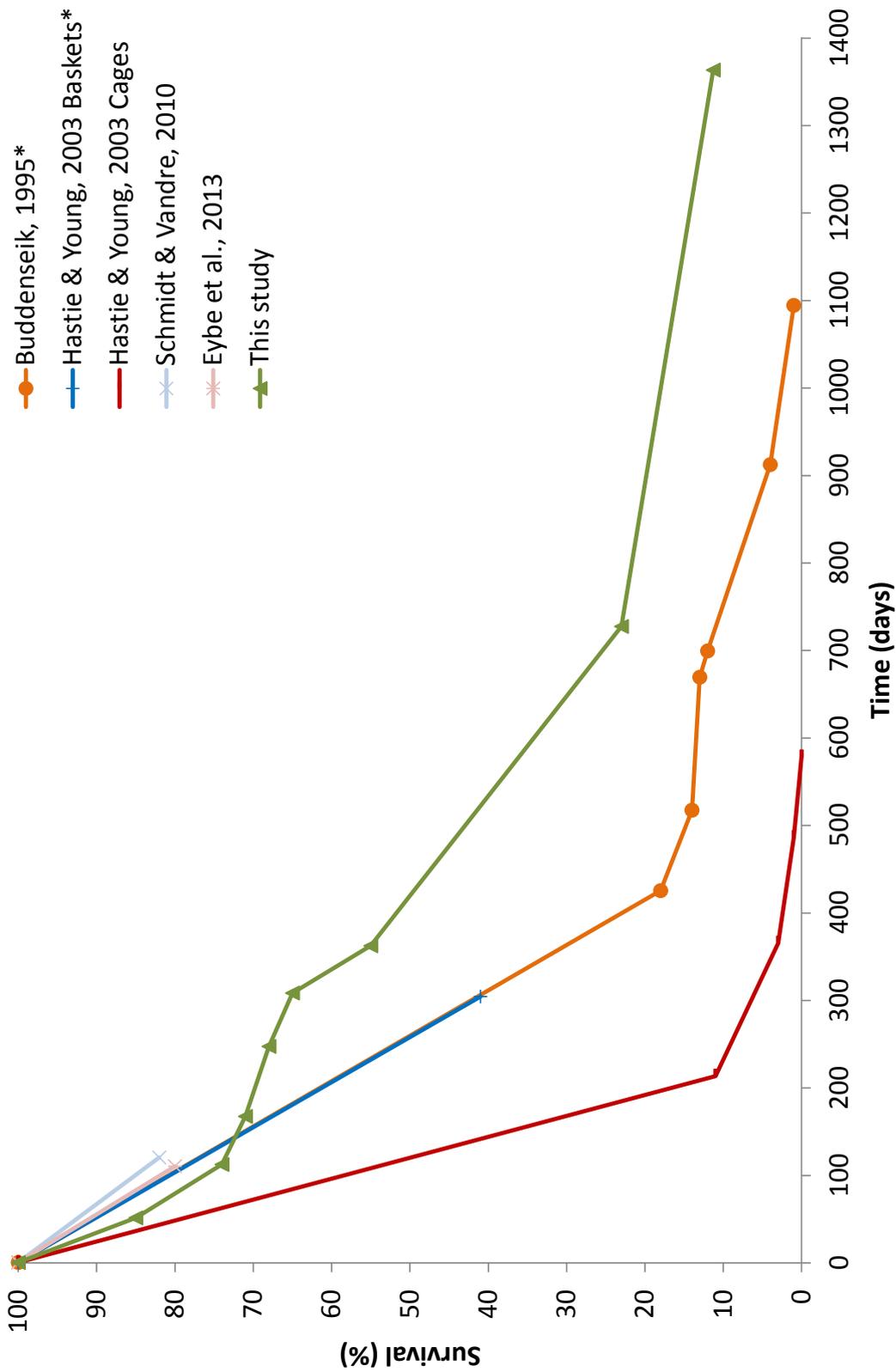


Fig. 7.1: Scatter plot showing survival of juvenile *M. margaritifera* from this study (to date) and other published studies showing that medium-term survival (up to 3.5 years here) remains the highest compared with other (currently published) rearing programmes.

flow rate, ammonia and total phosphorus (from organic matter) concentrations, biomass from biofilm and organic content, and dissolved oxygen (DO). Dissolved oxygen and flow were the most important habitat parameters affecting juvenile survival at this facility using this system. This agrees with findings of previous studies in the wild on pearl mussel rivers (Geist & Auerswald, 2007; Liberty *et al.*, 2007; Quinlan *et al.*, 2014). The species requires near-pristine habitat conditions and because juveniles inhabit the top 5 - 10 cm of substrate for the first few years of their post-parasitic lives, they are particularly vulnerable to degraded substrate conditions. Biological limitations (discussed in section 7.2.2 below) potentially make them vulnerable to low DO concentrations and high suspended solids loads and this is why poor exchange between the hyporheic zone, or high suspended solids loads are particularly damaging to juveniles. These issues are major contributors to juvenile mortality and likely the reason juvenile mussels are absent from the majority of pearl mussel rivers.

The Loss On Ignition reported in Chapter 4 compared favourably with functional pearl mussel rivers (Tarr, 2008) indicating that, whilst Windermere is a mesotrophic lake, the amount of organic matter may not be a cause for concern. However, the combination of small substrate size (and therefore smaller interstitial spaces) and the amount of organic matter created very low flow conditions in the 0.25 - 1 mm treatment cleaned monthly (Fig. 4.10) which had a detrimental effect on DO (Fig. 4.5). Low DO and high suspended solids can cause juvenile mussels to reduce their filtering activity. In these investigations, larger substrates which allowed for flow rates of between approximately 0.23 - 2.42 L/min appeared to maintain sufficient DO concentrations for high juvenile survival.

Ammonia and total phosphorus concentrations did not differ significantly between the treatments and did not appear to affect juvenile survival in this system. It is possible that the biofilm community adhered to the substrate may have contained nitrifying bacteria which could convert ammonia into less toxic compounds, but biofilm community structure was not considered during this study. Additionally because the system is not a static or recirculating one, ammonia build-up should not have occurred in the same way as was observed by Eybe *et al.* (2013) in their incubator system. Although the total phosphorus bound up in organic matter within the interstices was very high, these concentrations do not represent available phosphorus, but do indicate the enriched state of water in Windermere. Whilst outside of the scope of these investigations, a better understanding of juvenile diet is required to see

how much of the organic content (including biofilm) is usable for juveniles and how the algal and bacterial species and concentrations compare with those found in recruiting pearl mussel rivers.

Additional studies on juvenile density within the aquarium system described here are required to see if output can be increased. Eybe *et al.* (2013) found that survival was unaffected at densities between 200 - 400 juveniles but that individuals were significantly larger in treatments containing fewer individuals. The current investigations found no size-dependent over-winter survival so regular monitoring of survival and growth at different densities would provide additional information about if the speed of growth during early juvenile stages affects survival later in life.

7.2.2 Biology and ontogeny of feeding structures in juvenile freshwater pearl mussels

Gill ontogeny in *M. margaritifera* is slower than documented for other bivalves e.g. Veniot *et al.* (2003), Kovitvadhi *et al.* (2007), Cannuel *et al.* (2009), Trump (2010). This is not surprising because the species is long-lived and slow growing and may partially explain why juvenile *M. margaritifera* are particularly sensitive and difficult to rear in captivity. Younger and smaller juveniles are more active than older and larger ones indicating that they must expend more effort 'foraging' for food (pedal feeding) compared to larger individuals which have a more developed gill pump and are capable of filter feeding. This hypothesis is backed-up by ultrastructural observations of young juveniles (< 13 - 16 months old) which show simple, unreflected gills with no obvious method of transporting food particles from these filaments towards the mouth. Whilst the full suite of cilia and cirri are present even in the youngest individuals investigated (1 month old), particle transport and sorting processes cannot be well established given the simple arrangement of filaments, lack of oral groove and the under-developed labial palps. Particle transport towards the mouth may occur via water currents near the gill filaments at this stage, but further investigation is required to confirm this. Gill reflection and therefore the onset of the more developed and functional condition begins around 13 - 16 months old (> 1.2 mm in length) although it is not until approximately 25 - 34 months old (> 3 mm in length) that the oral groove develops and the outer demibranch begins to form. The adult gill condition was not observed even in the largest/oldest individuals

considered for this study (44 months old, 8.9 mm long) because juveniles still did not possess the tissue bearing ostia attached to the abfrontal surface of filaments. Four stages of development are proposed for *M. margaritifera* (Table 5.6 on page 148) although additional data from a wider size and age range from different populations would help confirm and refine these stages.

Measurements of inter-cirral distance (distance between laterofrontal cirri couplets) and the number of cilia per laterofrontal cirrus from individuals at various ages and developmental stages indicates that juveniles are capable of retaining very small particles (<2 μm). This builds upon previous findings that adult *M. margaritifera* preferably ingest particles <4 μm (Baker & Levinton, 2003) and provides an important insight into why the species may be particularly vulnerable to degraded habitats. The major impacts facing pearl mussel streams are aggravated erosion delivering fine particulate matter into the interstitial zone and increased nutrient loading leading to eutrophication. Juvenile mussels inhabit the interstices for around the first 5 - 10 years of their post-parasitic life (Moorkens *et al.*, 2007) and are therefore vulnerable to sub-optimal substrate conditions during this time. Fine silt and organic matter can clog interstices decreasing water exchange with the shallow hyporheic zone and reduce DO and food delivery (see section 7.2.1). High levels of solids (both organic and inorganic) in the interstices may cause juveniles to expend more energy attempting to clear unsuitable particles by increasing the production of pseudofaeces, or reduce filtering activity to avoid inhalation of these particles. Reduced filtration due to high particulate concentration has been noted by several authors (see Jørgensen, 1996; Barnes, 2006) and references therein) and can lead to anaerobic respiration, reduced metabolism, decreased tissue pH and reduced oxygen consumption, which may be particularly exaggerated in species like *M. margaritifera* which requires high DO concentrations (De Zwaan & Wijsman, 1976; Chen *et al.*, 2001). Due to their gill morphology allowing effective capture of very small particles (<2 μm), increased silt loading and primary production should be considered major stressors for the species.

For this investigation juveniles from several age cohorts (2012, 2014 and 2015) from a single population reared at the FBA Freshwater Pearl Mussel Ark were used. The study would have benefitted from having a complete time series of juveniles as additional observations could have been made on developments such as gill reflection and how quickly it happens (gap from 16 - 20 months old), development of the oral groove and onset of outer

demibranch development (gap from 20 - 34 months), the mode and timing of development of interfilamentary junctions (gap from 34 - 44 months) and the abfrontal tissue bearing ostia (+ 44 months). This study was limited to observations on structures particularly thought to be involved in feeding and focused mainly on gill ontogeny. Further work is required on other pertinent features such as siphonal development and a more comprehensive consideration of mantle ciliation and labial palp development and ciliation. Finally, relatively low numbers of measurements and counts were taken when considering the number of cilia per laterofrontal cirrus, inter-cirral spacing and interfilamentary spacing. Increasing the number of measurements taken and the number of populations over which observations are made would help refine the stages of development outlined in Chapter 5. Additionally, similar studies on other margaritiferids and other freshwater mussel families would be interesting and may contribute information on ecological requirements/limitations of different species as well as phylogenetic positioning.

7.2.3 Using fluorescence marking as a monitoring tool for juvenile mussels

The fluorochrome calcein was used to mark young juveniles and was successful at producing marks that lasted until at least 244 days post-marking. The suggested marking protocol is to immerse juveniles in calcein at a concentration of 60 mg/L calcein over three days. This was the first known attempt to mark such a slow-growing bivalve in this way and the pilot study attempted the longest known immersion of juveniles over 24 days. Even at the highest concentration considered in this pilot (120 mg/L), this immersion duration was not acutely toxic to juvenile mussels although the marks created were not significantly brighter than the suggested marking procedure above. Fluorescence marking can be a useful tool for a wide variety of applications including monitoring juveniles in captivity, monitoring shell growth rates, linking growth patterns with environmental conditions, or distinguishing juveniles from protected sites to tackle poaching (Bolton & Dey, 1979; Cáceres-Puig *et al.*, 2011; Ambrose *et al.*, 2012). It can also be a useful tool for monitoring augmentation methods (Eads & Layzer, 2002), particularly where augmentation is taking place in populations with some natural recruitment to distinguish introduced from resident juveniles.

Substrate investigations (Chapter 4) have shown that flow through substrates is important for DO delivery. Therefore a slightly modified aquarium which uses the same concept of an upper and lower chamber as described in Chapter 3, and which recirculates calcein-labeled water so that there is flow through the substrate may decrease stress, increase feeding activity (and calcein uptake) and increase survival. It is recommended that this modification is made to the system for future marking activities for juvenile *M. margaritifera*.

Also described here is the first known attempt to mark glochidia encysted within fish gills. Whilst results were variable and appeared to be affected by the specific fish from which glochidia originated, this work outlines a potentially time-saving way to batch-mark the glochidial stage. Future work should focus on the effects on mark identification and intensity of increasing the concentration of the calcein bath, increasing the exposure duration, or increasing the recovery period post-marking beyond that used in this study (0.5 % calcein bath for 3.5 minutes followed by 24 hour recovery period before being sampled). Additionally, marking fish with dietary calcein has produced clear and consistent marks on salmon scales when fed over 5 consecutive days (Honeyfield *et al.*, 2006) and may be an alternative for marking the late glochidial stage just before excystment. Exposure of female adult mussels to calcein-labeled water before brooding, as calcium concretions are being sequestered within the gills (Silverman *et al.*, 1985) may be another effective way of creating marked glochidia without exposing immature life cycle stages directly to calcein. It is not known how long marks would persist in encysted glochidia and if they would still be present in juveniles on excystment but this concept warrants further consideration. Exposure of calcein to direct sunlight has been shown to decrease mark intensity (Honeyfield *et al.*, 2008) but loss of fluorescence should be minimal because, depending upon the method of marking, the only potential exposure to sunlight would be when glochidia are released from the female mussel (before attachment to fish gills) or upon excystment from the host fish (before establishment in the river bed).

Low juvenile survival observed in the pilot in 2010 and the main immersion investigation in 2012 are likely due to the system in which juveniles were maintained post-experiment not providing sufficient flow and DO rather than due to calcein toxicity. Findings from the 2012 and 2015 substrate experiments have led to improvements being made to the aquarium system so marking activities via immersion in the future should produce marks of the same or better intensity and increase long-term juvenile survival.

7.3. Conclusions and practical implications

These investigations have demonstrated several factors contributing to the vulnerable nature of juvenile *M. margaritifera* during the first few years of their post-parasitic life, which are summarised in Table 7.1. A more complete understanding of the factors affecting survival and growth, timings of key biological development and stressors, and the conditions in which juveniles are maintained are critical to improving the success of rearing programmes and increasing the numbers of juveniles for augmentation purposes. This is particularly important when wild population levels are critically low, as is the case for several populations held at the FBA Freshwater Pearl Mussel Ark.

As shown in Table 7.1, the first two years post-excystment have several high risk periods. Factors affecting juveniles during this period include initial viability of juveniles post excystment (have they achieved sufficient development and do they possess sufficient nutritive reserves to be viable juvenile mussels?), lack of developed gills requiring an active feeding mode (pedal feeding), low DO concentrations and high primary production during summer months potentially leading to decreased filtration rates due to stressful environmental conditions (Jørgensen, 1990) and the onset of significant morphogenesis as gill reflection begins and progresses (Table 5.6). After this first two-year period the number of risk factors decreases somewhat as biological development progresses but higher summer temperatures will always present an elevated risk for juveniles (lower DO and higher primary production) particularly in environments suffering from nutrient enrichment. Ensuring adequate flow and low suspended solids concentrations during these periods will help minimize stress and mortality. In the wild, additional risk factors are present such as more extreme high temperatures in summer (smaller streams and rivers are more sensitive to air temperature), a more variable flow regime throughout the year leading to additional opportunities for juveniles to be washed out, or for low-flow conditions to cause low DO conditions. Hauer (2015) reviews hydro-morphological management techniques employed in pearl mussel rivers which can lead to better interstitial flow and the stabilization of juvenile mussel habitat.

The major conclusions from this thesis and their practical implications (below) should inform captive rearing practices going forward and particular focus should be given to rearing larger numbers of juveniles through the initial high-risk two year period:

Table 7.1: Summary of main periods during the first approximately 4 years post-excystment outlining whether the mortality risk to juvenile freshwater pearl mussels is deemed to be low, medium or high and which factors may contribute to increased mortality during those periods. *N.B. The mortality risk depends upon juvenile size and developmental stage and may therefore occur at different times in other captive rearing systems. Practitioners should consider how their individual rearing systems affect juvenile development and may therefore affect the timing of high-risk periods.*

Timing	Age (months)	Time of year	Mortality risk	Important considerations and potential risk factors
First growth season	0 – 4	June – October	High	<ul style="list-style-type: none"> • Insufficient reserves laid down as glochidia. • Minimally-effective filter-pump so must be very active to forage - high metabolism and DO requirement. • Lower DO concentrations due to high summer temperatures. • Higher primary production of potentially unsuitable algal species leading to decreased filtration rate (clamming).
First winter	4 – 11	October – May	Low	<ul style="list-style-type: none"> • No significant ontogenic changes. • Minimal temperature and DO risk factors. • Primary production lower than in summer so less energy required to clear excessive/unsuitable algal loads.
Second growth season	11 – 16	May – October	High	<ul style="list-style-type: none"> • Gill reflection begins (size dependent) – may require excess energy/increased metabolism. • Lower DO concentrations due to high summer temperatures. • Higher primary production of potentially unsuitable algal species leading to decreased filtration rate (clamming).
Second winter	16 – 23	October – May	High	<ul style="list-style-type: none"> • Gill reflection begins/is ongoing (size dependent). May require excess energy/increased metabolism.
Third growth season	23 – 28	May – October	Medium/High	<ul style="list-style-type: none"> • Development of oral groove and outer demibranch begins. Unsure of other ontogenic changes during this period – requires further investigation. • Lower DO concentrations due to high summer temperatures. • Higher primary production of potentially unsuitable algal species leading to decreased filtration rate (clamming).
Third winter	28 – 40	October – May	Low	<ul style="list-style-type: none"> • Outer demibranch development progresses (size dependent). May require excess energy/increased metabolism.
Fourth growth season	40 – 45	May – October	Medium	<ul style="list-style-type: none"> • Lower DO concentrations due to high summer temperatures. • Higher primary production of potentially unsuitable algal species leading to decreased filtration rate (clamming).
Fourth winter season	45 – 52	October – May	Low	<ul style="list-style-type: none"> • Minimal known risk factors - requires further investigation.

- **The culture method described in this thesis using a down-welling, flow through system with 1 -2 mm substrate cleaned monthly is a successful and low maintenance system.** The substrate size allows sufficient flow through substrate interstices to prevent clogging and the size is distinct from newly-excysted juveniles making sampling faster and more effective. The system allows juveniles to be exposed to a natural temperature regime and natural fluctuations in food-availability which helps combat domestication selection (Jones et al., 2006) and is preferable when considering release into native rivers.
 - ***M. margaritifera* requires high DO concentrations and flows capable of providing adequate scope for nutrition and removal of waste products.** Whilst investigations into the biological limits of DO concentrations for juvenile mussels were outside of the scope of this study, juveniles survived better in treatments which provided the most similar DO concentrations to the water column. DO concentrations averaging 8.24 mg/L (85 % saturation) were adequate to achieve high juvenile survival (81 % \pm 8) over the first two months post-excystment. Larger substrates provided higher rates of flow which are crucial to maintaining high DO concentrations whilst delivering food and removing waste products. Practical considerations for rearing programmes are important to avoid undue stress to juveniles, such as replacing sieves removed for cleaning with blank (just substrate) sieves to maintain water flow and DO delivery.
 - ***M. margaritifera* is capable of filtering particles <2 μ m and thus requires low suspended solids concentrations.** Gill and cilia/cirri morphology in *M. margaritifera* enable the species to filter particles <2 mm. This makes individuals vulnerable to high suspended solids concentrations as juveniles must either remove unsuitable particles via elevated production of pseudofaeces, or close in order to avoid inhaling these particles. Valve closure for extended periods of time has important implications for oxygen consumption and metabolism and creates the potential for anaerobic respiration. These implications may be particularly severe for juvenile mussels which may not have significant somatic reserves upon which to draw, thus making them at risk of starvation and mortality. Captive rearing programmes should aim to reduce turbid conditions. The use of sediment traps or settling ponds could reduce the supply of smaller (<2 μ m) suspended sediments (inorganic particles) to juveniles whilst the provision of appropriate filter sizes for the water source could assist in removing particles larger than the useful dietary range for *M. margaritifera*.
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- **Initial size of juveniles and collection time may have implications for juvenile vitality and survival.** Data from 2011, 2012 and 2015 indicates that time of collection during the drop-off period may have important implications for initial size and survival. Eybe et al. (2015) have already outlined that juveniles from the first few days of the excystment period grew less and displayed lower survival but this work could be extended to investigate the growth and survival of juveniles from across the entire excystment period. This information benefits rearing programmes because effort can then be targeted on collecting and rearing juveniles most likely to survive.
- **Calcein is a suitable fluorophore for marking juvenile mussels.** The method described is a useful one for improving monitoring in captivity and also for identification of captive reared juveniles if used for augmentation in rivers where juvenile mussels already exist.

7.4. Suggestions for future work

Findings outlined in this thesis raise several new questions about juvenile development and how this affects survival in captivity. More detailed information about juvenile development and vigor upon excystment should help make captive rearing programmes more efficient by ensuring high-quality juveniles are reared in systems which allow them to thrive. Building upon the initial work on juvenile development (Chapter 5) and adopting methods which emulate environmental conditions outlined in Chapters 3 & 4 should allow rearing programmes to achieve high survival provided that sufficient biofilm/diet is provided. It is suggested that additional work is required in the following areas:

7.4.1 Captive rearing systems

- Different stocking densities within sieves should be trialled to see if this can be increased without affecting survival. If higher densities are possible this would increase the potential yield of juveniles without impacting rearing effort.
- Larger substrate size clasts should be considered and the effects on DO should be quantified. Providing juveniles with a choice of substrates within which to bury would also provide a useful insight into whether substrate preferences/requirements change with age/size. These studies could inform future culture methods by demonstrating whether juveniles could be moved up size clasts as they increase in length in order to maintain a size difference between juveniles and substrate, thus facilitating quicker and easier sampling and monitoring activities.

- Little is known about the potential benefits of biofilm to juvenile mussels as both a food source and as a potential reservoir of nitrifying bacteria which could facilitate the breakdown of nitrogenous waste products. Investigation is required to establish if there is a difference in the structure and species assemblages of biofilm communities in recruiting 'vs' non-recruiting pearl mussel systems to assess their potential benefits for juvenile mussels.
- The natural diet of *M. margaritifera* is unknown and whilst some rearing programmes have had success culturing juveniles with artificial diets (Eybe *et al.*, 2013), knowledge of preferred diets at different ages would provide invaluable information to both those working on captive breeding of *M. margaritifera* and practitioners working on the ecology and restoration of wild populations.
- The effects on behaviour of low DO concentrations (both stable low DO and the effect of low DO spikes) should be investigated in tandem with quantifying lethal low DO concentrations for juvenile mussels at different ages/sizes. This would inform both captive rearing programmes and provide a benchmark against which to measure the suitability of potential reintroduction sites for juveniles in the wild.
- In a study in Northern Ireland, Wilson *et al.* (2012) found small but significant levels of genetic differentiation between captive-bred juveniles and samples from their source river indicating a possible founder effect. To date there have been no studies which have resulted in a recommendation of a minimum cohort size of adult mussels to maintain genetic integrity when propagating *M. margaritifera*. These are important considerations as juveniles selected for captive conditions and loss of heterozygosity will directly affect the genetic fitness of wild populations once juvenile augmentation begins (Jones *et al.*, 2006). Additionally, genetic selection may take place if juveniles are selected for vigor depending upon timing of excystment. Genetic studies should be undertaken before juvenile selection to ensure there is no loss of heterozygosity.
- Histological studies investigating lipid and polysaccharide presence/abundance would further inform data on collection time and provide information about the energetic reserves of juveniles excysting fish at different times. It would also provide additional information to developmental studies of juveniles post-excystment to gather more detailed information about the factors limiting juvenile survival. In turn, this would inform decisions about juvenile collection for captive rearing programmes in order to maximise juvenile vitality, survival and rearing effort.

7.4.2 Juvenile ontogeny

- In this thesis, data were collected from a total of 68 individuals across the age classes sampled. The number of individuals from each class could be increased in order to improve the quality of descriptions and different populations should be sampled to ensure that any structures observed or the timing of developments are not population-specific.
- The additional age/size classes mentioned in section 7.2.2 above should be sampled in order to gain a better understanding of development during these periods. This includes sampling individuals older than 44 months to learn when the tissue bearing ostia develops on the abfrontal surface of the gills. Additionally, further samples of adult mussels should be taken for more complete descriptions to be made of the adult gill condition.
- More measurements of structures such as length of laterofrontal cirri, inter-cirral distance, number of cilia/laterofrontal cirrus and ostia size should be taken from more individuals encompassing several populations to refine the preliminary conclusions outlined in this thesis. Descriptions from more individuals from different populations would also help refine the four stages of development and the timing of key developments.
- The link between feeding activity/behaviour and ontogeny should be further investigated with more individuals to enhance our understanding of how these factors affect each other. Particular attention should be given to whether individuals which are inactive upon encystment differ morphologically from active individuals and whether development is linked to food quality/quantity.

7.4.3 Fluorescence marking

- Different methods of marking different life cycle stages should be explored as a way of improving the efficiency of marking activities. This could include immersing adult female mussels calcein-labeled water so they sequester labeled calcium in concretions (Silverman *et al.*, 1985) which would then be transferred to developing glochidia, providing dietary calcein to fish with encysted glochidia, or, using a longer calcein bath in the osmotic induction method for fish with encysted glochidia.

7.5. Final remarks

This work provides a timely synthesis of important factors to consider for captive rearing programmes and outlines information on juvenile ontogeny which has important implications for juvenile survival both in the wild and in captivity. The last critical review of different captive rearing techniques and their success for *M. margaritifera* was six years ago (Gum *et al.*, 2011) but focused on intensive methods which grew mussels to at least 1 mm before placing them in gravel boxes or sheet cages within rivers. Recently the 2nd International Seminar for Rearing Unionoid Mussels in Luxembourg (Thielen *et al.*, 2015) provided a useful forum for the growing number of captive rearing programmes to communicate successes and failures of a number of rearing systems on a small number of mainly European unionoids. As *M. margaritifera* numbers decline in the wild, the means of producing sufficient numbers of captively-reared juveniles to make a difference to the restoration of wild populations becomes increasingly important. This thesis is an important addition to the growing body of knowledge available on captive rearing of *M. margaritifera* and outlines the importance of tailoring captive conditions to juveniles' developmental stage, especially during the first two years of post-parasitic life when juveniles are at their most vulnerable.

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Supplementary materials

Published papers

Effects of substrate size and cleaning regime on growth and survival of captive-bred juvenile freshwater pearl mussels, *Margaritifera margaritifera* (Linnaeus, 1758)

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Abstract The freshwater pearl mussel is critically endangered and most English populations are at risk of extinction unless conservation measures are implemented immediately. The study objectives were to test a culture system for rearing *Margaritifera margaritifera* in captivity, and to investigate the effects of substrate size (0.25–1 and 1–2 mm) and cleaning regime (weekly and monthly) on survival and growth. In total, 1207 and 518 juveniles were reared to 362 (12 months) and 758 days (25 months), respectively. After 362 days, survival was significantly higher in 1–2 mm substrate treatments cleaned monthly ($55 \pm 6\%$) and lowest in 0.25–1 mm substrate cleaned weekly ($14 \pm 3\%$). Growth was significantly higher

in 1–2 mm substrates cleaned weekly (length = 1.15 ± 0.21 mm) and lowest in 0.25–1 mm substrates cleaned monthly (length = 0.83 ± 0.23 mm). Juveniles from most treatments did not display size-dependent over-winter survival, but a significant correlation was found between shell length and survival in the 0.25–1 mm weekly treatment. This low-maintenance system utilised features of previously described systems and growth and survival rates were comparable to, if not better than, other studies culturing *M. margaritifera*. The system could be scaled up to rear significant numbers of juveniles in captivity.

Keywords Margaritiferidae · Captive rearing · Low-maintenance system · Mussel conservation

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Introduction

Freshwater molluscs are among the most endangered invertebrates in the world (Machordom et al., 2003; Primack, 2006) and are disproportionately imperilled compared with other groups (Williams et al., 1993). Understandably, the number of studies on freshwater bivalves has increased substantially over the past 30–50 years with the quickest growing subject area being ecology and conservation (Haag, 2012; Lopes-Lima et al., 2014). Unsustainable pressures such as habitat modification, unnatural silt and nutrient input, pressures on host fishes and deteriorating water quality are responsible for the decline of the freshwater pearl

mussel, *Margaritifera margaritifera* (Linnaeus, 1758). This species declined by over 90 % in the twentieth Century (Bauer, 1988) prompting urgent conservation action from both Government agencies and NGO's to improve pearl mussel habitat and, in some countries, to begin captive rearing activities for populations particularly at risk. The type of conservation strategy employed by different counties (i.e. emphasis on catchment improvements compared with captive breeding) depends upon a variety of factors including size of remaining populations, catchment size and types of pressure. In England, there are approximately 12 populations of *M. margaritifera*, all of which are in decline (Chesney & Oliver, 1998). In 2007, the decision was taken to remove a subset of individuals from the most imperilled English populations for captive rearing whilst catchment improvements, including pearl mussel habitat restoration, took place. The Freshwater Pearl Mussel Ark is a captive rearing programme funded by Freshwater Biological Association (FBA), Natural England and the Environment Agency, with the FBA managing captive rearing activities. The overall objectives of the project are to hold sub-populations from target rivers to protect against local population extinction, and to rear juvenile mussels for release into natal rivers.

A major benefit of ex-situ mussel culture is that environmental parameters can be controlled to optimise habitat conditions and ultimately increase juvenile survival. Percentage survival of larval and juvenile stages in the wild is low (Young & Williams, 1984) even in sustainable populations. Captive rearing can offer a short-term solution to boost population size until natural recruitment levels can be re-established in the wild. Captive rearing is not a long-term solution for declining populations and must be coupled with catchment-wide habitat improvements to reduce pressures on pearl mussel habitat. Substantial improvements in habitat quality are required to allow more sustainable levels of juvenile survival and enable demographic recovery of wild populations.

Research in the early 2000's into optimising captive rearing conditions for freshwater mussels (mainly by North American and some European practitioners) has led to the near perfection of these techniques for certain species (Lopes-Lima et al., 2014). Captive rearing of particularly sensitive species such as *M. margaritifera* however has proven slightly more

problematic and has required significant investigation. Several different methods of propagating *M. margaritifera* have been trialled in Europe (Gum et al., 2011) including bankside encystment and immediate release of encysted salmonids (Altmüller & Dettmer, 2006), rearing juveniles in trays or baskets (Hastie & Young, 2003; Taylor, 2007; Lange & Selheim, 2011; Scriven et al., 2011; Eybe et al., 2013; Lavictoire et al., 2014), suspending cages containing juveniles in raceways or rivers (Buddensiek, 1995; Schmidt & Vandr , 2010), allowing juveniles to excyst directly into raceways (Preston et al., 2007; Moorkens, 2011) and holding juveniles in boxes containing water and placing them in incubators (Lange & Selheim, 2011; Eybe et al., 2013). Whilst previous studies have investigated growth and survival of *M. margaritifera* juveniles under various culture conditions (Buddensiek, 1995; Hruška, 1999; Schmidt & Vandr , 2010; Lange & Selheim, 2011; Eybe et al., 2013), they have lacked specific information on periodic growth and survival rates over extended periods of time, mainly due to low numbers of surviving individuals. Hruška (1999) detailed the rearing of 30,000 individuals to over 3 years old but did not include information on original numbers or survival rates. Further studies with periodic monitoring and a standardised reporting system are required to better understand the factors affecting growth and survival of very young (<1 year) juveniles in captivity.

Previous studies focussing on captive rearing of mussel species (mainly North American) have found that certain environmental conditions in culture are important for optimal growth and survival. These include substrate size (Beaty & Neves, 2004; Liberty et al., 2007) and depth (Yeager et al., 1994; Beaty & Neves, 2004; Jones et al., 2005), maintenance (cleaning) regime (O'Beirn et al., 1998; Liberty et al., 2007), diet (Gatenby et al., 1997; Lange, 2005; Kovitvadhi et al., 2006; Schmidt & Vandr , 2010; Eybe et al., 2013) and mussel stocking density (Eybe et al., 2013).

This investigation sought to test an experimental culture system for *M. margaritifera* using a flow-through system with water sourced from a mesotrophic lake. Substrate size and cleaning regime were tested to identify optimal conditions for the species within this system and to assess which method provided the highest level of survival.

Materials and methods

Experimental work was undertaken at the Freshwater Biological Association headquarters in Cumbria, UK. Water was sourced from Windermere, a large mesotrophic lake, within a catchment with historic records for *M. margaritifera*. Water used in experiments had particles larger than 20 μm removed using a Hydrotech 501 filter. Water temperature followed the lake's natural temperature regime.

A pilot study, carried out between June 2011 and April 2012, informed experimental design for the work described here. Data collection took place over a 12-month period from June 2012. Two different substrate mixes (0.25–1 and 1–2 mm) and two cleaning frequencies (weekly and monthly) were tested, giving a total of four treatment types. Substrate was sourced from around the FBA facility on the western shore of Windermere. Before use, substrate was air-dried before it was sieved to the required clast sizes (either 0.25–1 or 1–2 mm).

A down-welling, flow-through system was designed which supplied filtered lake water to juveniles at a rate of approximately 67 ml s^{-1} . Thirty-six square holes were cut out of a styrene sheet fixed to the sides of a glass aquarium (995 mm \times 357 mm \times 510 mm). *Artemia* sieves (Hobby, Germany) with a mesh size of 0.9 mm were adhered to the styrene sheet to create a fixed support structure for the experimental sieves. Removable experimental sieves (mesh size 0.18 mm) were inserted into the fixed sieves, providing the sole pathway for water flow. Water entered the top of the system via a spray bar, passed through the sieves containing juveniles and substrate, and exited from the bottom of the system through the down-pipe (Fig. 1).

Nine replicates of each of four treatments (36 experimental sieves in total) were set up. Each sieve contained one of the experimental substrate mixes to a depth of approximately 1 cm (50 g dry weight). Treatment sieves containing substrate were exposed to flowing lake water for a minimum of 21 days prior to the start of the experiment to allow biofilm development on the substrate, as per Gum et al. (2011). Juveniles propagated from a single population provided by the FBA's Freshwater Pearl Mussel Ark project (Sweeting & Lavictoire, 2013) were used. One hundred newly excysted active juveniles were added to each experimental sieve (total 3600 individuals). For each sieve, 30 individuals were selected at random

and measured (length and height to nearest 50 μm) before being added. The position of experimental sieves within the aquarium was assigned on a random basis so that each column within the aquarium had one of each of the four treatments. Sixteen days after the experiment commenced, the surface of the styrene sheet was siphoned and 11 juveniles were found indicating escapement. *Artemia* sieves with a 0.3-mm mesh were placed over the experimental sieves so juveniles could not escape.

Every week, sieves in the weekly cleaning treatment (18 sieves in total) were removed from the aquarium and substrate was gently emptied into a glass container. Substrate was elutriated to suspend organic particles and the elutriate poured through a 0.18 mm mesh sieve to retain any suspended juveniles. Sieves were inspected under a low power microscope ($\times 20$) and any juveniles replaced into the experimental sieve along with the substrate. The 0.3 mm sieve (cover) and 0.9 mm sieve (fixed within aquarium) were also cleaned before the experimental sieve was replaced. The same process was repeated on a monthly basis for treatments requiring monthly cleaning.

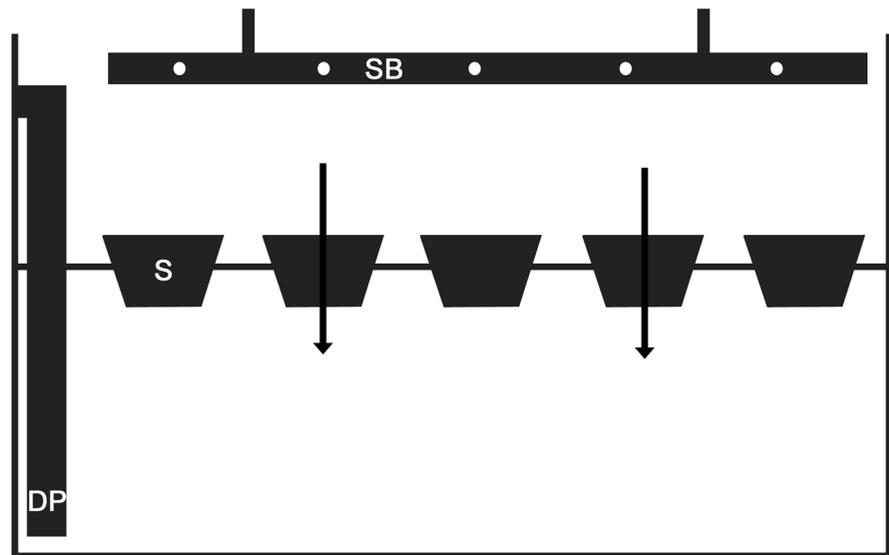
Approximately every 2 months (51, 112, 167, 247, 308 and 362 days post-excystment), the numbers of surviving juveniles and dead shells from each sieve were recorded. In addition, 30 live individuals from each sieve were chosen at random and measured. Where fewer than 30 individuals remained, all juveniles were measured. Dead juveniles were measured and removed from sieves. Sampling in this manner constituted a cleaning event as organic matter was removed during sampling.

Sampling for growth and survival ceased after 12 months but the system and cleaning regimes were retained for a further 13 months. Sieves were sampled for the final time at 758 days post-excystment. Survival and size were recorded as before but instead of being returned to sieves, all juveniles were removed to a modified salmon egg tray containing substrate measuring 1–2 mm as described in Sweeting & Miles (2010). Statistical analysis on juvenile size and survival are reported for the first 12 months only, unless otherwise stated.

Data analysis

Central Limit Theorem (Elliott, 1993) was applied to assume normality where appropriate. Standard

Fig. 1 Experimental set-up. Water enters at the top of the system via a spray bar (SB) and flows through sieves (S) containing substrate and juveniles (arrows show direction of flow). Water exits via the downpipe (DP)



deviation values are provided after mean values. One-way Analysis of Variance (ANOVA) with post hoc Tukey's HSD tests were used to assess the significance of survival, survival rates and size between treatments on the same sampling occasion when data were normal. Where data were not normal Kruskal–Wallis tests were employed. Two-way ANOVA's were used to investigate the interaction of substrate size and cleaning regime and their effects on both survival and size in treatments on day 362. Repeated Measures ANOVA's with pairwise comparisons were used to test survival between 0 and 362 days to see if survival changed at specific times. For Repeated Measures ANOVA's a Greenhouse–Geisser correction was applied if the assumption of sphericity was not met. Student's *t* tests were used to compare length pre- and post-winter to help establish if juveniles displayed size-dependent over-winter survival within treatments. Spearman's Rank Correlation Coefficient tests were used to test the significance of correlations between shell length and height, shell growth and temperature, and mean survival and shell length.

Juvenile length and survival across rows and columns in the aquarium were tested in June 2013 to rule out any bias due to sieve positioning (lateral and top-to-bottom positioning within the aquarium). There was no significant difference in survival between different columns ($F_{(8,27)} = 0.196$, $P = 0.989$) or rows ($F_{(3,32)} = 0.025$, $P = 0.994$) indicating that any

significant results between treatments were not due to sieve positioning. The same was true for mean juvenile length in June 2013; there were no significant differences between different columns ($F_{(8,27)} = 0.097$, $P = 0.999$) or rows ($F_{(3,32)} = 0.163$, $P = 0.920$).

Initial size in June 2012 was also tested to ensure juveniles in each sieve had the same starting length. Starting lengths of individuals in all 36 sieves were not significantly different ($F_{(35,1044)} = 1.35$, $P = 0.083$) at the beginning of the experiment (mean length = 0.40 ± 0.02 mm).

Results

Size

Spearman's Rank Correlation Coefficient tests found juvenile length and height were significantly correlated ($P < 0.001$); 0.25–1 mm weekly treatment ($r_{s(1216)} = 0.980$), 0.25–1 mm monthly treatment ($r_{s(1837)} = 0.965$), 1–2 mm weekly treatment ($r_{s(1876)} = 0.974$) and 1–2 mm monthly treatment ($r_{s(1888)} = 0.968$). As such only the length parameter was used for analysis in this study.

Length of juveniles on each sampling occasion was considered in turn to establish any effects of the different treatments over time (Fig. 2). Juvenile lengths were all significantly different ($P < 0.001$)

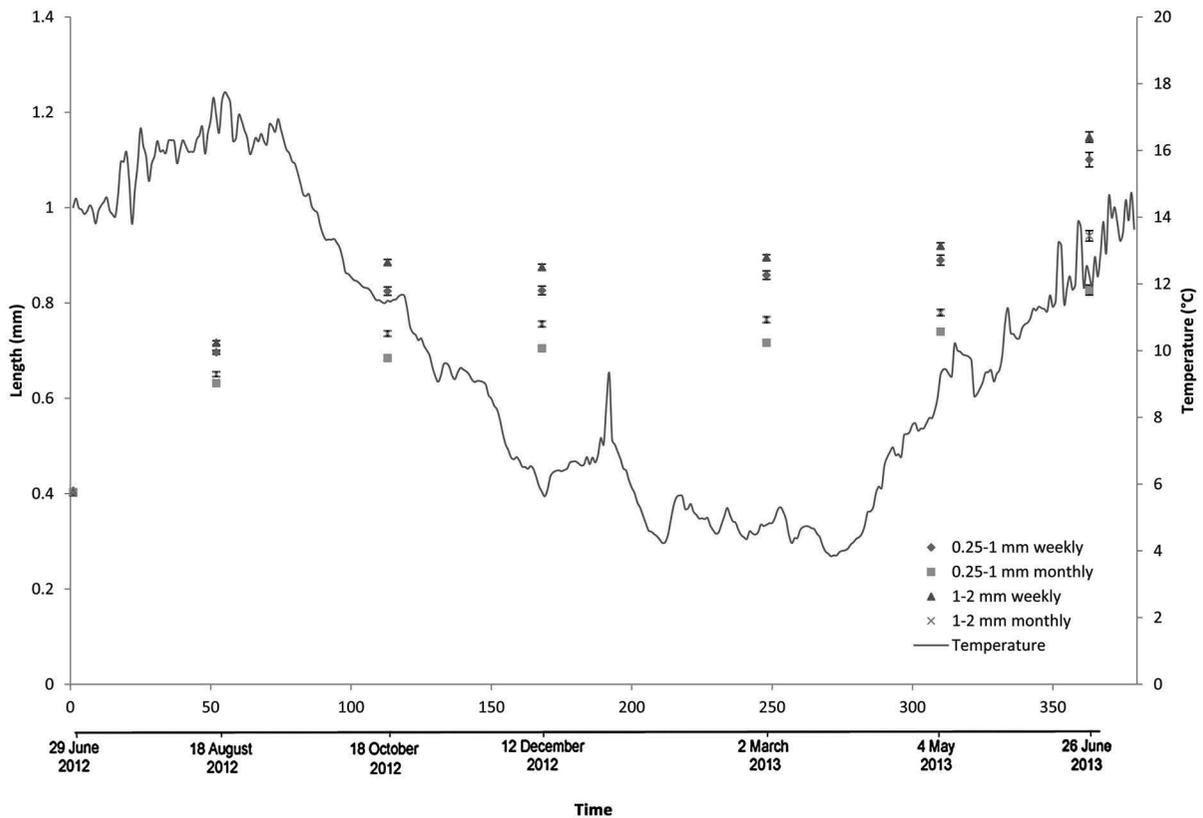


Fig. 2 Juvenile length (mm) with SD bars, and daily mean temperature (°C) during the course of the experiment. The x-axis is provided both in days since experiment commenced and per month to show how growth relates to time of year

among treatments on days 51 ($F_{(3,1076)} = 77.295$), 112 ($F_{(3,964)} = 195.723$), 167 ($F_{(3,951)} = 158.522$), 247 ($F_{(3,941)} = 175.247$), 308 ($F_{(3,906)} = 162.465$) and 362 ($F_{(3,883)} = 167.377$). The same pattern was observed throughout the experiment; 1–2 mm weekly > 0.25–1 mm weekly > 1–2 mm monthly > 0.25–1 mm monthly. Table 1 summarises these results giving mean length (mm) on each sampling occasion. A 2-way ANOVA was carried out for the June 2013

sample (362 days) and a significant interaction was found between substrate size and cleaning regime ($F_{(1,883)} = 7.414$, $P = 0.007$). Analysis of simple main effects found significant interactions at all levels.

Shell growth in *M. margaritifera* is positively correlated with temperature ($r_{s(214)} = 0.76$, $P < 0.001$). Apparent cessation of growth was observed below approximately 10°C (Fig. 3). Mean daily growth rates during the warmest period (June–August

Table 1 Mean length ± SD (mm) of juveniles on different sampling occasions in order of largest to smallest

	51 days	112 days	167 days	247 days	308 days	362 days
1–2 mm weekly	0.72 ± 0.07	0.89 ± 0.09	0.88 ± 0.10*	0.90 ± 0.09	0.92 ± 0.10 [^]	1.15 ± 0.17
0.25–1 mm weekly	0.70 ± 0.07	0.84 ± 0.11	0.86 ± 0.11*	0.87 ± 0.10	0.90 ± 0.12 [^]	1.10 ± 0.17
1–2 mm monthly	0.65 ± 0.08	0.74 ± 0.10	0.76 ± 0.10	0.77 ± 0.10	0.78 ± 0.11	0.94 ± 0.18
0.25–1 mm monthly	0.63 ± 0.07	0.69 ± 0.10	0.71 ± 0.10	0.72 ± 0.10	0.74 ± 0.11	0.83 ± 0.16

Size was significantly different between all treatments ($P < 0.001$). Tukey’s HSD tests showed significant differences within each sampling point ($P < 0.05$) except where indicated 167 days (* $P = 0.31$) and 308 days post-excystment ([^] $P = 0.17$)

2012) were almost 11 times higher than during the coolest period (February–April 2013). Average growth per day highlighted that growth slowed over winter but did not halt completely (Table 2).

Survival

We report survival rates comparable to, and in most cases greater than, previous studies of a similar duration on other species of freshwater mussel (Fig. 4a), and specifically *M. margaritifera* (Fig. 4b). In this investigation, higher survival was observed in the 1–2 mm monthly treatment with an average of over 55 % survival after 12 months and 23 % after 25 months. The lowest survival was in the 0.25–1 mm weekly treatment with the other two treatments displaying intermediate survival (Tables 3, 4; Fig. 5).

Survival over time

Survival differences within treatments over time were tested with Repeated Measures ANOVAs to see if

there were specific times when survival changed. Mean survival was different over the course of the experiment for all treatments ($P < 0.001$); 0.25–1 mm weekly ($F_{(2,021,16,171)} = 1147.196$), 0.25–1 mm monthly ($F_{(6,48)} = 315.484$), 1–2 mm weekly ($F_{(2,126,17,008)} = 324.543$) and 1–2 mm monthly ($F_{(2,030,16,243)} = 167.912$). Post hoc tests revealed that survival was the same only in the 1–2 mm weekly treatment between days 112 and 167 ($P = 0.086$) and in the 0.25–1 mm weekly treatment between days 167 and 247 ($P = 0.122$), days 247 and 362 ($P = 0.128$) and days 308 and 362 ($P = 0.288$).

Survival rate

Survival rates were considered to further examine data taking into account the high initial mortality observed in the 0.25–1 mm weekly treatment. Survival rates between the treatments were significantly different ($P < 0.002$) on days 51 ($F_{(3,32)} = 128.303$), 112 ($F_{(3,32)} = 41.388$), 167 ($F_{(3,32)} = 9.743$), 308

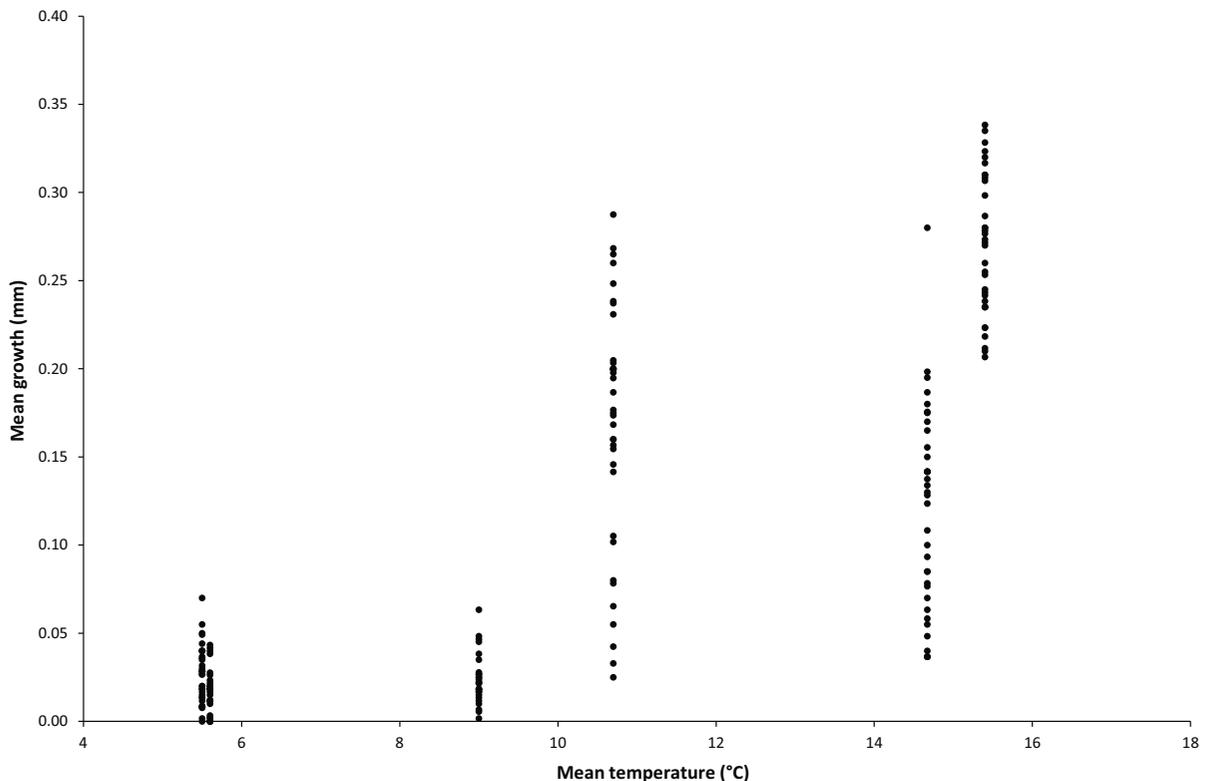


Fig. 3 Mean shell growth against daily mean temperature for the six sampling occasions

($F_{(3,32)} = 6.553$) and 362 ($H_{(3,32)} = 15.179$) but were not statistically different on day 247 ($F_{(3,32)} = 0.619$, $P = 0.608$).

When mean survival rate was plotted against mean shell length, no relationship was found for either of the 1–2 mm treatments (Fig. 6). In the 0.25–1 mm treatments however, there was a positive relationship, which is stronger in the treatment which is cleaned weekly. Spearman's rank correlation coefficient tests found no significant correlation in the 0.25–1 mm monthly treatment ($r_{s(4)} = 0.515$, $P = 0.296$) but there was in the 0.25–1 mm weekly treatment ($r_{s(4)} = 0.947$, $P < 0.01$). Larger individuals displayed significantly higher survival compared with smaller individuals in this treatment.

Over-winter survival

To establish whether juveniles displayed size-dependent over-winter survival, *t* tests were carried out comparing juvenile size pre- and post-winter (October 2012 and April 2013). Across all treatments, juveniles were significantly larger post-winter ($P < 0.001$); 0.25–1 mm weekly ($t_{(272)} = -4.377$), 0.25–1 mm monthly ($t_{(522)} = -5.239$), 1–2 mm weekly ($t_{(538)} = -3.717$), 1–2 mm monthly ($t_{(530)} = -5.027$). However, there were more juveniles in larger size classes in April 2013 compared to October 2012. This implies that, rather than there being high mortality of smaller juveniles over winter, individuals have grown, thereby pushing them into larger size classes.

Table 2 Average growth per day (µm) for individuals in each treatment and mean daily temperature (± SD) between sampling points

Treatment	0–51 days	51–112 days	112–167 days	167–247 days	247–308 days	308–362 days
Mean daily temp. (°C) over period	15.4 (1.0)	14.7 (2.0)	9.0 (1.7)	5.6 (1.1)	5.5 (1.5)	10.7 (1.3)
0.25–1 mm weekly	6.15	2.10	0.42	–0.01	0.66	3.67
0.25–1 mm monthly	4.18	1.01	0.31	0.15	0.27	1.76
1–2 mm weekly	6.80	2.52	–0.24	0.22	0.46	4.00
1–2 mm monthly	4.58	1.58	0.35	0.11	0.21	3.20

Negative values are due to sampling error and do not indicate shrinkage

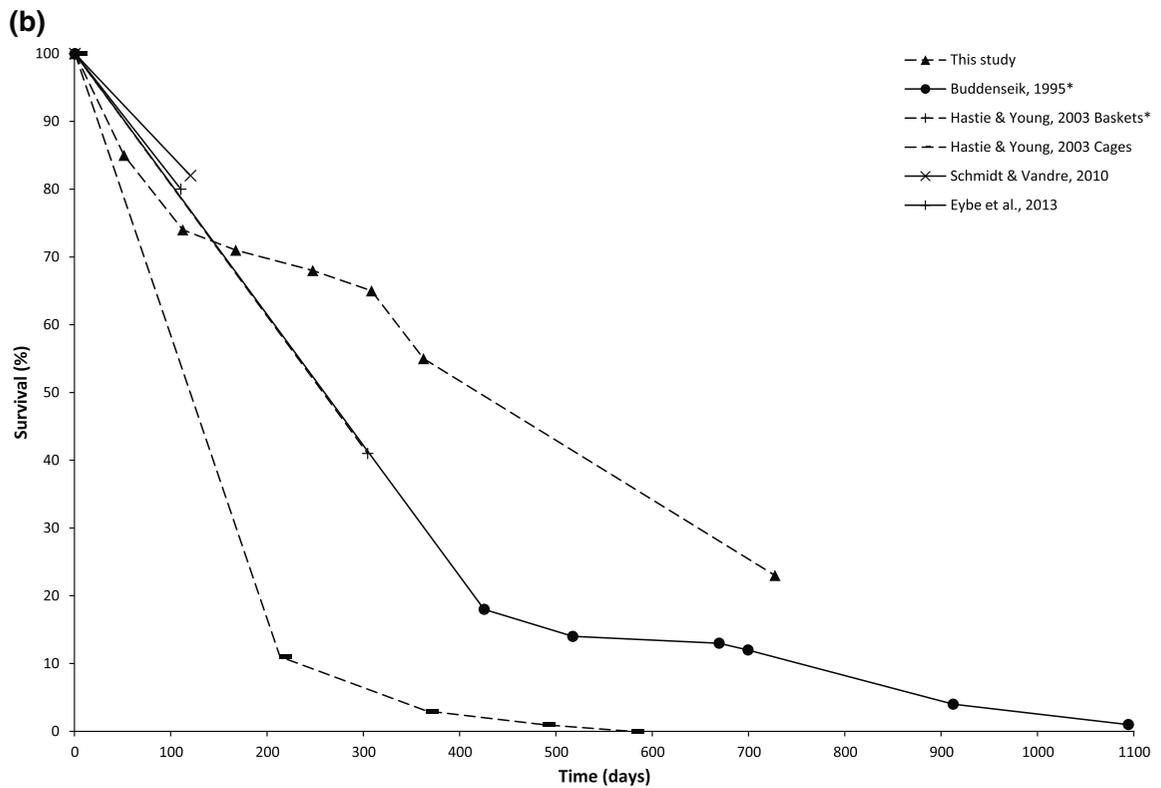
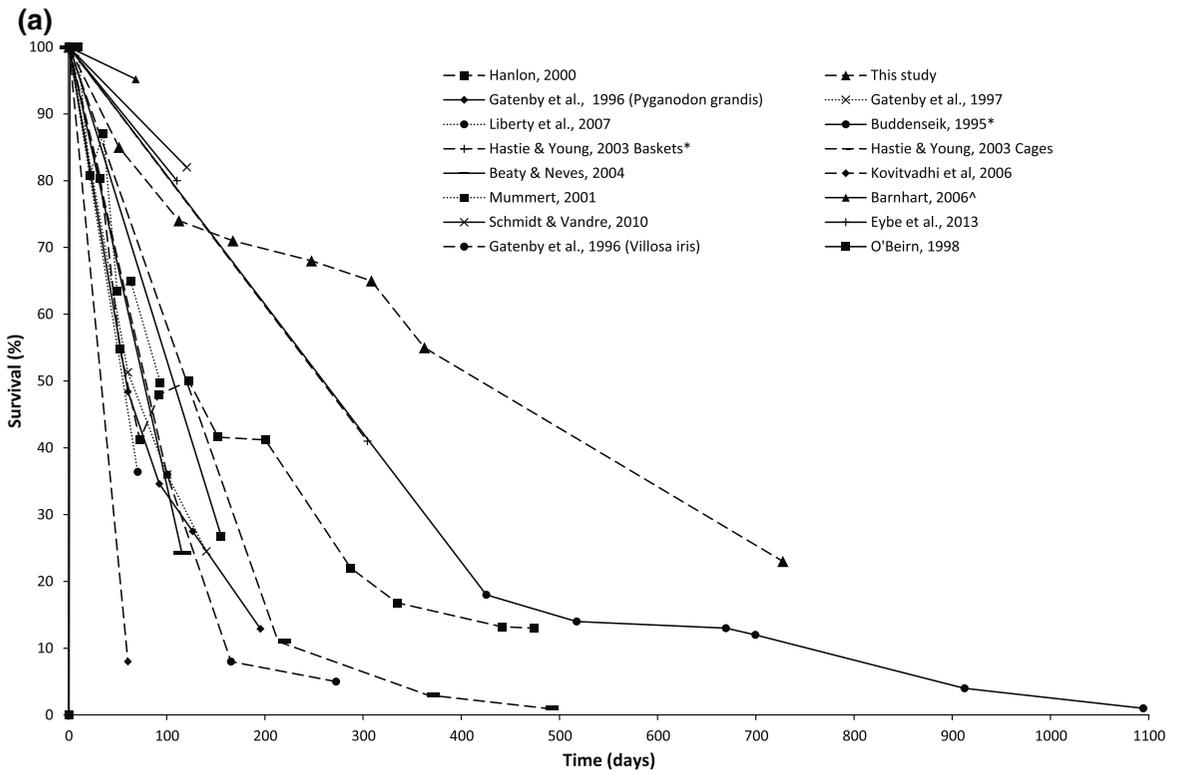
Table 3 Summary survival statistics. Total number of individuals surviving, percentage survival and the range of percentage survival for all treatments in June 2013

Treatment	Total no. survived	Survival (%)	Survival range (%)
0.25–1 mm weekly	123	14	8–19
0.25–1 mm monthly	253	28	19–40
1–2 mm weekly	333	37	18–47
1–2 mm monthly	498	55	45–63

Table 4 Mean number of juveniles surviving (± SD) at each sampling point in order of highest to lowest survival. Survival was significantly different between all treatments. Tukey's HSD tests showed significant differences within each sampling

point ($P < 0.05$) except where indicated on days 51 (* $P = 0.93$) and 112 ($P = 0.97$). Results have been rounded to whole juveniles

	51 days	112 days	167 days	247 days	308 days	362 days
1–2 mm monthly	85 ± 4	74 ± 4	71 ± 4	68 ± 3	65 ± 3	55 ± 6
1–2 mm weekly	76 ± 6*	51 ± 5 ^Δ	50 ± 6	48 ± 6	46 ± 6	37 ± 9
0.25–1 mm monthly	75 ± 4*	51 ± 8 ^Δ	43 ± 8	40 ± 8	33 ± 7	28 ± 6
0.25–1 mm weekly	40 ± 7	18 ± 4	16 ± 3	15 ± 3	13 ± 3	14 ± 3



◀ **Fig. 4** Mean survival in **a** other published bivalve captive rearing studies and **b** more specifically in *M. margaritifera* studies. Mean survival up until July 2014 has been provided for this study. *Asterisk* indicates figures based upon estimates; Buddensiek (1995) figures taken from survival graphs; Hastie & Young (2003) Baskets estimated a sample of known volume and multiplied up to indicate likely survival in total volume. *Hat* indicates only one treatment selected for illustrative purposes; this study investigated survival of 8 different species over different timescales. For all studies where several treatments are reported, only the best survival results are reported here for comparison

Discussion

The objective of this study was to test the effectiveness of the proposed culture system for rearing juvenile mussels (*M. margaritifera*) and to examine the effects of different substrate sizes and cleaning regimes on growth and survival. Here, we describe a culture method employing some features from previous studies (e.g. Hastie & Young, 2003; Lange, 2005; Barnhart, 2006; Preston et al., 2007; Gum et al., 2011) to create a low-maintenance system which utilises a natural temperature regime. The proportion of mussels escaping experimental containers was minimal (2.5 %) and can be prevented in future by placing covers over containers from the beginning. Juveniles were reared to an age of 758 days (25 months) with optimum treatments achieving 55 % mean survival after 12 months and 23 % after 25 months. No other captive rearing facility has published this level of survival over similar timescales for *M. margaritifera* (Fig. 4b). Growth during the first 362 days (12 months) was comparable with other studies (e.g. Hruška, 1999; Scheder et al., 2011; Scriven et al., 2011; Eybe et al., 2013). Juveniles in this study achieved growth rates of between 170 and 220 % (0.68–0.89 mm) during the first growth season, which compares favourably with Hruška (1999) reporting 250 %, and Eybe et al. (2013) with rates between 150 and 200 %. While it is not possible to directly compare growth from different captive rearing programmes due to differences in culture systems, temperature regimes, handling, and population-specific growth rates, the desired outcome of growth and survival from the techniques used in this study are positive compared with other reported methods.

This investigation concurred with other studies which have found that juveniles display a large size

range after a relatively short period (Beaty & Neves, 2004; Barnhart, 2006; Schmidt & Vandr e, 2010). By the end of the first year, some individuals were 2.5 times larger than others. This pattern has been shown to continue as juveniles get older with some individuals reaching over 9 times the length of conspecifics by 5 years old (Sweeting & Lavictoire, 2013).

Significantly higher survival was observed in larger substrates (1–2 mm) which concurs with previous studies on unionids (e.g. Liberty et al., 2007). Substrate size preference appears species-specific but may also be affected by the culture system. For example, different substrate size preferences were found for *Villosa iris* (Lea, 1829) in different culture systems in Virginia. Superior growth and survival were found in substrates <0.2 mm in a recirculating system (Hua et al., 2013) but higher survival was recorded in substrates between 0.5 and 0.85 mm in a flow-through system (Liberty et al., 2007). *M. margaritifera* is slower growing and has a longer juvenile stage compared to most other freshwater mussels, so rearing techniques from other species may have limited relevance. There remains a lack of understanding of the specific habitat requirements (physical, chemical and biological) for *M. margaritifera*, both in the wild and in captivity. For captive rearing programmes to become more successful a greater understanding of the factors limiting survival is needed.

Individuals in the 0.25–1 mm weekly treatment showed size-dependent survival, something which was not apparent in the other treatments. This may be because this treatment appeared to be the least suitable for *M. margaritifera* (displaying the highest mortality), so only the largest individuals were able to survive. If smaller substrate sizes impeded water flow to an extent where food or oxygen supply was not sufficient then size-dependent survival would also have been observed in the 0.25–1 mm monthly treatment. Likewise, if a weekly cleaning regime was too stressful for juveniles, size-dependent survival would have been observed in the 1–2 mm weekly treatment, but it was not. It appears that the combination of small substrate size and frequent (weekly) cleaning does not provide suitable conditions for *M. margaritifera* juveniles to thrive. This finding is important when considering catchment management and habitat improvements to ensure that enough coarse substrate is available for juveniles.

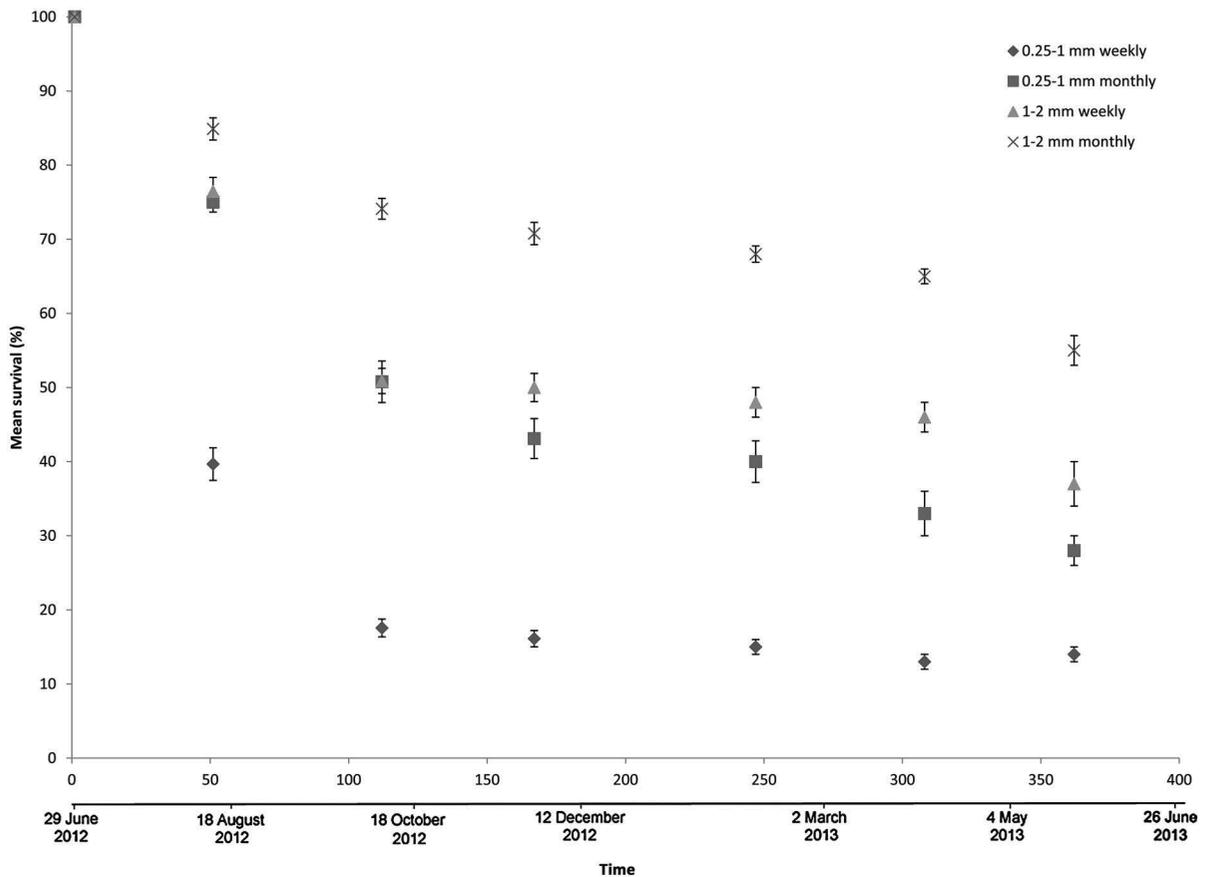


Fig. 5 Mean juvenile survival for each treatment with SD bars. The x-axis is provided both in days since experiment commenced and per month to show how survival relates to time of year. Juvenile survival between treatments was significantly different on all sampling occasions ($P < 0.001$); 51 days ($F_{(3,32)} = 128.30$), 112 days ($F_{(3,32)} = 148.285$), 167 days ($F_{(3,32)} = 145.296$), 247 days ($F_{(3,32)} = 140.117$), 308 days

($F_{(3,32)} = 145.350$) and 362 days ($F_{(3,32)} = 64.670$). The same pattern in survival was observed on all sampling occasions; 1–2 mm monthly >1–2 mm weekly >0.25–1 mm monthly >0.25–1 mm weekly. A 2-way ANOVA showed no significant interaction between the effects of substrate size and cleaning regime on survival in June 2013 after 362 days ($F_{(1,32)} = 0.805$, $P = 0.376$)

Cleaning of substrate on a weekly basis has a detrimental effect on survival compared to monthly cleaning (Table 4). Differences in survival rates between treatments were significant which infers that observed differences in survival were not artefacts of high initial mortality in the 0.25–1 mm weekly treatment after approximately 112 days. Poorer survival and growth in treatments cleaned more frequently has been documented in studies on different mussel species (O’Beirn et al., 1998; Liberty et al., 2007) due to stress or accidental damage/loss during sampling.

Whilst survival was compromised in treatments cleaned more regularly, growth was found to be significantly higher, contradicting findings of studies

on some unionid species (e.g. Liberty et al., 2007). This higher growth rate may be because cleaner substrate conditions allow pedal-feeding juveniles to forage for more or better quality food. Whilst this finding is interesting, higher growth rates for *M. margaritifera* should not be sought at the expense of survival in captive rearing programmes. It appears that most captive-bred species of mussel require enough cleaning to remove fine particles in order that normal foraging behaviour is not affected, but too much cleaning may cause stress and damage/loss, leading to higher mortality (O’Beirn et al., 1998). As reported in other studies (e.g. Buddensiek 1995), juvenile growth was found to be negligible at low temperatures and near-cessation (approximately $0.3 \mu\text{m}$ per day) of

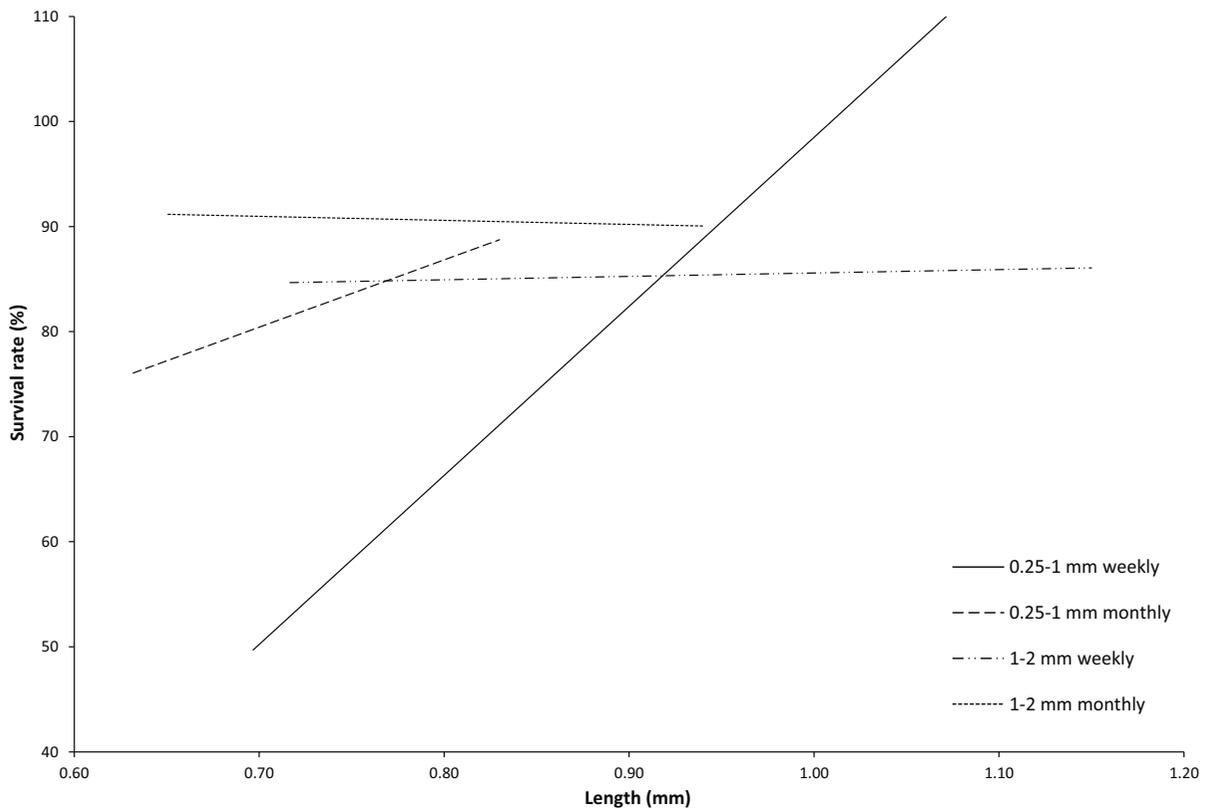


Fig. 6 Mean survival rate (%) against mean shell length (mm) for each treatment. Survival rate was >100 % on one occasion in the 0.25–1 mm weekly treatment due to sampling error

growth occurred below 10°C, corroborating the findings of Hruška (1999).

Across all treatments, mortality was highest during the first growth season (June–October 2012), after which mortality was relatively low over winter. This implies that survival is relatively stable when temperature (and therefore metabolic rate) is low. This was unexpected as it was assumed that mortality would increase over winter for those juveniles lacking sufficient nutritional reserves. Buddensiek (1995) not only found high mortality during the first few months post-excystment but also found complete mortality of smaller mussels (<0.7 mm) over-winter; a result which has not been replicated in this study. Size-dependent over-winter survival was not observed in this investigation, contrasting with the findings of Buddensiek (1995).

It is unclear if there is any intraspecific competition within sieves at a density of 100 juveniles in 34 cm³ of substrate, and testing different juvenile densities is an

aspect which requires further investigation. Eybe et al. (2013) found significantly higher growth in containers with 200 mussels suggesting that density-dependent competition was occurring in treatments with higher numbers. Barnhart (2006) achieved good survival for several North American freshwater mussel species at densities of 2000 individuals in small cups. Similarly Beaty (1999) found no density-dependent effects for *V. iris*. Higher densities however may lead to higher instances of fungal infection which can spread rapidly and kill large numbers of juveniles (L. Lavictoire, pers. observation). This system may safeguard against the spread of fungal infection because juveniles are contained in separate, removable sieves. Eybe et al. (2013) found fungal infections could be problematic and could spread rapidly, especially in containers with a density of 500 individuals per 500 ml water.

Culture conditions which allow water to flow through interstitial spaces but do not allow too much fine organic or particulate matter to infiltrate can

supply juveniles with suitable habitat conditions with good levels of oxygen and food (Liberty et al., 2007). The benefits of rearing juveniles in substrate >1 mm diameter could be improved oxygen and food supply and the more efficient removal of potentially toxic ions such as ammonia or nitrite found to be a limiting factor for juvenile survival (Eybe et al., 2013). Further investigations considering interstitial dissolved oxygen concentration, nitrite and ammonia concentration, and flow characteristics through different substrates and types of culture system (e.g. down-welling versus laminar flow) are required to better understand the habitat requirements of young juveniles. Investigation is also required to establish the natural diet of *M. margaritifera* and the importance of different algae and bacteria species as food items for juveniles. Whilst diet was not studied during this investigation, a comprehensive list of recorded phytoplankton in Windermere can be found in Reynolds & Irish (2000).

The exact age (or size) at which juvenile *M. margaritifera* metamorphose and switch from pedal-to filter-feeding is unknown but observations made during this experiment suggest that juveniles were still pedal-feeding at 12 months old but had switched to filter-feeding by 25 months old. This down-welling system is therefore suitable for juveniles which are pedal- as well as filter-feeding. Further investigation is required to establish if substrate requirements are different for pedal-feeding versus filter-feeding juveniles.

Although mussel culture systems have been somewhat perfected (Lopes-Lima et al., 2014) culture of *M. margaritifera* remains challenging. This culture system was successful in rearing 1207 juveniles to 12 months old and 518 juveniles to 25 months old. Juveniles were easy to find when sampling due to the small size of containers. This is important when designing culture systems to maximise efficiency. If scaled up, this system could potentially rear up to 2000 juveniles to 12 months old to a size of >1 mm using 1–2 mm substrate cleaned monthly, requiring minimal maintenance time (approx. 3 h/month). This work has informed breeding practices for the Freshwater Pearl Mussel Ark project at the FBA. A rearing protocol using 1–2 mm substrate cleaned approximately every 2–3 weeks in modified fish-egg trays (described in Sweeting & Miles, 2010) was introduced for all new juvenile cohorts collected from 2013. Initial results show good numbers of juveniles surviving after

24 months. Early success in this modified system allows tentative optimism that aspects of this investigation can be used to scale up propagation of threatened populations at this facility and could be replicated elsewhere.

Captive rearing programmes are an important activity to safeguard the most vulnerable populations and provide more time for catchment restoration to improve pearl mussel habitat in the wild (Gum et al., 2011), especially where problems are diverse and difficult to solve. It is important to understand the limiting factors of juvenile culture and to maximise survival in captivity but these initiatives should not replace restoration activities in the wild.

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