Biology and ecology of the introduced snail *Microxeromagna armillata* in south eastern Australia

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Frontispiece: Active *Microxeromagna armillata* adult (top) and inactive adult and juvenile *M. armillata* (bottom) in leaf litter of a citrus orchard.

Dedicated in loving memory to my dad,

Laurence Cowley Lush

3-4-1927 to 29-5-2006

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Abstract

Microxeromagna armillata (Lowe, 1852) is a snail introduced snail to Australia which has established populations in the Riverland and Sunraysia citrus growing regions. Citrus exported from these regions to the USA has been rejected due to contamination with *M. armillata*, causing significant economic losses. The life history, phenology and activity of *Microxeromagna armillata* has not been studied in Australia: this forms the basis of this thesis.

Microxeromagna armillata employs an iteroparous egg laying strategy in semi-field conditions and lays approximately 500 eggs per year. Field populations can reach high densities (~4000 snails/m^2), particularly during the winter months when juvenile recruitment occurs. Snails reach sexual maturity at ~ 6mm in shell diameter and can grow to this size from a juvenile stage (2mm) within six weeks. *Microxeromagna armillata* can reproduce successfully by self-fertilisation, and juveniles are able to aestivate with little reduction in subsequent fecundity. These traits make control of this pest a significant challenge. Leaf litter is the preferred habitat of *M. armillata*, but snails do move in the tree canopy. Cues for snail activity in the leaf litter and canopy appear to differ, as does the size of active snails in these areas. *Microxeromagna armillata* activity was low in the tree canopy during harvest compared to post harvest, intimating that fruit contamination is either occurring infrequently or post-harvest. Copper trunk bands were shown to minimise snail movement into the canopy and may be an important preventative measure.

These findings have changed the recommendations for *M. armillata* management in citrus groves of south eastern Australia.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Lush, A.L. and Baker, G.H. (2003) A novel method for the field study of mollusc movement using mucus trails. *In* Slugs and snails: Agricultural, Veterinary and Environmental Perspectives. BCPC Symposium Proceedings No. 80

Lush, A.L. (2004). Field and postharvest control of snails in citrus. Final report for Horticulture Australia Ltd project CT00040. South Australian Research and Development Institute (SARDI) pp 69

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Please note: in the print copies the only pages coloured are the ones with photographs.

Chapter 1 General introduction and thesis outline

The Mollusca is the second largest phylum of the animal kingdom, forming a major part of the world fauna. The Gastropoda is the only class of molluscs to have successfully invaded land [1], and many terrestrial molluscs have become successful world travellers [2]. Most of these travelling species have had no impact on humans, but several species have become significant as agricultural pests and as vectors of parasites. Of the most economic importance in Australia are the pests of agricultural and horticultural crops, with species from the Helicidae and Hygromiidae families being the most significant.

Species of these families were introduced with European settlement and quickly became significant pests of crops and gardens [3]. *Theba pisana* (Müller), *Cernuella virgata* (da Costa), *Prieoticella barbara* (Linnaeus) and *Cochlicella acuta* (Müller) are all pests of grain crops in southern Australia [4, 5]. Large numbers of these snails aestivate on the heads and stalks of cereals, contaminating grain and damaging machinery during harvest. Although *C. acuta* and *C. virgata* feed on detritus, *P. barbara* and *T. pisana* are reported to damage crop seedlings and ornamental plants [5]. *Cantareus aspersus* (Müller), the common garden snail, is also considered to be a pest of crops, gardens, orchards and stored crops in Australia [6]. Literature in Australia has focused on these five species of introduced snails [5-8].

Recently, another introduced species, *Microxeromagna armillata* (Lowe) has become economically significant in citrus [9]. *Microxeromagna armillata* has been found by U.S.A. quarantine authorities in shipments of navel oranges and, as this species is not present in the U.S.A., the fruit has been refused entry. Although *M. armillata* does not damage the fruit or the citrus tree, it is a quarantine concern for the U.S.A., which is the key export market for Australian navel oranges. Access to this market for Australian citrus began in the early 1990's and has proved to be lucrative for Australian citrus growers.

A key goal for the Australian citrus industry is to protect and enhance market access to the USA. Fruit rejection due to *M. armillata* contamination has caused substantial economic losses for growers, packers and exporters within the citrus industry across south-eastern Australia. If *M. armillata* is detected in export shipments, fruit is either repacked and re-exported to another country, or fumigated and destroyed in the USA, at a considerable cost to the industry. Management of *M. armillata* has become a key priority for the Australian citrus industry.

Microxeromagna armillata is exotic to Australia [1] and is thought to originate from the European region of the Palaearctic. It has been previously classified in the Helicidae but is now generally accepted as a member of the Hygromiidae [2, 10]. Literature regarding this species is scarce, mainly relating to a description of the species [11, 12], taxonomic placement [10, 11], and its geographic distribution in Europe [10, 11, 13-15] and Australia [1]. It is clear that further research needs to be conducted before management strategies can be developed and implemented in Australian citrus orchards.

In response to this need, a research program was initiated to investigate the biology, ecology and control of *M. armillata* in south-eastern Australian citrus orchards. Research presented in this thesis contributes to the wider program and is focused on answering three central questions:

i. **What are the life history traits of *M. armillata*?** Chapter 2 describes the life history traits of *M. armillata*, including reproductive lifespan, number and size of offspring and the influence of body size on these characters (section 2.3). Mode of reproduction is investigated experimentally (section 2.4), while the effects of aestivation and adult density on *M. armillata's* life history traits are explored in sections 2.5 and 2.6, respectively. A central theme in this chapter is the inter-relatedness of *M. armillata's* life history characteristics, and their changing relationships under different influences.

ii. **What is the phenology and spatial distribution of *M. armillata* within citrus orchards in south-eastern Australia?** The preferred habitat of *M. armillata* is identified and population dynamics monitored over time at differing locations in Chapter 3. These results are compared with the general biology of *M. armillata* described in the laboratory (Chapter 2) and are also discussed in the context of fruit contamination and risk assessment.

iii. **When and where is *M. armillata* active in citrus orchards?** The presence of *M. armillata* in the tree canopy is monitored in citrus orchards, and development of a new technique that facilitates monitoring of *M. armillata* activity is detailed in Chapter 4. The activity of *M. armillata*, and the size of active snails, is described with reference to the relative risk of fruit contamination at different times of year. Several options for preventing snail movement into the tree canopy are reported.

Finally, Chapter 5 provides a general discussion of the research findings, linking results from the three chapters to propose a management strategy for *M. armillata*. In light of this discussion, avenues for future research are also proposed in the final chapter.

Chapter 2 Life history traits of *Microxeromagna armillata*

2.1 General Introduction

Understanding the biology of an organism underpins its effective management. The most fundamental aspects of an organism's biology are generally characterised as those that relate to its reproduction and development, which are termed life history traits. Traits such as length of life, body size and age at maturity, number of offspring produced, number of reproductive events and the time intervals between these events, all describe the life history of an organism [16-18]. As variations in life history traits can drive population change, it is important that the traits are fully understood in order to effectively develop a population management strategy for pest species. Terrestrial molluscs exhibit a diverse range of life history traits [16, 19]. The life history traits of *M. armillata*, such as mode of reproduction, length of life, number and size of offspring, and size and age at maturity, have not been previously described. Hence, research in this chapter focuses on development of a culturing technique for *M. armillata* (section 2.2), the fecundity characteristics of *M. armillata* (section 2.3) and its mode of reproduction (section 2.4). In addition, several factors which may influence the fecundity characteristics of *M. armillata*, and hence impact on population dynamics, are explored. These include body size (section 2.3), aestivation (section 2.5), and snail density (section 2.6).

2.2 Development of a culture technique for *Microxeromagna armillata*

2.2.1 Introduction

Molluscs can lay eggs in and on many different substrates such as soil, trees, leaf litter, and water [16, 19], although most hygromiid snails lay eggs in soil [20-22]. *Microxeromagna armillata* inhabits both terrestrial and arboreal environments in citrus orchards, but it is not known where it lays its eggs. The three possible substrates for egg laying in citrus orchards include the tree, soil, and leaf litter. The following experiment aims to determine on which of these substrates, if any, *M. armillata* lays its eggs.

2.2.2 Materials and methods

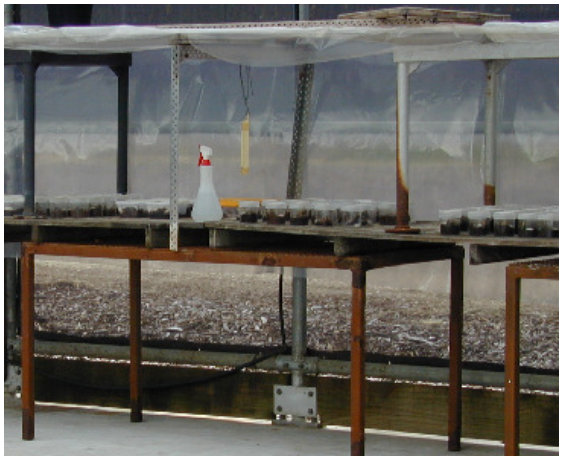
Forty snails were paired and placed on four different substrates in vented polycarbonate jars (200mL) (Figure 2-1a and b). The substrates included citrus twigs cut into 3 cm lengths (approximately 1.5 cm in diameter), moist soil at 3-5 cm in depth, and decaying citrus leaves (Figure 2-1a). Soils from two citrus growing regions were used as substrates, namely sandy soil (Riverland) and loamy sand (Sunraysia). Five jars were set up for each substrate. Each week they were checked for eggs, and the number of clutches and eggs per clutch were recorded. Food consisted of a 1:1:1 mix of rolled oats, milk powder and calcium carbonate, and was provided on a small plastic plate (Figure 2-1a). After the substrates were searched for eggs, they were remoistened and returned to the original containers. These containers were kept in a shade house and exposed to natural environmental conditions, but protected from rainfall. Each egg clutch was placed in a Petri dish on moist filter paper, and the numbers of eggs were counted using a dissecting microscope. Egg size was also measured using a dissecting microscope at 30x magnification.

Figure 2-1: Polycarbonate vented jars used to culture *Microxeromagna armillata*

a) *M. armillata* cultured on (from left) soil with decaying citrus leaves, decaying citrus leaves and citrus twigs. Food (white) is provided on a plastic plate.

b) top view of vented polycarbonate jars

c) Shade-house area where *M. armillata* was cultured



2.2.3 Results and Discussion

Eggs were laid in both the Riverland and Sunraysia soils, but no eggs were laid on either the citrus leaves or twigs (Table 2-1). *Microxeromagna armillata* laid single eggs and multiple egg clutches (mean numbers of eggs per clutch ± sd 10.2 ± 8.88, n = 38, pooled soil substrate data) from 0.5 to 2.5 cm deep in the soil. Mean egg diameter across clutches was 1.25 mm (± 0.05 sd, n = 38).

Within each soil type the number of eggs laid per pair was highly variable, and while more pairs laid a greater number of eggs on Sunraysia soil, no significant difference was found in the numbers of pairs laying eggs (Fishers exact test, P=0.167) nor the number of eggs laid per snail (Mann-Whitney U = 4, df=5, P= 0.072) on the different soil types. Soil type was broadly characterised in this experiment. Soil type and moisture content are known to influence snail breeding behaviour (eg *C. virgata* [23]) and further experimentation with full characterisation of soil chemical and physical attributes may yield differences in the breeding behaviour of *M. armillata*.

Table 2-1: Mean number of *Microxeromagna armillata* eggs laid per snail pair on different substrates.

Substrate	Mean number of eggs ± standard deviation
Riverland Soil	17.2 ± 28.6
Sunraysia Soil	83.0 ± 119.6
Citrus Leaves	0
Citrus Twigs	0

The most common place to find eggs of *M. armillata* in the field is in soil. Eggs have been observed in soil at both Riverland and Sunraysia citrus orchards, but on occasion egg clutches were also observed in leaf litter. It is thus possible that this experiment did not provide suitable conditions for *M. armillata* to lay eggs in leaf litter. Humidity and depth of leaf litter could be important factors. Low humidity may also have prevented egg laying on citrus twigs, but as no eggs were ever observed in citrus trees, this suggests the tree is an unlikely egg-laying substrate. However, as *M. armillata* readily laid eggs in Sunraysia soil, this was chosen as the substrate for culturing *M. armillata* in subsequent experiments.

2.3 Reproductive lifespan, number and size of offspring, and the influence of body size on key fecundity characteristics of *Microxeromagna armillata*

2.3.1 Introduction

Whilst no life-history traits have been described for *M. armillata* in the literature, there is a significant body of work on the life history traits of terrestrial molluscs. The key traits generally under consideration are length of life, reproductive lifespan, number and size of offspring and also the factors which may influence these characteristics, such as genetic, environmental, and physiological factors. Using this information we can test the theories of life history evolution, as well as understanding and predicting the population dynamics of individual species. Length of life/reproductive lifespan and number and size of offspring are key traits under investigation for *M. armillata*.

Terrestrial gastropods can live up to 19 years and, although many are long lived, more than half of existing records involve short-lived species [16]. Longevity in gastropods is negatively correlated with shell reduction, meaning that slugs or thinly shelled snails have shorter life-spans [24]. Exposure to high temperatures is also negatively correlated with longevity, as

species occupying arid environments tend to have shorter lives than those in other climates [24]. In addition, body size is found to be positively correlated with longevity as small snails and slugs live shorter lives than larger species. Although these trends are based on all the information available, it is important to note that only a minute proportion of the terrestrial molluscs have been studied in this context, and exceptions to these trends can be found [16, 24]. Within the Hygromiidae, species that have been studied are relatively short-lived, having annual or biennial lifecycles.

The relative relationship between juvenile and adult mortality is thought to control the evolution of the reproductive lifespan [25]. When the mean and variance of adult mortality increase, decreasing the reproductive lifespan, semelparity should arise, while iteroparity is brought about from trends in the opposing direction [25]. Snails within the Hygromiidae have been found to be both semelparous (producing offspring during only one year/season/periodic change in time) and iteroparous (survive and reproduce over successive years/seasons/periodic changes in time). The lifespan of *M. armillata* is not known, and the life history characteristics it has evolved have not been elucidated. The prediction that *M. armillata* would be short lived and semelparous could be made from the review of longevity by Heller [24] but this needs further investigation as exceptions to these general rules have been noted.

Age is difficult to determine in many molluscs, and as such the most commonly studied trait is size at maturity. This is one of the most studied life history traits in many organisms [17, 25, 26]. Optimal age and size at maturity are said to occur when equilibrium is reached between the benefits and costs of maturation at different sizes and ages [25]. Maturation at an earlier age decreases generation time, with the benefit of an increase in the probability of juvenile survival, as juveniles do not remain in this stage for long (see Stearns [25] for review). With shorter generation time also comes an increase in fitness, as offspring are born sooner and begin reproducing earlier [25]. On the other hand, delayed maturity may permit further growth,

which is highly correlated with an increase in fecundity [25-27]. However, this trend is not universal. Leather [28] found within lepidopterans that larger individuals were not necessarily more fecund than smaller ones and suggested that longevity, and the factors influencing it, would have a greater effect on fecundity. Similarly, no positive relationship was found between size and several reproductive traits in water striders, suggesting that size was selectively neutral [29].

Several studies have investigated the relationship between body size and fecundity within the Gastropoda. In Australia, Baker [30] found positive correlations between shell size and total number of eggs produced in both *T. pisana* and *C. virgata*. However, these positive correlations did not apply consistently for snails collected from different field sites, suggesting an environmental rather than size effect. Similarly no positive correlations have been found with shell height and fecundity for *C. acuta* in laboratory cultures [31]. In Britain, positive correlations were found between adult size and date of first clutch in *T. pisana*, but no correlation between size and total reproduction was found [32]. Large adults of the snail *Cepaea nemoralis* (Owen) were found to lay larger clutches, although no correlation was found between size and total egg production [33]. However, it is important to note that studies of this nature in terrestrial molluscs do not generally extend further than recording total egg production. Additional characteristics such as egg size and viability may also be influenced by adult size and not be illustrated by these studies.

The number of offspring produced by an organism can have a substantial influence on how a population changes over time. The number and size of offspring it produces represent the reproductive investment outlaid by an organism. There is a substantial body of research (Stearns [25] for review), which focuses on the evolution of fecundity and other life history traits in various organisms. Analysis of the evolution of offspring number and size is dominated by two models, one that discusses conflicts of interest, while the other emphasises trade-offs [25]. Both models begin with the Lack clutch hypothesis (references in Stearns [25])

which is based on a clutch size that optimises parental fitness while allowing for the effects of clutch size on offspring survival. Both of these frameworks agree that, where the reproductive effort provides decreasing returns or mortality is positively correlated with reproductive investment, an intermediate level of reproduction is demonstrated [25]. This is essentially demonstrated by iteroparity. The main advantage of iteroparity is the increased likelihood of successful reproductive events if one event fails due to environmental conditions; this is known as a bet-hedging or spreading-of-risk strategy [25]. In direct contrast to iteroparity, semelparity is characterised as 'a single suicidal reproductive effort' [25]. The advantages of semelparity are less obvious, but have been linked to evolution in relatively unstable environmental conditions [16, 34]. However, Yom-Tov [35] showed that sympatric species could evolve both semelparity and iteroparity under the same environmental conditions. Both iteroparity and semelparity are seen within the Mollusca, but assigning different species to these categories is not always clear-cut. *Theba pisana* has been found to have both annual and biennial lifecycles within the same environment [36]. Similar traits have been found in the Hygromiidae, with species including *C. virgata* [37], *Xeropicta vestalis* (Pfeiffer), *Helicella itala* (Linnaeus) and *Monacha cartusiana* (Müller)(reviewed by Heller [16]). However, Heller [16] classed these as semelparous as, although they can live for more than one year, these species only reproduced during one period. This classification of semelparity will be followed throughout this thesis. *Cernuella virgata*, whilst semelparous under this definition, has a relatively long reproductive period of up to eight months [30]. This may help to spread the risk within a season, rather than between the seasons as seen in iteroparous species.

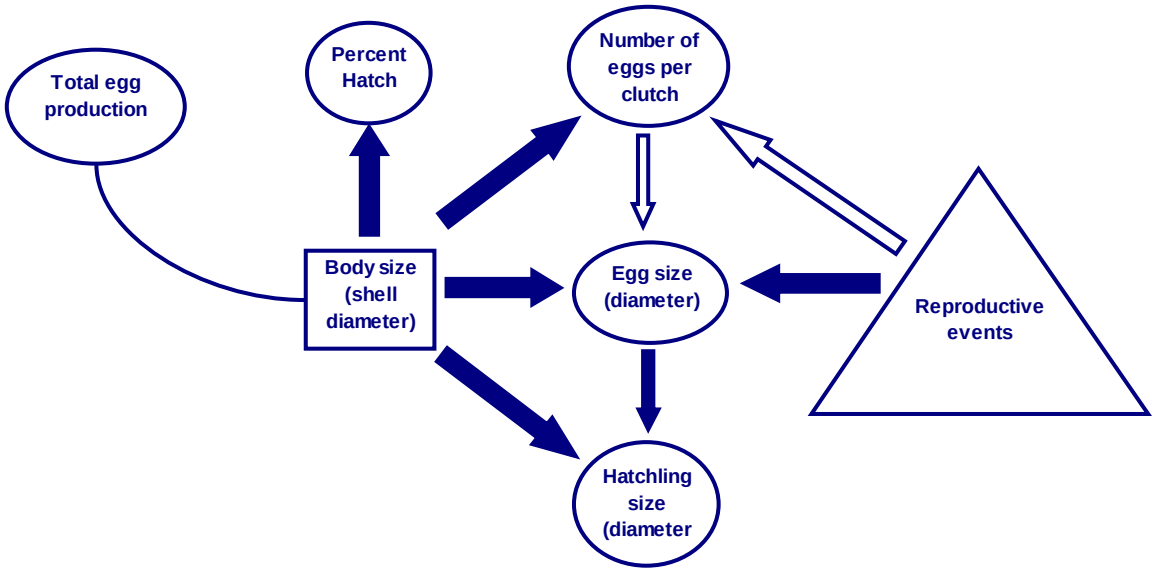
The number of eggs within a clutch varies greatly within the Mollusca, and is generally related to body size. Baur [38] neatly demonstrated that a relative measure of egg size adjusted for body size has been shown to correlate not only with how many eggs a snail species lays, but how they lay their eggs. This study found that snails with an egg diameter greater than 17% of its shell breadth/length laid eggs singly, while those with relatively smaller eggs laid them in batches. Irrespective of body size, factors such as altitude [39], inter- and intra-specific

competition [40] and time of season [30] can influence number of eggs laid per clutch within a species.

As the number of eggs per clutch varies between and within species, so can egg size, and a trade-off is often evident between these two traits [25]. Egg size in terrestrial molluscs can range from 0.4mm diameter [41] to 51mmx31mm [16]. In snails, egg size is strongly correlated with the size of the parent, but in slugs this correlation is less significant [16]. As well as interspecific differences in egg size, intraspecific variation has also been documented. *Arianta arbustorum* (Linneaus) egg volume ranged from 5.5 – 26.3 mm^3 , but within clutch variation was significantly smaller than among clutch variation [42]. Egg size has also been shown to vary depending on the time of the reproductive season, with the slug *Limax maximus* (Linnaeus) producing smaller clutches of larger eggs as it ages (Rollo 1983, cited in Heller 2001[16]). Heller [16] reviewed egg size in gastropods, with the 14 hygromiids studied showing egg sizes ranging from 1 - 3 mm. There are four snails within this group that are closely related to *M. armillata* in terms of body size; *Ashfordia granulata* (Alder)(9mm), *Pseudotricha rubiginosa* (7mm), *Perforatella bidentata* (Gmelin)(9mm) and *Trichia sericea* (Drapanaud)(7.5mm), with egg sizes of 1, 1.6, 1.7x2, and 1 mm, respectively. With this information, the most we could predict about *M. armillata* is that it may lay eggs between 1 and 2 mm in diameter, based on the relationship between body size and egg size in related species [16]. By using these estimates of egg size in the model developed by Baur [38], the relative measures of egg size to body size obtained range from 12 – 25%. This range overlaps the 17% cut off point identified by Baur [38], which means that we are unable to predict if *M. armillata* will lay eggs singly or in batches. In addition, there are no clear cut rules for predicting which reproductive strategy an organism may employ (references in Heller [16]). More research is clearly needed to describe these characteristics for *M. armillata*.

As discussed, fecundity characteristics are often interrelated, and these interactions can heavily influence the life history of an organism and how it responds to change. Determining which fecundity characteristics and tradeoffs are the most tightly linked in *M. armillata* will enable further understanding of the factors most affecting the life history of *M. armillata*. In addition, body size has been highlighted as a major influence on a variety of reproductive traits in many organisms, and may play an important role in the life-history of *M. armillata*. From this review of the literature, I hypothesise that the fecundity characteristics of *M. armillata* are interrelated, and influenced by body size, in a variety of ways (Figure 2-2). This model assumes that *M. armillata* lays more than one egg per clutch, and more than one clutch per year.

Figure 2-2: Expected relationships between life history traits of *Microxeromagna armillata*. Filled arrows represent a positive relationship, open arrows represent a negative relationship, and line connector represents no relationship.



To test this hypothesis and to describe the general reproductive characteristics of *M. armillata*, an experiment was designed to answer three main questions:

1. **What are the general reproductive characteristics of *M. armillata*?** Is *M. armillata* semelparous or iteroparous? How often does *M. armillata* produce eggs and in what quantity? How many offspring does *M. armillata* produce?
2. **Do these reproductive characteristics change with time/number of reproductive events?**
3. **Are life-history traits inter-related and/or influenced by body size in *M. armillata*?**

2.3.2 Materials and Methods

Sixty laboratory reared *M. armillata* were paired and enclosed in vented polycarbonate jars (200 ml) with 150 ml field soil collected from Nangiloc, Victoria and several decaying citrus leaves (cf Figure 2-1a). Pairs of *M. armillata* ranged in mean shell diameter from 4.8 to 8.3 mm, with pairs made of snails with the most similar size. Snail shell size was measured across the widest part of the shell according to the procedure outlined in Smith and Kershaw [1]. Snails were at least nine months old at the beginning of this experiment. Pairs were provided with additional food *ad libitum* that consisted of a 1:1:1 mix of rolled oats, milk powder and calcium carbonate. Pairs were housed under a transparent waterproof cover in a shade house (Figure 2-1c) except during laboratory inspection. Over a 75-week period (May 2002 - October 2003) the soil in each container was searched for egg clutches weekly, remoistened with water and returned to the container. Each week a small amount of new soil was added to the container and in cases where fungal growth was excessive, all soil was replaced. For each pair the number of clutches laid was recorded and individual clutches were placed on moist filter paper in Petri dishes. These were stored at 16°C in darkness for at least 12 hours. Storing the clutches on moist filter paper overnight allowed for maximum water

absorption by the eggs, ensuring that any difference in egg size measured was due to differences in structure rather than water content.

The number of eggs per clutch was counted using a dissecting microscope and a sub-sample of the eggs was measured to record egg diameter (Table 2-2). Initially egg diameter was measured at 10x magnification but it became clear that this level of measurement would not be fine enough to detect differences in egg diameter. Measurements were then taken at 30x magnification and egg sizes of those measured at 10x magnification were not included in any analysis. The number of eggs to be measured for each sample size (number of eggs per clutch) was calculated to ensure a minimum detectable difference of 0.1mm, according to the equations outlined in Zar [43, pp 105-109].

Table 2-2: Number of *Microxeromagna armillata* eggs measured per egg clutch

Number of eggs per clutch	**10**	**15**	**20**	**25**	**30**	**40**	**50**	**60**
Number of eggs measured	10	11	14	16	18	20	24	25

In 2002, a sub-sample of clutches from each pair was transferred into individual wells of a tissue culture tray (6 wells by 4 wells) lined with moist filter paper, covered and placed in an incubator at 16°C and constant darkness. Trays were checked daily for hatching, and filter paper in the tissue culture wells was remoistened as required to provide a humid environment similar to moist soil. In 2003 this procedure was repeated with several modifications. All of the clutches produced, rather than a sub-sample, were monitored for hatchling emergence and hatchling shell diameter was also measured. Hatchling shell diameter was measured across the broadest part of the shell as outlined in Smith and Kerswaw [1].

Fecundity can be expressed in a variety of ways, generally taking the form of reproductive output per unit of time. In the context of this experiment, time can be meaningfully expressed

in three ways – length of the experiment (75 weeks), season (year one or year two) and week (week of egg laying or week of experiment). The life history characteristics and other variables chosen to study in this experiment included:

- **Number of eggs laid per snail** – when both snails in a pair were still alive, they were assumed to contribute equally to the number of eggs laid.
- **Number of clutches laid per snail** – when both snails in a pair were still alive, they were assumed to contribute equally to the number of clutches laid
- **Number of eggs per clutch**
- **Mean egg size**
- **Mean hatchling size**
- **Proportion of eggs hatching**
- **Clutch hatch time –** time, in days, between first egg hatching and last hatching within a clutch.
- **Number of snails** – all containers began with two snails, but this could change with mortality.
- **Shell size**:
    - **Initial snail size (2002)** – shell diameter at the beginning of the experiment.
    - **Initial snail size (2003)** – shell diameter for each snail was measured prior to egg laying in 2003.
    - **Progressive snail size**– Mean shell diameter per pair was measured approximately every two weeks.
- **Week of experiment (date)** – allows for examination of seasonal influences.
- **Week of egg laying** – number of weeks that the pair/snail has been egg laying, e.g. week 5 would be the fifth week since egg laying commenced.

Comparisons within fecundity characteristics between years (2002, 2003) were undertaken using t-tests or Mann-Whitney U-tests as appropriate. Relationships between fecundity characteristics were investigated using multiple linear regressions or Pearson's correlation where appropriate. All analyses were conducted using SPSS for Windows 11.5.

2.3.3 Results and Discussion

General reproductive characteristics

Microxeromagna armillata exhibited an iteroparous egg laying strategy, laying eggs in both years of this experiment. Mortality of *M. armillata* was low during the first year (30% at December 2002) and at end of the experiment just over half of the snails had died (58%). Hence, the lifetime fecundity of *M. armillata* cannot be reported, as the remaining snails may have reproduced in a third year if maintenance and monitoring had continued. All pairs laid eggs, with almost 1000 eggs and 50 clutches laid on average per snail throughout the experiment (Table 2-3). Eighty-seven percent of all clutches laid were multiple egg clutches, comprising eggs of approximately 1.2 mm in diameter. A relatively low proportion of egg hatching was seen overall and, whilst the incubation time could not be precisely determined from this experimental design, observations suggest an incubation period of 1 – 3 weeks at 16^{o}C. Eggs in a clutch hatched, on average, within 6 days of each other (clutch hatch time, Table 2-3). Percentage hatch for individual clutches ranged from 0 – 200%. Several snails had a tendency to lay double embryo eggs, and hence had a hatch rate greater than 100%. Size at first reproduction was not recorded during this experiment, but subsequent dissections suggest that *M. armillata* is capable of reproduction at approximately 6mm in shell diameter (Figure 3-7).

Table 2-3: Reproductive traits of *Microxeromagna armillata* recorded over 75 weeks.

Reproductive characteristic	Mean ± sd	N
Eggs per snail	992 ± 360	31
Clutches per snail	47 ± 19	31
Eggs per clutch	21.54 ± 16.08	2491
Egg diameter (mm)	1.209 ±0.117	27937
Percent hatching	57.9 ± 41.7	835
Clutch hatch time (days)	5.9 ± 4.6	540

These traits form the basic reproductive characteristics of *M. armillata*, and some interesting comparisons can be made with species of a similar size and habitat. Baur [38] reviewed reproductive characteristics of small-sized snail species living in leaf litter, (reproduced here in Table 2-4, with addition of data for *M. armillata*), and while some of *M. armillata*'s characters are similar, such as size at maturity, egg size and relative egg size, others such as clutch size and lifetime reproduction are remarkably dissimilar.

Baur [38] hypothesised that very small-sized snails shared a suite of reproductive traits, in particular that snails with a relative egg size (egg size/size at maturity) above 17% tended to lay eggs singly, while species below this mark lay multiple egg clutches. *Microxeromagna armillata* utilises both egg laying strategies, and as such is similar to other snails of its size (eg *C. septemspirale, D. rotundatus*, Table 2-4).

Larger Hygromiid species, such as *C. virgata*, have a much lower relative egg size [Heller 16, 7.5 - 10% calculated from 30], yet *M. armillata* and *C. virgata* produce a similar number of eggs relative to their body size (1:165 and 1:144 respectively, shell diameter(mm):number of eggs laid). Hence, *M. armillata* maintains a very high investment in egg size relative to body size, particularly when total reproduction is taken into account. This is particularly surprising since *M. armillata* demonstrated an iteroparous egg laying strategy, compared to the semelparous *C. virgata*, and the risk of egg failure is spread over a longer period of time. However, these comparisons assume that egg diameter, and indeed body size, scale linearly with fitness characteristics which may not be the case.

Table 2-4: Summary of reproductive characters of small sized snail species living in leaf litter. Reproduced from Baur [38].

Subclass (Order)	Species	Locality	Size at maturity[a] (mm)	Egg size (mm)	Relative egg size[b]	Clutch size (range)	Lifetime reprodu-ction	Type of study[e]	Source[f]
Prosobranchia	*Cochlostoma septemspirale*	S-Germany	7.0	1.0	14.3%	5 (3-11)	-	L	(1)
(Mesogastropoda)	(Razoumowsky)								
Pulmonata	*Carychium tridentatum*	England	2.2	0.4-0.5	20.5%	1	5-6[c]	F/L	(2)
(Basommotophora)	(Risso)	S-Germany	1.5	0.32x0.41	24.3%	1	-	F	(3)
Pulmonata	*Cochlicopa lubrica* (Müller)	S-Sweden,	4.5	1.1x1.2	25.5%	1	0.6-3.4[d]	F	(4)-(6)
(Stylommatophora)		Poland							(7)
	Vertigo pussila (Müller)	Poland	-	-	-	1(1-2)	5-30[c]	L	(8)
		Sweden	1.9	0.5	26.3%	1	10[c]	L	
	Discus rotundatus (Müller)	Germany	6.0	1.0	16.6%	3 (2-5)	2-5[c]	L	(9)
		S-Sweden	-	-	-		0.2-3.5[d]	F	(5)
	Punctum pygmaeum (Draparnaud)	Sweden	1.3-1.4	0.41x0.50	32.7%	1	6[d]	L	(10)
	***Microxeromagna armillata* (Lowe)**	**Australia**	**5.8-6.0**	**1.2**	**20%**	**22 (1-97)**	**992[c]**	**L**	**(11)**

a) Shell length or shell breadth, b) Egg size/size at maturity, c) Number of eggs, d) Number of young per year, e) L= laboratory study, F = field study, f) Source: (1) Prince (1967); (2) Morton (1954); (3) Doll (1981); (4) Gugler (1963); (5) Wareborn (1979); (6) Uminski & Focht (1979); (7) Pokryszko (1987); (8) Baur (unpublished data); (9) Frömming (1954); (10) Baur [38]; (11) this study.

Influence of reproductive year on life history traits.

As *M. armillata* laid many clutches over two years, the distribution and characteristics of these eggs can be studied through time. In 2002, egg laying began in late autumn (mean = 28/5/2002 ± 1.6 weeks sd) and ceased in mid-spring (mean = 01/10/2002 ± 3.6 weeks sd). Egg laying resumed again in mid-autumn 2003 (mean = 7/4/2003 ± 3 weeks sd) and finished in late winter (mean = 25/8/2003 ± 6.1 weeks sd). The timing of egg laying is similar to that recorded for *T. pisana* across southern Australia [36], and *T.pisana*, *C. virgata* [30] and *C. acuta* [31] in laboratory cultures, although these species only reproduced in one year. Most hygromiid snails are thought to be semelparous, although some variability in life cycles is seen (*C. virgata, Xeropicta vestalis, Helicella itala, M. cartusiana*; reviewed by Heller [16]). This is generally due to a biennial lifespan rather than biennial breeding periods, as in *M. armillata*.

Microxeromagna armillata laid most of its eggs in the first few weeks of egg laying each year. The number of eggs laid per snail decreased gradually over time, in a similar pattern to that observed in *C. virgata, T. pisana* [30], and *C. acuta* [31]. In *M. armillata* this pattern was similar for both 2002 and 2003 (Figure 2-3) and, as *M. armillata* laid the same number of eggs and clutches per snail during both years, this is not surprising. Neither the number of eggs nor the number of clutches laid per snail differed between the years, but differences were seen between years with other fecundity characteristics (Table 2-5).

Figure 2-3: Mean number of eggs laid by *Microxeromagna armillata* individuals in 2002 and 2003 breeding seasons standardised for week of egg laying.

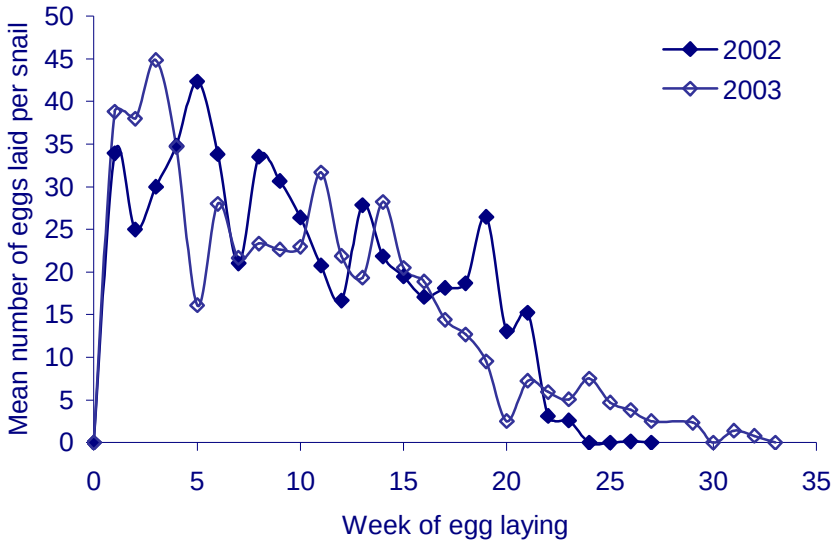


Table 2-5: Reproductive characteristics of *Microxeromagna armillata* in 2002 and 2003.

Mean values significantly different between years are followed by different letters and highlighted in bold type.

Reproductive Characteristic		**2002**	**2003**
Number of weeks between first and last egg laying	Mean± sd	18 ± 4	20 ± 7
	N	31	29
	Test Statistic	Mann-Whitney U = 352.5, P= 0.150	
Number of eggs laid per snail	Mean± sd	523 ± 171	502 ± 295
	N	31	29
	Test statistic	Mann-Whitney U = 481.5, P= 0.647	
Number of clutches laid per snail	Mean± sd	23± 6.7	25± 16.8
	N	31	29
	Test statistic	Mann-Whitney U = 441.5, P= 0.906	
Mean number of eggs per clutch	Mean± sd	**23^{a}± 15**	**20^{b}± 17**
	N	1357	1134
	Test statistic	Mann-Whitney U = 687757, P< 0.001	
Mean egg size (mm)	Mean± sd	**1.217^{a}± 0.103**	**1.230^{b}± 0.129**
	N	14693	13244
	Test statistic	t-test[1], P< 0.001	
Mean hatchling size (mm)	Mean± sd	-	0.976±0.103
	N	-	7108
Percent of eggs hatching	Mean	**86.9^{a}± 18.1**	**50.5^{b}± 42.8**
	N	170	665
	Test statistic	t-test[2] t = 18.599, df =522, P< 0.001	
Clutch hatch time (days)	Mean± sd	**4^{a}±2**	**6^{b}±5**
	N	80	460
	Test statistic	Mann-Whitney U = 12444, P< 0.001	

[1] Conducted in Microsoft Excel, not SPSS for Windows, [2] Arcsine transformed data

In 2003, fewer but larger eggs per clutch were laid in comparison to 2002. This indicates a potential trade-off between egg size and number of eggs per clutch, which is a common trend seen in other animals. This will be explored further in the subsequent section 'Relationships among fecundity traits and maternal shell size'.

A higher proportion of eggs hatched in the first year compared to the second year (Table 2-5) and, while the total number of eggs laid remained the same in both years, fewer offspring were produced in the second year. This decline in reproductive output could be a function of age, and *M. armillata* may have a predetermined number of eggs it is able to produce, or more infertile eggs could be laid with increasing age to provide food for newly emerging hatchlings, thereby increasing their fitness. Newly emerged hatchlings have been observed by the author eating unhatched eggs in previous experiments, and this could be an important source of nutrients for offspring with decreased fitness (i.e. that are laid at the end of the reproductive lifespan), and would give snails hatching first within a clutch a distinct advantage. Hatchlings of the land snail *A. arbustorum* also exhibit egg cannibalism, and Baur [44] demonstrated that hatchlings fed a cannibalistic diet experienced accelerated growth and higher survival, compared to those fed on lettuce. This may be one reason why *M. armillata* invests so heavily in relative egg size. To investigate this further, the time taken for all eggs to hatch within a clutch was measured to determine the level of hatching asynchrony (clutch hatch time). Clutch hatch time increased in the second year compared to the first, giving rise to a greater opportunity for egg cannibalism. *Microxeromagna armillata* may be able to regulate egg development time, the level of hatching asynchrony and therefore the propensity for intraclutch cannibalism, but this could not be demonstrated in this study.

Relationships among fecundity traits and maternal shell size

Numbers of eggs and clutches laid

Although initial shell diameter did not affect the total number of eggs laid during the experiment, it influenced how these eggs were laid over time. Snails larger at the beginning of the experiment laid significantly more eggs than snails smaller in the first year (adj R^2 = 0.164, P= 0.014; Figure 2-4). Although this relationship was significant, the proportion of variation accounted for is low, indicating that other factors contributed to predicting/explaining the number of eggs laid per snail. When initial snail size in 2002 was regressed against the number of eggs laid per snail in 2003, no significant relationship was found, but the trend was for snails smaller in 2002 to lay more eggs in 2003 (Pearson corr = -0.286, P= 0.066). A stronger and negative relationship was found between the number of eggs laid per snail in 2003 and initial snail size in 2003 (adj R^2 = 0.219, P= 0.007; Figure 2-5). As a strong positive relationship was found between initial snail size in 2002 and initial snail size in 2003 (adjR^2 = 0.648, P< 0.001), it is reasonable to assume that snails which were small at the beginning of 2002 were also smaller at the beginning of 2003, and hence it can be said that smaller snails in 2002 laid fewer eggs in 2002 but more eggs in 2003 compared to larger snails. This indicates that size at onset of reproduction may have significantly influenced the reproductive strategy of individual snails and that the production of offspring in each season was tailored to maximise fitness.

Smaller snails began egg laying later than larger snails (Figure 2-6) and spent less time laying eggs during the first year (adj R^2 = 0.283, P= 0.001). Conversely, larger snails laid fewer eggs in the second year and spent less time laying them (adj R^2 = 0.171, P= 0.016), which could indicate that there was a finite amount of reproductive resources available to the snail during its lifetime. Food rich in nutrients such as calcium, which is often a key component of snail eggs, was provided to snails ad libitum so it is unlikely that this resource limited the

reproductive capacity of these snails, but it is important to note that calcium is not the only nutritional requirement. Smaller snails may delay reproduction in favour of more growth, if an increase in size confers a resulting increase in offspring fitness. The relationships between body size and offspring size will be explored later in this section. A confounding factor in this experiment was the lack of information on size at first reproduction, which would assist in interpreting these data more conclusively. A strong positive relationship was found between the numbers of eggs and clutches laid (Figure 2-7), and the relationship between snail size and number of clutches laid mimics those previously described for the number of eggs laid.

Initial snail size in 2002 did not significantly affect the number of egg clutches laid per snail throughout the experiment, but a positive correlation was found between initial snail size in 2002 and the number of clutches laid in 2002 (Pearson corr. = 0.288, P= 0.05). A negative correlation was found between initial snail size in 2002 and number of clutches laid in 2003 (Pearson corr. = -0.337, P= 0.037). These relationships, although weak, are not surprising considering the relationship between snail size and seasonal egg production.

Figure 2-4: Mean number of eggs laid per *Microxeromagna armillata* adult in 2002 as a function of initial shell diameter 2002 (adj R^2 = 0.164, P= 0.014).

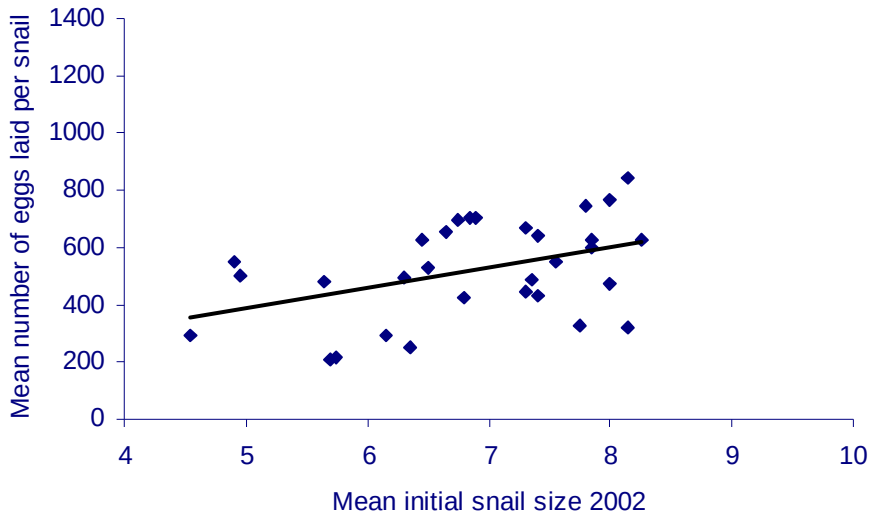


Figure 2-5: Mean number of eggs laid per *Microxeromagna armillata* adult in 2003 as a function of initial shell diameter in 2003 (adj R^2 = 0.219, P= 0.007)

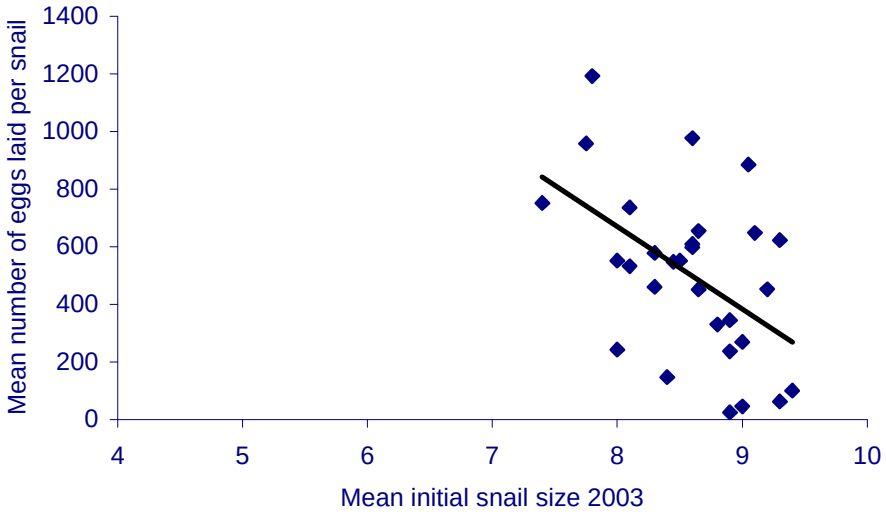


Figure 2-6: Onset of egg laying in 2002 as a function of initial experimental *Microxeromagna armillata* size 2002. (adj R^2= 0.389, P< 0.001)

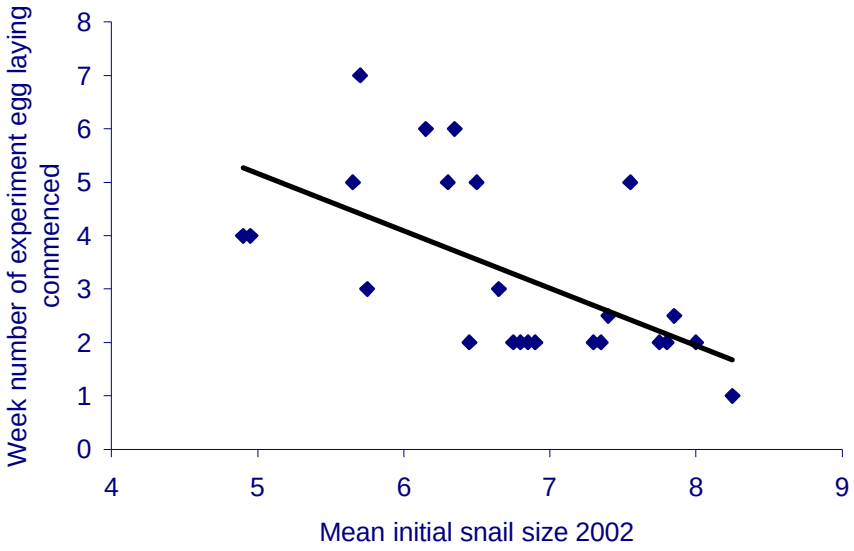
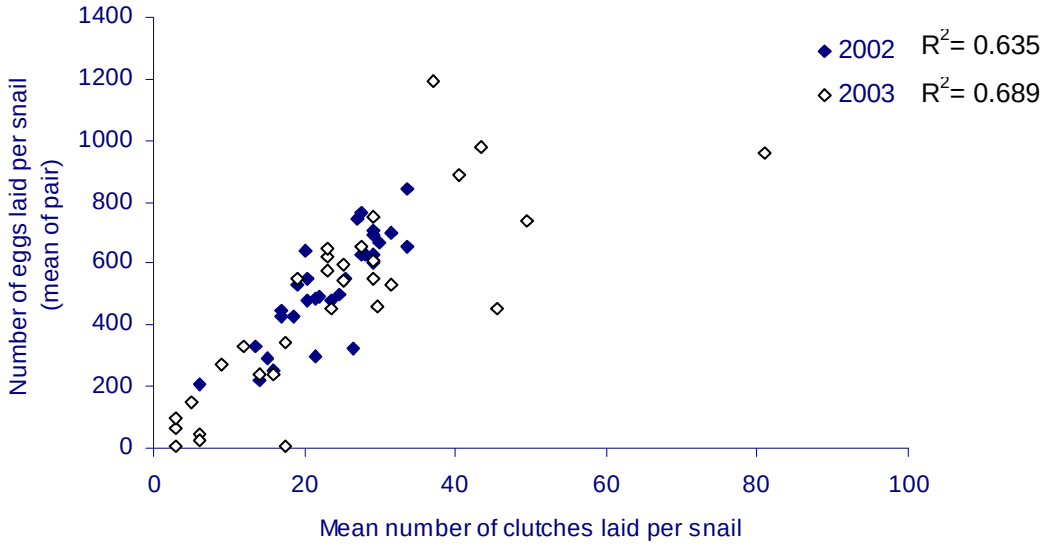


Figure 2-7: Relationship between the number of clutches and eggs laid per *Microxeromagna armillata* adult.



Number of eggs per clutch

A well-documented phenomenon in many organisms, particularly birds, is a progressively decreasing clutch size with successive clutches, while a positive relationship between body size and number of eggs per clutch has also been documented [33]. Both of these terms were included in a step-wise linear regression, and both terms assisted in predicting the number of eggs laid per clutch in each year (Table 2-6). However, while these terms are significant in the model, the proportion of variation explained is very low.

Table 2-6: Linear regression analysis of the impact of *Microxeromagna armillata* size and successive reproductive events on number of eggs per clutch in 2002 and 2003.

Regression analysis	Terms in model	β	t	*P*
2002 number of eggs per clutch: Adj R^2 = 0.062, F=45.544, P<0.0001, n=1357	Week of laying	-0.023	-8.714	<0.001
	Log initial snail size (2002)	0.130	4.907	<0.001
2003 number of eggs per clutch: Adj R^2=0.108, F=64.221, P<0.0001, n=1134	Week of laying	-0.317	-10.744	<0.001
	Log initial snail size (2002)	0.065	2.197	0.028

This indicates that either the relationship may not be linear or that other factors are more important in predicting the number of eggs laid in a clutch. Re-examination of the data indicated that a linear relationship was indeed the best fit. When initial snail size (2003) was substituted for initial snail size (2002) against the 2003 clutch data, it was not a significant term in the model. This could be a result of the relatively small size differences between the snails at the beginning of 2003 compared to 2002, as previously discussed, but it may also reflect the importance of size at onset of intial egg laying. While this was not measured precisely, snails smaller at the beginning of the experiment exhibited a different egg laying strategy to snails larger in the first year, and this may play an important role in determining clutch size.

Egg size

Several factors, which are likely to influence egg size, such as maternal snail size, week of laying, number of eggs per clutch and number of snails were included in a multiple regression analysis. All of these factors, except for number of snails, were significant in the model and assisted in predicting mean egg size in both 2002 and 2003 (Table 2-7). A consistent factor impacting on egg size in many organisms is the number of eggs laid per clutch, and a relationship between these two traits is seen in molluscs [45, 46], although it is not always consistent [32]. This relationship is present in *M. armillata*, although other factors such as parent size and successive reproductive events had a stronger relationship with egg size within each year.

Table 2-7: Linear regression analysis of fecundity characteristics which significantly predicted mean egg size of *Microxeromagna armillata* in both 2002 and 2003.

Regression analysis	Terms in model	β	t	*P*
2002 mean egg size: Adj R^2 = 0.339, F = 163.490, P <0.001, n = 951 clutches	Initial snail size (2002)	0.382	14.072	<0.001
	Week of laying	0.302	11.116	<0.001
	Number of eggs per clutch	-0.222	-8.418	<0.001
2003 mean egg size: Adj R^2 = 0.362, F =187.679, P<0.001, n = 987 clutches	Week of laying	0.419	15.464	<0.001
	Initial snail size (2002)	0.305	11.882	<0.001
	Number of eggs per clutch	-0.267	-9.918	<0.001

Experimental week was significant in both models when interchanged with week of egg laying, indicating that there could be a seasonal as well as a successive clutch effect on mean egg size. Co-linearity between these variables prevented simultaneous inclusion of both factors in analyses.

In 2002 initial snail size was the most influential term in the model predicting egg size, but in 2003 week of egg laying was the most significant term in the model (Table 2-7). This regression model was rerun with initial snail size (2003) instead of initial snail size (2002) for

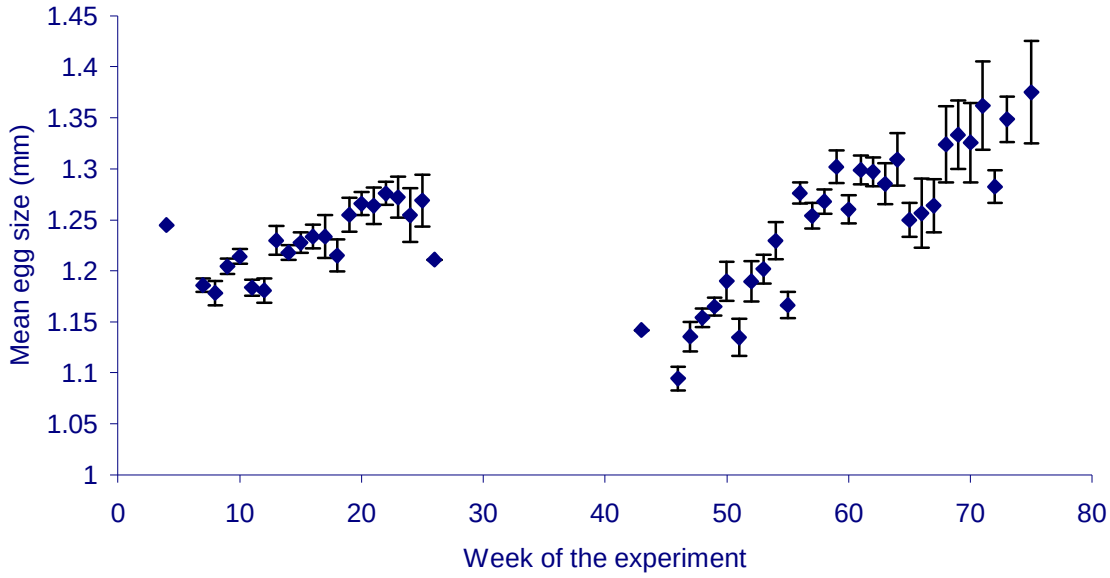
year 2003 data, and surprisingly this decreased the measure of variation explained by the model (adj R^2 = 0.333) but not the significance (P<0.001). This could be due to the relative difference in snail size measurements between the years. A large amount of growth is only represented by a small increase in shell diameter when snails are larger and therefore differences in actual shell volume/size are difficult to infer from diameter. This would have been more prevalent in 2003 when snails were larger, than in 2002 when shell diameter measurements may have more accurately reflected snail growth. It is also possible that older snails have varied more in how they have allocated their resources than younger snails. Thus a linear measure of snail size is less likely to truly reflect what resources a snail has to partition towards reproduction (egg size).

When progressive snail size was substituted for initial snail size in the model it was also a significant predictor of egg size for both 2002 (adj R^2 = 0.264, F =24.243, P<0.001, n = 195) and 2003 (adj R^2 =0.298, F =67.184, P<0.001, n = 469). In 2002, progressive snail size was the most important characteristic in the model followed by week of laying and number of eggs per clutch, which is similar to the previously fitted model (Table 2-7). In 2003, progressive snail size was the least important term following week of laying and number of eggs per clutch. This difference in the relative importance of predictors could add weight to the argument that size less accurately reflects reproductive fitness in older snails, but the sample size was substantially decreased using progressive snail size in the model and thus the power of the model to accurately predict mean egg size was decreased. Initial snail size was measured for all snails, whereas progressive snail size was measured fortnightly, and when included as a term in the model reduced the total number of clutches analysed.

Within each reproductive period (2002, 2003) shell diameter and egg size increased over time, but it is interesting to note that while the shell diameter of snails increased linearly throughout the whole experiment, egg size did not (Figure 2-8). Hence, the relationship between egg diameter and shell diameter changes throughout the life of *M. armillata* in this

experiment. We can conclude that egg diameter is not simply a physiological by-product of an increase in shell diameter, as may be expected to be the case. It is size at the onset of reproduction in each year that has the most influence on egg size and the reproductive strategy employed, rather than shell diameter throughout the season. Smaller eggs in larger clutches were laid early in the year and that the resulting offspring grew throughout winter and spring suggests these progeny are better positioned to survive the stresses of summer months, when mortality rates may be higher. Larger eggs in smaller clutches may provide more nutrients to offspring hatching in spring that have less time to grow before summer [46].

Figure 2-8: Mean *Microxeromagna armillata* egg size recorded during each week of the experiment. Error bars represent standard error of the mean; note y-axis does not originate at zero.



Hatchling size

The inter-relationships between the higher level fecundity characteristics and hatchling size were investigated for 2003 only, as hatchling size was not recorded in 2002. Egg size had the strongest relationship with hatchling size, closely followed by initial snail size in 2002 when

data were analysed with multiple linear regression (Table 2-8). Both of these regression terms were positive, as is the case in many species. Interestingly, when initial snail size in 2003 was substituted in the model for initial snail size in 2002, it was a significant term but did not improve the overall model (R^2 = 0.181, F= 32.785, P< 0.001). Even relatively small differences in adult size translated into differences in offspring size in 2003, but initial size in 2002 still had an impact on offspring size more than one year after egg laying began. Due to co-linearity, and the strong relationship between initial size in 2002 and 2003, it is difficult to determine which is the most influential measure. In analyses of the fecundity characteristics described previously, initial size in 2002 explained more variation in characteristics recorded in 2003 than initial size in 2003, indicating that perhaps it is size at maturity, rather than size at onset of egg laying each year, which has the most influence on reproductive productivity.

Table 2-8: Linear regression of fecundity characteristics with mean hatchling size of *Microxeromagna armillata* in 2003.

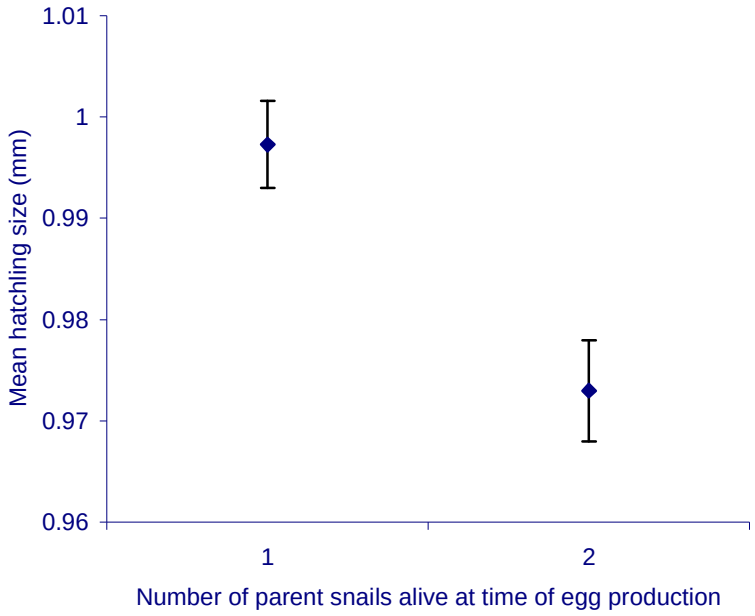
2003 hatchling size	Terms in model	β	t	*P*
Adjusted R^2 =0.225, F =26.141, P<0.001, n =433 clutches	Mean egg size per clutch	0.313	5.854	<0.001
	Initial snail size (2002)	0.220	4.468	<0.001
	Number of snails	-0.144	-3.323	<0.001
	Week of laying	-0.133	-2.678	0.008

The two remaining terms in the model, number of snails and week of laying, both showed a significant negative relationship with mean hatchling size, which was surprising (Table 2-8). Number of snails had the strongest negative relationship with mean hatchling size and indicates that snails which were alone due to mortality of one snail in the pair had larger offspring (Figure 2-9), even though egg size did not increase (cf Table 2-7, number of snails not a significant factor). This indicates that snails may be able to partition nutrients to eggs differently depending on their environment or reproductive status. If only one snail remains in a pair, then any eggs laid must be either a product of self fertilisation or cross fertilisation that utilised stored sperm, either of which could trigger a change in life history tactics relating to

the provision of nutrients to eggs. It is also possible that a decrease in population density could trigger an increase in offspring size. Larger individuals may have more energy or an increased level of fitness to disperse further to find a mate. The difference in hatchling size between isolated and paired parents may also be a function of time, as more snails were "alone" at the end of the season when eggs and hatchling are naturally bigger (week of laying), although if this was the case the number of parent snails would be expected to significantly predict mean egg size. As investigation of isolation effects on fecundity characteristics was not planned as part of this experiment, the number of samples and the time at which mortality occurred was variable, making interpretation of these findings difficult.

As the fecundity measures were examined in finer detail, e.g. from total egg production to hatchling size, more complex relationships were discovered. Positive relationships have been highlighted between egg size and week of laying, and egg size and hatchling size, yet a negative relationship was found between hatchling size and week of laying. Similarly, while egg size and number of eggs per clutch were negatively correlated, no relationship was found between hatchling size and number of eggs per clutch. This highlights the complexity of interactions between life history traits, and indicates that egg diameter does not always scale linearly, or at the same strength, with nutrient content of the egg or fitness benefit to the hatchling.

Figure 2-9: Effect of the number of *Microxeromagna armillata* adults per container on mean hatchling size in 2003. Bars represent standard error of the mean; note y-axis does not originate at zero.



Clutch hatch time

Clutch hatch time spanned from 0 (all hatched within 24 hours of collection) to 29 days, which is a surprisingly large range, and there may be a fitness benefit for this species to have such asynchrony in egg development (e.g. bet hedging) that was not highlighted by this study. The number of eggs per clutch was the only significant predictor of clutch hatch time (adj R^2= 0.169, F = 94.255, p< 0.001, n = 458), and this was a positive relationship. The time taken for a clutch to hatch (hatching asynchrony) may increase with increasing number of eggs within a clutch based on the additional time it takes to lay more eggs, although there is no evidence that this time is substantial for *M. armillata*. As the number of eggs per clutch increases, egg size decreases and the resulting hatchlings may be at a fitness disadvantage. Hatching asynchrony promotes egg cannibalism [47 and pers.obsv.] by first hatching sibs which may

increase the fitness of hatchlings in clutches with a high number of eggs. Although the number of eggs per clutch was the only predictor in this model, other unknown factors can influence clutch hatch time as was indicated by the difference in clutch hatch time between the years (Table 2-5).

Percent egg hatch

Percentage egg hatch was not significantly described by any measured fecundity parameter in 2002 (adj R^2 = 0.009, F = 1.184, P= 0.325, n = 80). This is surprising as body size was expected to influence hatching success. The 2003 data did not meet the assumption of normality and could not be analysed using multiple linear regression. Transformation did not improve the dispersion of the data. When only clutches from which some eggs hatched were considered in analyses, the week of experiment had a significant negative relationship with percentage hatch (adj R^2 = 0.022, n = 395, P= 0.001). However, the proportion of variation explained by this variable was so small that it is likely to be an artefact of analysis rather than a descriptor of a real effect.

2.3.4 Summary

Research in this section has addressed the three main questions highlighted in the introduction:

1. **What are the general reproductive characteristics of *M. armillata*?**

Microxeromagna armillata exhibited an iteroparous egg laying strategy during this experiment, laying an average of 992 eggs per snail during a two-year period, with a cessation in egg laying during summer. Heller [16] found that most snails which are agricultural pests exhibited semelparity, but he also conceded that the application of semelparous - iteroparous traits or r – K selected species theory had not yielded any broad generalisations within terrestrial

molluscs. When this experiment concluded, 42% of snails were still alive and may have survived to reproduce in following years with continuation of the experiment. The length of life for *M. armillata* has not been documented previously. As the snails used in this experiment were 8 – 10 months old prior to its commencement, this demonstrates that *M. armillata* can live for more than two years in a semi-field environment. *Microxeromagna armillata* utilises an intermediate strategy when considered against smaller and larger snail species. Smaller species generally lay single egg clutches and have a higher relative egg size to body size than larger species, which lay multiple egg clutches [38]. In *M. armillata*, the investment in relative egg size is high, yet it lays both single and multiple-egg clutches and has a similar relative reproductive output to larger snail species.

2. Do these reproductive characteristics change with time or reproductive event?

The reproductive traits of *M. armillata* changed with time and reproductive event on two different scales. Firstly, some life history characteristics of *M. armillata* differed between years, while other traits varied within years – these will be discussed below (question 3). With regard to differences in traits between years, a greater range of egg sizes were seen and clutches were smaller in the second year, although no difference was seen in the number of eggs in clutches laid in each of the two years. Laying a greater number of smaller clutches with larger eggs in the second year of reproduction could be a mechanism to increase offspring fitness, as reproductive resources may decline over the life of *M. armillata*. Evidence of declining reproductive resources with age may also be demonstrated by the decreasing percentage egg hatch with successive years. *Microxeromagna armillata* could be laying more infertile eggs to provide additional food for hatchlings which feed on conspecific eggs. This would be further promoted by the increase in hatching asynchrony within the clutches in the second year, as was shown by the increase in clutch hatch time. However, it is possible that these results are a consequence of laboratory study, which could extend fecundity beyond what would be described in the natural environment, resulting in an increase in infertile eggs. Surprisingly, it was recorded that some clutches had greater than 100% hatching, indicating

that twinning was occurring. This was generally confined to particular snail pairs that had a tendency to lay double eggs throughout the course of the experiment, rather than the whole experimental population.

3. **Are life-history traits inter-related and/or influenced by body size in *M. armillata*?**

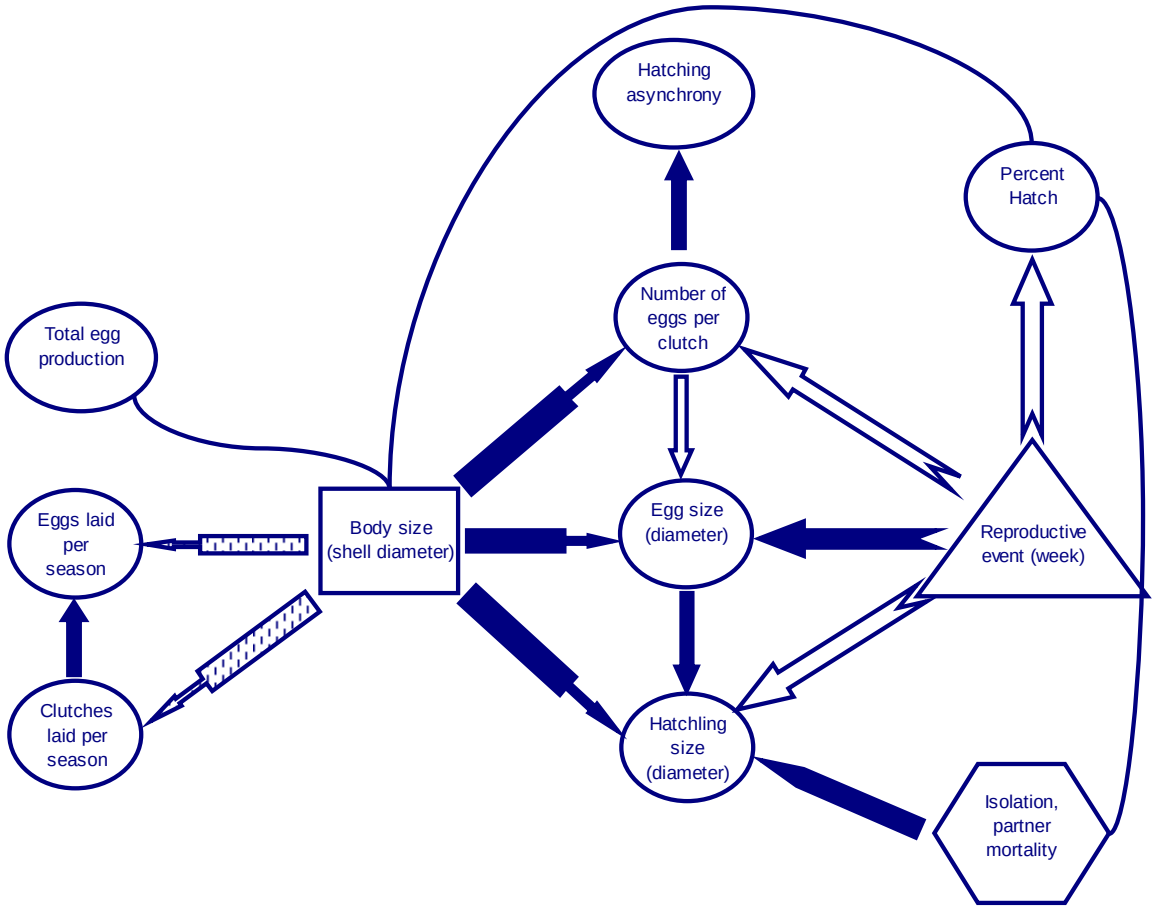
A number of life history traits were interrelated, and snail shell size also affected some reproductive characteristics of *M. armillata* (Figure 2-10). These relationships were more complex, and often differed, from those predicted in the initial model for *M. armillata* (Figure 2-2).

While initial shell diameter did not influence how many eggs were laid per snail during the experiment, it did significantly influence when eggs were laid. Smaller snails at the beginning of the experiment laid fewer eggs in the first year compared to the second and *vice versa* for larger snails. This is again an indication that *M. armillata* may have finite reproductive resources during its life-time, and allocates them accordingly, even when food is not a limiting factor. Snail size and egg size were positively correlated, as expected, and other factors such as week of egg laying (+ relationship) and number of eggs per clutch (- relationship) also played a significant role in predicting egg size during both years of the experiment. The effect of Initial snail size (2002) had a significant influence on reproductive characteristics of the same animals in the second year, further highlighting its importance, although these relationships were not always straightforward.

Whilst egg size increased with snail body size within each season, this relationship was not linear throughout the experiment, illustrating that egg diameter is not just a physiological by-product of shell diameter/maternal size. Larger eggs in smaller clutches, laid towards the end of the reproductive season would provide greater resources to hatchlings that may be more

susceptible to starvation, desiccation and predation over the summer months than their counterparts laid earlier in the season. Snails hatching early in the season may experience at least 6 months of growth prior to the summer period and initial hatchling size may not be as important for survival. This strategy can only hold true if larger eggs give rise to larger hatchlings; this was found to be the case in *M. armillata*.

Figure 2-10: Relationships among life-history traits established for *Microxeromagna armillata*. Filled arrows represent positive relationships, open arrows represent negative relationships, patterned arrows represent a positive then negative relationship with successive year, line connector represents no relationship.



Hatchling size was positively correlated with egg size and body size of the parent snail, but this relationship was confounded by the number of snails present when eggs were laid (Figure 2-10). This provides evidence that the size of *M. armillata* hatchlings can be influenced by either social factors (isolation/socialisation), or the quality of reproductive resources available (use of stored sperm or self-fertilisation), even if egg size is not. Either mechanism is influencing how nutrients were partitioned to eggs laid by isolated snails, as larger hatchlings arose from the eggs which were similar in size to those laid by paired snails. Social isolation [48] and self-fertilisation [49] have been shown to influence reproductive characteristics, and either or both of these mechanisms could be operating in *M. armillata*. While isolation did influence hatchling size, no relationship was found between isolation and the proportion of eggs hatching which might have been expected. It is not known if *M. armillata* can reproduce by self-fertilisation, and if so how this may influence the fecundity characteristics of this species. This forms the basis for later research (section 2.4).

No factors measured in this experiment impacted on the percentage hatch of *M. armillata* in a convincing manner, with the exception of experimental year. Further research is needed to quantify additional factors, such as soil moisture, temperature, and the presence of pathogens and predators, which may influence the proportion of eggs hatching.

The proportion of variation described by the regression models presented in this experiment was generally low, considering the large sample sizes, although often highly significant. Unless mortality occurred, measurements are based on the mean of a pair, and individual variation may confound the results of these studies. Accounting for this and quantifying other descriptors, such as parental hatchling size, parental growth rates, mating frequency, genetic history and environmental conditions, may assist in describing the factors which influence the reproductive characteristics of *M. armillata* with more accuracy.

2.4 Mode of reproduction

2.4.1 Can *Microxeromagna armillata* produce offspring using self-fertilisation?

2.4.1.1 Introduction

One of the most complex life history traits of molluscs is their mode of reproduction. All pulmonates are hermaphrodites, having both male and female reproductive systems, and can either be simultaneous or sequential hermaphrodites. In broad terms, simultaneous hermaphrodites have both male and female reproductive systems functioning within the same season, whilst sequential hermaphrodites function as one sex in one season (or early in the season), and then switch for the following season (or later in the same season) [50]. The majority of terrestrial molluscs are simultaneous hermaphrodites, with the male reproductive system maturing before the female reproductive system, which is generally thought to limit the capacity for self-fertilisation, although overlap does occur and autosperm can be stored [19, 51].

Although most terrestrial snails and slugs are obligate outcrossers [19], several species are capable of self-fertilisation [50, 51]. *Arianta arbustorum*, a helicid snail, can successfully self-fertilise, but with a reduction in fitness of offspring [52]. This trend is not universal as *Deroceras agreste* (Linnaeus) was found to be two to four times more fecund when animals self-fertilised compared to when they reproduced by outcrossing (reviewed by Heller [16]), with an additional increase in the longevity of offspring.

Although genetic diversity is compromised in self-fertilising snails even if fecundity is not, the associated trade-off is an increased ability for colonisation irrespective of mate-finding costs. Species that self-fertilise may be more effective colonisers [16], but no correlation between breeding system and habitat diversity has been found [53]. A trade-off between genetic diversity and dispersal may be reached in some snails and slugs which are predominantly out-crossers, but have the capacity to self fertilise. In this way, they can both maintain genetic diversity within a population and maximise dispersal, without the risk of reproductive isolation [16, 19, 50, 51].

Most hygromiids are thought to be incapable of self-fertilisation [19], but there is a lack of experimental evidence to confirm such a sweeping generalisation. With increasing reports of 'exceptions' to the autosterile rule [49], it is clear that more extensive studies of reproductive strategy should be conducted within this family. In previous research (section 2.3), it was shown that an individual *M. armillata*, which lost its partner early in the experiment, could still produce viable eggs the following year. This could be explained by the use of stored sperm from previous matings or it could suggest that *M. armillata* can self-fertilise. The possibility that *M. armillata* can produce viable offspring using self-fertilisation is investigated in this section.

2.4.1.2 Materials and Methods

Juvenile snails (n = 20), which ranged in shell diameter from 2.3 to 3.9 mm (mean = 3.1mm), were taken from a laboratory culture on the 29th of January 2003. Each snail was isolated in an individual container, with soil up to three centimetres deep and food as previously described (section 2.2.2). The snails in each container were checked for mortality each week and by the 18th of March 2003, 50% of these snails had died, leaving only 10 isolated snails. This number was insufficient to provide a paired control for the isolated snail group, so a

further 10 snails were taken from the laboratory culture, paired and placed in containers with soil and food as described previously. These snails ranged in size from 2.4 mm to 3.3 mm (mean = 2.7 mm). As the isolated snails had been provided with food and regularly moistened soil over a longer period of time, they had grown significantly more than those in the laboratory culture. However, once the snails removed from the laboratory culture on the 18th of March were provided with moist soil and food ad libitum, they grew quickly and reached a similar size to those isolated initially.

The soil in the containers was examined for the presence of eggs weekly, remoistened with water and returned to the container. Eggs were counted, measured and transferred to individual wells of tissue culture trays that were lined with moist filter paper according to the procedure outlined in section 2.3.2. Trays were placed in an incubator at 16℃ in darkness. The eggs in these trays were checked daily for hatching and, if hatching had occurred, the hatchlings were removed and measured using a binocular microscope (30x magnification). The percentage of eggs hatching was also recorded. This experiment ended on the 1/12/2003 after several weeks had passed without additional egg lay. The fecundity characteristics of paired and isolated snails were compared using a t-test or Mann-Whitney U test as appropriate. Multiple linear regression analysis was used to investigate relationships among fecundity characteristics. All analyses were conducted using SPSS for Windows11.5.

2.4.1.3 Results and Discussion

The first eggs were laid in May 2003. In the first week of egg laying, four out of five snail pairs, and five of the 10 isolated snails laid eggs. These clutches were monitored for hatching, and four out of the six clutches laid by the isolated snails in this week produced viable offspring. This is the first time that *M. armillata* has been proven to reproduce successfully by self-fertilisation. Snails in pairs laid approximately five times as many eggs and clutches per snail as isolated snails (Table 2-9), showing that snails which reproduced using self-fertilisation have a much lower reproductive output.

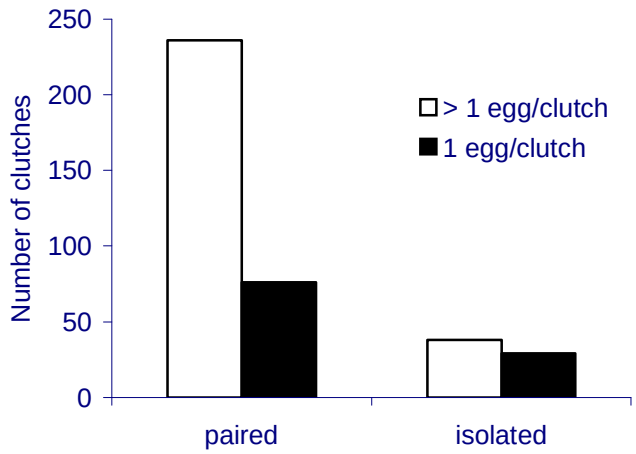
Isolated snails laid a higher proportion of single egg clutches than paired snails (Cross tabulation χ^2 = 10.069, df =1, P= 0.002; Figure 2-11), although no significant difference was seen between the numbers of eggs per clutch in isolated or paired snails overall (Table 2-9). This could be a bet-hedging strategy which reduces the risk of clones mating when they are laid singly rather than in multiple clone clutches, but at the same time, laying both single and multi-egg clutches could provide a better risk spreading strategy for isolated snails. Multi-egg clutches may provide insurance against desiccation and predation.

Table 2-9: Reproductive characteristics of isolated and paired *Microxeromagna armillata*. Mean values that are significantly different are followed by different letters and highlighted in bold type.

Reproductive Characteristic		Isolated	Paired
Number of eggs laid per snail	Mean± sd	**118.6**[a] ± 113.48	**596.5**[b] ± 162.86
	N	10	5
	Test statistic	t-test, t=-6.677, df=13, P= 0.001	
Number of clutches laid per snail	Mean± sd	**6.9**[a] ± 4.86	**31.5**[b] ± 15.73
	N	10	5
	Test statistic	t-test[†], t=-4.533, df =13[1], P= 0.001	
Mean number of eggs per clutch	Mean± sd	15.8 ± 14.3	20.6 ± 5.5
	N	10	5
	Test statistic	Mann-Whitney U = 12, P=0.109	
Mean egg size (mm)	Mean± sd	1.19 ± 0.13	1.23 ± 0.03
	N	10	5
	Test statistic	t-test, t=-552, df=13, P=0.591	
Mean hatchling size (mm)	Mean± sd	**0.92**[a] ± 0.05	**0.99**[b] ± 0.02
	N	7	5
	Test statistic	t-test t=-3.162, df=10, P=0.01	
Percent of eggs hatching*	Mean± sd	41.97 ± 14.22	44.93 ± 7.24
	N	7	5
	Test statistic	Mann-Whitney U=17.00, P= 0.935	
Clutch hatch time (days)	Mean± sd	5.57 ± 2.76	5.80 ± 1.09
	N	7	5
	Test statistic	t-test, t=-0.174, df = 10, P= 0.866	

[†] log transformed data, *arcsine transformed data

Figure 2-11: Proportion of single eggs to multiple egg clutches laid by paired and isolated *Microxeromagna armillata*.



Paired and isolated snails laid the same number of eggs per clutch and eggs of the same size, but isolated snails produced smaller hatchlings than paired snails. This is contrary to earlier results, where a negative relationship was found between number of snails and hatchling size (Figure 2-10). The reason for this change in the nature of the relationship between experiments is unclear. In the first experiment snails had not been isolated since the juvenile stage, and it is assumed that they had mated previously. As such, eggs resulting from these snails may have been fertilised using stored sperm rather than self-fertilisation.

Isolated and paired snails produced egg clutches that had similar mean hatching success (Table 2-9), although percentage hatch varied more for clutches produced by isolated snails (isolated: 0 – 88% hatching; paired: 24 – 64% hatching). Whilst all isolated snails laid eggs, no hatchlings were produced by three of the ten isolated snails. It is surprising that no significant difference in percentages of eggs hatching was found between the groups, as this is often seen in other species [49]. The factors which influence hatching in *M. armillata* are largely unknown, and it is possible that the difference in growth rates of snails in the two

groups influenced the proportion of eggs hatching. It is also possible that paired snails were reproducing using self fertilisation and that forms of social interaction between snails, other than mating, influenced the reproductive characteristics when compared to isolated snails [48, 54].

The inter-relatedness of fecundity characteristics was examined in this experiment, to determine if the relationships are similar to those documented for *M. armillata* in earlier experiments (Figure 2-10), and to highlight any effects of isolation on the relationships.

Number of eggs per clutch

Whilst the number of eggs per clutch did not differ between isolated or paired snails, the relationship between traits differed among these groups. When the number of eggs per clutch was greater than one, week of laying was negatively correlated with the number of eggs per clutch in paired (Pearson Corr.= -0.404, $P<0.001$, n=236), but not isolated snails (Pearson corr. = -0.149, P=0.372, n=38). The relatively low, highly significant correlation between the number of eggs per clutch and week of egg laying indicates that the lack of relationship in isolated snails may be due to low statistical power. As most snails in the experiment began laying eggs at the same time, the terms week of egg laying and week of experiment were parallel in their effect on the model.

Egg size

Maternal size at onset of egg laying, week of the experiment, and number of eggs per clutch were entered into a regression model to investigate their relationship with mean egg size for both paired and isolated snails. All predictors were significant terms in the subsequent linear regression for paired snails, but no relationship was found between snail size and egg size in isolated snails (Table 2-10). When week of egg laying was substituted for week of the year, or when shell diameter at different stages of the experiment was substituted for shell diameter at

onset of egg laying, no substantial changes were seen in the outcomes of the model for mean egg size.

Table 2-10: Linear regression analysis of characteristics which can significantly predict mean egg size in isolated and paired *Microxeromagna armillata*.

Regression analysis	Terms in model	β	t	*P*
Isolated: Adj R^2 = 0.297, F = 14.762, P <0.001, n = 66 clutches	Number of eggs per clutch	-0.397	-3.789	<0.001
	Week of laying	0.349	3.326	<0.001
Paired: Adj R^2 = 0.416, F =69.578, P<0.001, n = 290 clutches	Week of laying	0.563	11.976	<0.001
	Number of eggs per clutch	-0.280	-6.096	<0.001
	Shell size at egg laying	0.127	2.709	0.007

The relationships between number of eggs per clutch, week of laying and egg size, as described by the models here, are similar to those described previously for *M. armillata* (Figure 2-10). Eggs laid by paired snails increased in diameter with maternal body size, but no relationship was found between body size and egg size laid by isolated snails. This suggests that the benefits of a larger body size may be negated when self-fertilisation is employed, and provides further evidence that egg size is not simply a physiological by-product of body size, but can be manipulated by the parent.

Hatchling size

The relationship between mean hatchling size and mean egg size, number of eggs per clutch, week of the year, number of snails and snail size at onset of egg laying was investigated using multiple linear regression. All predictors were entered in the model, with the most non-significant terms removed sequentially until all remaining terms were significant predictors of mean hatchling size (Table 2-11). A positive relationship between egg size and hatchling size was seen in both isolated and paired snails, and this seems to be a consistent relationship in *M. armillata*. Hatchling size increased with week of egg laying in paired snails, which is

contrary to a previously observed relationship (Table 2-8), and the reason for this is unclear. Similarly, a positive relationship was found between number of eggs per clutch and hatchling size for isolated snails. A relationship between hatchling size and number of eggs per clutch has not been previously identified. Those trends in paired snails which differed from previously described trends (Figure 2-10) are particularly surprising as these experiments were conducted simultaneously in 2003 and snails were exposed, as far as was practical, to identical environmental conditions.

Table 2-11: Linear regression analysis of characteristics which can significantly predict mean hatchling size in isolated and paired *Microxeromagna armillata*.

Regression analysis	Terms in model	β	t	*P*
Isolated: Adj R^2 = 0.343, F = 10.413, P <0.001, n = 37 clutches	Number of eggs per clutch	0.685	4.563	<0.001
	Mean egg diameter	0.312	2.074	0.046
Paired: Adj R^2 = 0.216, F =23.417, P<0.001, n = 164 clutches	Mean egg diameter	0.263	2.740	0.007
	Week of egg laying	0.254	2.649	0.009

Surprisingly, snail size was not a significant predictor of hatchling size, indicating that the relationship between body size and hatchling size can be uncoupled. In previous results (section 2.3.3) single snails laid the same size eggs but had larger hatchlings, whereas in this case single snails laid smaller eggs and had smaller hatchlings overall. Single snails in the previous experiment (section 2.3.3) may have been reproducing by cross fertilisation using sperm stored from previous matings, and by self fertilisation, which may have confounded results. It is also important to note that no studies have been undertaken on the compatibility and mating frequency of *M. armillata*, and these factors may influence results obtained from arbitrarily paired snails.

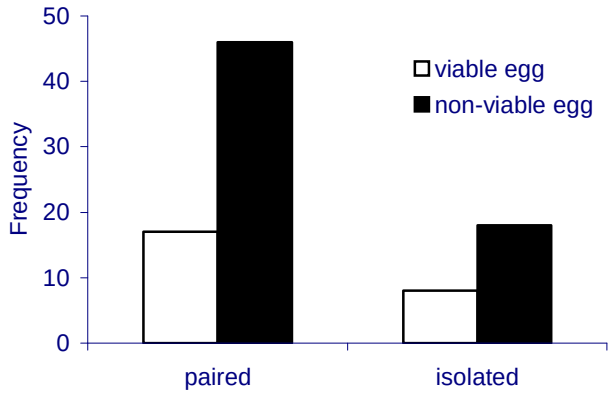
Clutch hatch time

The number of eggs per clutch significantly predicted clutch hatch time of clutches from both paired and isolated snails, but shell diameter also predicted clutch hatch time in paired snail clutches (Isolated: log transformed data, adj R^2 = 0.666, n = 36, F = 70.752, p<0.001; Paired: adj R^2=0.369, n=158, F=46.86, P<0.001). The relationship between shell diameter at the onset of egg laying and clutch hatch time was negative (t =-3.594), while as the number of eggs per clutch increased so did clutch hatch time (t = 9.316) in clutches from paired snails. Smaller adult snails may increase the clutch hatch time of their eggs, which could lead to increased cannibalism and provide extra nutritional resources for those hatching first, although this was not seen in a previous experiment with a larger sample size (section 2.3.3). The relationship between number of eggs per clutch and clutch hatch time was of a similar strength to that observed previously (section 2.3.3).

Percentage hatch

Percentage hatch was analysed (arcsine transformed data) for relationships with the aforementioned fecundity characteristics, but the data were not normally distributed. This was, in part, due to the relatively high number of single eggs per clutch. As a result, the percentage hatch for single eggs was examined separately, and no significant difference was found between percent hatch of single eggs laid by paired or isolated snails (χ^2 = 0.131, P= 0.718, df =1; Figure 2-12).

Figure 2-12: Egg hatching in single egg clutches laid by paired and isolated *Microxeromagna armillata*.



When the linear regression was repeated excluding single-egg clutches from the analysis, the data did not meet the assumptions associated with linear regression. The factors which may influence percent hatch were therefore analysed using Pearson's correlation, and no factors predicted percent hatch of eggs from isolated snails which may have been due to low statistical power. Week of laying (Pearson corr.= 0.156, P=0.037, n=132), number of eggs per clutch (Pearson corr.= -0.313, P<0.001, n=132) and egg size (Pearson corr.= 0.173, P=0.02, n=132) were correlated with the percentage hatch of eggs from paired snails. These fecundity characteristics are inter-related which makes interpretation of their true effect on hatching percentage difficult to determine.

2.4.1.4 Summary

The ability of *M. armillata* to reproduce via self-fertilisation is an important life history characteristic. This is the first time that a hygromiid snail has been recorded as being capable of self-fertilisation. The ability to reproduce by self-fertilisation significantly increases the colonising ability of *M. armillata*, when compared to snails which can only reproduce by cross

fertilisation, and also enhances the ability of the species to survive through periods of extremely low density. However, this increase in colonising ability is not without some tradeoffs. Isolated snails produce approximately five times fewer eggs, smaller eggs and smaller offspring. The relationship between body size and egg size was also uncoupled, so that lesser benefit was gained from increased size in snails reproducing via self-fertilisation. Isolated snails laid a higher ratio of single to multiple egg clutches than paired snails and this may be a bet-hedging strategy that spreads the risk of clones mating and/or minimises the risk of predation. The relationships between fecundity characteristics often differed between paired and isolated snails, and this provides some evidence that self-fertilising snails use different reproductive strategies. Hence, these patterns are not simply due to a reduction in overall fecundity.

2.4.2 The effects of self-fertilisation on fecundity characteristics of second generation isolated *Microxeromagna armillata*.

2.4.2.1 Introduction

A change in some fecundity characteristics due to self-fertilisation has been previously illustrated (section 2.4.1.3), but one of the unanswered questions is how long do the effects of self-fertilisation persist. If the negative effects of possible inbreeding depression persist in the population after subsequent out-crossing, then the benefits of self-fertilisation may be diminished. Few studies in molluscs have investigated the effects of inbreeding depression or self-fertilisation past the first generation (although see Baur & Baur [54]). The following experiment was designed to investigate the effects of inbreeding on the growth and fecundity characteristics of second generation self fertilised individuals. In addition, the life history traits of *M. armillata* recorded in this experiment are examined for interrelatedness and relationships compared to those outlined previously for *M. armillata*.

2.4.2.2 Materials and methods

Juvenile snails (n=60) were selected from the offspring of adult snails that had been isolated for at least 6 months to a maximum of 1 year. These juvenile snails were assumed to be products of self-fertilisation, since a high proportion of *M. armillata* reproduced in this manner when isolated in previous experiments (cf section 2.4.1). Forty of these snails were paired (SFP group), while the remaining 20 were kept isolated (SFS group). Snails were not paired randomly but were chosen on the basis of different parentage where possible, to avoid sib matings. As a control, another 60 juvenile snails were collected from a laboratory culture in which adult snails were paired up to and including at the time of egg laying, and assumed to be progeny of cross fertilisation. Forty of these juveniles were paired (CFP group) and the remaining 20 were kept isolated (CFS group).

Thus, the treatment groups comprised:

i. SFP – progeny of self fertilisation in pairs

ii. SFS – progeny of self fertilisation held singly

iii. CFP – progeny of cross fertilisation in pairs

iv. CFS – progeny of self fertilisation held singly.

The diameter of the shell was measured for all snails, then snails were placed on field-collected soil, two centimetres deep, in small cylindrical containers (6cm high, 4cm diameter). At intervals throughout the experiment, snails were photographed using a camera mounted on a dissecting microscope. The shell diameter was measured from these photographs at a later date using the OlysiaEasy® photographic software (2001, Soft Imaging System, Germany). The containers were covered with gauze secured by rubber band. The containers were kept outdoors in a shade-house, with exposure to normal environmental conditions, but were protected from rainfall events and manually watered as in previous experiments. Snail mortality was assessed weekly, and the soil inspected for the presence of eggs until 1/12/2003.

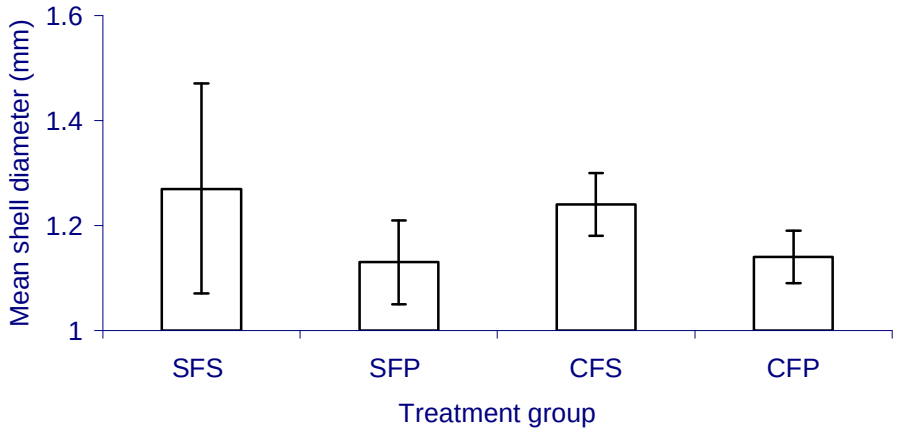
Additional fecundity characteristics were measured and analysed as previously described (Sections 2.3.2, 2.4.1.2). Cox regression and Kaplan- Meir analysis were used to compare survival among treatments. SPSS for Windows 11.5 was used for all analyses.

2.4.2.3 Results and discussion

Body size, as indicated by shell diameter was significantly different among groups at the beginning of the experiment (Figure 2-13). As previous experiments indicated that initial snail size can significantly influence life history traits (Section 2.3.3), this needed to be taken into account in subsequent analysis. However, the unequal variance of initial snail size among

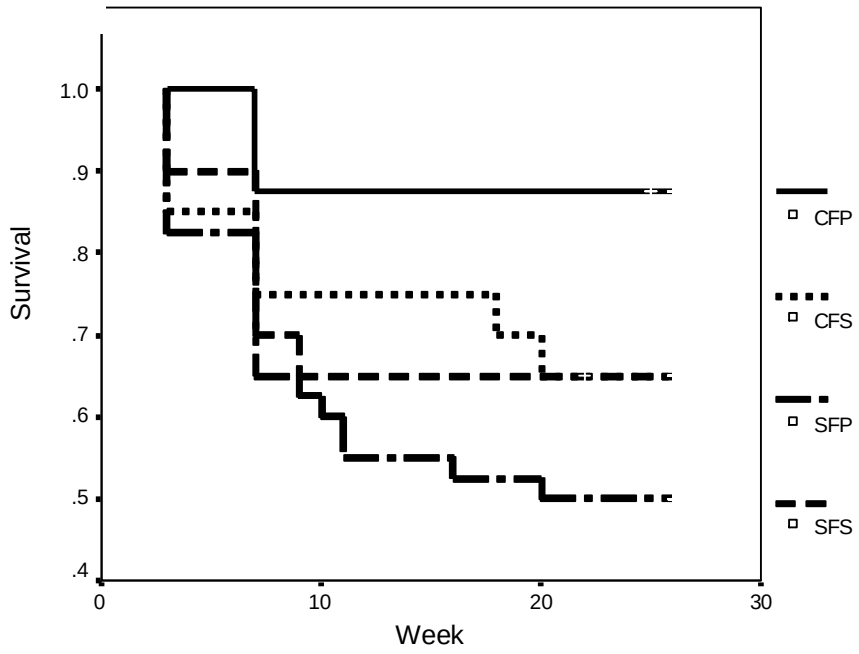
groups, and often unequal and low sample sizes, made statistical analysis a challenge. Comparisons among groups were not always statistically possible, but this is discussed on a case-by-case basis.

Figure 2-13: Initial shell diameter of *Microxeromagna armillata* across treatment groups (15/5/2003). SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = Cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair. Error bars represent standard deviations: n = 20 for all groups, mean shell diameter used for snails within a pair.



Snails in the CFP group had a higher survival rate during the experiment than all other groups (Figure 2-14), but these groups could not be statistically compared using Cox Regression as the assumption of proportionality of hazards between groups was not met. This did not change with transformation or categorisation of the data. Analysis using the Wilcoxon (Gehan) statistic indicated that survival of the treatment groups differed significantly (test statistic = 12.487, df=3, P=0.0059). While comparisons between specific groups cannot be made it is likely that, at the least, survival of CFP snails is significantly higher than SFS snails.

Figure 2-14: Survival function for *Microxeromagna armillata,* self-fertilised progeny, isolated (SFS), self-fertilised progeny, paired (SFP), cross fertilised progeny, single (CFS) and cross fertilised progeny, paired (CFP) snails (n =20 for single snails and n=40 for paired snails).

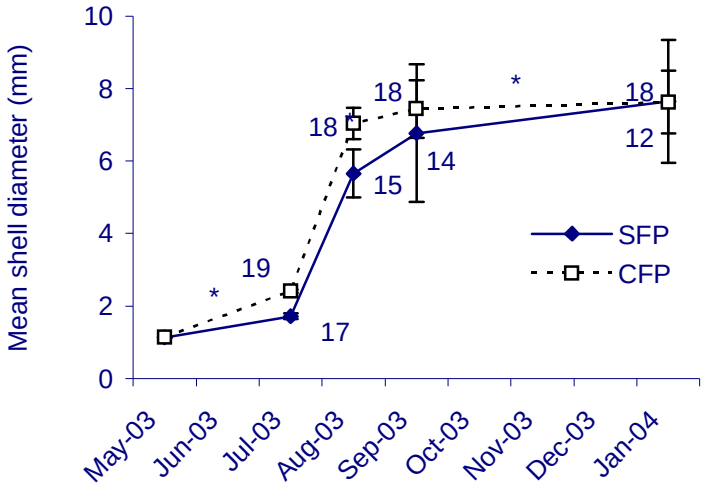


Snail growth

Snails in all groups increased in shell size over time (Figure 2-15, Figure 2-16) and the shell growth rates of snails (slope of the lines) were calculated between sampling times and compared across treatments. Comparisons were able to be made between SFS and CFS and between SFP and CFP groups, as initial shell diameter in these groups were not significantly different (**SFS v CFS**: Mann-Whitney U test, df =1, P= 0.933. **SFP v CFP**: t-test, t = -0.674, P= 0.505).

From May to July (week 0 - week 7) the growth rate of snails in the CFP group was higher than those in the SFP group (ANOVA, $F = 16.839$, $P<0.001$). From July to August (week 7 to 11) no significant difference occurred in growth rates between the treatment groups (Mann – Whitney $U = 103$, $P = 0.259$) and growth rates were highest overall during this period (Figure 2-15). From August to September (week 11 – 15) growth rates again differed between treatments, with the SFP treatment showing the fastest rate of growth (ANOVA, $F = 20.89$, $p<0.001$). This was also the case in the final sampling period from September to January of the following year (week 15 – 34) (ANOVA, $F=9.077$, $P = 0.005$). The higher growth rate of CFP snails during the initial period could be due to the increased fitness and nutritional reserves present in snails which are products of cross fertilisation. In the last two sampling periods, the growth rate of CFP snails was lower than SFP snails, and this coincided with the onset of egg laying in the CFP group. Snails in the CFP group began egg laying on average one month earlier than those in the SFP group (mean date CFP = 11/8/03, SFP= 14/9/03, t-test, $t = 9.099$, $df = 18$, $P<0.001$) and it is likely that growth slowed during egg laying as resources such as calcium were reallocated from shell growth to egg provisioning. Also, the CFP group had more snails lay eggs than any other group ($\chi^2 = 15.385$, $df = 3$, $P = 0.002$). Egg laying may have occurred later in the SFP group as an extended period of growth which optimises the relationship between shell size and offspring size (cf section 2.3.3) may provide an advantage, although the benefit of increased body size in self-fertilising snails may be lessened when compared to cross-fertilising snails (section 2.4.1.3).

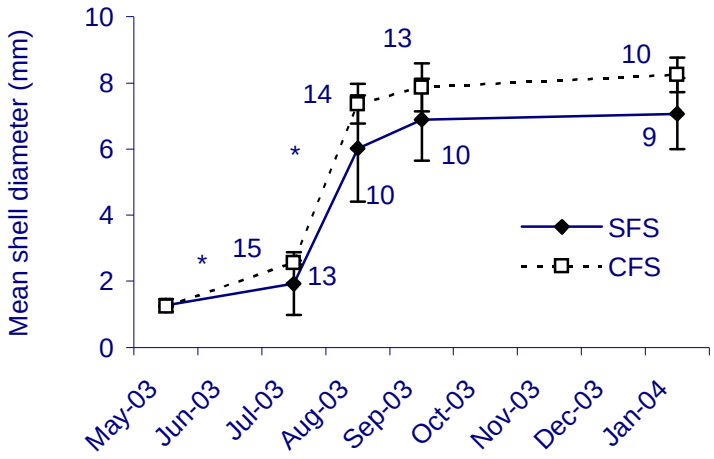
Figure 2-15: Changes in mean shell diameter of *Microxeromagna armillata* in SFP (self fertilised progeny, pair) and CFP (cross fertilised progeny, pair) during the experiment. Error bars represent standard deviation; numbers next to points represent number of samples. * symbol between points indicates growth rates (slope of the lines) are significantly different between treatments



The growth rates of snails in the SFS and CFS groups were significantly different during two of the four time intervals (Figure 2-16). CFS snails grew faster than SFS snails during both the first (Mann Whitney U = 45.5, P= 0.015) and second (ANOVA: F = 6.519, P= 0.018) sampling periods. Growth rates during the final sampling periods were not significantly different between treatments (ANOVA: F = 1.415, P= 0.248 and F = 1.0901, P= 0.311 respectively). The higher growth rates of CFS snails during the first two sampling periods could be due to higher fitness or increased nutritional reserves associated with snails that are a product of cross fertilisation. However, it is interesting to note that growth rates were similar in the last two sampling periods. Onset of egg laying occurred at the same time for SFS and CFS snails (date at first reproduction: t –test, t = -0.585, df =12, p=0.569) and hence any

change in partitioning of calcium resources to egg laying rather than growth also occurred at the same time.

Figure 2-16: Mean shell diameter of *Microxeromagna armillata* in SFS (single self fertilised snail) and CFS (single cross fertilised snail) during the experiment. Error bars represent standard deviations; numbers alongside points are number of samples. * symbol between points indicates growth rates (slope of lines) are significantly different between treatments

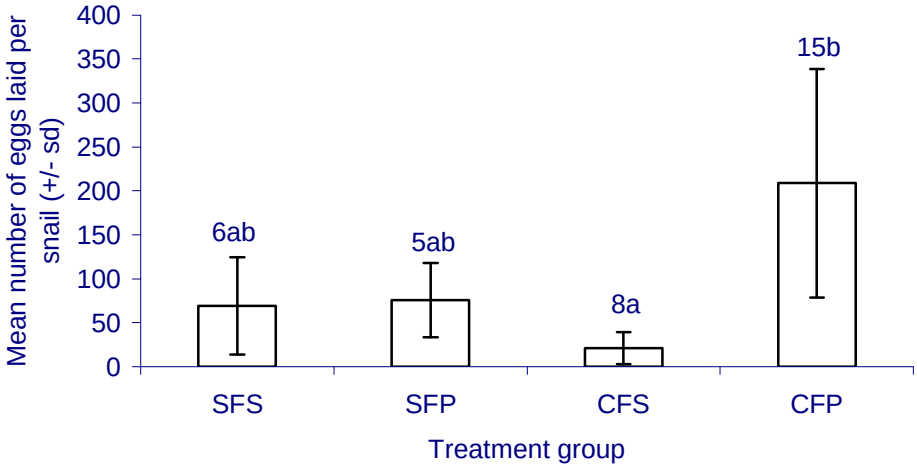


Number of eggs laid

When analysis of fecundity characteristics was undertaken, the initial shell diameter of those snails that laid eggs was compared across treatment groups. Variances in initial shell diameter were equal as assessed by Levine's test of equality of error variances ($F = 1.987$, $df1 = 3$, $df2 = 39$, $P = 0.132$, reciprocal transformed data). This enabled the use of this parameter as a covariate in ANCOVA in the comparison of the mean number of eggs laid per snail across groups. However, initial shell diameter was not a significant term in the ANCOVA model and was subsequently removed. The numbers of eggs laid per snail differed among

groups (ANOVA: log transformed data, df=3, F = 7.559, P<0.001). Post hoc testing showed that CFP snails laid more eggs than CFS snails (Games-Howell procedure for unequal sample sizes, P= 0.004, Figure 2-17), which supports previous results (section 2.4.1.3). CFP snails laid more eggs per snail than SFP snails, although these groups could not be compared statistically. It is possible that this difference is a result of inbreeding depression rather than a difference in initial snail size, as initial size was found to influence the timing rather than the number of eggs laid in a previous experiment (section 2.3.3). Examining the fecundity characteristics of snails produced by mating a self-fertilised with a cross fertilised snail may assist in elucidating this relationship. While some possible effects of inbreeding depression can be seen (CFP >>SFP), it would seem that pairing with a partner who is also a product of self-fertilisation may still be better than reproducing alone. Studies with a larger sample size are necessary to further investigate these trends.

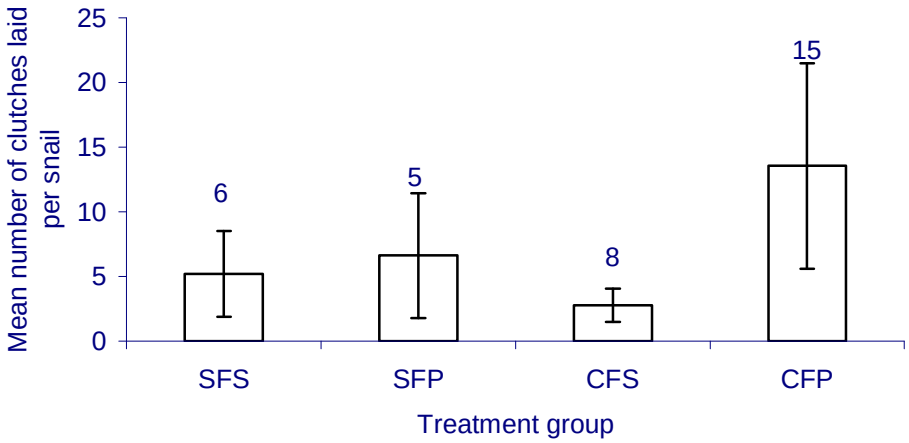
Figure 2-17: Mean number of eggs laid per *Microxeromagna armillata* across treatment groups. SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair. Error bars represent standard deviations; the number above the bars represents sample size, while treatment groups with differing letters are significantly different at p<0.05.



Number of clutches

The number of clutches laid per snail (Figure 2-18) was investigated using ANCOVA, but testing of the covariate x treatment interaction (initial shell diameter x group) was significant, violating ANCOVA assumptions. As a result, the number of clutches laid per snail was not analysed statistically. However, there was an apparent relationship between the mean number of eggs laid per snail and the mean number of clutches laid per snail in SFS (Pearson Corr. = 0.949, p= 0.004) and CFP (Pearson Corr. = 0.923, P<0.001) groups, but not within the SFP (Pearson Corr. = 0.717, P= 0.173) and CFS (Pearson Corr. = 0.492, P= 0.216) groups. The differences in these correlations among groups may be due to the employment of different reproductive strategies, but the small sample size makes this difficult to discern confidently.

Figure 2-18: Mean number of clutches laid per *Microxeromagna armillata* across treatment groups. SFS = self-fertilised single snail, SFP = self-fertilised snails in a pair, CFS = Cross-fertilised single snail, CFP = cross-fertilised snails in a pair. Error bars represent standard deviations; numbers above bars represents number of replicates.

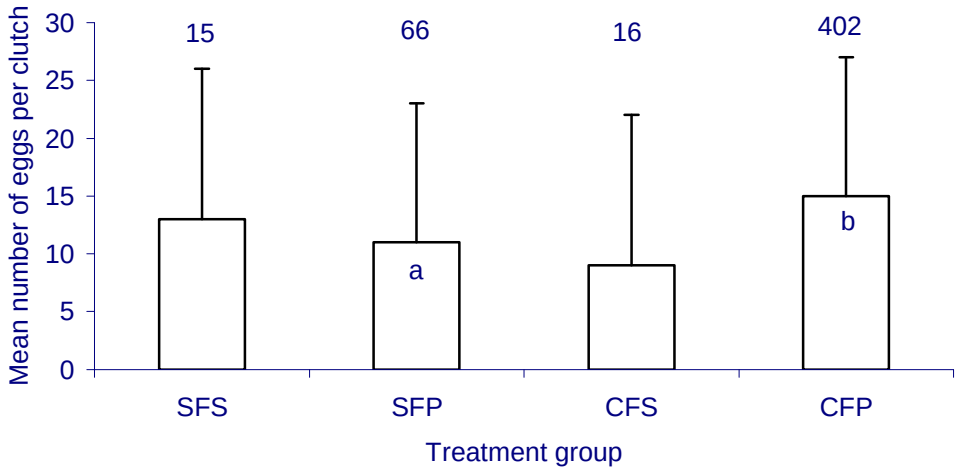


Number of eggs per clutch

All treatments had the same ratio of single to multi-egg clutches (Cross tabulation analysis: χ^2 = 4.148, n = 509, df = 3, P= 0.303), which is in contrast to results obtained in the previous experiment (section 2.4.1.3) where isolated snails laid a higher ratio of single to multi-egg clutches. This differing result may predominantly be due to a smaller sample size in this experiment compared to the previous experiment, but it is possible that this difference may be due to the socialisation of snails (as juveniles) in the earlier experiment. Previous contact with another snail may influence the reproductive strategy [54], such that snails invest fewer resources (lay more single eggs) with the expectation that interactions with other snails will re-occur in the future.

The number of eggs laid per clutch could not be compared across all groups due to violation of ANCOVA assumptions, but SFP and CFP treatment groups could be compared, as they did not differ in initial shell diameter (t-test, t = 1.164, P= 0.124). Snails in the CFP treatment laid significantly more eggs per clutch than those in the SFP treatment (t-test, t = -2.488, P= 0.013; Figure 2-19).

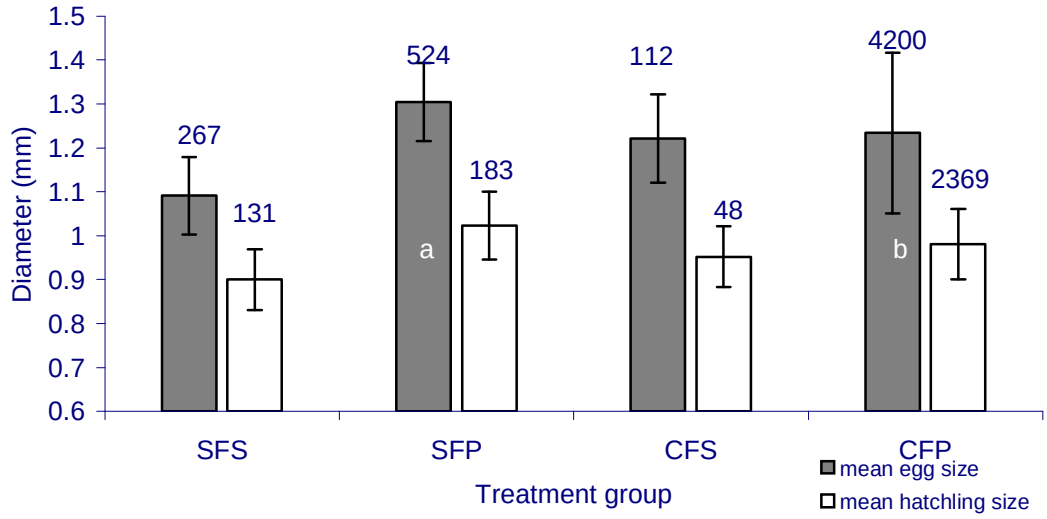
Figure 2-19: Mean number of eggs per clutch laid by *Microxeromagna armillata* across treatment groups. SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = Cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair. Error bars represent standard deviation, numbers above bars represent sample size. Bars containing different letters are significantly different from each other.



Egg and hatchling size

Although SFP snails laid fewer eggs per clutch than CFP snails, the eggs they laid were larger (t-test, t = 5.70, P<0.001; Figure 2-20). Thus production of fewer eggs of larger size might compensate for any negative effects of self fertilisation as a reproductive strategy. Hatchlings of SFP snails were larger than those of CFP snails which supports this hypothesis (Figure 2-20), although statistical comparison could not be undertaken. Though SFP hatchlings were bigger than CFP hatchlings, the ratio of egg to hatchling diameter was similar in the two groups (77 and 78% respectively), indicating that these groups share a similar strength relationship between egg size and hatchling size.

Figure 2-20: Mean egg and hatchling size of *Microxeromagna armillata* across treatment groups. SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = Cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair. Error bars represent standard deviations; sample size is given above the bars. Different letters represent significantly different groups.



Percentage of eggs hatching and clutch hatch time

The percentage of eggs hatching was low overall during this experiment, with a mean of only 36% for the CFP group (Figure 2-21). This result was similar to that recorded in a previous experiment investigating self-fertilisation (42 - 45%, Table 2-9), and lower than recorded in the initial fecundity experiment (58%, Table 2-3). CFP had the highest, and SFS had the lowest, percentage hatch when all cases, and when restricting analysis to those clutches in which some hatching occurred(%hatch > 0)(Figure 2-21). CFP and SFS groups were expected to be the most and least fit groups respectively, so this result is not surprising. Clutch hatch time ranged from 4 to 6.6 days across treatment groups (Figure 2-22), which is comparable to previous experiments (Table 2-5, Table 2-9). There was no evidence that self-fertilising snails produced eggs with higher hatching asynchrony, which would promote cannibalism, than cross fertilising snails.

Figure 2-21: Percent egg hatching of *Microxeromagna armillata* across treatments. SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = Cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair.

Error bars represent standard deviations; sample size is given above the bars.

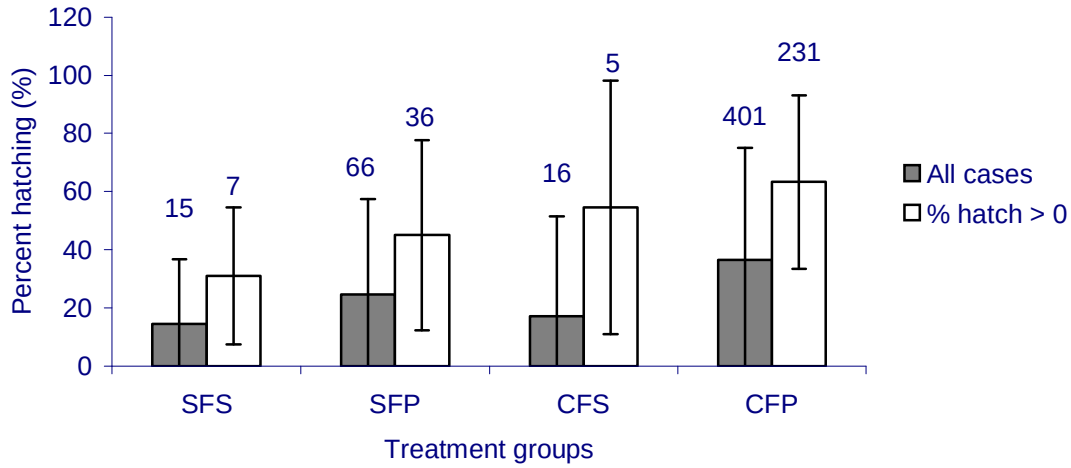
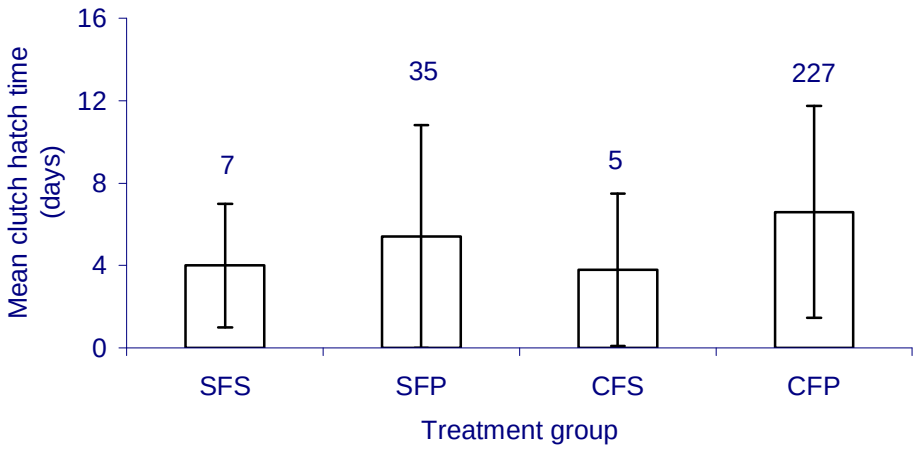


Figure 2-22: Mean clutch hatch time of *Microxeromagna armillata* across treatment groups. SFS = self-fertilised progeny, single snail, SFP = self-fertilised progeny, snails in a pair, CFS = Cross-fertilised progeny, single snail, CFP = cross-fertilised progeny, snails in a pair. Error bars represent standard deviations; sample size is given above the bars.



Interrelatedness of fecundity characteristics in CFP group

As in previous experiments, data collected were examined for inter-relatedness of fecundity characteristics. This was only undertaken within the CFP group due to the small sample size of other groups. Multiple linear regression was used to investigate the relationship between the numbers of eggs laid per clutch, week of egg laying, and growth rate (growth rate during weeks 0 – 7, 7 – 11, 11 – 15 were tested in separate models). Neither growth rate, nor week of egg laying significantly predicted the number of eggs laid per clutch in the CFP group (adj R^2 = 0.001, n = 402, F = 1.267, P= 0.283). This is in contrast to previous experiments where the number of eggs per clutch decreased with increasing week of egg laying (Sections 2.3.3, 2.4.1.3). Egg laying was monitored over a shorter period and for a smaller sample size in this experiment than in previous studies, which may have reduced the ability to demonstrate a trend in number of eggs per clutch.

Mean egg size was predicted (adj R^2= 0.148, F = 35.118, P<0.001, n = 394) by the number of eggs in a clutch (β = - 0.122, P<0.001) and week of egg laying (β = -0.376, P<0.001). The trend for egg size to decrease with increasing numbers of eggs within a clutch has been reported previously (sections 2.3.3, 2.4.1.3). However, in the CFP group the relationship between egg size and week of laying was negative, rather than positive as seen in other experiments. This experiment began in May and, as the snails were juveniles at this time, egg laying began much later in the year than would have been observed if snails were sexually mature in May. Reaching sexual maturity half-way through the period suitable for egg laying may change the reproductive stategies that *M. armillata* employs and hence alter the relationships between fecundity characteristics. Growth rate was not a significant term in the model which is surprising as mean egg size could be a likely trade-off with growth rate, as a consequence of resource partitioning. As speculated in previous experiments, it may be size at onset of egg laying which is a stronger influence on egg size throughout the egg laying period rather than growth rate, or progressive snail size.

Mean hatchling size was predicted (adj R^2 = 0.292, F = 32.06, P<0.001, n = 227) by mean egg size (β = 0.434, P<0.001), number of eggs per clutch (β = -0.212, P<0.001) and growth between weeks 7 to 11 (β = 0.115, P<0.041). The positive relationship between egg size and hatchling size has been discussed in previous experiments, as has the negative relationship between number of eggs per clutch, egg size and hatchling size. Of most interest in this regression model is the effect of growth (during weeks 7 to 11) on hatchling size. A higher growth rate immediately prior to the onset of egg laying (week egg laying commenced; mean± sd; 12 ± 0.83) would result in a larger body size that has been shown to increase hatchling size in previous experiments. Snails with a higher growth rate immediately prior to egg laying may also be the most efficient at utilising the nutrients in the food which, once growth has slowed and reproduction begun could make them more efficient at providing nutrients to their offspring.

The percent of eggs hatching was predicted (arcsine transformed data: adj R^2 = 0.209, F = 53.275, P<0.001, n = 397) by the week of egg laying (β =0.452, P<0.001) and the number of eggs in a clutch (β = 0.115, P= 0.011). When only clutches in which at least some hatching occurred were considered, the number of eggs per clutch (st β = -0.397, P<0.001) and week of egg laying (β = 0.326, P<0.001) were still significant terms in the model (adj R^2 = 0.303, F = 50.234, P<0.001, n = 227), although in the case of number of eggs per clutch the direction of the relationship had changed. An increase in the number of eggs per clutch is related to the chance of a clutch hatching, but when only clutches in which some hatching occurred were included in analysis, a higher number of eggs within a clutch decreased the percentage of eggs that ultimately yielded hatchlings. With a greater number of eggs in a clutch, *M. armillata* may lay more unfertilised eggs which provides additional food for offspring, although this could not be demonstrated within the scope of this experiment. Clutch hatch time was only predicted by the number of eggs in a clutch (adj R^2 = 0.204, n = 229, F = 59.477, P<0.001), a relationship which has been observed consistently throughout this series of fecundity experiments (sections 2.3.3, 2.4.1.3).

2.4.2.4 Summary

The pattern of snail growth was similar for snails in all groups throughout the experiment, with the highest growth rates seen during weeks 7 – 11. Further work is needed to relate growth rates with the underlying physiological processes of *M. armillata*, as the timing of measurement was arbitrary in this experiment. Progeny of cross-fertilised snails (CFP, CFS) had a higher rate of growth during the first seven weeks of the experiment when compared to progeny of self-fertilised snails (SFP, SFS), which may be due to inbreeding depression. SFP snails began egg laying later than CFP and this explains why growth of SFP snails was higher during the last weeks of the experiment (weeks 11-34). This was not the case with SFS and CFS snails as both growth rates and onset of egg laying were the same during weeks 11 – 34. Self-fertilised snails may delay reproduction, which increases body size and hence increases fitness of their offspring and/or maximises the potential of finding a mate.

Cross-fertilised paired (CFP) snails had lower mortality and a higher proportion of pairs laying eggs than all other groups. Snails in the CFP group laid more eggs than those in the SFP group, showing that even when the progeny of self-fertilised snails are given the opportunity to mate there is a reduction in fecundity. Inclusion of an additional treatment, crossing a self-fertilised snail with a cross fertilised snail, could clarify these results. Surprisingly, the progeny of SFS snails tended to lay more eggs per snail than progeny of CFS snails, and it is possible that self-fertilising snails have a tendency to produce offspring that in turn are more inclined to reproduce by self-fertilisation. Progeny of SFP snails laid fewer eggs per clutch and larger eggs than the progeny of CFP snails, which resulted in larger hatchlings. A greater investment in fewer eggs may assist in overcoming any loss of offspring fitness due to inbreeding depression.

2.5 Can *Microxeromagna armillata* aestivate and does this affect its reproductive characteristics?

2.5.1 Introduction

Many species of terrestrial molluscs have the ability to aestivate, which entails a slowing of activity and/or metabolism, during summer [19]. The ability to aestivate during times of unfavourable environmental conditions can substantially benefit a species. Snails are at greatest risk of mortality due to desiccation in arid environments, and aestivation reduces this risk [55]. Aestivation is not only a behaviour used to avoid arid environments. High temperatures, and/or lack of suitable food, can also cause aestivation or prolonged cessation of activity. It is assumed that aestivation results in a loss of fitness for an organism, due to the energy required to change the physiological processes of the metabolic system and maintain metabolic depression [56], but this loss may only be temporary. Much of this may depend on the length of inactivity, the adaptability of an organism, and the ability to acquire resources post-aestivation. For example, the cost of an organism decreasing its metabolic rate for a few days would be much less than for a few years. It is not known if *M. armillata* can aestivate, but many helicid and hygromiid snails have this ability (Pomeroy [57] and references therein). Laboratory observations throughout this project indicate that adult *M. armillata* can remain inactive successfully for many months when conditions are not suitable for movement, feeding or reproduction. It is not unusual for adult snails to undergo long periods of inactivity, but few studies have looked at the capacity of juveniles to do the same. The aim of this experiment was firstly to determine if juvenile *M. armillata* could survive long periods of inactivity, and secondly if a period of inactivity has any effect on subsequent growth and fecundity.

2.5.2 Materials and methods

Hatchlings produced by snails in 2002 (cf section 2.3, 2002/2003 fecundity experiment) were kept in tissue culture trays and stored at room temperature soon after egg hatching. No food, water or light was provided. Hatchlings were stored in this manner for 7 - 10 months (eggs laid on 3/7/2002 – 24/9/2002). On the 15th of May 2003 hatchlings were sprayed with water and those that did not become active were assessed as dead. Approximately 70% of hatchlings survived. Sixty of these snails were then placed into rearing containers: 40 snails were paired (20 pairs, AP group) and 20 snails were isolated (AS group). The shell diameter of each hatchling was measured at this time. The rearing containers and measurement of growth and fecundity characteristics have been previously described (section 2.4.2.2). The control groups for this experiment were the cross fertilised isolated (CFS) and cross fertilised paires (CFP) groups described in section 2.4.2, as this experiment was conducted concurrently and these snails had not undergone any substantial periods of inactivity. Parentage of the hatchlings that had undergone the period of inactivity was not known, but as most of the adults in laboratory culture had been part of a pair in 2002 (cf section 2.3.3) it is assumed that juveniles were a product of cross-fertilisation. This experiment ended on 1/12/2003. Kaplan-Meier and Cox regression analyses were used to investigate differences in survival between groups. T-tests, cross tabulation and Mann-Whitney U tests were used to analyse differences in fecundity characteristics between groups where appropriate. SPSS for Windows 11.5 was used for all analyses.

2.5.3 Results and discussion

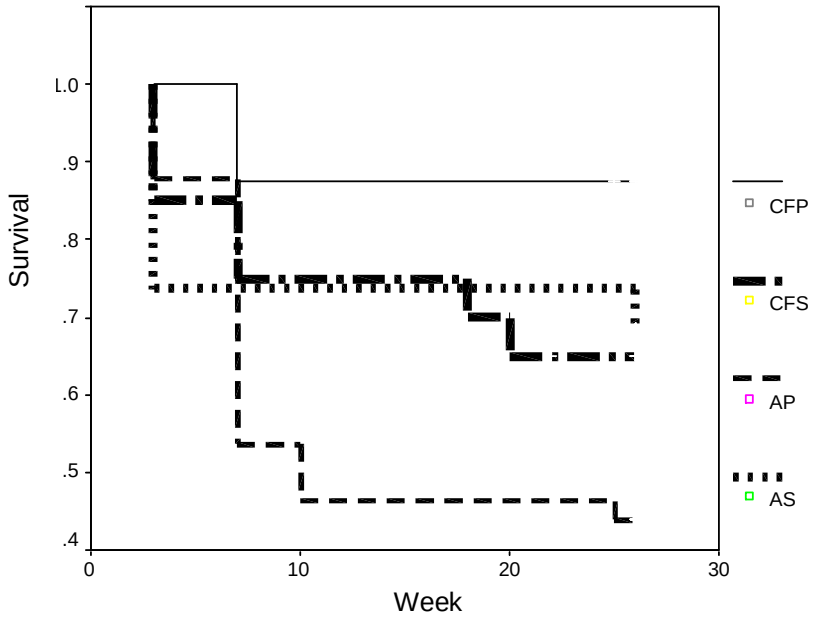
Some *M. armillata* juveniles survived for at least 10 months without food, water or light and responded quickly to subsequent changes in the environmental conditions. The heartbeat of inactive hatchlings could be seen through the shell when snails were examined under a microscope. After exposing the snails to water their heartbeats noticeably increased in speed, until the foot extended from the shell, when the heart then maintained a steady rhythm. Some

hatchlings were active within 10 minutes of water application, but other snails were not active for ~1 hour.

The characteristics of aestivated and non-aestivated snails were statistically compared, whilst controlling for initial shell diameter. The initial shell diameter of hatchlings was compared between the groups using ANOVA, but Levine's test of equality of error variances indicated significant differences among treatments ($F = 11.045$, $df1 = 3$, $df2 = 76$, $P<0.001$). Transformation did not improve the dispersion of the data. Nonparametric analysis indicated differences among the groups (Kruskal-Wallis: $\chi^2 = 21.075$, $df=3$, $P<0.001$). However, when groups were compared on a paired basis no significant differences were found in initial snail size between AS and CFS (Mann-Whitney $U = 129.5$, $P= 0.089$) and AP and CFP groups (t-test, $t = -0.101$, $P= 0.920$) which meant that the characteristics of these groups could be compared without incorporating initial shell diameter as a covariate in subsequent analyses.

Snails in the CFP group had a higher survival rate during the experiment than all other groups (Figure 2-23), but these groups could not be statistically compared using Cox Regression as the assumption of proportionality of hazards among groups was not met. This did not change with transformation or categorisation of the data. Analysis of groups using the Wilcoxon (Gehan) test demonstrated significant differences among groups (test statistic= 15.578, $df=3$, $P=0.0014$), which is most likely due to the greater survival rate of snails in the CFP group compared to the AS group.

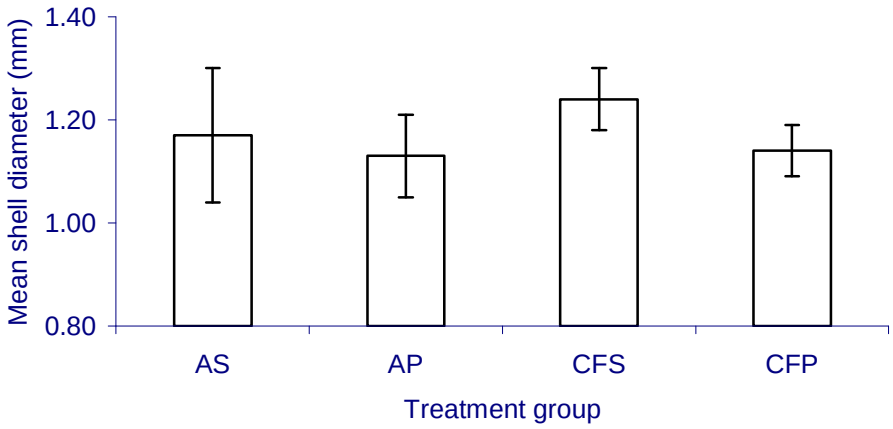
Figure 2-23: Survival function for *Microxeromagna armillata* aestivated isolated (AS), aestivated paired (AP), cross fertilised single (CFS) and cross fertilised paired (CFP) snails (n=20 for single snails and n=40 for paired snails).



Snail growth and egg laying

Previous research has shown that shell growth occurs even when snails are allocating substantial resources to reproduction and egg laying (cf Figure 2-15, Figure 2-16). Hence it is likely that when snails are active their shells are growing (e.g. do not switch resource allocation to growth on or off, but alter the rate of this allocation). Although initial shell diameter differed between groups in this experiment, the shell diameter of the inactive snails was not consistently larger than those in the control groups (Figure 2-24). As the shell diameter of the snails after a long period without food, light or moisture was comparable to those newly hatched snails in the control group, it is reasonable to assume that the snails were indeed inactive for a substantial amount of this time.

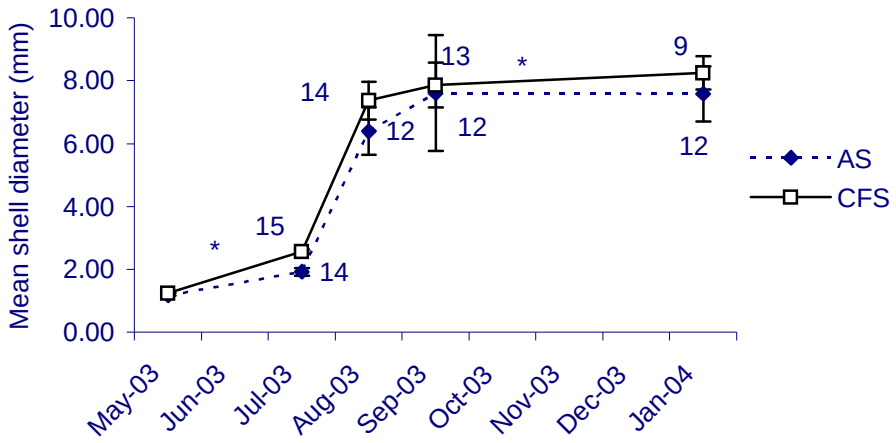
Figure 2-24: Mean initial shell diameter of *Microxeromagna armillata* across treatment groups. Error bars represent standard deviations; n = 20 for each group, mean shell diameter of snails within a pair used.



Mean shell diameter of AS and CFS groups changed over time, and the growth rate of snails in these groups differed in two of the four sampling periods (Figure 2-25). AS snails had a lower initial growth rate than CFS snails (t-test, t = -2.222, df = 27, P= 0.035) which indicates a decrease in fitness during the prolonged period of inactivity. CFS snails also had a higher growth rate during the final sampling period than snails in the AS group, which suggests that the effect on growth persists for a considerable part of the remaining life span.

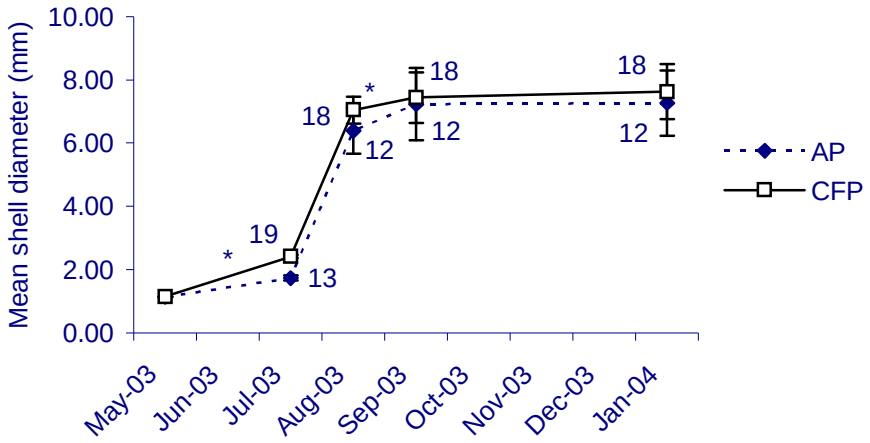
There was no difference between groups in the onset of egg laying (t-test, t = -0.487, df =9, P= 0.838), nor the mean number of eggs laid per snail (t-test, t = 0.547, df =10, P= 0.0597) during this time (see later discussion, Table 2-12). Twice as many snails in the CFS group (8) laid eggs as in the AS group (4). A decrease in the overall growth rate would eb expected as resources were directed away from growth and into egg production. However, this was not observed for CFS snails. That fewer AS snails may have reproduced than CFS snails indicates a reduced ability to provide resources to self fertilised offspring as a consequence of prior aestivation.

Figure 2-25: Mean shell diameter of *Microxeromagna armillata* AS (aestivated, isolated) and CFS (cross fertilised, isolated) hatchlings over time. Error bars represent standard deviations; numbers next to points represent sample number; * symbol between points indicates growth rates (slopes of the lines) are significantly different between treatments.



CFP snails grew faster than AP snails during the first sampling period (Mann Whitney U = 49, P< 0.001, Figure 2-26), which is a sign of greater fitness. But a lower growth rate of CFP snails was seen during the third sampling period (week 7 to 11, t-test = 3.332, df = 28, P= 0.002). As no difference was seen in the timing of first egg laying (t-test, t = 10647, df = 5.314 (equal variances not assumed), P= 0.157), it would be reasonable to assume that there is no difference in resource partitioning between the aestivated and non-aestivated snails. However, even though no difference in the onset of egg laying was seen between the groups, significantly more CFP pairs laid eggs than any other group (Cross tabulation: χ^2 = 12.160, df = 3, P= 0.007). As more CFP snails laid eggs, the overall growth rate of snails in the group would be expected to decrease relative to AP snails, as resources were partitioned from shell growth to egg laying, accounting for the slower growth rate during August to September.

Figure 2-26: Mean shell diameter of *Microxeromagna armillata* AP (aestivated, paired) and CFP (cross fertilised progeny, paired) snails over time. Error bars represent standard deviations; numbers near points represent sample number. * symbol between points indicates growth rates (slopes of the lines) are significantly different between treatments.



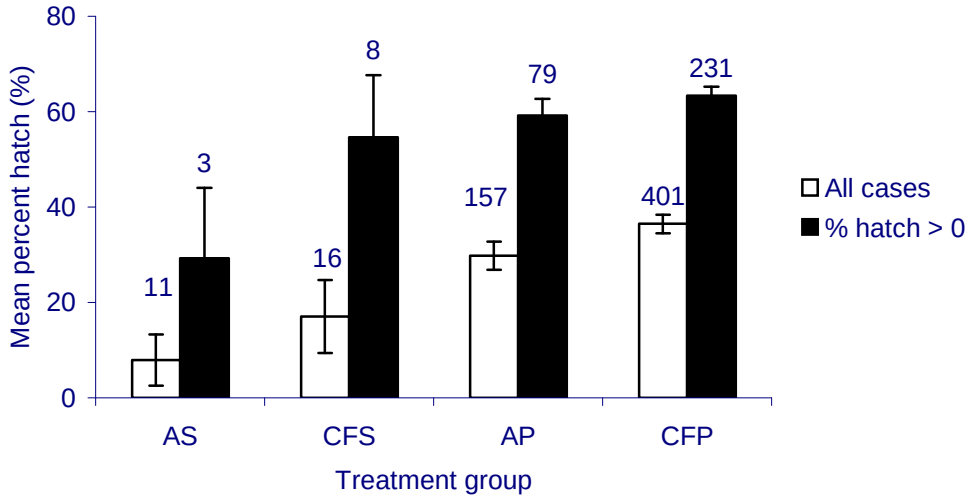
Comparison of reproductive characteristics among groups

The reproductive characteristics of aestivated and non-aestivated snails were similar within the isolated and paired groups (Table 2-12). The only significant differences were between the AP and CFP groups, in hatchling size and percentage hatch. Paired aestivated snails produced larger hatchlings than non-aestivated snails (Table 2-12), and paired aestivated snails produced a higher number of clutches where no hatching occurred at all (Figure 2-27). When only clutches that had some hatching were considered, there was no significant difference in percentage hatch (Figure 2-27). Although aestivated snails gave rise to hatchlings larger than those of CFP snails, no difference in egg size was evident between the groups. This indicates a fundamental difference in reproductive strategy or simply shifts in resource partitioning.

Table 2-12: Reproductive characteristics of aestivated and non –aestivated *Microxeromagna armillata*, which have been isolated or paired. AS = aestivated isolated, AP = aestivated pair, CFS = cross-fertilised single (non-aestivated), CFP cross-fertilised pair (non-aestivated). Mean values are significantly different when followed by different letters and are highlighted in bold type.

Reproductive Characteristic		AS	CFS	AP	CFP
Number of eggs laid per snail	Mean	29.5	21.25	200.83	208.56
	St. dev	35.35	18.23	153.79	130.08
	N	4	8	6	15
	Test statistic	t = 0.547, P= 0.597		t = -0.117, P= 0.908	
Number of clutches laid per snail	Mean	2.75	2.75	13.08	13.53
	St. dev.	2.06	1.28	10.42	7.93
	N	4	8	6	15
	Test statistic	t= 0.000, P= 1.000		t = -0.108, P= 0.915	
Mean number of eggs per clutch	Mean	10.73	7.73	15.35	15.42
	St. dev.	11.244	11.720	11.424	12.033
	N	11	22	157	405
	Test statistic	t= 0.702 P= 0.488		t = -0.60, P=0.952	
Mean egg size (mm)	Mean	1.275	1.265	1.262	1.257
	St. dev.	0.116	0.094	0.093	0.097
	N	10	22	157	395
	Test statistic	t = 0.246, P=0.808		t= 0.578, P= 0.563	
Mean hatchling size (mm)	Mean	0.904	0.936	**1.001** [a]	**0.985** [b]
	St. dev.	0.064	0.047	0.059	0.060
	N	3	8	79	231
	Test statistic	t = -0.905, P= 0.389		t = 2.061, P=0.04	
Percent of eggs hatching	Mean	7.98	21.38	**29.78**[a]	**36.23**[b]
	St. dev.	17.8	35.976	37.057	38.605
	N	11	22	157	404
	Test statistic	Mann U =105, P=0.560		Mann U =28432, P= 0.046	
Clutch hatch time	Mean	1.33	3.13	6.55	6.59
	St. dev.	2.398	3.091	4.802	5.137
	N	9	8	80	229
	Test statistic	t = -1.344, P= 0.199		t=-0.054, P= 0.957	

Figure 2-27: Percent hatching of *Microxeromagna armillata* clutches (all clutches and clutches where % hatch >0) across treatment groups. AS = aestivated isolated, AP = aestivated pair, CFS = cross-fertilised single, CFP cross-fertilised pair. Error bars represent standard error of the mean; numbers above bars indicate sample size.



Although twice as many CFS snails laid eggs compared to AS snails, no other differences in reproductive characteristics were evident between AS and CFS groups. The effect of a long period of hatchling inactivity seems limited to a decrease in early growth rate, and a lower proportion of snails subsequently using self-fertilisation as a reproductive strategy. With more replication in this experiment, further differences between treatments may have been found. Nevertheless, this study provides strong evidence of the resilience and adaptability of *M. armillata*.

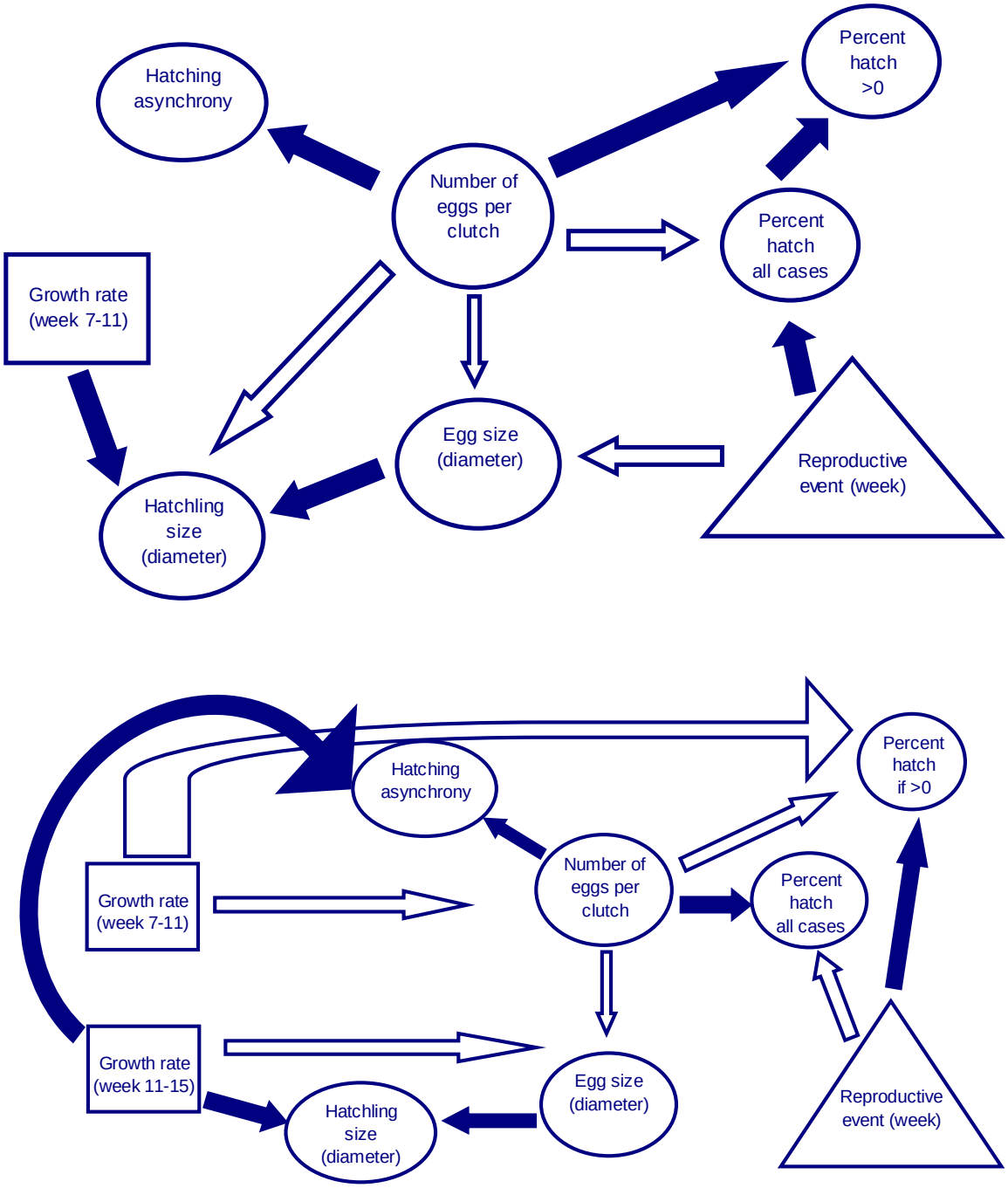
Interrelatedness of fecundity characteristics in the AP group

The relationships between fecundity characteristics have already been described for the CFP group (section 2.4.2.3), but the sample size of the AP group allows for further investigation. A weak but significant correlation was found between growth rate during weeks 7 – 11 and the number of eggs per clutch (Pearson corr. –0.268, P= 0.001, n = 157). This inverse relationship between growth and number of eggs per clutch was not evident in the CFP group and may be a function of decreased fitness in aestivated snails. However, the amount of variation explained by the model is low and it would be unwise to emphasise this relationship. The number of eggs per clutch had a negative relationship with egg size, as did the growth rate between weeks 11- 15 (Table 2-13, Figure 2-28). The negative relationship between egg size and numbers of eggs per clutch has been consistent in earlier fecundity experiments and thus appears to be a constant trade-off. Within the AP group, snails that had a slower growth rate during the last sampling period laid larger eggs, indicating a further trade-off between resources allocated for growth and reproduction.

Table 2-13: Multiple linear regression analysis of the interrelatedness of fecundity characteristics of aestivated, paired *Microxeromagna armillata* (AP).

Regression parameters	Terms in model	β	t	p
Egg size: adj R^2 = 0.214, F = 15.153, p<0.001, n = 157	Number of eggs per clutch	-0.359	-5.054	<0.001
	Growth rate weeks 11 – 15	-0.289	-4.074	<0.001
Hatchling size: adj R^2 =0.275, F=15.785, p<0.001, n = 79	Egg Size	0.539	5.288	<0.001
	Growth rate weeks 11 – 15	0.356	3.5	<0.001
Percentage hatch all cases (arcsine transf.) adj R^2 =0.351, F=42.919, p<0.001, n= 156	Week of egg laying	0.597	9.159	<0.001
	Number of eggs per clutch	0.162	2.492	0.014
Percentage hatch >0 (arcsine transf.) adj R^2 =0.417, F=19.366, p<0.001, n=78	Week of egg laying	0.512	5.226	<0.001
	Number of eggs per clutch	-0.332	-3.427	<0.001
	Growth rate weeks 7 - 11	-0.306	-3.266	0.002
Clutch hatch time	Number of eggs per clutch	0.580	6.656	<0.001
Adj R^2 = 0.402, F = 27.53, p<0.001, n = 80	Growth rate weeks 11 - 15	0.259	2.978	0.004

Figure 2-28: Relationships between fecundity characteristics in control (CFP, top) and aestivated (AP, bottom) snails. Filled arrows represents a positive relationship, open arrows indicate a negative relationship.



The growth rates of aestivated snails had a greater influence on the range of fecundity characteristics than was seen in the control group overall (Figure 2-28), and from these relationships it appears that aestivation is not without its costs. While *M. armillata* is recovering from a period of physiological stress, the relationship between growth and reproduction is more tightly bound.

2.5.4 Summary

Microxeromagna armillata juveniles have the capacity to aestivate under dry conditions from hatching, and can quickly become active again in the presence of moisture. In this study, *M. armillata* juveniles successfully survived aestivation for up to 10 months, with relatively little mortality.

In general, the growth of *M. armillata* followed a sigmoidal curve, and it is interesting to note that no differences were found between the growth rates of the different groups when growth rates were highest (week 7 – week 11). Given adequate nutrition and conditions favouring activity, the rate of growth is still apparently influenced by physiological conditioning. In this experiment, this period corresponded to snails ~2mm in size growing to sexual maturation at ~ 6.5mm in size. A greater frequency of measurement during this period may assist in understanding the growth pattern of *M. armillata* with respect to its underlying physiological changes.

The growth and fecundity characteristics of aestivated snails differed from those of their non-aestivated counterparts. Twice as many isolated control snails laid eggs compared to isolated aestivated snails, and the control group grew at a faster rate during two of the four sampling periods. No other differences were seen in their fecundity characteristics. The delay in reproducing by self-fertilisation in aestivated isolated snails, compared to the control group, could prevent the reduction in offspring fitness associated with this method of reproduction.

More control paired snails laid eggs than aestivated paired snails. Control snails grew faster than aestivated snails initially, but had a lower growth rate prior to the commencement of egg laying. Aestivated paired snails produced larger hatchlings than control snails from eggs of the same size, but key fecundity relationships remained similar to those highlighted in previous experiments (e.g. the relationship between egg size and hatchling size, section 2.3.3). In a greater number of instances, growth rates provided significantly greater prediction of fecundity characteristics of aestivated snails compared to those of the control group. It appears from this work that the relationships between growth and reproduction are more tightly bound in animals which have undergone physiological stress, with aestivated snails adjusting their reproductive strategies accordingly.

2.6 Does adult density affect the reproductive characteristics of *Microxeromagna armillata*?

2.6.1 Introduction

One of the keys to controlling a pest population is to understand not only its general biology and life history traits, but also other factors which impact on these traits. This can help predict how a population may respond to change, such as an increase in mortality due to application of a control measure [58]. One mechanism shown to influence population regulation, is population density. High population densities have been correlated with reduced fecundity and increased mortality (reviewed by Smith [58]).

In *C. aspersus*, negative associations have been found between density and body size, and density and growth rates [59]. Similar trends were found in *C. nemoralis* and *C. hortensis*, with growth rates of juveniles reduced at high densities [60]. Several authors have proposed that these associations are due in part to reduced snail activity caused by the increased presence of mucus trails in the case of land snails, and chemical conditioning of the water in aquatic snails [59-62]. In contrast, Pomeroy [63] speculated that food was a limiting resource in the case of *C. virgata*, while Butler [64] proposed a combination of these factors.

While the exact mechanism by which density influences land snail population dynamics is still a subject for debate, its importance is widely acknowledged. While there is a substantial body of work regarding density-dependent population regulation, not only in molluscs but also in other taxa, there are other studies which have failed to find any correlations between density and mortality, fecundity or growth rates. An increase in growth and natality rates was seen with increases in density in studies of the aquatic snail *Biomphalaria glabrata* (Say)(reviewed by Thomas, Goldsworthy et al [62]), while in a laboratory study with *C. acuta*, no correlation

was found between snail density and fecundity [31]. Documenting the influence of density on population regulation can also be hampered by time lags in observation of effects and the influence of environmental setting.

Density dependent population regulation has been demonstrated in some mollusc species, but has not been shown for *M. armillata*. The aim of this experiment was to identify any effect of *M. armillata* density on its fecundity characteristics, such as the number of eggs and clutches laid per snail, egg size hatchling size, hatching asynchrony (clutch hatch time) and percentage hatch.

2.6.2 Materials and methods

Six hundred and thirty snails were collected from Nangiloc in Victoria, with shell diameters ranging from approximately 4 to 7mm. These snails were then randomly allocated to containers (14.5 x 9 cm) filled with soil to a depth of two centimetres, at densities of 2, 4, 8, 16, 32 and 64 snails per container (5 containers per density). This gave a range of snail densities of 153 snails/m^2 to 4904 snails/m^2. Half of the lid area was removed and covered with thin gauze for ventilation (Figure 2-29). Decaying citrus leaves were provided as food and shelter, with additional food provided ad libitum as previously described (section 2.2.2).

Figure 2-29: Containers used to investigate the effect of density on the fecundity characteristics of *Microxeromagna armillata*. The white powder visible in containers Y and R is the food source provided to *M. armillata*.

Each week, mortality was assessed and soil checked for eggs. Soil was then remoistened and replaced in the container. Egg clutches were counted and the number of eggs per clutch was determined. Ten eggs per clutch were measured using a binocular microscope, and the entire clutch was then transferred to a well in a tissue culture tray. A sub-sample of clutches from the different densities was monitored for hatching, with hatchling size, hatching percentage and clutch hatch time recorded.

2.6.3 Results and discussion

Snail density had few effects on the fecundity characteristics of *M. armillata* (Table 2-14). The number of eggs laid per snail did not vary significantly with increasing density, nor did the number of clutches laid per snail. However, a weak but significant relationship was found between the number of eggs laid per clutch and snail density (Table 2-14). Further examination of the data showed a small number of very large clutches (60 to 300 eggs)

occurring in the high density treatments. The number of eggs recorded per clutch in this situation may be an overestimation, as separating distinct clutches when sorting soil was difficult at high snail and egg densities. When these outliers were removed from the analysis no relationship was found between the number of eggs per clutch and snail density (Pearson's correlation = 0.031, P= 0.205).

A significant but weak negative correlation was found between egg size and initial snail density (Table 2-14), although when the progressive snail density, which adjusted for mortality, was used for comparison, no significant association was found (Pearson's correlation = 0.067, P= 0.176). Snails at higher densities may lay smaller eggs, and this effect may persist after snail density decreases. However, as the variation that is explained by the variables is low, this cannot be stated with confidence from the data set. No correlation was found between snail density and hatchling size, which is not surprising considering the weak relationship between egg size and snail density.

The percentage of eggs hatching was negatively associated with initial snail density (Table 2-14) and this relationship strengthened when progressive snail density was substituted (Pearson's correlation = -0.502, P= 0.001). This result suggests that density has a role in population regulation for *M. armillata*, effected by an increase in the number of unfertilised eggs laid by snails at high densities. Research by Baur [65] focussed on the relationship between adult density and cannibalism between egg clutches. Baur's study showed that with decreasing distances between egg clutches, between clutch cannibalism increased, and acted as a density-dependent population regulation mechanism. Between clutch cannibalism was not measured in the present work but, if this occurs in *M. armillata*, then laying fewer fertilised eggs per clutch at high densities could decrease the risk of fertile eggs being eaten by hatchlings from other clutches. Hatching asynchrony in some land snail species promotes intra-clutch cannibalism [44, 47, 66-68], and, while newly hatched *M. armillata* have been

observed feeding on conspecific eggs, no change in hatching asynchrony (clutch hatch time) was seen with snail density (Table 2-14).

A confounding influence in this experiment was the variability in size of snails used Snail size has been shown to significantly influence the fecundity characteristics of *M. armillata* (section 2.3) and, while snails were randomly allocated to treatments, the size range of snails was relatively large. This may have precluded any subtle effects of density from being revealed, while potentially masking any effect of density on egg laying of differently sized snails. Similarly, as all eggs were removed from the containers for measurement, any effects that juvenile recruitment may have had on the egg laying population could not be described. In addition to snail size and juvenile recruitment, weekly soil disturbance and the densities previously experienced by snails used in this experiment may also have confounded results. These issues need to be addressed before the full effects of snail density on fecundity in *M. armillata* can be determined.

Table 2-14: Life history traits of *Microxeromagna armillata* at different adult densities.

Reproductive characteristic		Snail density (number of snails per container)					
		2	**4**	**8**	**16**	**32**	**64**
Number of eggs laid per snail	Mean ± sd	92 ± 38.57	51.85 ±28.46	124.48 ±90.12	97.40 ±59.99	90.99 ±63.88	53.53 ± 35.58
	N	4	5	5	5	5	5
	Test statistics	Linear Regression: adjR^2 =0.005, F = 1.154, P= 0.292					
Number of clutches laid per snail	Mean ± sd	4.5 ± 2.04	2.2 ± 1.34	5.23 ± 4.02	4.05 ± 2.54	3.5 ±2.43	1.78 ±1.22
	N	4	5	5	5	5	5
	Test statistics	Linear Regression: adjR^2=0.056, F = 2.073, P= 0.114					
Number of eggs per clutch	Mean ± sd	20 ± 14	24 ± 16	24 ± 16	24 ± 18	26 ± 19	30 ± 31
	N	36	44	209	324	560	571
	Test statistics	**Pearson's correlation = 0.113, p<0.001**					
Egg size (mm)	Mean ± sd	1.178 ±0.09	1.183 ± 0.059	1.175 ± 0.072	1.164 ± 0.072	1.163 ± 0.077	1.164 ± 0.064
	N	222	230	921	1219	2053	2018
	Test statistics	**Pearson's correlation = -0.074, P= 0.04**					
Hatchling size (mm)	Mean ± sd	1.025 ± 0.071	1.055 ± 0.120	1.056 ± 0.088	1.059 ± 0.080	1.061 ± 0.096	1.057 ± 0.088
	N	71	182	494	549	789	558
	Test statistics	Linear regression: adjR^2= -0.007, F=0.07, P= 0.792					
Percent of eggs hatching	Mean ± sd	0.93 ± 0.03	0.89 ± 0.11	0.92 ± 0.1	0.89 ± 0.19	0.88 ± 0.14	0.82 ± 0.24
	N	3	9	35	42	51	48
	Test statistics	**Pearson's correlation = -0.207, P= 0.004**					
Clutch hatch time (days)	Mean ± sd	4.33 ± 1.15	3.11 ±0.78	3.00 ± 1.06	2.90 ± 1.72	3.29 ±1.83	3.19 ± 1.36
	N	3	9	35	42	51	47
	Test statistics	Pearson's correlation = 0.041, p=0.579					

2.7 General Discussion

The life history traits of *M. armillata* have been studied for the first time in this body of research. I conclude that *Microxeromagna armillata* is highly fecund, utilises an iteroparous egg laying strategy, can reach sexual maturity in six weeks and live for more than two years in a semi-field environment. Given the wide intra-specific variation in reproductive strategies exhibited by other terrestrial snails, it is reasonable to assume that these traits are plastic, and differences may be seen between populations and environments [36, 39, 69, 70].

Microxeromagna armillata is capable of reproducing via self-fertilisation, although some loss in fecundity occurs when this strategy is utilised. Snails reproducing in this manner laid fewer, smaller eggs than their cross-fertilising counterparts, resulting in smaller offspring. Surprisingly, no difference was found between the percent hatching of self-fertilised or cross-fertilised eggs, indicating that self-fertilisation is relatively successful when it is employed. This is not generally the case, as *A. arbustorum* [49] and *C. aspersus* [71] show a severe reduction in fecundity when self-fertilisation is employed. It is unknown whether the overall reduction in fecundity of *M. armillata* is a result of inbreeding depression or a lack of social facilitation, as even predominantly self-fertilising species can demonstrate reduced fecundity when isolated [48, 54]. This merits exploration with further work. Second generation progeny of self-fertilised snails laid more eggs per snail than those whose parent was not a product of self-fertilisation, indicating that snails with a family history of self-fertilisation may be more likely to utilise this reproductive strategy sooner rather than waiting for a potential mate. Chen [49] hypothesised that *A. arbustorum* displayed inbreeding avoidance behaviour, as the rate of self-fertilisation in isolated snails decreased over successive seasons. This type of behaviour may be transferred epi-genetically or genetically so that offspring of self-fertilised snails display increased inbreeding avoidance behaviour.

Microxeromagna armillata can aestivate and has this ability upon hatching. It is not known whether this is common in hygromiids, as little research has been conducted with hatchlings, but it has been reported from the Achatinidae [72]. Fewer aestivated snails laid eggs than snails which had not aestivated, but the overall fecundity of these groups may still be similar when whole of life reproduction is taken into account. Aestivated juveniles may delay reproduction and allocate resources to growth, and thereby increase fecundity (and/or survival) in following seasons [73]. For aestivated snails which laid eggs in the first season, the rate of growth interacted at a stronger level with their fecundity traits when compared to non-aestivated snails. Adult snail density had little effect on the fecundity characteristics of *M. armillata*, although at high density the percentage of eggs hatching decreased. Adjustments to the experimental design may clarify the role of density in population regulation, although it is not uncommon for few effects to be seen [31].

The results indicate that *M. armillata* is a very resilient organism which is able to use a suite of bet-hedging strategies. This became increasingly apparent as the inter-relatedness of life-history traits was examined. Relationships between traits in *M. armillata* were not always consistent among the experiments undertaken, which is an indication of *M. armillata*'s ability to respond to changes such as aestivation and isolation.

The most comprehensive data gathered on *M. armillata's* life history traits (section 2.3) uncovered some interesting relationships. Snail size significantly affected some life history traits of *M. armillata*, but it did not influence how many eggs were laid per snail. Rather it influenced how these eggs were distributed through time. Smaller snails began egg laying later and laid fewer eggs in the first season than larger snails, a trend which was reversed in the second year. A strong positive relationship was found between body size, egg size and hatchling size, illustrating that the trade-off between growth and reproduction has a wider impact on traits other than total egg production. While these relationships are strong they were not universal, indicating the ability of *M. armillata* to adjust maternal investment, or at

least are conditional on the prevailing environment. In this case, the relationship between egg size and hatchling size is the most interesting. It appears that the nutritional content of eggs changes in certain circumstances. This has been demonstrated in *A. arbustorum* [46], where nutrient content does not always scale equally with egg size. This highlights the importance of detailed experimentation when describing the reproductive strategies of an organism, especially as these trade-offs may be species specific [74].

In many organisms there is a significant trade-off between the number of eggs per clutch and the size of eggs [45, 46, 75, 76] and this was demonstrated for *M. armillata.* It has been hypothesised that egg size increases (and clutch size decreases) throughout the season due to a decrease in nutritional quality of food [33, 46], but this relationship was observed in *M. armillata* despite abundant provision of highly nutritious food. Snail size influences egg size, and as *M. armillata* grew throughout the life of the experiment, this confounds the relationship between egg size and clutch size. However, the relationship between egg size and body size was not linear throughout life, only within each breeding season, indicating that fecundity is not solely size-specific. This intimates that it is an adaptive strategy to lay fewer, larger eggs towards the end of each reproductive period, quite possibly in response to environmental variables, although this study did not exclude the influence of a limited number of fertilised ova which would imply more investment in albumen and oviductal gland products to fewer eggs. Offspring born early in the season have the opportunity for a longer period of growth prior to summer inactivity than those born later in the season. Smaller hatchlings may have a higher rate of mortality during summer aestivation, so increasing hatchling size later in the season would be beneficial.

The characteristics that *M. armillata* displays would favour rapidly colonisation of favourable environments and attainment of high population densities. Colonisation ability is maximised by the capability to survive long periods of time without food and water, and to reproduce via self-fertilisation. *Microxeromagna armillata* is also highly adaptive, with the capacity to employ

various reproductive strategies. The trade-offs between growth and reproduction, and the relationships between fecundity characteristics are complex in *M. armillata*, as in many other organisms. The rapid growth, ease of culturing, long breeding periods, sequential egg laying and plastic reproductive strategies make *M. armillata* an interesting model organism for further study of life history evolution.

Chapter 3 Phenology and spatial distribution of *Microxeromagna armillata*

3.1 Introduction

In the previous chapter, some life history characteristics of *M. armillata* (and factors affecting them) were described and discussed using laboratory experimentation. Examination of life history traits in this manner has greatly increased the biological knowledge of *M. armillata*, and has provided a basis for development of a management strategy. However, some studies have highlighted significant differences in life history traits within a species between laboratory and field environments [77]. For this reason, it is important to confirm, where possible, the results seen in the laboratory with field-collected data. Accurately describing populations of *M. armillata* in the field and how changing life history traits may influence the population structure is important in order to implement appropriate control strategies.

Traditionally, populations are described in the initial phase by demography and the construction of life tables (Stearns 1992[25] for review). Such an approach is problematic in the case of *M. armillata* for several reasons. Firstly, experiments in Chapter 2 have shown that *M. armillata* can lay multiple clutches of eggs over a long period (up to 33 weeks), juveniles can reach sexual maturity in just three months and remain inactive without great fitness loss for at least ten months, adults can reproduce in more than one year, and no external shell characteristics such as ribs or a thickened aperture are observed when snails are sexually mature, as occurs in other snail species [20, 78]. These characteristics make it difficult to determine distinct cohorts and ages of *M. armillata* and hence the construction of life tables is a significant challenge . Secondly, very little information is available on age specific survivorship of *M. armillata*. Taking into account these obstacles, the most logical point of entry for description of field populations is simply to describe the phenology of *M. armillata* in citrus orchards (section 3.2).

In addition to phenology, the spatial distribution of *M. armillata* within the citrus orchard also has important implications for snail management. Prior to a management strategy being implemented, it is essential to understand where *M. armillata* is most abundant in the orchard and if this changes with season (section 3.3). This will ensure that any control measures are correctly targeted.

3.2 Phenology of *Microxeromagna armillata*

3.2.1 Introduction

Snail age is impossible to determine precisely from snail shell size (cf section 2.5), but the size of field-collected snails may give an indication of their sexual maturity, and provide a key to describing the phenology of *M. armillata.* In laboratory studies, the size of *M. armillata* at the onset of egg laying was recorded (Chapter 2), but recording this characteristic in the field is difficult. One of the techniques that has been used with other snail species to predict the onset of the breeding season is the correlation of shell size with sexual maturity [19]. In particular, the albumen gland has been shown to swell when snails become sexually mature [19, 70, 78], and changes in albumen gland size over time were positively correlated with shell size in *T. pisana* (Baker and Hawke 1990) and *C. acuta* (Baker et al. 1991b). This technique will be used with field collected *M. armillata* to determine size at sexual maturity at different times of the year, and assist in interpretation of the phenology of *M. armillata* in the field. If a clear correlation can be found between size and sexual maturity irrespective of season, then this could be developed into a decision making tool for citrus growers. With the high potential for population increase that *M. armillata* demonstrated in the laboratory (Chapter 2), it would be beneficial for citrus growers to be able to suppress adult *M. armillata* numbers before the breeding season begins. Identifying when juvenile recruitment occurs in the field and at what size *M. armillata* is capable of reproduction would allow this to be achieved with more accuracy. *Microxeromagna armillata* has been observed most frequently in the leaf litter underneath citrus trees and hence this was the substrate chosen for sampling.

3.2.2 Materials and methods

One orchard block with a population of *M. armillata* was selected from both the Riverland and Sunraysia citrus growing regions. Leaf litter under ten trees was sampled by collection from four 30cm^2 quadrats placed randomly underneath each tree in each orchard. Surface soil to a depth of approximately 5mm was also collected from each quadrat using a trowel. This ensured that any snails that fell from the leaf litter during collection were harvested, and that any juveniles still present in the topsoil after egg hatching were collected. Leaf litter and soil from each quadrat were placed in calico bags and transported to the laboratory. Leaf litter underneath the trees was sampled every 4 – 6 weeks at each orchard.

Each sample was searched for snails underneath a magnifying lamp (1.5x magnification). Snails collected from each sample were drowned overnight in water to facilitate extension of the foot from the shell in any snails collected live. Snails were considered to have been alive at collection when the foot extended from the shell during drowning. After 24 hr the water was decanted and replaced with 80% ethanol for preservation. The numbers of live and dead snails were counted under a dissecting microscope and each snail shell was measured using a dissecting microscope graticule at 10x magnification. Shell size was measured using the same procedure as in Smith et al [1].

As snail shells may persist in the environment for some considerable time [79], only those shells that still demonstrated full colour, and were not cracked or damaged in any way were included in analyses. This assisted in standardising the age of shells collected across the sampling dates. The density of live snails was calculated to give the number of snails per square metre of leaf litter. Size – frequency histograms were constructed for live and dead snails at each of the sampling dates, with snails grouped into 1mm size categories, e.g. up to 1mm, 1.01mm to 2mm, 2.01mm to 3mm and so on.

A sub-sample of live snails from each sampling date collected from the Riverland and Sunraysia sites were dissected to investigate the size of reproductive organs. The shell diameter and length of the albumen gland were measured for each snail using a dissecting microscope and graticule at 10x magnification. Snails were left in 80% ethanol for at least one month before dissection to allow for any shrinkage of the albumen gland and to standardize this across samples.

3.2.3 Results and Discussion

The density of live snails collected from leaf litter in the Riverland orchard was generally highest in the winter months, reaching a peak of 2582 snails/m^2 in July 2000 (Figure 3-1). Significant increases in snail density occurred from June to July 1999, February to March 2000, and May to July 2000. Increases in snail density indicate that either juvenile recruitment or migration is occurring. Examination of the size – frequency histograms for these time periods suggest that juvenile recruitment has occurred from June to July 1999, and March to July 2000 (Figure 3-2). The increase in snail density observed from February to March 2000 (Figure 3-1) evidently did not result from juvenile recruitment, but from snails migrating into the leaf litter (cf Figure 3-2). The increase in numbers of snails in the 3.01 – 9mm shell diameter range was relatively high and unevenly distributed across size classes indicating that the increase cannot just be attributed to progression from one class into another through growth, but is likely the result of migration. However, the growth rates of size classes in the field is unknown and further field experimentation is needed to state this with confidence.

Juvenile snails (1.01 – 2mm) were collected on all sampling dates, although often in low numbers (Figure 3-2). This could indicate that *M. armillata* lays eggs throughout the entire year, but a more likely explanation is that a proportion of juveniles aestivate over the summer months, or remain generally inactive (section 2.5). Live hatchling snails (ca 1mm in shell diameter) were rarely included in the samples (cf Table 2-5). However, dead snails in this size

class were frequently collected, (Appendix A, Figure A-1) and this could indicate either a bias in the sampling procedure, or a high mortality rate of small juveniles (<1mm) in the field. While snail shells may persist in the environment for a considerable period of time, it is reasonable to assume that smaller shells breakdown at a faster rate than larger shells [79]. This confounds the sampling results and makes the comparison of live and dead snails across size categories and time, difficult. For this reason, discussion in this section will focus on data for live snails, with the information regarding dead snail size for the Riverland and Sunraysia orchards presented in Appendix A.

Microxeromagna armillata kept in laboratory culture were shown in earlier experiments to be capable of reaching greater than 10mm in shell diameter, but no snails over 9.01mm were collected from the field, and relatively few were greater than 8.01mm. This most likely indicates the effect that unlimited food and a diet rich in calcium has on the growth of *M. armillata* in the laboratory, although it is possible that large snails may have been present in an area which was not sampled.

Albumen gland measurements were taken from snails with a shell diameter greater than 4mm, and clear differences in gland size were observed in snails of different sizes from the Riverland site (Figure 3-3). Generally, snails with a shell diameter greater than 6.3mm had large albumen glands, while snails less than 5.6mm in shell diameter had small albumen glands. In between 5.6 and 6.3mm shell diameter, the albumen gland varied from small to large and could be considered as being in the process of gland maturation. This is particularly evident in February 2000 (Figure 3-3) and is consistent with the onset of reproduction in following months (Figure 3-2). Dissection of snails from the Sunraysia site showed similar results, these are presented in Appendix A.

Figure 3-1: Density of live *Microxeromagna armillata* in leaf litter collected from a Riverland citrus orchard. Four 30cm^2 quadrats of leaf litter were sampled per tree for 10 trees on each sampling date. Error bars represent 95% confidence intervals.

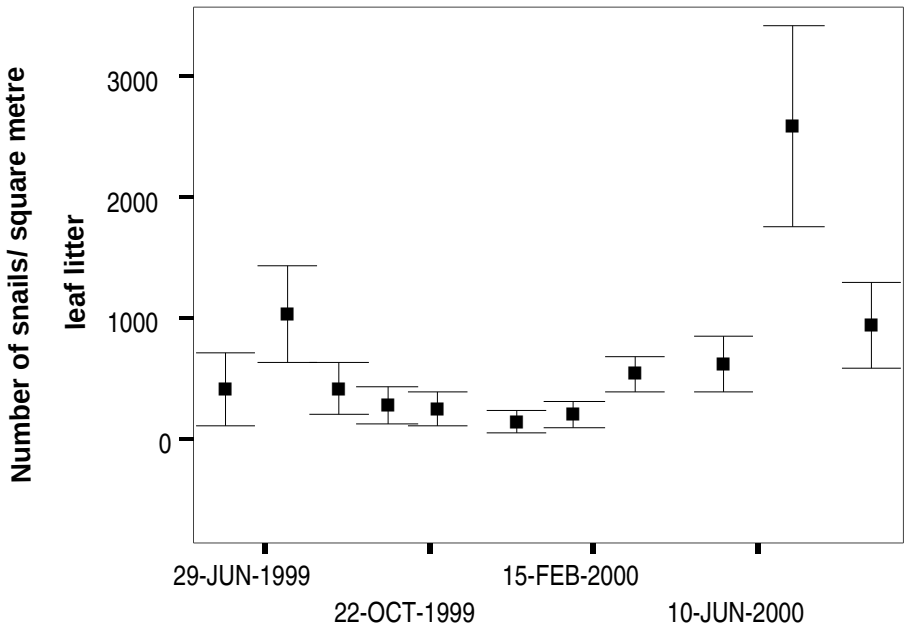


Figure 3-2: Size frequency histograms of live *Microxeromagna armillata* from the Riverland orchard, June 1999 to August 2000. Note change of scale on 6/7/00 and 30/8/00

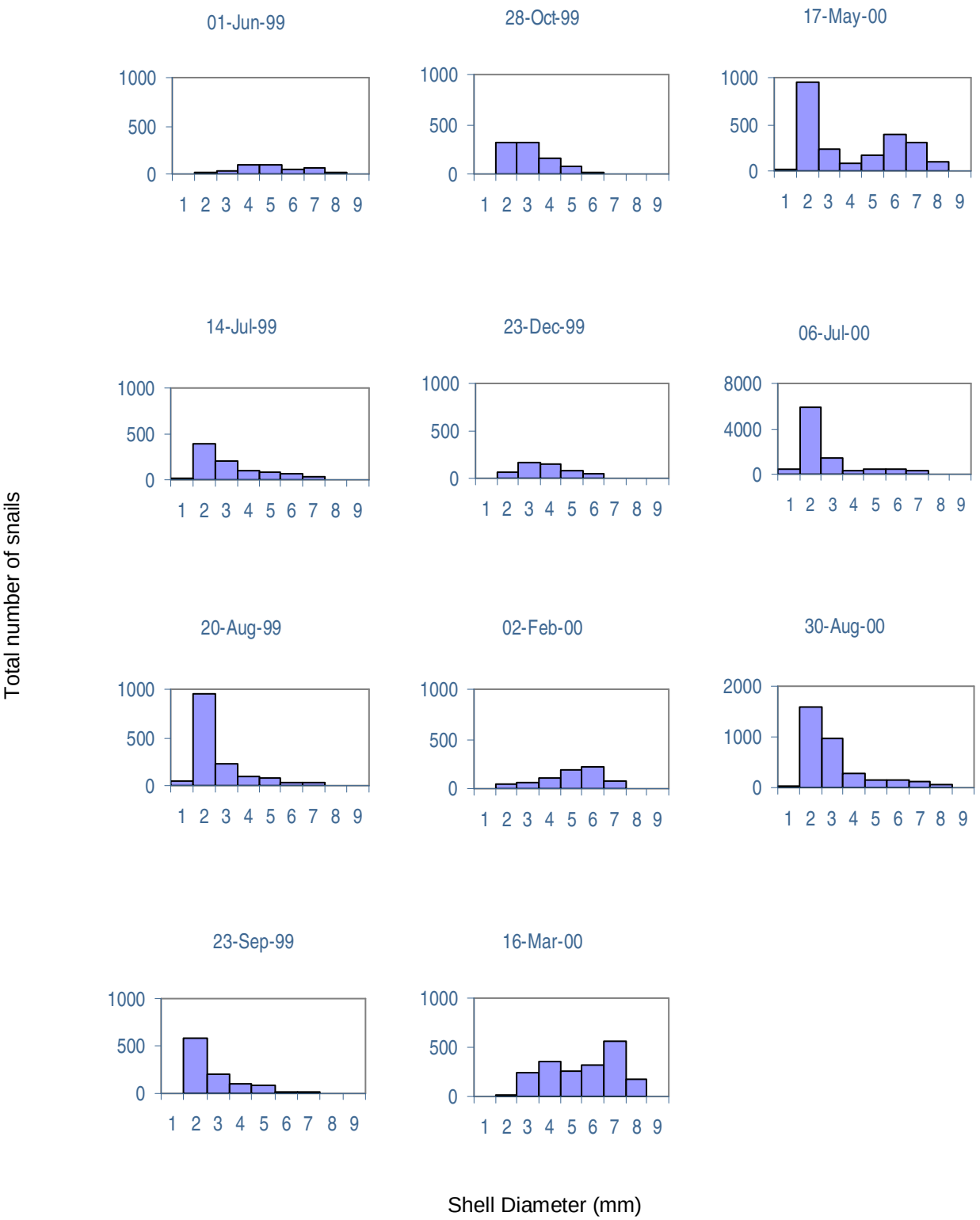


Figure 3-3: Length of albumen gland as a function of shell diameter for *Microxeromagna armillata* collected from a Riverland orchard.

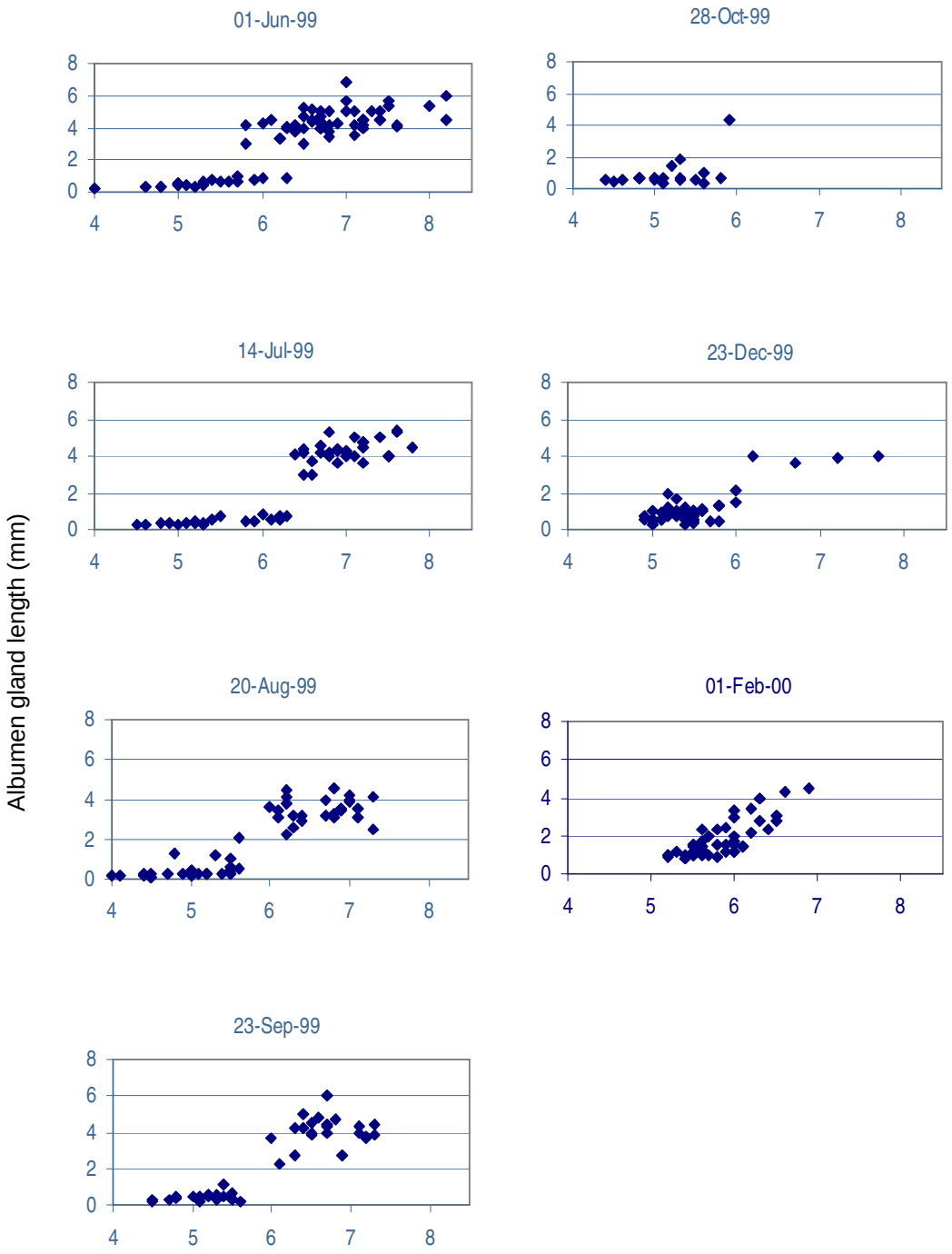


Figure 3-4: Density of live *Microxeromagna armillata* in leaf litter in a citrus orchard in Sunraysia. N = number of 30cm^2 quadrats of leaf litter sampled on each sampling date.

Error bars represent 95% confidence intervals; note scale on x-y axis differs from Figure 3-1.

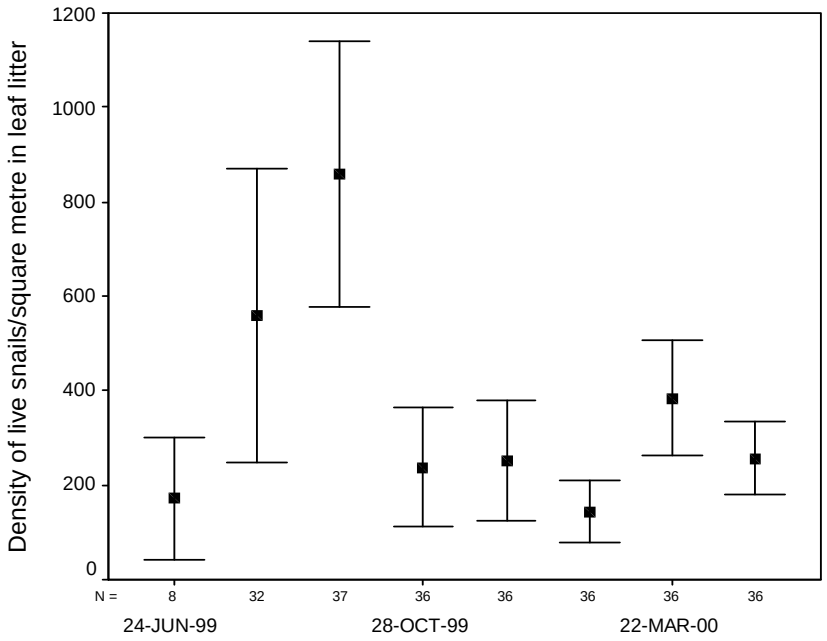
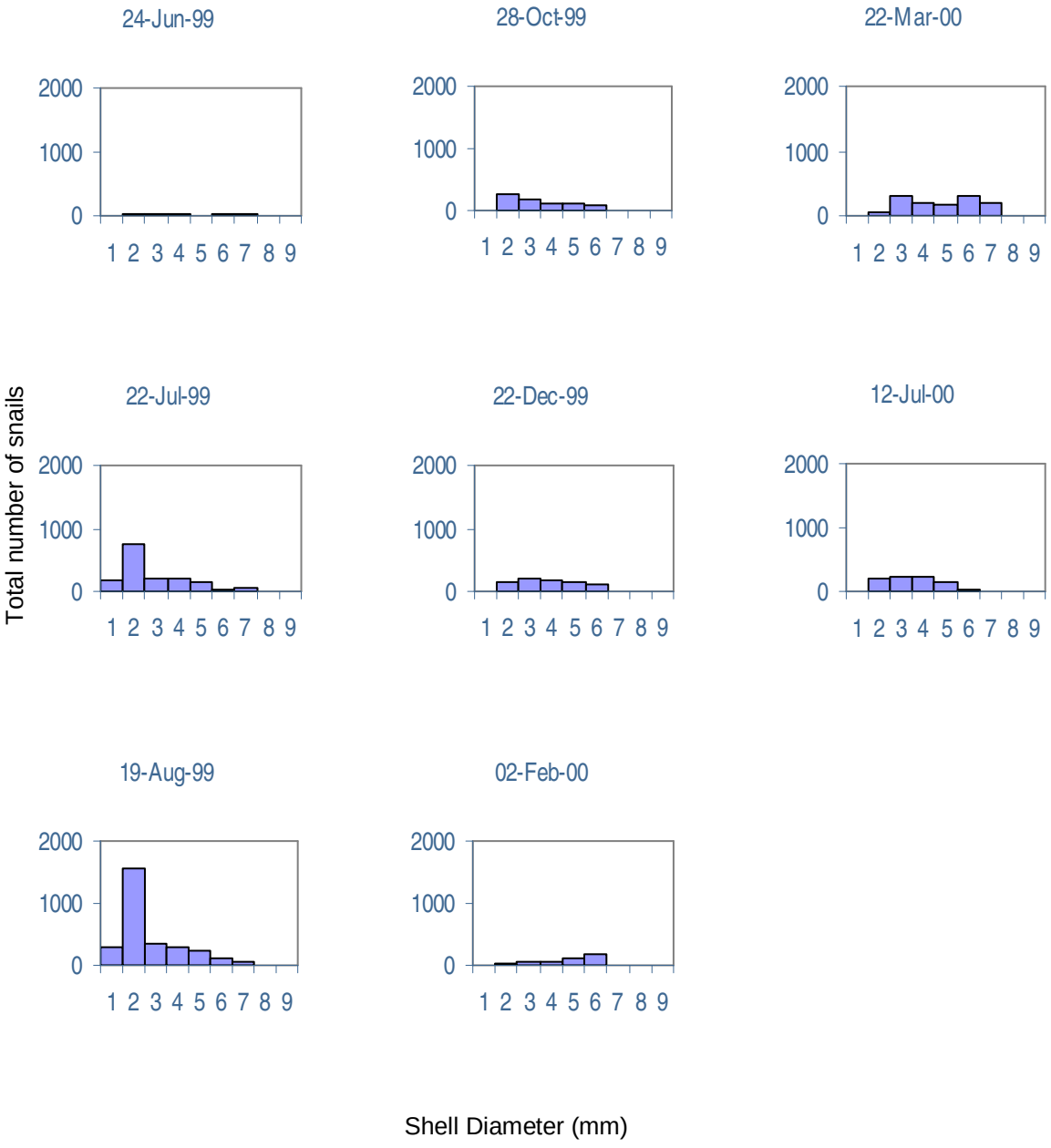


Figure 3-5: Size frequency histograms for live *Microxeromagna armillata* collected from the Sunraysia orchard, June 1999 to August 2000



In the Sunraysia orchard, snail density increased during the winter months of 1999 and from February to March in 2000 (Figure 3-4). Examination of the size-frequency distributions during these periods indicates that juvenile recruitment occurred during the winter months of 1999, and migration was responsible for the increase in density from February to March 2000 (Figure 3-5). This is similar to results seen for the Riverland orchard, but no increase in snail density was observed from March to July 2000 at the Sunraysia site. Few juveniles were seen in July 2000 (Figure 3-5), which contrasts with both the previous year, and results from the Riverland orchard (Figure 3-2). Examination of the size-frequency distributions for the Sunraysia site shows a substantial increase in the total number of dead snails in March and July 2000 (Appendix 1b). A disproportionate number of large snails (>4mm) were dead compared to smaller snails (<4mm) when comparisons with live size-frequency distributions are made (Figure 3-4). This could be due to the confounding nature of dead snail collection as previously discussed, but a more likely cause is a difference in management practices between the orchards. In the Sunraysia orchard, snail bait was applied on 3 – 4 occasions during autumn 2000, compared to only one application at the Riverland site. Application of snail bait could be responsible for the substantial increase in the numbers of dead snails at the Sunraysia site in March and July 2000, and as the majority of these snails were large, it could also account for low juvenile recruitment in July. Applications of molluscicidal bait could not be withheld at these sites as all blocks with fruit scheduled for export, and a population of *M. armillata*, must be baited. Repeating this work in navel orchards not targeted for export and with no molluscicde applications would assist in describing the phenology of *M. armillata*.

3.3 Spatial distribution of *Microxeromagna armillata*

3.3.1 Introduction

Microxeromagna armillata was abundant in leaf litter under citrus trees (Figure 3-1, Figure 3-4), but it is important that other orchard substrates are examined, for several reasons. Firstly, it is not known when or where fruit contamination with *M. armillata* occurs in the orchard, and to infer the most likely source of contamination the relative abundance of *M. armillata* on different orchard substrates must be investigated. Secondly, migration into the leaf litter sub-population occurred in both the Sunraysia and Riverland orchards from February to March, and it is important to determine the source of these adults. Other citrus blocks surrounded those orchard blocks previously under study (section 3.2), but it seems unlikely that migration would occur between adjacent citrus blocks in a biased manner. Adjacent blocks are separated from those under study by a small track, roughly the equivalent of an inter-row space. *Microxeromagna armillata* is present in these adjacent blocks. One-way migration into the leaf litter from these adjacent blocks is unlikely due to the uniform nature of the landscape, resources and snail population, and was not studied in this project. However, migration into the leaf litter could be occurring from another orchard substrate such as the tree canopy or inter-row area. The relative abundance of *M. armillata* on orchard substrates other than leaf litter could provide an insight into ways to prevent fruit contamination. Research in the following section investigates the spatial and size distribution of *M. armillata* within a citrus orchard in autumn, winter and spring.

3.3.2 Materials and Methods

In 2003, five different orchard substrates were searched for *M. armillata* in autumn, winter and spring (Table 3-1). Twenty trees were randomly sampled in each season from a navel citrus block in Nangiloc, Victoria.

Table 3-1: Orchard substrates sampled, and sampling methods used, to examine the distribution of *Microxeromagna armillata* in a citrus orchard

Orchard substrate	Sampling method	Comments
Leaf litter under tree canopy	Collection, 30x30cm quadrat	Leaf litter and 0.5cm topsoil were collected from within a 30x30cm quadrat, placed in a calico bag and transported to laboratory for sorting. Each quadrat was sub-sampled (25%) in the laboratory prior to hand sorting
Area in between orchard rows (grassy sod)	Collection, 30x30cm quadrat	Plant material and the top 0.5cm of soil were removed from a 30x30cm quadrat and transported to the laboratory for hand sorting. Quadrats were not sub-sampled.
Tree trunk and major branches	Visual search	Tree trunks were examined for the presence of *M. armillata* from the base of the trunk to a height of 2 metres.
Outer tree canopy: Twigs, leaves and fruit	Visual search, 30x30cm quadrat	10 quadrats were searched around the outer edge of the tree canopy. If fruit was on the tree, the number of fruit per quadrat and snails per fruit was recorded. Quadrats were searched to a depth of approximately 30cm.
Fruit on the ground	Visual search	Each piece of fallen fruit underneath the tree canopy was examined to give a mean number of snails per piece of fallen fruit per tree.

Samples of leaf litter and grass collected were hand searched for snails at 1.5 times magnification in the laboratory. Snails were counted, assessed as live or dead, and shell size determined under a dissecting microscope. Sampling in autumn could not be conducted in the one day due to inclement weather, and as a result sampling was split over two days, one week apart. A sub-sample of live snails collected in autumn and spring were dissected to measure the length of the albumen gland as previously outlined in section 3.2.2.

3.3.3 Results and Discussion

Approximately 330 snails/m^2 of leaf litter were recorded in autumn, which rose to 4000 snails/m^2 of leaf litter in spring (Figure 3-6). This population increase could either be a result of migration into the area and/or juvenile recruitment. An increasing number of smaller snails were found in the leaf litter over this autumn to spring period, which suggests the population increase was largely due to recruitment of juvenile snails (Figure 3-8), as *M. armillata* lays eggs during winter (cf Section 2.3). This also reflects the results in section 3.2.3, where juvenile recruitment had occurred during winter at the Riverland and Sunraysia orchards (1999/2000). It is possible that juvenile recruitment is due to immigration, but as previously discussed this seems unlikely.

Albumen gland measurements from autumn and spring 2003 (Figure 3-7) also mirrored those seen at the Riverland orchard in 1999/00 (Figure 3-3). Irrespective of season, snails less than 5.6mm in diameter had appreciably smaller albumen glands than those greater than 6.3mm in diameter. Dissections in autumn showed a greater number of large albumen glands in snails between 5.6 and 6.3 mm shell diameter than in spring. This could indicate that more snails are maturing sexually in autumn whereas in spring differences in albumen gland maturity could indicate different cohorts. As *M. armillata* has the ability to remain inactive for long periods of time, and to grow to maturity in just a few months (cf section 2.5), separating cohorts is difficult. However, large but immature *M. armillata* in spring would seem better served by conserving their energy for the likely aestivation over summer than animals maturing sexually 3 – 4 months before egg laying occurs. The relationship between body size and fecundity in *M. armillata* discussed in section 2.3 indicates that further investment in growth in spring is a more effective strategy than maintaining sexual readiness for a length period prior to reproductive activity.

A significant difference in snail density was seen between the leaf litter and grassy sod areas (Figure 3-6). Snail numbers were much higher in the leaf litter at all times of year when compared to the grassy sod, which indicates that leaf litter is the preferred habitat. Eggs were observed in soil at the base of plants in the sod area, but it seems that only a small number of snails are utilising this area. Size – frequency histograms of snails collected from the grassy sod (Figure 3-9) indicate that juvenile recruitment is occurring in this area, as snails ≤1mm in shell diameter were present. It is unlikely that these hatchlings would be capable of migrating long distances.

Figure 3-6: Mean number of live *Microxeromagna armillata* per square metre in leaf litter and grassy sod. Error bars represent standard deviations.

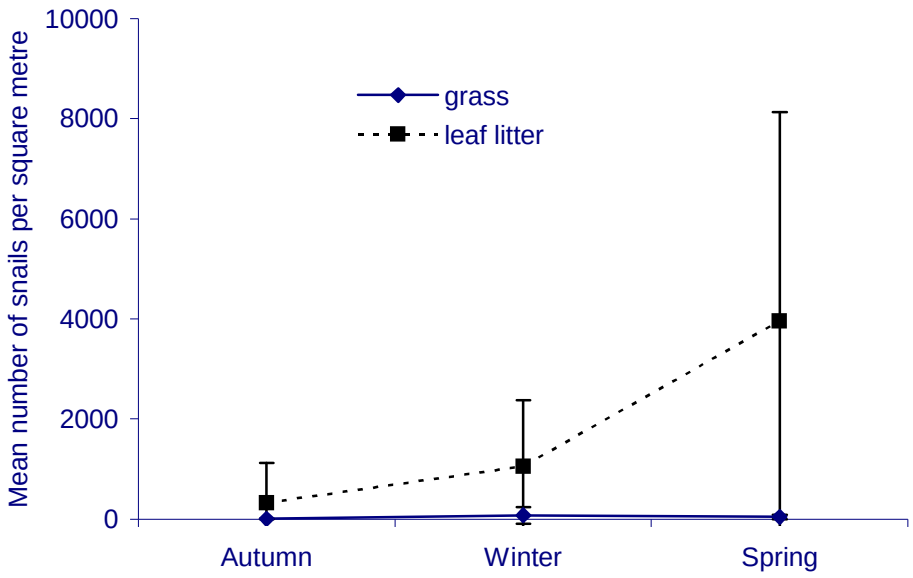


Figure 3-7: Length of albumen gland as a function of shell diameter for *Microxeromagna armillata* collected from a Sunraysia orchard in autumn and spring 2003.

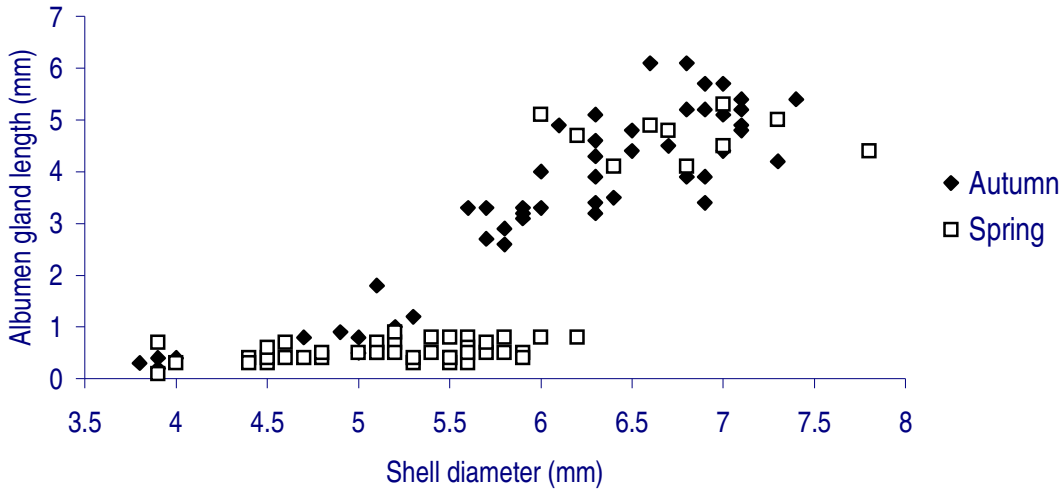


Figure 3-8: Size frequency histograms of *Microxeromagna armillata* collected in leaf litter from a Sunraysia orchard in autumn, winter and spring 2003

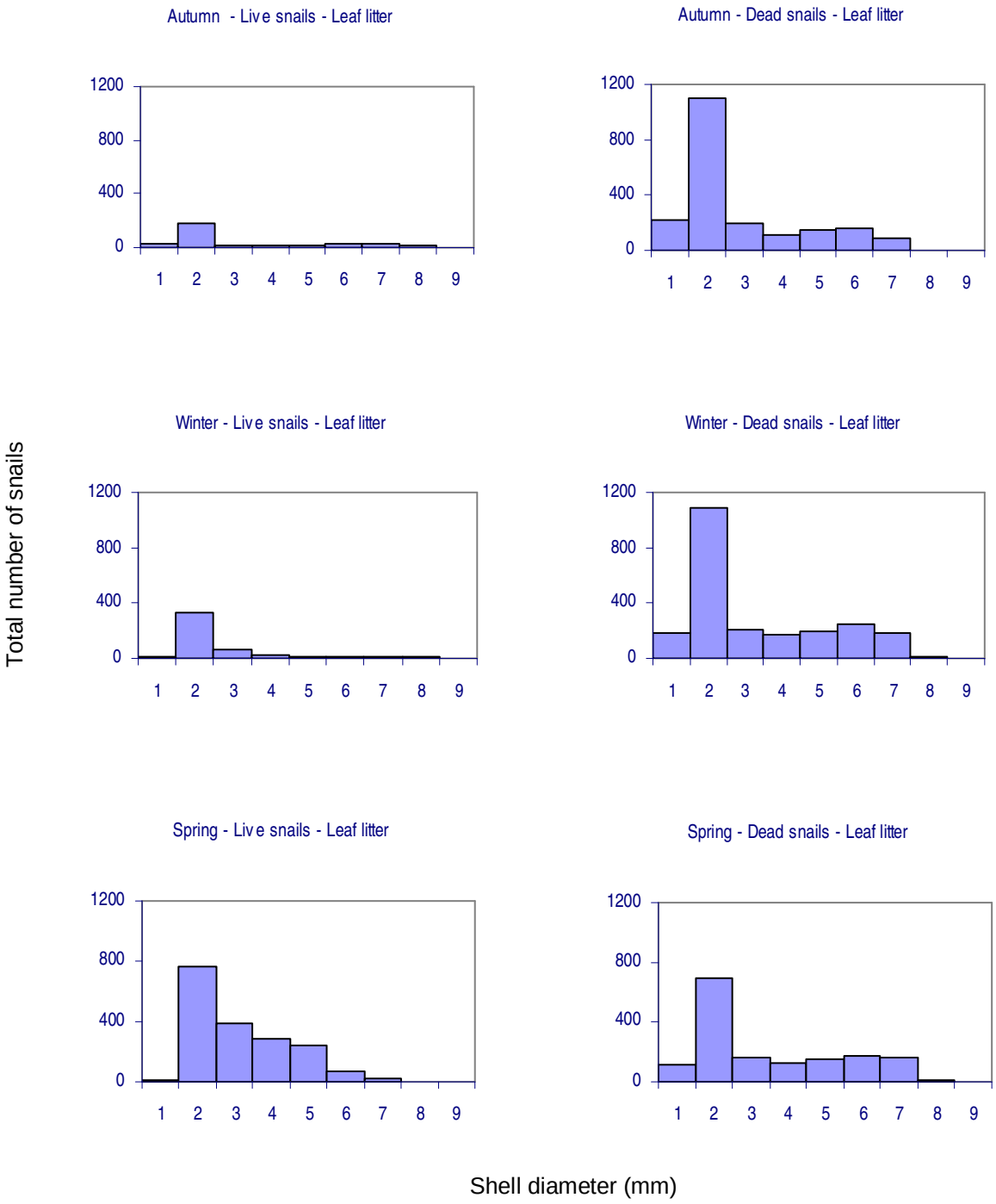
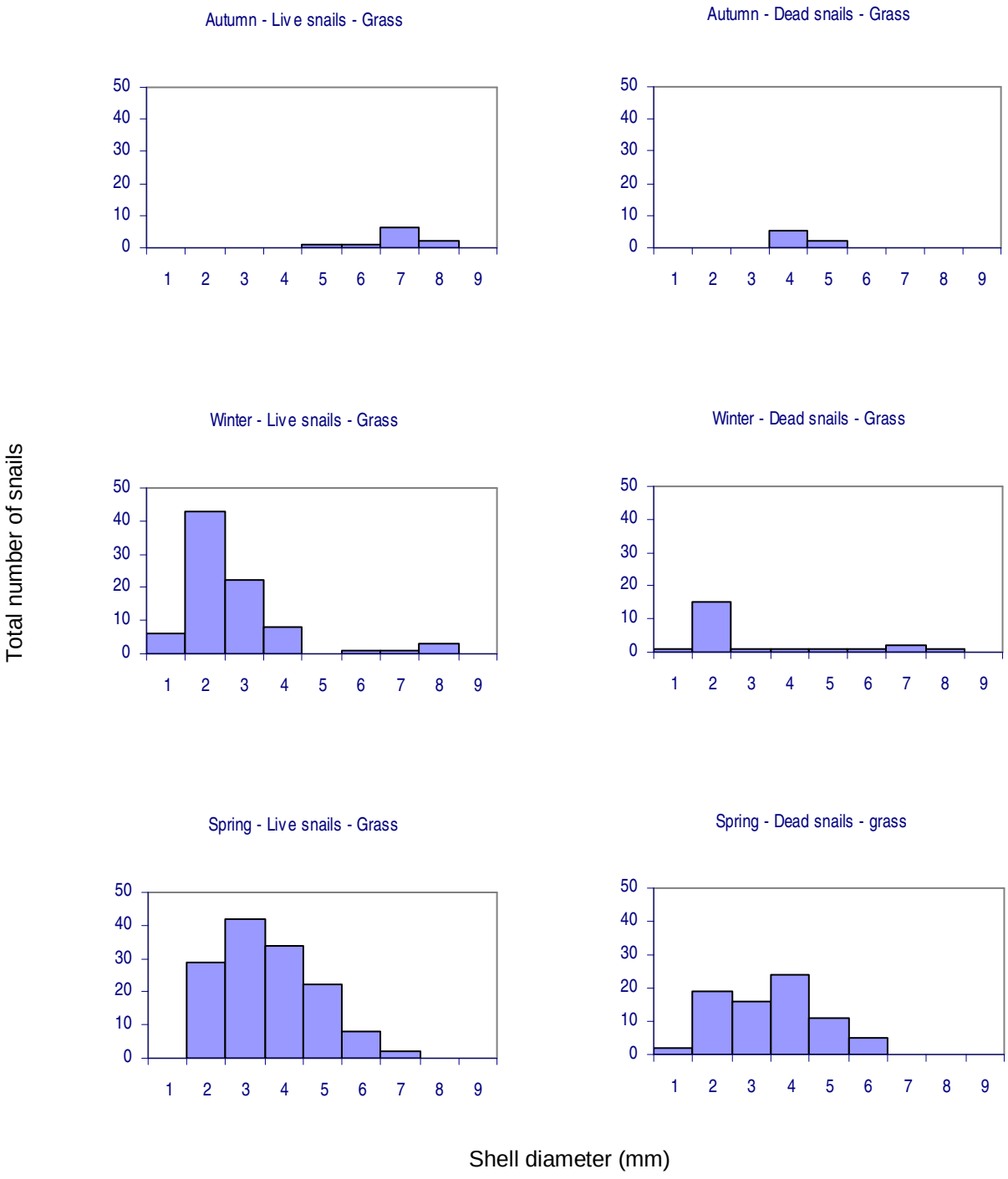
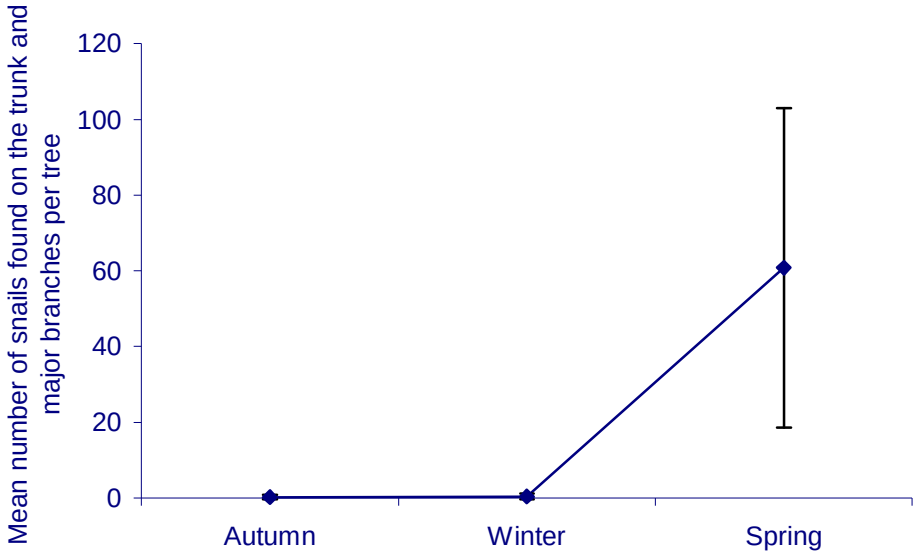


Figure 3-9: Size frequency histograms of *Microxeromagna armillata* collected in grass from a Sunraysia orchard in autumn, winter and spring 2003



The mean number of snails found on the trunk and major branches varied between seasons in 2003 (Figure 3-10). Very few snails were found on these substrates during autumn and winter, and as *M. armillata* lays eggs in soil during this period (Chapter 2, sections 3.2.3, Figure 3-2, Figure 3-4) this is not surprising.

Figure 3-10: Mean number of *Microxeromagna armillata* found on the trunk and major branches of Navel trees during autumn, winter and spring. Error bars represent standard deviation.



Microxeromagna armillata was more abundant on the trunk and major branches in spring compared to autumn and winter. In spring, egg laying had slowed considerably in the laboratory (Figure 2-3) and with warmer weather in the field, it is likely that *M. armillata* is moving into the tree canopy to shelter from summer heat. An increase in *M. armillata* abundance in the tree canopy would increase the risk of fruit contamination in spring, compared to autumn and winter. The majority of the navel orange harvest is completed by spring, but some late-harvest varieties may be more susceptible to snail contamination of fruit.

Investigation of the outer tree canopy area showed the same trend in snail abundance as the trunk and major branches (Figure 3-11). No snails were found in the outer canopy (or on fruit) during autumn and winter sampling, but *M. armillata* was found on this substrate during spring. This indicates that snails are more abundant further out into the canopy during spring, which may increase the risk of snail contamination of fruit.

Figure 3-11: Mean number of *Microxeromagna armillata* found per canopy quadrat (n = 10 quadrats from 20 trees). Error bars represent standard deviations.

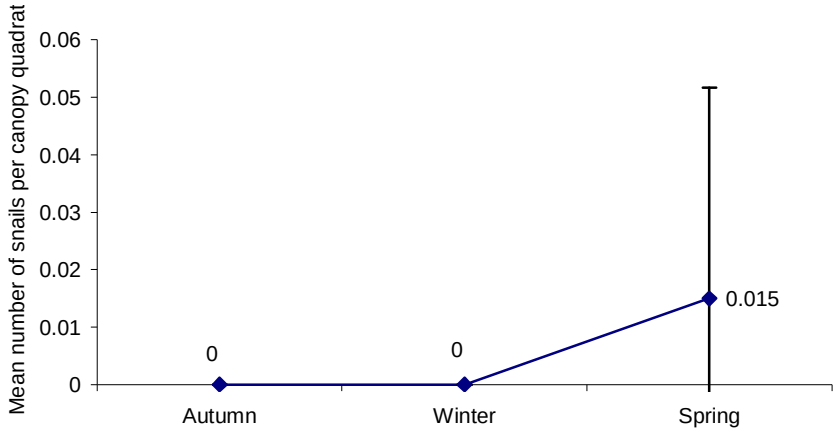
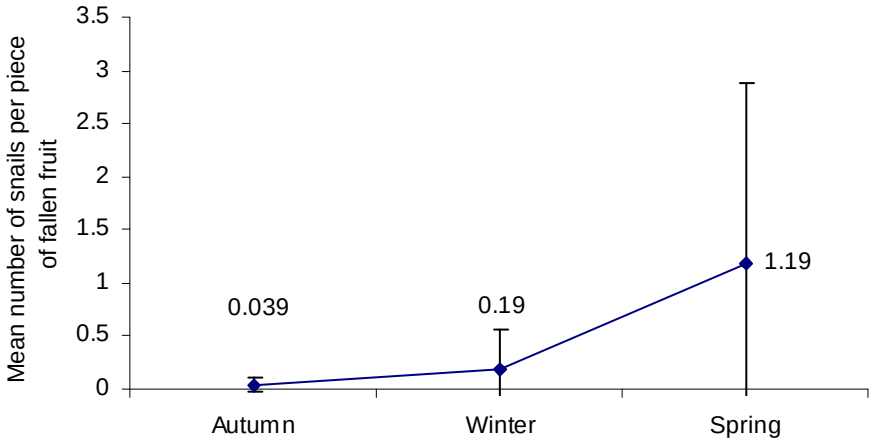


Figure 3-12: Mean number of *Microxeromagna armillata* found per piece of fallen fruit underneath the tree canopy (n=20 trees). Error bars represent standard deviations.



Low numbers of snails were found on the trunk and in the canopy in autumn and winter, yet this is when the majority of the navel orange harvest takes place. If the risk of snail contamination of fruit on the tree is very low during this time, it is possible that snail contamination is occurring in another manner. An alternative mechanism of snail contamination is the harvesting of fallen fruit. It is strongly recommended within the citrus industry that fallen fruit is never harvested as this increases the risk of bacteria, fungi and fruit fly contamination of fruit. However, as picking staff are paid by the weight of fruit picked, harvesting fallen fruit is often a temptation. In all seasons sampled, the risk of finding a snail on a piece of fallen fruit was much higher than finding a snail in the outer canopy of a tree (Figure 3-12, Figure 3-11). This could be a possible pathway for snail contamination of fruit destined for export, so it is important that fallen fruit is never harvested to minimise the risk of snail interception by quarantine authorities.

3.4 Summary

The phenology and spatial distribution of *M. armillata* has been described here for the first time. *Microxeromagna armillata* can reach very high densities in leaf litter, although this varies between seasons and years in Australian citrus orchards. *Microxeromagna armillata* is sexually mature once a shell diameter of 6.3 mm is reached, and snails of this size were present during all sampling periods throughout the study. General trends such as juvenile recruitment are comparable between orchards at different locations. The majority of juvenile recruitment (1 – 2mm in shell diameter) occurs in the winter months although juveniles are present in the leaf litter at all times of year. Juvenile recruitment occurred in the laboratory in winter and juveniles could remain inactive for long periods of time - both of these traits are supported by field results in this chapter but, although unlikely, egg laying and juvenile recruitment in orchards during the summer months cannot be excluded.

Microxeromagna armillata is more abundant in leaf litter under the citrus trees than in the grassy inter-row area at all times of year sampled, which infers that leaf litter is a preferred habitat. *Microxeromagna armillata* is more abundant in the trees during spring than autumn and winter, but *M. armillata* is still more likely to be found on fallen fruit in the leaf litter than on fruit in the tree. The risk of snail contamination of fruit is highest if fallen fruit is harvested, but if proper picking practices are adhered to, then the risk of snail contamination of fruit is highest in spring when *M. armillata* is more abundant on the tree canopy. Most navel orange varieties would have been harvested by this time, but late-harvest varieties may be particularly vulnerable to snail contamination.

Chapter 4 Movement of *Microxeromagna armillata* in citrus orchards

4.1 Introduction

The study of movement is fundamental to the understanding of the basic biology and ecology of snails. In the case of *M. armillata* it is also of vital importance to determine how and when snails are likely to contaminate citrus fruit. No research has been conducted on the movement of *M. armillata* or the behaviour leading to fruit contamination.

Temperature and moisture are thought to be the most important factors influencing snail activity [19], and are closely interrelated. Generally speaking, snails are inactive at high temperatures, and conditions of low environmental moisture and humidity. Although there are upper and lower limits, activity is promoted under moderate temperatures and relatively high humidity. Several species of terrestrial snails, including many of those that have been introduced into Australia, inhabit environments that experience high temperatures. One of the characteristics of these snails is their climbing behaviour, with snails climbing off the ground and onto fence posts and grain stalks to avoid the surface heat of the soil [5]. These snails will then aestivate until environmental conditions are more conducive to activity. A similar adaptation is seen in other parts of the world. In certain harsh seasonal climates, snails burrow to avoid high soil surface temperatures [80].

In addition to temperature and moisture, light has also been identified as a factor influencing movement in terrestrial molluscs [81-85]. Many species are nocturnal (references in Asami [81]), and change the expression of behaviours such as egg laying, hibernation [84] and duration of movement [86] in response to photoperiods.

Most of the ecological research focussing on terrestrial molluscan activity has been centred on movement at ground level, with limited studies on arboreal snails, or snails which inhabit both niches. However, there are several exceptions to this. A study on the arboreal distribution of the land snail *Euhadra amaliae* (Kobelt) not only demonstrated that snail movement increased with increasing moisture, but that the spatial distribution of snails in the tree changed in response to moisture [87]. In drier conditions *E. amaliae* rests on leaves, but during or immediately after rainfall, higher proportions are found on trunks and branches. The tree climbing behaviour of *Cerithidea rhizophorarum* (Adams)(Potamididae) was found to change with season in a mangrove forest in Japan [88]. This species aggregated on particular trees irrespective of the tree's measured physical traits. Studies on the behaviour of *Cerithidea anticipata* (Iredale) in Australian mangrove forests suggest the climbing behaviour is due to the avoidance of physiological stress rather than avoiding predators [89].

In addition to environmental variables, the movement of terrestrial molluscs can be influenced by other factors. The stage of maturity has been shown to affect dispersal in *Achatina fulica* (Bowditch), with activity of juveniles and adults differing significantly [90]. Juvenile *A. fulica* dispersed the longest distances, with young and old adults travelling shorter distances. Snail size was also found to have a significant effect on distance travelled in the minute land snail *P. pygmaeum*, with smaller snails travelling shorter distances than larger specimens [41]. In contrast Carne [23] found that *Cernuella virgata* and *Cochlicella acuta* juveniles moved further than their adult counterparts.

The distribution of vegetation within the immediate environment also plays a role in the dispersal of molluscs. Movement of *T. pisana* is greater in areas of sparse vegetation compared with thick vegetation [91]. This was characterised further in *Sitala jenynsi* (Pfieffer), with dispersion being impeded by shrubs, edible plants, and variable vegetation density [92]. The mechanisms and factors affecting molluscan movement are complex and in many cases

can be species specific [82]. In order to understand and predict the movement of *M. armillata* within the citrus orchard, it is essential that further research be conducted in this area.

As *M. armillata* moving into the tree canopy is of primary concern to Australian citrus growers, mechanisms that minimise this movement are of paramount importance. The most logical approach to achieve this is to decrease snail numbers in the field. The control of molluscs has been studied widely and is generally separated into three categories, biological, cultural, and chemical control. Biological control is not a realistic option for management of *M. armillata* mainly due to the high cost of sourcing and testing biological control agents, so cultural and chemical control mechanisms are the primary focus. Chemical control of terrestrial gastropods in Australia is generally attempted with the use of metaldehyde or methiocarb based products applied as pellets, dusts, or sprays [5], although recently an iron EDTA based pellet has become available in Australia [93]. These molluscicides are most commonly applied in pellet form, and the effectiveness of these pellets relies on the movement and feeding of the target snail and slug species. There are many complex factors which influence the activity of molluscs, and hence there are many associated problems with the chemical control of molluscs. The effectiveness of field applications is influenced primarily by the behaviour of the molluscs and the environmental conditions [19]. Previous research has shown that *Microxeromagna armillata* populations were not significantly affected by the application of various molluscicide baits, and this is thought to be due to a combination of behavioural, environmental and chemical factors [9]. An optimum solution for citrus growers is to employ a treatment that is independent of these factors, which are often beyond their control.

Cultural methods of control such as handpicking, cultivation, burning, crop rotation and windrowing [5, 94-96] have all been used to successfully control molluscs populations in broad acre cropping systems. These cultural control mechanisms cannot be used for snail control in citrus orchards. There are two main types of cultural control methods used to

control snails in citrus, skirt pruning and trunk banding. Skirt pruning involves removing the lower limbs and branches of the citrus tree, so that there are no other access points to the canopy other than the trunk. This method of pruning significantly reduced the damage by *Cantareus aspersus* in Californian citrus orchards [97] and is now standard practice for Australian growers exporting citrus to the USA [98].

Trunk banding is often used in conjunction with skirt pruning in the USA. This involves placing a barrier, generally copper foil or copper sheeting, around the tree trunks. Copper is a well-known repellent of snails [19], although the exact mechanism of this effect is not well understood. Copper barriers can prevent movement of *C. aspersus* into the canopy for up to 5 years if the barrier is well maintained [97]. This method of cultural control takes advantage of the trunk being the only access point for snails to enter the canopy, and relies on good weed control. Copper bands are not used in Australian citrus orchards and the effect of copper banding on the movement of *M. armillata* has not been investigated.

Research in this chapter focuses on the presence of snails in the tree canopy (section 4.2), development of a technique to monitor snail activity and estimate active snail size (sections 4.3 and 4.4), the activity levels and size of *M. armillata* active on different orchard substrates in different seasons (section 4.5), and the prevention of snail movement using copper bands and sprays (section 4.6).

4.2 Presence of snails in the tree canopy

4.2.1 Introduction

All trees in blocks scheduled for fruit export to the USA are skirted as described above. This means that the only access point for snails to contaminate fruit on the tree is via the trunk and major branches. Studies in the previous chapter on the spatial distribution of *M. armillata* within citrus orchards provided an indication of the abundance of snails in the tree canopy (section 3.3). In earlier work trunk sampling was only undertaken on three occasions. Further sampling is required to more definitively determine patterns of snail abundance in the citrus tree canopy. Hence the aim of this experiment was to identify when, and how many, snails were present in the tree canopy during a two-year period.

4.2.2 Materials and Methods

Two Washington navel citrus blocks in Nangiloc, Victoria were monitored for the incidence of snails on the trunk and major branches. Thirty trees were monitored in block 1 and ten trees were monitored in block 2. The trees in block 1 were 5 – 10 years older than those in block 2. To assess the number of snails on the trunk and major branches, visual counts were conducted. Each tree was searched from the base of the trunk to approximately 180cm high on the major branches. As searching progressed around the trunk, the major branches were also searched to a radius of 40cm out from the tree trunk. Counts were generally conducted on a monthly basis.

4.2.3 Results and Discussion

Numbers of snails found on the trunk and major branches of navel trees in block 1 varied greatly over time (Figure 4-1). Two periods of relatively high snail numbers on trunks occurred between 4/10/2001 and 14/3/2002 and again the following spring between 16/10/2002 and 19/2/2003 to a lesser extent. These periods of high snail numbers correspond with the warmer months of the year and suggest that *M. armillata* is ascending the tree to gain shelter from the summer heat. These results are mirrored in younger trees (Figure 4-2).

Figure 4-1: Mean number of *Microxeromagna armillata* on the trunk and major branches of Navel orange trees (block 1) in Nangiloc, Victoria. Error bars represent standard deviations, n = 30 trees

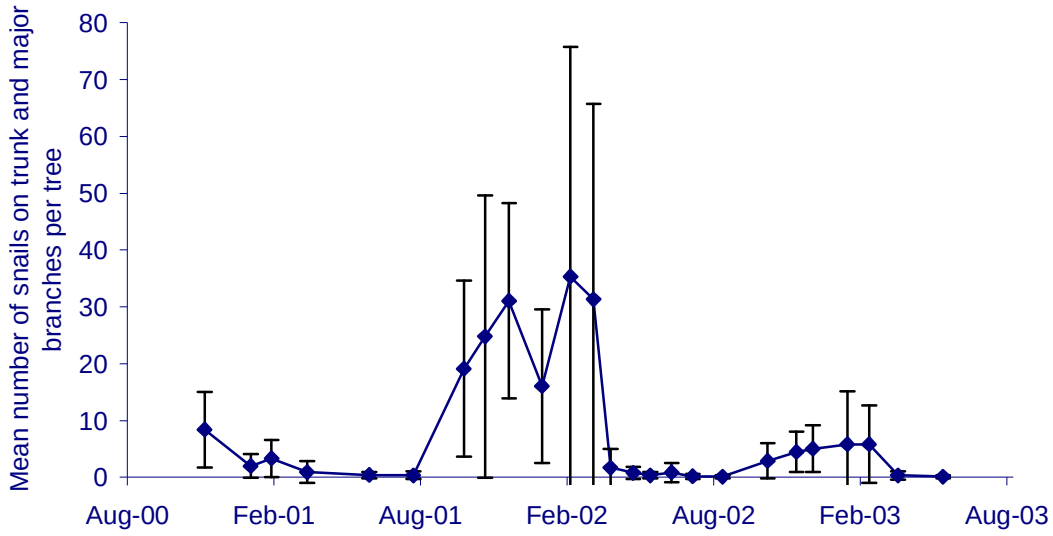
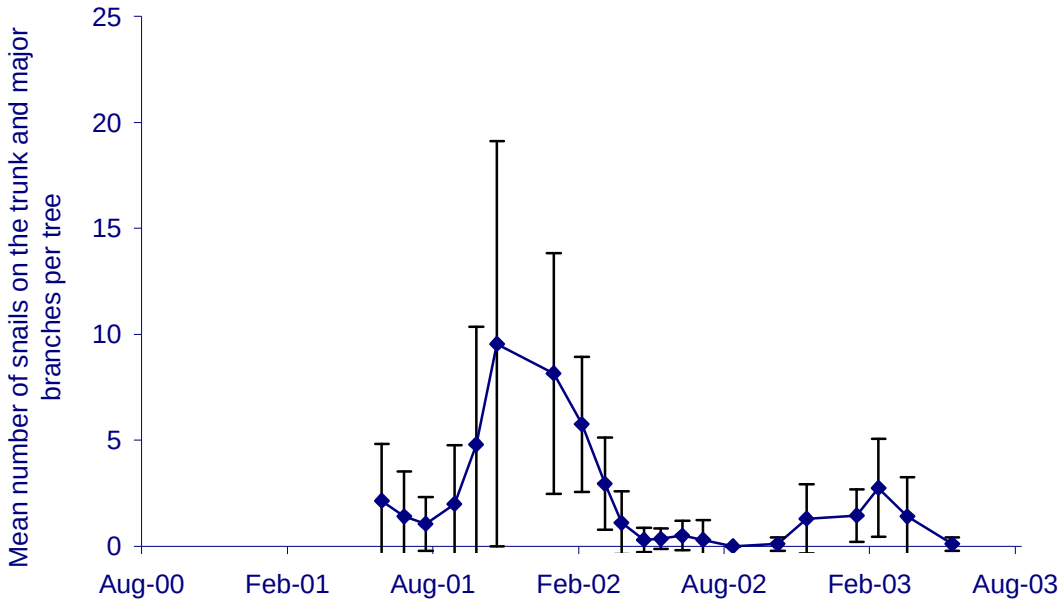


Figure 4-2: Mean number of *Microxeromagna armillata* on trunk and major branches of Navel orange trees (block 2) in Nangiloc, Victoria. Error bars represent standard deviations, n = 10 trees.



Snail abundance was lower in block 2, but the trend over the seasons was the same as describerd for block 1 (Figure 4-2).

In each block, when snails were present in trees, the variation between trees was high. This variability could be in part due to *M. armillata* preferring to climb some trees to others. Tree structure, type and position of irrigation system, and leaf litter distribution on the orchard floor are all likely to play a role. In these experiments it is interesting to note that overhead sprinklers were used block 1, and under tree sprinklers in block 2. With almost identical patterns in snail movement over the two-year periods in both blocks, it is unlikely that these irrigation systems influence seasonal trends in snail movement.

Very few snails were found on the trunk and major branches during fruit harvest (June/July) in 2001 and 2002, which mirrors the results found in previously described work (cf section 3.3). Few snails were seen on the trunk and major branches in autumn and winter, but high numbers were found in spring. This could indicate that either snail contamination of fruit is not occurring while fruit is on the tree, or as snails are not present on the trunk, they are concentrated in the outer canopy. Research in section 3.3 suggests that snail contamination of fruit is not occurring while it is on the tree, but further investigation is needed before this can be stated with confidence.

4.3 Development of method to study snail activity

4.3.1 Introduction

Counts of snails on tree trunks (section 4.2) only provide snapshots in time of snail numbers and thus an indication of seasonal trends in snail activity. To investigate short-term movement in the tree canopy, movement of individual snails needs to be studied. Various techniques have been employed to study mollusc movement, including mark-release-recapture [91, 99, 100], time lapse photography [101, 102], harmonic radar [103], and radio transmitters [90, 102]. These methods can often disrupt normal movement and are time consuming, expensive and difficult to use with cryptic and small species. The small size and cryptic nature of *M. armillata*, and its movement in a variety of habitats (soil surface, leaf litter and trees), makes the study of its movement a significant challenge. As such, an alternative method to those outlined above was sought.

The presence of mucus trails has been used as an indicator of slug and snail activity in crops and gardens, although the duration of trail persistence in the environment is thought to be low [19]. All snails use mucus for locomotion that results in production of a mucus trail, and provides a ‘map’ of their movement. This has been exploited in laboratory studies to

investigate the trail following capabilities of the slug *Limax psuedoflavus* [104] - slugs moved over plastic, which when subsequently dusted with chalk, highlighted mucus trails and provided a map of their movement. This use of chalk dusting to map movement would be impractical to use in a field situation, but the use of mucus trails to map snail movement has merit.

Mucus trails have been seen on branches in the citrus canopy although it can't be determined when this movement occurred. Low mucus persistence would limit the scope of any movement study that uses mucus trails as its base, and high mucus persistence could confound studies of short-term movement. Mucus persistence has yet to be quantified experimentally. Of particular interest is the persistence and detection of mucus trails on materials that can be placed in the field for monitoring movement. Mucosal secretions from gastropods, although primarily composed of water, contain large glycoprotein and glycosaminoglycan molecules [105]. These molecules can be highlighted in histological samples using the stain Alcian blue, which has been used to characterise secretion of glycosaminoglycans in the tissues of the slug *Deroceras reticulatum* (Müller) [106] and the snail *A. fulica* [107]. I was interested in the potential of this stain to be used to track the movement of *M. armillata* in the field and to determine the most likely route and timing of fruit contamination. The following section outlines the development of a new technique to monitor snail movement in the tree canopy and leaf litter, which involved development of a staining procedure, checking inhibition of snail movement, investigating mucus trail persistence in a semi-field environment, and testing the technique in the field.

4.3.2 Materials and methods

4.3.2.1 Development of staining procedure

This experiment was designed to determine if Alcian blue could be used to stain mucus trails of *M. armillata* and, if so, whether the technique could be used on materials placed in the field. Flagging tape (Strength Mining, white PVC flagging tape, 25mm width) was chosen as the test material as it is can be easily attached to most surfaces, in particular tree trunks and branches. Forty-five strips of flagging tape were marked in the laboratory with mucus trails of *M. armillata*. A strip was considered marked when at least two snails had travelled 2.5cm on it. As each snail moved on the tape its progress was marked at adjacent points with permanent pen at regular intervals. After marking, tape strips were immersed in solutions of Alcian blue 8GX (Sigma) in water (0.1, 0.5 or 1%) for varying lengths of time (1, 2.5 or 5 mins). Tape strips were then removed and de-stained by agitating in a beaker of water for approximately thirty seconds. This was repeated twice with fresh containers of water. Tape strips were hung on a rack and excess stain removed by spraying with a water rinse bottle until the rinse water was clear. Tapes were then air-dried at room temperature and numbers of stained trails per tape strip recorded. The total numbers of stained and non-stained trails were compared across dye concentrations and immersion times, using a log linear model for multi-dimensional contingency tables.

4.3.2.2 Inhibition of snail movement by flagging tape

A choice test was performed to establish if flagging tape inhibited snail movement. Forty citrus twigs (approximately 10 cm long and 4 mm diameter) were banded with tape on one end and placed parallel on a support so that the majority of each twig was suspended 1.5 cm above the bench top by each end. One active field collected *M. armillata* was placed in the centre of each twig so that each snail had to cross a banded or non-banded section to leave the twig. As each snail left a twig the surface (banded or non-banded) it crossed was recorded.

4.3.2.3 Persistence of mucus trails on flagging tape in semi-field conditions

The main factors that are thought to influence mucus persistence are sunlight, moisture and dust. To test the influence of these factors, 85 strips of tape were marked with mucus trails laid down by juveniles of *M. armillata* (1-2 mm in diameter) on a 12 x 2.5 cm area of tape. To check for any contamination that resembled mucus trails, an equal area on each strip was left unmarked. Five marked strips were allocated to each of three treatments (control, irrigation and dust), which were stained and assessed on six different dates (Table 4-1), although on day 32 only two treatments were stained and assessed. All tape strips were attached vertically to an outdoor bench and exposed to sunlight (but not rain) for the duration of the experiment. Tapes allocated to the irrigation treatment received irrigation applications for 5 minutes at a rate of approximately 6 litres/min. Tape strips undergoing the dust treatment were covered with soil for 1-2 mins. Treatments were applied according to the schedule outlined in Table 4-1. The presence/absence and intensity of trails (clear trails, faint trails or no trails) was recorded after staining with 1% Alcian blue for 5 mins. Data were analysed using Chi-squared goodness of fit tests.

Table 4-1: Treatment schedule of tape strips stained on day 0 to 32.

	Irrigation		Dust	
Stained on day	Number of applications	Applied on day (s)	Number of applications	Applied on day (s)
0	1	0	1	0
3	1	3	1	3
7	2	3, 5	2	3, 5
12	5	3,5,7,10,11	5	3,5,7,10,11
14	6	3,5,7,10,11,12	6	3,5,7,10,11,12
32	6	3,5,7,10,11,12		

4.3.2.4 Persistence of mucus trails on flagging tape in field conditions

In order for this banding and staining technique to be used as an effective monitoring tool, it is important to establish the length of mucus trail persistence on bands in the field. This will establish the length of time that snail movement/activity can be successfully monitored in a field situation without band replacement. To investigate mucus trail persistence, fifteen trees were randomly chosen and banded with flagging tape around the major branches in a citrus orchard in Nangiloc, Victoria, Australia. A band of tape pre-marked with mucus trails of *M. armillata* was applied to each of the major limbs of each tree selected. Pre-marking was conducted in the laboratory as previously described. In addition to the bands in the trees, two white ceramic tiles (200 x 200 x 6 mm) were wrapped with a strip of pre-marked flagging tape and placed in leaf litter underneath the canopy of each tree. One pre-marked piece of tape was placed on either the upper or lower surface of each tile.

Bands and tiles from five trees were removed after each duration of 16 hours, 13 days and 26 days. The five bands removed after 16 hours had been exposed to maximum and minimum temperatures of 16.5 and 6.8 °C, respectively, and two hours of overhead irrigation from 12:00 am to 2:00 am at an unknown rate. Bands removed after 13 days had been exposed to average daily maximum and minimum temperatures of 18.5 and 7.0°C respectively, 7.2 mm of rain, and one irrigation. Bands removed after 26 days had been exposed to average daily maximum and minimum temperatures of 17.6 and 6.0 °C respectively, 11.2 mm of rain and two irrigation events. After removal, bands were stained with 1% Alcian blue for 5 mins and the number of stained trails recorded. To determine if mucus trail persistence was affected by duration of exposure to orchard conditions and/or the surface of band application, a log linear analysis for multi-dimensional contingency tables was performed on the data.

4.3.3 Results

4.3.3.1 Development of staining procedure

Ninety-three percent of pre-marked trails were stained overall (Table 4-2). No significant effect was seen with concentration (χ^2 = 0.49, df=2, P = 0.783), or immersion time (χ^2=0.28, df=2, P = 0.871), and no interaction was seen between the two factors (χ^2=12.08, df=8, P = 0.148). No changes in stained trail intensity were observed for tapes treated with varying concentration and immersion times.

Table 4-2: Number of mucus trails stained on flagging tape with Alcian blue at different concentrations and immersion times. (Number of strips per treatment combination =5)

Concentration of alcian blue (%)	Immersion time (mins)	Total number of trails pre-marked	Total number of mucus trails stained
0.1	1	17	17
	2.5	12	11
	5	21	20
0.5	1	14	12
	2.5	27	26
	5	16	15
1	1	24	21
	2.5	12	11
	5	11	10

4.3.3.2 Inhibition of snail movement by flagging tape

Microxeromagna armillata did not show any aversion to crossing over a band of flagging tape, with equal numbers crossing over banded (20) and non-banded (20) twig sections.

4.3.3.3 Persistence of mucus trails on flagging tape in semi-field conditions

Mucus trails from juvenile *M. armillata* were successfully stained with Alcian blue after 32 days in the control group (Table 4-3), although there was a reduction in trail intensity at day 32 (χ^2 = 4.29, df=1, P= 0.0384). Dust treatments had no effect on trail detection or intensity, but significant differences were seen as a result of irrigation. After five irrigation treatments and 12 days sunlight, trail intensity decreased significantly (χ^2 = 10.00, df=1, P= 0.0016), and with six irrigation treatments and 32 days sun exposure no trails were detected. This was significantly different from the control (χ^2 = 10.00, df=1, P= 0.0016).

Table 4-3: Number of tape strips showing stained mucus trails after exposure to sunlight, irrigation and dust treatments. C – clear trails, F – faint trails, NT – no trails

	Control			Irrigation			Dust		
Day	**C**	**F**	**NT**	**C**	**F**	**NT**	**C**	**F**	**NT**
0	4	1	0	5	0	0	4	1	0
3	5	0	0	5	0	0	5	0	0
7	5	0	0	4	1	0	5	0	0
12	5	0	0	0	5	0	5	0	0
14	5	0	0	0	3	2	5	0	0
32	2	3	0	0	0	5			

All tape strips were checked for contamination (staining of areas other than mucus trails) in the unmarked area of the tape. Some contamination was seen on tapes that had been irrigated, but this staining was circular in pattern and could easily be distinguished from mucus trails. This pattern of contamination accumulated with further irrigation treatments and was thought to be caused by glycoproteins present in the water. No contamination was seen on the control or dust treated strips.

4.3.3.4 Persistence of mucus trails on flagging tape in field conditions

Overall the persistence and subsequent staining of mucus trails after exposure to field conditions was high (Table 4-4). Log-linear analysis (Table 4-5) shows that duration of exposure and application surface influenced the persistence of mucus trails in the field.

Table 4-4: Proportion of mucus trails stained on flagging tape after exposure to field conditions on a tree-surface (T), upper- tile surface (UL) and lower- tile surface (LL). n = number of tape strips examined, s = number of stained trails, p = number of pre-marked trails

	Surface					
Day	**T (s/p)**	**n**	**UL (s/p)**	**n**	**LL (s/p)**	**n**
1	0.91 (96 /105)	27	0.8 (12/15)	5	1.0 (17/17)	5
13	0.98 (41/42)	13	0.76 (13/17)	5	0.67 (12/18)	5
26	0.89 (39/44)	15	0.78 (18/23)	5	0.06 (1/17)	5

Table 4-5: Summary of analysis using a log-linear model on the number of stained and non-stained trails recorded after differing lengths of field exposure and application surfaces

	df	**χ^2**	**p**
Duration of exposure	2	20.70	<0.001
Application surface	2	117.41	<0.001
Exposure*surface	8	218.3	<0.001

Decreased persistence of mucus trails on lower-tile tapes was seen over time (Table 4-4). Increasing levels of contamination were seen on tile bands over time: this was particularly evident on bands placed on the underside of tiles. These bands were in direct and continuous contact with moist plant material, by-products of which responded to staining with alcian blue.

It is possible that mucus trails persisted on these surfaces, but the level of background contamination made these difficult to detect. A stain was considered to be caused by contamination if it was an irregular shape, or of a length and width that could not have been caused by a snail. Bands placed on top of tiles also showed some contamination from water droplets, although this did not affect detection of pre-marked mucus trails. Persistence of mucus trails on bands placed in the tree was particularly high, with little reduction over time (Table 4-4).

4.3.4 Discussion

A staining procedure was successfully developed which can mark *M. armillata* trails laid in the field. Evaluation of the staining procedure indicated that an advantage could be gained by decreasing the concentration of Alcian blue and immersion time of tapes (Table 4-2). Using a lower rate and immersion time would provide a cost saving by limiting the amount of dye needed, and decreasing assessment time. The persistence of mucus trails on flagging tape is crucial to the successful application of this technique to monitor snail activity. Mucus trails from juvenile snails can persist for at least 32 days when exposed to sunlight, and a minimum of 14 days with a combination of sunlight and dust treatments. However, the most crucial factor is the effect of moisture. Most snail species are active in moist conditions, and the persistence of trails in these conditions is essential in order to accurately assess levels of activity and movement. This experiment demonstrated that mucus trails could persist after five irrigation treatments and 12 days exposure to sunlight. Only after 6 irrigation treatments and 14 days sunlight, did the persistence of mucus trails decline. In a citrus orchard, a standard irrigation would be conducted at a rate of 5mm/h [98], whereas in this experiment, irrigation treatments were conducted at 360,000mm/h. The persistence of mucus trails from juvenile snails under these conditions was unexpected and indicates that, over similar time periods, irrigation treatments in the field and rain events will not significantly affect trail persistence.

The final step in the progression of this technique was assessment in the orchard. Mucus trails persisted well in the orchard environment, although this varied with application surface and time of exposure. Band contamination on the underside of tiles became a significant issue after 13 days field exposure. This contamination was most likely a result of close prolonged contact with moist plant material and soil, by-products of which may have contained complex glycoproteins. However, a high number of additional mucus trails were found on bands on the upper tile surface, which means that snails in the orchard are moving on top as well as underneath the tile surface. Contamination on the tile underside will not pose a significant threat to the success of this technique as a monitoring tool in the field. This technique allows the short-term movement of *M. armillata* to be investigated in the tree canopy and leaf litter for the first time.

4.4 Development of a method to estimate size of active snails

4.4.1 Introduction

The size of snails moving in the canopy may have an impact on the risk of fruit contamination and knowing this information may assist in developing a management strategy to prevent snails from moving in this area. Although the size of snails which shelter in the orange navel is not known, it is logical that smaller snails are likely to penetrate further into the navel and remain sheltered during post harvest treatments. With development of a technique to record snail trails laid in the field it could be possible to estimate snail size by the width of the trails that they produce. The relationship between snail shell diameter and trail width has not been investigated previously with any slug or snail species. Intuitively, larger snails should have a bigger foot and leave a wider mucous trail. If this is the case and a good relationship exists between shell diameter and trail width, then shell diameter may be predicted from trail widths recorded in the field. The following section of research presents the development of a calibration curve for predicting snail size by the width of snail trails laid on flagging tape.

4.4.2 Materials and Methods

The shell diameter of 26 snails was measured using a dissecting microscope equipped with an eyepiece micrometer. Each snail was then allowed to move across a strip of flagging tape thereby leaving a mucus trail. Each strip of flagging tape was then stained with Alcian blue in the same manner as outlined in section 4.3. Trails stained on the flagging tape were then photographed at 7x magnification using a camera mounted on a dissecting microscope. Trails stained on tape were photographed to enable image enhancement that would help to clearly define the edges of each trail. Although clearly distinguishable with the naked eye, under the microscope light reflected from the white flagging tape made distinguishing between the blue stain and the white background difficult. The width of each trail was measured from the

photographs, at five random points along its length, using the OlysiaEasy® (2001, Soft Imaging System, Germany) program. Obvious irregularities in trail widths, such as turns in a trail, were not measured. Regression analysis was then performed on snail shell diameter and mean snail trail width.

4.4.3 Results and Discussion

A strong relationship was found between snail shell diameter and trail width (adj R^2 = 0.855, F = 141.112, P<0.001; Figure 4-3). Confidence intervals for inverse prediction (predicting shell diameter from trail width) were calculated using the procedure outlined in Zar [43 pp 342-344]. This enables parameters of the regression analysis to be used in predicting snail size from trails laid on flagging tape (Figure 4-4).

Figure 4-3: Relationship between *Microxeromagna armillata* shell diameter and trail width. Error bars represent standard deviation of measurements within a trail.

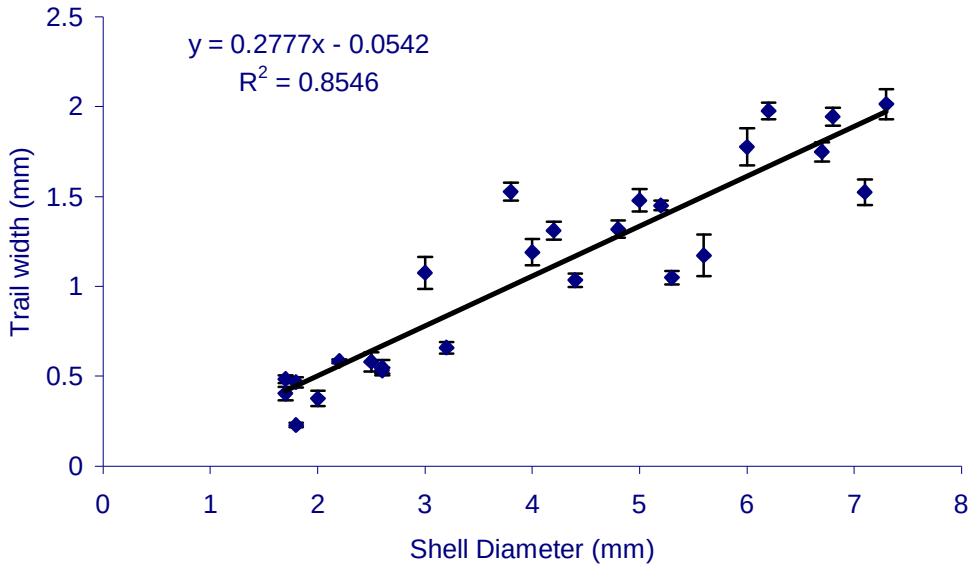
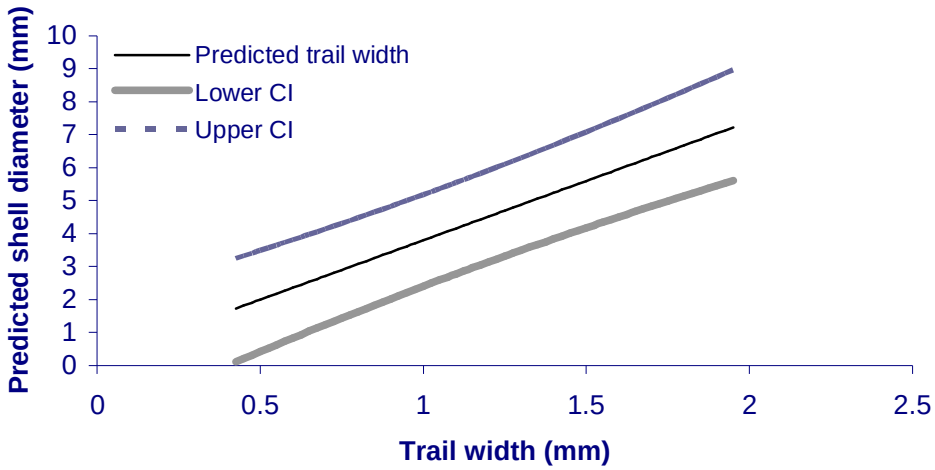


Figure 4-4: Inverse prediction of shell diameter based on trail width (90% CI)



The confidence intervals associated with this inverse prediction are relatively wide (Figure 4-4), and this is in part a function of the crude nature of inverse prediction and the variation in the primary relationship between shell diameter and trail width. An increase in the precision of measurement is unlikely to decrease the confidence limits, and as mucous composition is known to vary with environmental conditions [108], physiological status of the animal [105], and type of locomotion (Pearce 1989 cited in Luchtel and Deyrup-Olsen 2001) and trail width would exhibit natural variation irrespective of shell size. However, the ability to estimate snail size from trails on flagging tape in the field will be valuable in evaluating the risk of fruit infestation, and increasing the understanding of *M. armillata* ecology. This is explored further in section 4.5.

4.5 Activity and size of snails moving in the orchard

4.5.1 Preliminary field trial

4.5.1.1 Introduction

With successful development of a technique to monitor snail activity and the size of active snails, the next progression was to apply the technique in the field. Snail trails on different orchard substrates (trunk, lower branches and upper branches), were studied to provide a clearer picture of how *M. armillata* moves in these areas and may contaminate fruit.

4.5.1.2 Materials and Methods

Five trees were randomly selected in the orchard and 10 flagging tape bands were placed around the trunk, and all major branches originating from the trunk, at varying heights. Of these 10 bands, five were pre-marked with mucous trails as outlined in section 4.3. An average of 5 trails were pre-marked per flagging tape band (range 3 to 7). Pre-marked bands were 80 cm in length: 50cm allocated for covering the branch circumference, 15cm containing pre-marked trails, and 15cm for a control section.

All bands were removed from the trees after one week (applied 30/4/03, removed 7/5/03), which included an irrigation event. After removal, tapes were submerged in 1% Alcian blue for five minutes and de-stained according to the procedures described in section 4.3. For pre-marked pieces of tape, the numbers of stained and non-stained trails were recorded. The number of additional trails found on unmarked flagging tape bands was also recorded.

4.5.1.3 Results and Discussion

Of the 134 pre-marked mucous trails, only one trail was not stained after a week of field exposure (mean proportion stained = 0.99, ± 3.33 sd, number of tapes n=25). The persistence of mucus trails in the field was very high during this one-week period, and was not compromised by an irrigation event. These results clearly indicate that mucus trails can persist on flagging tape despite exposure to moisture, dust and sunlight in the field. The high proportion of stained pre-marked trails recorded show that this technique can be used with confidence to monitor snail activity in the field.

Snail activity in the trees was very low, with only one additional trail found on an un-marked band area. As the mucus trail from this snail was present on a lower branch band and no trails were recorded on the trunk band below, or upper branch band above, the movement of this snail must have been relatively localized. The presence of moisture in the orchard (irrigation) did not trigger widespread movement in the canopy, indicating that moisture is not the only cue stimulating snail activity. It is possible that snail activity was occurring in the leaf litter underneath the tree rather than in the tree canopy, as this time of year corresponds to the onset of the breeding season for *M. armillata* (cf section 2.3). *Microxeromagna armillata* lays its eggs in soil and activity would be expected in this area. Further experiments will investigate the relative activity and size of active snails in leaf litter and tree canopy environments, to ensure that all orchard surfaces are examined for activity.

4.5.2 Pre-harvest snail activity

4.5.2.1 Introduction

Very low levels of snail activity were seen in the tree canopy during the preliminary field trial and comparisons with activity in the leaf litter could not be made. Snail activity in the leaf litter needs to be assessed to provide a reference point for snail activity in the tree canopy. It is also possible that movement in the canopy was not monitored for a sufficient amount of time to allow for snail movement, as environmental conditions may not have been conducive for snail activity during this one-week period. For these reasons, the following experiment was designed to assess snail activity on all orchard substrates for up to 26 days.

4.5.2.2 Material and methods

Fifteen trees were randomly chosen and banded with flagging tape around the trunk and major branches in a citrus orchard in Nangiloc, Victoria, Australia. All branches stemming from the trunk were banded so that any snail moving up into the tree or down the tree would have to cross the flagging tape bands (Figure 4-5). The mean height of bands placed on the tree were as follows; ground to trunk – 14.25cm, ground to lower branch – 47.25 cm, ground to upper branch – 63cm. Measurements were taken following the surface of the tree rather than vertically as height above the ground surface. A band of tape pre-marked with mucus trails of *M. armillata* was applied to each of the major limbs of each tree selected. Pre-marking was conducted in the laboratory as previously described. To investigate the activity of snails on the orchard floor two white ceramic tiles (200 x 200 x 6 mm) were wrapped with flagging tape in such a manner that each edge of the tile was covered with flagging tape and strips of flagging tape ran diagonally across the centre of each tile (Figure 4-5). Two tiles were placed in leaf litter underneath the canopy of each banded tree. Bands were applied on 21/5/2003 and 22/5/2003. Bands and tiles from five trees were removed after each duration of 16 hours (22/5/2003), 13 days (6/6/2003) and 26 days (17/6/2003). Pre-marked tape pieces were

applied to each tree surface at the beginning of the experiment and removed for testing at each of the time periods. The results of these tests have been reported in section 4.3.2.4. The five bands removed after 16 hours had been exposed to maximum and minimum temperatures of 16.5 and 6.8 °C, respectively, and two hours of irrigation from 12:00 am to 2:00 am at an unknown rate. Bands removed after 13 days had been exposed to average daily maximum and minimum temperatures of 18.5 and 7.0°C respectively, 7.2 mm of rain, and one irrigation episode. Bands removed after 26 days had been exposed to average daily maximum and minimum temperatures of 17.6 and 6.0 °C respectively, 11.2 mm of rain and two irrigation events. After removal, bands were stained with 1% Alcian blue for 5 mins and the number of stained trails recorded. Visual counts of the number of snails on the trunk and major branches on band application and removal were also undertaken as previously described in section 4.2.2. In analysis, the number of trails per surface per tree were pooled so that each surface within a tree, rather than each tape, was considered as a replicate. The data were considered in this way to account for the diffusion of snail activity between the trunk (1 tape) and branch surfaces (e.g. 3 tapes as in Figure 4-5).

Figure 4-5: Navel tree banded with flagging tape to assess snail movement in the canopy (left) and tile banded with flagging tape to assess snail activity in the leaf litter.

As little activity was seen on many surfaces during each sampling period, these sampling periods were pooled to estimate snail size. Ten pieces of flagging tape from tiles placed in the leaf litter were randomly chosen for evaluation. If there were five or fewer trails on a tape piece then the width of all trails were measured. If five or more trails were present, then the tape was partitioned into 2 cm sections. Sections of tape were randomly chosen and trail widths were measured for all trails within a section. Sections continued to be examined until the width of at least five trails had been measured. Trail widths were measured using OlysiaEasy® (2001, Soft Imaging System, Germany) at three points from within each trail. The width of all trails found on the trunk, lower branches and upper branches was measured. Snail size was estimated from trail width using the regression equation: $y = 0.2777x - 0.0542$ (cf Figure 4-4). Pearson correlations and analysis of variance were used to analyse data where appropriate.

4.5.2.3 Results and Discussion

Seventy-five percent of the trees sampled after 16 hours showed signs of snail activity, with the majority of the trails found on tape in the leaf litter. The number of trails on each surface increased during the 26-day experiment (Table 4-6). The number of trails should increase linearly if snails were equally active each day, but this was not the case, indicating that activity varied from time to time. Within the tree structure, no snail movement occurred in the upper branches until the last sampling date. At this time, fruit on the tree was only a few days away from harvest. Snail activity in the upper branches was very low indicating a small risk of snail contamination of fruit during this time.

Table 4-6: Mean number of *Microxeromagna armillata* mucus trails (±sd) stained on flagging tape applied to different orchard surfaces and after varying field exposure times. n=5 for all combinations.

	Time of exposure (days)		
Surface	**0.66**	**13**	**26**
Leaf litter	2.8±3.6	15±7.7	97±73
Trunk	0.4±0.6	2.6±4.8	2.6±3.7
Lower branches	0.2±0.5	0.6±0.9	2.8±3.8
Upper branches	0.0±0.0	0.0±0.0	0.4±0.6

As the leaf litter surface was sampled using a different method to that used for the tree canopy, it is invalid to compare the data directly. In light of this, the mean numbers of trails per orchard surface and exposure time were examined using Pearson's correlation. Snail activity in the leaf litter was only correlated to activity on the trunk during 0.66 days (Pearson coefficient =0.948, P= 0.014) and not over the longer time periods. This indicates that activity occurs more frequently in the leaf litter than in the tree. The minimum correlation coefficient which would be significant with a sample size of five is 0.878 [43 Appendix B Table B.17],

which is quite high. An increase in experimental sample size would decrease the minimum significant correlation coefficient and increase the ability to detect associations between variables. This was addressed in further experiments (sections 4.5.3,4.5.4).

If activity occurs on the tree trunk, then activity on the lower branches is likely since the trail numbers on these surfaces were correlated for the 13 and 26 day time periods (*13 days*: Pearson coefficient = 0.948, P= 0.014. *26 days*: Pearson coefficient = 0.923, P= 0.025). Activity in the upper branches was only observed after 26 days and was correlated with activity on the lower branches (Pearson coefficient = 0.881, P= 0.048).

There are three areas which can be directly compared to check for differences in snail activity; the trunk, lower branches and upper branches. As bands for the 0.66 days exposure were removed before the 13 and 26-day bands were applied, the latter tapes would not have recorded the same activity period. Therefore the data for 0.66 days was excluded and ANOVA analyses restricted to on the number of trails per day for the trunk and lower branches at 13 and 26 days. Only two trails were found on the upper branches in the entire 26-day period and were not included in the analysis.

No significant differences were found between number of stained trails per day on the trunk and lower branches, between 13 and 26 days exposure, and no interaction was found between tree surface and time period (Table 4-7). Snail activity in the canopy is patchy; otherwise differences in the numbers of trails per day recorded over time would have been seen. When snail activity occurs on the trunk, activity is also likely on the lower branches. Activity in the canopy is not consistently correlated with movement in the leaf litter, which indicates that environmental or behavioural cues for activity are different between the leaf litter and canopy.

Table 4-7: ANOVA of number of stained trails per day for trunk and lower branches (tree surface) at day 13 and 26 (time of exposure)

Dependent Variable- number of trails per day				
Source	df	Mean Square	F	p
Corrected Model	3	0.020	0.449	0.722
Intercept	1	0.257	5.665	0.030
Tree surface	1	0.027	0.587	0.455
Time of exposure	1	0.002	0.041	0.843
Tree surface*Time of exposure	1	0.033	0.718	0.409
Error	16	0.045		
Total	20			

No snails were found on the trunk and major branches at the time of band application when visual counts were undertaken. As a result, analysis of the relationship between trunk counts undertaken at the time of band application and snail activity on the different orchard surfaces could not be performed. The low numbers of snails on the trunk and major branches during this experiment are in accord with the visual counts undertaken in previous experiments at this time of year (section 4.2.3). Snails were found on the trunk and major branches at band removal (mean number of snails per tree ± sd, 0.0833 ± 0.289). The number of snails on the trunk at band removal was compared to the number of trails per day on the different orchard surfaces using Pearson's correlation. No significant correlations were found between the number of snails on the trunk and major branches and snail activity on the leaf litter (Pearson corr. = -0.086, n =12, p= 0.791), trunk (Pearson corr. = -0.180, n =12, P= 0.575) and lower branches (Pearson corr. = -0.108, n =12, P= 0.738). Snails at this time of year may be moving up and down the tree for short periods and not remaining on the tree for any great length of time. This type of snail movement would explain the lack of correlations between the numbers of snails on the trunk and major branches and snail activity in the different orchard surfaces. *Microxeromagna armillata* begins egg laying in the laboratory in early autumn (cf section 2.3).

As egg laying only occurs in the soil and leaf litter (section 2.2), it is likely that *M. armillata* would not be present in the tree canopy for sustained periods of time.

The mean size of snails active during the pre-harvest period varied between orchard surfaces (Figure 4-6). A trend in increasing shell size with increasing height above the ground was seen, although this trend was not continued into the upper branches (Figure 4-6). Only two trails were recorded from the upper branches, which is too small a sample to compare statistically with other surfaces.

Figure 4-6: Mean estimated shell diameter of *Microxeromagna armillata* active on different orchard surfaces pre-harvest. Error bars represent standard error; numbers above bars represent the sample size.

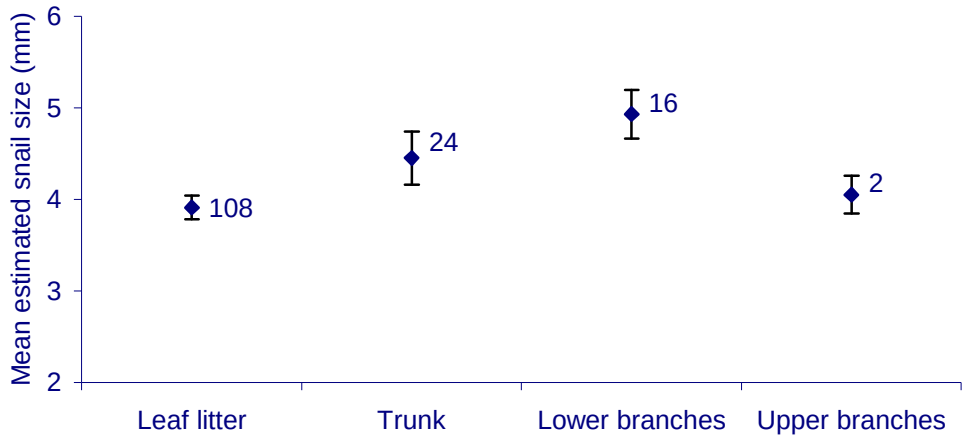


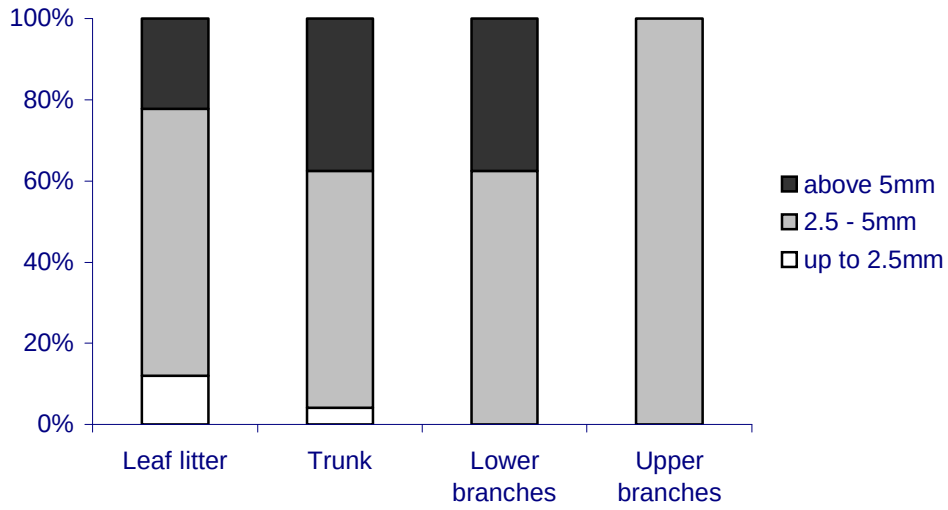
Table 4-8: Analysis of variance of estimated snail size between orchard surfaces pre-harvest

Dependent Variable: Estimated snail size

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected Model	17.891	3	5.964	3.432	0.019
Intercept	489.908	1	489.908	281.924	<0.001
Orchard surface	17.891	3	5.964	3.432	0.019
Error	253.708	146	1.738		
Total		150			
Corrected Total	271.599	149			

Significant differences were found between the sizes of snails active on the different orchard surfaces (Table 4-8). Post-hoc testing was conducted using Hochberg's GT2 procedure that takes into account unequal sample sizes. Trails deposited on the tiles indicates that snails active in the leaf litter were significantly smaller than those active on the lower branches (P= 0.017). No other differences in snail size between orchard surfaces were found. When the size distribution of active snails in the different areas is examined (Figure 4-7), no snails below 2.5mm in diameter were recorded moving in the lower or upper branches and only 10 % of those snails active on the trunk were below 2.5mm in diameter. In contrast, 20% of the snails active in the leaf litter were smaller than 2.5mm. Sixty percent of the snail population collected from leaf litter at this time (cf section 3.3) was less than 2.5mm in size (Figure 3-8). Snails of this size were only responsible for approximately 20% of recorded movement in the leaf litter, and accounted for none of the movement in the lower and upper branches during this experiment. This indicates that it is the larger snails in the population that are moving greater distances although they are a relatively small percentage of the total population. Smaller snails may be moving the same distance as larger snails, but active on different substrates or moving in a different pattern.

Figure 4-7: Size distribution of *Microxeromagna armillata* active on different orchard surfaces during pre-harvest



4.5.2.4 Summary

Snail activity during the pre-harvest period (late May through mid-June) was lowest in the upper branches of the canopy and low in the canopy overall. Snails that were active in the canopy were generally larger than snails active in the leaf litter. Activity in the orchard did not increase linearly over time indicating that snail activity is patchy and movement is initiated by distinct environmental and/or behavioural conditions. Activity in the tree is not always correlated with activity in the leaf litter, and different cues for movement in different areas of the orchard are likely. Snails moving into the tree canopy were not remaining there for long periods of time as was indicated by the low visual counts. It is unlikely that snails are climbing into the tree to shelter from inclement weather (aridity) and the drivers of movement into this area remain unknown.

The risk of fruit contamination in the pre-harvest period (early may to mid June) may be regarded as low, which concurs with results found in section 4.2. Applying snail control measures is recommended at this time as few snails are to be expected in the canopy but active in the leaf litter.

4.5.3 Snail activity during harvest

4.5.3.1 Introduction

Monitoring snail activity pre-harvest highlighted a low risk of fruit infestation via fruit in the tree, but harvest is the crucial time for prevention of snail contamination of fruit, and hence snail movement in the orchards during this time needs to be investigated. The trial design from the pre-harvest experiment (section 4.5.1) was enhanced to include a greater number of trees and activity was assessed over a 7-day period. The harvest period sampling was conducted in the week following the conclusion of the previous experiment as this coincided with harvesting of the fruit. Some selective picking had already taken place, but fruit was still present on most trees. The aim of this experiment was to provide an indication of snail activity and the size of active snails in the orchard during harvest.

4.5.3.2 Materials and methods

Thirty trees were randomly chosen from a 20x20 tree area in a Nangiloc orchard (previously described). Flagging tape bands were applied to each of these trees on the trunk (1 band) and branches (1 band on every major branch). Only upper branches were banded rather than lower and upper branches as in the previous experiment. Bands were applied at mean heights as follows; ground to trunk – 17.56mm, ground to upper branches – 71.5cm. A tile wrapped in flagging tape as described in section 4.5.2.2, was placed in the leaf litter under each tree. The bands were applied on 17/6/03 and removed 7 days later on 24/6/2003. Pre-marked flagging tape bands were also applied on 2 branches per tree, and one pre-marked

piece per tile. The placement of the pre-marked tape on the tile alternated between the top and the underside of the tile with successive trees. On removal, bands were transported back to the laboratory where they were stained with Alcian blue according to the procedure outlined in section 4.3. The proportion of pre-marked trails that were stained on tape pieces across the different surfaces was evaluated using contingency tables.

When tiles were transported back to the laboratory, a freshly wrapped tile (no exposure to snail trails) was placed in between each exposed tile in the stack to check for any cross contamination of mucus trails between tiles during transportation. Pre-marked tapes were also transported to and from the field site without exposure to the orchard environment to further evaluate the staining technique. Visual counts of the number of snails on the trunk and major branches were undertaken on band application in the same manner as previously described.

To estimate the size of active snails in the orchard, ten pieces of flagging tape were randomly chosen from each surface sampled. If there were five or fewer trails on a tape piece, then the widths of all trails were measured. If five or more trails were present, then the tape was partitioned into 2cm sections. Sections of tape were randomly chosen and trail widths were measured for all trails within a section. Sections continued to be examined until the width of at least five trails had been measured. Trail widths were measured using OlysiaEasy® (2001, Soft Imaging System, Germany) at three points within each trail, with the mean of these widths used for comparison.

4.5.3.3 Results and Discussion

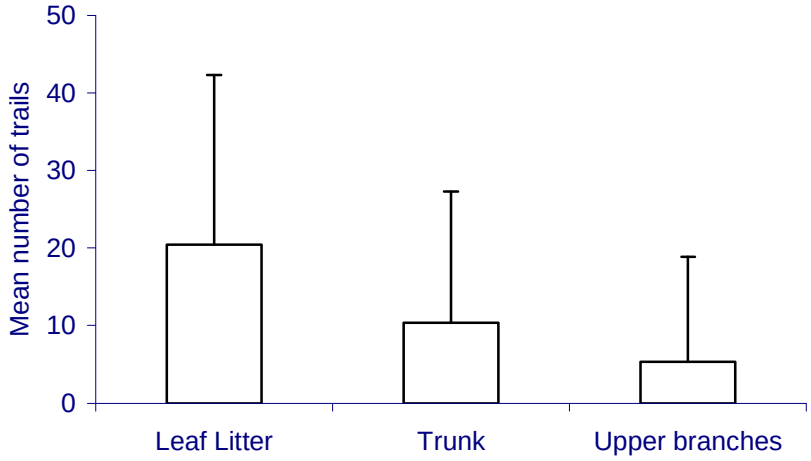
4.5.3.3.1 Evaluation of staining method

The percentage of pre-marked trails stained was high on all surfaces, ranging from 83% (lower tile surface) to 93% (tree surface). The proportion of stained trails to pre-marked placed on the bottom of tiles was compared to those placed on the top of the tiles and no significant differences were found between them (Fisher's exact test, one tailed P= 0.833). These data were then pooled and compared with the proportion stained to pre-marked on the branch surface. No significant difference was found between the proportions stained across the tile and branch surfaces (Fisher's exact test, one tailed, P= 0.800). The field data were then pooled and compared with the proportion of stained to marked trails on control tapes transported to and from the field with no exposure. No significant difference was seen between the control and the exposed tapes (Fisher's exact test, one tailed P= 0.786). The tape on unexposed tiles stacked between exposed tiles was exposed to stain, but no trails were stained indicating that cross contamination between stacked exposed tiles does not occur. This work confirms the validity of the technique to be used as an assessment tool for snail activity in the orchard.

4.5.3.3.2 Evaluation of snail size and activity

Of all the trees sampled, 80% had snail activity in the leaf litter, with the number of trails per tile ranging from 2 to 83. When there was no snail activity in the leaf litter under a tree, no trails were found on trunk or branch tapes for that tree. Overall, activity on the trunk surfaces (64% of trees) was lower compared to the leaf litter (80% of trees). Almost half of the trees sampled (47%) had no snail activity in the upper branches.

Figure 4-8: Mean number of *Microxeromagna armillata* trails per tape on different orchard surfaces during harvest. Error bars represent standard deviation; n = 30 trees**.**



Activity in the branches was generally low, although four extreme outliers were seen in the data. This may indicate that some trees are more susceptible to snail activity in the upper branches, and it is possible that there is some physical aspect to the trees that promotes snail movement. This could be the structure of the tree, how irrigation affects water movement in the canopy, and/or the growth of moss on the limbs. Although the number of trails on each surface can give a relative indication of snail activity, it is also possible in the cases of these outliers that the trails counted came from only a few snails moving over the same tape many times, thus leading to overestimating activity.

Activity on each of the different surfaces was examined using Pearson's correlation (Table 4-9). Activity on the trunk and in the leaf litter was positively correlated, as was activity on the trunk and upper branches. Activity in the leaf litter was not significantly correlated with activity in the upper branches. It appears that there are other factors that make snails more likely to move higher in the tree to the upper branches as previously discussed. Snails could be

moving from the outer canopy area down the tree and crossing over the upper branches, in addition to travelling up the trunk into the tree. However, as few snails are found in the outer canopy area of the tree (section 3.3), this is less likely.

Table 4-9: Pearson correlations between numbers of stained trails per orchard surface during harvest (17/6/2003 – 24/6/2003). Significant correlations are highlighted in bold, n = 30

Surface comparison	Pearson corr.	p
Leaf litter v trunk	**0.598**	**<0.001**
Leaf litter v branch	0.34	0.066
Trunk v branch	**0.883**	**<0.001**

The mean numbers of trails found on the trunk and upper branches were then compared using a t-test. No significant difference was seen between activity on the trunk and upper branches (t = 1.263, df = 58, P=0.212), but the number of trails found on the upper branches was consistently lower than on the trunk. The number of snails counted visually on the trunk and major branches was compared to snail activity on the orchard surfaces using Pearson correlation (Table 4-10). Snail numbers on the trunk and major branches were low overall (mean = 0.2333, sd = 0.568). Activity on the trunk was positively but weakly correlated with the number of snails counted on the trunk and major branches at band application, but again was not correlated with activity in the leaf litter nor the upper branches. Snails active on the trunk were not moving on the upper branches, which further indicates that snails active in the canopy are only moving small distances. Snail presence on the trunk was not correlated with activity in the leaf litter, which provides support for the hypothesis that some trees were preferred for snail activity over others, and that the cues for snail movement in the leaf litter are different to those for movement in the canopy.

Table 4-10: Pearson correlation of the numbers of *Microxeromagna armillata* counted on the trunk and major branches at band application and number of snail trails per day across orchard surfaces during harvest. Significant correlations are highlighted in bold, n = 30

Snail numbers on trunk and major branches correlated with the mean number of trails per day on:	**Pearson corr.**	**P**
Leaf litter	0.324	0.081
Trunk	**0.385**	**0.036**
Branch	0.057	0.765

The size of active snails did not differ between the orchard surfaces (Figure 4-9, Table 4-11). Snails active in the upper branches tended to be larger than snails active in the leaf litter and on the trunk, but snails active on the trunk and leaf litter surfaces were similar in size. Similar to the pre-harvest results, larger snails moved higher in the canopy compared to the trunk and leaf litter and no snails less than 2.5mm in shell diameter were moving in the upper branches (Figure 4-10). The size distribution of active snails on the leaf litter and trunk were similar, but the percent of snails smaller than 2.5mm in diameter moving on the trunk was higher than that seen pre-harvest. Pre-harvest and harvest results will be compared in section 4.5.5.

Figure 4-9: Mean estimated shell diameter of *Microxeromagna armillata* active on different orchard surfaces during harvest. Error bars represent standard error; numbers above columns represent sample sizes.

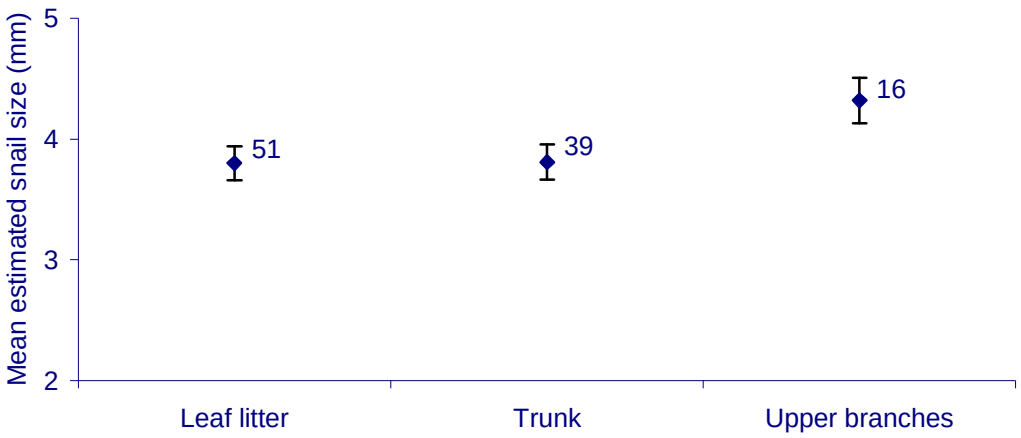
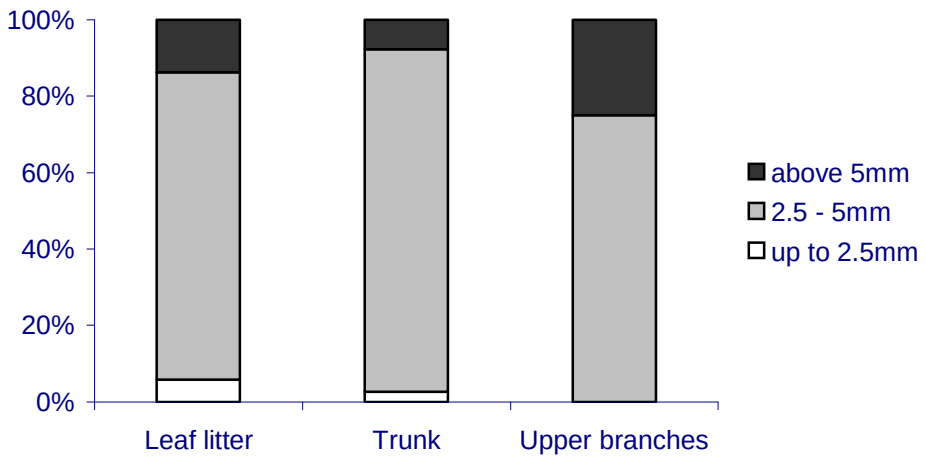


Table 4-11: Analysis of variance of estimated *Microxeromagna armillata* size between orchard surfaces during harvest (log transformed data).

Dependent Variable: log estimated shell diameter

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected Model	.040	2	.020	3.055	0.051
Intercept	49.161	1	49.161	7471.995	<0.001
Orchard surface	0.040	2	0.020	3.055	0.051
Error	0.730	111	0.007		
Total	53.991	114			
Corrected Total	0.771	113			

Figure 4-10: Size distribution of *Microxeromagna armillata* active on different orchard surfaces during harvest



Fewer than 10% of snails active on the trunk and leaf litter were less than 2.5mm in diameter, but snails of this size comprised approximately 80% of the population collected from leaf litter (Figure 3-8). Conversely, 90 – 100% of snail movement on all orchard surfaces came from snails greater than 2.5mm in diameter (Figure 4-10), although snails of this size only accounted for approximately 20% of the total leaf litter population (Figure 3-8). This confirms that the larger snails in the population were moving more than smaller snails during harvest in the areas sampled, as was also seen in the pre-harvest period.

4.5.3.4 Summary

Snail activity during harvest was low in the canopy overall but lowest in the upper branches of the tree. Snails that were active in the upper canopy were larger than snails active on the trunk and in leaf litter. The risk of fruit contamination at this time would be low which confirms the results found in section 3.3, but several trees had unusually high activity in the upper

branches. Attributes of a tree canopy may make it more favourable for snail activity, but these remain unknown and warrant further investigation.

The low numbers of juvenile snails active in the leaf litter and on the trees is surprising considering their relative abundance in leaf litter. This may be an artefact of the experimental design as juveniles may be moving in an area not covered by the sampling, namely under the leaf litter surface. Leaf litter in citrus orchards can be quite deep and juvenile snails may prefer to move within the leaf litter rather than on the surface. If this is the case, even recording activity on the underside of tiles placed in the leaf litter, as well as on the surface, may not have been sufficient to capture juvenile movement. But additional factors can also influence snail movement and these will be discussed further in section 4.5.4.

Snail control applied during a one-week harvest period would be unlikely to significantly influence snail movement into the canopy, so application of molluscicides before this period would be recommended. For late harvest navel varieties, snail control in June may prove beneficial as snails are active in the leaf litter and, although egg laying will have begun, the majority of the egg laying period is still ahead (section 2.3).

4.5.4 Snail activity post-harvest (Spring)

4.5.4.1 Introduction

Examining the movement of *M. armillata* in the canopy during spring is important for several reasons. Firstly, spring has shown the highest level of snail presence on the trunk and major branches (Figure 4-1, Figure 4-2) and can provide a reference point for data collected in autumn and winter. Secondly, although the fruit in the block under study has already been harvested, late Navel varieties are still harvested in spring. It is unlikely that snail movement in a tree canopy would by significantly different just based on variety, although architectural

factors may play a role. Even though harvest has already taken place, using this variety as a model may indicate trends for snail movement for late harvest navel varieties. Fruit on the tree may encourage snail movement into the canopy which would make this approach invalid, but this does not seem to be the case (cf Figure 4-1, Figure 4-2). Identifying the movement patterns of *M. armillata* during this time would enable calculation of the risk of fruit contamination for these navel varieties. The following experiment quantifies the activity and size of *M. armillata* on orchard surfaces during spring.

4.5.4.2 Materials and methods

Twenty trees were randomly chosen from a 20x20 tree area in a Nangiloc orchard (previously described). These trees were banded with flagging tape around the trunk and around each branch at two different heights. The mean height of bands were as follows: ground to trunk = 19cm, ground to lower branches = 52.58cm, ground to upper branches = 112.92cm. Lower and upper bands were placed on the same branch prior to any subsequent branching of the limb. Applied in this manner, activity on the lower and upper branches can be compared directly as activity is not diluted by minor branching. A tile wrapped in flagging tape was placed in the leaf litter under each tree. Bands were applied on 29/10/03 and removed seven days later on 5/11/2003. Pre-marked flagging tape bands (as described in section 4.3) were also applied to two branches per tree and one tape per tile. The placement of the pre-marked tile piece on the tile alternated between the top and the underside of the tile with successive trees. On removal, bands were transported back to the laboratory where they were stained with Alcian blue according to the procedure outlined in section 4.3. The proportion of stained to pre-marked trails were recorded and assessed using contingency tables to evaluate the staining method, and the total number of additional trails on each tape was recorded to investigate snail activity in the orchard.

There were areas of high dye coverage that made counting the number of snail trails difficult on a large proportion of the tapes assessed. This dye coverage could have been due to contamination or complete snail trail coverage. When this occurred, smaller sections of tape were examined for snail trails. Each tape piece was divided in to 2cm lengths and numbered. Numbers were then randomly selected and the corresponding section of tape was examined for snail trails. This was repeated 5 times for each piece of tape. If the random number corresponded to the area of tape around the knot a new random number was selected. The length of tape was recorded and the number of trails per tape was estimated. When assessing an entire tape, individual trails were counted, but when the tape was assessed using 2cm sections, only portions of some trails were counted. The remaining portions of these trails could have been recounted in other sections leading to an overestimation of the number of trails per tape. To account for this, a random sample of tapes for each surface was evaluated using 2cm sections and the proportion of trails crossing the right hand side of each tape section was compared to the total number of trails in the section. This can then be used to adjust trail estimates so that the number of trails per tape is not overestimated. Five 2cm sections on branch (n = 14), trunk (n=10), and tile (n=5) tapes were assessed for the proportion of trails crossing the right hand side of the tape section. Cross tabulation analysis was used to compare the proportion of trails crossing the right hand side of a section with the total number of trails per section, across orchard surfaces.

Visual counts of the number of snails on the trunk and major branches were undertaken on band application according to the procedure outlined in section 4.2. Analysis of variance was used to compare the snail activity on the different tree surfaces.

Snail sizes were estimated as outlined in section 4.4. A Kruskal-Wallis non-parametric analysis of variance was used to compare the estimated shell diameter of active snails on different orchard surfaces. Non-parametric multiple comparisons for data with unequal sample

sizes were used to determine which orchard surfaces differed from each other, according to the procedure outlined in Zar [43 pp 223-226].

4.5.4.3 Results and Discussion

The percentage of pre-marked trails that were stained was high on all surfaces, with a range of 90% (lower tile surface) to 98% (upper tile surface). The proportion of stained trails to pre-marked trails placed on the bottom of tiles was compared to those placed on the top of the tiles and no significant differences were found between them (Fishers exact test, one tailed P= 0.833). The proportion of stained to pre-marked trails placed on the lower branches was compared to those placed on the upper branches and no significant differences were found between them either (Fishers exact test, one tailed P= 0.833). The data for both tile surfaces were then pooled and compared with the proportion of stained to pre-marked on the pooled branch surface data. No significant difference was found between the proportions stained across the tile and branch surface (Fishers exact test, one tailed, P= 0.757). The staining efficiency remained high across all surfaces, which means that this technique can be used to measure snail activity with confidence.

No significant differences in the proportion of trails crossing the right hand side of a section were found between the trunk, tile or branch tapes (χ^2 = 2.143, df=2, P= 0.342). When all surfaces were pooled, 38.5 % of the trails crossed over the right hand side of the sections assessed. In further analyses, the number of trails per tape calculated using the section method was reduced by 38.5% to adjust for any overestimation due to double counting.

4.5.4.3.1 Evaluation of snail activity in the orchard

Similarities in snail activity across the orchard surfaces were investigated using Pearson's correlation (Table 4-12). No correlation was found between activity in the leaf litter and any other surface, but activity on all tree surfaces was highly correlated with one another. The

strong correlation between activity on the trunk and branch surfaces indicates that snails which are moving on the trunk, are also moving on the lower and upper branches. The lack of a relationship between activity in the leaf litter and activity in the tree again indicates that there are different triggers for snail movement on these surfaces.

Table 4-12: Pearson's correlations of activity (number of trails) on different orchard surfaces. Significant correlations are highlighted in bold.

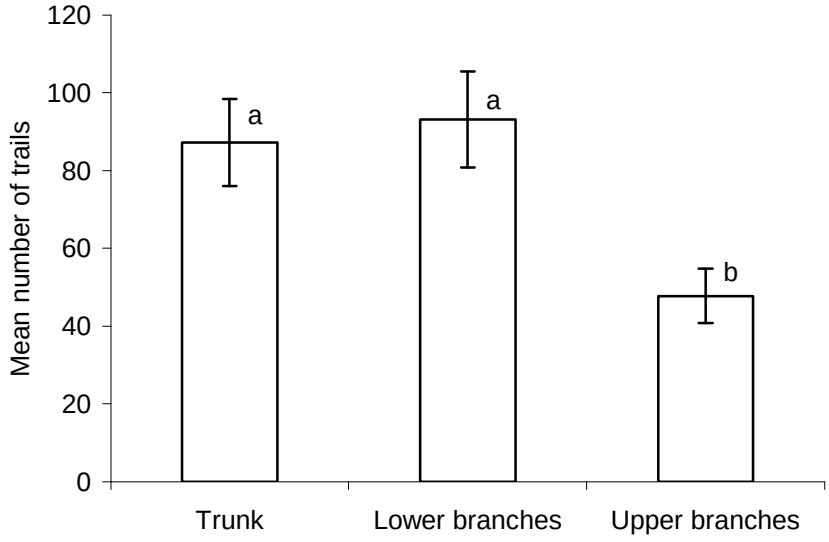
Orchard surfaces compared	Pearson corr.	P	N
Leaf litter v trunk	0.089	.751	15
Leaf litter v lower branches	.113	.687	15
Leaf litter v upper branches	-0.091	.746	15
Trunk v lower branches	**0.844**	**<0.001**	20
Trunk v upper branches	**0.720**	**<0.001**	20
Lower v upper branches	**0.772**	**<0.001**	20

Significant differences in snail activity were found between the tree surfaces in this spring sampling (Table 4-13, Figure 4-11). Comparison of means using Tukey's test, indicated that activity in the upper branches was significantly lower than activity on both the trunk and lower branches (P= 0.026, and P= 0.009 respectively)(Figure 4-11). No significant difference was found between activity on the trunk and lower branches (P= 0.916). For all tree surfaces the counts were variable, indicating active snails prefer some trees to others. Other studies have reported similar results [88], but the variability observed in the present study could in part be due to variability in snail distribution in the leaf litter. Snail activity in the canopy was higher than previously seen during pre-harvest and harvest sampling (these will be compared directly in section 4.5.5).

Table 4-13: Analysis of variance for number of *Microxeromagna armillata* trails stained per tree surface

Source	Type III Sum of Squares	df	Mean Square	F	P
Corrected Model	24374.800	2	12187.4	5.572	0.006
Intercept	347016.150	1	347016.2	158.648	<0.001
Tree surface	24374.800	2	12187.4	5.572	0.006
Error	124678.050	57	2187.3		
Total	496069.000	60			
Corrected Total	149052.850	59			

Figure 4-11: Mean number of *Microxeromagna armillata* trails on different tree surfaces during one week in Spring. Error bars represent standard error, n = 20 for all surfaces, differing letters above columns indicate significant differences.



Visual counts of the number of snails on the trunk and major branches (mean ± sd; 49.6 ± 25.57) were related to snail activity on the orchard surfaces using Pearson correlation (Table 4-14).

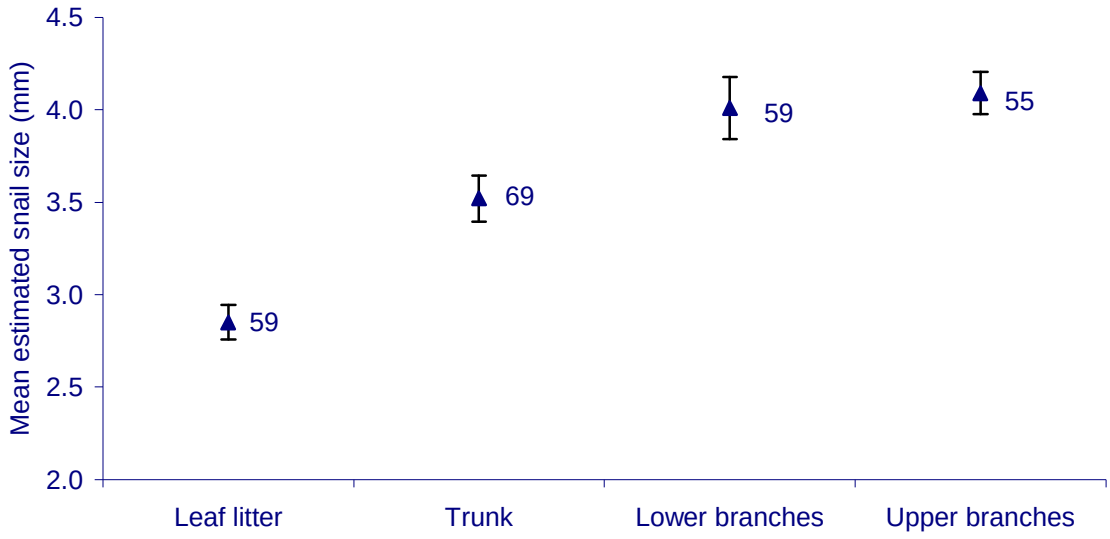
Table 4-14: Correlation between number of *Microxeromagna armillata* on the trunk and major branches and *Microxeromagna armillata* activity (stained trails) on different orchard surfaces in Spring. Significant relationships are highlighted in bold type

Snail numbers on trunk and major branches correlated with the mean number of trails per day on:	**Pearson corr.**	**P**	**N**
Leaf litter	0.195	0.487	15
Trunk	0.286	0.222	20
Lower Branches	**0.493**	**0.027**	**20**
Upper Branches	**0.566**	**0.009**	**20**

Significant correlations were found between snail numbers on the trunk and major branches and activity on the lower and upper branches. No correlation was seen between the number of snails found on the trunk and major branches and activity on the trunk, which indicates that once snails enter the tree canopy via the trunk, they spend more time moving on the upper and lower branches than on the trunk surface. This could indicate that snails moving into the canopy from the leaf litter were not returning during the one-week time period. The high numbers of snails found on the trunk and major branches when bands were applied substantiates this hypothesis, as does the relatively high number of snails found in the outer canopy of the trees (cf Figure 3-11).

The mean size of active snails differed between orchard areas during this spring sampling (Kruskal Wallis H = 56.251, df = 3, P<0.001), with the size of active snails increasing with increasing height above the ground (Figure 4-12).

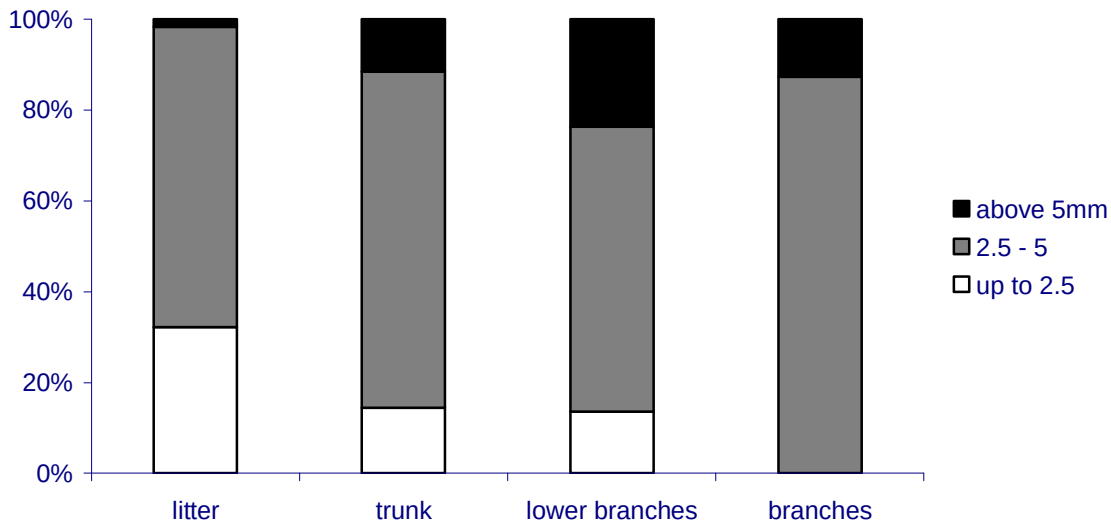
Figure 4-12: Mean estimated shell diameter of active *Microxeromagna armillata* on different orchard surfaces in spring. Error bars represent standard error; numbers in columns are the number of snail trails used to estimate snail size.



Snails active on the upper branches were significantly bigger than snails active in the leaf litter ($Q = 7.222 >> Q_{4,\ 0.05}\ 2.639$) and snails active on the trunk ($Q = 3.34769 >> Q_{4,\ 0.05}\ 2.639$). No significant differences were found between snail sizes on the upper and lower branches ($Q = 1.85 < Q_{4,\ 0.05}\ 2.639$). Snails active on the lower branches were significantly bigger than those active in the leaf litter ($Q = 5.335 >> Q_{4,\ 0.05}\ 2.639$), but were not significantly different from those active on the trunk surface ($Q = 1.38 < Q4_{4,\ 0.05}\ 2.639$). Snails active on the trunk were significantly bigger than those active in the leaf litter ($Q = 4.2526 > Q_{4,\ 0.05}\ 2.639$).

The size of active snails in the orchard increased with increasing height above the ground indicating that larger snails were active in the higher tiers of the orchard. This aspect of the data was investigated further by looking at the size distribution of snails moving in the different areas (Figure 4-13).

Figure 4-13: Size distribution of *Microxeromagna armillata* active on different orchard surfaces in spring



No snails smaller than 2.5mm in diameter were found active in the upper branches, while the size distribution of snails active on the other surfaces were broadly similar. Approximately 20% of snails active on the leaf litter, trunk and lower branches were less than 2.5mm in size, although almost 60% of the population sampled from the leaf litter (section 3.3.3) were in this size class. In contrast, more than 50% of active snails on all orchard surfaces were larger than 5mm in shell diameter, although this group forms less than 10% of the leaf litter population (Figure 3-8). Again this indicates that the majority of snails active on any given tree surface were from the largest-sized part of the population.

4.5.4.4 Summary

Snail activity was high during spring on all orchard surfaces, although fewer trails were recorded in the upper branches than in other areas of the tree canopy. Larger snails moved higher in the tree canopy, a similar pattern to that found during the pre-harvest and harvest sampling periods. The risk of snail contamination of fruit in spring is much higher than during

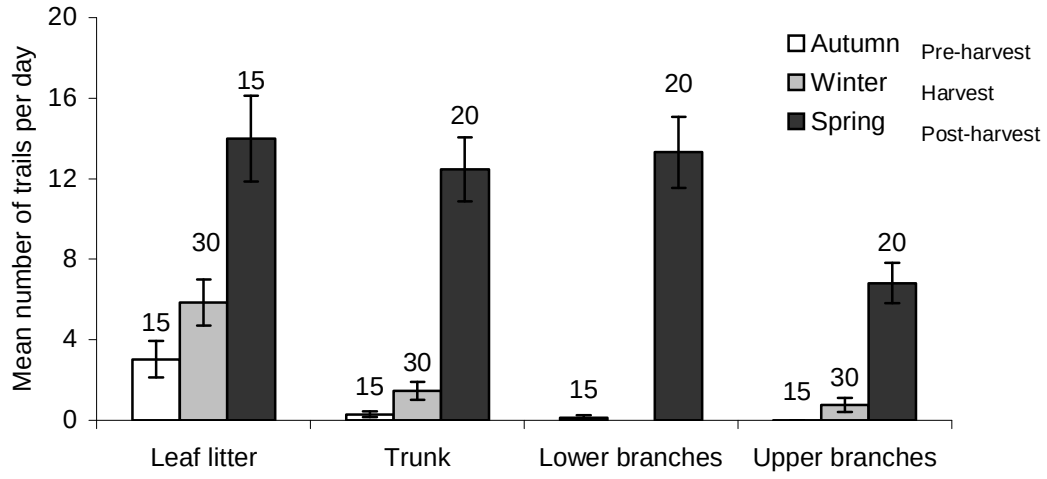
the pre-harvest and harvest periods. Snail activity in the canopy was relatively high and the number of snails counted on the trunk and major branches was also high. This indicates that snails were moving into the canopy and staying in that area for longer periods of time than previously seen, increasing the risk of fruit contamination. Spring was also the only time when snails were found in the outer edges of the tree canopy (Figure 3-11), an observation which supports these results.

4.5.5 Comparing snail activity between seasons and orchard surfaces

Comparison of snail activity on different orchard surfaces, between seasons, will help to evaluate which period has the highest risk of fruit contamination with *M. armillata*. The estimated number of trails per day was calculated so that direct comparisons could be made using the sampling data that had been collected. Analysis of variance was performed on the number of trails per day to investigate any effect of orchard surface, sampling date, and any interaction between orchard surface and sampling date. Due to the dispersion of the raw data, analysis of variance could not be used, and transformation of the data did not improve the dispersion. When ranked data were analysed, the assumptions of ANOVA were not met and non-parametric analysis was also problematic. As a result, graphical analysis of the data is presented.

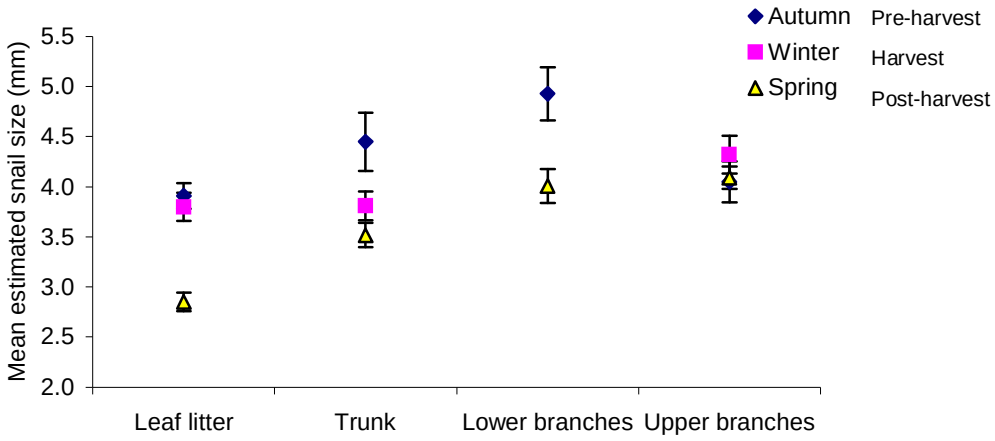
Activity in spring was much higher than in pre-harvest and harvest periods on all orchard surfaces, but particularly on surfaces within the citrus trees (Figure 4-14). Activity during pre-harvest and harvest was primarily in the litter, although some snails were active in the citrus trees.

Figure 4-14: Mean number of *Microxeromagna armillata* trails found per day across orchard surfaces and seasons. Error bars represent standard error; numbers above columns represent sample sizes.



On all sampling dates, snail size increased with increasing height, with the exception of the upper branches in pre-harvest and spring (Figure 4-15). In general, active snails in the pre-harvest period were larger than those in winter and spring sampling periods. This corresponds to the findings in section 3.3, which showed a higher proportion of the population was greater than 5mm in the autumn when compared to winter (harvest) and spring leaf litter samples.

Figure 4-15: Estimated shell diameter of *Microxeromagna armillata* in pre-harvest (autumn), harvest (winter) and post harvest (spring) active on different orchard surfaces. Error bars represent standard error; numbers in columns represent the number of trails sampled to estimate snail size.



Summary

Activity on all orchard surfaces was higher in spring, but of particular concern from an industry viewpoint was movement in the upper branches as this activity was most likely to lead to snail contamination of fruit. Movement of snails in the lower and upper branches can occur in pre-harvest and harvest periods, but the level of movement in these areas is low when compared with spring. Active snails are smaller in spring which could also mean that there is an increased risk of snails entering and sheltering in the navel. *Microxeromagna armillata* was found in the outer canopy in spring, which heightens the risk of fruit contamination during this period (cf section 3.3).

4.6 Prevention of snail movement

4.6.1 Introduction

Numbers of pest snails are commonly reduced by the application of molluscicides, usually applied in pellet form. Application of molluscicidal baits can give variable results that depend on bait type and active ingredient, snail activity and feeding and bait breakdown: all of which can be influenced by season and prevailing environmental conditions. Previous research has shown that *M. armillata* field populations were not substantially affected by the application of molluscicidal baits, and this is thought to be due to a combination of factors [9]. Until further research into the failure of these applications is conducted with *M. armillata* in citrus groves, the only option for citrus growers is to prevent snail movement into the canopy of citrus trees, and hence decrease the risk of fruit contamination.

Copper trunk bands are used to prevent *C. aspersus* movement in American citrus groves [97], but have not been employed in Australian citrus orchards. Several similar products are available in Australia for use in the home garden and include a copper foil band and a copper adhesive tape. However, *C. aspersus* adults are significantly larger than *M. armillata* adults and it is not known how this may affect the efficacy of the tree banding. Smaller snails are better able to exploit gaps between the bands and the tree, which are common if the tree trunk is not perfectly cylindrical. Recently a copper silicate spray, Socusil, was registered in Australia for prevention of snail movement in citrus and vines. These products have either not been evaluated with respect to *M. armillata* movement (copper foil and adhesive tape), or tests have yielded inconclusive results (Socusil; Lush [9]). Investigating the potential of these treatments to prevent *M. armillata* movement into the tree canopy forms the primary focus of research in this section (sections 4.6.2, 4.6.3).

In addition to minimising the movement of *M. armillata* in the tree canopy, it is also important to consider that *M. armillata* may also contaminate fruit post-harvest. Post-harvest contamination is most likely to occur while fruit is stored in picking bins on the orchard floor. Prevention of snail movement into picking bins would be beneficial to minimise the risk of fruit contamination, and also to prevent the spread of *M. armillata* from orchard to orchard via empty bins. As copper is a well known repellent of snails [19], copper sprays applied to picking bins may minimise any movement of *M. armillata* leading to contamination of fruit post-harvest. The potential of copper sprays as repellents on plastic surfaces is explored in section 4.6.4.

4.6.2 Effect of Copper banding and Socusil on presence of *Mixcroxeromagna armillata* in the citrus tree canopy

4.6.2.1 Introduction

The following experiment reports on the efficacy of copper banding as a means to prevent snail movement into the canopy, and compares this cultural control with Socusil, a copper silicate spray registered for use in Australia as a trunk barrier.

4.6.2.2 Materials and Methods

Thirty trees were randomly selected from a Navel citrus block in Nangiloc, Victoria. Three treatments; Socusil spray, copper bands and a control, were randomly allocated to these trees in a completely randomised design. The Socusil spray (Copper silicate 0.28% active) was applied neat to the tree trunks, with application extending from the base of the tree trunk to the trunk crown. The copper band treatment consisted of manufactured copper sheeting, cut into strips which completely encircled the citrus tree trunks and gave a minimum band width of 10cm. These bands were then fastened around the straightest portion of the trunk using a tongue and groove design (Figure 4-16). The copper band was moulded to the tree trunks by hand where obvious gaps existed between the band and the trunk. Treatments were applied on 8/6/2001. Visual counts of snails on the trunk and major branches were

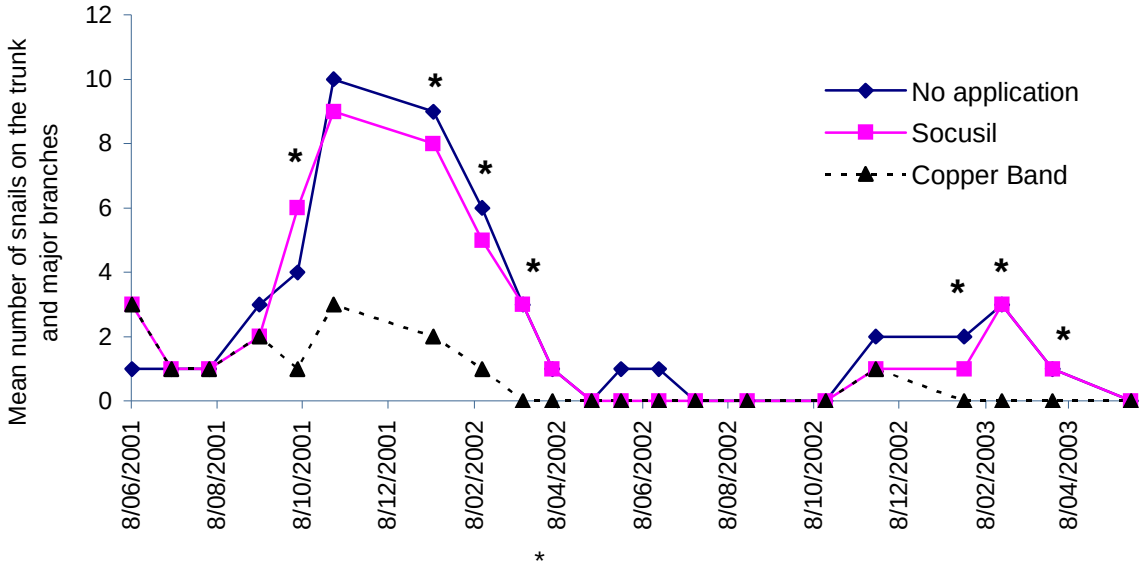
undertaken (cf section 4.2.2 for method description) prior to applying the treatments and at varying intervals until 22/05/2003. Differences in snail trunk counts between the treatments were determined at each date using analysis of variance, or Kruskal-Wallis non-parametric analysis of variance. Analyses were conducted on log-transformed data.

Figure 4-16: Copper band applied to the trunk of a Navel orange tree

4.6.2.3 Results and Discussion

The mean number of snails found on the trunk and major branches significantly differed among treatments on seven of the twenty-one sampling dates (Figure 4-17, Appendix B). During this experiment there were two periods where snail numbers were high in the canopy, namely spring and summer in each of the two years. Throughout the experiment, and in particular the periods of peak snail abundance, trees that had been treated with Socusil were infested with snails to the same extent as control trees. An exception to this was the one occasion when the Socusil treated trees had more snails on the trunk than the non- treated trees (30/10/2001). It is possible that Socusil could have been effective during the first few sampling dates after application, but as snail numbers were low this could not be determined.

Figure 4-17: Mean number of *Microxeromagna armillata* snails counted on the trunk and major branches of Navel orange trees after application of copper bands and Socusil. Stars indicate sampling dates where treatments are significantly different, n = 30 at all dates. Standard deviations and test statistics are presented in Appendix B.



In direct contrast to Socusil, trees that had copper bands applied to the trunk had significantly fewer snails in the tree canopy than untreated trees. This difference occurred on six occasions, coincident with the times of greatest snail abundance and demonstrated that copper bands were successful at reducing snail movement into the canopy. Furthermore, copper bands can remain effective for at least two years. As the application of copper bands is quite labour-intensive and costly, these bands would need to exhibit effectiveness over more than two seasons to make application economical [97]. However, for growers who have specific high value crops targeted for export, copper banding could provide extra insurance against snail contamination, and reduce the need for molluscicide application.

4.6.3 Effect of copper bands and copper adhesive tape on snail activity on tree trunks

4.6.3.1 Introduction

Cheaper alternatives to copper sheeting are currently available for use in the home garden, and these products can now be evaluated with greater accuracy using the banding and staining technique outlined in section 4.3. The ability of copper foil bands and copper adhesive tape, available for purchase in the home garden market, to prevent *M. armillata* movement into the citrus canopy is evaluated in this section.

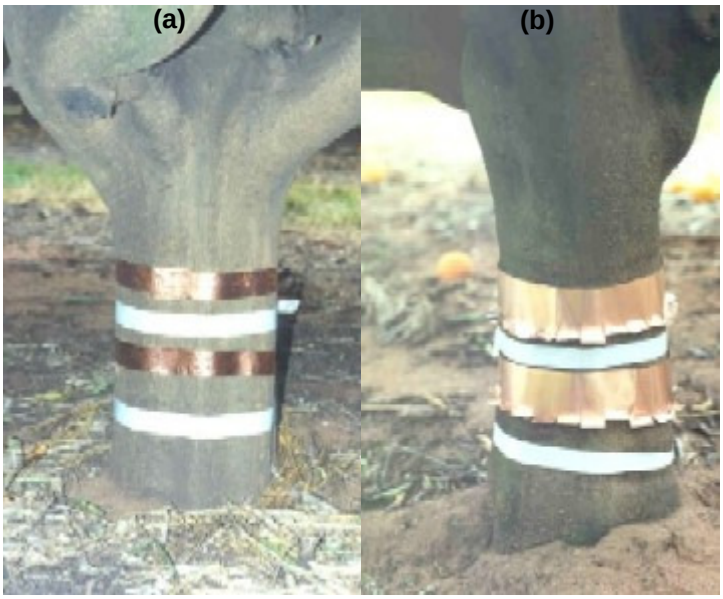
4.6.3.2 Materials and Methods

Ten navel trees were randomly selected from a 20x20 tree block in Nangiloc, Victoria. Five trees were banded around the trunk at two different heights with an adhesive copper tape (Safer®, snail and slug copper barrier tape, 3 cm width; Figure 4-18a). Below the lowest band, and in between the bands, flagging tape was applied to the tree to establish the level of snail activity in the different areas. This experimental design was repeated for the second treatment, a copper foil product (Snail Barr ®, Custom Copper, 7.6cm width; Figure 4-18b). This experiment was conducted from 17/6/2003 to 24/6/2003 (winter) and repeated on 30/10/2003 to 5/11/2003 (spring). The copper bands were left in place in the intermediate period, and flagging tape was reapplied again in spring. If any copper bands had been compromised during that time this was noted and they were repaired to the original standard. In spring, a third piece of flagging tape was applied above the highest copper band on the trunk to add a further measure of snail activity.

Flagging tape was removed after seven days, transported to the laboratory and stained with 1% Alcian blue to detect snail mucous trails. The numbers of trails per tape were counted after staining, and in cases where the number of trails was very high, the section sampling method was used, as described in section 4.5.4.2. Flagging tape bands placed in between each copper band treatment were compared to flagging tape placed below the copper bands, which gave an indication of snail movement up into the tree canopy. Any trails found on the

flagging tape placed in the middle of the copper bands had to have resulted from snails' crossing over a copper surface. Flagging tape was placed around randomly selected tree trunks (n=30) that did not have any copper banding and left for a seven-day period to serve as a control measure of snail activity.

Figure 4-18: Copper adhesive tape (a) and copper foil bands (b) applied to Navel orange tree trunks in winter. White flagging tape was placed below and in between the copper bands to assess snail activity.



4.6.3.3 Results and Discussion

All copper bands, both adhesive and foil, were still in place on the tree trunks one week after application when flagging tape was removed in winter. In spring, four months after application, one adhesive tape band had fallen off the tree, but all other bands remained in place. Hundreds of snails were found in gaps between the underside of copper foil bands and the trunk surface in spring, and a few snails were also found resting on the copper foil bands. Snails observed were approximately 3 – 5 mm in shell diameter. No snails were found resting on the copper adhesive tape.

The mean number of trails found on flagging tape placed in different tree positions varied with copper band type and with sampling time (Table 4-15).

Table 4-15: Mean number of *Microxeromagna armillata* trails on flagging tape placed below, in between, or above copper bands on Navel tree trunks. Means ± standard deviation followed by differing letters are significantly different across rows, n=5 for all samples except those marked with *, where n = 30.

		Flagging Tape position		
Season	**Band Treatment**	**Below**	**Middle**	**Above**
Winter	Copper Adhesive	31.60± 43.17a	0.00± 0.00b	
	Copper Foil	10.40± 14.29a	1.40± 1.95a	
	No band		10.33*± 6.88	
Spring	Copper Adhesive	298.29± 243.41a	149.37± 175.33a	29.90± 33.59a
	Copper Foil	59.96± 8.11a	13.00± 12.21b	13.00± 14.37b
	No band		61.97*± 56.40	

Significantly more trails were found below the copper adhesive tape than in between the copper tape during winter (Mann-Whitney U = 2.5, P= 0.032), but no differences in the numbers of trails were found between tape positions in Spring (ANOVA log transf., df=2, F=3.59, P= 0.06). This is in direct contrast to results seen for the copper foil bands where no differences were found between the number of trails above and in between copper foil bands in winter (t-test, t = -1.395, p=0.201), but in spring significant differences were seen (ANOVA, df=2, F=6.101, P=0.015). A greater number of trails were found below the copper foil bands than in the middle of the bands (Tukey's test, P= 0.026) and above the bands (Tukey's test, P= 0.026).

Comparisons were then made between the numbers of trails found on control tapes and those found in the different positions surrounding both copper products. In winter, no differences were found between the numbers of trails found below copper adhesive tape, below copper foil and the control tapes (ANOVA log transf., df =2,F=0.563, P= 0.574). A similar result was seen when comparisons were made between the number of trails found on control tapes and those from the middle of the copper adhesive and copper foil bands (Kruskal-Wallis, *H*= 6.890, P= 0.03, no differences found in post-hoc testing).

In spring, significant differences were found in the number of trails found below the copper products and control (ANOVA log transf. data; df=2, F=4.138, P= 0.027). Post hoc testing showed significantly more trails below the copper adhesive tape than the copper foil (Hochberg, P=0.024) while both copper treatments did not differ from the number of trails found on the control tapes (Hochberg: copper foil, p=0.374; copper adhesive, P=0.123).

Significant differences were also found between treatments when the number of trails on tape placed above the copper products was compared with the control (ANOVA: df=2, F=7.566, P= 0.002). A greater number of trails were found on control tapes than those above both copper foil (Hochberg, P= 0.007) and copper adhesive (Hochberg, P= 0.039) bands.

The copper adhesive tape successfully prevented snail movement into the tree canopy in winter, with no snail trails found between the copper tape barriers (section 4.6.3.3). In spring, four months after band application, the adhesive tape did not prevent all snail movement, as there was no difference in the number of trails found in between the bands and the number of trails below the bands. However, while not statistically significant, fewer trails were found on tape placed above the adhesive bands. This could indicate that the upper band did deter movement as snails, which crossed one copper barrier, were less likely to cross a second barrier. However, it is more likely that snails moved more in between the bands trying to find a route up the trunk without crossing a copper barrier. Once the upper barrier was crossed then

snails went straight up the trunk with no impediment. This is substantiated by the comparison of tape placed on un-banded trees to tape placed below the first copper band. Significantly more trails were seen on tape placed below the copper band than those on the control trees, indicating that the copper band increased movement in this area. Snails coming in contact with the copper barrier are likely to search around the trunk for another point of access to the canopy if the copper is repellent. This would increase the number of trails per tape when compared to the control where snails were not repelled by a copper barrier. This indicates that the adhesive tape had some repellent properties in spring, but not sufficiently to prevent snail movement into the canopy. Increasing the width of the adhesive tape barrier may improve the effectiveness of this product.

The copper foil bands were not as successful as the copper adhesive tape in preventing snail movement into the canopy. Some trails were found on flagging tape in between copper bands in winter (although significantly less than below the copper bands), and no difference was seen between snail activity in the different band positions in spring. This indicates that only four months after application, the copper foil bands were ineffective. In spring, large numbers of snails were found sheltering between the copper band and the trunk, indicating that there were sufficient gaps for snails to move underneath the bands. The copper foil bands did not mould to the tree trunk as well as the adhesive tape, and gaps could be seen. This is the likely cause for the failure of the copper foil bands to prevent snail movement into the canopy.

4.6.4 Repellency of Copper products applied to plastic

4.6.4.1 Introduction

The movement of *M. armillata* into the tree canopy is thought to be the most likely cause of fruit infestation, although post-harvest infestation of fruit may occur. Post-harvest fruit infestation is most likely to occur while fruit is stored in picking bins on the orchard floor, particularly if these are stored overnight. To minimise the risk of fruit contamination and to prevent the spread of *M. armillata* from orchard to orchard, prevention of *M. armillata* contamination of picking bins would be advantageous. The following research investigates the potential of several copper products, as picking bin treatments, to prevent snail contamination.

4.6.4.2 Materials and Methods

Sheets of high-density polyethylene (2mm thick) were cut into 5cm by 5cm squares. This plastic was chosen to replicate that used in the production of picking bins. Each plastic square was randomly allocated to a treatment and immersed in the treatment until completely covered. Each treatment was replicated 15 times, and the experiment was conducted twice. The treatments used in this experiment were: water (control), copper silicate (Socusil, no dilution), cuprous oxide (Norshield, 200g/100L) and copper adhesive tape (described in 4.6.3). The latter was added as a treatment only in the second experiment. After immersion the squares were placed on sheets of paper towel to dry. After each square was dry, a small adhesive paper circle (1cm in diameter) was added in the centre to act as a refuge for the snails. An active field collected snail (> 4mm diameter) was placed on the refuge and their presence or absence on the refuge was recorded 15 minutes and 24 hours after placement. Snails had previously been kept inactive in the laboratory (conditions of very low humidity and free water) until immediately preceding the experiment when water was sprayed onto the snails to stimulate activity.

4.6.4.3 Results and discussion

Fifteen minutes after the snails were placed in each refuge in the first experiment, the majority had moved out of the refuge, and consequently no significant differences were found among treatments (X^2 <0.001, df =2, P= 1.00). When the experiment was repeated and copper adhesive was added as a treatment, similar results were seen, with the majority of snails moving out of the refuge within the first 15 mins after placement. No significant differences were seen among treatments when chi-square analysis was performed (X^2=<0.001, df =3, P= 1.00).

Application of the copper based products; copper silicate, cuprous oxide, and copper adhesive tape, to plastic thus did not deter snail movement over small areas. This would limit the benefit of applying these products to picking bins in order to prevent snail contamination. However, as snails were active when they were placed in the refuge, and possibly under stress from handling, the drive to move away and into a more sheltered area may have been greater than any repellent effect of the copper. This could also explain the ineffectiveness of the copper treatments in this experiment. In section 4.6.3, copper adhesive tape prevented snail movement in winter when snail climbing pressure was low, but not in spring when snail pressure was high. Cunningham and Pniewska [109] found in a similar study, that Socusil when applied to a Petri dish showed 100% repellence over a 24-hour period. This is in direct contrast to the results found during this experiment. The type of plastic used may explain this difference. Socusil may dry evenly on the petri dish surface when compared to the high-density plastic surface. After drying, the Socusil coating could not be seen, so it is not known if any pooling or droplet effect occurred. If this had occurred, *M. armillata* may have been able to navigate the plastic surface avoiding the Socusil droplets. Further testing of these compounds on different surfaces is needed before they can be recommended for use as repellents on alternate surfaces such as picking bins.

4.6.5 Summary

The use of copper trunk bands to prevent snail movement into the canopy of citrus trees has some promise. Copper sheeting decreased snail abundance in the citrus trees for up to two years, and may have been effective past the life of the experiment. This product was the most effective of all methods tested. Copper adhesive tape strips prevented snail movement into the canopy in winter, but became compromised in spring. Increasing the width of the copper adhesive tape strips on the trunk may improve repellence during periods of high snail pressure. Copper foil bands were not as effective as copper adhesive tape and although they did reduce snail movement into the canopy, they became compromised four months after application.

Copper products applied to plastic did not show any repellence of snail movement in a simple laboratory assay, and this is contrary to previous results . Further testing, particularly in the field would be valuable in determining the prospects for this group of products.

4.7 Discussion

The movement of *M. armillata* in citrus orchards has been described for the first time, concurrent with development of a novel method to study this movement. *Microxeromagna armillata* was found to climb citrus trees and the exhibition of this climbing behaviour changed over time. Different degrees of climbing behaviour are seen within gastropods [eg 110], with variation in climbing behaviour observed between closely related species [82]. *Microxeromagna armillata* could be classified as an intermediate climber– moving through both terrestrial and arboreal environments. Visual counts of *M. armillata* on the trunk and major branches of citrus trees were higher in spring and summer, while counts were low in autumn and winter (section 4.2). While *M. armillata* was not often found by searching trees in autumn and winter, snails were found to be active in trees. Snails were moving on the trunk and lower branches during autumn (pre-harvest) and winter (harvest) but rarely moved into the upper canopy during these periods. As visual snail counts were low during these periods it appears that *M. armillata*, while moving up into trees, was not remaining in the canopy for long periods of time. It is not known why *M. armillata* moves into the trees during this period, especially as egg laying occurs in soil then. *Microxeromagna armillata* may climb to avoid physiological stress [19], but the short term nature of this movement would suggest otherwise. *Microxeromagna armillata* may be responding to a vertical temperature gradient and moving in the canopy only at night [82], but if this is the case only a small proportion of the snail population responds to such cues. In any case, this level of movement in the upper canopy area was minimal, making the risk of snail contamination of fruit low during autumn and winter.

Snail activity was higher in spring on all orchard surfaces, including the upper branches of citrus trees (section 4.5.5). In addition, snail counts on the trunk and major branches were also high, indicating that snails were moving into the canopy and staying in that area. This period poses the highest risk for snail contamination of fruit, and late harvest Navel varieties

may be more susceptible as harvest coincides with the spring activity in the trees. *Microxeromagna armillata* may climb and reside in the tree in spring to avoid physiological stress [19] as is seen in other species such as *C. virgata, T. pisana, C. acuta* and *P. barbara* [5] which climb on to the heads and stalks of cereal crops in late spring/summer. Visual counts on the trunks and major branches remained consistently high during the summer months in the two orchards studied which supports this hypothesis. The level of snail activity in the canopy during spring was high, but it is not known if this is due to more snails moving from the litter into the tree canopy or recurring activity of *M. armillata* in the tree canopy. Further research into the activity patterns of *M. armillata* during spring and summer is needed to confirm aestivation of *M. armillata* during this time.

Larger snails moved higher in the canopy at all times of the year. Controlling the largest snails in the population prior to fruit harvest would thus help to minimise the risk of fruit contamination. Adult and juvenile snails have often been shown to differ in their movement characteristics [23, 41, 82, 90]. *Microxeromagna armillata* juveniles are generally surrounded by a plentiful source leaf litter in this system, and if this is the main food source of *M. armillata*, the need for movement may be decreased. Adult *M. armillata*, while also surrounded by an abundant food source, may benefit more from greater dispersal in the context of finding a suitable mate and perhaps oviposition sites.

Activity of snails in the leaf litter often did not correlate with activity in the canopy, indicating that there are different cues for movement in the leaf litter and movement into the trees. Large variation in snail activity was seen between trees, particularly on the branch surfaces. This could be due to the patchiness of snail distribution on the orchard floor (Chapter 3), but as snail activity in the leaf litter was often not related to activity in the tree canopy this is unlikely. Some snails have been shown to prefer the shady side of trees [89], and trees which offer more shade or have a branch structure which promotes pooling of water, may be preferred by *M. armillata*. The arboreal snail *E. amaliae* has also been shown to climb some trees in

preference to others, even choosing to climb trees that they do not feed on directly in preference to those they do [111]. Watada and Wada [111] hypothesised that this may be due to the presence in the trees of algae and fungi, which also forms part of this snail's diet. This may be one of the reasons that *M. armillata* climbs trees, as moss and fungi were often observed on citrus trees. Little is known about the diet of *M. armillata*.

Trees closest to irrigation sprinklers (over-head and under-tree) generally receive more water, which may promote higher relative humidity and increased snail climbing behaviour. In order to better predict when and which trees *M. armillata* will climb, further investigation of the relationship between a tree's physical characteristics, orchard management practices (eg irrigation, pruning) and snail activity is essential. An increasing trend in Australian citrus production is the optimisation of irrigation efficiency with more growers installing drip irrigation and/or open hydroponic farming systems. The movement of *M. armillata* may differ in these systems.

Movement of *M. armillata* into the tree canopy can be suppressed by the use of copper trunk bands. Trunk bands made from copper sheeting showed the most promise as they reduced snail numbers in the tree canopy over a two-year period. Copper foil and copper adhesive tape trunk bands also demonstrated a repellent effect on *M. armillata*, but these were not as effective as those made from copper sheeting. The use of trunk bands in citrus orchards would involve a considerable investment by the grower; hence the effectiveness of copper bands over a longer period is desired. The copper sheeting trunk bands may have provided longer-term protection against snail movement into the tree canopy (> 2 years), but this could not be assessed in the scope of this study.

Chapter 5 General Discussion

The objective of this thesis was to investigate the biology and ecology of *M. armillata* in south eastern Australian citrus orchards, with a view to providing a foundation for development of a management strategy against this pest. To achieve this aim, research focussed on answering three main questions:

1. What are the life history traits of *M. armillata*? (Chapter 2)
2. What is the phenology and spatial distribution of *M. armillata* in citrus orchards? (Chapter 3)
3. When and where is *M. armillata* active in citrus orchards? (Chapter 4)

Here, key results from each chapter are discussed with respect to developing recommendations for pest management and identifying future research options. The discussion of future research options is also extended to encompass the less applied aspects of my research program.

I found that *M. armillata* is highly fecund, and utilises an iteroparous egg laying strategy, with the majority of egg laying occurring during the winter months in the laboratory (Chapter 2). High levels of juvenile recruitment in the field and dissection of field collected *M. armillata* further confirm winter as the key breeding period (Chapter 3). This is contrary to previous advice available to citrus growers which indicated that snails were inactive during winter [98]. Prior to this research program, standard snail management practice involved applying molluscicides in late June to July when fruit harvest begins and snail assessments are undertaken. However, after studying the lifecycle of *M. armillata*, a superior approach could be to apply molluscicides in late autumn. Application at this time, targets mature snails prior to egg laying, and would assist in preventing juvenile recruitment into the population. Snails were also found to be relatively more active in the leaf litter compared to the tree canopy in

autumn, which is a key factor in the success of molluscicide application. It is important that activity is assured when baits are applied and this is especially important in autumn, as activity may be patchier at this time compared to winter, when conditions are more consistently moist. I have recommended bait application in late autumn to the citrus industry, with the proviso that monitoring for snail activity is conducted, and this is now common practice.

The beginning of the breeding season, and hence the best timing of molluscicide bait applications, may vary from year to year with the changing environment and snail population. For growers to make an informed assessment of the need and timing of molluscicide applications, a decision making tool is needed. To serve this need, the size of snails in the field can be used as an indicator of the onset of breeding. In both field and laboratory environments, *M. armillata* became sexually mature and capable of egg laying at approximately 6mm in shell diameter. This can provide a crude predictive tool for population growth throughout the season and give citrus growers a guide to target control applications. If the majority of snails found in early autumn are large (5-6mm), then egg laying is likely to occur early in the year and molluscicide applications should begin as soon as consistent snail activity is seen. This requires growers to monitor snail populations for both size and activity early in the year rather than waiting until fruit harvest has begun in winter. This is a key aspect of the management of *M. armillata*. If egg laying commences early, the resulting hatchlings may be able to grow to maturity and reproduce prior to spring. With two generations of snails in one year, the population would increase at a much greater rate than if only one new generation was recruited, and the risk of fruit contamination would increase considerably. To further refine this predictive tool, generations of snails should be monitored in the field to confirm the onset of reproduction. Incorporation of survival rates of *M. armillata* at different sizes, ages and fitness levels would further benefit prediction of population change. The mortality rates of *M. armillata* in the field are unknown and this warrants further investigation for several reasons. Firstly, prior to this research *M. armillata* was assumed to be semelparous and for this reason molluscicide baiting was not recommended in spring, when

adults would die after reproduction. As *M. armillata* has now been identified as iteroparous, applying molluscicides in spring may have some merit. Monitoring survival rates of mature adults over time would confirm the reproductive strategy used by *M. armillata* and hence validate the current recommended baiting strategy. Secondly, it is important to quantify the level of juvenile recruitment into the population so that population growth can be predicted with more accuracy. Determining juvenile mortality and the factors which influence it would assist in this process.

Copper foliar applications, while not registered for use against snails, are often applied to citrus trees in early autumn to enhance snail control [98]. However, I have shown that in late summer and early autumn snails move from the tree canopy into the leaf litter, possibly after an aestivation period, and few snails are present in the tree canopy in late autumn (Chapter 4). This makes the application of copper foliar sprays for snail control redundant in autumn. While the efficacy of foliar copper sprays for snail control is questionable at best, copper sprays are integral in preventing disease and their use could be continued for this reason. Growers applying copper sprays could schedule applications to maximise disease prevention rather than snail control, which has commonly been the case.

Few snails were found in the tree canopy during harvest, although low levels of activity were recorded. *Microxeromagna armillata* was rarely found on fruit during the course of my studies and it is still not known how fruit contamination occurs, or what size snails infest fruit. However, while the risk of fruit contamination on the tree was low in winter, high numbers of snails were found on fallen fruit under the tree canopy, indicating that harvesting fallen fruit increases the risk of snail contamination considerably. It is recommended that fallen fruit is never harvested for this reason. This is also recommended for control of other pests such as fruit fly as well as a range of bacterial and fungal diseases [98]. Higher levels of activity were seen in the tree canopy during spring (Chapter 4), hence late harvest varieties may be more susceptible to fruit contamination. Growers planning to export fruit from these varieties need

to monitor and implement a snail control strategy well prior to their harvest period to decrease the risk of contamination.

Some key areas of research need to be continued to provide a more complete management strategy for citrus growers. As contamination of fruit is the central problem, movement of *M. armillata* in the tree canopy should be investigated further. Utilising the new method developed for recording mucous trails, activity of snails closer to the fruit should be measured to quantify the probability of fruit becoming contaminated whilst on the tree. Activity measured on the upper branches in this study can only be used as a guide since fruit, in many cases, were more than a metre away from these sites. Another area of significant interest is the cue(s) for movement by *M. armillata*. *Microxeromagna armillata* activity occurred at different times in the arboreal and terrestrial environments, suggesting that the cues for movement in these niches are different. Identifying the triggers of snail movement in the canopy would assist in predicting when the risk of fruit contamination is highest. This appears to be different for snails of different sizes and this should be taken into account in future research. Similarly, *M. armillata* appeared to climb some trees in preference to others. This may be due to structural differences between trees, proximity to irrigation sprinklers, or a food source for *M. armillata* present in some trees rather than others. Understanding the driving force behind movement in the canopy during harvest, and the factors affecting it, may improve control.

Movement of snails into the tree canopy can be minimised for up to two years by application of copper bands to the tree trunk, which replicates results seen in America with *C. aspersus* [97], although efficacy varied with band type. Using trunk bands in conjunction with the application of molluscicides in late autumn would assist citrus growers in preventing snail contamination of fruit. Copper products applied to picking bins may provide some protection against snail contamination after harvest and spread of *M. armillata* from orchard to orchard, although this was not demonstrated by this study.

Control with molluscicides in the field has generally been poor [112] and, while copper bands are an effective tool, the high investment required to install and maintain bands may make them unattractive to citrus growers. Enhancing the efficacy of baits and improving the potential of trunk barriers would have significant benefit to citrus growers. Investigating the effect of bait application rates and methods, bait attraction and palatability, and bait breakdown in irrigated systems may provide information which can be used to improve the current management strategy. Similarly, development of low maintenance trunk barriers, and/or effective trunk sprays may result in an increase of technology uptake by growers and ultimately reduce the risk of fruit contamination. Conducting this work in conjunction with further investigation of *M. armillata* movement could lead to development of a warning system for snail movement into the canopy, similar to frost risk warnings. At such times a disposable trunk barrier or trunk spray could be applied to protect against short-term snail movement into the canopy during harvest, or alternatively the harvest could be suspended for the risk period. This strategy may still be time consuming and costly, but could prove beneficial in years when egg laying starts early and population growth peaks at harvest. In these years it is unlikely that applying molluscicides alone will decrease the risk of fruit contamination to satisfactory levels.

Prior to export, all citrus blocks must be inspected for snails and a risk assessment prepared [98]. This inspection is conducted by visual searching. A disparity was seen on several occasions between sampling methods used to assess the risk of snail contamination. When visual counts were zero, snail activity was still recorded in some instances using the method developed to detect mucous trails. Utilisation of this new method in risk assessment would yield a more accurate result, and may decrease the risk of contamination of export produce. This method may also prove beneficial when sampling large (or difficult to reach) areas of low snail density, as a limited outlay is required to survey presence/absence, and resources can be focused on more intensive sampling at a later stage if necessary. Detection of mucous trails on/in export containers could also provide quarantine authorities with an early indication of any mollusc contaminants, which are increasingly common with high levels of global trade

[2]. It is also possible that this technique may be useful in semi-aquatic systems as mucus trails are more persistent in moist conditions than previously thought [19, 113]. Whilst Alcian blue is not species specific, mixed mollusc populations may be distinguished by trail width, if adult/juvenile trail widths do not overlap, but identifying species specific mucosal factors and evaluating a range of mucosal stains could increase the applications of this technique.

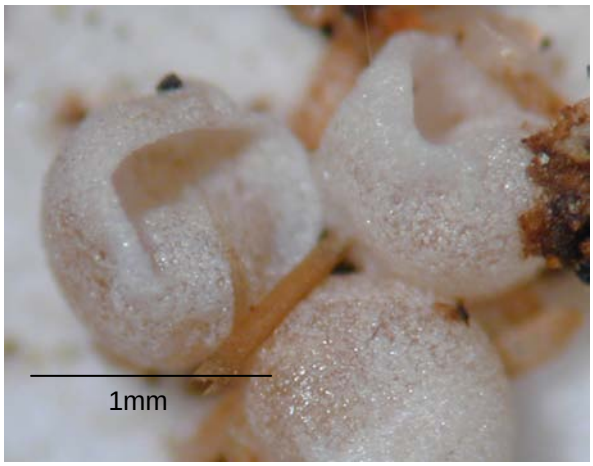
Another key area of research in the development of a management strategy for *M. armillata* is investigation of natural enemies in the field. Whilst *M. armillata* is an introduced snail and as such there is not expected to be specialist predators or parasitoids in Australia, generalist predators may play an important role in population regulation. Carabid and staphylinid beetles have been identified as important predators of slugs at the egg, juvenile and adult stages in Europe [114-116]. Other species from these insect families may be equally important for snail population control in Australia. Some molluscicides have been shown to cause mortality to non-target organisms including carabid beetles, although this is still a subject for much debate [117, 118]. As carabid beetles may also be important predators of other citrus pests, any potentially harmful effects of bait applications must be taken into account.

As is the case in most agricultural systems, an integrated approach to pest control is needed. Combining cultural, chemical and biological control methods with detailed biological and ecological knowledge of the pest species provides the best chance for effective pest management. Whilst this process has begun for *M. armillata*, further research is needed to provide a comprehensive integrated management strategy. It is also important to note that *M. armillata* is only a contamination threat in citrus, but *M. armillata* may feed on other living plant tissue. Further studies of the food range of *M. armillata* are needed to provide an indication of the threat *M. armillata* poses to other cropping systems.

Whilst the primary aim of this research was to contribute to development of a management plan for *M. armillata*, aspects of this research have a much broader scope. In particular, aspects of the life history research warrant further consideration.

Limited experimental work was conducted on the ability of *M. armillata* to aestivate. This is a fascinating area of work with implications not only for snail management, but for the understanding of physiological processes in general. Very young juveniles can survive for at least 10 months without water, but the maximum duration for aestivation has not been measured. *Microxeromagna armillata* eggs were also observed to be tolerant to desiccation. Whilst culturing *M. armillata* some eggs became severely dehydrated, so much so that the egg shell was folded in on itself (Figure 5-1). A high proportion of these eggs successfully hatched after rehydration.

Figure 5-1: Desiccated eggs of *Microxeromagna armillata*



This indicates that *M. armillata* has some capacity to withstand desiccation even at the embryonic stage, and can adapt its physiological processes according to the egg environment. Several authors have investigated this phenomenon [119-121], but only for a limited species range and desiccation rate. Eggs and juvenile snails are often assumed to be

extremely susceptible to desiccation, without any experimental data provided. Observations for *M. armillata* to date dispute this assumption, making this a stimulating avenue for future research.

Whilst investigating the inter-relatedness of *M. armillata's* fecundity characteristics, some complex relationships were found among life history traits. Of particular interest are the cues which affect maternal resource allocation. Maternal growth rates (juvenile and adult), juvenile aestivation, past reproductive and social experience, and use of self-fertilisation all influenced how resources were allocated to offspring production and some relationships among life history traits. In many cases, my experiments were not designed to specifically investigate the inter-relatedness of life history traits and their changing nature, but rather the general characteristics of *M. armillata*. Carefully targeted experiments are needed to test life history evolution hypotheses. This is a fruitful research area that would benefit by the use of *M. armillata* as a study organism. *Microxeromagna armillata* appears to have a repertoire of reproductive strategies which it can utilise, and hence it is an excellent model organism for use in exploring these strategies and the factors affecting them in more detail.

Whilst more study is needed to provide a comprehensive management plan for *M. armillata*, research presented here has provided a strong foundation, with initial recommendations, for snail management. As is commonly the case, more questions have arisen than could be addressed within the scope of this thesis. This presents an exciting challenge for research in the future.

Appendix A: Size-frequency distributions of shells and albumen gland lengths of *Microxeromagna armillata*.

Figure A-1: Size frequency histograms of dead *Microxeromagna armillata* collected from the Riverland orchard, June 1999 to August 2000.

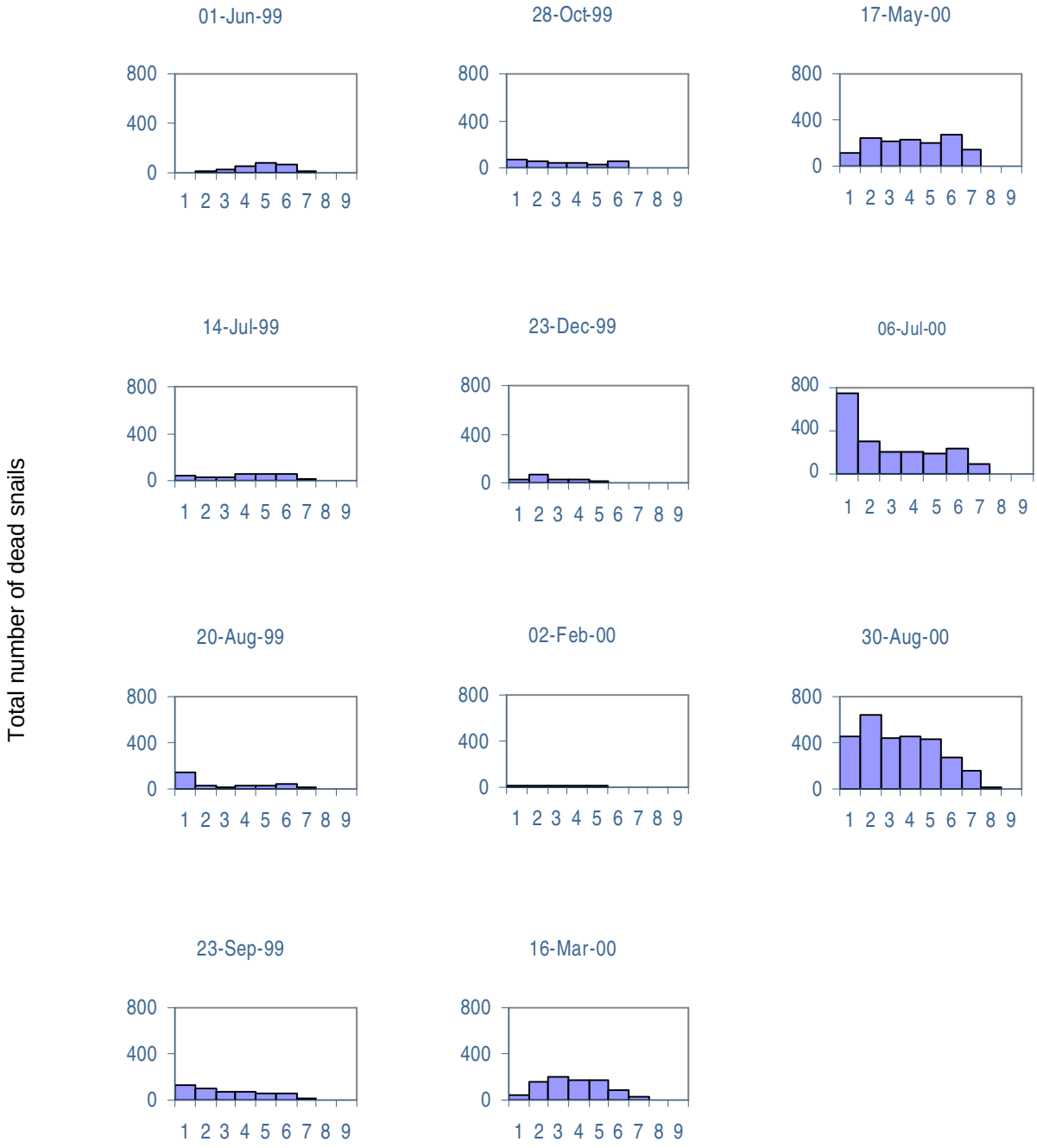


Figure A-2: Size frequency histograms of dead *Microxeromagna armillata* collected from the Sunraysia orchard, June 1999 to August 2000.

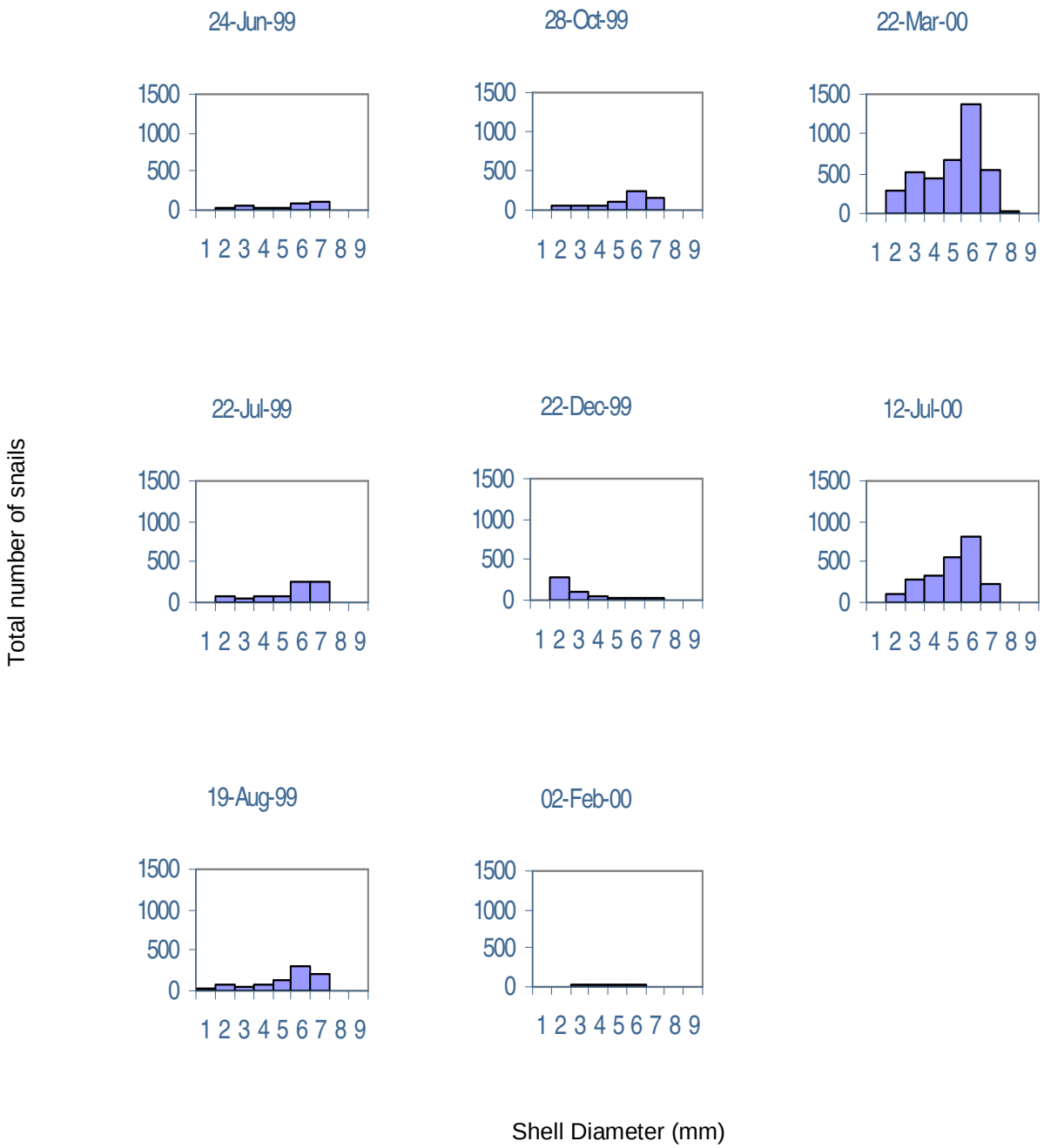
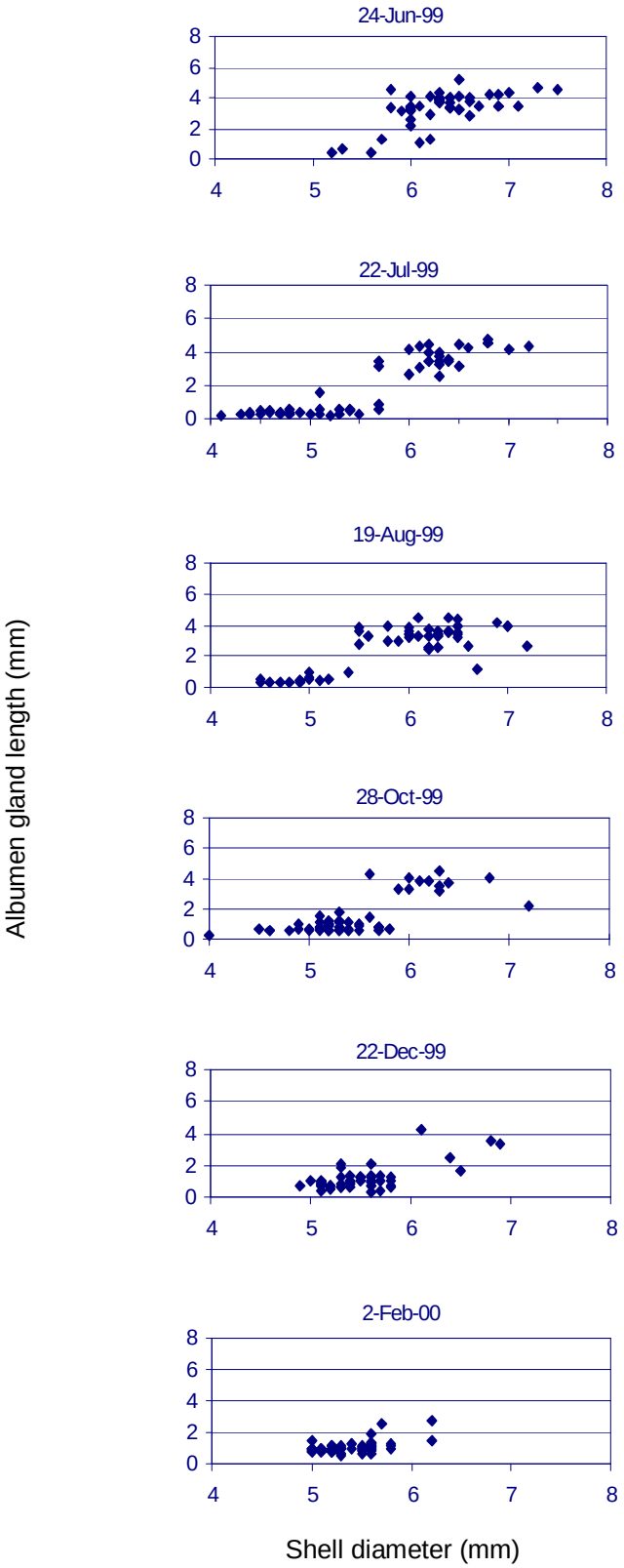


Figure A-3: Length of albumen gland as a function of shell diameter for *Microxeromagna armillata* collected from a Sunraysia orchard.



Appendix B: Effect of copper trunk treatments on numbers of *Microxeromagna armillata* on the trunk and major branches of citrus trees.

Table B-1: Mean number of *Microxeromagna armillata* on the trunk and major branches of Navel trees after application of copper bands and Socusil. Comparison of treatments within each date was made using analysis of variance (Tukey's post-hoc testing) or Kruskal-Wallis non-parametric analysis of variance as appropriate. Means (± standard deviation) followed by differing letters are significantly different and are highlighted in bold type, total n = 30 (n=10 each treatment) and df =2 on all dates.

Date	Control (no band)	Socusil	Copper band	Test statistic
8/06/2001	1.4 ± 1.2	2.9 ± 3.5	2.5 ± 2.1	F= 0.839, p=0.443
6/07/2001	1.4 ± 1.3	1.4 ± 2.8	0.7 ± 1.1	F=0.899, p= 0.419
2/08/2001	1.1 ± 1.2	1.0 ± 1.4	0.9 ± 1.1	F=0.086, p= 0.918
7/09/2001	2.5 ± 1.7	1.5 ± 3.6	1.5 ± 2.9	F=2.662, p= 0.088
4/10/2001	**3.5^{ab} ± 2.2**	**6.1^{a} ± 7.5**	**1.3^{b} ± 1.5**	**F=5.578, p=0.009**
30/10/2001	10.4 ± 12.7	8.7 ± 5.4	3.1 ± 3.3	*H* =5.145, p=0.076
9/01/2002	**8.7 ^{a} ± 5.6**	**7.6 ^{a}± 6.0**	**1.6 ^{b}± 2.3**	**F=9.730, p=0.01**
13/02/2002	**6.4 ^{a} ± 3.4**	**5.1 ^{a} ± 3.0**	**1.4 ^{b} ± 2.2**	**F=12.56, p<0.001**
14/03/2002	**3.0 ^{a} ± 2.2**	**2.9 ^{a} ± 2.2**	**0.4 ^{b} ± 1.0**	**F= 6.5, p= 0.05**
4/04/2002	0.8 ± 0.9	1.4 ± 1.9	0.3 ± 0.7	F=1.941, p= 0.163
02/05/2002	0.4 ± 0.7	0.2 ± 0.4	0.0 ± 0.0	*H*=3.329, p=0.189
23/05/2002	0.5 ± 0.5	0.2 ± 0.4	0.0 ± 0.0	*H*=6.845, p=0.03*****

19/06/2002	0.7 ± 0.8	0.3 ± 0.5	0.1 ± 0.3	*H*=4.255, p=0.119
15/07/2002	0.2 ± 0.4	0.4 ± 1.3	0.0 ± 0.0	F=0.662, p=0.524
21/08/2002	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
16/10/2002	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3	
21/11/2002	1.6 ± 2.1	1.0 ± 1.1	0.6 ± 1.3	F=0.948, p=0.4
23/1/2003	**1.6 [a] ± 1.1**	**1.3 [a] ± 1.4**	**0.2 [b] ± 0.4**	***H*= 8.55, p=0.014**
19/02/2003	**3.0 [a] ± 2.1**	**2.5 [a] ± 2.6**	**0.1 [b] ± 0.3**	***H*=11.471,p=0.003**
27/03/2003	**1.3 [b] ± 1.0**	**1.5 [a b] ± 2.5**	**0.2 [a] ± 0.4**	***H*=6.763, p= 0.034**
22/05/2003	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0	

***** Post-hoc testing for nonparametric analysis of variance was undertaken according to the procedure outlined in Zar [43 pp223-226] and groups were not significantly different.

References

1. Smith, B.J. and R.C. Kershaw, *Field guide to the non-marine molluscs of south eastern Australia*. 1979, Canberra: Australian National University Press. 285.
2. Robinson, D.G., *Alien Invasions: The effects of the global economy on non-marine gastropod introductions in to the United States.* Malacologia, 1999. **41**(2): p. 413-438.
3. Beesley, B.L., G.J.B. Ross, and A. Wells, eds. *Mollusca: The Southern Synthesis. Fauna of Australia*. ed. B.L. Beesley, G.J.B. Ross, and A. Wells. Vol. 5. 1998, CSIRO Publishing: Melbourne. Part A xvi 563, Part B viii 565-1234.
4. Baker, G.H., *The biology and control of white snails (Mollusca:Helicidae) introduced pests in Australia. Division of Entomology Technical Paper*. 1986, Commonwealth Scientific and Industrial Research Organisation, Australia.
5. Baker, G.H. *Damage, population dynamics, movement and control of pest Helicid snails in southern Australia*. in *Slugs and Snails in World Agriculture*. 1989: British Crop Protection Council.
6. Furness, G.O., *Survey of snails on citrus in the Riverland of South Australia.* Australian Journal of Experimental Agriculture and Animal Husbandry., 1977. **17**: p. 1036-1039.
7. Charwat, S.M. and K.A. Davies, *Laboratory screening of nematodes isolated from South Australia for potential as biocontrol agents of Helicid snails.* Journal of Invertebrate Pathology, 1999. **74**: p. 55-61.
8. Pomeroy, D.E. and H.M. Laws, *The distribution of introduced snails in South Australia.* Records of the South Australian Museum., 1967. **15**: p. 483-494.

9. Lush, A., *Small brown snail control in citrus orchards*, in *Prime Notes*. 1999, Department of Primary Industries: Brisbane, Queensland 2000.

10. Hausdorf, B., *Uber die Verbreitung von* Microxeromagna armillata *(Lowe, 1852) und* Xerotricha conspurcata *(Draparnaud, 1801) in Griechenland und der Turkei.* Malakologische Abhandlungen, 1990. **15**(6): p. 55-62.

11. Giusti, F. and G. Manganelli, *A new Hygromiidae from the Trryhenian Islands of Capraia and Sardinia with notes on the genera* Xeromicra *and* Xerotricha *(Pulmonata:Helicoidea)(**)(Studies on the Sardinian and Corsical Malacofauna, VIII).* Boll. Malacologico, 1989. **25**: p. 23-62.

12. Lowe, R.T., Helix caperta *car.* armillata. The Annals and Magazine of Natural History, 1852. **Series 2 - 9**(50): p. 112-120.

13. Perejo, C., et al., *Contribution to the knowledge of the terrestrial Malacological fauna between Rivers Henares, Jarama and Tajuna in the Communidad de Madrid.* Graellsia, 1993. **49**: p. 77-85.

14. Almodovar, A., et al., *Terrestrial snails (Gastropoda, Pulmonata, Stylommatophora) from the southeast of the region of Madrid.* Boletin de la Real Sociedad Espanola de Historia, 1996. **92**: p. 127-138.

15. Munoz, B. and C. Parejo, *Malacofauna from Extremadura, Spain. I. Superfamilies Helicoidea and Hygromioidea (Mollusca:Gastropoda).* Bol.R.Soc.Esp.Hist.Nat.(Sec.Biol.), 1994. **91**: p. 185-197.

16. Heller, J., *Life history strategies*, in *The biology of terrestrial molluscs*, G.M. Barker, Editor. 2001, CAB International: New York. p. 413-445.

17. Stearns, S.C., *Trade-offs in life history evolution.* Functional Ecology, 1989. **3**: p. 259-268.

18. Nylin, S., *Life history perspectives on pest insects:What's the use?* Austral Ecology, 2001. **26**: p. 507-517.

19. Godan, D., *Pest slugs and snails*. 1983, Berlin: Springer-Verlag. 445.

20. Staikou, A. and M. Lazaridou-Dimitriadou, *Aspects of the life cycle, population dynamics, growth and secondary production of the snail* Monacha cartusiana *(Muller, 1774)(Gastropoda Pulmonata) in Greece.* Malacologia, 1990. **31**(2): p. 353-362.

21. Chatfield, J.E., *The life history of the helicid snail* Monacha cantiana *(Montagu), with reference also to* M. cartusiana *(Muller).* Proceedings of the Malacological Society of London, 1968. **38**: p. 233-245.

22. Baker, G.H., *Helicidae and Hygromiidae as pests in cereal crops and pastures in southern Australia*, in *Molluscs as Crop Pests*, G.M. Barker, Editor. 2002, CABI Publishing: New York. p. 193-216.

23. Carne, V.L., *Ecology of Mediterranean snails in southern australian agriculture: A study of* Cernuella virgata *and* Cochlicella acuta *on the Yorke Peninsula*, in *School of Agriculture and Wine*. 2003, University of Adelaide: Adelaide. p. 333.

24. Heller, J., *Longevity in molluscs.* Malacologia, 1990. **31**(2): p. 259-295.

25. Stearns, S.C., *The evolution of life histories*. 1992, Oxford: Oxford University Press. 249.

26. Roff, D.A., *The evolution of life histories; theory and analysis*. 1992, New York: Chapman and Hall. 535.

27. Roff, D.A., *Trade-offs between growth and reproduction: an analysis of the quantitative genetic evidence.* Journal of Evolutionary Biology, 2000. **13**: p. 434-445.

28. Leather, S.R., *Size, reproductive potential and fecundity in insects: things aren't as simple as they seem.* Oikos, 1988. **51**(3): p. 386-389.

29. Klingenberg, C.P. and J.R. Spence, *On the role of body size for life-history evolution.* Ecological Entomology, 1997. **22**: p. 55-68.

30. Baker, G.H., *Production of eggs and young snails by adult* Theba pisana *(Muller) and* Cernuella virgata *(da Costa) (Mollusca: Helicidae) in laboratory cultures and field populations.* Australian Journal of Zoology, 1991. **39**: p. 673 -679.

31. Baker, G.H., B.G. Hawke, and B.K. Vogelzang, *Fecundity of* Cochlicella acuta *(Muller) (Mollusca:Helicidae) in laboratory cultures.* Invertebrate Reproduction and Development, 1991. **20**(3): p. 243-247.

32. Cowie, R.H., *The lifecycle and productivity of the land snail,* Theba pisana *(Molluscs:Helicidae).* Journal of Animal Ecology, 1984. **53**: p. 311-325.

33. Wolda, H. and D.A. Kreulen, *Ecology of some experimental populations of the landsnail* Cepaea nemoralis *(L.). II. Production and survival of eggs and juveniles.* Netherlands Journal of Zoology, 1973. **23**(2): p. 168-188.

34. Schaffer, W.M., *Optimal reproductive effect in fluctuating environments.* The American Naturalist, 1974. **108**(964): p. 783-789.

35. Yom-Tov, Y., *Life history tactics in two species of desert snails.* Journal of Arid Environments, 1983. **6**: p. 39-41.

36. Baker, G.H. and B.K. Vogelzang, *Life history, population dynamics and polymorphism of* Theba pisana *(Mollusca:Helicidae) in Australia.* Journal of Applied Ecology, 1988. **25**: p. 867-887.

37. Baker, G.H. *Population dynamics of the white snail,* Cernuella virgata *(Mollusca:Helicidae) in a pasture cereal rotation in South Australia*. in *Proceedings of the 5th Australasian Conference of Grassland Invertebrate Ecology*. 1988.

38. Baur, B., *Growth and reproduction of the minute land snail* Punctum pygmaeum *(Draparnaud).* Journal of Molluscan Studies, 1989. **55**: p. 383-387.

39. Baur, B. and C. Raboud, *Life history of the land snail* Arianta arbustorum *along an altitudinal gradient.* Journal of Animal Ecology, 1988. **57**: p. 71-87.

40. Rollo, C.D., *Consequences of competition on the time budgets, growth and distributions of three species of terrestrial slugs.* Researches in Population Ecology, 1983. **25**: p. 44-68.

41. Baur, A. and B. Baur, *Individual movement patterns of the minute land snail* Punctum pygmaeum *(Draparnaud)(Pulmonata:Endodontidae).* Veliger, 1988. **30**(4): p. 372--376.

42. Baur, A., *Within-and between-clutch variation in egg size and nutrient content in the land snail* Arianta arbustorum. Functional Ecology, 1994. **8**: p. 581-586.

43. Zar, J.H., *Biostatistical analysis*. Fourth ed. 1999, New Jersey: Prentice-Hall Inc.

44. Baur, B., *Possible benefits of egg cannibalism in the land snail* Arianta arbustorum *(L.).* Functional Ecology, 1990. **4**: p. 679-684.

45. Madec, L., et al., *Size-fecundity relationships in the land snail* Helix apsersa: *preliminary results on a form outside the norm.* Invertebrate Reproduction and Development, 1998. **34**(1): p. 83-90s.

46. Baur, A. and B. Baur, *Seasonal variation in size and nutrient content of eggs of the land snail* Arianta arbustorum. Invertebrate Reproduction and Development, 1997. **32**(1): p. 55-62.

47. Baur, B., *Cannibalism in gastropods*, in *Cannibalism. Ecology and evolution among diverse taxa*, M.A. Elgar and B.J. Crespi, Editors. 1992, Oxford University Press: New York. p. 102-127.

48. Vernon, J.G., *Low reproductive output of isolated, self-fertilizing snails: inbreeding depression or absence of social facilitation.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1995. **259**(1355): p. 131-136.

49. Chen, X., *Self-fertilisation and cross-fertilisation in the land snail* Arianta arbustorum *(Mollusca, Pulmonata: Helicidae).* Journal of Zoology, London, 1994. **232**: p. 465-471.

50. Heller, J., *Hermaphroditism in molluscs.* Biological Journal of the Linnean Society, 1993. **48**: p. 19-42.

51. Tompa, A.S., *Land snails (Stylommatophora)*, in *The Mollusca; Reproduction*, A.S. Tompa, N.H. Verdonk, and J.A.M. Van Den Biggelaar, Editors. 1984, Academic Press: London. p. 47-140.

52. Chen, X. and B. Baur, *The effect of multiple mating on female reproductive success in the simulatneously hermaphroditic land snail* Arianta arbustorum. Canadian Journal of Zoology, 1993. **71**: p. 2431-2436.

53. Foltz, D.W., H. Ochman, and R.K. Selander, *Genetic diversity and breeding systems in terrestrial slugs.* Malacologia, 1984. **25**: p. 593-606.

54. Baur, B. and A. Baur, *Social facilitation affects longevity and lifetime reproductive success in a self-fertilizing land snail.* Oikos, 2000. **88**: p. 612-620.

55. McQuaid, C.D., G.M. Branch, and P.G.H. Frost, *Aestivation behaviour and thermal relations of the pulmonate* Theba pisana *in a semi-arid environment.* Journal of Thermal Biology, 1979. **4**: p. 47-55.

56. Storey, K.B., *Life in the slow lane: molecular mechanisms of estivation.* Comparative Biochemistry and Physiology Part A, 2002. **133**(3): p. 733-754.

57. Pomeroy, D.E., *Dormancy in the land snail,* Helicella virgata *(Pulmonata:Helicidae).* Australian Journal of Zoology, 1968. **16**: p. 857-869.

58. Smith, G.C., *An analysis of the form of density dependence in a simulation model of a seasonal breeder undergoing control.* Ecological Modelling, 1997. **95**: p. 181-189.

59. Dan, N. and S.E.R. Bailey, *Growth, mortality and feeding rates of the snail* Helix aspersa *at different population densities in the laboratory, and the depression of activity of helicid snails by other individuals, or their mucus.* Journal of Molluscan Studies, 1982. **48**(3): p. 257-265.

60. Cameron, R.A.D. and M.A. Carter, *Intra- and interspecific effects of population density on growth and activity in some Helicid land snails (Gastropoda:Pulmonata).* Journal of Animal Ecology, 1979. **48**: p. 273-246.

61. Yom-Tov, Y., *Field experiments on the effect of population density and slope direction on the reproduction of the desert snail* Trochoidea (Xerocrassa) seetzeni. Journal of Animal Ecology, 1972. **41**: p. 17-22.

62. Thomas, J.D., G.J. Goldsworthy, and R.H. Aram, *Studies on the chemical ecology of snails: the effect of chemical conditioning by adult snails on the growth of juvenile snails.* Journal of Animal Ecology, 1975. **44**: p. 1-28.

63. Pomeroy, D.E., *Some aspects of the land snail,* Helicella virgata, *in South Australia.* Australian Journal of Zoology, 1969. **17**: p. 495-514.

64. Butler, A.J., *A shortage of food for the terrrestrial snail* Helicella virgata *in South Australia.* Oecologia, 1976. **25**: p. 349-371.

65. Baur, B., *Population regulation in the land snail* Arianta arbustorum*: density effects on adult size, clutch size and incidence of egg cannibalism.* Oecologia, 1988. **77**: p. 390-394.

66. Baur, B., *Do the risks of cannibalism and dessication influence the choice of oviposition sites in the land snail* Arianta arbustorum*?* Journal of Zoology, London, 1988. **216**: p. 495-502.

67. Baur, B., *Inter-population differences in propensity for egg cannibalism in hatchlings of the land snail* Arianta arbustorum*.* Animal Behaviour, 1994. **48**: p. 851-860.

68. Baur, B. and A. Baur, *Proximate factors influencing egg cannibalism in the land snail* Arianta arbustorum *(Pulmonata, Helicidae).* Oecologia, 1986. **70**: p. 283-287.

69. Baker, G.H., *The life history, population dynamics and polymorphism of* Cernuella virgata *(Mollusca:Helicidae).* Australian Journal of Zoology, 1988. **36**: p. 497-512.

70. Baker, G.H., B.G. Hawke, and B.K. Vogelzang, *Life history and population dynamics of* Cochlicella acuta *(Muller) (Gastropoda:Helicidae) in a pasture-cereal rotation.* Journal of Molluscan Studies, 1991. **57**: p. 259-266.

71. Lucarz, A., *Evidence of an egg-laying factor in the prostatic secretion of* Helix aspersa *Muller.* Comparative Biochemistry and Physiology Part A, 1991. **100**(4): p. 839-843.

72. Runham, N.W., *Mollusca*, in *Reproductive biology of invertebrates*, K.G. Adiyodi and R.G. Adiyodi, Editors. 1993, Jacaranda Wiley Ltd, Queensland, Australia. p. 311.

73. Tsitrone, A., *Delayed selfing and resource reallocations in relation to mate availability in the freshwater snail* Physa acuta*.* The American Naturalist, 2003. **162**(4): p. 474-488.

74. Booth, D.T., *Composition and energy density of eggs from two species of freshwater turtle with twofold ranges in egg size.* Comparative Biochemistry and Physiology Part A, 2003. **134**: p. 129-137.

75. Madec, L., C. Desbuquois, and M. Coutellec-Vreto, *Phenotypic plasticity in reproductive traits: importance in the life history of* Helix aspersa *(Mollusca:Helicidae)*

in a recently colonized habitat. Biological Journal of the Linnean Society, 2000. **69**: p. 25-39.

76. Shanbag, B.A., R.S. Radder, and S.K. Saidapur, *Maternal size determines clutch mass, whereas breeding timing influences clutch and egg sizes in the tropical lizard,* Calotes versicolor *(Agamidae).* Copeia, 2000. **4**: p. 1062-1067.

77. Bezemer, T.M. and N.J. Mills, *Clutch size decisions of a gregarious parasitoid under laboratory and field conditions.* Animal Behaviour, 2003. **66**: p. 1119-1128.

78. Baker, G.H. and B.G. Hawke, *Life history and population dynamics of* Theba pisana *(Mollusca:Helicidae) in a cereal-pasture rotation.* Journal of Applied Ecology, 1990. **27**: p. 16-29.

79. Cadee, G.C., *Bioerosion of shells by terrestrial gastropods.* Lethaia, 1999. **32**(3): p. 253-260.

80. Livshits, G.M., *Ecology of the terrestrial snail* Brephulopsis bidens *(Pulmonata: Enidae): mortality, burrowing and migratory activity.* Malacologia, 1985. **26**(1-2): p. 213-223.

81. Asami, T., *Divergence of activity patterns in coexisting species of land snails.* Malacologia, 1993. **35**(2): p. 399-406.

82. Tilling, S.R., *Activity and climbing behaviour: a comparison between two closely related landsnail species,* Cepaea nemoralis *(L.) and* C. hortensis *(Mull).* Journal of Molluscan Studies, 1986. **52**: p. 1-5.

83. Ford, D.J.G. and A. Cook, *The effects of temperature and light on the circadian activity of the pulmonate slug,* Limax pseudoflavus *Evans.* Animal Behaviour, 1987. **35**: p. 1754-1765.

84. Bailey, S.E.R., *Circannual and circadian rhythms in the snail* Helix aspersa *Muller and the photoperiodic control of annual activity and reproduction.* Journal of Comparative Physiology, 1981. **142**: p. 89-94.

85. Lewis, R.D., *Studies on the locomotor activity of the slug* Arion ater *(Linnaeus). I. Humidity, temperature and light reactions.* Malacologia, 1969. **7**(2-3): p. 295-306.

86. Ford, D.J.G. and A. Cook, *The modulation of rhythmic behaviour in the pulmonate slug* Limax pseudoflavus *by season and photoperiod.* Journal of Zoology, London, 1994. **232**: p. 419-434.

87. Watada, H. and K. Wada, *Arboreal distribution of the land snail* Euhadra amaliae. Venus, 1996. **55**(2): p. 123-137.

88. Ohtaki, H., E. Maki, and K. Tomiyama, *Tree climbing behaviour of the snail* Cerithidea rhizophorarum *(Gastropoda:Potamididae).* Venus, 2002. **61**(3-4): p. 215-223.

89. McGuiness, K.A., *The climbing behaviour of* Cerithidea anticipata *(Mollusca: Gastropoda): The roles of physical and biological factors.* Australian Journal of Ecology, 1994. **19**(3): p. 283-289.

90. Tomiyama, K. and M. Nakane, *Dispersal patterns of the giant african snail* Achatina fulica *(Ferussac)(Stylommatophora:Achatinidae), equipped with a radio transmitter.* Journal of Molluscan Studies, 1993. **59**: p. 315-322.

91. Baker, G.H., *Dispersal of* Theba pisana *(Mollusca:Helicidae).* Journal of Applied Ecology, 1988. **25**: p. 889-900.

92. Kasigwa, P.F., *Dispersion factors in the arboreal snail* Sitala jenynsi *(Gastropoda:Ariophantidae).* South African Journal of Zoology, 1999. **34**(4): p. 145-153.

93. Young, C. *Metal chelates as stomach poison molluscicides for introduced pests,* Helix aspersa*,* Theba pisana*,* Cernuella virgata *and* Deroceras reticulatum *in Australia*. in *Slug and Snail pests in Agriculture*. 1996: British Crop Protection Council.

94. Cowie, R.H., *Apple snails (Ampullariidae) as agricultural pests: their biology, impacts and management*, in *Molluscs as Crop Pests*, G.M. Barker, Editor. 2002, CABI publishing: New York. p. 145-192.

95. Glen, D.M. and R. Moens, *Agriolimacidae, Arionidae and Milacidae as pests in west European cereals*, in *Molluscs as Crop Pests*, G.M. Barker, Editor. 2002, CABI Publishing: New York. p. 245-254.

96. Reuda, A., et al., *Vaginulidae in Central America, with emphasis on the bean slug* Sarasinula plebeia, in *Molluscs as Crop Pests*, G.M. Barker, Editor. 2002, CABI Publishing: New York. p. 115-144.

97. Sakovich, N.J. and B. Bailey, *Skirt pruning and tree banding as snail controls.* Citrograph, 1985: p. 18-20.

98. Moulds, G. and B. Tugwell, *Citrus Growing Manual: A manual for quality decision making*. 1999, Berri: J.C. Irving Printers.

99. Fenwick, A. and M.A. Amin, *Marking snails with nail varnish as a field experimental technique.* Annals of Tropical Medicine and Parasitology, 1982. **77**(4): p. 387-390.

100. Gosselin, L.A., *A method for marking small juvenile gastropods.* Journal of the Marine Biology Association, 1993. **73**: p. 963-966.

101. Howling, G.G. and G.R. Port, *Time-lapse video assessment of molluscicide baits.* Monograph British Crop Protection Council, 1989. **No. 41**: p. 161-166.

102. Bailey, S.E.R., *Foraging behaviour of terrestrial gastropods: integrating field and laboratory studies.* Journal of Molluscan Studies, 1989. **55**(2): p. 263-272.

103. Lovei, G., et al., *Harmonic radar - a method using inexpensive tags to study invertebrate movement on land.* New Zealand Journal of Ecology, 1997. **21**(2): p. 187-193.

104. Cook, A., *Trail following in slugs: the stimulus, its reception and the behavioural response.* Ethology Ecology and Evolution, 1994. **6**: p. 55-64.

105. Luchtel, D.L. and I. Deyrup-Olsen, *Body Wall: Form and Function*, in *The biology of terrestrial molluscs*, G.M. Barker, Editor. 2001, CAB International: Oxon, United Kingdom. p. 147-178.

106. Triebskorn, R. and D. Ebert. *The importance of mucus production in slugs' reaction to molluscicides and the impact of molluscicides on the mucus producing system*. in *Slugs and Snails in World Agriculture*. 1989. Guildford, U.K.: British Crop Protection Council.

107. Jeong, J., et al., *Localization and characterization of acharan sulfate in the body of the giant African snail* Achatina fulica. Comparative Biochemistry and Physiology Part B, 2001. **130**: p. 513-519.

108. Lincoln, B.J., T.R.E. Simpson, and J.L. Keddie, *Water vapour sorption by the pedal mucus trail of a land snail.* Colloids and Surfaces B: Biointerfaces, 2004. **33**(3-4): p. 251-258.

109. Ichinose, K., *Influence of age and body size on alarm responses in a freshwater snail* Pomacea canaliculata. Journal of Chemical Ecology, 2002. **28**(10): p. 2017-2028.

110. McCoy, K.D. and T.D. Nudds, *Interspecific variation in climbing by gastropods: implications for transmission of* Parelaphostrongylus tenuis. American Midland Naturalist, 1997. **137**(2): p. 320-328.

111. Watada, H. and K. Wada, *Tree-species preference of the arboreal snail* Euhadra amaliae*.* Venus, 1998. **57**(1): p. 29-37s.

112. Lush, A.L., *The biology, ecology and control of the small brown snail,* Microxeromagna armillata *in citrus orchards of south eastern Australia*, in *Horticulture Australia*. 2001, South Australian Research and Development Institute: Adelaide. p. 39.

113. Lush, A.L. and G.H. Baker. *A novel method for the field study of mollusc movement using mucus trails*. in *Slugs and Snails Agricultural, Veterinary and Environmental Perspectives*. 2003. Canterbury, Kent: British Crop Protection Council.

114. Kromp, B., *Carabid beetles in sustainable agriculture: a review on pest control efficacy, cultivation impacts and enhancement.* Agriculture, Ecosystems & Environment, 1999. **74**(1-3): p. 187-228.

115. Digweed, S.C., *Selection of terrestrial gastropod prey by cychrine and pterostichine ground beetles (Coleoptera: Carabidae).* Canadian Entomologist, 1993. **125**(3): p. 463-472.

116. Asteraki, E.J., *The potential of carabid beetles to control slugs in grass/clover swards.* Entomophaga, 1993. **38**(2): p. 193-198.

117. Bieri, M. *The environmental profile of metaldehyde*. in *Slugs and snails; Agricultural, Veterinary and Environmental Perspectives*. 2003. Canterbury, Kent: British Crop Protection Council.

118. Meredith, R.H. *Slug pellets- risks and benefits in perspective*. in *Slugs and Snails: Agricultural, Veterinary and Environmental Perspectives*. 2003. Canterbury, Kent: British Crop Protection Council.

119. Bayne, C.J., *Survival of the embryos of the grey field slug* Agriolimax reticulatus*, following dessication of the egg.* Malacologia, 1968. **9**(2): p. 391-401.

120. Bayne, C.J., *A study of the dessication of egg capsules of eight gastropod species.* Journal of Zoology, London, 1968. **155**: p. 401-411.

121. Yom-Tov, Y., *The biology of two desert snails* Trochoidea (Xerocassa) seetzeni *and* Sphincterochila boissieri. Israel Journal of Zoology, 1971. **20**: p. 231-248.