

**THE STRUCTURE OF HYDROPYLE CELLS IN
DORMANT EGGS OF THE SOUTH AFRICAN BROWN
LOCUST, *LOCUSTANA PARDALINA* (WALK.)**

Sharon Saacks

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DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

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ABSTRACT

Water regulation in drought-resistant eggs of the brown locust *Locustana pardalina* (Walk.) may be mediated by specialised serosal cells in the hydropyle region. These cells are thought to both supply water to the embryo and to waterproof the egg during very dry periods. The characteristics of hydropyle cells in quiescent eggs were examined at intervals during desiccation at 15-22% RH, and again after rehydration. Embryos were pre-catareptic and any pre-existing diapause condition had terminated.

Egg water contents and the sizes of cells and nuclei decrease with desiccation, and increase after rehydration. Deformable cell and nuclear membranes accommodate size changes. Surface microvilli increase in length with rehydration and cytoplasmic vacuoles become larger, indicating absorption of water. Organelle integrity improves upon rehydration while autophagy remains common.

Evidence of the production and secretion of a waterproofing protein substance, while expected, was not found. However, electron dense vesicles present at the apical boundaries suggest that the cells may nonetheless produce some substance in that region. Dehydrated and rehydrated cells show signs of desiccation damage, but this does not affect cell functioning as embryos from rehydrated eggs developed to hatching. The results indicate that, in addition to structural protection, hydropyle cells may have molecular protection against desiccation damage, and may be able to undertake limited repair upon rehydration.

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CHAPTER 1 INTRODUCTION

Locustana pardalina (Walker) is endemic to the semi-arid Karoo region of South Africa, has the highest outbreak frequency of any African plague locust, requiring the use of chemical control measures almost annually in South Africa (Price and Brown, 1997), and has the potential to affect the food security of nine southern African countries (Brown and Keiser, 1997). Short life cycles, high fecundity and highly gregarious behaviour produce regular outbreaks (Price and Brown, 2000). Adults display phase polymorphism with solitary females producing mostly diapause eggs, gregarious females producing non-diapause eggs, and females in an intermediate, or transiens phase, producing pods that contain a mix of diapause and non-diapause eggs (Matthée, 1951). Diapause is already a form of dormancy but non-diapause brown locust eggs also have a dormant state, termed quiescence. Eggs of both phases are resistant to prolonged periods of drought and can remain viable for as long as 31-36 months (Price and Brown, 2000) provided that the soil receives at least 25mm of rain before the water content of the egg falls below 40% (Matthée, 1951), allowing dehydrated eggs to replace lost moisture. The eggs will then return to a state of dormancy, either quiescence or a deeper diapause state, until conditions are favourable and diapause has finally terminated. These characteristics allow for the accumulation of eggs in the soil over a number of seasons (Matthée, 1951) and synchronised hatching under suitable rainfall conditions, usually at the end of a drought period, contributes to outbreaks and swarming behaviour (Matthée, 1951; Price, 1987; Gehrken and Doumbia, 1996).

Dormancy during a long, hot dry period can be stressful for the eggs as they are particularly vulnerable to desiccation due to their high surface to volume ratio (Gehrken and Doumbia, 1996). Brown locust eggs have well developed mechanisms for retaining water in the field (Matthée, 1951; Shulov and Pener, 1963): eggs are laid in protective pods that are sealed with a froth plug and these are deposited approximately 10 cm below the surface of the soil, which is typically sandy and therefore highly permeable (Briggs et al., 1997). Solitary females, the most common form of adult during drought periods and those laying proportionately more diapause eggs (Matthée, 1951), show some adaptive

behaviour in that they will preferentially lay their eggs at the base of a slope (Price, 1987) where the soil is more likely to hold moisture for longer. The foam casing and plug of the pods significantly reduce the rate of drying in the pods (Petty, 1973.). The plug is particularly thick and strong and is able to resist lateral crushing by the surrounding soil (Petty, 1973) thus maintaining the pathway for water and oxygen supply to the eggs. It also provides a buffer between the pod and the harsh environmental conditions at the soil surface (Price, 1987). In addition, cuticle membranes of the eggs themselves are tough and impermeable and a waterproofing wax layer is produced that is sandwiched between the layers of the unspecialized cuticle, as well as a waterproofing protein (or similar substance) layer adjacent to the hydropyle cuticle (Matthée, 1951).

The relationship between the diapause condition and drought tolerance is, in general, variable (Tauber et al., 1986; Danks, 2000) and remains undefined for *L. pardalina* eggs. However, diapause in most species is thought to improve survival in predictable, adverse environmental conditions (Tauber et al., 1986) such as desiccation, anoxia, and freezing, and is mostly believed to be genetically or developmentally programmed (Hand and Podrabsky, 2000). The diapause condition in *L. pardalina* is supposedly dependent on maternal phase, which is, in turn, primarily dependent on environmental conditions and age (Matthée, 1951). However, female phase is labile and can be reversed (Matthée, 1951; Price, 1982). It has been shown for *Schistocerca gregaria* that the proportion of diapause to non-diapause eggs from a female is affected if that female is gregarized, even if such exposure occurs as late as at the time of laying itself (Hägele et al., 2000). Matthée (1978) has also shown that exposure to high temperatures can induce diapause in *L. pardalina* eggs after oviposition, which may not be that uncommon as temperatures in the soil at egg pod depth can apparently reach 45-60°C (Matthée, 1978).

The cells of the hydropyle region at the posterior tip of the eggs are specialised, enlarged serosal cells that secrete the specialized cuticle of hydropyle by the sixth day after oviposition. They are responsible for water regulation in the eggs (Matthée, 1951), thereby directly influencing embryonic development and survival. During dry periods, passive diffusion of any available water to

dehydrated eggs occurs through the hydropyle cells. In normal development, or when conditions are favourable after a dry period, the hydropyle cells actively pump water to the developing embryos after replacing lost moisture (Matthée, 1951). They are also thought to produce and secrete a water-soluble proteinaceous substance that seals the apical surface of the cells, preventing unnecessary water loss during periods of desiccation (Matthée, 1951). Given the pivotal role these cells play in both the development and protection of the developing embryo, via the regulation of water supply and loss, it would be essential that they are able to survive drying and regain functional integrity following drought periods.

The biology and control of *L. pardalina* is well researched and documented, but not of hydropyle cells specifically. Matthée's 1951 seminal work on brown locust eggs included a histological study of hydropyle cells with the light microscope, while Slifer and Sekhon (1963) showed a few micrographs that included sections of hydropyle cells in their electron microscope study of the hydropyle cuticle in diapause eggs of the grasshopper, *Melanoplus differentialis*. No work has been done on orthopteran eggs in general during recent years. In addition, descriptive studies of fine structure are no longer popular as current research is more focused more on elucidating biochemical events in cells (Bowers and Maser, 1988). However, given the lack of information on the structure of hydropyle cells and on preparation protocols for fixing and embedding resistant material, such as orthopteran eggs, this study may provide a starting point from which further detailed studies can be made. Such studies are becoming increasingly important due to increased concern over control methods in ecologically sensitive areas such as the Karoo biome (Price and Brown, 1997; Brown and Keiser, 1997; Thomas, 2000). More information about egg and embryonic development is needed to facilitate the design of accurate models for predicting swarming events (Hanrahan and Horne, 1997) that would enable more effective control.

Mechanisms of desiccation tolerance need to be understood for the metabolic engineering of sensitive cells (Potts, 2001), including mammalian and human cells (Guo et al., 2000; Turnnacliffe et al., 2001) of medical significance, such as blood platelets (Wolkers et al., 2002). Desiccation of larvae and whole organisms such as

numerous insects, tardigrads, and soil-dwelling collembolans (Hinton, 1960; Danks, 2000; Holmstrup et al., 2001), often with reference to freeze tolerance (Holmstrup and Zachariassen, 1996; Bayley et al., 2001), is well known but the effect of drying on the structure of individual animal cells is not. The ability of plant cells to withstand drying, and even complete desiccation, has been extensively documented. Walters et al. (2002) have noted that the physical and physiological properties of cells change when water is removed and these changes are often characterised by reduced size or lack of integrated metabolism. Structural responses to drying such as cell wall folding, are very prominent and easily observed in plant cell preparations (Van der Willigen et al., 2001), and a number of structural changes in plant cells that may be attributed to desiccation damage have been identified (Sherwin and Farrant, 1996; Farrant et al., 1999; Van der Willigen et al., 2001). Most of the work on desiccation tolerance in plants is aimed at one of two objectives: engineering drought tolerance in plants of economic and humanitarian significance (Alpert and Oliver, 2002); and perfecting desiccation of plant seeds for storage (Vertucci and Farrant, 1995). Engineering either seeds or mammalian cells for storage utilizes a very rapid drying process – “ultradrying” (Pammenter et al., 2002) – which is quite unlike drying under drought conditions in the field. The effect of drying on the vegetative tissue of drought tolerant plants is also quite commonly studied, and is probably of greater relevance to this project.

In general, typical adaptations to dehydration in both insects (Danks, 2000) and plants (Vertucci and Farrant, 1995; Sherwin and Farrant, 1996) include mechanisms to either: 1) tolerate water loss, perhaps by entering anhydrobiosis where metabolism “ceases”; 2) acquire water; and/or 3) limit loss, either through ecological, mechanical and/or physiological/biochemical adaptations. Dehydrated tissues suffer potential damage to membranes, causing them to rupture or leak, and to proteins by degradation. (Danks, 2000; Wesley-Smith et al., 2001). Tolerant plants have been shown to have certain characteristics that allow cells to avoid irreversible desiccation damage. They can minimize mechanical damage from loss of turgor, maintain integrity of macromolecules and membranes and minimize and repair chemical damage. They are able either to repair this damage, and/or have

protective mechanisms against damage (Vertucci and Farrant, 1995; Sherwin and Farrant, 1996).

1.1 Definitions

1.1.1 Dormancy states: diapause and quiescence

There are two main kinds of dormancy in insects: *quiescence* and *diapause*. “Dormancy” is a general term referring to a condition of interrupted growth, development and reproduction. “Quiescence” and “diapause” are more specific terms, referring to different types of dormancy. In quiescence, development stops due to recurring or currently adverse conditions that are unfavourable for growth and reproduction, such as low temperatures or extreme dryness (Gordh and Headric, 2001). Diapause by definition is a typically long-term physiological condition or state of restrained morphological development (Gordh and Headric, 2001) under direct or indirect control (Danks, 2000). Cellular metabolic depression is a feature of both states and downregulation is achieved much faster under the diapause condition (Hand and Probrabsky, 2000). An organism can enter and leave quiescent states repeatedly during its life, as conditions change. Diapause occurs only once and will persist even when favourable conditions become available, whereas quiescence is terminated when conditions become favourable. A certain level of diapause development (physiogenesis), that can only take place within an optimal range of conditions that is characteristic to the species (Wigglesworth, 1972 and 1974; Yamashita and Hasegawa, 1985), needs to occur before diapause can end.

1.1.2 Drought tolerance vs. desiccation tolerance

Alpert and Oliver (2002) suggest that, in plants, drought tolerance and desiccation tolerance are operationally and conceptually different and proposed definitions that separate the two that are as follow: Drought tolerance refers to tolerance of suboptimal water availability, i.e. that which is low enough to reduce plant performance. Tolerance would include ways of maintaining cell water content.

Desiccation tolerance is a form of drought tolerance but refers to water availability that is low enough to cause complete drying to equilibrium with the air. Tolerance involves surviving the near complete loss of water. Desiccation tolerance is rare in vegetative tissue and appears rather in seeds and pollens where tolerance is developmentally programmed as opposed to environmentally induced. It has not yet been determined whether brown locust eggs reach full desiccation, as defined above. However, it seems likely that most events that occur during development are dependent on environmental factors, implying that drought tolerance is the more likely type of response in these eggs.

1.2 Background

1.2.1 Brown locust biology and development

Locustana pardalina displays a particularly complex course of development (Uvarov, 1966) that follows different pathways depending on moisture (rainfall) conditions and the physiological state of the egg (Matthée, 1951). Eggs are supplied with sufficient water at oviposition to allow for development to diapause or quiescence (Shulov and Pener, 1963). Eggs can either enter a state of facultative diapause, or respond to immediate dry conditions by assuming a state of quiescence or both (it is possible for both states to be present in the same egg at different times). Female phase, which is primarily a density-dependent phenomenon, is a major determinant of the type of egg laid by *L. pardalina*. There seem to be a number of possible factors that are involved in the transmission of “information” on the females crowding experience to the embryo: current speculation (based on studies on *S. gregaria*) is that the signal may be via ecdysteroid levels either in the eggs (Tawfik et al., 2002) or the foam plugs (Saini et al., 1995;), although this is still not shown to be definite (Hägele et al., 2004). McCaffery and others (1998) showed that there is some gregarizing factor in *S. gregaria* foam plugs that is active for up to one day.

Eggs start developing soon after they have been laid but neither diapause nor non-diapause eggs absorb any water while the hydropyle complex is developing. Once the hydropyle cells are fully differentiated (on about the sixth

day after oviposition), non-diapause eggs will absorb available water but diapause eggs will not (Matthée, 1951). Development will proceed until anatrepsis when non-diapause eggs will either enter a state of quiescence (Q1) if the soil has been dry, or hatch within 10-15 days if 10-25mm of rain has fallen (Price and Brown, 2000). If quiescent eggs are moistened again, they will regain turgidity within approximately 24 hours and then continue developing till hatching, provided they have absorbed enough water to initiate catatrepsis and conditions are favourable. If the eggs have not absorbed sufficient water for catatrepsis to proceed before being exposed to dry conditions again, the embryo will develop a little further and the eggs will revert to the quiescent state, identified as the second quiescent stage (Q2), until conditions are favourable.

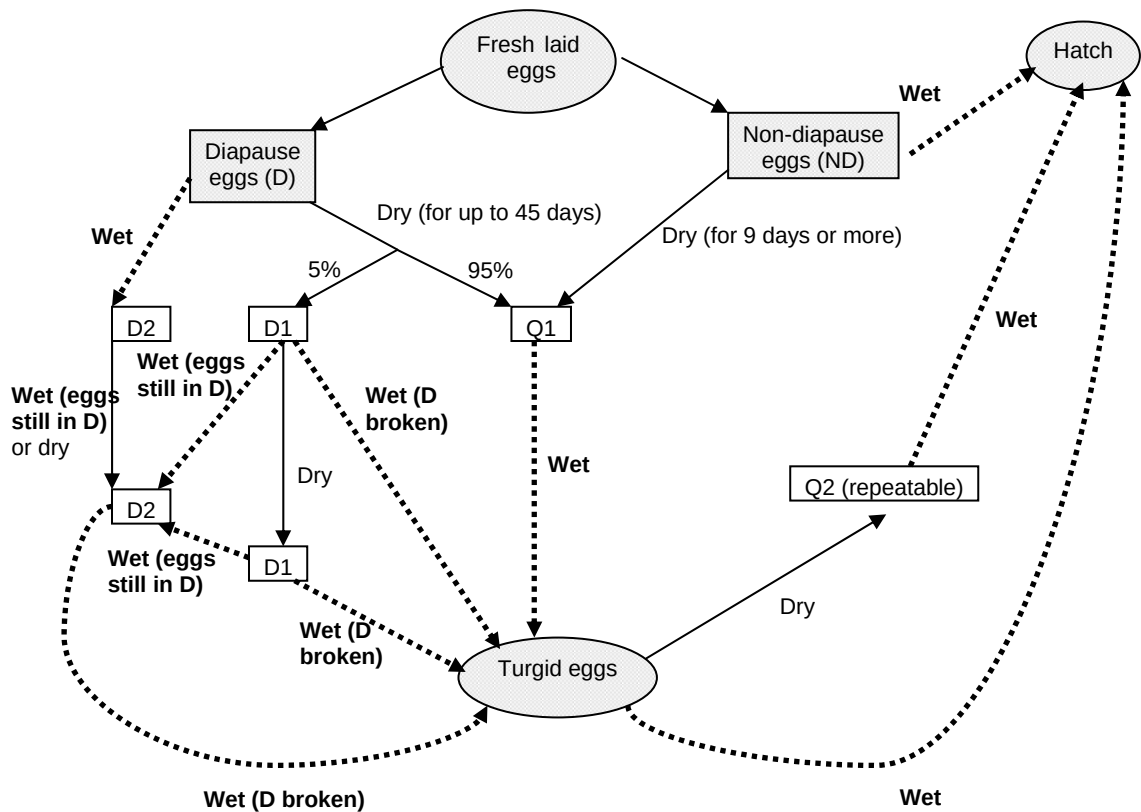


Figure 1. Developmental routes that can be taken by *Locustana pardalina* eggs, as determined by environmental and physiological conditions (adapted from Matthée, 1951).

Similarly, in diapause eggs, the diapause state (D1) is reached at about the eighth or ninth day of development (depending on moisture conditions), at which

point the anatreptic embryos in quiescent (Q1) and diapause eggs are morphologically identical (Stage 30; Matthée, 1951). Like many other species, morphogenesis is suspended during anatrepsis when somites and other axial structures are present and the embryo is about to undergo revolution - tissue differentiation has not yet begun at this stage. It appears as though development cannot be arrested during phases of intense mitotic activity (Lees, 1955). Diapause eggs will take up water prior to the elimination of diapause to replace that lost through evaporation. Uptake in these instances does not break diapause; instead development may proceed to a second diapause level, termed D2 (known as “true” diapause), at which stage embryos are morphologically identical to the second quiescent stage Q2. Morphological changes between D1 and D2 (or Q1 and Q2) are slight; there is a marginal change in size and further segmentation of the tarsus. Further uptake of water only occurs after termination of diapause, even in favourable conditions. Once diapause is broken, the eggs behave as if quiescent and will follow the same developmental pathway. The flow diagram in Figure 1 best illustrates all the possible developmental routes that *L. pardalina* eggs may follow.

1.2.2 The hydropyle structure and function

Newly laid acridid eggs usually contain too little water to complete development. Insect egg coverings, however, become practically impermeable after the yellow cuticle has formed and only absorb water through a limited region of the cuticle, the hydropyle complex. This structure is located at the posterior tip of the egg and consists of the hydropyle cells and the covering layers of secondary cuticle that they form – the yellow and white cuticles of the hydropyle (see Figure 6). Hydropyle cells are specialised, enlarged serosal cells, which are formed from part of the embryonic blastoderm [in addition to the serosal cells, the blastoderm forms the germ band (Kaltoff, 1976; Heming, 2003)]. They secrete the hydropyle cuticle within the first six days after laying in *L. pardalina* (Matthée, 1951). Both layers of the hydropyle cuticle have a pore canal system that allows free movement of water through the structure, and all water enters (and sometimes leaves) the egg through it. Regulation of intake appears to be governed by hydropyle cells (Matthée, 1951).

Slifer (1958) has demonstrated the importance of the hydropyle complex in *Melanoplus differentialis*. The hydropyle cells of this species secrete a waxy layer that covers and completely waterproofs the hydropyle cuticle, an action that is thought to induce diapause. Under natural conditions, the hydropyle cells may also be responsible for terminating diapause by breaking down the wax layer enzymatically (Lees, 1955). This implies that the hydropyle complex is involved in the initiation, maintenance and termination of diapause in *M. differentialis* eggs, and that the occurrence of diapause (or its absence) is dependent on the changes that take place in the hydropyle (Slifer and Sekhon, 1963). Conversely, the uptake of water and diapause appear to be independent in *L. pardalina*.

Hydropyle cells of brown locust eggs are thought to secrete a proteinaceous covering substance that is permeable to water, even soluble in it, but impermeable in dry conditions (Uvarov, 1966). Matthée (1951) proposed that this substance is secreted by the hydropyle cells within seven to 10 days of being laid, and within 14 days of any subsequent wetting episode that results in water absorption by the egg (at local ambient temperatures of around 35°C and dry conditions ranging from zero to 60% RH). In freshly laid eggs this sealing action would coincide with the onset of embryonic dormancy, i.e. quiescence or diapause. In older quiescent or diapause eggs, hydropyle cells supposedly become dormant after secretion of this layer. This system allows for the pores in the hydropyle cuticle to be sealed during periods of desiccation, and for unobstructed water absorption upon contact with water.

Initially, water absorption may be passive (i.e. while the cell is in a shrunken, dehydrated state and subject to an osmotic gradient). Once turgid, or at “normal” size, no water enters via diffusion but proceeds with active absorption (Lees, 1955) if conditions are favourable and there is no diapause condition. Hinton (1981) argued that there was very little evidence for active uptake in insect eggs, and that cells competent to absorb water will continue to do so until the hydrostatic pressure of the egg rises enough to hinder further uptake. It has, however, been found to be an active process in *Locustana* eggs (Matthée, 1951; Edney, 1957). Matthée (1951) found that active uptake in *L. pardalina* eggs is dependent on aerobic respiration - a lack of cellular energy results in no water uptake. The rate of exchange is

determined by the hydropyle cells and this, in turn, by the properties of the cells (Matthée, 1951); for example, hydropyle cells of diapause eggs do not actively absorb water whether there is waterproofing or not, because the cells are inactive (Lees, 1955).

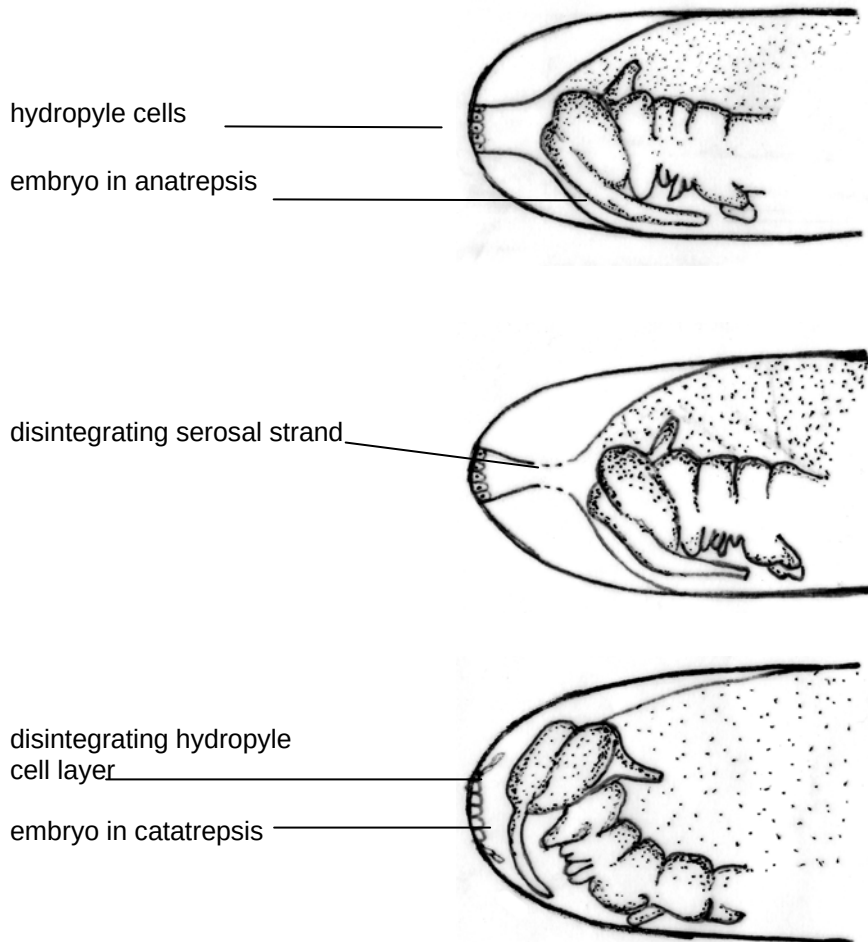


Figure 2. Development from anatrepis to catatrepsis in *L. pardalina* (after Matthée, 1951): **A.** Turgid egg with embryo in anatrepis, serosal strand un-stretched and still connecting embryo to hydropyle; **B.** Pre-catatreptic (late anatrepis) embryo, serosal strand stretched but still intact; and **C.** Embryo entering catatrepsis, serosal strand broken off and revolution of embryo about to begin.

Water absorbed by the cells is secreted into the area between the hydropyle cells and the embryo head, stretching the serosal strand (Figure 2). This pocket of water serves to meet embryonic water requirements as well as playing a part in

embryonic revolution, or catatrepsis (Matthée, 1951). When the pocket of water above the embryo head is large enough to push the embryo back and stretch the serosal strand to a certain point critical to the initiation of catatrepsis, the embryo will start to turn. Hydropyle cells remain intact and attached to the surface of the hydropyle after the serosal strand has broken (Matthée, 1951). After catatrepsis has occurred, it is not necessary that further water be taken up to complete development in *L. pardalina*. However, if there is contact water available, the egg will continue to absorb water prior to hatching (Matthée, 1951).

1.3 Aims and objectives

The objective of this study was to describe the structure of the hydropyle cells of brown locust eggs with a view to better understanding the mechanisms of egg development and responses to environmental stresses. Specific aims were: to examine hydropyle cells of *L. pardalina* eggs during development under favourable conditions and to describe the structural change that occurs within the cells during that development; to subject quiescent *L. pardalina* eggs to drying regimens and to observe and describe structural changes in the hydropyle cells during desiccation; and to rehydrate desiccated eggs and to observe and describe hydropyle cells after rehydration.

CHAPTER 2 MATERIALS AND METHODS

2.1 Introduction

It was originally intended that the structure of hydropyle cells of freshly laid diapause and non-diapause eggs would be observed and described at regular intervals during normal development without desiccation. The results from this would have provided a comparative baseline both for this study against the structure of hydropyle cells from desiccated eggs, and for a successive study on structure and physiology of diapause and non-diapause eggs by I. Kambule (University of the Witwatersrand). However, the hoppers that were bred out from the original eggs supplied by PPRI were very small, fragile and infertile, and this portion of the study had to be abandoned (see Appendix 1).

When assessing responses to drying, the ultimate test should be whether an organism can resume independent functioning after dehydration and rehydration. (Walters et al., 2002). While the experimental design used in this project included tests for viability (hatching / no hatching) after periods of drying and subsequent rehydration, physical structure was used as the primary indicator of cellular “functioning”. The extent of water loss during drying was also examined. Physical differences or changes in dehydrated and rehydrated cells might not necessarily imply irreversible damage, but they would indicate consequences of drying or protective strategies, and may suggest maintenance of function during the processes (Pammenter et al., 2002).

For water content measurements, there are a number of different ways to express data but mass-based measures are the most common: data for this study are thus presented. While practical for within-species comparisons, Sun (2002) notes that the use of mass-based parameters in between-species comparative studies has shortcomings. Varying proportions of macromolecules that have different hydration properties may affect equilibrium water contents of eggs of different species. This needs to be borne in mind for future work.

As regards histological techniques, standard fixation techniques (gluteraldehyde with post-fixing in osmium tetroxide) were used for this study.

While the morphology of chemically fixed cells is generally acceptable (Fields et al., 1997), conventional aqueous fixing protocols produce rehydration artefacts in partially hydrated or dehydrated tissue that distort the ultrastructure. For this reason, many current studies using plant material commonly employ anhydrous fixation techniques, such as osmium tetroxide vapour (Dickson and Elleman, 1985) and freeze-substitution (Fields et al., 1997; Wesley-Smith et al., 2001). Some workers, however, consider the results from standard, cryo- and vapour fixation techniques to be very similar (Sherwin and Farrant, 1996; Farrant et al., 1999) and maintain that if the same technique is used on all tissues in the experimental series, as in this study, comparisons are valid.

2.2 Locust cultures

Locustana pardalina eggs were provided by the Locust Research Unit at the Plant Protection Research Institute (PPRI), Pretoria, South Africa. Pods had been laid in August 2002 and April 2003 and eggs were an average of 26 weeks old (range 12 - 40 weeks) when used. Adult locusts in the transiens / solitary phase had been maintained on bran and wheat grass in glass-fronted cages. The adults that produced the older eggs had been kept outdoors during the day and indoors at night, while those that produced the younger eggs were mainly housed indoors. A mean ambient temperature of 27°C was maintained in the cages with additional heating provided by a light bulb situated above the clear lid of the cage. The cages had an upper and lower compartment, separated by a metal grid floor, with the locusts confined to the upper compartment. The insects had constant access to flower pots filled with moist soil that were fitted into holes in the grid floor. These were dampened regularly to encourage oviposition: while brown locust females typically lay their eggs in dry sand in the field, they will preferentially oviposit in moist soil if available. The pots were collected as soon after egg-laying as possible and kept at 2 - 4°C to suppress further development. It must be noted that periods between oviposition and collection were not consistent. Collection of the pots was done by removing them through the lower compartment, and they were

immediately replaced with fresh soil-filled pots. All eggs were removed from the pods at the start of the experiments.

2.3 Egg collection and separation

Pods were removed from the surrounding soil in the flower pots and eggs were extracted and separated using fine brushes. One pod (45 eggs) was taken from the 40-week-old pods and six pods (239 eggs) were used from the 12-week-old pods (this distribution was determined by the availability of pods; there were far fewer 40-week than 12-week-old pods). All the eggs were slightly shrivelled at removal, indicating a loss of moisture during the storage period, and in most fine cracks were visible in the outer chorion (see Figure 4a). It was intended that the experiments be conducted on diapause and non-diapause eggs separately, so that any differences in responses to drying between the two states of dormancy could be noted. While egg pods from transiens / solitary adults should have contained a mix of diapause and non-diapause eggs (Matthée, 1951), it was not certain that any of the pods would still have any diapause eggs, given their age. Diapause is eliminated in most eggs within 36 – 95 days, or longer, under favourable conditions. Prolonged desiccation may shorten the time taken for diapause to “break”: Matthée (1951) found that diapause could be broken in 95% of eggs within a maximum of 45 days, with the diapause breaking in the majority of eggs within 20 days of desiccation, at a relative humidity (RH) of 60%. He further found that diapause had terminated in 97 - 98% of eggs from pods that had been stored at room temperature for six months, that these were in a state of quiescence and that quiescent eggs behave similarly, regardless of whether they originated from diapause or non-diapause eggs. The benefit of this is that quiescent eggs used in any experiment need not be the same age, or of the same stock.

There appear to be no existing guidelines for separating diapause from non-diapause eggs that have been removed from mixed pods, without allowing the eggs to develop till hatching. Matthée (1951) had moistened pods immediately after laying and, after the third day, separated the eggs on the basis of size (larger eggs were assumed to be non-diapause, due to absorption of water). Alternatively, to

obtain only diapause eggs, he sampled eggs from numbered pods and later (after continued incubation and hatching) discarded all preparations that had been derived from mixed pods, while retaining those that came from diapause-only pods. Gehreken and Doumbia (1996) used a similar method for determining diapause and non-diapause eggs of the grasshopper, *Oedaleus senegalensis*. This is a very expensive method, in egg pod terms, and the limited number of eggs available for this project precluded its use.

In newly laid eggs, then, those that fail to absorb water after the third day are considered to be diapause, but older eggs, even those in the diapause state will absorb enough water to replace what they have lost during storage. Without knowing just how much water the individual eggs had lost during storage, it was impossible to define what the replacement amount would be for each diapause egg. It could be safely assumed that any eggs that did not absorb any water after wetting were either in a diapause state and had not lost any water during storage, or were dead. It was decided that those eggs that did not absorb any water and otherwise appeared healthy, would be classified as diapause.

Ten randomly selected eggs provided a pre-experiment baseline and the remaining eggs were incubated at 30-31°C on moist soil (15ml distilled water to 50ml autoclaved soil) for 48 hours in order to rehydrate them and to identify any diapausing eggs. This hydration regimen was employed in all instances where hydration and incubation was required during the experimental process. Three eggs did not show a change in mass and were most likely diapause eggs as they appeared healthy: these were omitted from the study.

2.4 Dehydration and rehydration

The twelve-week-old eggs were randomly divided to form two groups while the 40-week-old eggs constituted a group on their own. To examine the effects of drying and rehydration on the hydropyle cells, each group of hydrated and weighed eggs were placed first on to dry soil in open Petri dishes, and then into a glass desiccating chamber on a wire mesh platform over 500g activated silica gel (Figure 3). The lids of the desiccators were sealed with vacuum grease silicon (Unilab) and

then placed in a growth room at an ambient temperature of 29.7 - 31°C. Water content and rate of water loss was determined gravimetrically: a Precisa 125A SCS balance (± 0.1 mg) was used throughout. Relative water contents were calculated using the equation: $RWC = [WC(100-WC_F)]/[WC_F(100-WC)] \times 100$ (after Sun, 2002) where WC_F is water content at full turgor and WC the content at the time of sampling.

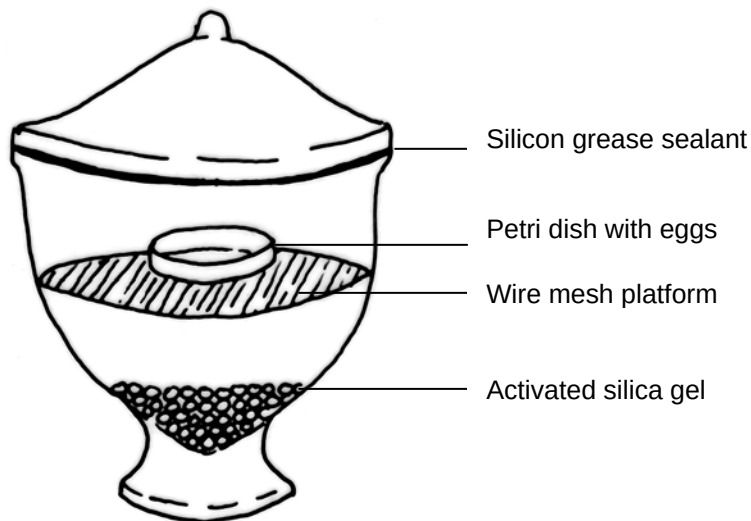


Figure 3. Schematic diagram of experimental apparatus; desiccating chamber with grid platform over activated silica gel.

Eggs in the first (40-week-old eggs) and second (12-week-old eggs) chambers were used to track the effects of drying on the hydropyle. A minimum of five randomly selected eggs were collected at regular intervals over a 14-day period for the first chamber and 10 eggs over a seven day period for the second chamber. The eggs were then rehydrated on moist soil for 48 hours, and sampled again. The remaining eggs were left to incubate until hatching to check for viability. Rehydrated eggs from the second chamber were very prone to fungal growths that could not be remedied through treatment with 2% NaOCl. It was therefore not possible to incubate the remaining eggs from this chamber till hatching. However, eggs from the same batch as these had been used to generate one generation of breeding adults (see Appendix 1) at the university, so that the viability of these eggs prior to the experiment had at least been determined. All remaining healthy eggs from the second chamber were therefore sampled after

rehydration. Preliminary analysis of changes in egg mass during the drying process was conducted, detailed tables for which can be seen in Appendix 2. After weighing, embryos and hydroyles were dissected out from the eggs under 2% glutaraldehyde for staging and processing for microscopy.

The third chamber contained 12-week-old eggs that were used to track the extent and rate of water loss in *L. pardalina* eggs during drying. These were not sampled, but individual eggs were weighed at regular intervals prior to and during a 23-day period drying period. Changes in water content were analyzed using Kruskal-Wallis non-parametric ANOVA followed by Dunn's multiple comparison test.

2.5 Microscopy

Embryos were prepared for viewing with both light and scanning electron microscopes, while hydroyles were embedded in resin for both light and transmission electron microscopy.

2.5.1 Egg dissection and fixing

The chorion was first removed from sampled eggs: eggs were placed on paper towel to dry until small cracks appeared in the chorion and then transferred to a glass dish containing HistoClear (orange oil) for one hour (after Slifer, 1963), followed by rinsing in several changes of distilled water until no oil droplets rose to the surface. The chorion could then be peeled off with fine camel-hair brushes. Many of the older eggs from PPRI already had extensive cracks in the chorion (Figure 4A) and this step was therefore unnecessary. In these, the chorion could be removed immediately after sampling, using the brushes.

The posterior tips of the eggs (about 2mm that contain the hydroyle) were prepared for light and transmission electron microscopy (TEM). Whole eggs were placed in a drop of phosphate buffered glutaraldehyde (2%) on a wax block on the stage of a dissecting microscope, and the posterior tips were removed with a sharp blade. Care was taken to avoid cutting into the embryo when making the incision, particularly in very desiccated eggs where the embryo head was often pushed up

against the hydropyle. In less desiccated eggs, the outline of the embryo, including eye spots, can easily be seen through the cuticle (Figure 4B). Before transferring the tips into 2% glutaraldehyde for overnight fixing, the cup-shaped hydropyle tips were checked to ensure that they were open and that the cut edges had not stuck together. Most often an air bubble would get trapped inside the sealed “cup” which was very difficult to free once the tissue has been fixed without causing damage. The following morning, samples were washed three times in phosphate buffered saline (PBS) for five minutes at a time, post fixed in 1% osmium tetroxide for 1 - 2 hours and washed in PBS again.



Figure 4. *Locustana pardalina* eggs **A)** after removal from the pod with the chorion still attached. Note the cracks in the chorion and the dented appearance of the anterior tip of the egg, indicative of its dehydrated state. Some of the frothy pod covering is still attached to the egg; and **B)** after removal of the chorion. The outline of the embryo can be faintly observed against the dark background. It has not yet undergone catatrepsis as the abdomen still extends to the anterior end of the egg (arrow) hyd: hydropyle region (bar = 1mm).

2.5.2 Electron microscopy for hydropyles

Following fixing, specimens were washed in PBS (3 x 5 minutes) and dehydrated in alcohol series (30%; 50%; 70%; 90%; 100% x 2 for 30 minutes each). Samples were infiltrated with increasing concentrations (1:3, 1:1, 3:1, 1:0) of epoxy resin (EPON 812) in propylene oxide for 30 minutes at each stage with rotation and then under vacuum. This gradual infiltration was used to enhance permeation of the

tissue as propylene oxide has a lower viscosity than EPON, particularly EPON 812, which polymerizes much faster than other resins (Mollenhauer, 1998). Hydropyle tips were all oriented in the same direction in the mould (Figure 5) so that, when cut, sections would show consistency with cells all oriented in the same plane.



Figure 5. Diagram of resin block showing orientation of hydropyle tissue within the block.

The blocks were left to polymerize overnight at 60°C and semi-thin (1µm) transverse sections were cut using a Reichert Ultracut ultramicrotome. Sections were stained with Toluidine blue and viewed: images were captured digitally for analysis. Thin sections (approx. 90 – 120nm thick) were stained with uranyl acetate and lead citrate, according to standard protocol, viewed at 80kV with a JEOL JEM 100S transmission electron microscope and photographed. Measurements were taken manually from micrographs

2.5.3 Embryo preparation and examination

Embryos were removed from the eggs by gently applying pressure to the anterior tip of the egg once the hydropyle had been cut off, thereby “pushing” the embryo out of the egg along with the yolk. The yolk and extra-embryonic membranes were then carefully and completely removed: these are very difficult to remove once fixed. Embryos were gelatinous and delicate immediately after removal and were left in the glutaraldehyde for a couple of minutes before handling. Embryos were transferred to a dish under liquid medium – either PBS or insect ringer – for cleaning. The embryos were then either photographed immediately after removal from the eggs, or fixed in 70% alcohol (after Matthée, 1951; Humason, 1967) for

further preparation. For SEM preparation, embryos were gradually taken to 100% alcohol, critical point dried and carbon and gold palladium coated, and mounted onto stubs. For light microscopy, whole mount preparations were used.

Matthée (1951) had squeezed embryos out of the eggs directly onto slides, allowed the residual yolk to air dry, thereby fixing the embryo to the slide and then kept the slides in 70% alcohol. Using this method, I found that embryonic features were very indistinct as the surrounding yolk and membranes had not been removed and it is not possible to do so once the embryo is stuck to the slide. Also, embryos frequently came loose from the slides and were lost.

Fixed embryos were stained either with haemotoxylin for approximately 11 minutes, or carmalum for 20 hours without rehydration, and dehydrated from 70% alcohol with three changes to 95% and two changes of absolute alcohol for half an hour each. Clearing and mounting whole embryos was often difficult. Preparations were cleared using a gradual increase in ratio of alcohol to xylene mixtures (80:20; 60:40; 50:50; 40:60; 20:80; and 0:100) for 20 – 30 minutes for each mixture. Embryos were then placed in well slides filled with xylene and impregnated with mountant by adding DPX to the xylene in the well, one drop a day, carefully mixing it in with the clearer till thickened, and then allowing the concentration to continue by evaporation (after Humason, 1967). Embryos were oriented in the well once the mixture has thickened, but before it has completely set. Coverslips were warmed before lowering onto the preparation.

2.6 Analysis of changes in hydropyle cell structure

The effect of drying and rehydration on hydropyle cell structure was assessed by examining the changes in morphology of cells from each treatment. Using semi-thin sections and digital images captured through the light microscope, measurements of whole cell and nucleus areas, hydropyle depths and the distance from the inside edge of the cuticle to the outer edge of the nucleus (see Figure 6) were taken using Simple PCI image analysis software (Version 5.1.0, Compix Inc.).

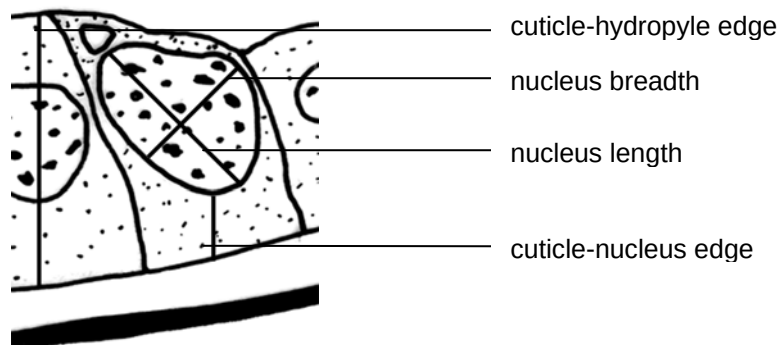
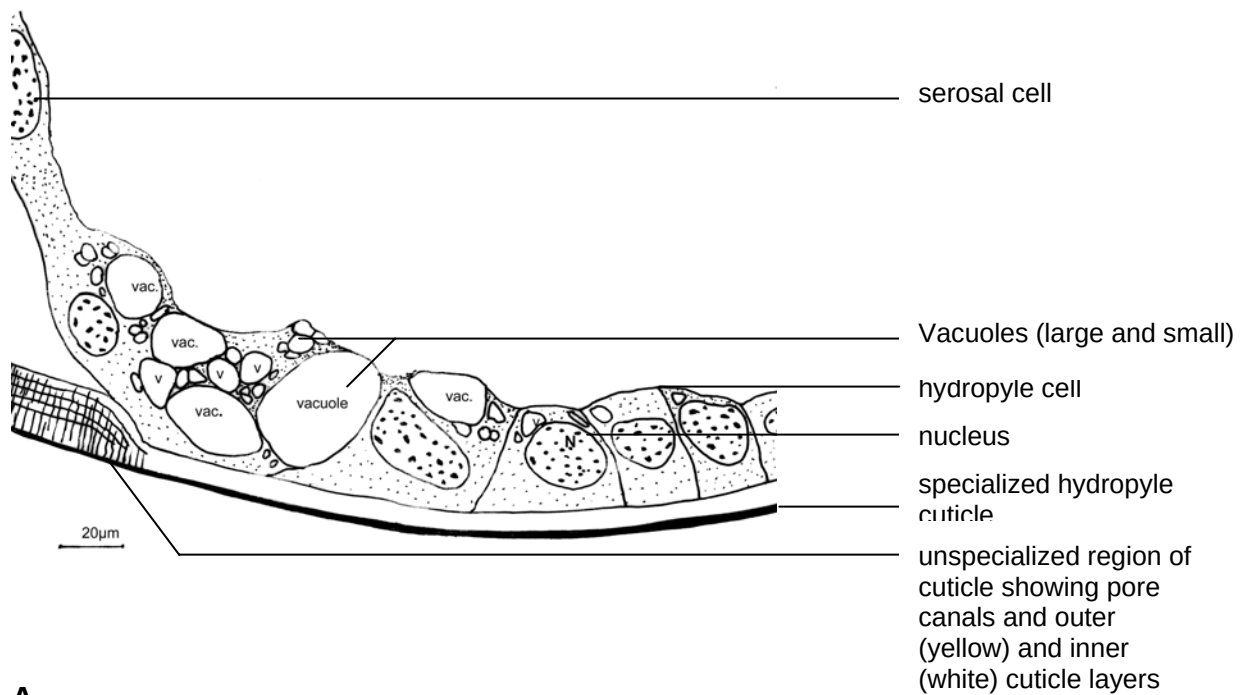


Figure 6. Schematic diagram of the hydropyle structure in *Locustana pardalina* eggs. **A.** Stretch of hydropyle cells showing the transition from specialized to unspecialized cuticle; heavy vacuolated cytoplasm, particularly toward the outside edge of the structure. Specific cell regions will be referred to as follows: the apical region is that closest to the cuticle, while the basal region is that closest to the egg contents (bar = 20µm). **B.** Enlargement of one hydropyle cell indicating the positions of measurements used for morphometric analysis.

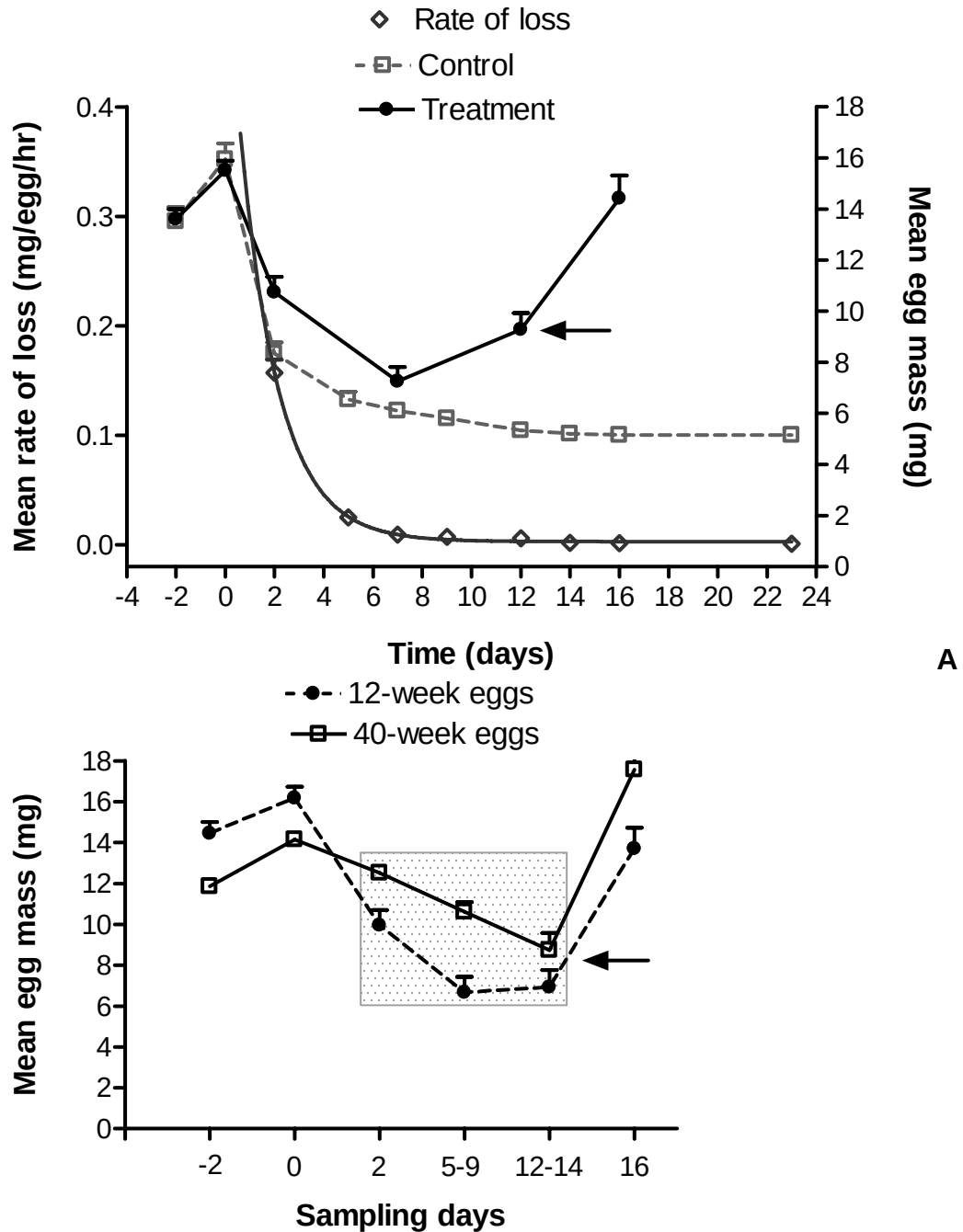
The system was calibrated to the appropriate magnification for each image. A minimum of five measurements (from five different hydropyle cells) for each measurement category was made per egg, with a minimum of five eggs representing each treatment level (i.e. early, intermediate and late desiccation, and rehydration). Data for cell area measurements had to be treated differently. Lateral cell boundaries were very often indistinct or not visible at all which meant that it was not possible to gather a decent body of data. Analysis of all other data for structure showed that there were no significant differences during the drying period, i.e. from day 2 onward (one way ANOVA) so data for these levels were pooled and cell measurements were analyzed on the basis of differences between dehydrated and rehydrated levels only using unpaired *t*-tests. All calculations were done using Graphpad InStat version 3.06 for Windows (Graphpad Software, San Diego, California). Data for pre-experiment and pre-hydrated eggs were omitted as sections from these levels were of poor quality and provided too few clear measurements for analysis: comments regarding these two treatment levels will therefore be made on the basis of qualitative observations only, as well as comments on the extent of vacuolation as this was not quantified.

CHAPTER 3 RESULTS

Preliminary analysis of changes in egg mass during the drying process revealed that there were no changes in either the 40-week-old eggs (Kruskal-Wallis non-parametric ANOVA) or the 12-week-old eggs (one way ANOVA) from the second day onward. The data from these two chambers was therefore pooled and treatment levels were determined according to how long the eggs had spent in the desiccators (see Appendix 2).

3.1 Water content

Figure 7 shows the effects of hydration, dehydration and subsequent re-hydration on *Locustana* eggs. Pooled data from the treatment chambers is shown in Figure 7a. In all eggs, mean egg mass increased due to water uptake when initially hydrated for 48 hours. Eggs had visibly increased in size after hydration and this swelling caused further splitting in the chorion. Twelve-week-old eggs showed the greatest losses ($P < 0.001$, Kruskal-Wallis) within the first 48 hours in the desiccating chambers and eggs from the control group showed an additional small ($P < 0.05$) loss on the ninth day, with no other changes. Forty-week-old eggs only showed a significant change in mass (compared to the hydrated state) on the fifth day in the desiccator ($P < 0.05$, Kruskal-Wallis). There were no further changes in the treatment groups after the first 48 hours drying until rehydration, when a significant increase in weight ($P < 0.001$) was observed again. The average rate of water loss in control eggs (Figure 7A) was similar to that shown by Matthee (1951): eggs showed a rapid rate of water loss during the first seven days, after which there was no change. Remaining eggs from the first chamber ("treatment" eggs, 40-weeks-old) that were incubated on moist soil at 30 - 31°C hatched after seven to ten days. No eggs hatched while in the desiccators.



B
Figure 7. A. Graph showing changes in egg water contents for “treatment” eggs (Chambers 1 and 2 pooled, $n=180$) and “control” eggs (Chamber 3, $n=60$). Rate of water loss shown was determined from the “control” eggs. Day -2 to 0 = initial hydration. For treatment group, days 2 to 14 = dehydration; day 14 to 16 (arrow) = rehydration. **B.** Data from eggs in Chambers 1 (40-week-old eggs, $n=33$) and 2 (12-week-old eggs, $n=68$) indicate that older eggs showed less extreme responses to desiccation. While they absorbed more water during initial hydration ($p<0.001$), losses were only evident after 5 to 9 days in the desiccator ($p<0.001$). The shaded area represents the days of drying during

which there was no statistical difference between eggs from different chambers. Error bars=SEM.

While average mass after rehydration was not statistically different to those after initial hydration, there were a number of eggs (44% in total) that gained more than 100% of what they had lost during dehydration. Some of the eggs had swelled so much that they had ruptured and showed signs of wound repair while others ruptured spontaneously when handled with a brush. Combined egg mortality rose sharply from zero to 40% during the intermediate period of desiccation and remained high for the duration of the experiment. Most of the mortalities occurred in younger (12-week-old) eggs.

Figure 7B shows the responses of eggs of different ages to dehydration. Older eggs absorbed more water during initial hydration and lost water at a slower rate than younger eggs, particularly during the first 48 hours. Relative water contents of older eggs appeared to be consistently higher than younger eggs. There was also consistently more variation in younger eggs at all stages prior to and during the experiment (see Appendix 2 for data).

3.2 Structure

3.2.1 Light Microscopy

Observations were made on as many sections as possible: only 34% of blocks that had been prepared (26 out of 77 blocks) yielded sections that could be used to take measurements and for observation with the light microscope. A number of fixation and embedding problems were evident: in most cases, the resin had not penetrated the cuticle, which meant that it pulled away from the outer edge of the tissue during sectioning, and in some instances penetration of resin from the cell-side of the hydropyle was also poor. The fixing and embedding protocols used seemed to have been more effective on the older eggs from the first chamber (67% success as opposed to 16% in the second chamber). A second EPON recipe (Dawes, 1988) had also been attempted, but the results were no different from those achieved from using the standard EPON recipe currently employed in the laboratory. The

problems with fixation and embedding made it very difficult to section the majority of blocks.

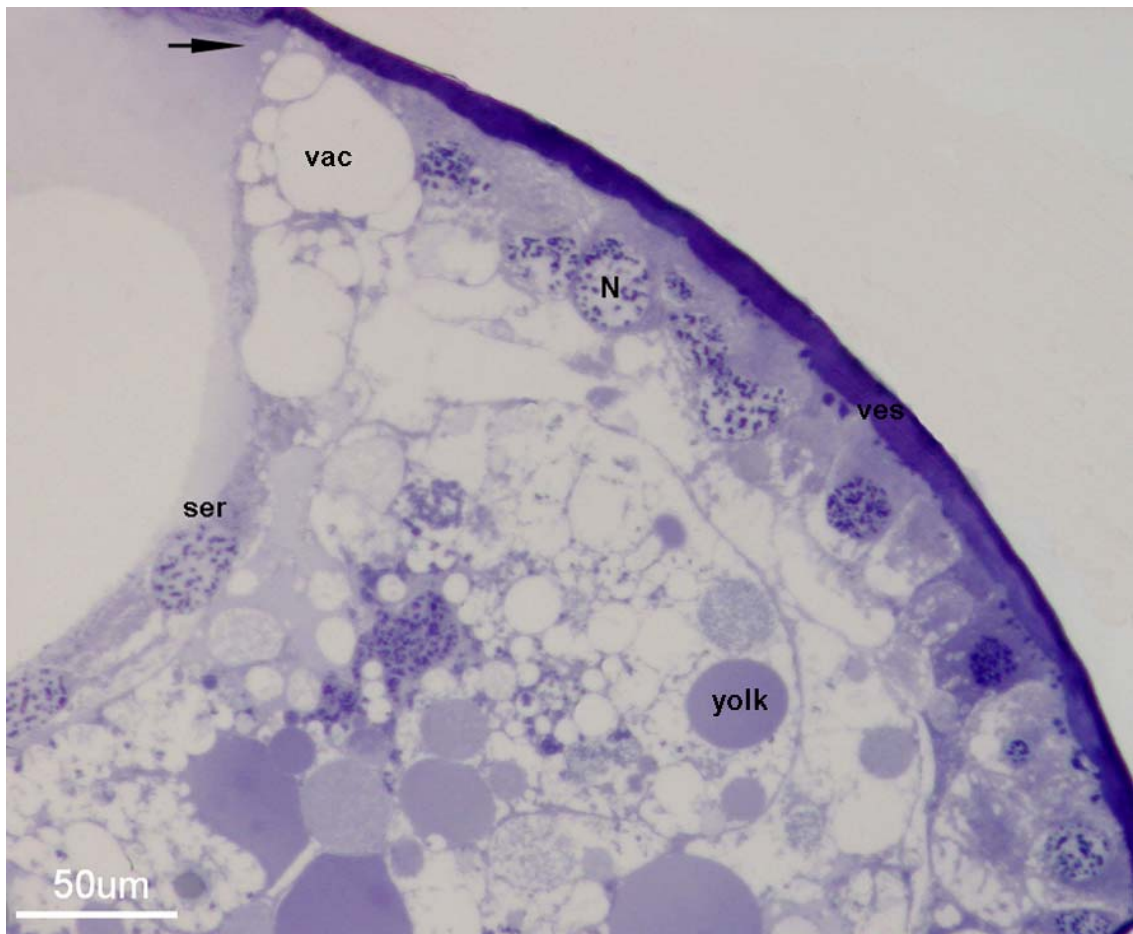


Figure 8. Light micrograph showing hydropyle structure made up of the specialized hydropyle cuticle with a row of hydropyle cells closely apposed to the inner edge. Serosal cells form part of the serosal strand, which is barely intact. Large vacuoles are prominent, but more so at the peripheral edge of the hydropyle. The transition to the unspecialized cuticle is only just visible at the top right of the micrograph (arrow). N: nucleus; ser: serosal strand and cells; vac: vacuole (bar=50µm).

The general appearance of hydropyle cells and the adjacent specialized cuticle is shown in Figure 8. The transition to the unspecialised yellow and white cuticle was clearly seen at either edge of the hydropyle. Cells of the hydropyle, which were mostly vacuolated, were arranged in a single layer below the specialized hydropyle cuticle and had a different appearance to regular serosal cells. Nuclei in all cells were generally centrally located, heterochromatin-rich,

large and either ovoid or spherically shaped. No nucleoli were visible. Heterochromatin bundles were evenly dispersed in the nuclei in most of the cells with some intercellular variation.

There was considerable variation in development between eggs that had just been removed from the pods and this variation remained evident throughout the experiments. It was observed in eggs from both treatment chambers: eggs of the same age, and even from the same pods, were therefore at different developmental stages prior to the experiments. This variation appeared to be confined to changes that occur during anatrepsis – i.e. from early to late anatrepsis. Differences were not easy to detect in the appearance of the embryos, which were all pre-catatreptic, but were apparent in the appearance of the hydropyle cells. Gross morphological changes in embryos were so slight as to be almost indistinguishable even through a compound microscope, although some could be resolved with the SEM (see below). Differences in cell morphology between eggs of the same age and treatment level were obvious (Figures 9A and B, 10A and B, and 10C, D and E), suggesting that development had progressed in some eggs and that this progression was noticeable in the hydropyle cells but not yet in the embryos.

Hydropyle cells from eggs that were perhaps in early anatrepsis had a compact shape with dense, smooth-looking cytoplasm surrounding a large nucleus (not shown). It is suspected that these few eggs had either been in a diapause or a primary quiescent (Q1) state at the beginning of the experiment. Cells from eggs that were at a later developmental stage were not as compact; they appeared to have elongated, with greater volume and therefore, cytoplasmic area (Figure 9A), and the cytoplasm had a more granular or coarse appearance. It is estimated that cells with this appearance were from eggs that were in late anatrepsis (or pre-catatrepsis) or Q2 in previously quiescent eggs. Cells from eggs in which catatrepsis was imminent or had begun were not all adjacently arranged below the cuticle (Figure 9B); some had either been pulled away, or were starting to detach from the cuticle, presumably having completed their function. Cells furthest from the cuticle had the most degenerated appearance in that their cytoplasm was scanty with a loose, highly interrupted appearance and nuclear content was sparse.

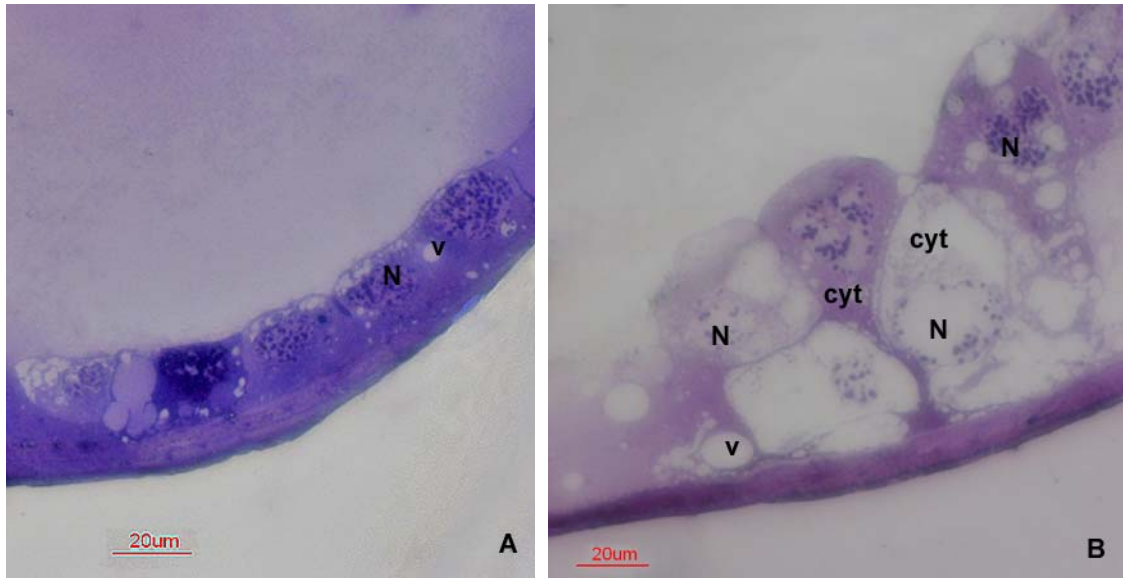


Figure 9. Light micrographs of cells from turgid (RWC 100%) pre-hydrated eggs (i.e. at day 0, after pre-hydrating for 48 hours) that show the variation in hydropyle appearance prior to the drying treatment. All sections shown were taken from older eggs from the first desiccating chamber and were therefore from the same pod. **A.** Vacuolated hydropyle cells still attached to the cuticle have large centrally-located nuclei with evenly distributed chromatin bundles that are typical to anatreptic eggs; and **B.** Hydropyle cells from an egg entering catatrepsis: cells have detached from the cuticle and are mostly degenerated. The area between the cuticle and the detached cells is heavily vacuolated. v: vacuoles; cyt: cytoplasm; N: nucleus (bar = 20µm).

Figures 10 and 11 show the general appearance of dried and rehydrated hydropyle cells as viewed with a light microscope. The chromatin in nuclei of eggs with the lowest water contents ($\leq 60\%$ RWC) appeared to be more condensed, with little variation between cells of the same egg (Figure 10E). Vacuoles of varying sizes were visible in cells from all eggs (except one) with the largest vacuoles appearing at the extreme outer edges of the hydropyle. The overall number of vacuoles did not appear to be different between dehydrated and rehydrated cells, but vacuole size appeared to have increased with rehydration. Cell area also increased with rehydration (see next paragraph), but the proportion of cell area occupied by vacuoles was not measured. From observation only, therefore, it seems that the absolute vacuolar area increases with rehydration, while the

proportion of cell area occupied by vacuoles may remain unchanged (in desiccated and rehydrated cells from eggs from the same developmental stage). The largest vacuoles in hydrated cells (Figure 11) were located between the nucleus and basal cell membrane, and also just outside the basal membrane. Smaller vacuoles could also be seen below the cuticle, and in some eggs that may have been at a different stage of development, these vacuoles were larger. The variation in vacuolar appearance between eggs may be attributed to the developmental stage of the egg, i.e. the extent of vacuolation in dehydrated cells depended on the amount of water that had been absorbed by the egg prior to storage and drying, which would have been related to the embryos' developmental status.

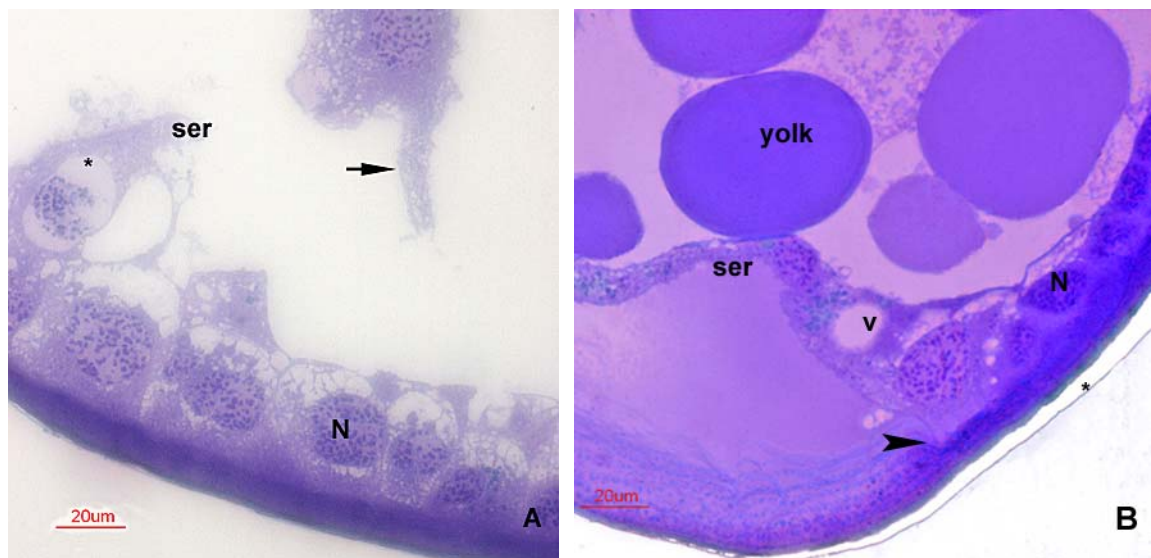
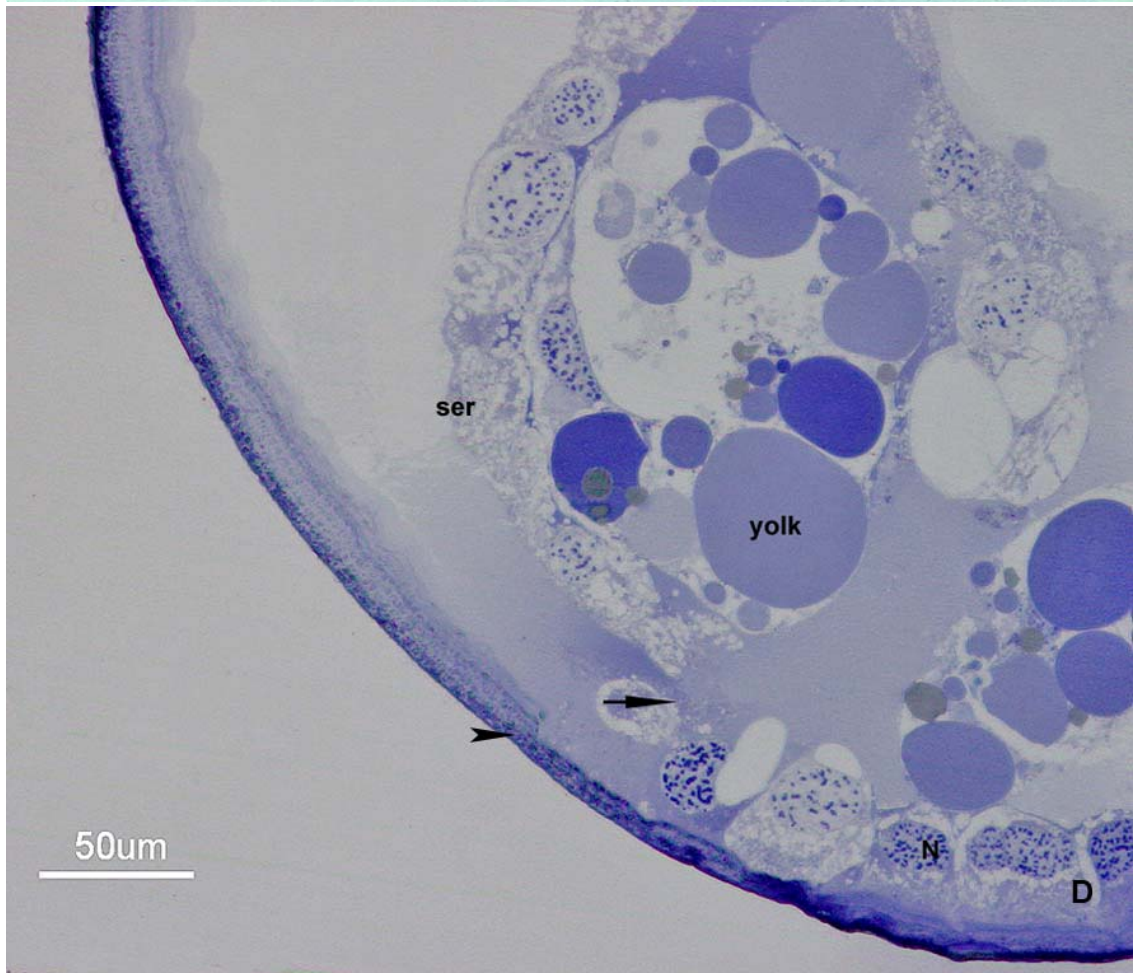
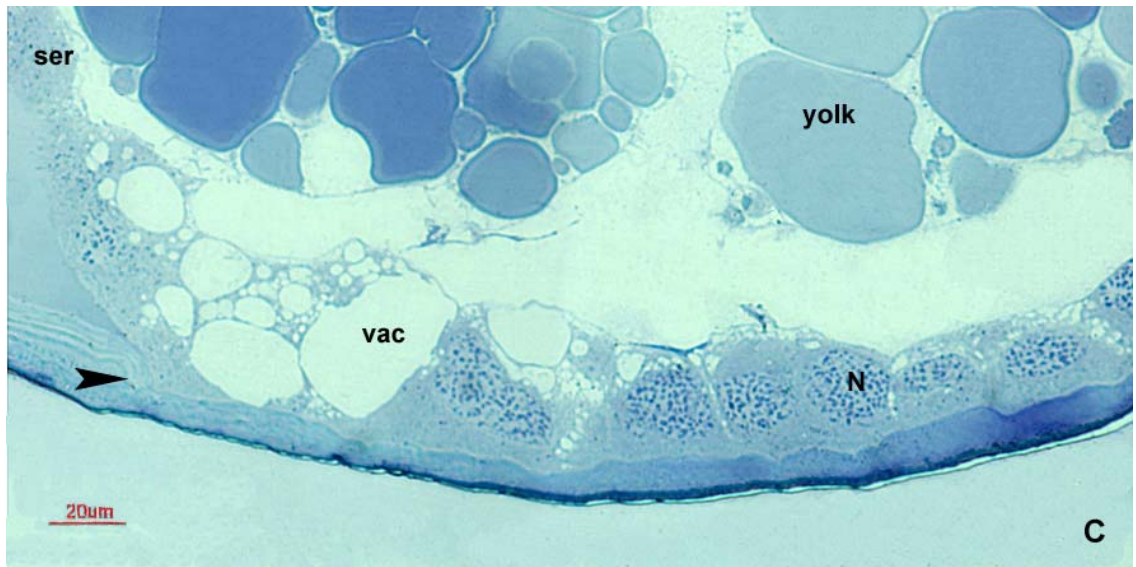
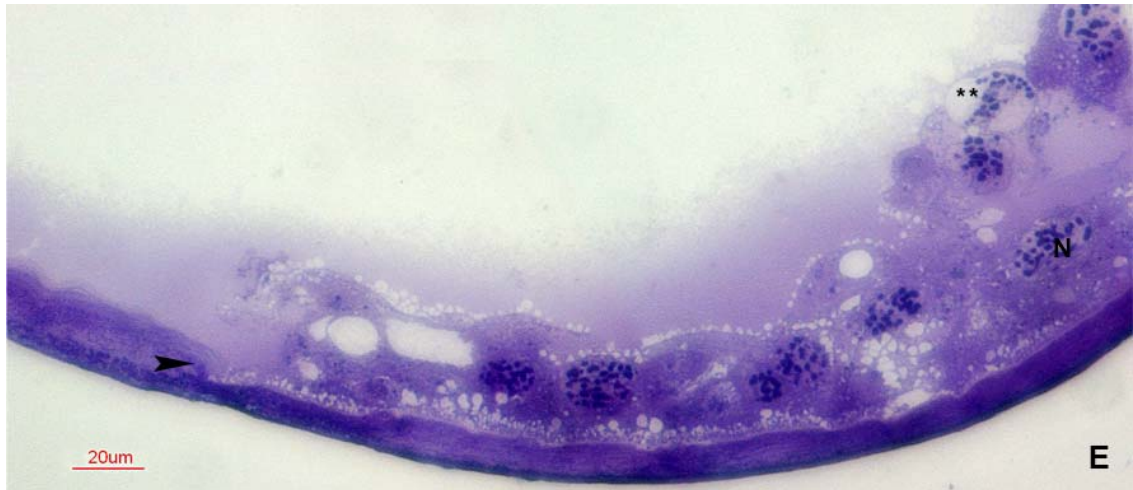


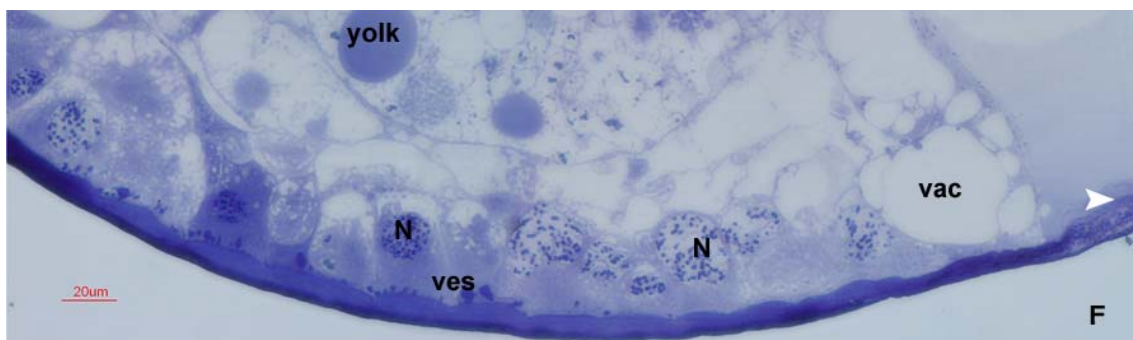
Figure 10. Light micrographs of dehydrated hydropyle cells. All hydropyles shown were sampled from 40-week-old eggs and were dried at 15% RH.

A. RWC 79% (bar = 20µm) and **B.** RWC 93% (bar = 20µm) are from eggs dehydrated for 2 days





C. RWC 89% (bar=20µm), **D.** RWC 73% (bar=50µm) and **E.** RWC 60% (bar=20µm) are from eggs dehydrated for 5 days.



F. RWC 91% (bar=20µm) was dehydrated for 9 days. The serosal strands of all these eggs were intact prior to dissection but D shows a strand that has broken during dissection (arrow). N: nucleus; ser: serosal cells or strand; ves: vesicles; * or vac: vacuoles; ** degenerating cells; arrowhead: transition from unspecialized to specialized cuticle.

Darkly stained granules were visible along the inside edge of the cuticle in seven eggs (Figures 8 and 11). Likewise, seven eggs displayed homogenously stained sub-cuticular intrusions (Figure 11); four eggs showed both granules and intrusions. None of these features were found exclusively in eggs from any particular treatment, but the sub-cuticular granules were found mostly in cells from 40-week-old eggs that had RWC greater than 90% (the exception was one egg at 54% RWC) and the dark intrusions were only observed in cells from eggs that had stretched serosal strands, i.e. were preparing for catatrepsis. This suggests that the

occurrence of both may be linked to the level of development rather than hydration.

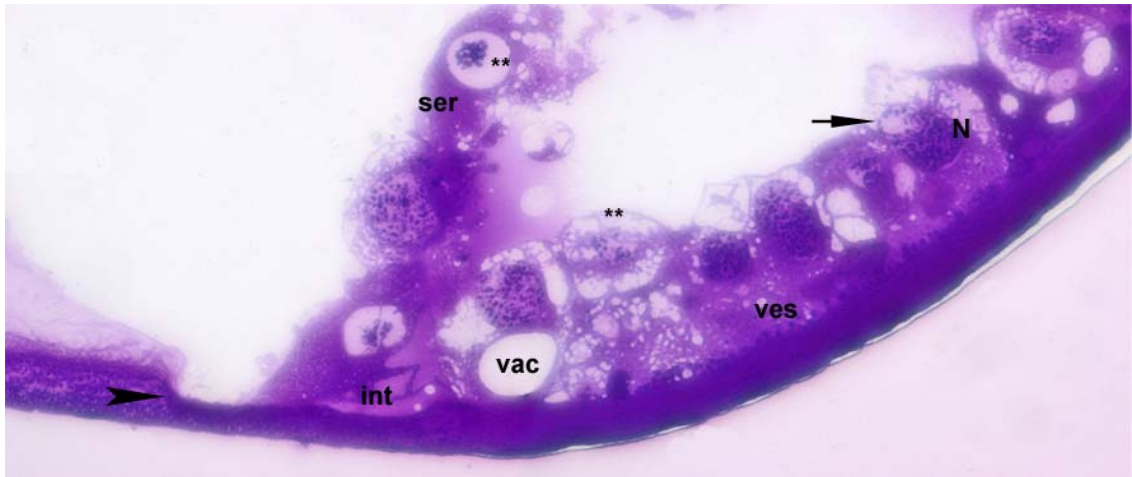


Figure 11. Light micrograph of hydrated hydropyle cells from a 40-week-old egg, RWC 117%. Densely stained vesicles are visible below the inner edge of the cuticle as well as homogenously stained intrusions. Vacuolation is extensive and many cells are starting to degenerate, particularly those that have detached from the cuticle, showing partially vacant nuclei (arrow) and cytoplasm. Catatrespsis had just begun in this egg as the serosal strand had broken. int: intrusions; vac: vacuole; ves: vesicles (bar = 20 μ m).

3.2.2 Embryonic development

As mentioned, there appeared to be subtle developmental differences between eggs of the same age, but identifying specific differences was difficult. Embryos were examined directly with the dissecting microscope, and then again either with a compound microscope (whole-mount embryos on slides), or with a SEM. Most embryos appeared to be at approximately the same developmental stage (Figure 12A), that of D or D', i.e. anatrepsis in either diapause or quiescent eggs (after Matth e, 1951). Morphological changes in embryos were most clearly seen in scanning electron micrographs (Figure 12B). Here the segmentation of the tarsus, which Matth e (1951) used as one indicator of the progression from diapause to "true" diapause, or to indicate further development in the quiescent egg, can be more clearly identified.

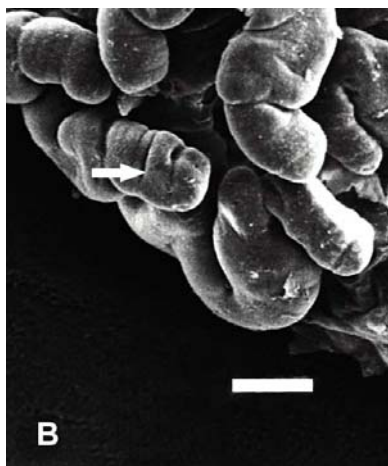
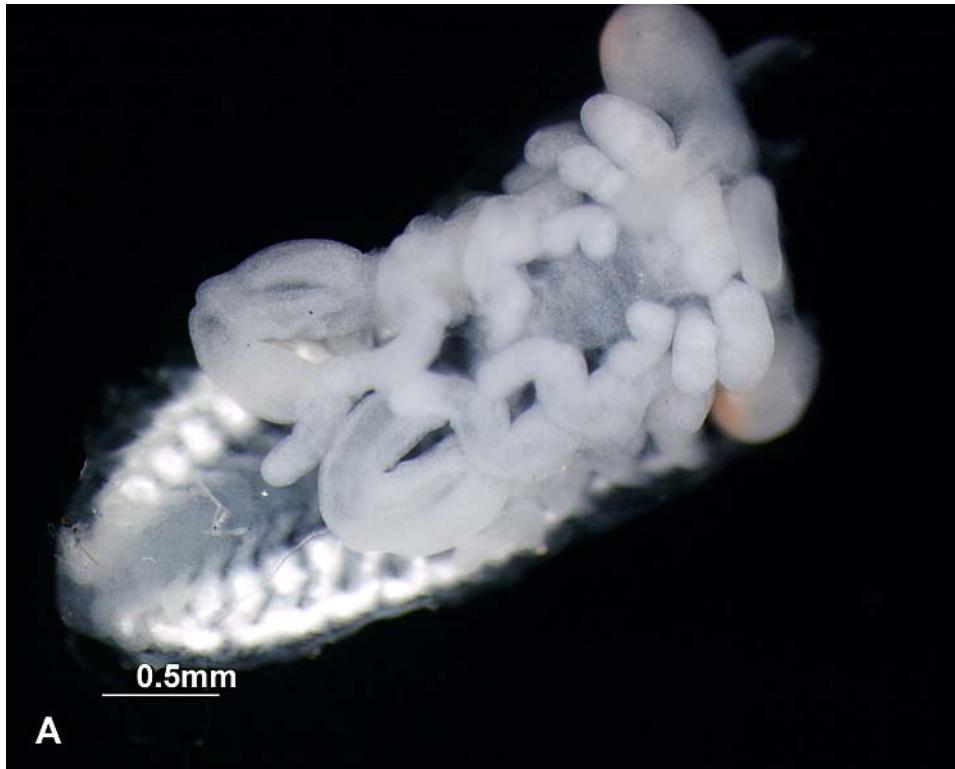


Figure 12. A. Light micrograph of anatreptic embryo after removal from egg. This stage corresponds to Matthée's (1951) diapause or quiescence stage in appearance (bar = 5mm); **B.** Further development to either a deeper diapause (D2) or secondary quiescent (Q2) stage can be resolved with the SEM. Segmentation of the tarsus is evident in this embryo (bar = 100 μ m).

More than half (69%) of the eggs had serosal strands that were stretched to varying degrees, which correlates to a pre-catatreptic stage, and six of the embryos looked as though they may have just started turning, but none had completed catatrepsis. While it appears, therefore, that there was some pre-experimental inter-egg variation in development, both within and between eggs of different ages and pods, and that this may have had an effect on the eggs ability to withstand desiccation, the variation seemed to be confined to those changes that occur between diapause and “true” diapause or, similarly, those that occur immediately

between primary and secondary quiescence, but prior to catatrepsis in normally developing eggs. The latter is the most likely developmental stage observed in the embryos in this experiment. Changes due to continuing embryonic development may have been occurring at a cellular level and therefore were not yet detectable in the gross morphology of the embryos. The preceding observations are purely descriptive and were not tested statistically.

3.2.3 Analysis of changes in hydropyle cell structure

Image analysis showed that cells and nuclei generally increased in size with rehydration. Changes in nuclei size appeared to be a function of changes in breadth, not length (Table 1), while increases in cell area were due to increases in both cellular breadth and length (Figure 13). Cells within the hydropyle region of the same eggs appeared to respond uniformly to treatments (see Appendix 3 for data).

TABLE 1. One-way analysis of variance of morphometric measurements from cells of various levels of desiccation and hydration.

Treatment	Nucleus area (μm^2)	Nucleus breadth (μm)	Hydropyle depth (μm)	Cuticle to nucleus (μm)
Early desiccation	290.99 ± 87.8^a	12.72 ± 2.8^a	21.35 ± 7.9^a	6.70 ± 2.4^a
Intermediate desiccation	306.39 ± 72.9^a	13.12 ± 2.0^a	29.07 ± 5.9^{ab}	8.47 ± 3.8^a
Late desiccation	287.14 ± 49.7^a	12.44 ± 2.6^a	30.98 ± 10.9^{ab}	8.40 ± 2.26^{ab}
Re-hydration	147.12 ± 65.8^b	17.87 ± 2.8^b	40.04 ± 11.7^b	15.79 ± 6.8^b

Each value represents the mean $\pm$ SD of measurements taken from at least five different cells, from at least five different eggs, per treatment. Treatments within each measurement with the same superscript are not significantly different ($P>0.05$).

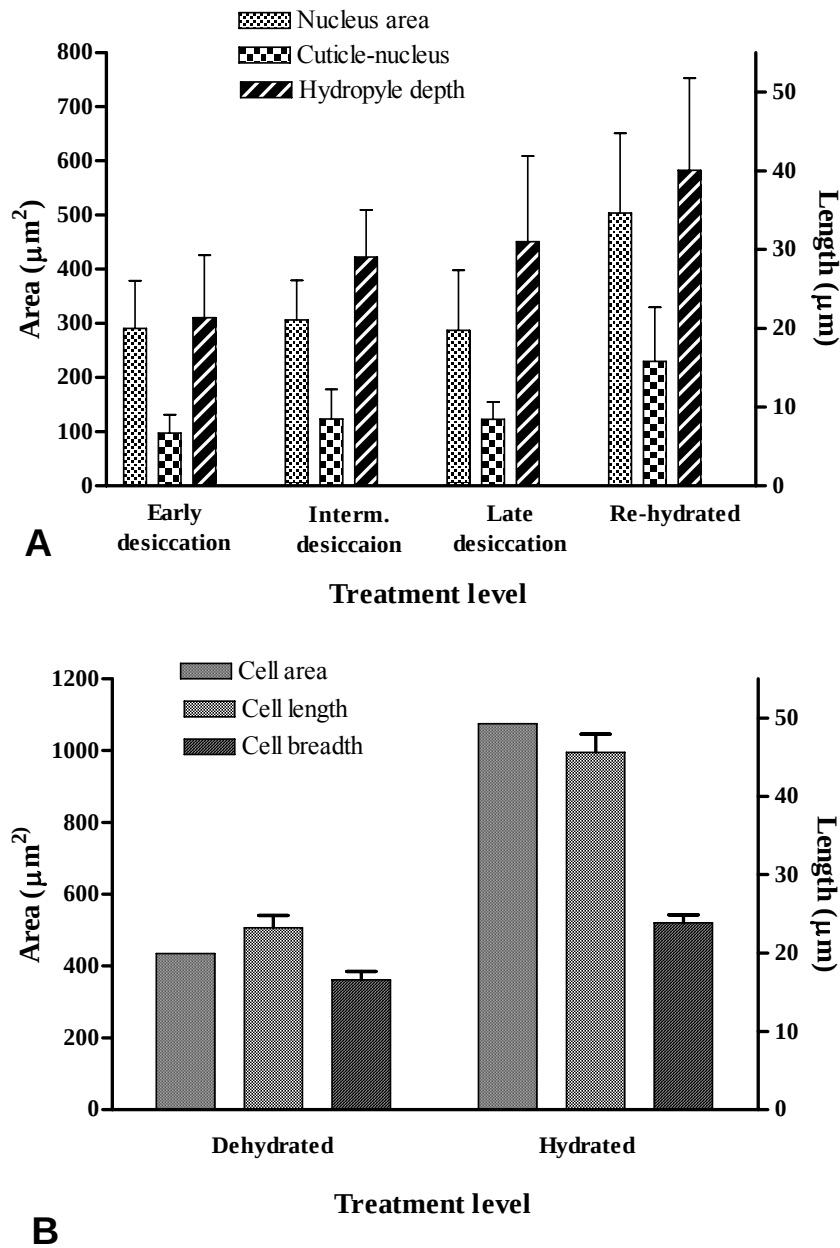


Figure 13. Graphs showing changes in cell morphology during dehydration and rehydration.

A. Within-cell measurements (data in Table 1); and **B.** Whole cell measurements where each value represents the mean $\pm$ SEM of measurements taken from at least thirteen different cells across five hydrated and six dehydrated eggs. All treatments within each measurement are significantly different ($P < 0.0001$); error bars = SEM.

3.2.4 Electron Microscopy

Thin sections from only eight blocks were suitable for viewing with the electron microscope (EM). Electron micrograph observations will be reported in terms of dehydrated versus rehydrated hydropyle cells, without specific reference to levels of desiccation. Given the small number of usable sections, statistical comparisons of data was not possible. The assumption used for this report then, is that while these micrographs cannot be regarded as defining representations of the differences between all dried and all hydrated hydropyle cells, they are representative of what may happen in some hydropyle cells, at certain stages of development, during extreme desiccation and subsequent rehydration.

Examination of the ultrastructure of hydropyle cells (Figures 14 and 15), both desiccated and rehydrated, revealed that both were similar in general appearance: the apical cell membrane was deeply folded to form microvilli that were visible below the white cuticle. There were no mitochondria visible within the folds of the microvilli in either dehydrated or rehydrated cells. Pores were visible in the laminated endocuticle (Figure 14A): this structure had a very similar appearance to that of the endocuticle of *Melanoplus differentialis* (Slifer, 1963). The lateral boundaries of hydropyle cells from both treatments had the highly interdigitated appearance typical of invertebrate cells (Figure 14B). Boundary membranes in the basal regions were less convoluted with septate junctions (Figure 14C). Apically located lateral junctions were not as clearly defined, but appeared to be zonula adherens junctions, while the remaining lateral junctions had a demasome-like appearance (not shown).

The darkly stained granules that were visible just below the cuticle in semi-thin sections were observed as electron dense vesicles of varying sizes (approx. 0.3 – 1.6 μ m), but appeared to be generally larger in rehydrated cells (Figure 15A). Likewise, the darkly-stained intrusions noted in semi-thins could be clearly seen as a compact, electron-dense matrix, perhaps extracellular, occupying spaces between microvilli (see Figure 14A), as apically located horizontal bands (Figure 15B), and also occupying intercellular spaces that appeared to form between lateral cell membranes (not shown).

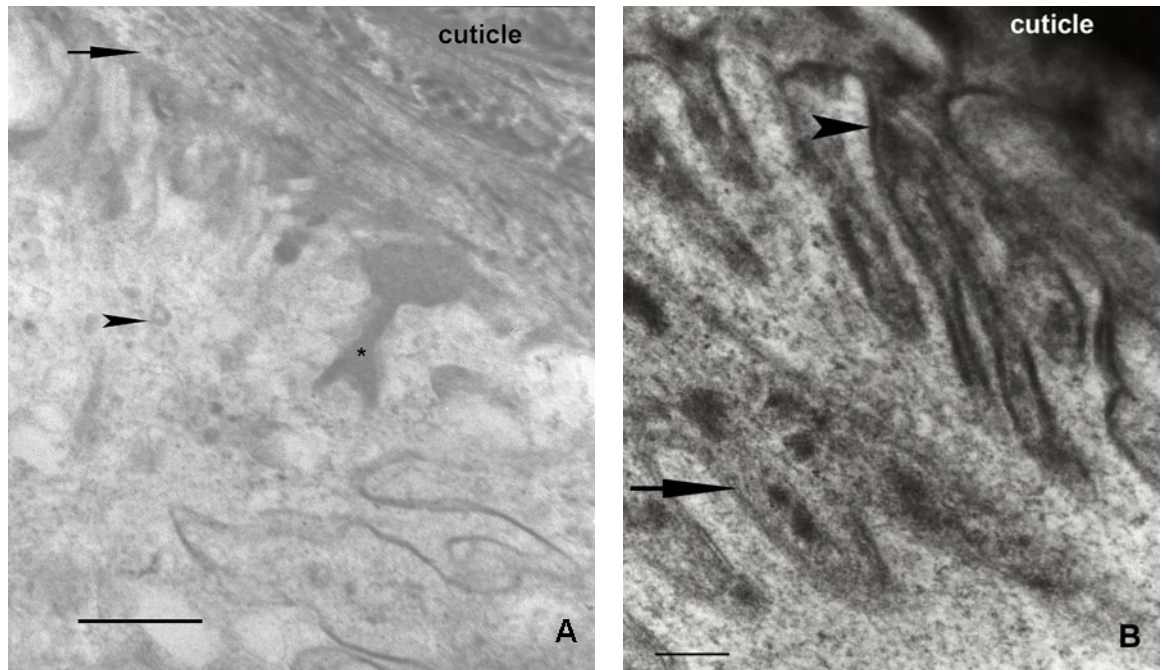
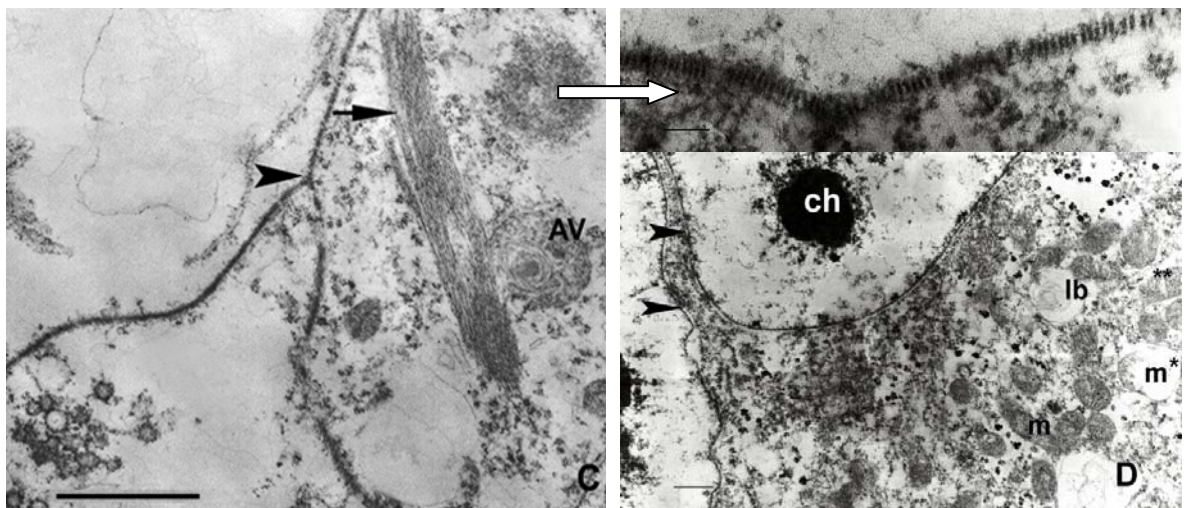


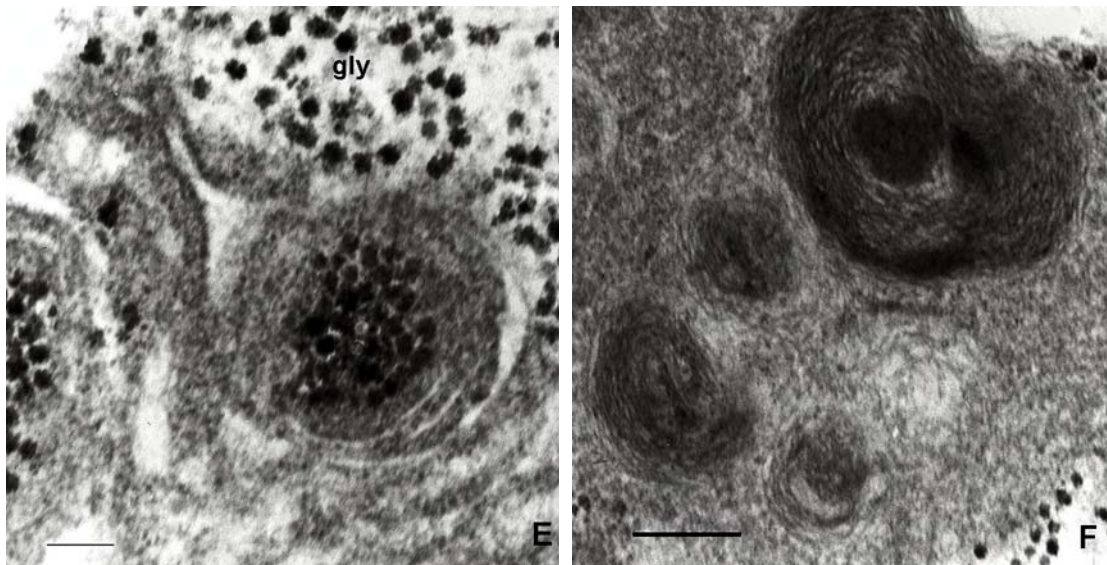
Figure 14. Electron micrographs of dehydrated hydropyle cells.

A. The inner layer of the specialized hydropyle cuticle (arrow) showing its layered structure. Deep folds are visible in the apical hydropyle cell membranes directly below the endocuticle, some of which are “filled” with electron dense material (*). The arrowhead shows what appears to be a microsome (bar = 1 μ m); **B.** Highly convoluted lateral hydropyle cell membranes (arrow) and deeply folded nuclear membranes (arrowhead) (bar = 0.67 μ m).



C. Septate junctions at the basal intersection of three hydropyle cells (arrowheads). The septa were each approximately 6 – 8nm thick [seen clearly in the enlargement (bar = 0.2 μ m)] and separated cell membranes by an even 20-30nm. Tapering bundle of fibrils

approximately 3 μ m long and 0.4 μ m wide (arrow), seemingly freely suspended within the cytoplasm, can be seen alongside autophagic vesicles. The grey shadows on these micrographs are areas of varying section thickness, a result of irregular resin penetration (bar = 0.67 μ m); **D**. Deeply folded nuclear membranes of dehydrated hydropyle cells (arrowheads) and highly condensed heterochromatin bundles. Cytoplasm contains swollen mitochondria with electron-transparent central regions and dense material at the periphery (m*) and others with dilated cristae, and cytoplasmic discontinuities (**). Lamellar bodies such as the one shown here may be a degenerated mitochondrion that has collapsed on itself (bar = 0.67 μ m).



E. Lamellar bodies that may be collapsed storage vesicles or autophagic vesicles of ER origin (bar = 0.4 μ m); and **F**. Lamellar bodies with dense concentric layers (bar = 1 μ m). AV: autophagic vesicles; ch: heterochromatin bundles; lb: lamellar body; m: mitochondria.

No nucleoli were visible in any of cells viewed with the EM. Few Golgi stacks were observed and the absence of saccules filled with electron dense contents on the maturing face of the stacks suggested that they were not very active. Vacuoles were slightly oval to round in shape with boundary membranes, although these were not always intact (Figure 15C). Clusters of electron dense granules were visible throughout the cytoplasm in both desiccated and rehydrated cells, although the distribution did not follow any uniform pattern (Figure 15D). These clusters ranged in size from 0.06 – 0.1 μ m and were observed freely

distributed in the cytoplasm, clustered around the perimeter of vacuoles, sequestered inside membrane-bound vesicles, and in some instances, forming dense bands around vacuoles just outside the basal membranes of hydropyle cells. These characteristics most closely fit the description and size of glycogen clusters.

In desiccated cells, microvilli lengths were 0.2 - 0.5 μ m. Nuclei appeared to be more folded than those observed in rehydrated cells, not so much with respect to the depth of the folds, but rather the degree (i.e. the apposing edges of the folds were closer together [Figure 14D]). Nuclear chromatin in most nuclei observed had a very condensed appearance. The mitochondria in desiccated cells had gaps that seemed consistent with dehydration and/or rehydration damage, cellular aging or fixation artifacts. Damage was also evident in the form of cytoplasmic gaps and as membrane discontinuities (Figure 14D). Endoplasmic reticulum (ER) with ribosomes appeared in short profiles, and spherical structures 150 - 600nm in diameter were noted below the apical cell boundary. It is not clear whether these structures were vesicles or dilated ER profiles viewed in cross section (Figure 14A). They had the appearance of microsomes in that they were of a similar size (measurements across ER profiles suggest that the normal diameter in cross section would be closer to 30 - 40nm) and appeared to have ribosomes attached to the outer surface (as described by Weiss, 1988). Whorls of concentrically arranged ER, while largely degraded, were noted in the most dehydrated cells. Figure 14C shows another arrangement that could be identified as desiccation-damaged ER, but the dimensions and general appearance suggest that it is more likely that this was a bundle of microfilaments, as described by Zissler and Sandler (1982). Coated pits and a number of infoldings were noted in the basal cell membranes, while coated vesicles 70 - 133nm diameter (not shown) were observed in the cytoplasm, suggesting biochemical traffic between the egg and hydropyle cells.

The most noticeable characteristic of dehydrated hydropyle cells was the increased autophagy of cytoplasm and organelles into membrane-bound vacuoles (Figures 14C and 15E). Lamellar bodies were also more common, suggesting increased membrane turn-over. The lamellar bodies shown in Figure 14E have the appearance of storage vacuoles that have at least partially emptied their contents, after which the surrounding membranes may have collapsed to form the layers

observed. Lamellar bodies with densely interleaved layers were also observed (Figure 14F); it is not clear whether these were lytic vacuoles or poorly fixed organelles. Rehydrated cells also contained autophagic vacuoles (Figure 15E), but they were not as numerous as in dehydrated cells.

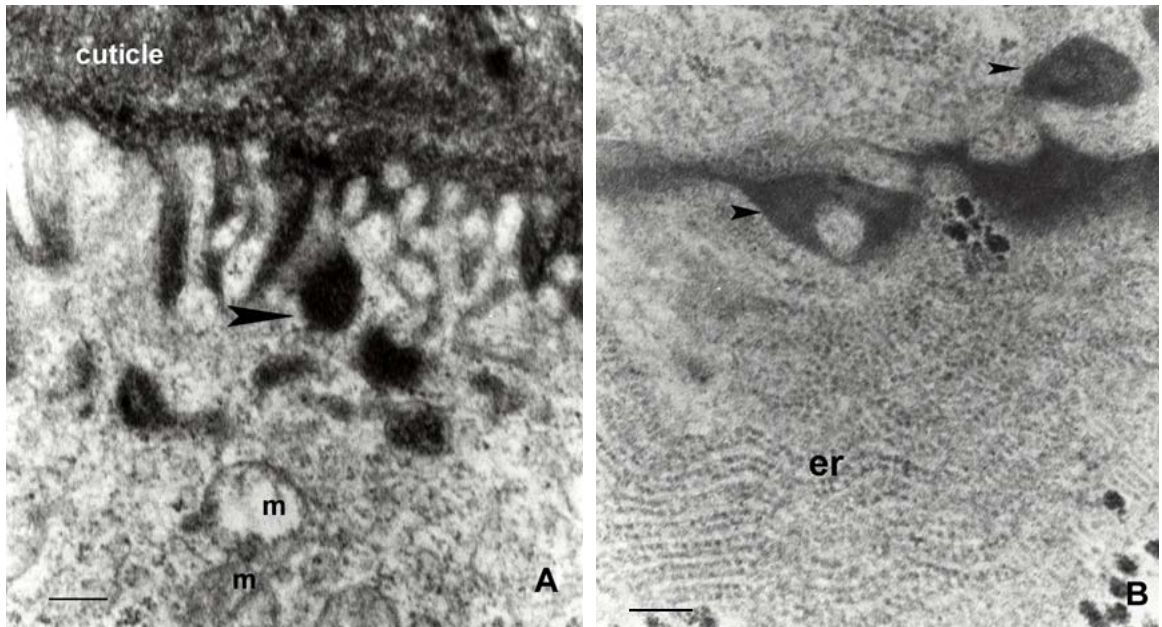
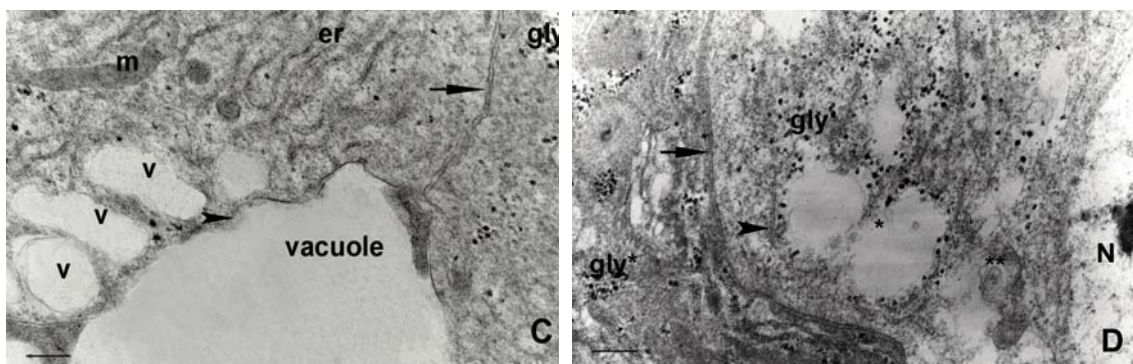


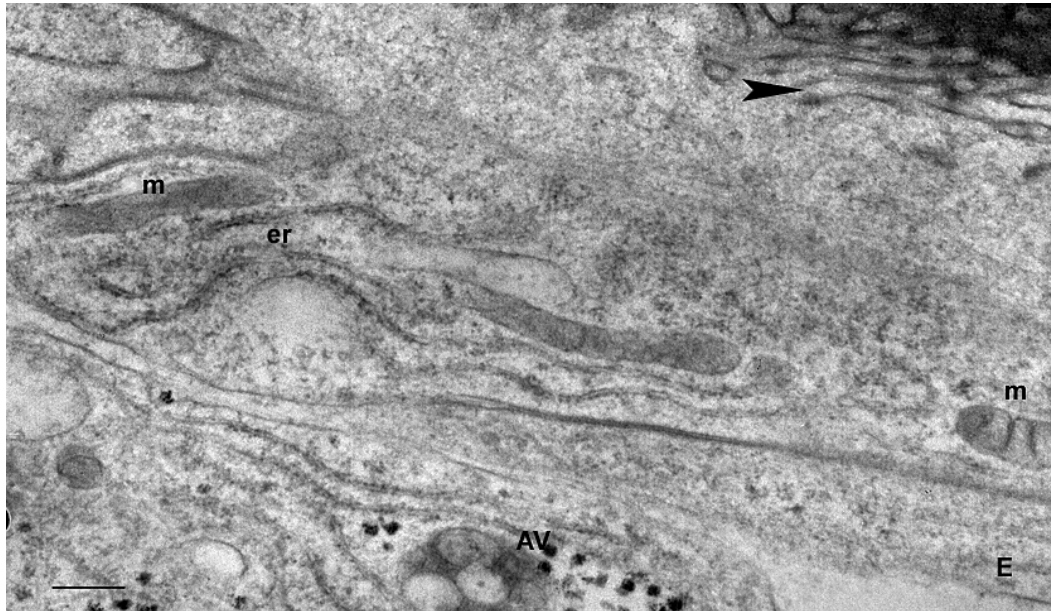
Figure 15. Electron micrographs of hydropyle cells from rehydrated eggs.

A. Electron dense vesicles just below the microvilli (arrowhead) at the apical surface and mitochondria that were still in a damaged or degraded state can be seen in the cytoplasm (bar = $0.33\mu\text{m}$); **B.** An electron dense intrusion or matrix running just below the cuticle (arrowheads) (bar = $0.33\mu\text{m}$).

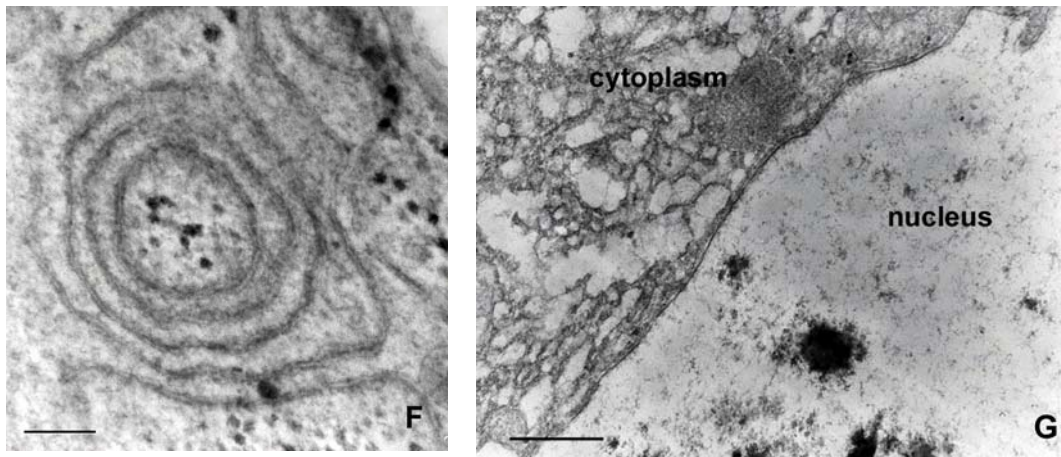


C. Larger vacuoles can be seen just inside this basal hydropyle cell membrane (arrowhead). Enclosing vacuole membranes are variously intact. Very large extracellular vacuoles can be seen below the basal membrane, toward the interior of the egg. Arrow: lateral cell membrane (bar = $1\mu\text{m}$); **D.** Glycogen rosettes distributed through the

cytoplasm in a scattered manner or clustered. Persistent evidence of damage or degeneration include short, disrupted ER profiles (arrowhead), discontinuous membranes (*) and swollen and disrupted mitochondria (**). Arrow: lateral boundary between two hydropyle cells –differences in the appearance of cytoplasm between adjacent cells was common (bar = 1 μ m).



E. The cytoplasm of rehydrated cells showed longer ER profiles, intact mitochondria and deeper surface folds (arrowhead) (bar = 0.5 μ m).



F. Whorl of concentrically arranged ER (bar = μ m); **G.** Nucleus and cytoplasm of a degenerating hydropyle cell that had detached from the cuticle. The nucleus is swollen with electron transparent contents, while the cytoplasm is very condensed and confined to a small band around the nucleus (bar = 0.67 μ m). c: cuticle; AV: autophagic vesicle; er:

endoplasmic reticulum; gly: glycogen; gly*: glycogen clusters; m: mitochondria; v: vacuole; ves: vesicles.

Rehydrated hydropyle cells seemed to regain full turgidity while retaining many of the features seen in dry cells. The most noticeable changes were that microvilli increased to 0.5 – 1µm in length, suggesting increased absorption. Mitochondria and endoplasmic reticulum were more abundant and appeared to have regained some structural integrity. Numerous mitochondria of varying conditions were present in the cytoplasm as well as longer endoplasmic reticulum profiles that were sometimes arranged in parallel. These were seen both between the apical boundary and nucleus, and between the nucleus and basal boundary (Figure 15E). Numerous long profiles of ER were observed close to the apical boundary (Figure 15B) and some shorter and more dispersed profiles of ER with ribosomes attached were noted (Figure 15D). The concentrically arranged whorls of ER observed in desiccated cells persisted in rehydrated cells (Figure 15F). Free ribosomes were also apparent in the cytoplasm of a few hydrated cells. Heterochromatin bundles were less condensed in the nuclei of some cells and the intercellular differences in chromatin appearance observed in light micrographs was also evident in electron micrographs.

Apart from the changes in the depth of the microvilli, the most noticeable difference in rehydrated cells was the larger size of the vacuoles, particularly those basally located. As mentioned, some cytoplasmic, mitochondrial and membrane discontinuities were still visible (Figures 15C, D and E) and these features could have been due to poor fixation. However, they were consistent across all cells viewed with the EM, some of which were clearly not affected by poor fixing or resin impregnation. This implies that some of the observed damage may well have been due to the rigors of drying or rehydration and/or cellular aging. To avoid confusing the possible contributing causes of cellular damage, and to ensure that any morphological comparisons made are valid, the discussion of ultrastructural changes will be restricted to features that are least influenced by preparation (example: presence, proximity, number or size of organelles etc.).

Cells that had started to degrade were evident in both dehydrated and rehydrated eggs. Figure 15G is of a hydropyle cell from a rehydrated egg that had moved some distance from the cuticle already and was in the process of disintegration. The nuclei of these cells had a distended appearance, with nucleic material confined to one end of the nucleus. The remainder of the space inside the nucleus was electron-transparent. The surrounding cytoplasm was restricted to a narrow band around the nucleus and this cytoplasm had a dense appearance, with no recognizable organelles visible. Outside of this band of cytoplasm, complete sub-cellular dissolution was evident.

Figures 15C and E are noticeably superior to others shown here. These were taken from sections from one particular rehydrated 40-week-old egg (RWC 145%). Apart from variations in resin penetration, sections were clear, cytoplasm seemed intact, and organelle integrity seemed robust. This egg was still in anatrepsis and the serosal strand was a bit stretched but intact. The only feature that separates this egg from the others is that uptake of water during pre-experimental hydration was very low - only 0.5mg.

CHAPTER 4 DISCUSSION

Almost all comments on structure made in this chapter are based on observations from sections taken from older eggs. Results from the older eggs were markedly better than those achieved from younger eggs. The lack of results from breeding attempts using the 12-week-old eggs suggests that these eggs were not very robust to begin with, which would explain their poor responses to the rigors of drying/hydration cycles and preparation protocols for microscopy. These eggs had been collected at the tail end of the breeding season at the PPRI in Pretoria during which time breeding cages were no longer taken outdoors during the daylight hours. While attempting to establish a successful breeding population at the university, I. Kambule (pers. comm.) found that viable eggs could not be produced when the cages containing breeding hoppers were kept indoors. It would appear that a certain amount of sunlight is necessary for the production of viable and robust eggs.

4.1 Water content

The pattern of changes in mean egg mass, as well as the changes in cell- and organelle size, shows that eggs lose water during dry periods, and absorb water again during periods of hydration, even after being subjected to harsh drying conditions. The rate of water loss during drying from this study showed a similar trend to those of Matthée (1951), but varied somewhat in scale. These differences could be due to, firstly, the lower relative humidities used in this study. Using quiescent eggs that had been hydrated until they were turgid, the combined mean rate of water loss shown by Matthée's eggs during the first 48 hours in the desiccator was approximately 0.0115 mg/egg/hr. These eggs had been kept at 35°C and, while Matthée did not specify the RH in his desiccator, he had been using 60% RH in all the other sections of his study. The greatest mean rate of loss shown by eggs in this study was 0.1571 mg/egg/hr measured after the first 48 hours for 12-week-old eggs in the control chamber at 22% RH. The lowest loss rate shown by these eggs during that same period was 0.0325 mg/egg/hr measured for 40-

week-old eggs at 15% RH. The lower values recorded for the older eggs could be attributed to their superior condition as rate of water loss from brown locust eggs is in part dependent on the physiological condition of the egg (Matthée, 1951).

Different relative humidities affect the rate of drying and time taken to reach equilibrium (Pammenter et al., 2002). *Locustana* eggs will lose water at all humidities below 100% RH (Matthée, 1951) and quite obviously, greater osmotic gradients would produce more rapid rates of drying and shorten the time needed for the egg to be in equilibrium with the surrounding air. Air humidity in *L. pardalinas* natural breeding environment fluctuates from approximately 95% in the early morning to 10% or lower in the afternoon, and in the soil, humidities lower than 20% are common (Matthée, 1951). While this is comparable to the humidity in the desiccators used in this study, the eggs used had been stripped of all their protective coverings: soil, pods, and foam plugs had been completely removed, and the chorion and waterproofing layers had been compromised. The unspecialized cuticle was most likely permeable by the time that the eggs were placed in the desiccators because the wax layer would have become discontinuous due to the extreme stretching experienced during initial hydration (Matthée, 1951), and the absence of the chorion on most of the eggs would have decreased their resistance to desiccation (Matthée, 1951). The chorion has a mesh-like structure with tangled struts that form small air spaces (Hartley, 1961 for *Locusta migratoria migratorioides*; Slifer and Sekhon, 1963 for *Melanoplus differentialis*; observed with EM for *Locustana pardalina* in this study but not shown) which readily fill with water (Hartley, 1961).

Repeated handling of the eggs also affects the rate of loss of egg mass (Shulov and Pener, 1963) and this would have been amplified in the eggs used in this project if the cuticle and sealing layers had already been compromised during stretching. Lastly, individual eggs were placed directly onto dry soil after hydration and not left on the soil that they had been hydrated on, which would have held some moisture for a little while.

Under field conditions, quiescent eggs that have absorbed sufficient water to become turgid will slowly dry out if insufficient water is available (Price, 1982). Shulov and Pener (1963) found that the mass of *S. gregalia* egg pods reduced

gradually over a 12-week period at 100%RH (but no contact water), with no obvious levelling off. While field conditions for *L. pardalina* egg pods are quite harsh (see Chapter 1) it seems unlikely that the disappearance of contact water would be instantaneous. There would possibly be some condensation on the surface of eggs in pods once the contact water had been removed (Shulov and Pener, 1963) and it seems logical that the humidity in the closed confines of the pods would be slightly higher than the surrounding soil. Also, water in an unsaturated soil is subject to adsorption and capillarity (Hillel, 1971), which would ensure that a thin film of water adhered to the surface of the eggs. Adsorbed water on the egg surface may even be present over the hydropyle area, depending on the angle of contact, due to capillarity. This remaining film of water would require a suction force greater than the forces of interfacial attraction to remove it (Childs, 1969).

Slow drying is required for most species to survive dehydration (Danks, 2000) as many of the adjustments (discussed in section 4.6) that provide protection against dehydration are specific, and the necessary processes take time and require energy. What is not known is how long it takes for the RH in *L. pardalina* egg pods to reach low levels (20% or lower) after they have received moisture from rainfall, or how long turgid eggs in their pods take to become air dry, i.e. in osmotic equilibrium with surrounding dry air, under typical dry field conditions. Vegetative plant tissue that takes longer than five days to become air dried under natural conditions would be classified as slow-drying. These plants therefore have more time to induce protective mechanisms under field conditions (Farrant et al., 1999). It would most likely take longer than 5-7 days shown here and by Matthée (1951) for individual eggs to stop losing significant amounts of water, assuming that this implies that the egg is in equilibrium with the surrounding air at this point. It is also likely that the highest losses (i.e. within the first 48 hours) would be much lower than those shown here and elsewhere (Matthée, 1951). It is therefore possible that eggs in the field actually have a longer time period to initiate, or accumulate, protective measures against the effects of desiccation stress.

From observations only, the lower limit of tolerance, in terms of water content, was somewhere between 50-70% RWC, not 40% as determined by

Matthée (1951). Sections that showed hydropyle cells that appeared dead or severely damaged were all from eggs with RWCs in this range. It is not clear how Matthée (1951) calculated egg water contents in his study and it is possible that the harsher drying conditions in these experiments could have contributed to these mortalities.

4.2 Variations between eggs

The observed variations in eggs – mass, relative water contents and development – of the same age are to be expected as collection dates were approximate, and in the case of the 12-week old eggs, which had apparently been collected over a two week period, the variation in collection periods could have been considerable. Even in studies where egg age is specified, there is likely be some variation as the oviposition-to-collection period is not always precise (Shulov and Pener, 1963), unless only those pods that are collected as the female is seen to oviposit are used. Variation between eggs of the same pod that were observed is also common (Shulov and Pener, 1963). Contributing factors, other than diapause or non-diapause conditions, may include relative position of eggs within the pods and the concomitant distribution of contact water within the pod (see Appendix 1 for observations made during this study), and variation in covering membrane integrity and, therefore, response to handling (Shulov and Pener, 1963).

Observed differences in cell morphology – without accompanying observable changes in embryos – between eggs of the same age and treatment level suggest that development had progressed in some eggs and that this progression was noticeable in the hydropyle cells but not yet in the embryos.

Matthée (1951) described how hydropyle cells absorb water, both passively and actively, until the embryo undergoes catatrepsis and the serosal strand has broken. After this, the cells will remain adjacent to the hydropyle, but will gradually degenerate. Hydropyle cell structure, as shown here, seems to support these findings: apical surface infoldings (microvilli) become deeper with hydration; hydropyle cells, their nuclei and their cytoplasmic vacuoles progressively increase in size with continued hydration (also Kambule et al.,

2005); and maximum vacuolation is achieved by the beginning of catatrepsis (Figure 11).

This pattern implies uptake of contact water by the hydropyle cells during development at least up until catatrepsis is underway and the strand has snapped. Hydropyle cells that show greater degrees of swelling and vacuolation may belong to eggs that are closer to catatrepsis. Those in which some or all hydropyle cells are variously detached from the cuticle could then be said to be yet closer to, or in, catatrepsis. While these features were evident in the hydropyle cells, there were no accompanying changes in the embryos that were observable at the gross level. Therefore, it may be possible to track subtle developmental changes by the structural appearance of the hydropyle cells when the structure of the embryo appears unchanged.

Noticeable differences seen in these sections were that the hydropyle cells of hydrated eggs did not necessarily remain in their position adjacent to the hydropyle cuticle throughout catatrepsis. It is suspected that as the serosal strand stretches, particularly during rapid uptake of relatively large amounts of water by eggs that are pre-catatreptic, the pulling action may cause some hydropyle cells to detach from the cuticle (Figures 9B and 11). Repeated wetting and drying cycles might also cause incremental detachment of hydropyle cells with each cycle.

There were a number of hydropyles from eggs in which the strand had broken during initial hydration. The embryos in these eggs must have been ready to enter catatrepsis at the initial hydration, after which too much water was lost during drying for the embryo to complete development to hatching. This is what tends to happen if eggs are exposed to very low humidities after initiation of catatrepsis (Matthée, 1951) that includes serosal strand breakage. Hydropyle cells appeared to have died but were more closely apposed to the cuticle (see Figure 10E) than cells from eggs in which the strand had broken only after the rehydrating process (Figure 11). Rapid drying sometimes prevents serosal strand breakage as the “cushion” of water between the hydropyle and embryo diminishes quite quickly, causing the strand to relax (Matthée, 1951), but this evidently was not the case for these eggs. This means that eggs in which the strand had broken, and which therefore did not survive desiccation, were from the outset at a slightly later

developmental stage than eggs that did survive desiccation and only had the strand break at the second hydration (or “rehydration”).

Clearly the developmental status of the embryo has some influence on its ability to survive desiccation stress. The appearance of the hydropyle cells, because of their close association with embryonic development, is largely determined by the stage of embryonic development

4.3 Absorption and secretory activity in hydropyle cells

Deeply folded cell membranes are, according to Slifer and Sekhon (1963), characteristic of surfaces that are absorbing. The infoldings that are seen in the apical cell membranes of hydropyle cells, which have been called microvilli here appear to be considerably deeper than the fine microvilli observed at the surface of hydropyle cells of *M. differentialis*, which are more common to cells that are secreting, not absorbing (as noted by Slifer and Sekhon, 1963). This suggests that the hydropyle cells in *L. pardalina* eggs may be more active in absorption rather than secretory activities.

It was expected that some signs of protein synthesis and secretion would be observed: this would support Matthée’s (1951) findings that hydropyle cells secrete a waterproofing protein (or similar substance) layer, a process which would have to occur repeatedly given the regular cycles of drying and wetting experienced by *L. pardalina* eggs. Evidence for protein synthesis and secretion would have included: the presence of a nucleolus (Weiss, 1983), which should be very obvious in EM (Hay, 1968); extensive rER; saccules with electron-dense contents on the maturing face of the Golgi apparatus as well as a general abundance of vesicles with electron-dense contents (Harrat et al., 1999); and mitochondria closely apposed to the rER-Golgi continuum (Weiss, 1983). In addition, cells engaged in large scale, active transport of materials across a membrane would have mitochondria held within the folds of the membrane (Weiss, 1983). While mitochondria in rehydrated cells were mostly found in the same peri-nuclear region as the ER, there were no other indicators of possible protein synthesis and secretion. However, the presence of vesicles at the apical

boundary suggests that there is some secretory activity at that surface (Figures 8, 10F, 11 and 14A).

Depression of transcription and translation in the nucleo-cytoplasmic compartment and within the mitochondria is one of the features of metabolic depression (Tauber et al., 1986; Hand and Podrabsky, 2000). Consequently, one would expect RNA synthesis to be substantially reduced. Quiescent hydropyle cells therefore have conflicting interests during periods of drying that follow hydration: they need to downregulate metabolic activity in the cells (which may already be quiet in diapausing eggs), and at the same time engage in metabolically expensive activities such as the production of waterproofing substances.

The absence of a nucleolus, while confusing, does not entirely exclude the possibility that the cell may be producing such a substance. Once serosal cells have stopped dividing, they attenuate and polyploidize their nuclei through endomitosis (Heming, 2003), hence the abundance of heterochromatin in the nuclei of hydropyle cells. However, embryonic cell cytoplasm is known to contain positional information or cytoplasmic determinants that control the behaviour of the cells (Wolpert et al., 1998) and it may be this characteristic that dictates production of the waterproofing substance for the hydropyle region. Matthée (1951) conducted histological tests to determine the composition of the waterproofing substance and commented that the positive stain for protein was very pale. It may be then that the amount of protein substance that is needed to waterproof that area is so small that large-scale production of proteins may not be necessary. Serosal cells are in general capable of producing and secreting proteins, as they secrete the cuticle and wax components of the surface layers as well as the hatching enzymes that degrade the cuticle (Heming, 2003).

4.4 Effects of hydration and rehydration on hydropyle cells

4.4.1 Egg, cell and organelle size

As previously mentioned, whole eggs, their hydropyle cells and the organelles in the cells show structural responses to varying moisture regimes, expanding when hydrated and shrinking when dried. Egg shrivelling is possibly an adaptation that

minimizes mechanical damage from turgor loss in the egg during drying. The cuticle does not become permeable during desiccation as the continuity of the wax layer over the unspecialised cuticle is not disrupted by folding and collapsing of the egg during desiccation (Matthée, 1951). The equivalent in tolerant plants would include structural features such as leaf folding and flexible cell walls (Farrant et al., 1999).

Likewise, hydropyle cells and nuclei are able to maintain shape and resist mechanical stresses of desiccation as they have a gel framework and are elastic, a feature typical to animal cells (Yost, 1972). Cell walls are highly deformable and can fold to accommodate changes in cell volume, thus avoiding the likelihood of damage from negative turgor. Membranes around nuclei, mitochondria and plastids in dry cells of tolerant seeds are also known to maintain shape and conserve membranes through folding (Walters et al., 2002).

4.4.2 General subcellular anatomy

Most hydropyle cells in this investigation showed signs of desiccation stress, but this was possibly seen in conjunction with the effects of cellular aging (given an egg age of approximately 40 weeks and the fairly advanced developmental status of the embryos, i.e. late anatrepis/ early catatrepsis) as well as fixation and/or rehydration artefacts. In cells that are showing degradation due to aging, cytoplasm becomes less dense with areas of lysis and degenerates to a point where the cells are partially empty but still have nuclei, which may appear to have lost some or all of their contents (Del Bene et al., 1998). Endoplasmic reticulum, while present, are less regularly arranged, have shorter cisternae (Harrat et al., 1999), and may be swollen or degenerating (Del Bene et al., 1998). Saccules on Golgi apparatus are transparent and mitochondria appear swollen with dilated cristae and twisted membranes (Harrat et al., 1999) or may be transformed into spheroidal bodies with dense lamellar material at the periphery and an electron-transparent central region (similar in appearance to that shown in Figure 14D). The appearance of areas of glycogen (as in Figure 14D) is a result of the change in cellular metabolism associated with aging (Harrat et al., 1999). There might therefore be a number of

factors contributing to the structural changes seen in hydropyle cells and these might be interchangeable.

Membrane continuity was variable in hydropyle cells from both dehydrated and rehydrated *L. pardalina* eggs. Membranes are usually the primary site of desiccation damage: dehydration of biological membranes results in fusion and lipid phase transitions that causes them to rupture or leak (Danks, 2002) so that structural and functional activity of the membranes are often irreversibly damaged (Crowe et al., 1984). In desiccated seeds and pollens, most damage is due to membrane leakage (Alpert and Oliver, 2002). Lesions or structural perturbations would be evidence of increased membrane breakdown and permeability, but it seems as though some disruptions may not be resolved with TEM (Wesley-Smith et al., 2001). Electrolyte leakage is commonly measured in plant tissue (using a conductivity meter) and rate of leakage calculated. This is used as an indicator of membrane integrity, and therefore the extent of membrane damage, in dry and rehydrated tissue (Sherwin and Farrant, 1996).

Electron micrographs of hydropyle cells from eggs that developed without drying cycles (I. Kambule, pers. comm.) show discrete vacuoles with clear, intact membranes. Vacuole membranes in cells that were dried and rehydrated showed varying degrees of continuity (Figures 15C and D). This did not seem to adversely affect the cells ability to supply water to the developing embryo: vacuoles were still able to expand during hydration and embryos were still supplied with sufficient water for development to hatching. Some mixing of vacuolar contents with the cytoplasm does occur, as is sometimes seen in rehydrated plant tissue (Wesley-Smith et al., 2001).

Autophagic vesicles that were observed seem to be common in desiccated cells and are often a feature of desiccated plant cells (Wesley-Smith et al., 2001). Cells use autophagy to break down cellular components to provide energy for survival during starvation. This is also a mechanism to remove worn-out organelles (Weiss, 1983), or in this case, ones that are not essential for survival, or those that are potentially susceptible to desiccation damage, or those that have been damaged during desiccation. Autophagy may be an important survival mechanism and appears to be triggered almost immediately by stress signals in plant cells (Wesley-

Smith et al., 2001). Plant cells subjected to sucrose starvation have been reported as using autophagy of cellular reserves to generate substrates for mitochondrial respiration. Hydrolysis of substrates during autophagy could cause an increase in osmotically active solutes within cells, and in so doing possibly reduce water loss (Wesley-Smith et al., 2001).

Other observed changes in organelles of hydropyle cells in brown locust eggs that have been associated with drying and/or rehydration in plant cells include the following: mitochondria swell and cristae are disrupted but regain normal structure after rehydration (Alpert and Oliver, 2002). Endoplasmic reticulum typically shows a varied response to desiccation stress and conformational changes such as the concentric whorls seen here (Figure 14F) are common. These whorls have been associated with cessation of growth in drought-stressed plant cells, with the inhibition of protein synthesis in heat-shocked plant cells, and are presumed to indicate the probable onset of metabolic stress (Wesley-Smith et al., 2001). Endoplasmic reticulum may also play a role in the formation of autophagic vesicles (Weiss, 1983) perhaps such as those seen in Figure 14E. Some profiles of ER have also been seen to remain fairly fragmented and dispersed in rehydrated seed axes (Wesley-Smith et al., 2001). Lastly, nuclei with condensed chromatin may also be a sign of desiccation injury, as observed in plant cells (Wesley-Smith et al., 2001). The similarities listed here suggest that some of the structural changes observed in hydropyle cells may very well have been caused by desiccation stress and that the cells have mechanisms of protection and/ or repair in place.

4.4.3 Vacuolation

Hydropyle cells from solitary brown locust eggs that have been laid and stored (at 2-4°C) for a very short period in dry soil, removed from pods and incubated on moist soil at 30-31°C, have a similar appearance to the cells observed in this study in that the nucleus is large with evenly dispersed heterochromatin bundles, is seen to increase in size and move further away from the cuticle edge with progressive hydration, and cell area also appears to increase with hydration (I. Kambule, pers. comm.). Noticeable differences between these and the older eggs used in this study are that: 1) the cytoplasm of younger eggs appears smooth and continuous as

opposed to granular and coarse as in the older cells used in this study; and 2) vacuolation is less extensive in younger eggs. After the first 48 hours of hydration a few large and discrete vacuoles were evident in the basal region of only some cells from young eggs and further hydration appeared to cause further vacuolation (I. Kambule, pers. comm.), but this was still far less than that observed in the older eggs. These differences could be attributed to the differences in breeding conditions and collection regimens employed for each, as well as to cellular responses to drying cycles.

Older eggs from the PPRI (Pretoria) stock had been subjected to an unknown number of wetting and drying cycles prior to use. For these eggs, soil in the laying pots had been moistened regularly to encourage oviposition (see Chapter 2) and the periods between oviposition and collection were varied, during which time the soil would most probably have been moistened fairly regularly. It therefore seems likely that some eggs (certainly non-diapause eggs) could have had the opportunity to absorb some water by the time they were collected from the cages and already had vacuolated hydropyle cells prior to storage, which is essentially a dry period. Additionally, vacuoles could remain reasonably intact during cellular drying (discussed below) and this would mean that older cells that had been through a cycle or two of wetting and drying would be vacuolated from the outset and would be proportionately more so after further experimental wetting and drying cycles. Variations in the appearance of cytoplasm between young and older eggs are most likely due to degradation associated with cellular aging, as discussed.

The combination of observed increases in microvilli length and vacuole size with hydration may support earlier speculation (Matthée, 1951) that hydropyle cells absorb exogenous contact water which possibly travels through the cytoplasm in vacuoles to supply the developing embryo. The fact that the relative area occupied by vacuoles does not decrease with drying suggests that this may be an anatomical feature of hydropyle cells that allows them to maintain water, or resist loss, as much as possible. The same trend in vacuole retention during drying has been observed in plant tissue and its function is to fill cytoplasmic space, thus minimizing mechanical damage due to loss of turgor (Farrant et al., 1999).

However, water in the vacuoles in plant cells is replaced with compatible solutes and this makes them increasingly sensitive to osmotic swelling upon rehydration. A very rapid rate of water uptake after extreme desiccation may cause imbibition-related damage to vacuoles (Wesley-Smith et al., 2001), which could cause the kind of rupturing of vacuole membranes seen in sections from hydropyle cells.

4.5 General discussion relating to structure

This study has shown that the primary role of hydropyle cells in *L. pardalina* eggs is most likely the provision of water to developing embryos. It is very likely that they are also responsible for waterproofing the hydropyle cuticle when necessary, as speculated by Matthée (1951). It was also shown that the eggs (and hydropyle cells) are capable of surviving rapid and extreme desiccation, even without the protective covering of the egg pod and in most cases, without a full chorionic covering.

The accepted test of tolerance is whether an independently functioning organism can resume normal activity upon rehydration (Pammenter et al., 2002; Walters et al., 2002). Brown locust eggs meet this criterion as unprotected turgid eggs lose an average of 38% of their water content during fourteen days drying at 15% RH and yet still develop to hatching after rehydration.

Hydropyle cells of the eggs show subcellular damage during drying and some reversal of damage after rehydration. Damage is most often measured by reversibility of changes (Walters et al., 2002) and is not necessarily indicated by differences in morphology, ultrastructure or metabolism between the dry and hydrated state, as these may just be consequences of water removal and may be completely reversible once water is provided. While it seems unclear whether changes are consequences of drying, or protective strategies, or signs of damage (Walters et al., 2002), desiccation remains a stress even in tolerant tissues. No tolerant organisms have an infinite life span in the dehydrated state and damage is accumulated in this state (Pammenter et al., 2002)

In theory, truly desiccation tolerant tissues will have all the mechanisms to tolerate all the anatomical, biochemical and biophysical changes that result when

cells go through cycles of dehydration and rehydration. More realistically, Vertucci and Farrant (1995) define true desiccation tolerance as the ability to maintain sufficient structural integrity during drying to repair damage when water becomes available. Furthermore, tissues that survive desiccation most likely employ a combination of strategies to limit damage, and that the level of tolerance can be seen as a function of the effectiveness of those strategies. Farrant et al. (1999) maintain that different mechanisms for tolerance are employed by plants that dry slowly and those that dry rapidly. It is proposed that *L. pardalina* eggs employ a combination of strategies that enhances tolerance to drying and facilitates survival of both slow and rapid drying. Behavioural, morphological, physiological, developmental and metabolic adaptations contribute to tolerance in *L. pardalina*, but Alpert and Oliver (2002) claim that, for plants, tolerance is mostly a result of cellular features, or properties, that play a major role in protection and repair.

Structural features that retard water loss are important elements in tolerance. Reducing the rate of water loss allows for the establishment of protective measures but when loss of water is rapid, there is usually no time to do so. In this latter scenario, plants have a constitutive protection mechanism during drying and rehydration that is always in place. This mechanism takes the form of constant levels of sucrose and late embryogenesis abundant (LEA) proteins. This does not prevent damage altogether though and repair mechanisms, evident as the synthesis of ‘recovery’ proteins called rehydrins, are also employed (Alpert and Oliver, 2002). Brown locust eggs are subject to repeated and irregular instances and periods of drying and appear to be able to withstand both slow (in the field) and rapid (under experimental conditions) drying regimens. Repeated cycles of dehydration and rehydration would in theory necessitate rapid protective responses (Farrant et al., 1999) and it seems likely that brown locust eggs, and in particular the hydropyle cells examined here, would have some sort of pre-existing protective mechanism in place to enable such a response, rather than one that has to be mobilised whenever the need arises.

Many organisms that withstand desiccation express the disaccharide trehalose (Guo et al., 2000) which can replace water around the phosphate head group of phospholipids, thereby stabilizing dry membranes (Crowe et al., 1984).

Insects can apparently also utilize trehalose without hydrolysis to glucose and in *M. differentialis* eggs it is the principal free, neutral sugar, with diapause eggs containing twice as much as infertile eggs (Randal and Derr, 1965). Beenackers et al. (1981) found that diapausing eggs of *Bombyx mori* show an increase in the accumulation of lipids that supposedly provide the energy required during diapause (via the oxidation of fatty acids).

Matthée (1951) similarly found that cytoplasm of hydropyle cells in quiescent eggs gave a slight positive stain for fats. It is not possible to confirm this finding here and further studies using cytochemical techniques for the detection of proteins and lipids (Harrat et al., 1999) are needed to identify macromolecule contents in hydropyle cells of eggs in different phases, stages of development and with varying hydration levels. It seems logical that diapause *L. pardalina* eggs would contain a supply of protective macromolecules as they would need to be able to endure periods of dormancy regardless of the environmental conditions. It is not entirely clear whether all brown locust eggs have the potential to be diapause eggs and may therefore all be supplied with necessary protective elements at oviposition, as it seems as though diapause can either be determined by female phase prior to laying or environmentally induced after laying (Matthée, 1978). It certainly seems that, in general, the diapause or quiescent condition in brown locust eggs would most likely confer added tolerance to drying as they have a low ‘metabolic mass’ of developed tissue. They would therefore already be metabolically “quiet” due to developmental status so that very little depression or downregulation of metabolism would be necessary before rates suitable for survival of anhydrobiosis were reached (Guppy and Withers, 1999).

From the results seen here it is also evident that the stage of embryonic development in *L. pardalina* has some bearing on the eggs ability to withstand drying. It is important that the embryo is still in anatrepsis at the initiation of desiccation and, more importantly, that the serosal strand is intact. In other words, catatrepsis might be imminent, but can’t actually have begun. Matthée (1951) made similar observations for *L. pardalina* and Shulov and Pener (1963) had also noted that *S. gregaria* eggs that had not reached late anatrepsis before drying, and then lost too much water during drying, would not develop past anatrepsis.

Similarly in plants there is an association between developmental status (usually late embryogenesis) and the effect of drying rates, as well as the lower limit of desiccation tolerance (Chandel et al., 1995; Vertucci and Farrant, 1995; Camplans et al., 1999).

4.6 Technique

Insect eggs in themselves are notoriously difficult to fix, regardless of their hydration status, partly because of the barrier provided by the resistant cuticle membranes (Zissler and Sandler, 1982). Successful penetration of egg tissue with resin has proved here to be as difficult to achieve as successful fixation. As mentioned in Chapter 2 and shown in Chapter 3, problems were experienced with both fixing and embedding procedures used on hydropyle tissue in this study. While these were not necessarily uniform in extent, the persistence of certain problems suggests that a particular set of preparation criteria needs to be established for this tissue. This would typically be achieved by making comparisons of specimens fixed and embedded by different means, as suggested by Bowers and Maser (1988).

Bowers and Maser (1988) also state that the measure of the quality of a fixation process is how well the fixed specimen reflects its living state. Van der Willigen et al. (2001) did not fix plant tissue (vegetative), but viewed sections by LM in a medium that was of comparable osmotic potential, which was measured psychrometrically. While it would be helpful to view hydropyle cells without fixing, it would also be useful to determine the osmolarity of the cells natural milieu so that biologically relevant parameters for fixation methods can be established. The point of this is would be to determine the conditions that minimize the artefactual distortions and volume changes, such as breaches in membrane integrity, cytoplasmic spaces, and swollen organelles that occur during fixation (Bowers and Maser, 1988). Tissue blocks as small as 1mm^3 can show non-uniform volume changes during fixation: glutaraldehyde causes shrinking while OsO_4 may cause swelling, and then dehydration, infiltration and embedding would cause shrinkage again (Hayat 2000). It may therefore be advisable to experiment with

buffers of varying osmolarities, perhaps using non-ionizing, osmotically active substances (or non-electrolytes, as suggested by Dawes, 1988) for use with glutaraldehyde in fixing hydropyle tissue.

Given the strong case made for anhydrous or freezing protocols, it may be also useful to attempt one or both of these on *L. pardalina* eggs so as to rule out the possible inclusion of rehydration artefacts in dried (or partially hydrated) tissue that are supposedly caused by rapid imbibition during fixing in an aqueous solution (as in Wesley-Smith et al., 2001). Fixing the tissue over osmium vapour may be an alternative method worth attempting as this technique is used on plant material by other workers at the university (D. Mycock, pers. comm.).

EPON (and in particular EPON 812 that was used in these experiments) is a high viscosity resin that polymerizes much faster than most other resins, which limits the time for infiltration into tissue. Propylene oxide, on the other hand, has a very low viscosity (Mollenhauer, 1988). Three plastic resin embedding protocols have recently been used in our laboratory: two different recipes for EPON resin (used in this study) and Spurr's resin (used by I. Kambule). Spurr resin mixtures are harder with a low viscosity that supposedly allow for more infiltration time, and this is the most common protocol for embedding plant tissue with their tough cellulose walls (Mollenhauer, 1988) and should theoretically be suitable for use on hard and resistant tissue (Komplarens, 1988), such as that of the hydropyle, but this has not been shown to be so (I. Kambule, pers. comm.).

The most successful sections were those of the 40-week-old eggs used in this study. While the preparation of all tissue was identical, the condition of the eggs themselves may have contributed to the variation in results seen here, so it seems reasonable to speculate that the procedure used here may be a good starting point for experimentation with recipes. The only adjustment that was made to the standard protocol used in the laboratory was to increase the number of steps in the infiltration process. The rationale was that a more gradual process that started with mixtures with higher propylene oxide ratios, and therefore lower viscosity, would improve penetration of the EPON 812. This may provide a reasonable basis for further work as it is obvious that the technique needs to be manipulated until the optimal protocol is reached.

Many of the sections showed signs of chatter, which is the most common sectioning problem with EPON (Mollenhauer, 1988). However, it was determined that, while rare, the cause of the chatter in this instance was primarily system-induced, as it was discovered that there were high frequency vibrations from external machinery that affected the ultramicrotome. There were also problems with movement in the resin sections under the electron beam as they were not properly supported at the edges, specifically at the cuticle edge where the resin had not penetrated (see Figure 10B). This caused extreme compression in some areas that would otherwise have been suitable for viewing and also made it difficult to photograph the cuticle as it simply shrivelled and folded over under the electron beam. If infiltration that secures the outer edge of the cuticle to the resin material is not possible, a support film could be used to decrease the drift and breakage of tissue caused by the electron beam, as described by Hayat (2000). Static electricity in the laboratory, which is common in the dry South African highveld winters, also proved problematic as it makes collection of the sections onto grids very difficult and causes extensive damage in the sections. Use of a humidifier in the lab, as well as perhaps a “roughing” of the grid surface by rinsing it in absolute acetone immediately before use (Hayat, 2000) might help to minimize these problems.

4.7 Conclusion

Based on observations made on the limited number of sections in this study, it seems that: 1) hydropyle cells provide water to developing embryos and may provide hydropyle cuticle waterproofing; 2) in addition to having structural and morphological features that retard water loss, *L. pardalina* eggs are able to protect against desiccation damage and/or repair damage upon rehydration, as the absence of either mechanism would have shown irreversible damage across all cells observed; and 3) the developmental status of the embryo affects tolerance – eggs that are in the later stages of anatrepsis are somehow better equipped to deal with more extreme conditions. Ongoing experiments, as suggested throughout this discussion, are needed to support these findings, particularly as this is the first study of its kind.

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APPENDIX 1

Development without incubation in freshly laid eggs

Egg pods that were intended for the section of study that involved incubation and development without desiccation were collected from first generation females that had been reared at the university from PPRI eggs. Eggs were removed from a few pods and incubated at 30 – 31°C on moist soil till hatching. The soil was kept moist by adding water every 24 hours with a dropper and fungal growth was treated by washing the affected eggs with a 2% sodium hypochlorite (NaOCl) solution, followed with a few rinses in distilled water, after which the egg was returned to the incubator. Hatched hoppers were reared under the identical conditions as the PPRI cultures, as described in Chapter 2, and pots were checked daily for pods (I. Kambule, pers. comm.).

Pods (seven) were removed from the pots after laying and incubated whole on moist soil at 30 - 31°C with 24 hour rotation and wetting. These eggs were much smaller than the PPRI eggs used in the desiccation experiments i.e. the parental eggs ($n=134$, $P<0.001$, Kruskal-Wallis). They were also exceptionally fragile and could only be handled after three days of incubation; even then, they ruptured upon contact with the brush. To minimize handling, I only attempted to separate diapause and non-diapause eggs in four pods, and then only after the seventh day of incubation (i.e. after the hydropyle cells would have differentiated). When the pods were opened, it was evident that there was differential uptake of water by the enclosed eggs, in that those situated at the periphery of the pod were more swollen than those toward the core. Also, eggs that had come loose from broken pods showed considerably greater amounts of swelling. Eggs were then weighed and incubated directly on moist soil for 48 hours during which they absorbed a significant amount of water ($P<0.0001$, Mann-Whitney Test). There were only eight out of 159 eggs that did not show any increase in mass, suggesting that they may have been diapause eggs. These were kept separate from the non-diapause eggs. Eggs from the remaining three pods were removed and incubated directly on moist soil at different stages that were determined by the state of the individual eggs, i.e. whether they were robust enough to be handled.

Five to eight eggs from each pod were randomly sampled on days zero, 2, 5, 7, 9, 12 and 14. Embryos (if any) and hydropyles were dissected out from the egg under 4% gluteraldehyde for staging and processing for microscopy. These incubated cultures were particularly prone to fungal infections and because of the delicate state of the eggs, treatment with 2% NaOCl resulted in very high egg losses. Hydropyles were fixed with the chorion intact. All attempts to remove the chorion in these eggs failed and repeated handling with the brushes caused extensive damage. It was also not possible to improve the quality of eggs produced by hoppers that had been bred from the original PPRI batch (I. Kambule, pers. comm.): while the eggs used were viable in that they could be incubated to produce live hoppers that were capable of breeding, no second generation hoppers were hatched.

Very few of the eggs that had been prepared for microscopy showed any signs of development of hydropyle cells at all. Even serosal cells were not to be seen in the majority of eggs. Of all the eggs sampled, only a few showed signs of embryonic development, with two embryos developing to the D/ D' stage, but these appeared to be very small when compared to the embryos removed from the older eggs in the desiccation experiments. Only two successfully prepared eggs, sampled at the ninth day of development, showed the presence of a hydropyle when viewed under the light microscope. Of these, only one appeared to have gone through a normal developmental process. The hydropyle region was reduced in extent, but the cells were nonetheless large with the general appearance noted in the desiccation experiments (see Chapter 3).

This section of the study therefore had to be abandoned. However, a subsequent study performed at the university by Kambule (in progress) does include observations of hydropyles in eggs incubated without desiccation and results from this study have been referred to in the main text.

APPENDIX 2

Preliminary analysis of egg mass data

TABLE 1A. Derivation of grouping for analysis

Desiccating chamber	Chamber 1	Chamber 2	Chamber 3
Eggs (n)	33	68	55
Age of eggs	Approx. 40 weeks	Approx. 12 weeks	Approx. 12 weeks
Status	“Treatment”	“Treatment”	“Control”
RH	15%	22%	22%
Sampling days	0,2,5,9,12,14*,16	0,2,5,7*,9	0,2,5,7,9,12,14,16,23
Initial grouping	0 = pre-experiment 2 = early ^a 5-9 = intermediate ^a 12-14 = late ^a 16 = rehydrated	0 = pre-experiment 2 = early ^b 5 = intermediate ^b 7 = late ^b 9 = rehydrated	None
Revised grouping** for pooled results	0 = pre-experiment 2 = early 5 = intermediate 9-14 = late 16 = rehydrate	0 = pre-experiment 2 = early 5-7 = intermediate 9 = rehydrate	None
* indicates day of re-hydration ** done according to number of days of drying ^a indicates NS (P>0.05) groups for eggs from Chamber 1 ^b indicates NS (P>0.05) groups for eggs from Chamber 2			

TABLE 2A. Statistical analysis (Kruskal-Wallis) of changes in mass of desiccated and hydrated eggs from Chamber 1 using initial grouping.

Comparison	P value
Pre-experiment vs. Hydrated	*** P<0.001
Pre-experiment vs. Early	ns P>0.05
Pre-experiment vs. Intermediate	ns P>0.05
Pre-experiment vs. Late	ns P>0.05
Pre-experiment vs. Rehydrated	*** P<0.001
Hydrated vs. Early	ns P>0.05
Hydrated vs. Intermediate	*** P<0.001
Hydrated vs. Late	*** P<0.001
Hydrated vs. Rehydrated	ns P>0.05
Early vs. Intermediate	ns P>0.05
Early vs. Late	ns P>0.05
Early vs. Rehydrated	ns P>0.05
Intermediate vs. Late	ns P>0.05
Intermediate vs. Rehydrated	*** P<0.001
Late vs. Rehydrated	*** P<0.001

Summary of data				
Group	Number of eggs (n)	Median	Minimum	Maximum
Pre-experiment	33	12.000	9.400	13.400
Hydrated	33	14.400	10.200	16.700
Early	5	12.500	11.900	13.200
Intermediate	8	10.650	8.600	12.500
Late	9	9.100	5.400	11.300
Rehydrated	5	17.900	14.600	20.100

TABLE 3A. Statistical analysis (ANOVA) of weight changes in desiccated and hydrated eggs from Chamber 2 using initial grouping.

Comparison	P value
Pre-experiment vs. Hydrated	ns P>0.05
Pre-experiment vs. Early	* P<0.05
Pre-experiment vs. Intermediate	*** P<0.001
Pre-experiment vs. Late	*** P<0.001
Pre-experiment vs. Rehydrated	ns P>0.05
Hydrated vs. Early	*** P<0.001
Hydrated vs. Intermediate	*** P<0.001
Hydrated vs. Late	*** P<0.001
Hydrated vs. Rehydrated	ns P>0.05
Early vs. Intermediate	ns P>0.05
Early vs. Late	ns P>0.05
Early vs. Rehydrated	ns P>0.05
Intermediate vs. Late	ns P>0.05
Intermediate vs. Rehydrated	*** P<0.001
Late vs. Rehydrated	*** P<0.001

Summary of data				
Group	Number of eggs (n)	Median	Minimum	Maximum
Pre-experiment	68	14.800	4.900	22.100
Hydrated	68	16.450	7.400	22.700
Early	11	9.700	5.800	15.800
Intermediate	12	6.000	3.500	11.500
Late	13	7.300	3.200	12.500
Rehydrated	22	13.400	5.200	22.500

ns = not significant

* = significant, 95% CI

*** = extremely significant, 99% CI

TABLE 4A. Analysis of weight changes in desiccated eggs before and after revised grouping (ANOVA).

Results for Chamber 1 vs. 2, or “treatment” groups are shown. Changes in eggs from Chamber 3 were consistently different from those in Chamber 1, but not from those in Chamber 2.

Dessication level	Initial grouping	Revised grouping
Early	ns P>0.05	ns P>0.05
Intermediate	*** P<0.001	ns P>0.05
Late	ns P>0.05	N/A

ns = not significant

*** = extremely significant, 99% CI

APPENDIX 3

Analysis of changes in cell morphology

TABLE 5A. Data for cell morphometrics

Statistical analysis	Data parameter	Dehydrated	Rehydrated
Unpaired <i>t</i> -test			
Cell area (μm^2)			
P<0.0001, <i>t</i> = 6.880(34 df)	Mean	435.01	1074.8
	# of points	23	13
	SEM	55.468	75.355
	Minimum	110.03	547.88
	Maximum	1160.2	1602.5
	Median	426.37	1105.9
Cell breadth (μm)			
P<0.0001, <i>t</i> = 4.419(34 df)	Mean	16.579	23.856
	# of points	23	13
	SEM	1.086	1.040
	Minimum	7.493	18.722
	Maximum	25.282	32.678
	Median	16.913	23.283
Cell length (μm)			
P<0.0001, <i>t</i> = 8.262(34 df)	Mean	23.237	45.636
	# of points	23	13
	SEM	1.576	2.291
	Minimum	12.803	29.308
	Maximum	41.689	59.384
	Median	23.075	46.826

Each value represents the mean $\pm$ SEM of measurements taken from at least thirteen different cells across five hydrated and six dehydrated eggs.

TABLE 6A. Data for subcellular morphometrics

Statistical values (One-way ANOVA)				
Comparison	Cuticle-nucleus	Nucleus area	Nucleus breadth	Nucleus length
Early vs interm.	ns P>0.05	ns P>0.05	ns P>0.05	ns P>0.05
Early vs late	ns P>0.05	ns P>0.05	ns P>0.05	ns P>0.05
Early vs rehyd.	* P<0.05	* P<0.05	* P<0.05	ns P>0.05
Interm. vs late	ns P>0.05	ns P>0.05	ns P>0.05	ns P>0.05
Interm. vs rehyd.	* P<0.05	* P<0.05	* P<0.05	ns P>0.05
Late vs rehyd.	ns P>0.05	* P<0.05	* P<0.05	ns P>0.05

ns = not significant, * = significant, 95% CI

Summary of data					
Group	# of eggs	SEM	Maximum	Minimum	Median

Cuticle – nucleus (µm)					
Early	5	1.053	3.317	9.491	7.254
Intermediate	7	1.419	4.501	14.604	7.459
Late	5	1.012	6.329	11.341	7.813
Rehydrated	5	3.062	10.693	27.659	13.233
Nucleus area (µm²)					
Early	5	39.266	151.31	384.36	314.61
Intermediate	7	27.540	192.67	409.48	328.91
Late	5	49.709	174.79	432.44	274.01
Rehydrated	5	65.795	313.62	725.03	488.17
Nucleus breadth (µm)					
Early	5	1.260	8.502	15.417	12.372
Intermediate	7	0.7735	9.316	14.710	14.111
Late	5	1.168	9.209	16.102	12.929
Rehydrated	5	1.260	13.311	20.755	18.040
Nucleus length (µm)					
Early	5	1.250	17.767	25.072	22.476
Intermediate	7	1.291	17.139	28.499	23.596
Late	5	2.376	15.595	28.468	21.113
Rehydrated	5	2.014	23.597	34.920	26.172

Each value represents the mean ± SEM of measurements taken from at least five different cells, from at least five different eggs, per treatment.