INVESTIGATION OF EGG DEVELOPMENT IN THE BROWN LOCUST, *LOCUSTANA PARDALINA* (WALK.) (Orthoptera: Acrididae).

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science.

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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

Locustana pardalina (Walk.) eggs have the ability to survive during drought. Diapause and quiescence, both types of dormancy, play a major role in contributing to brown locust survival under arid conditions by preventing immediate hatching and allowing build-up of eggs in the soil which contributes to swarming. This study investigated the water balance, hydropyle cell structure, tracking of development and the metabolic rate of eggs in different states.

The eggs have the ability to resist desiccation and to survive water loss when it occurs. *Locustana pardalina* eggs consist of 66 % water and can lose almost all the water during desiccation.

Hydropyle cell structure showed morphological structures such as lateral infoldings supporting evidence of active rather than passive water uptake. We showed that water absorption was immediate in non-diapause eggs and limited in diapause eggs. There was a general increase in hydropyle cell nuclear area and cell height during water absorption.

We measured the metabolic rate of diapause and non-diapause eggs and directly linked these to embryonic development. Day 6 after laying seems to be the point at which some of the embryos in anatrepsis enter diapause and others continue development. The metabolic rate of non-diapause eggs increased exponentially until hatching while that of laboratory and field diapause eggs maintained a low stable metabolic rate. Eggs subjected to alternate drying and hydration showed adaptability by stopping development, lowering their metabolic rate while still maintaining the embryo.

Locustana diapause and non-diapause eggs have the ability to control water absorption, resist desiccation and survive water loss. The maintenance of low stable metabolic rate of desiccation resistant diapause eggs contributes to the success of *Locustana* in harsh environments.

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CHAPTER 1 INTRODUCTION

The brown locust, *Locustana pardalina*, Walker (Orthoptera: Acrididae) occurs in the semi-arid regions of the central Karoo of South Africa and is therefore subjected to dry conditions. The majority of acridids are found in semi-arid geographic regions, implying that aridity must be favourable for these insects (Uvarov, 1957). The brown locust usually completes 2 - 4 generations per year and is therefore termed multivoltine (Lea, 1962). "The minimum requirements for life of the brown locust population are a little grass to eat, some bare ground on which to bask in the sun and enough wet or dry soil, in which to lay eggs"(Lea, 1962). The brown locust occurs over the entire Northern Cape, including parts of the western and Eastern Cape together with southern Namibia (D. Brown, pers comm.). Locust swarms may spread to most parts of the Karoo region in plague years. Large swarms occur sporadically every 7-11 years (Lea, 1968; De Villiers, 1988; Nailand & Hanrahan, 1993). Locust swarming will reoccur which is why its biology needs to be understood.

The brown locust occurs in two phases, the swarming / gregarious phase and the solitary phase. There is an intermediate phase known as transiens phase (Matthée, 1951). A feature distinguishing the brown locust from most other locusts is that the transiens / gregaria phase hopper is larger than the solitary phase hopper and has an orange and black colouration and a constricted pronotum. Solitary locust hoppers are of uniform colouration, usually green and grey while gregarious ones are brown, black on a yellow or orange background (Matthée, 1951). There is a correlation between the phase of the adult locust and the type of egg laid. Fully gregarised adult brown locusts are extremely active, flying both day and night and laying only non-diapause eggs in vast egg beds which may hatch to cause major swarm problems.

True solitarious forms are relatively sedentary and only undertake night flights (Price, 1988). They do not form swarms and are thought to lay mainly diapause eggs. These eggs can accumulate in the soil and will hatch after adequate rainfall, causing unexpected swarms. Both types of eggs have the ability to survive repeated dehydration and rehydration. Transience phase adults lay both diapause and non-diapause eggs (Matthée, 1951). Understanding the physiology of the brown locust eggs could be a key to understanding swarming.

The brown locust owes its success for survival in adverse conditions of the Karoo to a number of factors relating to its eggs and their development. On oviposition, the foam covering of the egg pod is reinforced with soil particles forming a hard case or protective covering which protects the eggs against soil compression and desiccation (Hinton, 1981b). Ewer (1977) conducted a study to determine functions of the foam plug in acridid eggs. The foam plug allows gaseous exchange between the egg mass and the soil surface and is also used as an escape route during hatching (Ewer, 1977). The brown locust has a relatively thicker and stronger froth plug than *Locusta migratoria migratorioids* (R. & F.) and *Schistocerca gregaria* (Forsk.) (Ewer, 1977). Petty (1973) reported that the foam covering of the brown locust reduces water loss from eggs under desiccation. Eggs in a pod lose water at a slower rate compared with eggs not protected by the pod (Petty, 1972).

The eggs themselves are also drought resistant. Eggs may survive in the soil up to three years (Lounsbury, 1910 and Faure, 1932).They have an impermeable cuticle and can survive up to 60 % water loss (Matthée, 1951). Hinton (1981b), for example, kept brown locust eggs at 40 ºC, 60 % relative humidity (RH) for over two years in the laboratory. Diapause eggs are a contributing factor for brown locust survival under arid conditions in that they prevent immediate hatching, allowing build-up of

eggs in the soil over a period of time. Diapause in these eggs ends after a period of 9 to 40 days after which they will hatch depending on the moisture content of the soil (Matthée, 1951). The return of favourable conditions, such as heavy rains, trigger simultaneous hatching causing outbreaks of locust swarms enabling them to migrate to alternative breeding sites (Matthée, 1951). An adaptation of the brown locust to arid environments with erratic rain is the absorption of small amounts of moisture to replace water loss without continued development (Matthée, 1951). When diapause is complete eggs may enter quiescence which can last for several years. This contributes to the survival of the embryo. Swarms arise after the build-up of solitary forms in the previous seasons (Price, 1988). The physiology of the egg may hold the key to swarming.

1.1 The egg and development of the embryo

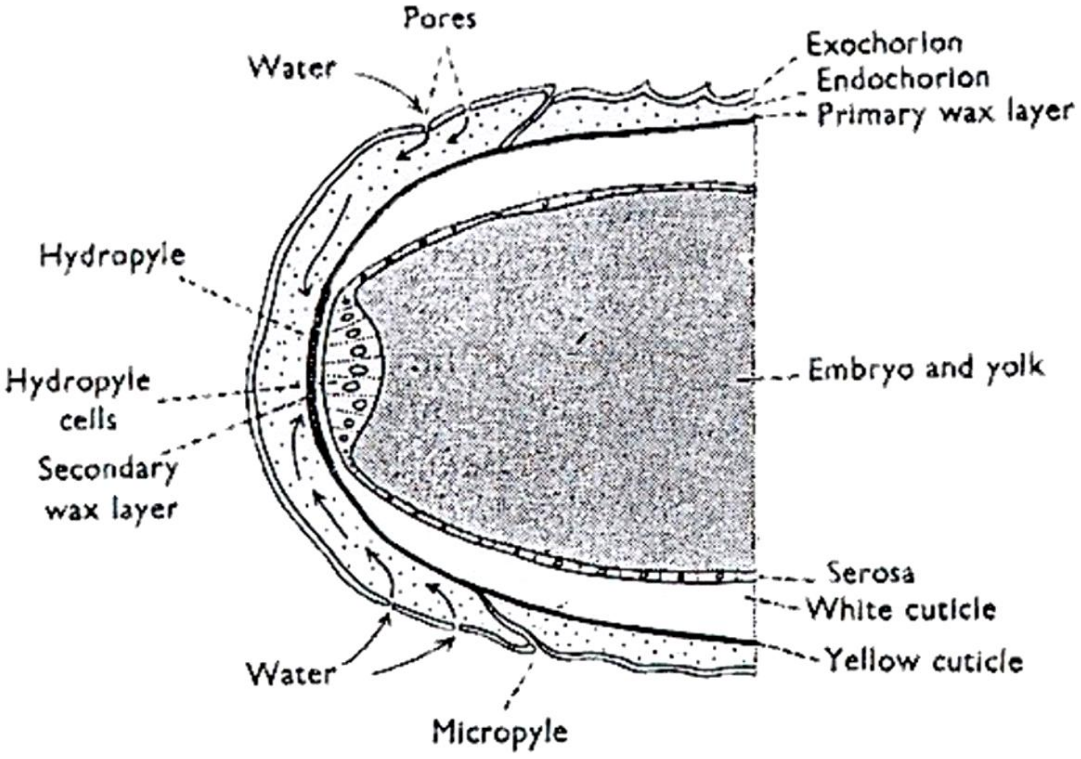


Figure 1. Diagram showing embryonic membranes at the posterior end of *Melanoplus differentialis* egg [after Lees (1955)].

The brown locust egg like that of *Melanoplus differentialis* has protective layers such as the chorion on the outside, followed by the secondary egg membranes (yellow and white cuticle) (Fig. 1). The chorion is the tough protective layer made of chitin. The chorion consists of two distinct layers, an outer exochorion and an inner endochorion (Fig. 1). Serosal cells are part of the embryo and they produce the white and yellow cuticle (Matthée, 1951). In the brown locust, the yellow and white cuticle are impermeable to water vapour and prevent desiccation (Matthée, 1951). The hydropyle is situated at the posterior end of the egg and acts as an area of water entry.

Most acridid eggs have micropyles in the chorion arranged in a ring near the posterior end of the egg (Hinton, 1981b). These are areas of sperm entry. Micropyles do not penetrate the cuticle layers secreted by the serosa of the egg.

The process of tracking development is best described by observing the external morphology of the embryo and dividing this into a number of stages (Chapman, & Whitman, 1968). Development follows these stages; development of the blastoderm, the appearance of the germ disc, differentiation into the protocephalon and protocorm, embryo segmentation and appearance of appendages, differentiation of appendages, blastokinesis, the completion of blastokinesis, a rapid growth phase leading to the completion of dorsal closure allowing the embryo to occupy the entire egg, deposition and differentiation of the cuticle on the first instar (Chapman, & Whitman, 1968). Riegert (1961) investigated the progression of development in non-diapause eggs of *Melanoplus billituratus*. Authors have come up with many different stages in egg development. Matthée (1951) describes more than 30 stages in the brown locust. There are 24 in *Melanoplus differentialis* and 20 in *Dociostaurus maroccanus* (Quesada - Moraga & Santago - Alvarez, 2000). Steele (1941) used 16 stages for *Austroicetes cruciata,* while Chapman & Whitman (1968) used 9 stages for

grasshopper embryos *Pyrgomorpha* and *Ornithacris*. The latter system was adopted by Wardhaugh (1978) for *Chortoicetes terminifera*. The lack of uniformity in the description of development has resulted in difficulty in producing a scheme that is common to all acridids. In staging the embryos Matthée (1951) initially looked at features such as germ band development, differentiation into the head and tail regions, formation of mouthparts and lastly the number of abdominal segments. Steele's (1941) classification was similar to that of Matthée (1951) except that it matched stages of development by describing the external morphology and the position of the embryo in the egg. Steele (1941) and Wardhaugh (1978) have linked embryo descriptions with illustrations for a better understanding of embryonic development. Matthée (1951) only classified embryos up to stage 30, i.e. before catatrepsis and more developed embryos to stage 30^{+}, whereas Wardhaugh (1978) continued with various stages of more developed embryos. For the purpose of this project embryos were classified according to both Matthée (1951) as he worked on *Locustana* and Wardhaugh (1978) who has better illustrations and worked on *C. terminifera* which has similar egg physiology to *Locustana*.

Following fertilization the zygote nucleus divides resulting in daughter nuclei which migrate to the periphery of the egg thereby forming the blastoderm (Johannsen & Butt, 1941). These nuclei further multiply producing thousands of nuclei, each nucleus surrounded by a small amount of cytoplasm and these are called energids. Some energids remain in the yolk to become vitellophages which are involved in yolk digestion as a nutrient source for the developing embryo. The energids, now called the blastomeres, form cytoplasmic strands into the yolk for nutrient uptake. The cytoplasmic strands begin to disappear and cell membrane formation is completed resulting in the formation of the blastoderm. Cells of the blastula thicken and enlarge

on the ventral side of the egg forming the germ band (Johannsen & Butt, 1941). The germ band, situated ventrally inside the egg, is where the embryo will develop. It forms the ventral regions of the future body namely head, body segments and appendages which later become increasingly well defined (Gullan & Cranston, 1994). The rest of the cells forming the blastoderm become part of a membrane (the serosa) that forms the yolk sac (Fig. 2). The serosal cells grow around the germ band thereby enclosing the embryo in an amniotic membrane.

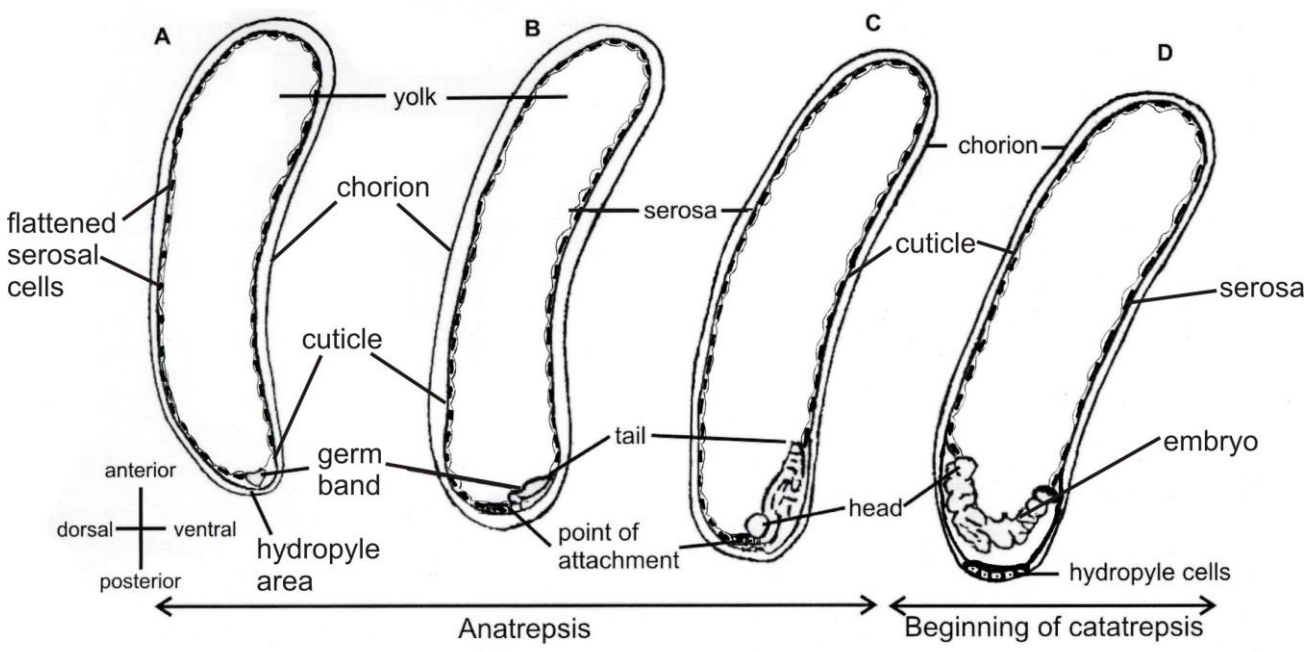


Figure 2. Developmental stages of a locust embryo showing various features not drawn to scale [modified from Lees (1955), Uvarov (1966), Chapman (1998)]. A - C anatrepsis, D the beginning of catatrepsis.

In most insects the germ band lengthens and folds into a sausage shape, with the ectoderm on the outside thereby producing a two-layered embryo. The lateral edges of

the germ band fuse along the dorsal midline of the embryo's body (Evans, 1984). The embryo rests ventrally on the surface of the egg with its head facing down towards the hydropyle (Fig. 2C).

There are two major stages of development namely anatrepsis and catatrepsis. Anatrepsis is the development of the embryo up to the point prior to the rotation of the embryo (Lees, 1955). The stage of anatrepsis is shown in figures 2A & B. At the end of anatrepsis, the serosal strand still connects the head of the embryo to the enlarged specialized serosal cells termed hydropyle cells. Diapause eggs remain in anatrepsis.

Catatrepsis begins when the embryo starts turning away from the ventral pole resulting in the stretching and breaking of the serosal strand (Matthée, 1951). This stage is assumed to be after stage 30^{+} according to Matthée (1951) (Fig 2D). Catatrepsis in non-diapause eggs occurs on the 6^{th} day, followed by the hatching of the hoppers which begins on the 10^{th} day at average temperature of 30 ºC. Similar movements of the embryo have also been documented in Ephemeroptera, Odonata, hemipteroid insects (Chapman, 1998) and in most Orthoptera, such as *M. differentialis* and *Locustana* (Matthée, 1951). In the brown locust development can be delayed up to the end of anatrepsis (Matthée, 1951).

1.2 Dormancy

Dormancy in insects refers to a period of interrupted growth or reproduction. Dormancy includes both diapause and quiescence. An organism may enter and leave quiescence repeatedly during its life while diapause usually occurs only once.

Quiescence is a short term, direct and reversible response (Blackenhorn *et al.*, 2001), resulting from recurring adverse conditions, such as temperatures above 25 ºC in *D. maroccanus* (Quesada - Moraga & Santiago - Alvarez, 2000) or unfavourable

moisture conditions, such as lack of water for over 9 days after laying in the brown locust (Matthée, 1951). Quiescent eggs can become turgid under wet conditions and eventually hatch or they remain in quiescence if kept dry (Faure, 1932).

Chapman (1998) defined diapause as a delay in development which has evolved in response to regularly appearing adverse environmental conditions. This, however, does not refer to immediately prevailing adverse environmental conditions, such as a sudden drop in temperature. Diapause is a genetically programmed developmental response that occurs at a specific stage for each species (Hahn & Delinger, 2007). The development of diapause is a response to environmental stimuli such as low temperature, long day length, sometimes high temperature or drought (Yamashita & Hasegawa, 1985), the state of food, temperature and the age of the parent (Chapman, 1998). Diapause, therefore is physiologically imposed and is coordinated with the environment (Yamashita & Hasegawa, 1985).

Daylength experienced by the mother is a good indicator of diapause induction (Yamashita & Hasegawa, 1985). Daylength is used as an environmental cue for diapause induction and termination by insects with facultative diapause. The daily cycle of light and dark changes precisely with the season of the year (Yamashita & Hasegawa, 1985).

Exposure of grasshoppers about to lay to short days results in the production of diapause eggs. For example, in the Senegalese grasshopper, *O. senegalensis,* high temperatures of 40 ºC and long photoperiods of (LD 14: 10 h) result in the production of non-diapause eggs, whereas low temperatures of 25 ºC and shorter photoperiods (LD 12: 12 h) yield diapause eggs (Colvin & Cooter, 1995). Matthée (1951) showed that exposure of the brown locust adults to short days at 35 ºC yielded diapause eggs.

There are two types of diapause: obligatory and facultative diapause. Obligatory diapause is a compulsory diapause at a specific point in development occurring in insects having one generation a year (univoltine) (Yamashita & Hasegawa, 1985). Insects having two or more generations a year, that is bi - or polyvoltine, may enter into facultative diapause which is induced by environmental conditions that indicate a coming adverse condition (Yamashita & Hasegawa, 1985). For example, *Chortoicetes terminifera* (Othoptera: Acrididae) is multivoltine and undergoes facultative diapause (Wardhaugh, 1980). This is also the case in the brown locust (Matthée, 1951).

In insects, egg diapause can occur at any stage of development from just before the formation of the blastoderm to the completion of development (Lees, 1955). In *M. differentialis* diapause commences 20 days after oviposition lasting for a few weeks to several months (Bodine, 1929). Diapause occurs during late anatrepsis in both *Locustana* (Matthée, 1951) and *C. terminifera* (Wardhaugh, 1978). Matthée (1951) also found that if favourable conditions of moisture and temperature prevail, the diapause embryo becomes slightly broader and longer but will not develop further until diapause is complete.

In most diapausing species only one developmental stage exhibits diapause (Chapman, 1998). In the genus *Aedes* diapause occurs in the fully developed embryo (Yamashita & Hasegawa, 1985). In eggs of the European Tetiigoniidae (Orthoptera) an initial diapause can occur just after blastoderm formation and a final diapause close to the end of embryonic development (Ingrisch, 1986).

In *C. terminifera* (Walk.), for example, diapause potential is determined by the environment acting on the parents during their nymphal stages (Wardhaugh, 1980) and on the eggs (Hunter & Gregg, 1984). This has also been documented in brown locust eggs (Matthée, 1951). The diapause of the brown locust eggs, for example, is

terminated by exposure to 35 ºC, 60 % RH for 45 days under dry conditions (Matthée, 1951). After the termination of diapause water is absorbed, when available, for the completion of embryogenesis in several species of grasshoppers such as *Austroicetes cruciata*, *M. differentialis*, the locusts *D. maroccanus* and the brown locust (Lees, 1955).

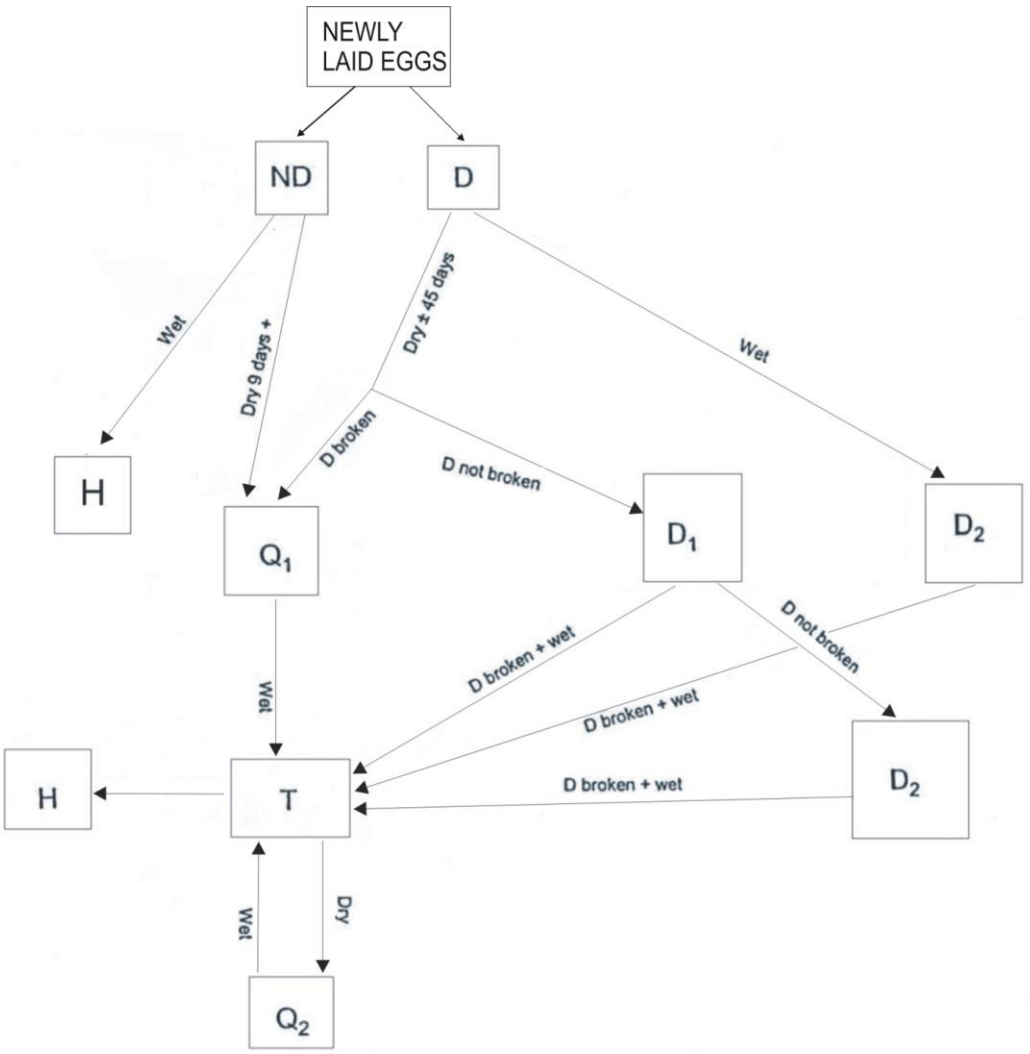


Figure 3. The interrelationship between non-diapause and diapause eggs [modified from Matthée (1951), Uvarov (1966)]. ND = non-diapause, D1 & D2 = 1st and 2nd diapause, Q1& Q2 = 1st and 2nd quiescent, T= turgid, and H = hatching.

A detailed study on egg diapause and quiescence in the brown locust has been described by Matthée (1951). Newly laid eggs can either be in diapause or non-diapause (Fig. 3). The fate of diapause (D) and non-diapause (ND) eggs is shown in Fig. 3. Development in the brown locust is promoted in both diapause and non-diapause eggs moistened immediately after oviposition. In the presence of contact moisture during incubation, non-diapause (ND) eggs develop without interruption and hatch (H) in 10 days at 35 ºC (Fig. 3). Non-diapause eggs can enter quiescence (Q) after the 9^{th} day of development; if eggs are kept dry (Matthée, 1951). This state continues only as long as the eggs are kept dry. If moistened at any point after day 9, development will be resumed. When the eggs in (Q) are moistened, they absorb water and become turgid (T). If turgid eggs are dried, they lose water becoming quiescent (Q) again. They may remain in this state for many months, but on sufficient wetting they become turgid and hatch (Matthée, 1951).

The behaviour of diapause eggs is different (Fig. 3). Diapause eggs moistened immediately after laying develop to the second diapause stage (D_2), regardless of conditions (Matthée, 1951). Diapause can be broken by keeping newly laid eggs dry for 45 days, after which the eggs develop if moistened or become quiescent (Q). After quiescence, the eggs follow the same course as non-diapause eggs (Matthée, 1951). Ninety - five percent of the freshly laid eggs will complete diapause if kept dry for ± 45 days after laying. In the other 5 %, diapause will not be eliminated, and these eggs may continue in diapause (D_1) (Matthée, 1951). If diapause is not broken, the embryos advance to the second stage of diapause (D_2). It should be stressed that quiescent and diapausing embryos are morphologically identical and their nature can only be discovered through their response to moistening (Matthée, 1951).

1.3 Water absorption and the hydropyle

Water uptake is a normal requirement for growth and development of orthopteran eggs (Lees, 1955). Water uptake usually occurs over a limited developmental period varying from species to species. It occurs prior to any significant development in *Camnula*, during early embryonic development in *Locusta*, after blastokinesis in *M. differentialis* (Chapman, 1998), before and after diapause in *C. terminifera* (Wardhaugh, 1980) and the brown locust (Matthée, 1951). When quiescent eggs of the brown locust start absorbing water as soon as they are moistened there is a marked size increase or turgidity within 24 hours. A day prior to hatching, there is a decline or cessation of water uptake through the hydropyle (Matthée, 1951).

Hinton (1981a) described three types of hydropyles, namely serosal hydropyles, serosal cuticle hydropyles and chorionic hydropyles. The brown locust and *M. differentialis* have serosal cuticle hydropyles which have been described by Matthée (1948, 1951) and Slifer & Sekhon (1963), respectively using light microscopy. The hydropyle, located at the posterior tip of the eggs, is essential for efficient water uptake in both *M. differentialis* and *L. pardalina* eggs (Matthée, 1951). It is a small circular area of modified serosal cells where a thinner layer of yellow cuticle has been secreted (Fig. 1). The serosal cuticle hydropyle in the brown locust has a proteinaceous substance, secreted by the hydropyle cells thus preventing unnecessary water evaporation through the hydropyle (Matthée, 1951). On moistening, this proteinaceous layer covering the hydropyle area dissolves and water is absorbed. When this layer is disrupted naturally or by artificial means, water enters the egg and development resumes. If water uptake is interrupted, there is re-secretion of this layer (Matthée, 1951). *Locustana* eggs have been shown to survive repeated dehydration

and rehydration conditions (R. Price, pers comm.). This is most unusual for insect eggs.

Matthée (1951) produced evidence through sets of experiments using brown locust eggs that the mechanism of water uptake is in part an active process dependent on respiration and in part due to passive diffusion of water through membranes of the hydropyle. Passive diffusion is significant during the early stages of water uptake before any stretching in the egg membranes. However, as eggs become turgid no more water is able to enter the egg through diffusion. The egg membranes, such as the serosal cuticle and the chorion, described in detail below, stretch without rupture as a result of the active water transfer from the external environment to the inside of the egg (Matthée, 1951).

The chorion is produced by the ovarian follicular cells and is composed of sclerotized proteins impregnated with wax materials (Hinton, 1981b; Evans, 1984; Nation, 2002). The chorion is brown and has no chitin (Slifer, 1945; Nation, 2002). Lipid layers found below the chorion prevent water loss (Biemont *et,al* 1981). The chorion is made up of 2 layers; the exochorion and the endochorion. The exochorion consists of material perforated by fine holes (Slifer & Sekhon, 1963). The endochorion is a meshwork layer consisting of interlocking tangled system of fine struts (Hartley, 1961). The yellow and the white cuticles are the protective material on the outside of the egg, below the chorion, which are secreted by the serosal cells (Matthée, 1951). The outer serosal cuticle is also known as the yellow or exocuticle and has no chitin. The inner cuticle, also known as the white or endocuticle consists of chitin and about 8 lamellae containing pore canals. Slifer & Sekhon (1963) reported seeing interdigital folds where the 2 layers of the cuticle meet. The innermost

layer of the endocuticle is the unspecialized dense endocuticle with thread-like structures known as wax filaments described by Slifer & Sekhon (1963).

The formation of egg membranes during development results in the reduction of water loss in the eggs. Brown locust eggs may survive a dramatic decrease in moisture content of approximately 63 % (Matthée, 1951), which is very unusual. In diapause eggs of brown locust, lost water is replaced by absorption of the available water through the hydropyle without further development occurring. In the presence of small amounts of water, the eggs become more turgid and once diapause is complete they become quiescent (Matthée, 1951).

Insect eggs tolerate only a certain fall in their moisture content below which they cannot survive no matter how resistant to desiccation they may be (Hinton, 1981b). To solve this problem, brown locust eggs have a secondary waxy layer on the yellow cuticle / white cuticle interface which is crucial in preventing water loss. This wax layer has a critical temperature above which water loss increases sharply. This temperature has been demonstrated to be 55 - 58 ºC in the brown locust (Matthée, 1951) and 42.5 ºC in *Rhodnius* (Beament, 1946).

Experiments on water loss performed in eggs of *Zonocerus variegatus* (L.) at 27 - 31 ºC and 70 - 80 % RH have shown that fluctuations in water loss and water uptake of *Zonocerus* eggs relate to the development of the serosal cuticle (Chapman & Page, 1978). Similarly in the brown locust as the cuticle is being laid down the waterproofing mechanism of the egg is increased (Matthée, 1951).

1.4 Metabolic rate during egg development

The diapause state is characterized by reduced metabolic rate and hence reduced oxygen uptake. This is a key survival mechanism which enables the insect to stretch

its food reserves to bridge the unfavourable period (Denlinger, 1978). Oxygen uptake is held at a minimum during the diapause period as has been shown by Bodine (1929) in *M. differentialis* eggs. Diapause eggs have the ability to significantly reduce their metabolic rate to a minimum for survival by switching from growth metabolic rate to one sufficient for maintenance (Hinton, 1981a).

Metabolic rate refers to the energy metabolism per unit time and can be determined from the total heat production of the organism. This gives information about the total fuel / energy used. In principle, it is the most accurate but technically very complex method. The rate of oxygen consumption by the eggs provides an index to the rate of general metabolism (Uvarov, 1966). Oxygen consumption and carbon dioxide output in *M. differentialis* eggs have been shown to rise steadily during early development reaching a maximum at about day 20. The embryo is still at anatrepsis during this stage, if it enters diapause the rate of respiration drops to a low level where it remains until development is resumed (Boell, 1935). Measurements of O_2 uptake and CO_2 output have been recorded in eggs of *M. differentialis* using the differential Bancroft micromanometer (Thompson & Bodine, 1936), in *Aulocara elliotti* (Orthoptera) using a Cartesian-diver microrespirometer into which individual eggs were placed and measured (Roemhild, 1965); in *Homorocoryphus Nitidulus vicinus* (Orthoptera: Tettigonidae) using differential capillary microrespirometers of modified Scholander & Stern-Kirk patterns (Hartley, 1971); in *Z. variegatus* (L.) (Modder, 1978) using Warburg – apparatus; in diapause and non-diapause eggs of *Eupholidoptera syrnensis* (Orthoptera: Tettigonidae) manometrically using the Warburg - apparatus (Ingrisch, 1987); using Warburg - apparatus in the silkworm *Bombyx mori* (Yaginuma & Yamashita, 1998) and using closed - system respirometry in *Manduca sexta* (Woods, 2009). Egg metabolism has not been extensively studied

with even fewer comparisons of the non-diapause and diapause states. In a few studies done on diapause eggs, it was found that oxygen uptake increased during post-diapause development (Boell, 1935; Thompson & Bodine, 1936; Roemhild, 1965) with lower oxygen uptake in diapause (Modder, 1978, Ingrisch, 1987). Woods (2009) found a rise in metabolic rate as development progressed. There is a similarity in the egg structure of *M. differentialis* to that of *Locustana* and it would therefore be interesting to investigate whether similar development metabolism occurs. However none of the studies have linked stage of embryo and their metabolic rate.

Locust swarming reoccurs and this is the reason the biology of egg survival, egg physiology and swarm build-up needs to be understood. The classical work of Matthée (1951) needs to be integrated with more recent studies on other locust egg staging, development and diapause. Long term survival and resistance to drought of the egg stage will be the key to understanding locust swarming.

1.5 Aims

This project was designed to consider the relationship between developmental structure, metabolic rate and the physiological state of brown locust eggs.

1a. To measure the percentage weight change in eggs in order to separate eggs into their physiological state.

b. To investigate water balance under different moisture conditions.

2. To investigate the structure of the hydropyle cells.

3. To track embryo development under laboratory conditions.

4. To measure the metabolic rate of non-diapause, diapause, quiescent and dead eggs.

5. To investigate the metabolic rate of eggs in response to dehydration and rehydration.

CHAPTER 2 MATERIALS AND METHODS

2.1 General methods used

2.1.1 Source of the eggs and breeding stock

Eggs were initially provided by the staff of the Locust Research Unit, Plant Protection Research Institute (PPRI) of the Agricultural Research Council. They had been obtained from brown locusts caught in the Karoo and reared in the laboratory. During March 2004 solitary adults were collected in the Karoo district, kept in the laboratory at the University of the Witwatersrand and maintained as a source of eggs.

The locusts were housed in cages (51.5 x 38.3 x 38) cm^3 under natural light with an additional 100 Watt Osram lamp to allow for temperature choice. The lamp was left on for 12 hours a day. The locusts were fed bran, freshly germinated wheat and freshly cut kikuyu grass three times a week. Two flower pots filled with sandy Karoo soil were placed on the floor of the cage as oviposition sites. Daily inspections were conducted once adults were mating to check for the presence of egg pods in the flower pots. Flower pots with holes in the soil (containing egg pods) were removed, dated and placed in the cold room at 2 - 4 ºC to arrest development. Eggs that had been in the cold room for more than several months were dissected to check their developmental stage. They had not developed passed catatrepsis.

2.1.2 Preparing and weighing eggs for the experiment

The egg pods were exposed by tipping the soil from the flower pot onto a paper towel. The egg pods were broken open and the foam covering the eggs removed using fine brushes. Individual eggs were placed in a 14 cm Petri dish on sterilized moist soil. The dishes with the eggs were then placed in an incubator at 30 ± 0.5 ºC for 24 hr. The moist soil was prepared as follows: 13 ml distilled water were added to 50 ml of

soil (modified after Matthée, 1951). The presence of excess moisture in the soil leads to fungal growth on the eggs (Petty, 1972) therefore the amount of water recommended by Matthée (1951) was reduced. Fungal growth was found to be detrimental to egg development. The fungus was removed by rinsing the eggs in 2 % sodium hypochlorite (NaOCl) (Slifer & Sekhon, 1963). The eggs were weighed individually using a camelhair brush to gently push each egg onto a piece of paper rolled it into a 'cone'. This was to avoid contamination and constant direct handling of eggs. The eggs were then placed on paper towel and camelhair brushes were used to remove the soil surrounding the egg. A small Petri dish lined with filter paper was placed on the Precisa 125A SCS balance. The balance was zeroed and the egg weighed. The mass of the egg was then recorded in milligrams (mg).

The removal of the chorion was essential when the metabolic rate of the eggs was being measured. Trial experiments on egg metabolic rate showed that eggs were susceptible to fungal attack on the intact chorion and this gave a false reading. Three methods were tested to remove the chorion. One of the easiest methods involved wetting the eggs with distilled water for a few seconds and drying them on paper towel. As cracks appeared on the chorion, fine camel hair brushes were used to peel off the chorion. A second method involved transferring eggs into a dish containing either liquid paraffin (Slifer, 1958) or Histoclear for 1 hour followed by rinses in several changes of distilled water until no oil droplets appeared on the water surface (J. Mitchell, pers comm.). The Histoclear did not break diapause but increased the visibility of the embryo within the egg. Fine camelhair brushes were used to peel off the chorion. The third method involved treating the eggs with 3 % NaOCl for 1 - 2 minutes (Slifer, 1945); followed by washing and removal of the chorion as mentioned above. This third method was the preferred method.

2.1.3 Incubation of eggs for hatching

Eggs were extracted from the egg pod and were then transferred to a Petri dish containing moist soil prepared as described previously (section 2.1.2). The eggs were arranged side by side on long straight ridges made on moist soil for efficient water absorption (Fig. 4; Slifer, 1958).

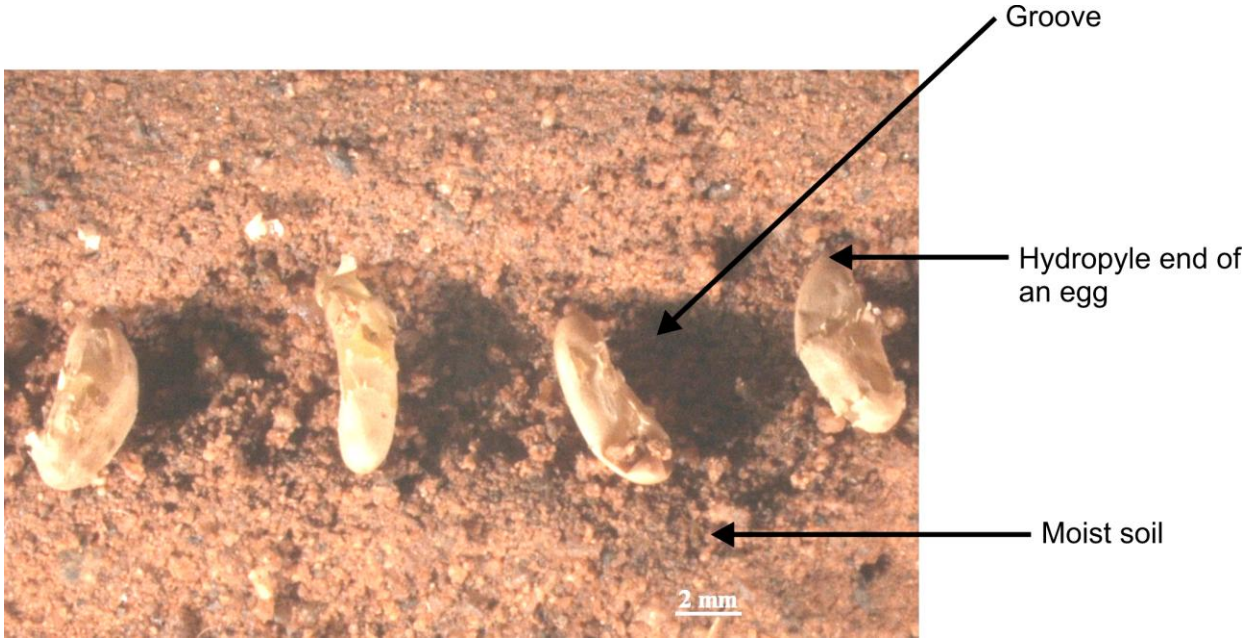


Figure 4. Dry eggs placed in a groove on moist soil for water absorption and hatching (after Slifer, 1958). Note the chorion is cracked.

2.1.4 Preparation for microscopy

The embryos and hydropyles were removed from two randomly chosen eggs as follows: the eggs were placed on a wax block and using the dissecting microscope the posterior tip of the egg, containing the hydropyle was excised using a blade. The embryos were gently squeezed out of the egg shell and the excess yolk around the embryo removed using fine needles. The relative position of the embryo to the hydropyle was confirmed. Whole embryos were fixed in 70 % alcohol (Humason, 1967) and rapidly photographed using a Nikon DXM 1200 digital camera mounted on a Nikon SMZ and a Zeiss Discovery V12 stereomicroscope.

In a trial run for transmission electron microscopy (TEM) studies hydropyles were fixed overnight in 4 % gluteraldehyde in phosphate buffer solution (PBS). Specimens were then washed in 3 changes of PBS. Post - fixation was done in phosphate buffered 1 % osmium for 1 - 2 hours. Specimens were washed in 3 changes of PBS for 5 minutes each and dehydrated through a graded alcohol series for 30 minutes each followed by 2 changes of propylene oxide for 30 minutes each. To improve EPON 812 resin (EMS, Premiere Technologies) infiltration, specimens were placed in the following concentrations of propylene oxide: Epon resin mix (3:1, 1:1, 1:3, 0:1) on a rotation mixer for 30 minutes. The specimens were then placed under vacuum to remove air bubbles and were then embedded in a plastic mould in the correct orientation and polymerized at 70 ºC for 18 hours.

Some of the tissue embedded in Epon became too brittle for sectioning and therefore hydropyles were embedded in Spurr's resin in subsequent experiments (after Spurr, 1969). The fixation, post - fixation and dehydration methods were all similar to the Epon resin protocol, with the exception that ethanol was used in the infiltration process instead of propylene oxide. Times were increased to1 hour each. Semi-thin sections prepared for light microscopy viewing were stained with toluidine blue and viewed with a Nikon DXM 1200 / SMZ microscope and digital camera. Ultra-thin sections were prepared for TEM and were stained with uranyl acetate and lead citrate, according to standard protocol and viewed and photographed with a JEOL JEM 100s TEM.

2.1.5 Determining metabolic rate

Carbon dioxide is used as the estimate of metabolic rate due to the efficiency of the analyzer. CO_2 analyzers are more sensitive than the current oxygen analyzers. The CO_2 emission of groups of 3 eggs was measured using a method similar to that

described by Woods & Singer (2001) (Figs 5 & 6). The CO_2 was measured using an infrared CO_2 analyzer (LI-CO 6262, Li - Cor, Lincoln, NE, USA).

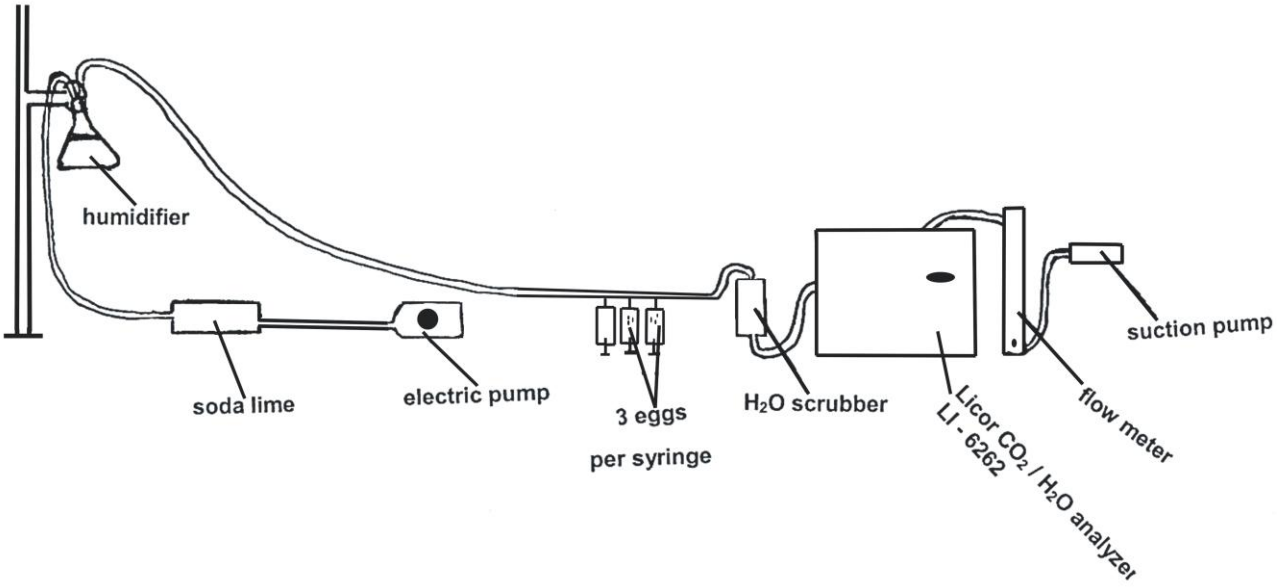


Figure 5. Experimental design for measuring metabolic rate of eggs.

Eggs were weighed, as described previously, before the analysis. They were then placed in groups of three in the respirometry chambers made from 30 ml glass syringes (Becton Dickson, Franklin Lakes, NJ, USA). A small hole was drilled through the wall of each syringe. A three way stopcock was placed between the syringe and the needle. All the connections were sealed with vaseline to prevent leakage. Three syringes were used for measurement (Fig. 5). The first syringe which was a control contained no eggs. Three eggs were placed in the second and the third syringes, away from the plunger using a fine brush. It was found from preliminary tests that three eggs were necessary per measurement to obtain reliable repeatable results.

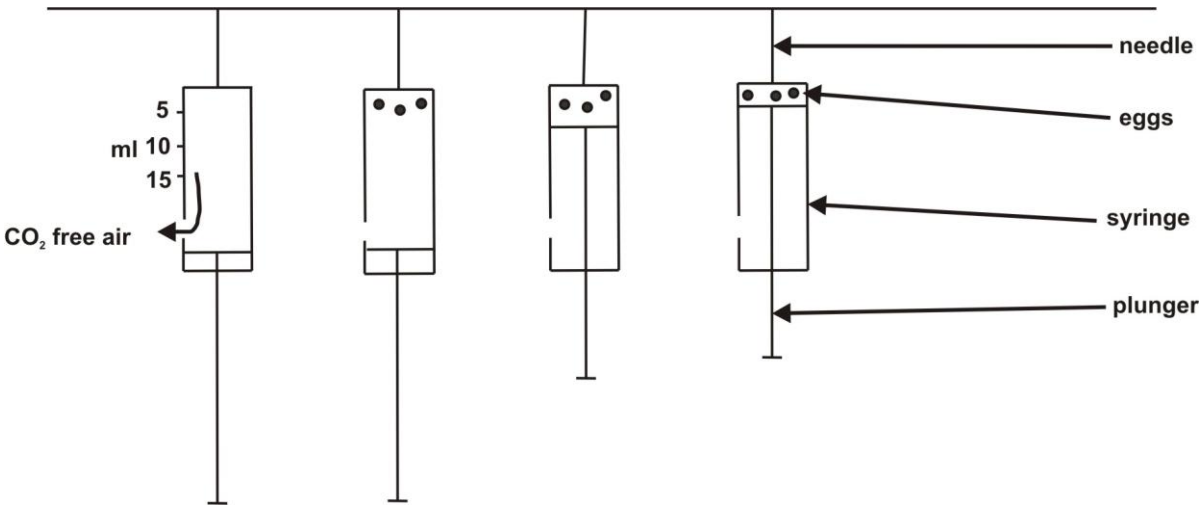


Figure 6. Sequence followed to measure metabolic rate of eggs (after Woods & Singer, 2001).

The plunger was placed away from the hole so that the chambers could be flushed with humid CO_2 free - air for 3 minutes (Fig. 6). At the end of the flushing period, the plunger was pushed down to the 15 ml mark, blocking the hole, so that all the purged air flushed through the needle. The stopcock was closed and the eggs were left in the sealed chamber of CO_2 free air for 25 minutes.

An air stream scrubbed of CO_2, using soda lime, and water, using magnesium perchlorate, was drawn through the CO_2 analyzer at a flow rate 40 ml / min, regulated by a calibrated flow meter. Into this air stream was delivered boluses of 5 ml of air from each syringe in sequence before the water scrubber. This was repeated. The amount of carbon dioxide in the injected sample was converted from ppm (parts per million) to µL by integration of the CO_2 curve. Datacan V (Sable systems, Las Vegas, NV, USA) program was used to record and analyze the data.

2.2 Separating diapause and non-diapause eggs

In the trial experiment, 334 eggs were incubated as described in sections 2.1.2 and 2.1.3. These eggs had been stored in sand in the cold room for more than 2 weeks prior to the start of the experiment. The following assumption was made: mass gain is equal to water absorbed. The eggs were extracted from the egg pods and weighed individually before and after 24 hours of incubation on moist soil at 30 ± 0.5 °C. Mass gain of the eggs was calculated using the following formula; $m_f - m_i =$ mass gain, where m_i = the initial mass prior to incubation and m_f = the final mass after 24 hours. A sample of the eggs was allowed to hatch to confirm egg viability and their diapause or non-diapause state. A similar preparation method was followed for eggs used subsequently. The initial mass and the final mass after 24 hours was compared using the t-test. This experiment was not carried further and was redesigned.

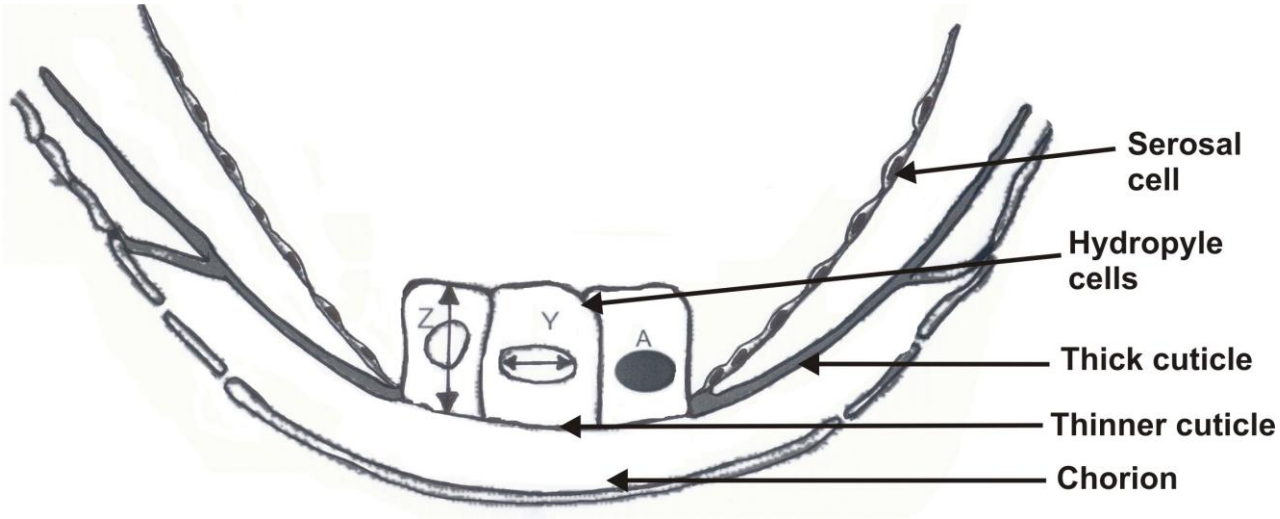
Subsequently, 64 eggs which had been kept at 4 °C for 43 days were incubated on moist soil at 30 ± 0.5 °C for 22 days and weighed on day 0, 2, 7, 9, 12, 14 and 22. 'Days' refers to the time period from the point when the eggs were extracted from the egg pods. The progress of development was observed. The eggs were subsequently divided into three categories; diapause, non-diapause and dead eggs. Eggs that turned into a dark brownish colour and were hard and brittle were regarded as dead. Once the eggs were dead they were removed from the data set. Hatching was expected between days 10 - 12, where this occurred the eggs were regarded as non-diapause eggs; after day 15 - 20 unhatched eggs were considered to be in diapause (Matthée, 1951). The average mass gain of eggs prior to hatching was calculated as mentioned previously. The mean egg mass was calculated for each day for each category and results were compared using t test and regression analysis.

2.3 Desiccation of eggs

Twenty-nine randomly chosen eggs which had been kept at 4 °C were placed on dry soil in a desiccator at 15 % RH, 30 ± 0.5 °C. This RH was achieved by placing silica gel in a desiccator. They were weighed at intervals of 0, 2, 5, 7, 9, 12, 14, 17, 19, 21, 23, 26, 30 and 33 days. After day 33, the eggs were rehydrated for 24 hours on moist soil at 50 % RH, 30 ± 0.5 °C. In order to calculate the percentage weight loss, the following formula was used; $\frac{m_i - m_f}{m_i}$ X 100 = mass loss, where m_i = initial egg mass at day 0 and m_f = final egg mass. To calculate the % of water in eggs the following formula was used: % of water in eggs = $\frac{live_{wt} - dried_{wt}}{live_{wt}}$ X 100, where ***live*$_{wt}$**, is the initial mass of fresh eggs and ***dried*$_{wt}$** is the final mass of the dried eggs.

2.4 Measurements taken from hydropyle cells

Each block was sectioned through the centre of the hydropyle to avoid oblique sections. Different regions of the hydropyle cells which were measured during the incubation period are shown in Fig. 7. The images were measured using the simple PCI high performance image analysis software (Version 5.1.01.011 Compix Inc., (1996-2002, Burnip & Gayler, Cranberry, USA).



A - nuclear area (shaded)

Y - nuclear diameter

Z - cell depth

Figure 7. Measurements taken from the hydropyle cells.

2.5 Metabolic rate of eggs correlated with embryonic development

Individual eggs were separated from the egg pod and placed in a Petri dish. The day of removal of the eggs from the pods just prior to the experiment was taken as day 0 of the experiment. Metabolic rate of eggs were measured first as described in section 2.1.5 and embryos were then dissected as described previously (section 2.1.4) to determine the stage of the embryo. Embryos were viewed with the Nikon DXM 1200 / SMZ microscope and photographed. Eggs in early stages of development were very fragile and therefore some of them were damaged during dissection, processing or photographing. Embryos were staged according to Matthée's (1951) and Wardhaugh's (1978) embryo classification. Embryos were used for light microscopy (LM) viewing while hydropyles were used for both LM and transmission electron microscopy (TEM) viewing. All day 0 eggs were grouped into non-diapause, as it was not possible to distinguish between diapause and non-diapause embryos at day 0

(Matthée, 1951). Some of the eggs which were used for metabolic rate measurements and left for hatching died due to stress of handling.

In a preliminary experiment it was found that most of the eggs were in non-diapause. We therefore did not have enough diapause eggs to make a comparison with eggs in other states. The experiment was therefore repeated. In order to be certain that these eggs were in diapause, they were treated as described below and we were also fortunate to find females laying eggs in the field at the end of summer.

2.5.1 Laboratory diapause eggs

Eggs laid by solitary females reared in the laboratory were moistened while in the soil and incubated at 30 ± 0.5 °C, 50 % RH and allowed 14 days to hatch (Fig. 37). This was done in order to be certain that eggs which remained in the egg pods were true diapause eggs. The laboratory eggs were then separated out of egg pods and kept in 3 separate batches each laid by different females. The metabolic rate of laboratory eggs was then recorded as described in section 2.1.2 between day 0 and 23. Day 0 is the time when the eggs were extracted from the egg pods.

2.5.2 Field diapause eggs

Field eggs were collected at the end of summer and on the collection site some locusts were dying which means they were old. Older females lay diapause eggs (Matthée, 1951). Egg pods were collected from De Aar & Pofadder in the Northern Cape in May 2008. These eggs were measured within a week of arrival from the field. Eggs were isolated and hydrated as described previously and placed in an incubator at 30 ± 0.5 ºC as described in section 2.1.2 and their metabolic rate measured from 0 to 28 days.

Some of the field eggs were kept on dry soil in the laboratory at room temperature and used as a control. A follow-up experiment was conducted to record the metabolic rate of eggs in quiescence.

2.5.3 Quiescent eggs

Eggs were obtained from batches laid on the following dates: 15 - 20 August, 10 - 20 August and 27 - 30 July 2007 and stored at 2 - 4 ºC. The eggs pods were removed from the soil and placed into 3 separate Petri dishes and labelled batch 1, batch 2 and batch 3 corresponding to the 3 pots mentioned above (Fig. 8).

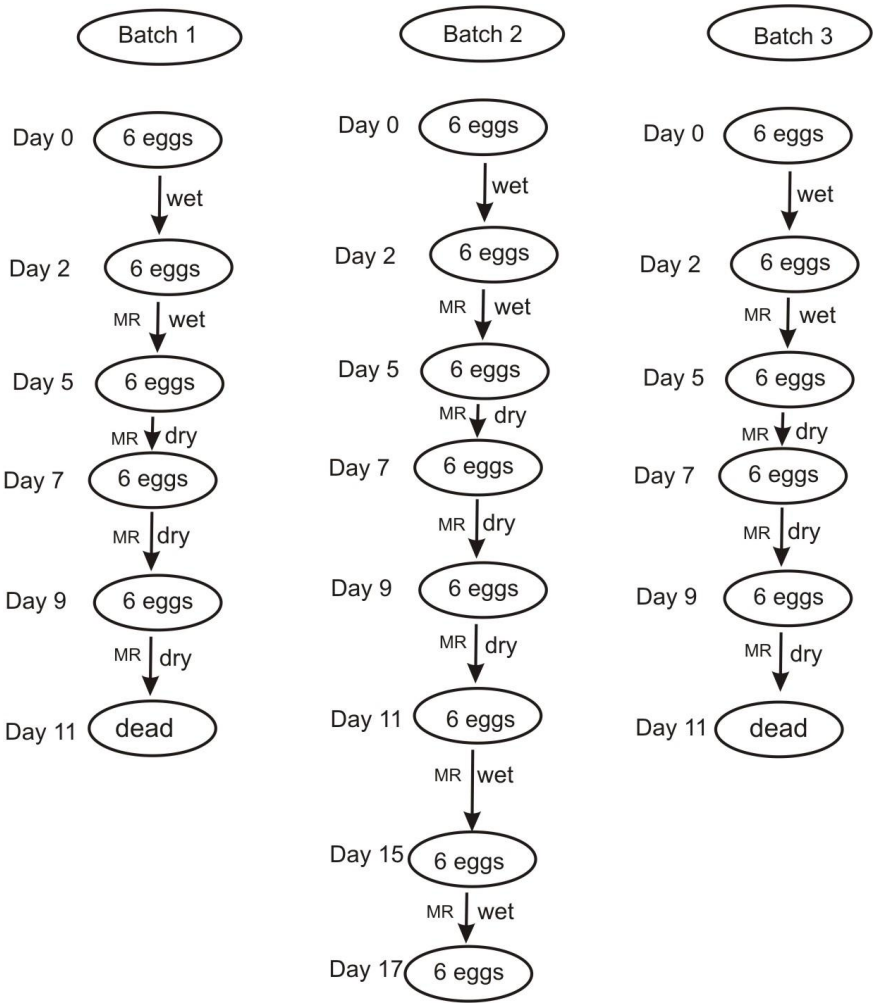


Figure 8. Experimental design for inducing developing eggs into quiescence. (MR = metabolic rate, wet = eggs on moist soil, dry = dry soil).

Batch 1 and 2 eggs were 63 and 68 days old respectively, before the experiment was started. Batch 3 eggs were 89 days old. The eggs were incubated as described in section 2.1.2. The metabolic rate of the eggs was recorded at day 2 and 5 of incubation. After day 5 eggs were then placed on dry soil in 3 separate Petri dishes at 15 % relative humidity and 30 ± 0.5 °C. The metabolic rate of these eggs was again recorded after eggs had been dehydrated for 2 and 4 days. Eggs were immediately placed on moist soil for 3 days under similar conditions of temperature. The metabolic rate was measured after eggs had been rehydrated for 4 and 6 days. The metabolic rate measurements were terminated after 17 days as eggs developed fungus. Eggs that had fungus or had died were discarded. Eggs remaining at the end of the experiment were incubated to observe hatching. A control was conducted with dead eggs.

2.5.4 Dead eggs

Dead eggs were obtained by placing 15 fresh eggs in boiling water for 5 minutes. In order to test that the eggs were dead, 1 % colourless tetrazolium dye was used on both live and dead eggs. Eggs were incubated in 1 % tetrazolium for 24 hours at room temperature in the dark (Bancroft & Gamble, 2002). The tetrazolium indicates respiratory activity. Colourless tetrazolium competes with the cell's normal electron acceptors and changes colour when reduced. In live eggs colourless 1 % tetrazolium is reduced to pink / red while in dead eggs the stain remains colourless (Bancroft & Gamble, 2002). Tetrazolium is not a vital dye and eventually kills the eggs at the end of having displayed their respiratory activity.

2.6 Alternate dehydration and hydration of eggs

Seventy eggs were separated from the egg pod and dehydrated on dry soil over silica gel in a desiccator at 30 ± 0.5 °C, 15 % relative humidity for periods of 0, 2, 5, 7 and 9 days (Fig. 9). Day 0 was the day eggs were extracted from the egg pod.

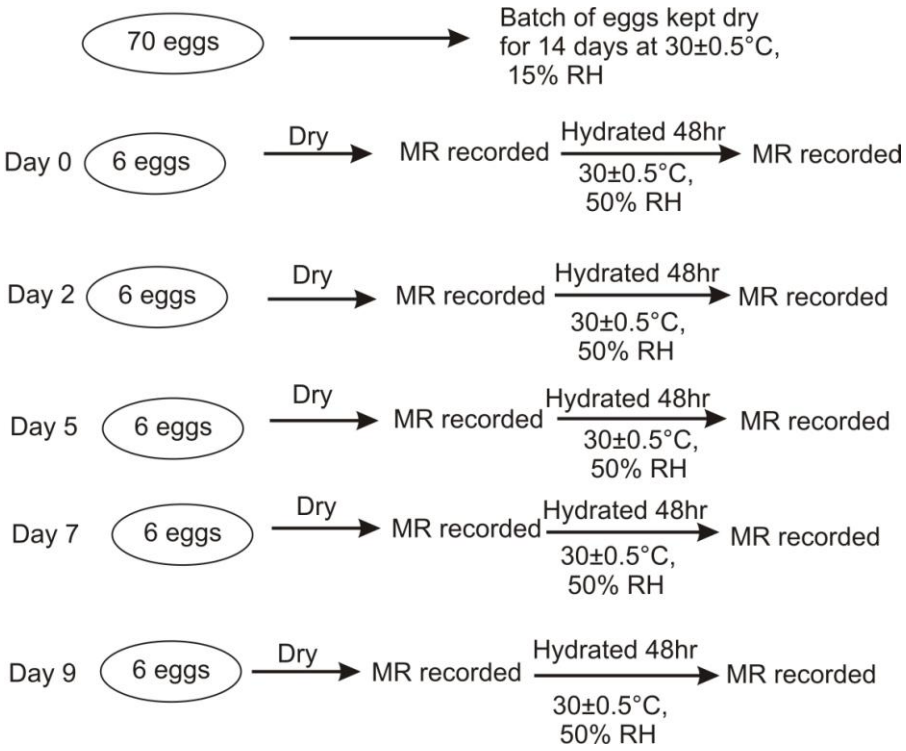


Figure 9. Experimental procedure of alternate dehydration and hydration.

Six eggs were sampled from the initial batch of dry eggs for each different day (Fig. 9) and the metabolic rate recorded (Section. 2.1.5). A non-parametric t-test was used to analyze the data.

CHAPTER 3 RESULTS

3.1 Separating diapause and non-diapause eggs

The physiological state of eggs when removed from the egg pod cannot be determined visually. It is important to separate diapause and non-diapause eggs for future developmental and physiological studies. Matthée (1951) used the visual method to differentiate between the physiological state of eggs after 3 days of incubation in moist conditions but this is not reliable. A pilot study to separate diapause and non-diapause eggs was conducted in which eggs were incubated on moist soil and weighed after 24 hours to see if they had absorbed water. The basic assumption made in this experiment was that the mass of the egg increases as water is absorbed. It was thought that 24 hours would be ideal for eggs to start absorbing water as suggested by Matthée (1951).

Eggs with an initial mass below 7 mg and those greater than 12 mg were discarded. When eggs below 7 mg were dissected they contained mostly yolk with no embryos and those heavier than 12 mg had started developing fungus. The eggs were divided into 2 batches. Some eggs absorbed water and increased in mass (Table1). These were assumed to be non-diapause eggs. They became turgid and the chorion of these eggs cracked open. All the other eggs that stayed the same and those that lost weight or gained 0.1 mg of water were assumed to be in diapause. Of the eggs assumed to be in diapause, 46 % decreased in mass, 29 % showed no change in weight and 14 % increased by 0.1 mg (Table 1)

TABLE 1. Mass of eggs (mg) after 24 hours on moist soil.

	Initial mass Mean ± SD; Range	Final mass Mean ± SD; Range	% mean mass gain	% mean mass loss
Diapause (n = 56)	8.5 ± 1.25; 7 - 11.9	8.3 ± 1.32; 6.9 - 8.9	2.96	2.4
Non-diapause (n = 113)	8.8 ± 1.5; 7 - 11.9	9.9 ± 9.9; 7.5 - 12.3	11.2	0

The overall results of the separation experiment were observed and analyzed (Table 1). The initial and final mean mass of both non-diapause and diapause eggs were significantly different ($t_{0.05, 112} = 8$, $P < 0.0001$), ($t_{0.05, 55} = 2.5$, $P = 0.008$). The mean initial mass of both diapause and non-diapause eggs did not differ significantly ($t_{0.05, 167} = 1.1$, $P = 0.1$). There was a significant difference between the final mass of both diapause and non-diapause eggs ($t_{0.05, 167} = 4.5$, $P < 0.0001$), although the final range in mass showed overlap (Table 1). In diapause eggs water absorption only occurs to replace water lost by evaporation (Matthée, 1951). Once this is achieved, water absorption is stopped**.**

The increased water absorption in non-diapause eggs promotes growth and development until hatching occurs. The mass of the egg increases as more water is absorbed. This explains the significant difference in the initial and final mass of non-diapause eggs.

The initial mass of eggs does not indicate whether an egg would be in diapause or non-diapause. From this experiment the change of weight over 24 hours was not sufficient to separate all the eggs into either diapause and non-diapause. This led to the second experiment which was conducted to see what happened over a longer

period of time so that the eggs could be more accurately separated into the two phases.

Eggs assumed to be in non-diapause were followed through and they hatched after 10 days of incubation. Diapause eggs were not expected to hatch until diapause was broken. For the second experiment, 64 eggs were used.

The total sample size of eggs that did not increase in mass and had not hatched by day 14 was 27 (= 42%), and that of eggs that increased in mass and hatched by day 14 was 13 (= 20%).

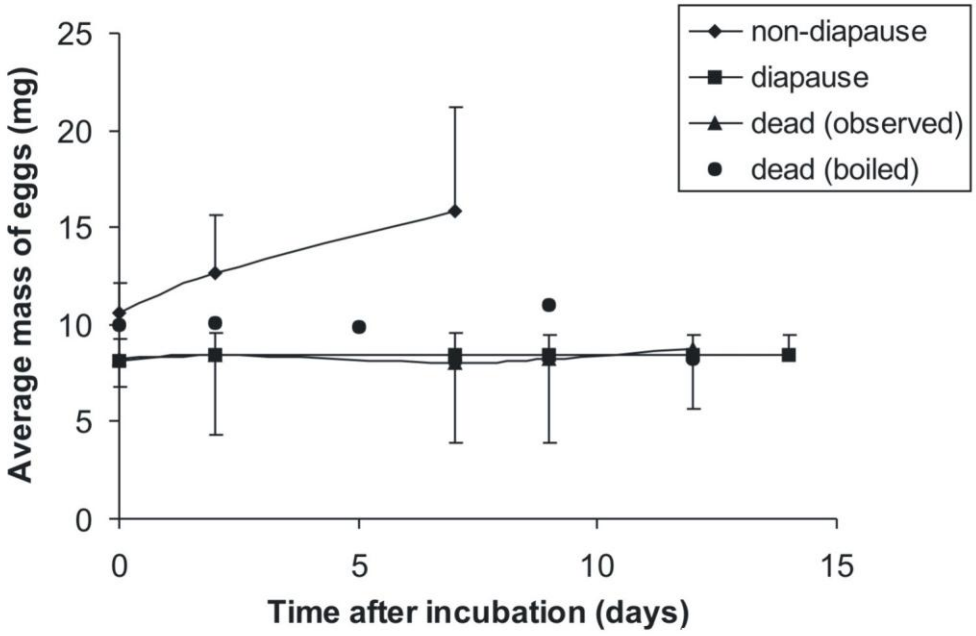


Figure 10. Mass gain (mean ± SD) of eggs at 30 ± 0.5 °C, 60 % relative humidity over 14 days.

Figure 10 shows that there was no correlation between change in mass and the number of days in dead (boiled) and eggs that died during the experiment (observed dead). Observed dead eggs began with a mean mass of 8.3 ± 1.4 mg and increased to 8.7 ± 3 mg with a sample size of 3 after 12 days of incubation (Table 2). At the end of the experiment 23 eggs were classified as dead using the criteria mentioned previously (section 2.2). An average mass gain was 0.5 mg after 14 days of incubation

(Fig. 10). Eggs that were killed by boiling had a relatively higher initial mean mass of 10 mg but by 12 days showed no difference to the observed dead eggs.

TABLE 2. Variation in measured mass of eggs (mg) in different states.

	Diapause	Non-diapause	Dead - observed	Dead - boiled
Days	Mass (x ± SD) n=sample size	Mass (x ± SD) n=sample size	Mass (x ± SD) n=sample size	Mass (x ± SD) n=sample size
0	8.1 ± 1.1, n=29	10.6 ± 1.5, n=3	8.3 ± 1.4, n=32	10 ± 0.9, n=6
2	8.4 ± 1.2, n=29	12.7 ± 3, n=3	8.5 ± 4.2, n=16	10.1 ± 0.4, n=6
5				9.9 ± 0.8, n=6
7	8.5 ± 1.1, n=29	16 ± 5.4, n=3	8 ± 4.1, n=10	8.1 ±, 0.8 n=6
9	8.4 ± 1.1, n=29	9.7, n=1	8.2 ± 4.3, n=10	11 ± 0.8, n=6
12	8.4 ± 1, n=29	all had hatched	8.7 ± 3, n=3	8.2 ± 0.9, n=6
14	8.4 ± 1, n=29		8.8 ± 1.5, n=3	
22	all alive		all dead	

Diapause eggs started with an initial mass of 8.1 ± 1.1 mg while non-diapause eggs had an initial mass of 10.6 ± 1.5 mg (Table 2). The variation of mass of diapause eggs was 8.1 - 8.5 mg with little change during the 22 days (Fig. 10). There was no significant difference between the total mean mass of diapause and observed (dead) eggs after 14 days of incubation ($t_{0.05, 10} = 0.2$, $P = 0.85$).

Non-diapause eggs increased in mean mass to 16 ± 5.4 mg after 7 days of incubation. The range of mass in non-diapause eggs was 11 - 16 mg. The average mass gain of these non-diapause eggs just before hatching was 5.2 mg (Table 2). All non-diapause eggs hatched after 10 days of incubation (Table 2). Hatching success was $\frac{3}{64}$ = 4.7 % of the original total. The percentage mortality was $\frac{32}{64}$ = 50 % and the percentage diapause was $\frac{29}{64}$ = 45 %.

3.2 Desiccation of eggs

This experiment was to investigate the effect of dehydration on egg mass and viability under controlled temperature and moisture conditions. In this experiment weight loss was assumed to be equal to water loss. Twenty nine randomly chosen eggs had an average initial mass of 13.9 ± 4.1 mg prior to desiccation (Table 3).

TABLE 3. Mass of eggs (mean ± SD) mg exposed to desiccation conditions of 15 % RH, 30 ± 0.5 ºC.

Time (days)	Mean mass (mg)	% initial mass	n
0	13.9 ± 4.1	100	29
2	8.9 ± 4.2	64	28
5	7.2 ± 3	51	28
7	6.6 ± 2.7	47	28
9	6.2 ± 2.4	45	28
12	5.7 ± 1.9	41	28
14	5.6 ± 1.8	40	28
17	5.6 ± 1.9	40	28
19	5.2 ± 1.5	37	28
21	5.1 ± 1.4	37	28
23	5 ± 1.4	36	28
26	4.9 ± 1.3	35	28
30	4.8 ± 1	34	28
33	9.8 ± 3.7	70	20

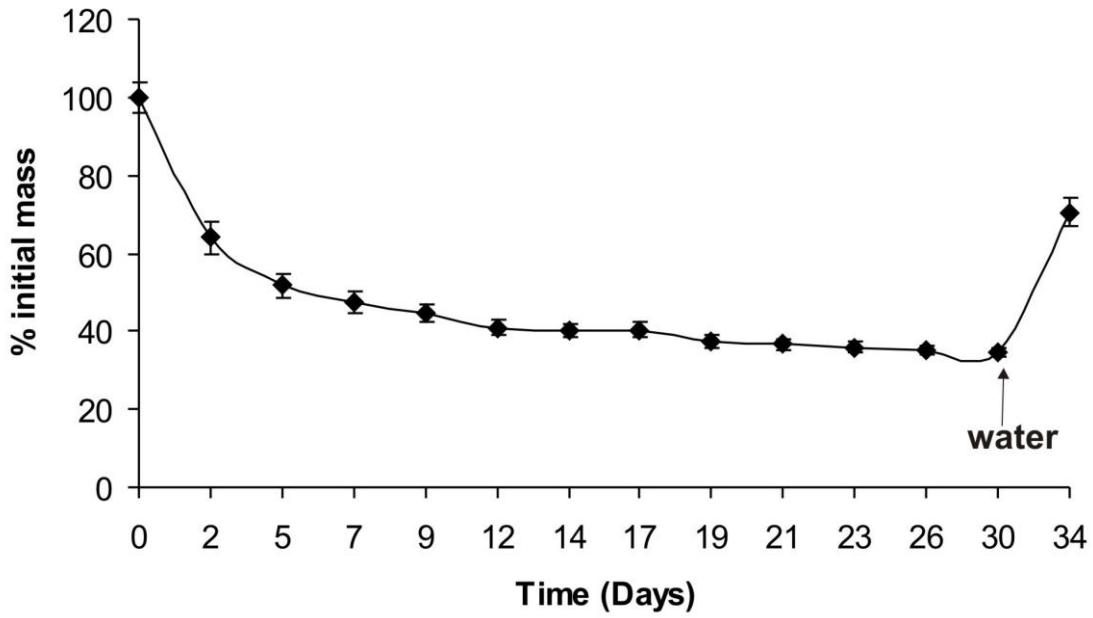


Figure 11. Mass loss (mean ± SD) of eggs during desiccation and subsequent hydration at day 30 (arrow).

There was an initial steep drop in weight loss until day 2 followed by a gradual decrease to 34 % of the initial mass at day 30 (Fig. 11). The average egg mass decreased to 4.8 ± 1 mg at day 30 which means that the eggs had lost an average of 9.2 mg of water (= 65 % weight loss) (Fig. 11). Twenty eggs dried in the oven at 60 ºC after 6 days had a constant dry weight of 4.28 ± 0.26 mg and a range of 3.8 - 4.8. The % water in the egg was 66.61.

On hydration the percentage initial mass increased by 70 % (Fig.11). Rehydrated eggs were found to have absorbed an average 9.7 ± 3.5 mg water after 1 day of hydration. Twenty eggs had absorbed water at the end of the experiment. The 8 eggs excluded fitted the criteria of dead eggs (section 2.2) were excluded from the experiment.

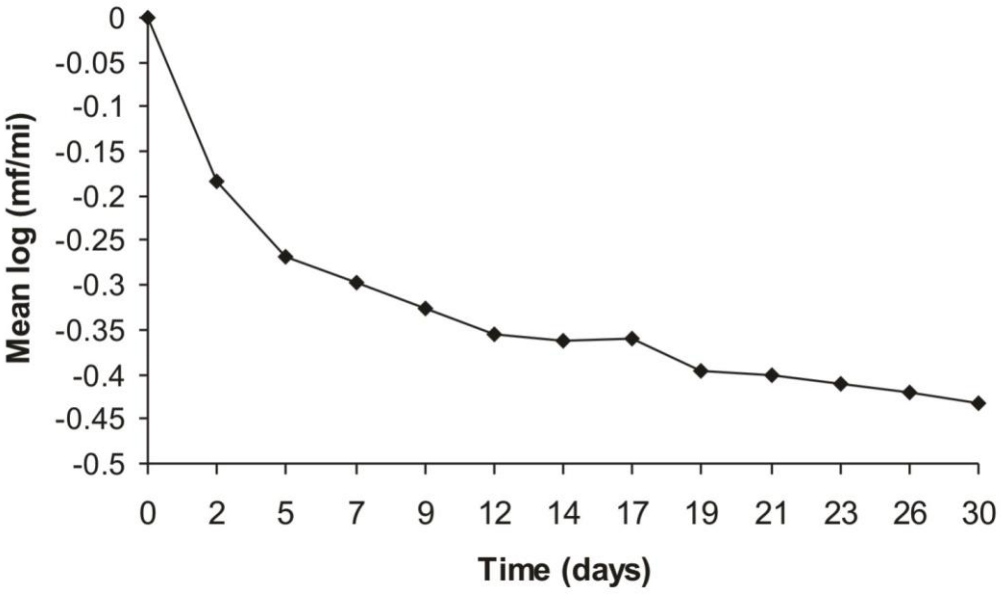


Figure 12. Rate of water loss over time.

There was an initial sharp drop in the rate of mass loss during the first few days of dehydration which later stabilized after day 2 (Fig. 12). There was an inverse relationship between the rate of mass loss during desiccation as opposed to a linear relationship found in insects (Hadley, 1994).

3.3 Changes in the hydropyle structure of eggs after hydration

The hydropyle is a region specialized for water absorption situated at the posterior tip of the egg and therefore facing downwards when eggs are laid. In *Locustana pardalina* there are no surface modifications on the chorion directly covering this part of the egg. Thick endochorion and thin exochorion cover the surface.

The outer layer of cells forming the embryo, the serosa, secrete a thin exocuticle, or yellow cuticle, and within that a thicker endocuticle, white cuticle (Matthée, 1951). The endocuticle is thinner in the hydropyle region than elsewhere. A secondary wax layer is also secreted by the serosa and plays an important role in preventing the egg desiccation (Matthée, 1951). The tough endocuticle consists of

about 8 lamellae containing pore canals which become clearer in later development. Below the endocuticle lies the unspecialized dense endocuticle with thread-like structures known as wax filaments described by Slifer & Sekhon (1963).

The structure of the hydropyle is as described as follows. In the normal developing eggs, the hydropyle cells remain attached to the hydropyle cuticle surface whereas the serosal cells are loosened from the cuticle (Matthée, 1951) (Fig. 13). The hydropyle seems to form a circular patch of cells. The number of hydropyle cells counted in cross section was as high as 25 across the widest portion. This would vary with different sections. Before the rotation of the embryo, embryonic cells remain attached to the hydropyle. The serosal cells remain connected to the edge of the hydropyle (Fig. 13). On rotation, the embryo becomes loosened from the hydropyle cells and the serosal strand snaps (Matthée, 1951).

The serosal cells are stretched into long thin strands but the hydropyle cells are generally cuboidal (Fig. 13). The nuclei of both hydropyle and serosa cells contained numerous darkly stained chromatin granules of variable sizes (Fig. 14), that is heterochromatin.

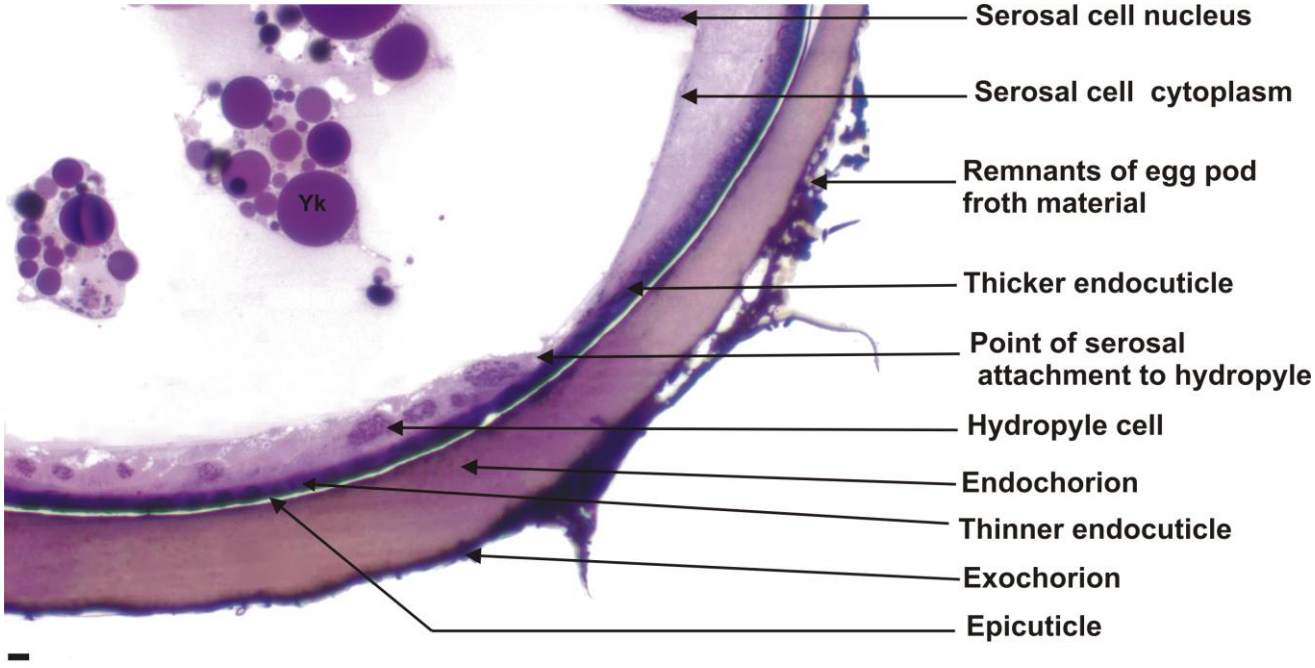


Figure 13. A low power micrograph of hydropyle with thin cuticle and the chorion at day 0. Yk = yolk (scale bar = 10 µm).

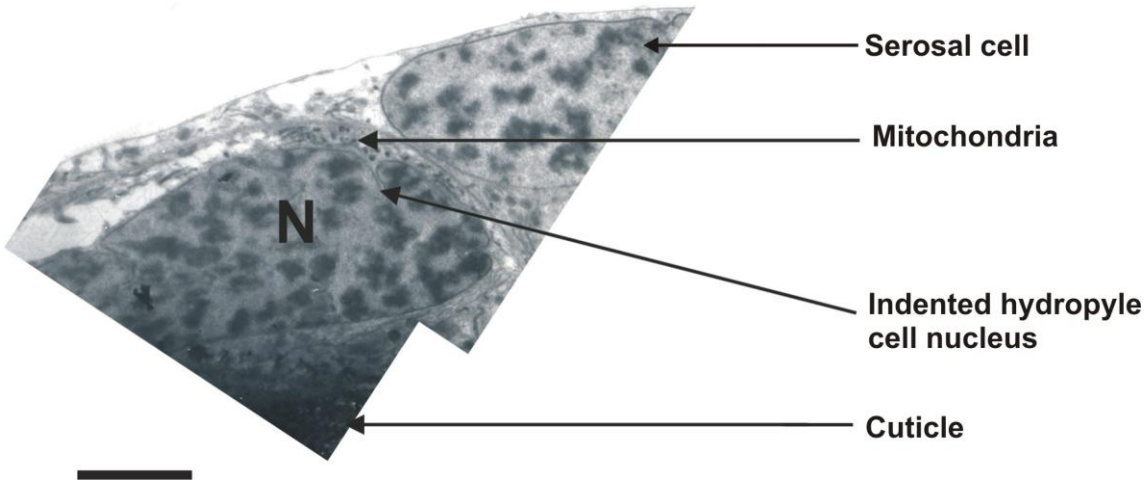


Figure 14. Micrograph of a cross - section through the serosal cell located at the edge of the cuticle at day 0 of incubation. Note the indented serosal cell nucleus (N) with chromatin material scattered inside the nucleus (scale bar = 2.5 µm).

Measurements of the hydropyle cells were taken as shown in Chapter 2 section 2.4 in order to record any change in size. There was a general increase in the nuclear area, nuclear diameter and the cell depth from day 0 to day 6 of incubation (Table 4, Fig. 15). Hydropyle cells have reduced in size and maybe dying at day 10 (Table 4,

Fig.15). However, in quiescent eggs at day 15, the hydropyle cells remain similar to those of day 10 (Fig. 15) with huge vacuoles (Fig. 26).

TABLE 4. Measurements (mean ± SD) of 4 hydropyle cells per egg using light micrographs.

Time after incubation (days)	Nuclear area (μm^2)	Nuclear diameter (µm)	Hydropyle cell depth (µm)	No. of eggs
0	160 ± 52	12 ± 3	23 ±12	3
2	564 ±18	19 ± 4	46 ± 4	3
6	405 ±14	22 ± 6	61 ± 2	4
10	246 ± 67	9.8 ± 2	27 ±9	2
15	224 ± 80	13 ± 3	26 ± 8	2

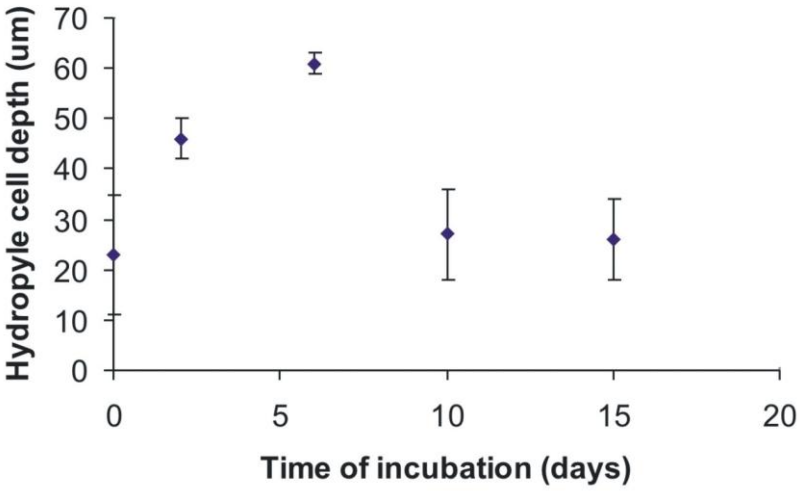


Figure 15. The cell depth (mean ± SD) of the hydropyle cells after different days of incubation.

A more detailed description of changes in structure are reported below and summarized in Table 5.

TABLE 5. Comparison of hydropyle cells of non-diapause eggs at different incubation periods.

	Day 0	**Day 2**	**Day 6**	**Day 10**
Cell shape	Relatively flattened & cuboidal	Cells increased cuboidal to columnar	Cells increased in thickness, columnar	More flattened to cuboidal
Cell height	No distinct cell boundary	Increased cell height	Distinct, increase cell height	Decreased cell height
Nuclei shape	Flattened to oval	More rounded	More rounded	Some flattened
Chromatin	Dispersed	Dispersed	Dispersed	Dispersed
Vacuole	Not distinct	Not distinct	Distinct	Distinct
Intercellular spaces	No	No	Large	Small to absent

Day 0

These were dry eggs that had been removed from the egg pod and had not been incubated on moist soil. The hydropyle cells were squamous to cuboidal with flattened nuclei (Figs 16 & 17). In several eggs the hydropyle cell cytoplasm was barely wider than the nucleus. The nuclei in the cells were arranged in a clear single layer (Fig. 16). Darkly stained tightly packed chromatin granules were observed in the nuclei (Fig. 16). The cytoplasm had no clear cell boundaries between cells in most sections observed with the light microscope and cells appeared to have a smooth surface adjacent to the embryo (Fig. 18). There were no intercellular spaces (Fig. 16) and no vacuoles in the cytoplasm visible with the light microscope but small ones were visible at the TEM level (Fig. 17). The TEM showed that the cytoplasm contained endoplasmic reticulum, mitochondria and dark granules (Figs 17 & 19). In some sections the cells appeared to overlap each other.

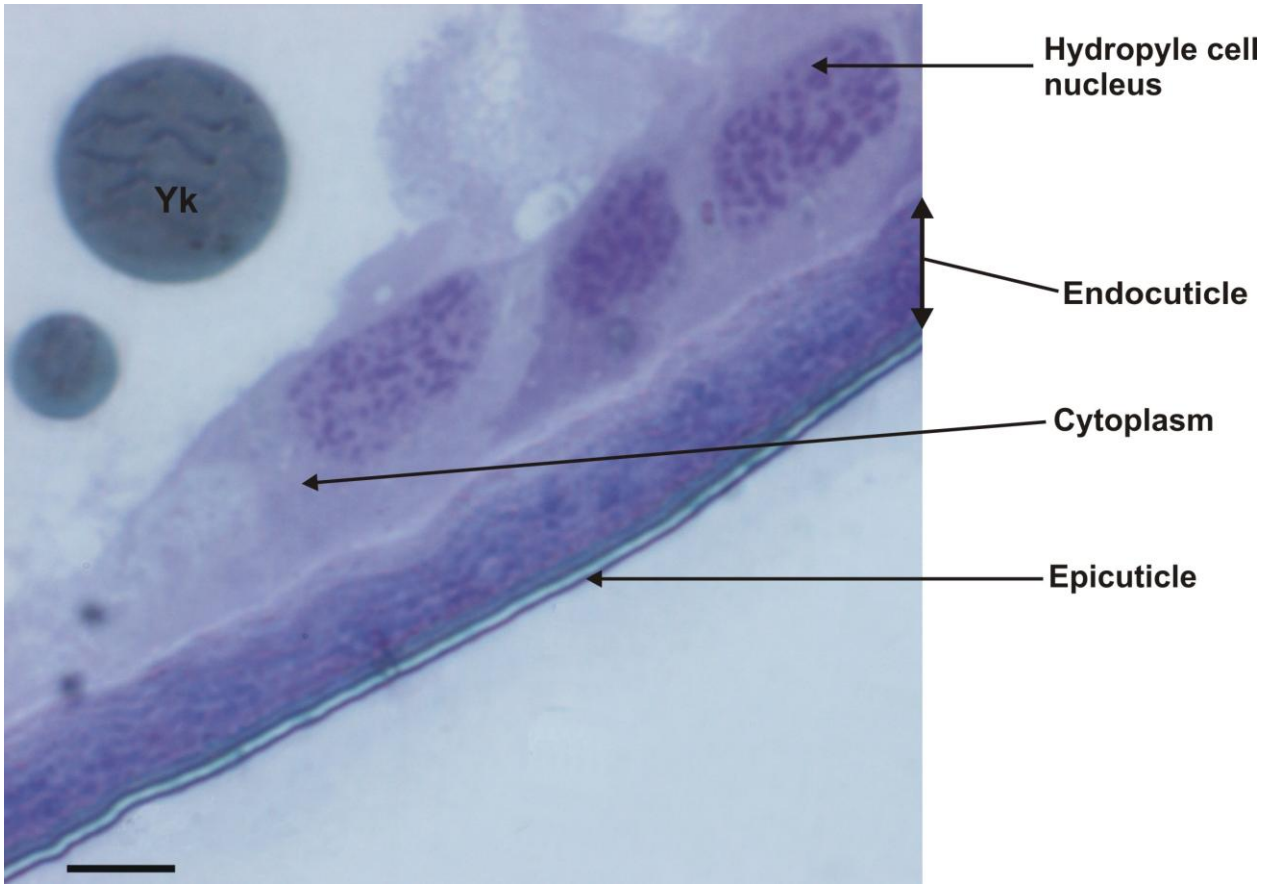


Figure 16. Light micrograph of hydropyle cells of a diapause egg after extraction from an egg pod, day 0. Note the toluidine blue patches on the nucleus (N). Yk = yolk granules (scale bar =10 µm).

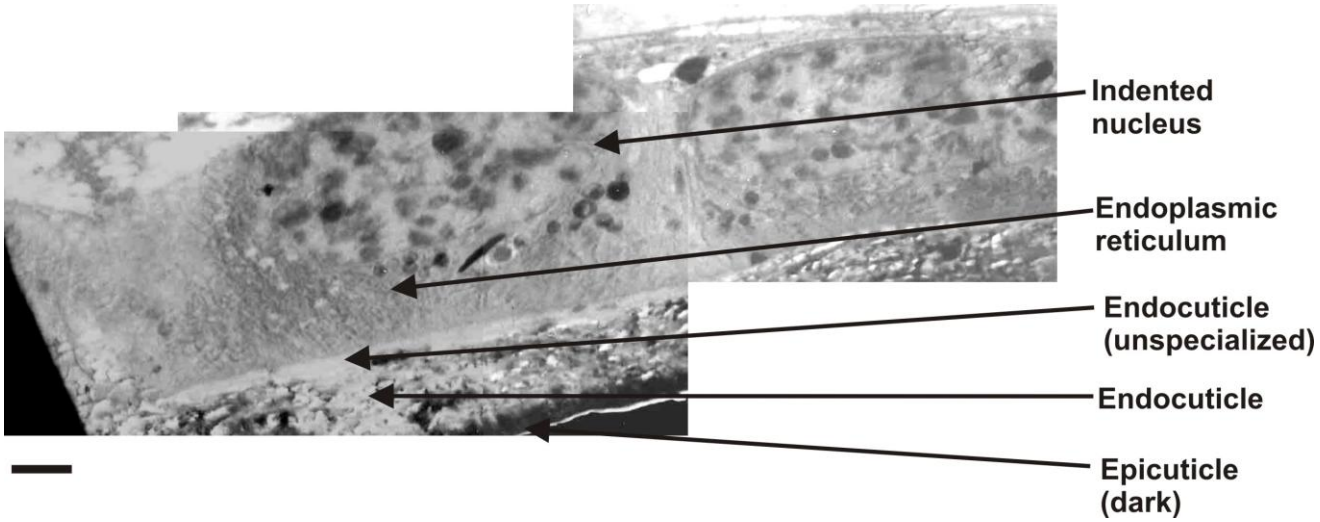


Figure 17. Transmission electron micrograph of thin sections through the hydropyle area of the same egg (scale bar = 2.5 µm).

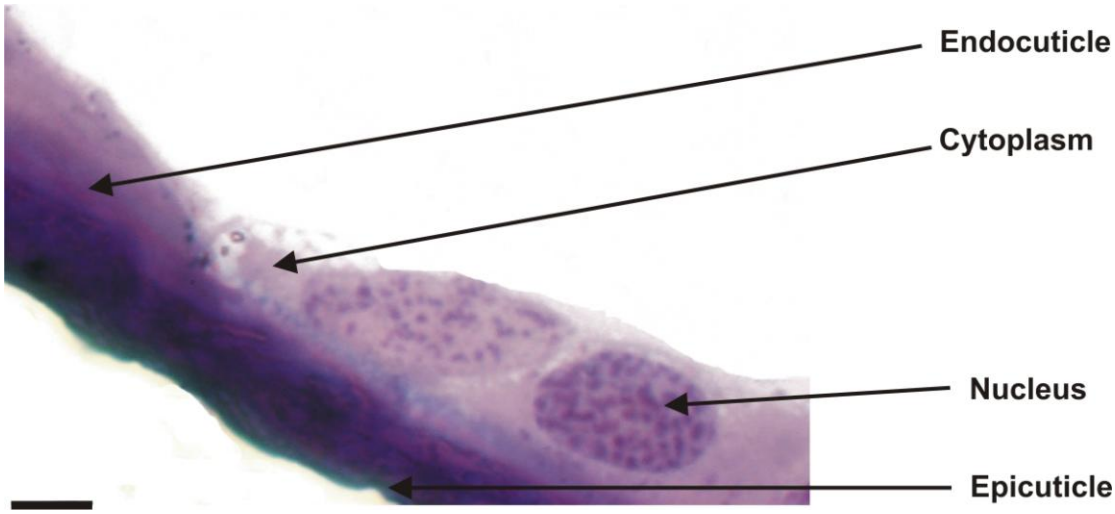


Figure 18. Light micrograph of a cross-section through the hydropyle area at day 0. Note the endocuticle is thinner next to the 2 hydropyle cells (scale bar = 10 µm).

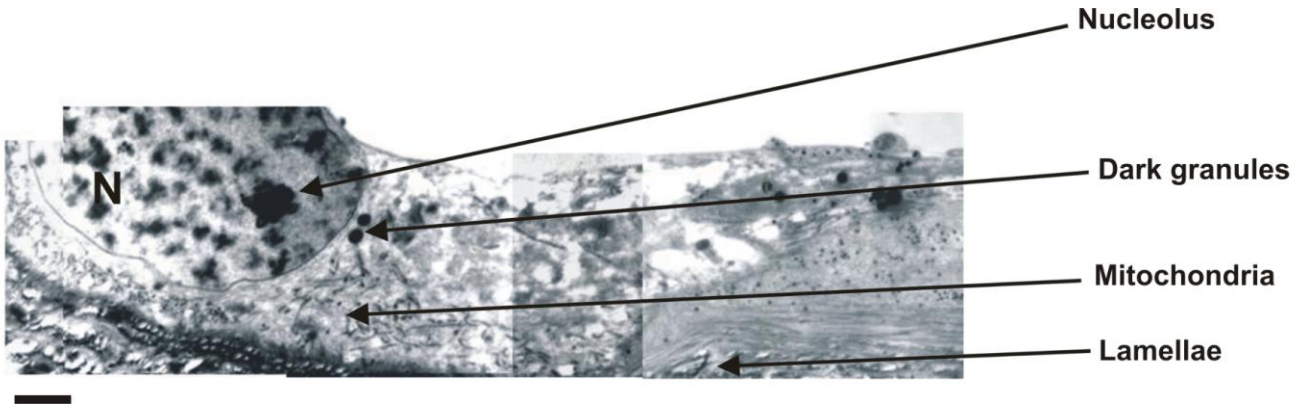


Figure 19. An electron micrograph of a thin cross section through the hydropyle area. Note the presence of the nucleolus and the dark granules (scale bar = 2.5 µm).

Dark granules were observed in the cytoplasm near the periphery of the nucleus (Figs 19 & 20). These dark granules appeared similar to those described by Balinsky (1985) in the egg of the butterfly, *Acraea horta*. The dark granules appeared to be associated with rough endoplasmic reticulum and therefore they could be related to secretion (Fig. 19). There appear to be mitochondria and endoplasmic reticulum in the cytoplasm (Figs 17, 19 & 20).

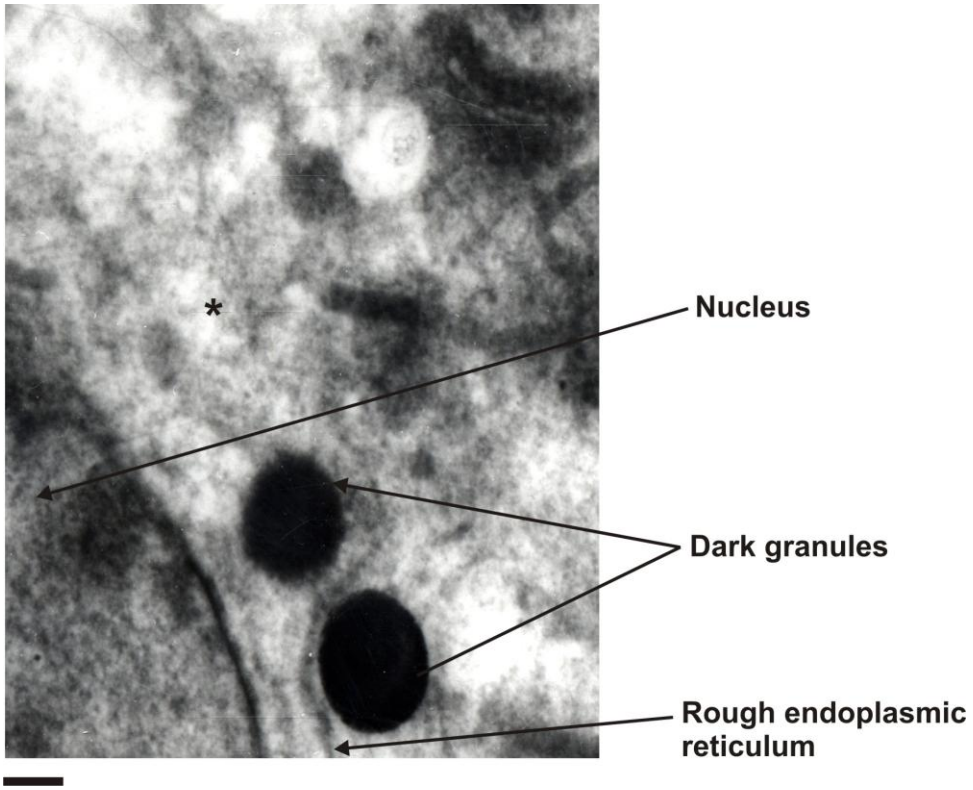


Figure 20. High power of section shown in Fig. 19 showing dark granules, the hydropyle cell nucleus, and strands of rough endoplasmic reticulum in the cytoplasm (*) (scale bar = 0.67 μm).

Days 2 and 3

The hydropyle cells increased in cell depth and became distinctly cuboidal to columnar (Figs 21 & 22). The cells seemed to overlap with nuclear position becoming less regular. The nuclei increased in size and became more rounded (Fig. 21). Chromatin granules were evenly dispersed. The surface next to the embryo was irregular with projections in some places. Boundaries between cells were clearer. There seemed to be spaces between the cytoplasm and the cuticle (Fig. 21).

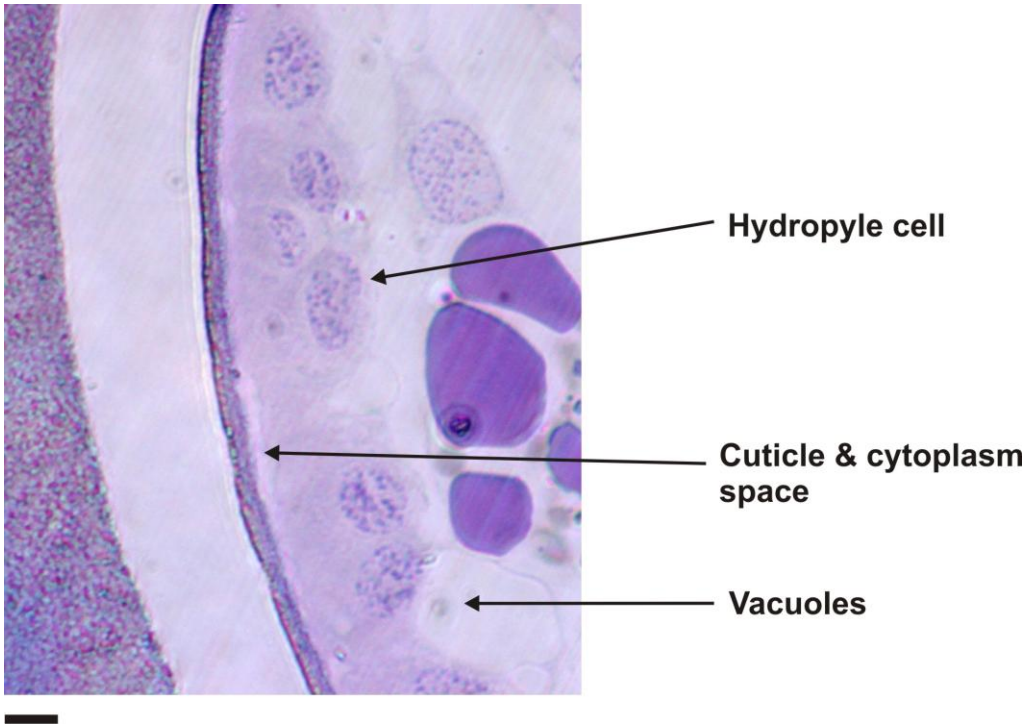


Figure 21. Light micrograph of a cross-section through the hydropyle area at day 2 of incubation. Note the less regularly aligned nuclei (scale bar = 10 µm).

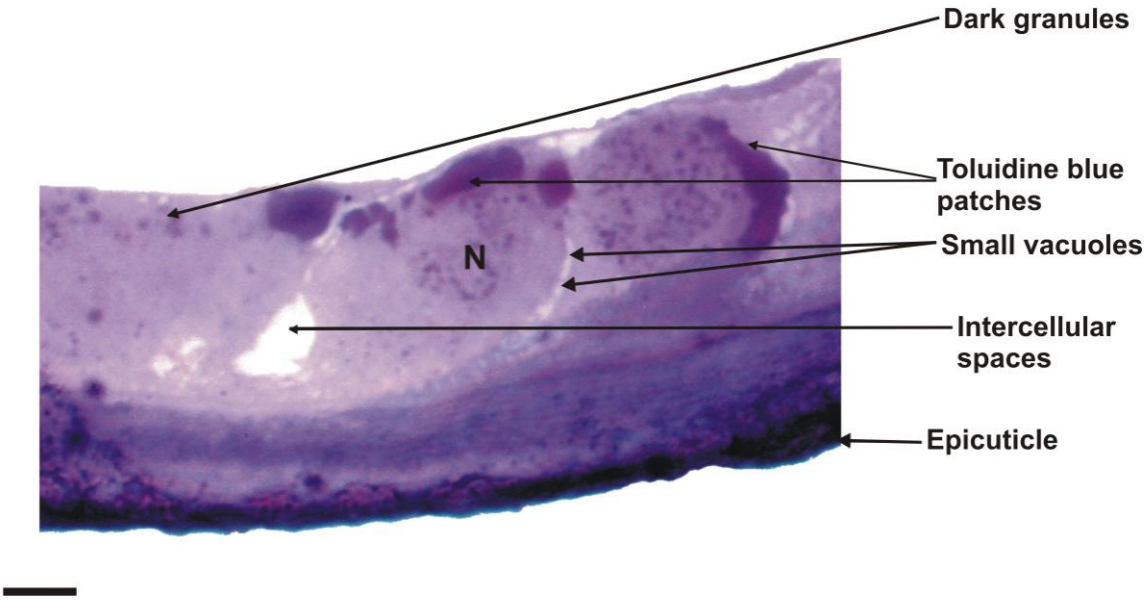


Figure 22. Section through hydropyle cells after 3 days of incubation showing small vacuoles, toluidine blue positive patches and dark granules (scale bar = 10 µm).

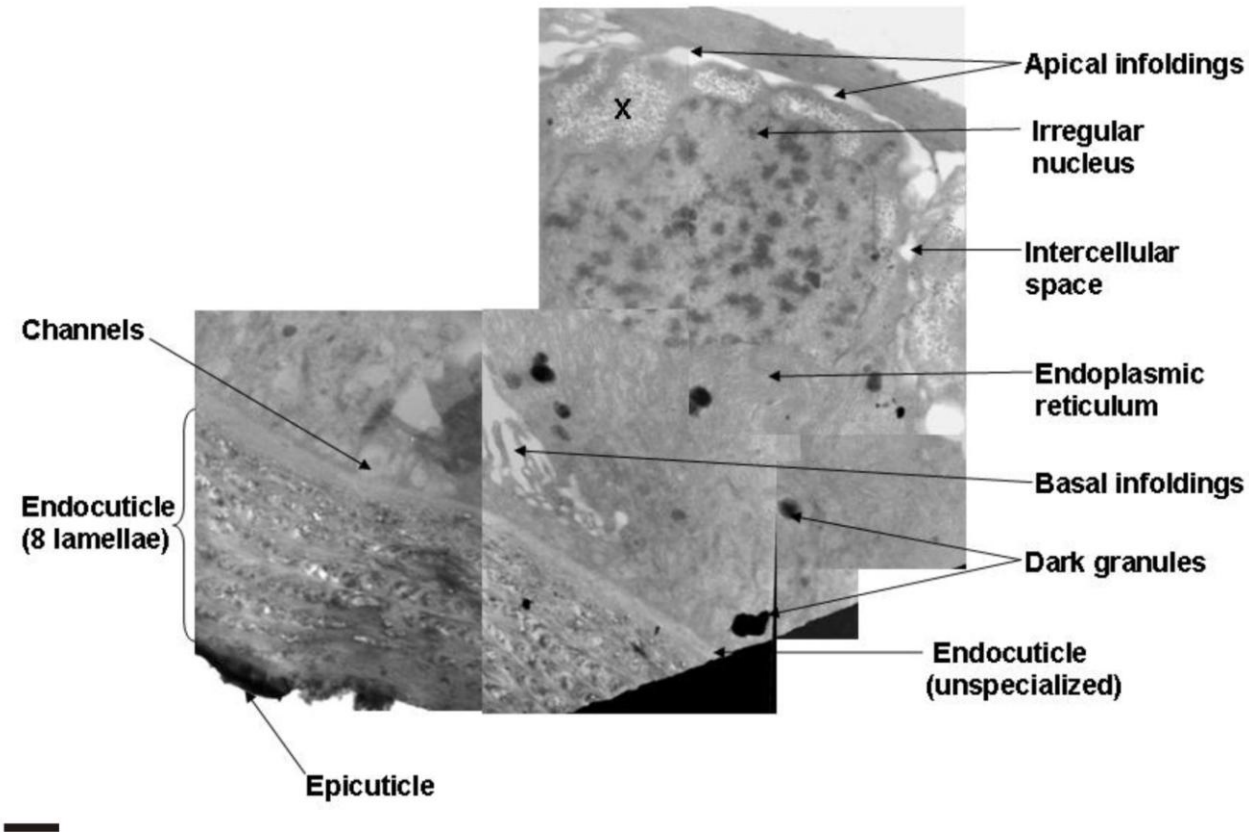


Figure 23. Thin section of Fig. 22 through the hydropyle area (scale bar = 2.5 µm).

The hydropyle cells had become more cuboidal and increased in cell depth between the cuticle and the nuclei (Figs 22 & 23). The embryo had not turned and therefore was still in anatrepsis. The structure of the chorion, epicuticle, endocuticle with 8 lamellae (Fig. 23), the number of hydropyle cells, the presence of serosa cells and the presence of vacuoles are as described previously. The nucleus was more rounded but nuclear indentations were seen clearly with the TEM (Fig. 23). There were large distinct irregular intercellular spaces between hydropyle cells (Figs 22 & 23) which were not present at day 0. There were distinct toluidine blue positive patches surrounding part of the nucleus (Fig. 22) in the cytoplasm, observed using the light microscope, and these could possibly be endoplasmic reticula with surface ribosomes containing toluidine blue staining material (Figs 22 & 23). At this stage the

endocuticle lamellae and the channels in the adjacent cytoplasm were observed (Fig. 24). It was expected that microvilli would be present in this tissue but none were found.

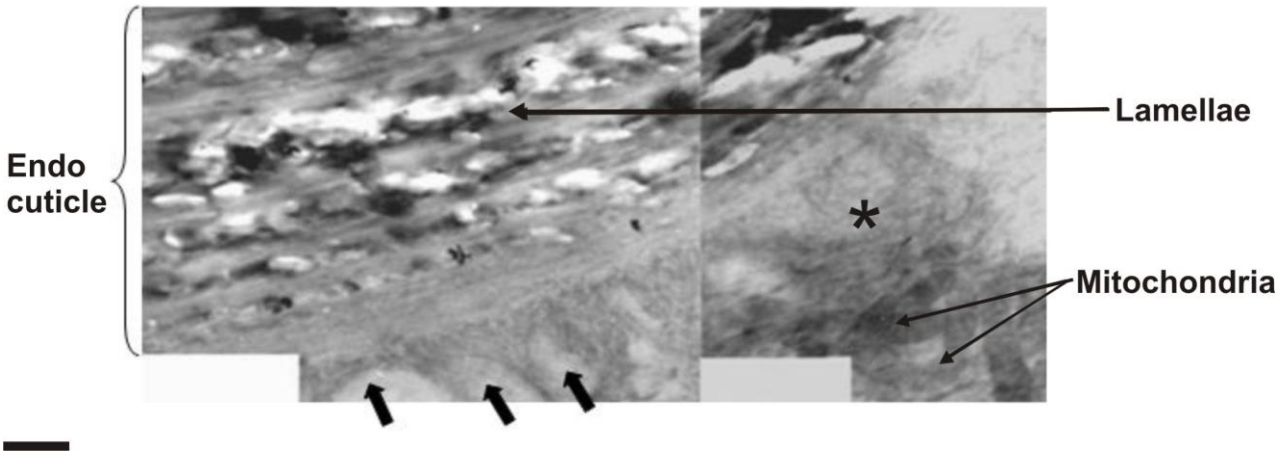


Figure 24. High power micrograph of Fig. 23 showing the endocuticle, channels (⟶) and mitochondria in the cytoplasm (*) (scale bar = 0.67 µm).

Days 5 and 6

At days 5 and 6 the hydropyles had 2 distinctly different appearances. In some, the hydropyle cells remained squamous with flattened nuclei while in others the hydropyle cells seem to have increased in size due to water absorption.

Figure 25 is an example of hydropyle cells with flattened nuclei showing no signs of water absorption. A distinct double layer of cells was visible (Fig. 25). The hydropyle cell shape was squamous and more flattened than previously (Fig. 25). The outer cell layer was very squamous in shape and cell boundaries were not clearly distinct (Fig. 25). The nuclei were flattened and oval (Fig. 25). Densely packed chromatin granules were observed with few minute intercellular spaces present in some sections.

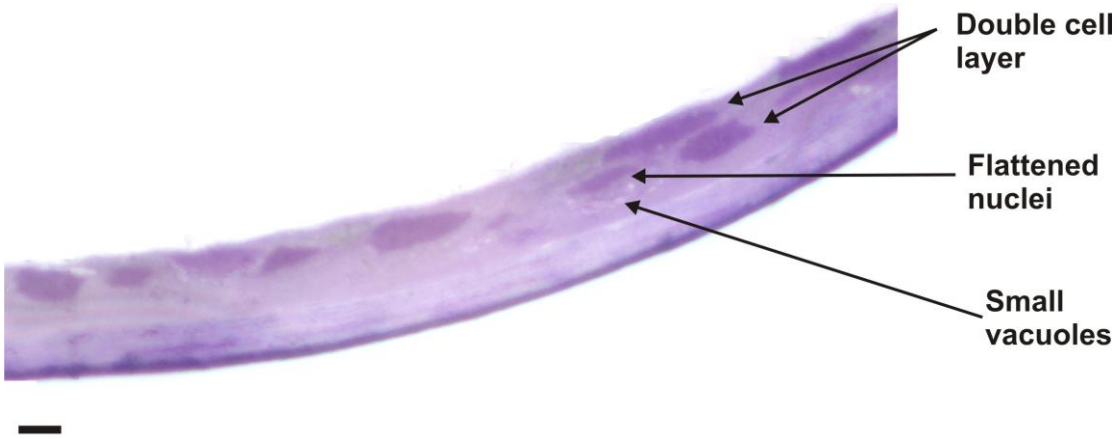


Figure 25. High power micrograph of a section through the hydropyle after 5 days of incubation (scale bar = 10 µm).

Matthée (1951) claimed the serosa changed by folding itself over the hydropyle cells creating a double layer of cells (Fig. 25). This could explain the double layer of cells and could also be a dissection artefact. Cells may be changing in size or dividing and thus forming what appears to be a double layer. This might explain the variation in measurements shown in Table 4.

In other hydropyles there was an increase in cell size. Distinct large and small vacuoles were observed in the cytoplasm at this stage of development (Fig. 26). The hydropyle cells with round nuclei have enlarged resulting in columnar cells (Fig. 26). It would seem that the hydropyle cells of *Locustana* were enlarging as development progressed.

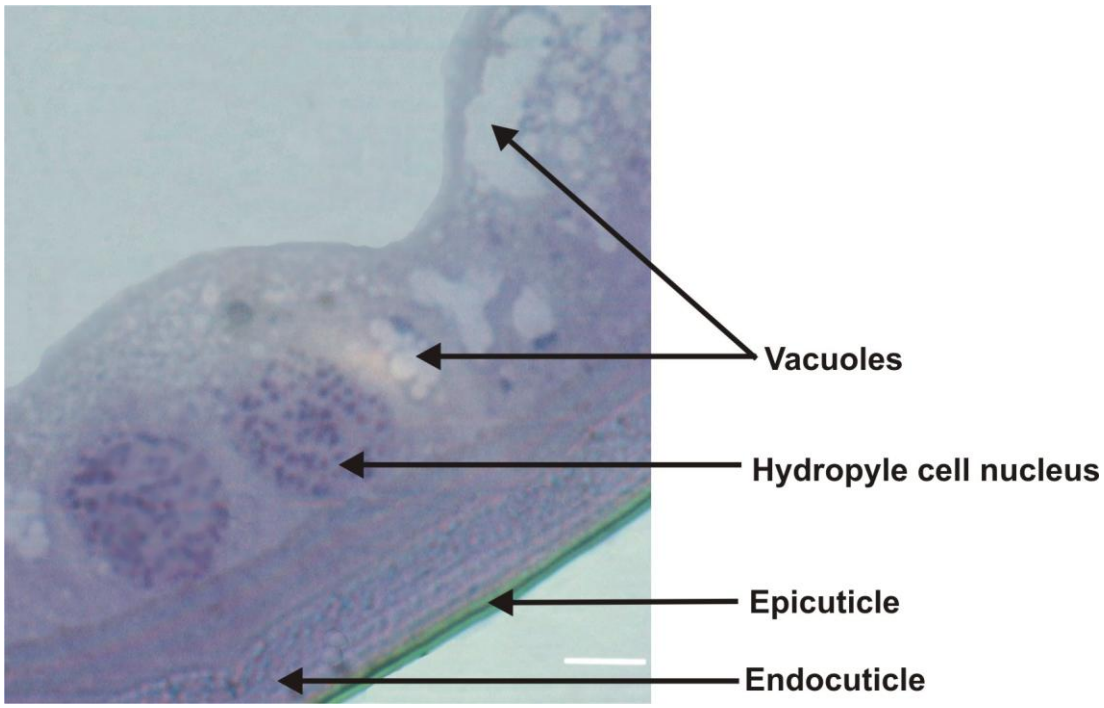


Figure 26. Light micrograph of hydropyle cells after 6 days of incubation (scale bar = 10 µm).

Day 10

At this stage the hoppers were about to hatch (Fig. 27). The hydropyle cells appeared cuboidal (Fig. 27). Some cells may have been alive while others possibly dying. Huge vacuoles were present (Fig. 27) and a few nuclei with very condensed chromatin were observed. Intercellular spaces found were narrower and small. The cytoplasm was found to be disintegrating in some cells. These are characteristics of necrotic cells (Fig. 28).

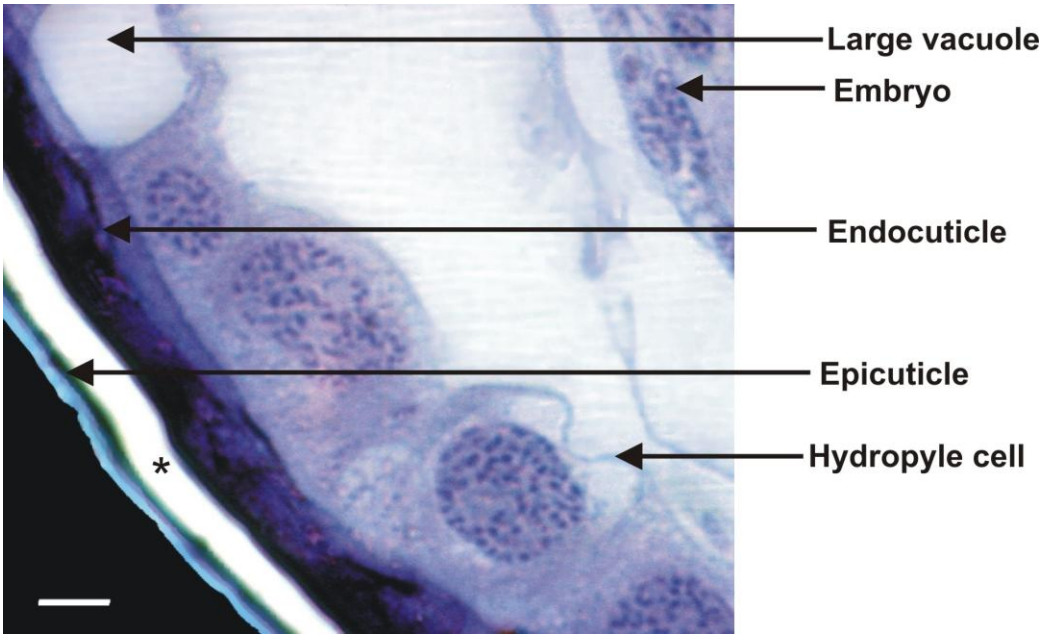


Figure 27. Light micrograph of hydropyle cells after 10 days of incubation. Cells probably still living (scale bar = 10 µm).

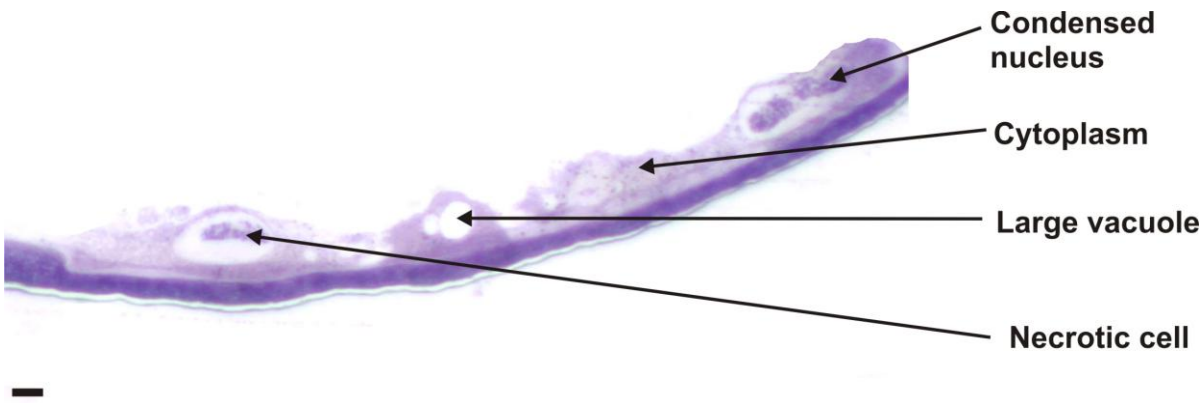


Figure 28. Light micrograph of necrotic hydropyle cells after extraction from an egg pod (scale bar = 10 µm).

Day 15

Cells were cuboidal to columnar (Fig. 29). The nuclei were more rounded and there were huge vacuoles present at the cell surface (Fig. 29). There was no increase in cell size. It was not clear whether these cells were undergoing necrosis. This embryo was found to be well advanced after dissection and had a very low metabolic rate (see section 3.6) and therefore was classified as quiescent.

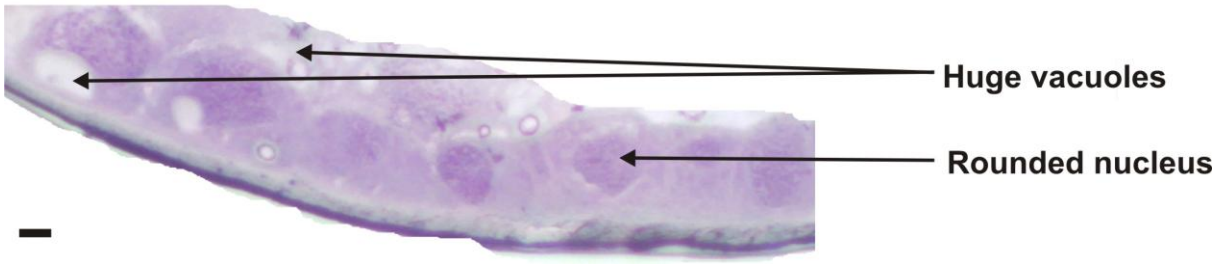


Figure 29. Light micrograph of hydropyle cells after 15 days of incubation (scale bar = 10 µm).

3.4 Overview of embryo development in *Locustana pardalina*

Figure 30 shows an outline of the overall development for *L. pardalina*. In this study we have enhanced description of Matthée's (1951) key stages relevant to this project.

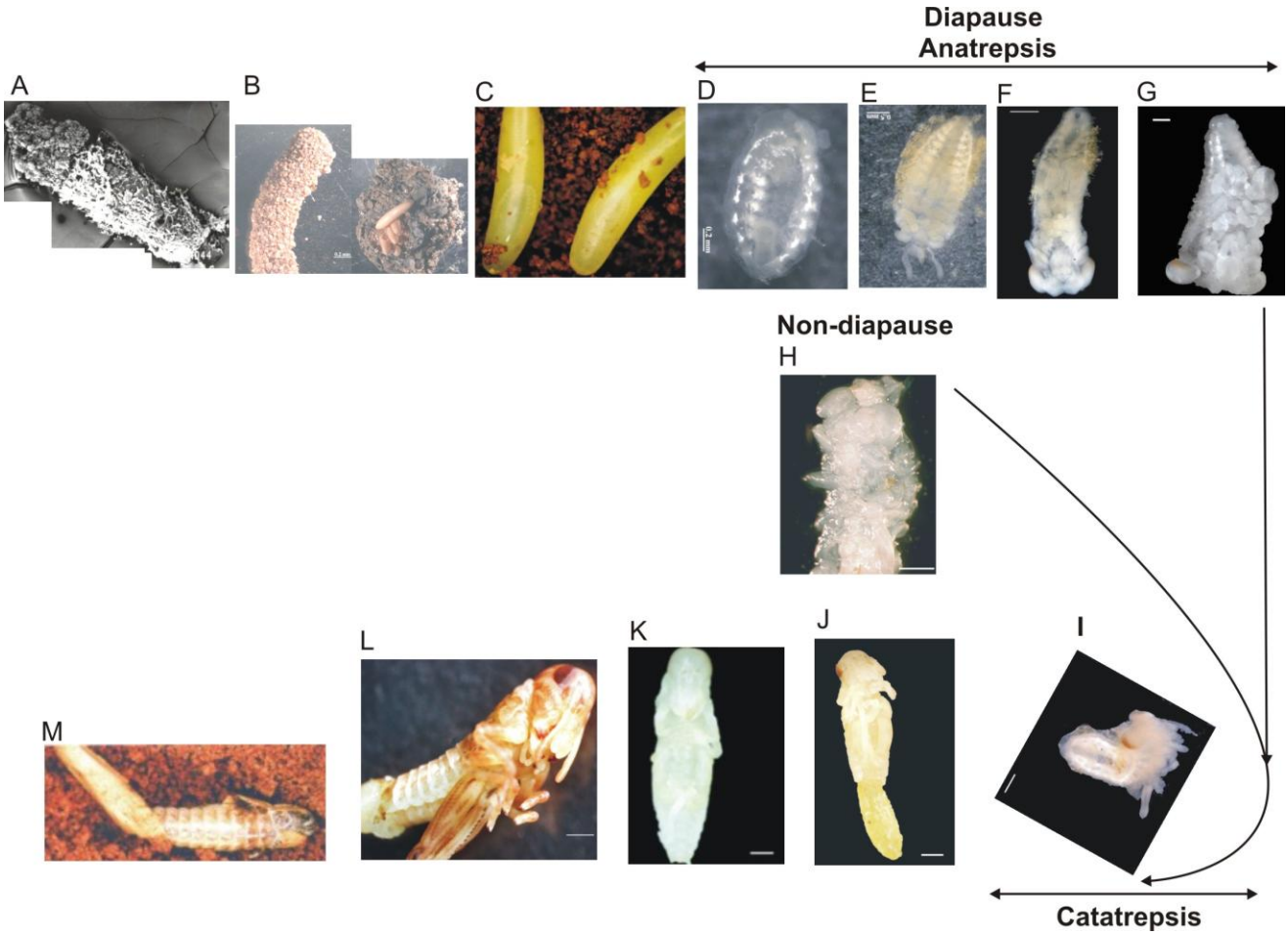


Figure 30. Photographic overview of development in *Locustana pardalina* (see text and Figs 30A to 30M for more detail).

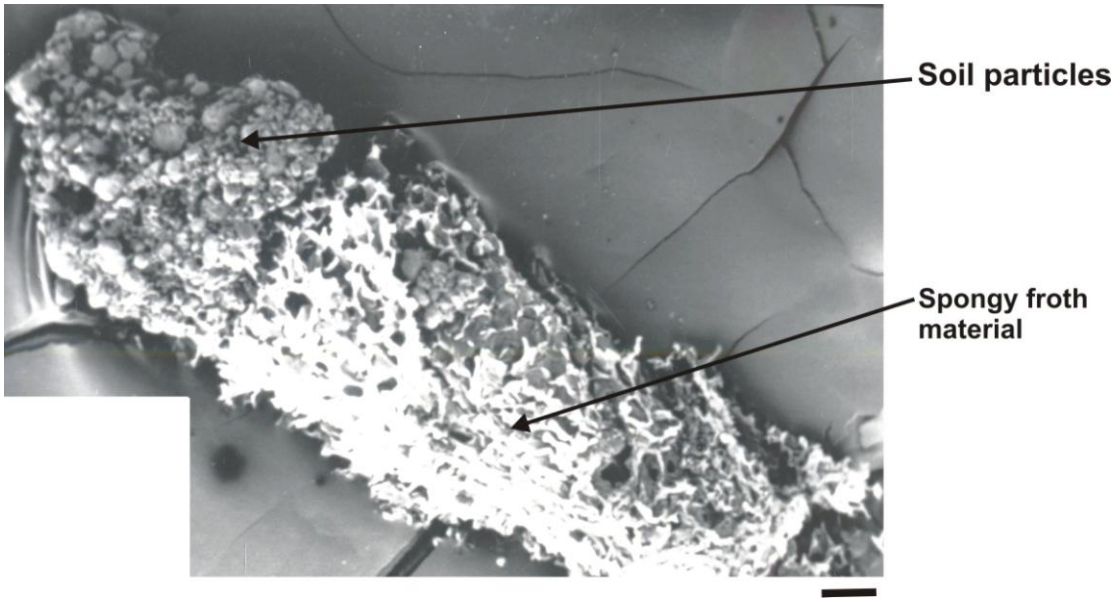


Figure 30A. Electron micrograph of an egg pod (scale bar = 100 µm).

An egg pod is made up of an egg mass and white spongy froth material encrusted with soil particles (Fig 30A). The outer wall of the egg pod is very compact but fragile when freshly laid and hardens over time. The egg pod together with the foam reduces the rate of water loss from the eggs mass considerably (Petty, 1972).

1. Egg pods and eggs

Locustana egg pods shown in Figure 30B were collected in 2 different sites one consisting of sandy clay soil and the other deep red loose sand. These were round and J – shaped, respectively. In a study done in the Bothaville district I, L and S shaped egg pods were found (Price, 1988). The type of soil in which the female lays the eggs has an influence on the egg pod shape. However, Price (1988) concluded that *Locustana* egg pods are predominantly J - shaped (Fig 30B) and were found in sand, loam and clay soil types. Loose sandy soils are associated with straight pods. Twisted and L - shaped pods occur in loam and clay soils (Price, 1988).

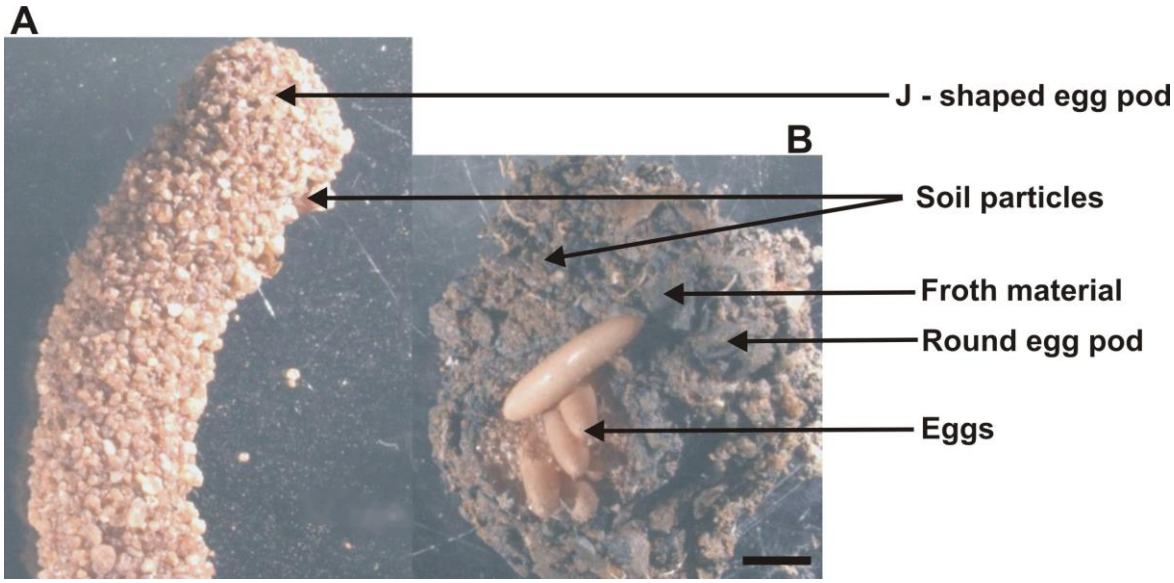


Figure 30B. Light micrograph of different shaped egg pods collected from different field collection sites. A= pod from Pofadder and B = pod from De Aar (scale bar = 0.2 mm).

Removal of the chorion is important when the metabolic rate is being measured and when tracking development of the embryo. The chorion is susceptible to attack by microorganisms. Freshly laid eggs are difficult to dissect successfully without killing the embryo. The most common and easiest method is to view the embryo while it is still inside the egg. This is made possible by soaking the egg in Histoclear for 30 minutes.

Chorion removal does not inhibit development as dechorionated eggs shown in Figure 30C have been shown to hatch successfully. The secondary wax layers around the egg together with the outer yellow and the inner white cuticles prevent water loss in the absence of the chorion (Matthée, 1951).

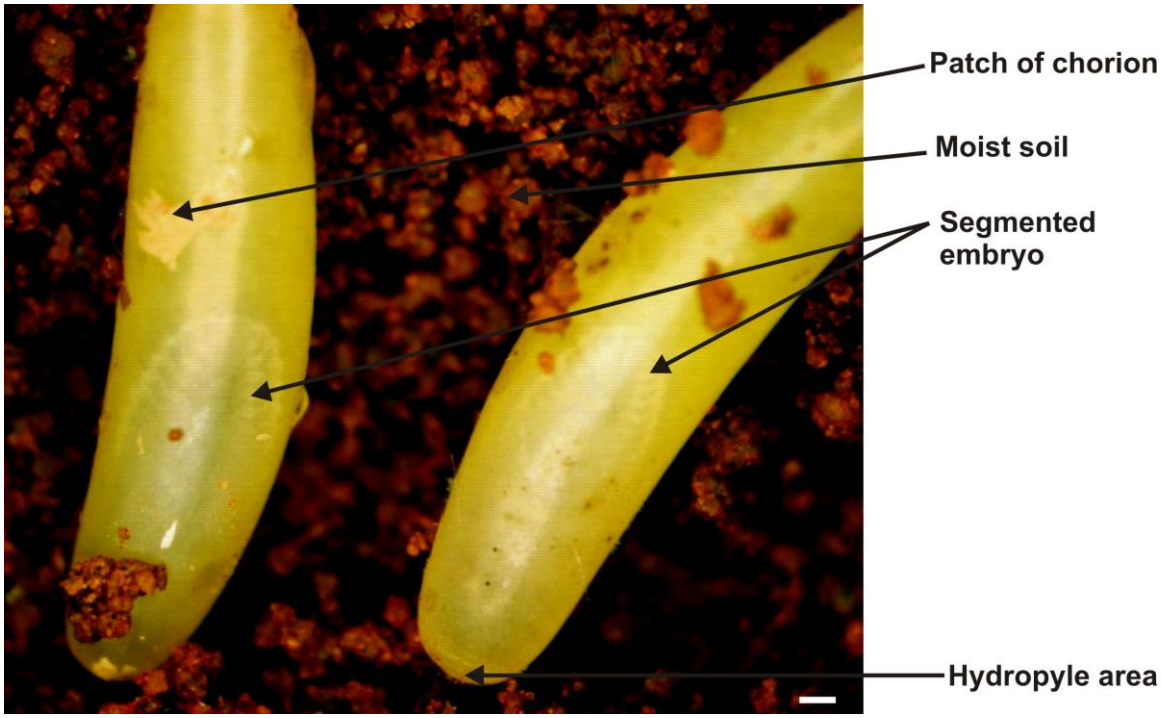


Figure 30C. Light micrograph of dechorionated eggs in anatrepsis (scale bar = 0.06 mm).

The serosal cuticle hydropyle situated at the posterior pole of the egg consists of hydropyle cells responsible for water absorption overlain by the cuticle (Matthée, 1951). The embryos are attached to the hydropyle cells through the serosal strand during anatrepsis.

Early stages of *Locustana* development were described by Matthée (1951). Stages of embryonic development covered in this project involve late anatrepsis to the end of catatrepsis. At late anatrepsis the embryo shows signs of abdominal segmentation (Fig. 30 D).

2. Later stages of anatrepsis

The embryo depicted is considered to be at stage 15 according to Matthée (1951) (Fig. 30D). The embryo has differentiated into the protocephalic and the protocormic regions shown in Figure 30 D. Segmentation had begun from the anterior to the posterior end. The segments could also telescope together making it difficult to count

them. Ten abdominal segments were counted. This marks the completion of abdominal segmentation. At this point the embryo is considered to be in stage 15 of development (Matthée, 1951) or stage III a (Wardhaugh, 1978).

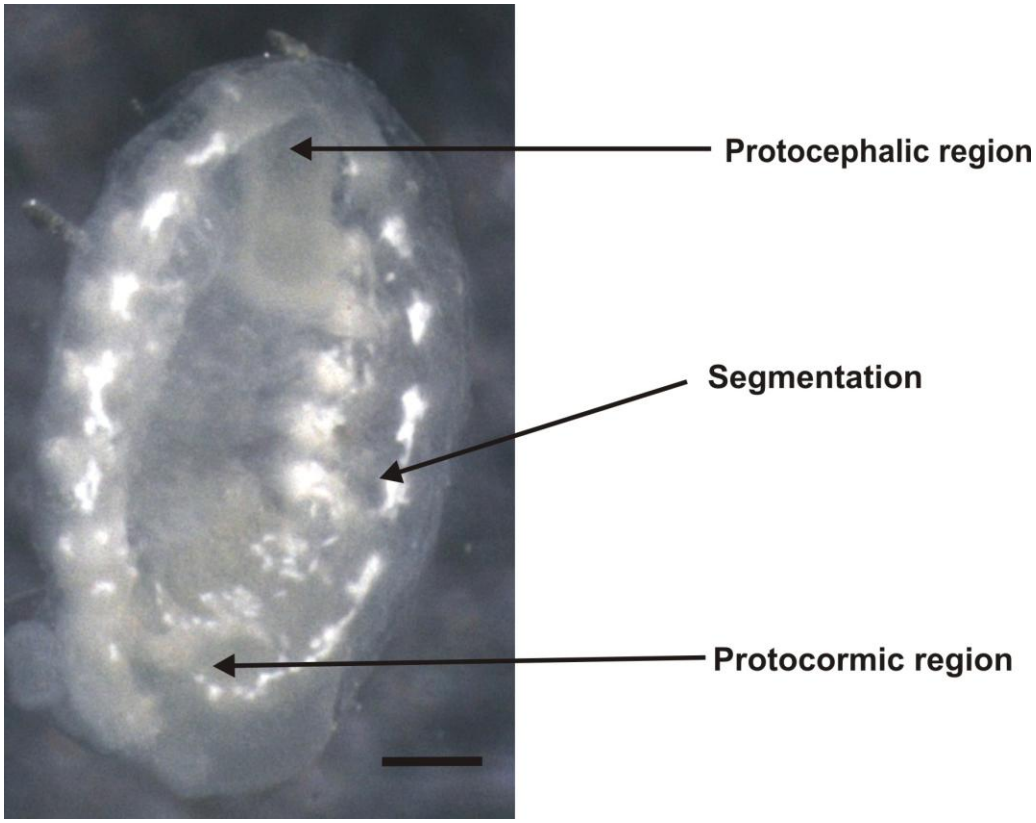


Figure 30D. Light micrograph of a segmented embryo (scale bar = 0.2 mm).

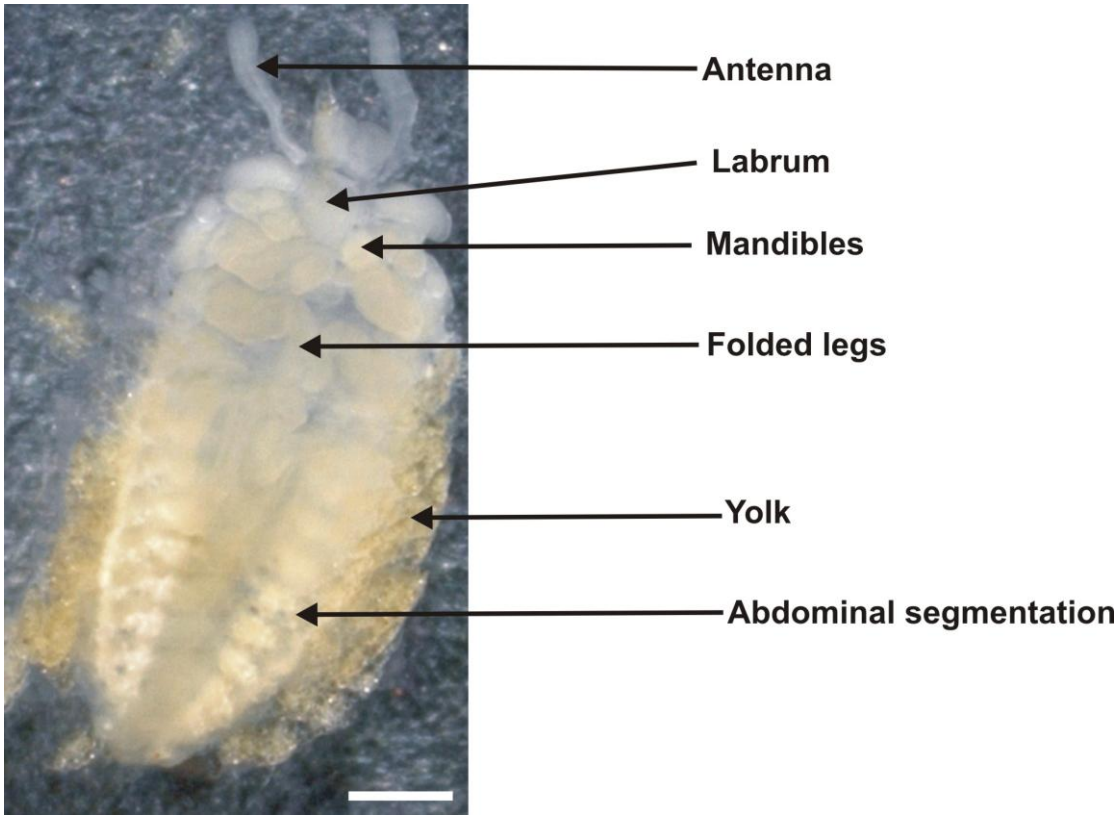


Figure 30E. Light micrograph of a non-diapause embryo (scale bar = 0.5 mm).

At this stage the appendages such as antennae are starting to show segmentation (Fig. 30E). Mouthparts such as mandibles, maxillae and labrum have started to develop. A small swelling between the procephalic lobes has developed into the labrum. Lateral swellings have developed into legs which are starting to show segmentation. Note that the yolk still surrounds the embryo. This embryo is considered to be at stage 21 of development (Matthée, 1951) or stage IV b2 (Wardhaugh, 1978).

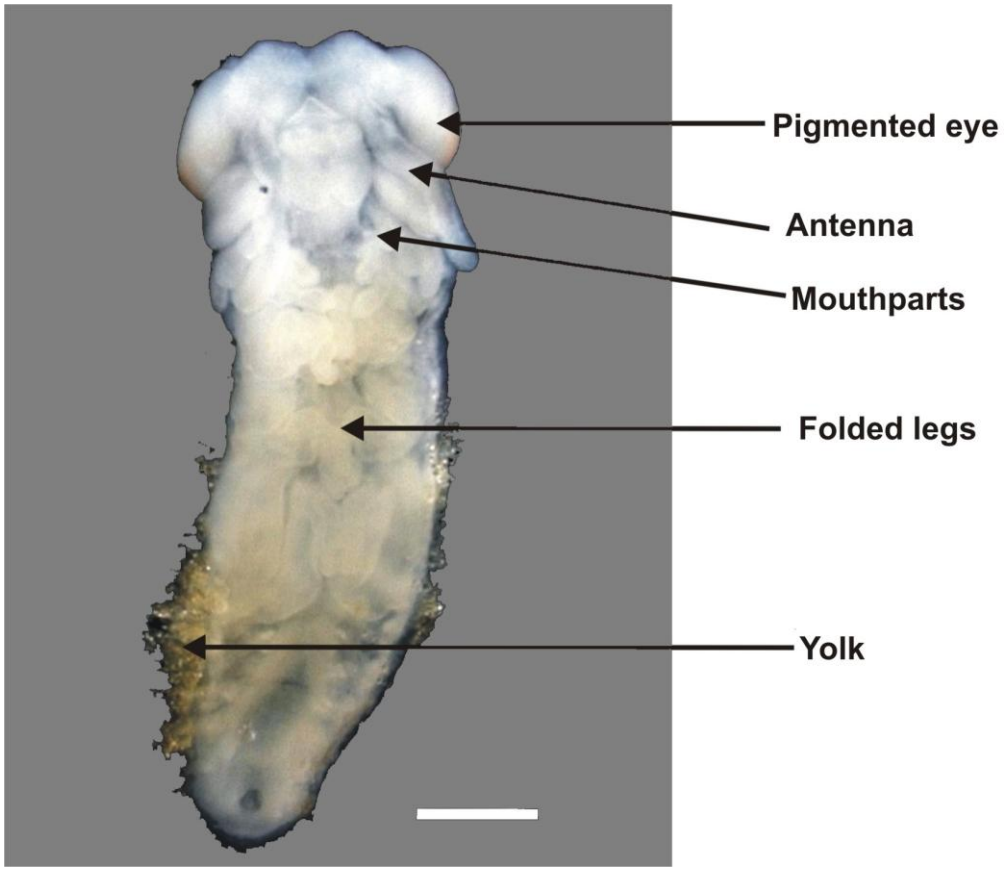


Figure 30F. Light micrograph of a diapause embryo (scale bar = 0.5 mm).

Yolk is still present on the dorsal surface of the embryo (Fig. 30F). Note the red - brown eye pigment in the dorsal part of the eye. This eye pigmentation is indicative of an embryo in diapause as non-diapause embryos lack eye pigmentation as in *C. terminifera* (Wardhaugh, 1978). The legs of the embryo have grown longer. The mouthparts such as the mandibles, maxillae and labrum have developed further (Figs 30F & 30G). This embryo is at stage 26 of development (Matthée, 1951) and stage IV c (Wardhaugh, 1978).

Matthée (1951) did not mention any eye pigmentation of the embryo in his classification and Wardhaugh (1978) mentions this feature in his description of *C. terminifera* embryo. In this study, eye pigmentation was observed in *Locustana* in diapause embryos as described above.

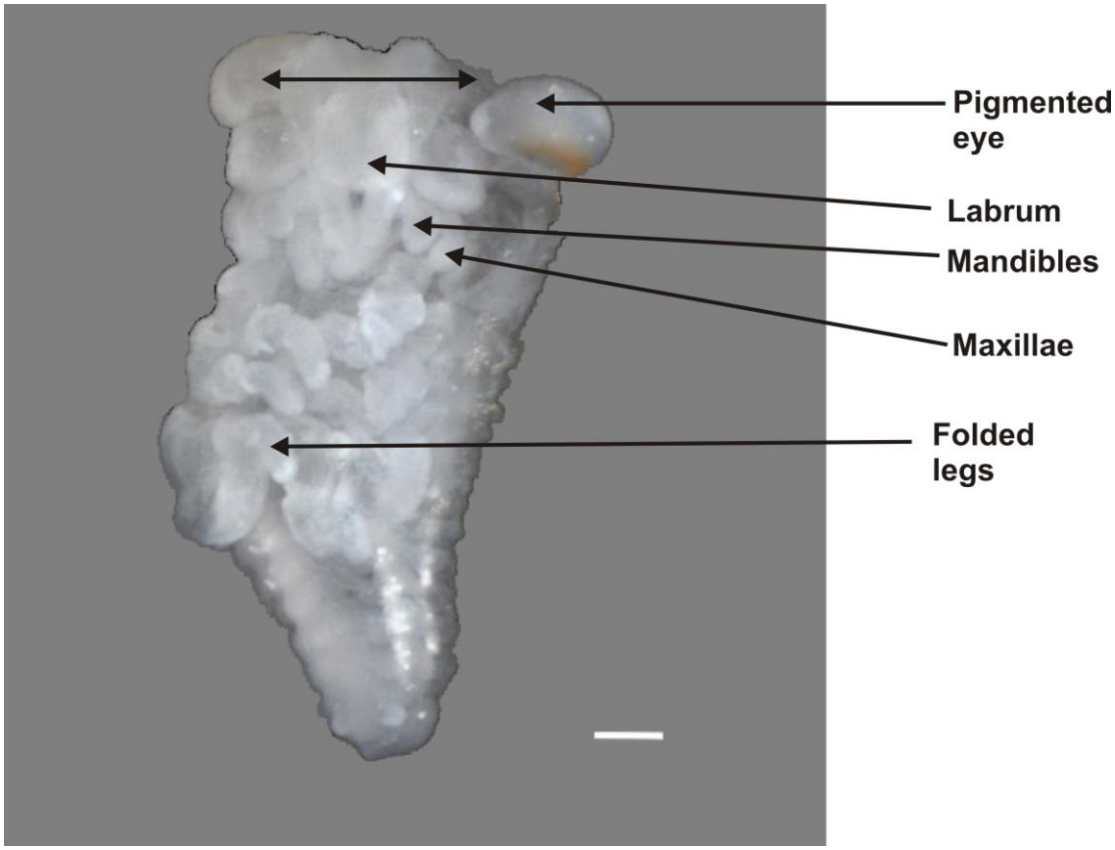


Figure 30G. Light micrograph of a diapause embryo (scale bar = 0.2 mm).

At this stage the eye is becoming heavily pigmented (Fig. 30G). The embryo has become broader (↔), compared with the embryo in Fig. 30F. This confirms Matthée's (1951) observation than once true diapause is reached there is no further development of the embryo but the embryo increases in width. This embryo is at stage 26 according to Matthée's (1951) classification and stage IV c (Wardhaugh, 1978). The embryo has been in diapause for an additional 9 days, compared with Fig. 30F.

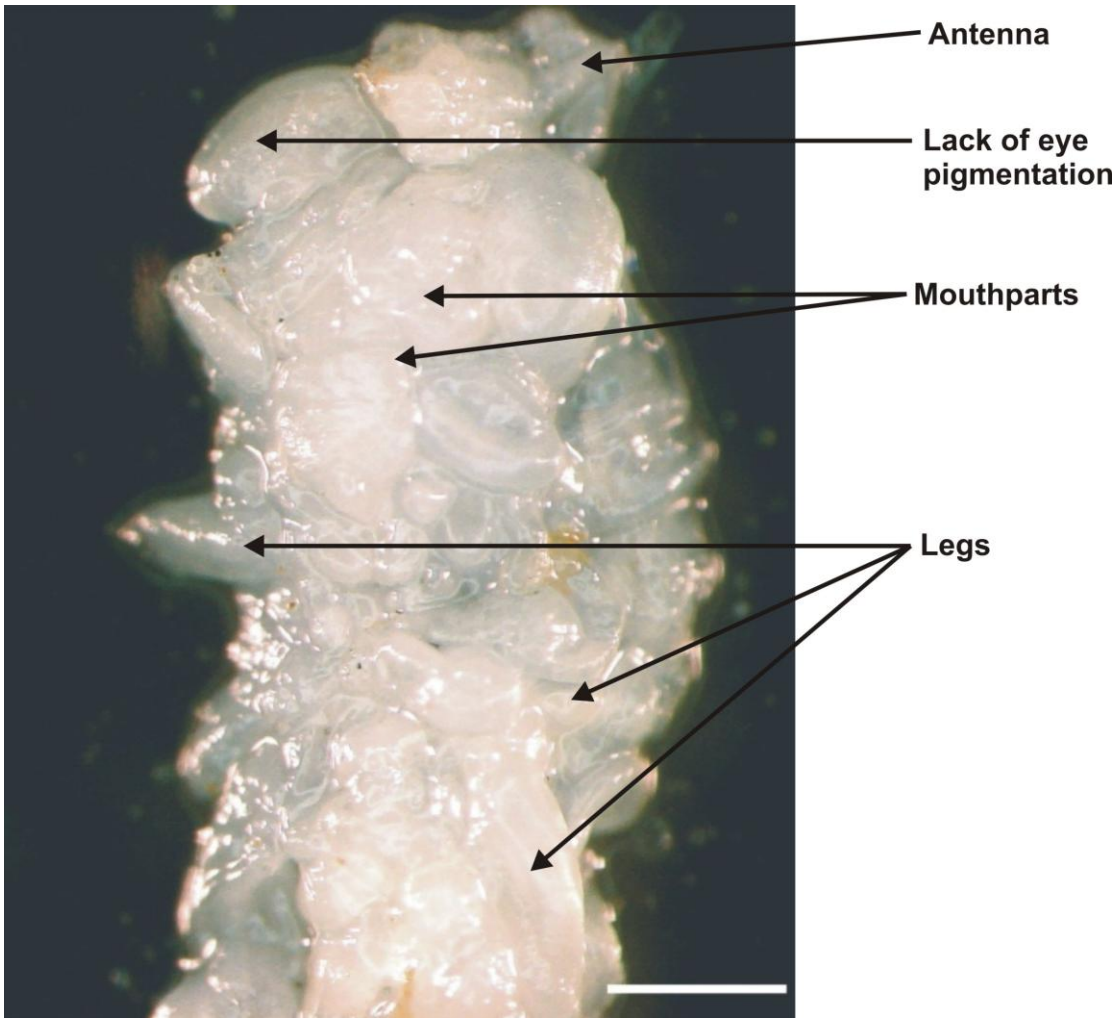


Figure 30H. Light micrograph of a non-diapause embryo (scale bar = 0.5 mm).

Rudiments of appendages are visible (Fig. 30H). This embryo is at a similar stage to Fig. 30F but lacks eye pigmentation and is therefore in non-diapause. Mouthparts are not as well developed as Fig. 30F. Appendages are not clearly visible as they are obscured by yolk (Fig. 30H). This embryo is at stage 26 according to Matthée's (1951) classification and stage IV c (Wardhaugh, 1978).

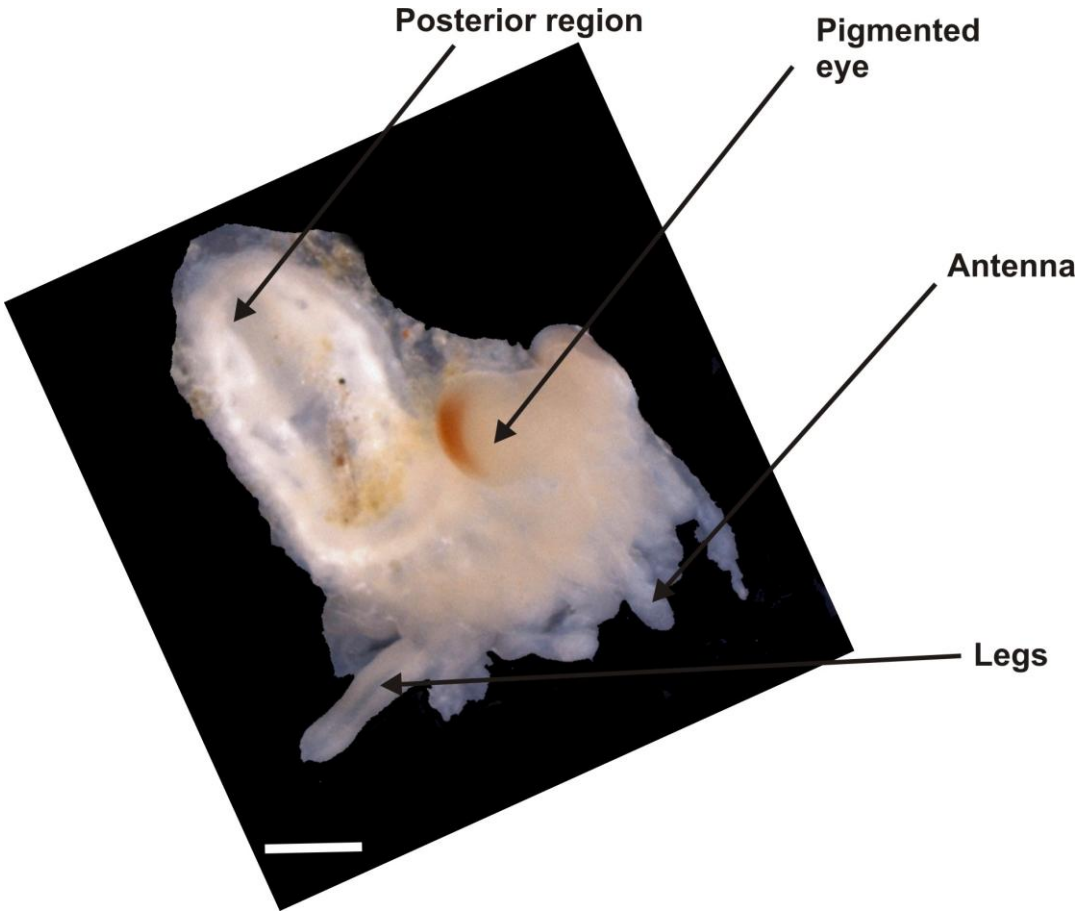


Figure 30I. Light micrograph of an embryo undergoing catatrepsis (scale bar = 0.2 mm).

At this stage the embryo is undergoing catatrepsis which occurs rapidly (Steele, 1941) (Fig. 30I). We were very lucky to find an embryo with catatrepsis partially completed. Catatrepsis is the process involving the embryo turning from the ventral pole of the egg to the dorsal region of the egg. This results in the stretching and breaking of the serosal strand (Matthée, 1951). Note that the body region of the embryo is slightly bent due to yolk resistance as observed in *Austroicetes cruciata* (Steele, 1941). After catatrepsis the embryo encloses the yolk (Steele, 1941).

Catatrepsis in *Locustana* occurs after stage 30^{+} (Matthée, 1951) and stage 20 in *M. differentialis* (Slifer, 1932). The *Locustana* embryo shown in Fig 30L is more advanced with segmentation of legs compared to the less developed *C. terminifera* during stage V of catatrepsis (Wardhaugh, 1978).

3. Catatrepsis and hatching

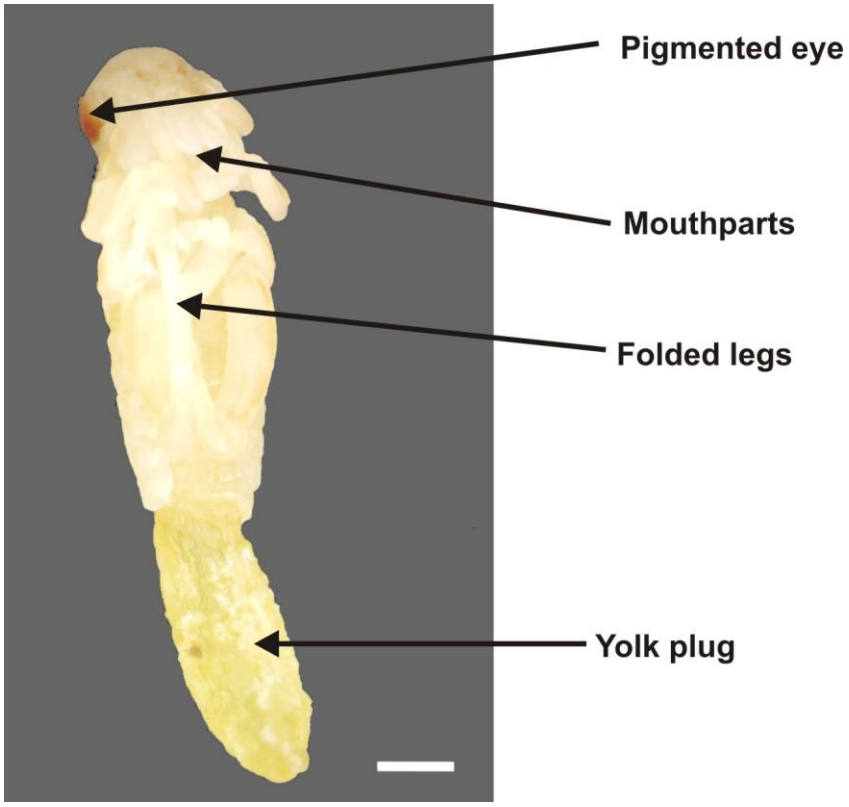


Figure 30J. Light micrograph of an embryo after catatrepsis (scale bar = 0.5 mm).

Note the pigmentation on the dorsal section of the eye is starting to form (Fig. 30J). There is progressive increase in pigmentation over parts of the body. The dorsal closure is incomplete. Note the yolk plug projecting from the dorsal portion of the embryo (Fig. 30J). The folded legs are N-shaped. This embryo is in stage 30^{+} of development (Matthée, 1951) and stage VII b (Wardhaugh, 1978).

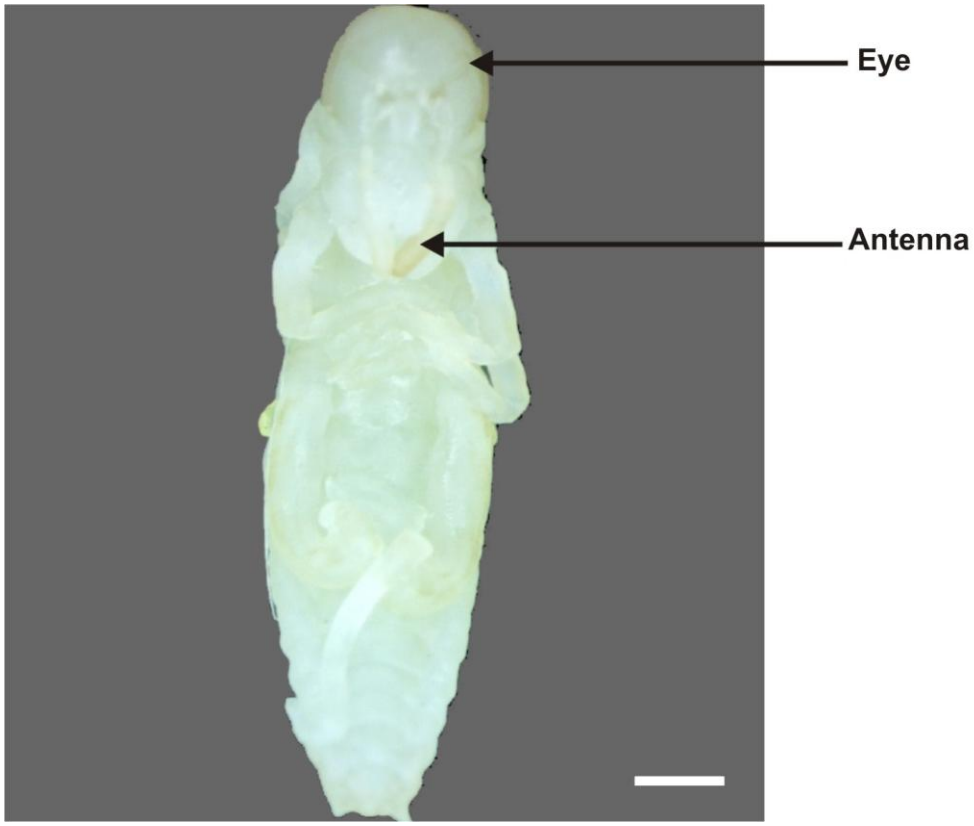


Figure 30K. Light micrograph of a non-diapause embryo (scale bar = 0.5 mm).

The dorsal closure has been completed, no yolk projection visible (Fig. 30K). This embryo is in stage 30^{+} of development (Matthée, 1951) and stage VIII b (Wardhaugh, 1978).

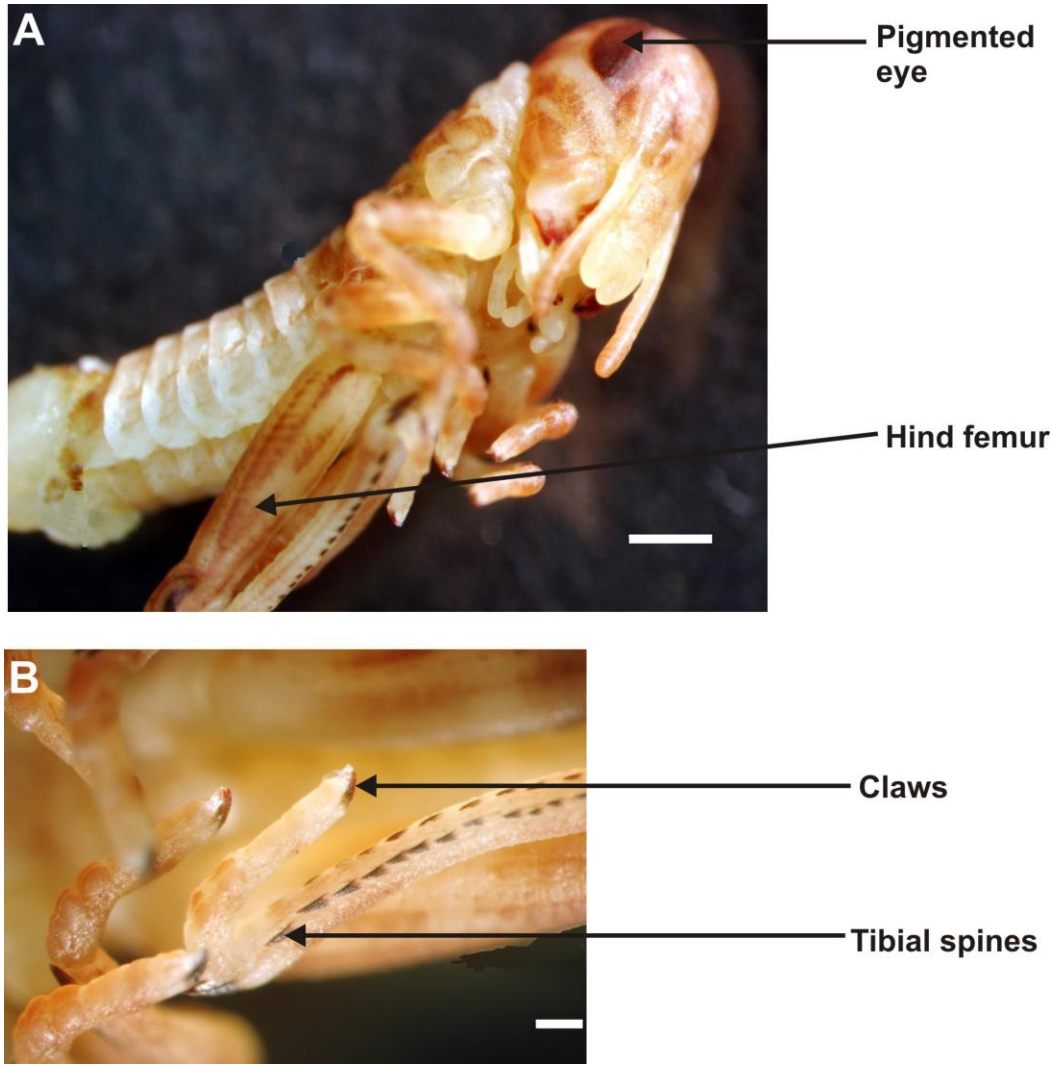


Figure 30L. (A) Light micrograph showing an embryo about to hatch (scale bar = 2 mm), (B) High power light micrograph showing the claws and tibia (scale bar = 0.2 mm).

The entire body of the hopper is strongly pigmented (Fig. 30L). Tibial spines, claws have darkened. Segmentation of antennae, mouthparts and legs are distinct. The hind femur has reached to about the end of the abdomen (Fig. 30L (A)), as observed at the end of catatrepsis in *A. cruciata* (Steele, 1941). The embryo is in stage 30^{+} (Matthée, 1951) and stage IX (Wardhaugh, 1978).

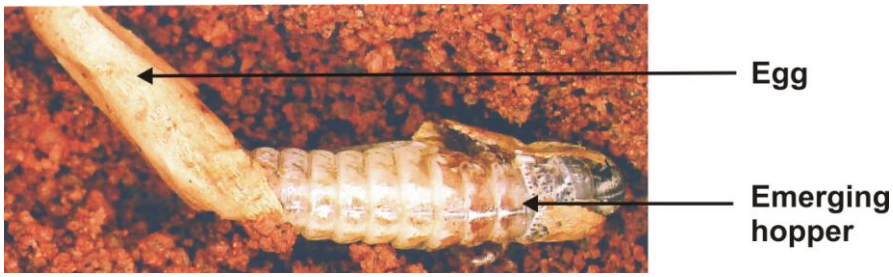


Figure 30M. Light micrograph of a 1st instar emerging from an egg.

The hopper is shown emerging from an egg (Fig. 30M). The cuticle appears light grey to the naked eye on hatching and darkens within an hour after moulting.

3.5 Metabolic rate of eggs correlated with embryonic development

All day 0 eggs were grouped into non-diapause (Table 6A), as it was not possible to distinguish between diapause and non-diapause embryos (Matthée, 1951 & section 2.5). The recorded metabolic rate was relatively low and on dissection the embryos were in the early stages of development and in anatrepsis (Fig. 31, p. 44). All embryos observed after 6 days of incubation were still in anatrepsis (Table 6A).

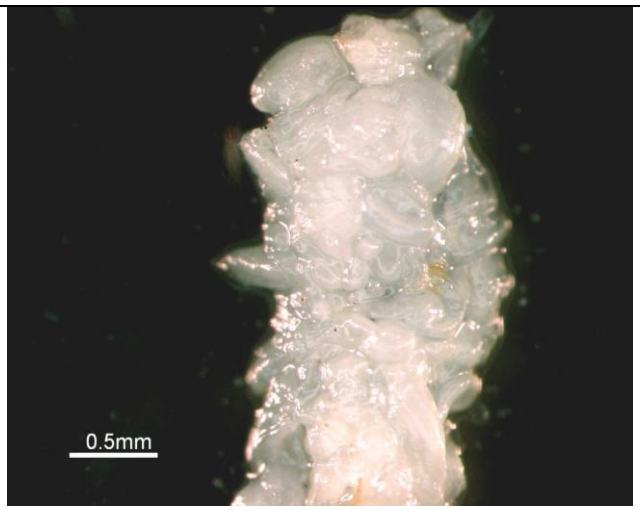
Day 6 seems to be the point at which some embryos remain in anatrepsis until diapause is triggered. Some of the embryos remained in anatrepsis (stage 15 to 29) and were therefore in diapause while others continued developing and entered catatrepsis (stage 30 onwards). There was a large variation in the metabolic rate at day 6 of incubation (Table 6A, Fig. 32, p. 45) followed by a variation in embryonic development at day 8 (Table 6A). A stage 30^{+} embryo which was close to hatching was found after 10 days of incubation (Table 6A).

Generally non-diapause eggs developed quickly after day 8 showing high metabolic rates and hatching after 10 days of incubation (Fig. 32, p. 45). These non-

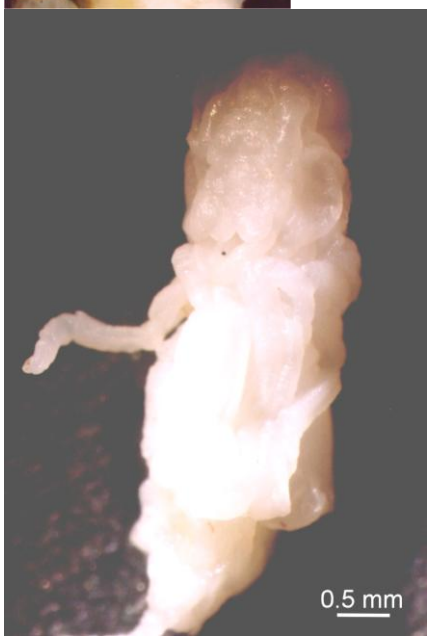
diapause eggs showed a linear increase in their metabolic rate of 0.32 μLCO_2 per day (r^2 = 0.49, P = 0.04) until hatching occurred (Fig. 31) but the variability was large (Fig. 32).

TABLE 6. Mean metabolic rate and corresponding embryonic development in *Locustana pardalina*, n = number of readings of metabolic rate at 3 eggs per measurement, (range).

Table 6A. Non-diapause

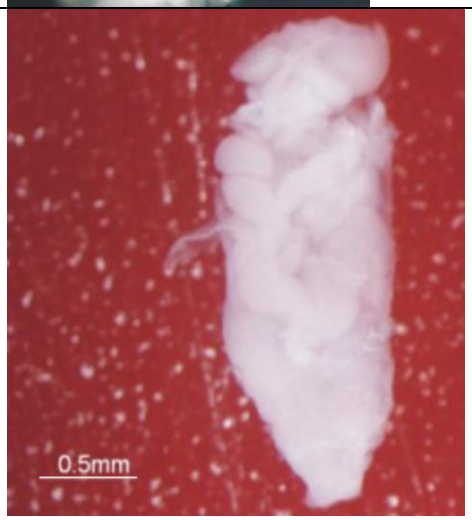
No. of days	**Embryo position relative to hydropyle**	**Mean metabolic rate (μLCO_2/mg/h)**	**Assumed stage of development (Matthée, 1951)**	**Sample of embryo pictures**
0	Embryo facing hydropyle = anatrepsis	2.02 (0.14 - 3.9) n = 11	Late stage 1	
1	Anatrepsis	2.52 (1 - 3.9) n = 5	Late stage 1	
2	Anatrepsis	3.52 (1.7 - 5.3) n = 7	2	
5	Anatrepsis	1.74 (1.4 - 1.96) n = 4	15	
6	Anatrepsis	5.32 (2.8 - 6.9) n = 5	25	

6	Anatrepsis		28	
8	Anatrepsis,	6.7 n = 1	15	

8	Catatrepsis		29	
	Anatrepsis		28	
	Catatrepsis		30^{+}	

9	Catatrepsis Anatrepsis	4.9 n= 1	30+ 24 Other stage found (27)	 
10	Close to hatching	9.2 n = 1	30+	

Table 6B. Diapause

No. of days	**Embryo position relative to hydropyle**	**Mean metabolic rate (μLCO_2/mg/h)**	**Assumed stage of development (Matthée, 1951)**	**Sample of embryo pictures**
8	Anatrepsis	1.22 (0.15 - 2.3) n = 2	18 28	
9	Anatrepsis	0.69 n=1	27	

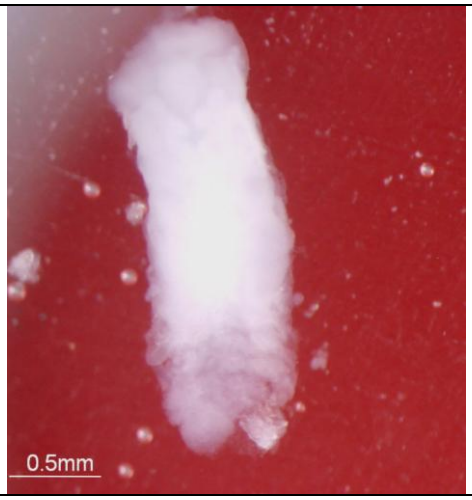
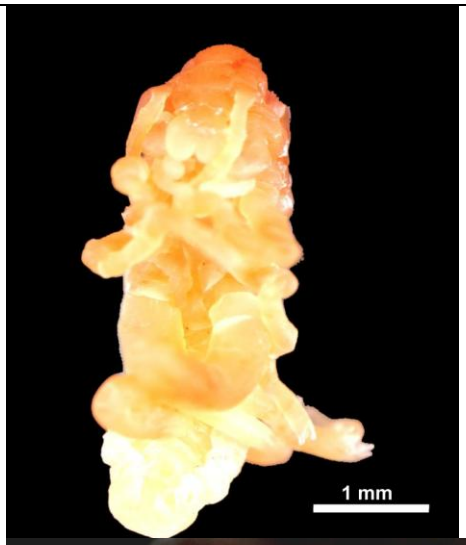
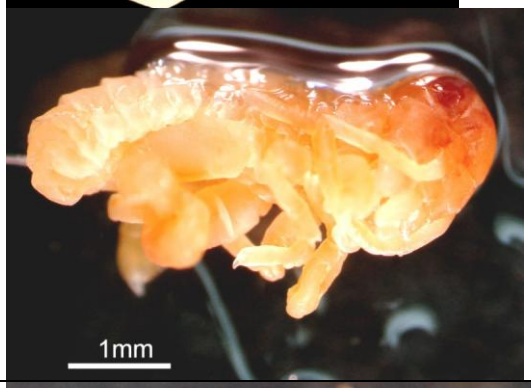
9	Anatrepsis		24	
10	Anatrepsis	1.12 n =1	24	
12	Anatrepsis	2.27 (1.73 - 3.1) n = 2	24	
15	Anatrepsis	1.42 n = 1	28 Other stage found (24)	
20	Anatrepsis	3.3 n = 1	24	
26	Anatrepsis	1.26 (0.59 - 1.92) n = 2	15	

Table 6C. Quiescence

No. of days	Embryo position relative to hydropyle	Metabolic rate (μLCO_2 /mg/h)	Assumed stage of development	Embryo picture
15	Catatrepsis	0.175 n = 1	30^+	
			30^+	
18	Catatrepsis	0.157 n = 1	30^+	

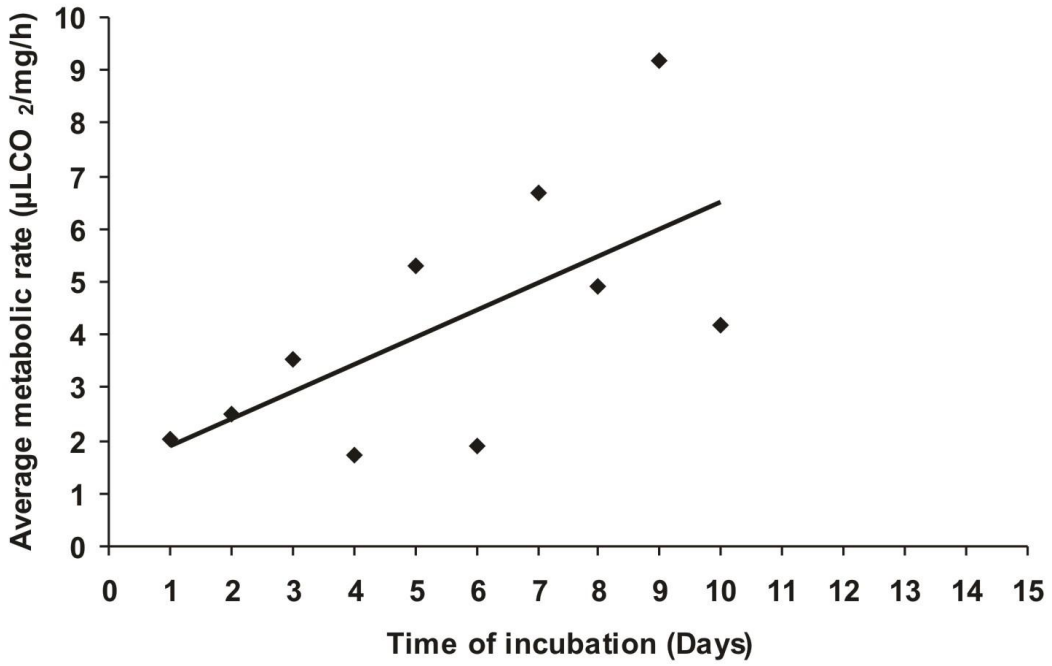


Figure 31. Average metabolic rate of non-diapause eggs of *Locustana pardalina* ($y = 0.51x + 1.4$, $r^2 = 0.42$, $p = 0.037$).

The metabolic rate of diapause eggs varied within a narrow range compared with non-diapause eggs (Table 6B). Although a variation was found in embryonic development stages, all 13 dissected embryos were still in anatrepsis and therefore in diapause (Table 6B). The common feature of the diapause eggs was the relatively low metabolic rate between 8 and 26 days after which the experiment was terminated (Table 6B). Non-diapause eggs after day 6 had an average of 5.3 μLCO_2 /mg/h while diapause eggs showed an average of 1.4 μLCO_2 /mg/h (Table 6A &B). All diapause eggs had a significantly lower metabolic rate than that of day 6 non-diapause eggs (Fig. 32) ($t_{0.05, 4} = 4$, $P = 0.02$). Day 6 was chosen due to the change and variability in the metabolic rate in non-diapause eggs. Most embryological changes occurred at day 6 of development.

Only 2 embryos were found to be in quiescence based on the stages of embryonic development (Table 6C). Their metabolic rates were lower than those of

diapause embryos (Fig. 32). Quiescent eggs had an average metabolic rate of 0.17 μLCO_2 /mg/h (Table 6C). Further experiments were conducted on diapause and quiescent embryos (see section 3.6 & 3.7).

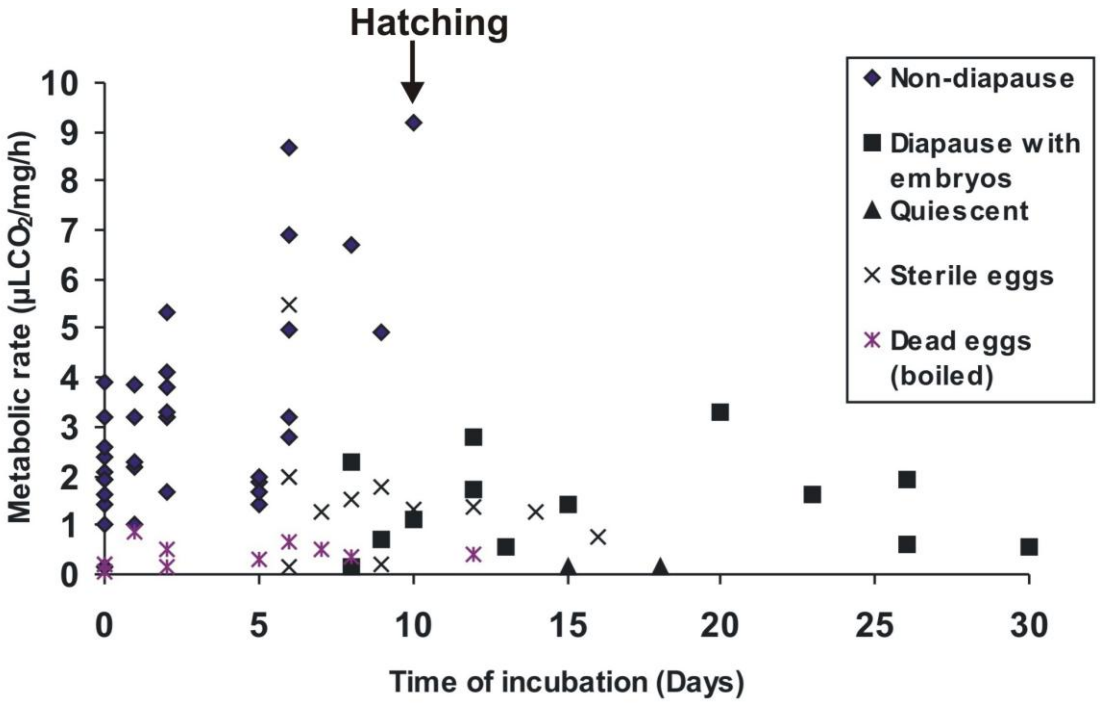


Figure 32. Metabolic rate of *Locustana pardalina* eggs during development.

On dissection some of the eggs had no embryos and were classified as sterile eggs. The average metabolic rate of the sterile eggs was (1.5 ± 1.4) μLCO_2 /mg/h (Fig. 32).

The average metabolic rate of dead eggs was (0.4 ± 0.2) μLCO_2 /mg/h (Fig. 32). This metabolic rate was used as a control. To make sure that the boiled eggs were dead, they were placed in 1 % colourless tetrazolium for 24 hours at room temperature in a dark cupboard. Live eggs were also incubated, as a control, at the same time. Live eggs became pink. Dead eggs did not change colour which would have indicated respiratory activity (refer to section 2.4). These eggs also fitted the

criteria of dead eggs mentioned previously (section 2.2). For further results see section 3.10.

3.6 Metabolic rate of diapause eggs

As one aspect of this study was dormancy, we wanted to work on more eggs in this state of diapause and quiescence. In order to increase the sample size of diapause eggs measured in section 3.5 (Table 6B), experiments were repeated on laboratory reared and field collected eggs.

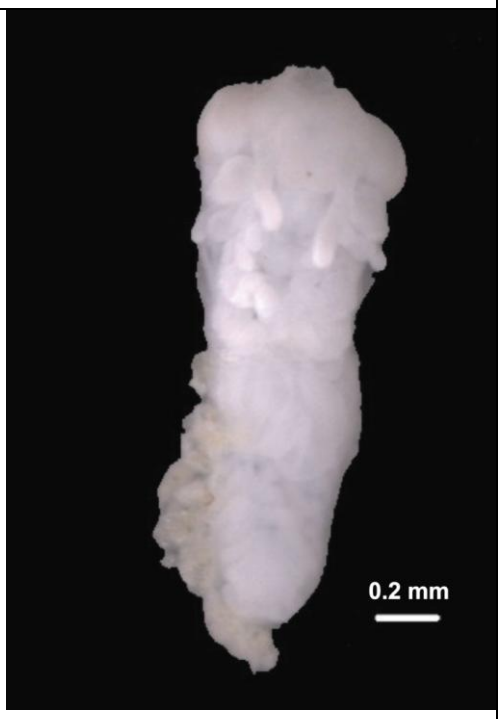
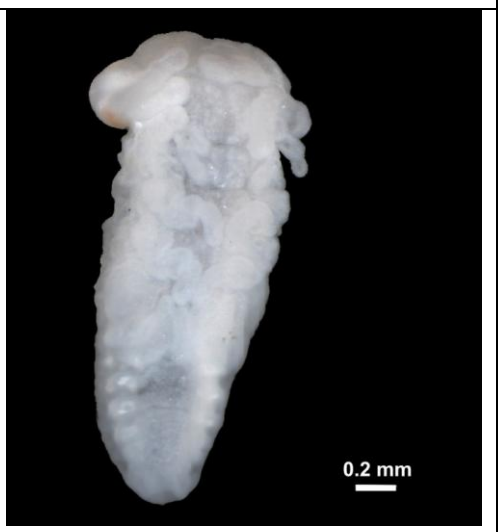
TABLE 7. Mean mass (mg) of individual laboratory and field diapause eggs used for metabolic measurements. Control eggs = dry field eggs

	Laboratory (wet) eggs	**Field (wet) eggs**	**Control (dry) eggs**
Mean	11.1	13.7	12.6
SD	1.8	3	2.2
Range	8.4 - 15	8.6 - 20.2	10 - 16.9

There was a variation in the mean egg mass of the diapause eggs (Table 7). Laboratory reared eggs had the lowest mean mass of 11 ± 1.8 mg compared with all other eggs. Mean egg mass of control eggs was slightly lower than field collected eggs (Table 7). This showed that field collected eggs placed on moist soil had hardly absorbed any water.

TABLE 8. Mean metabolic rate of laboratory diapause eggs kept on moist soil and corresponding embryonic development.

Time after 14 days of incubation	**Mean metabolic rate ($\mu LCO_2/mg/h$)**			**Assumed stage of development (Matthée, 1951)**	**Sample of embryo pictures**
	Batch 1	**Batch 2**	**Batch 3**		
0	3.9	2.6	2.4	20	 
1	3.88			20	
2	4.9	2.5		24	
5	1.3			24	

6			2.8	20	Damaged after measurement
7	1.9			29	
8		2.3		20	
12			1.73	24	
13			0.55	24	Damaged after measurement
21		1.63		20	
23	1.36	1.9		28	

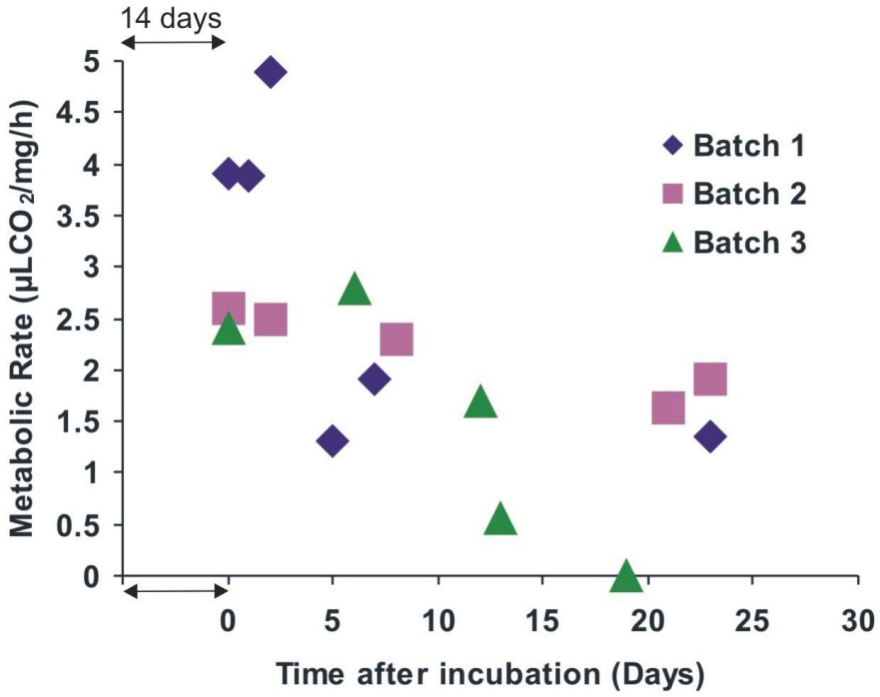


Figure 33. Metabolic rate of laboratory diapause eggs on moist soil.

These 3 batches of laboratory reared eggs were tested as described in section 2.5. Eggs which remained in diapause had a stable mean metabolic rate of 2.02 µLCO_2/mg/h in batch 2, 1.52 µLCO_2/mg/h in batch 1 and 2.18 µLCO_2/mg/h in batch 3. The average metabolic rate of diapause eggs was 1.9 ± 0.6 µLCO_2/mg/h (Fig. 33). The initial increase after 2 days of incubation in the metabolic rate in batch 1 (Fig. 33) may possibly imply that these eggs were coming out of diapause. The differences in the metabolic rate between all diapause eggs and non-diapause eggs at day 2 after incubation (section 3.5, Table 6B) was significantly different ($t_{0.05, 8} = 3.5$, P= 0.007).

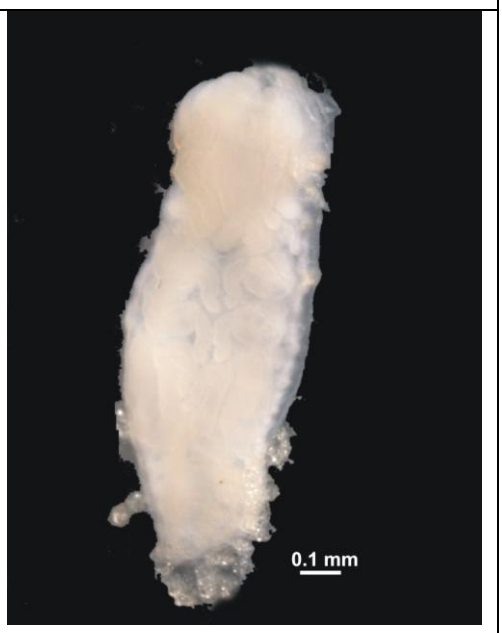
The metabolic rate was stable for all 3 batches for 15 days (Fig. 33). The low metabolic rate in batch 3 after 19 days of incubation was due to the fact that the eggs were becoming fragile and dying. However, one embryo dissected out of batch 3 after 19 days of incubation (Table 9) was a healthy viable embryo noted to be in stage 28.

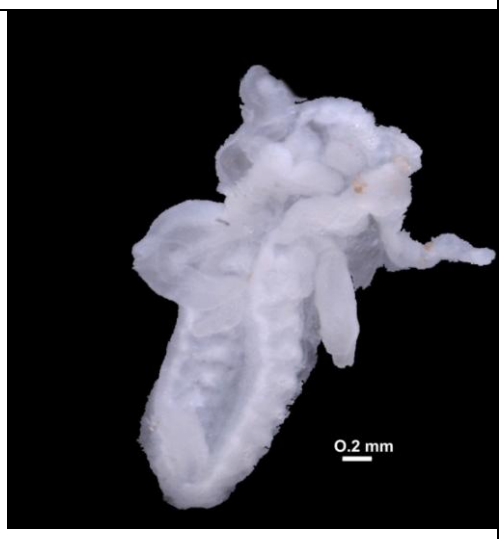
Eggs in batch 2 not used for measurements started hatching after 37 days from when they were initially watered. This confirms that eggs were alive and healthy

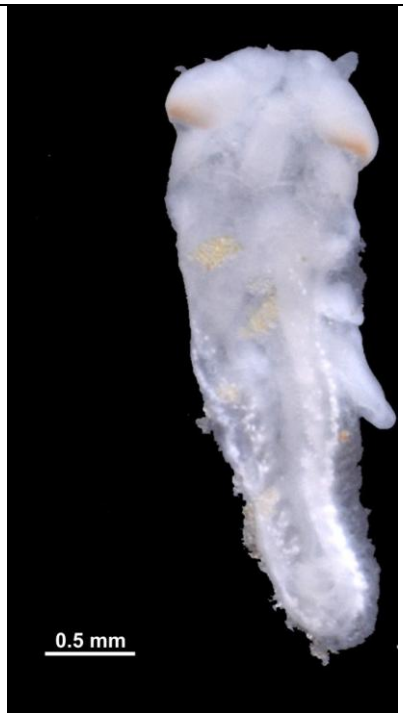
during the experiment. Eggs were first watered 21 days after being laid. Matthée (1951) reported that eggs remain in diapause between 9 and 40 days. Some eggs used in our experiment remained in diapause for 61 days.

Field egg batches 1 and 2 were collected in De Aar while field egg batch 3 was collected in Pofadder. They were laid on the 17th April 2008 in Pofadder and on the 18th May 2008 in De Aar. These egg pods were a mixture of diapause and non-diapause types. The bulk of the eggs were in diapause with a few non-diapause eggs hatching at day 11 after watering and separation. The remainder of the field collected eggs maintained a stable metabolic rate with a range 0.1 - 1.3 µLCO_2/mg/h (Table 9, Fig. 34) compared to 0.6 - 2.8 µLCO_2/mg/h of laboratory reared eggs.

TABLE 9. Mean metabolic rate of field eggs kept on moist soil and corresponding embryonic development.

Days	**Mean metabolic rate (μLCO_2/mg/h)**			**Assumed stage of development (Matthée, 1951) & Embryo picture**	
	Field 1	**Field 2**	**Field 3**		
0	0.026			27	 
1	0.88	0.14		28	
2		0.13	0.73	24	
4	0.77		0.75	27	

5		0.64		27	
6	0.39			24	
7		0.79		28	
8	1.3			24	
11	1.5		3.2	24	
12		4.3		28	
13	1.2		0.075	24	
14		0.61		24	
15	0.75		4	27	
16		0.57		28	
18	0.49		0.05	24	
19		0.62		24	
20	0.17		0.2	24	
22	0.12		0.34	27	
25			0.12	24	

26	0.3			27	
27		0.975		24	
28	0.11		0.058	28	

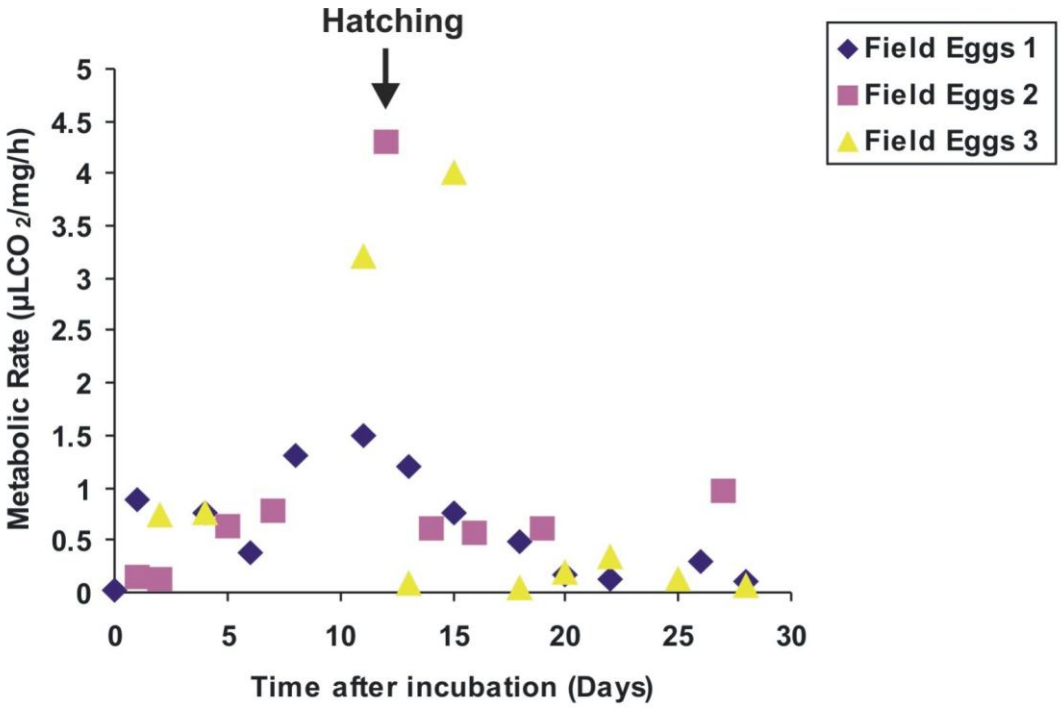


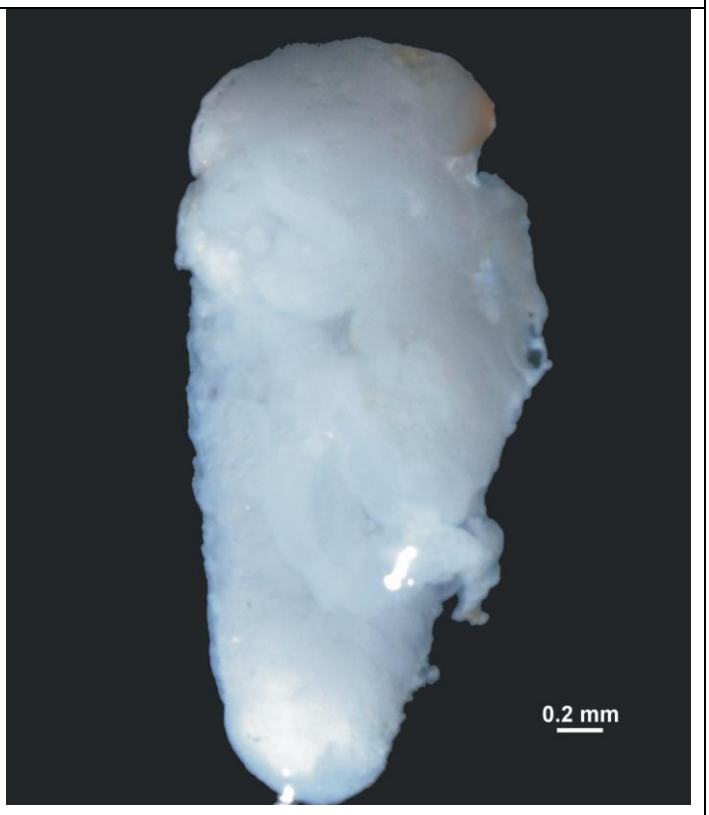
Figure 34. Metabolic rate of field eggs collected from Pofadder and De Aar, kept on moist soil.

The average metabolic rate of field collected eggs in diapause was 0.5 µLCO_2/mg/h (Fig. 34). The high metabolic rate recorded at day 12 was related to eggs coming out of diapause and being close to hatching (Fig. 34, Table 9). Hatching occurred with the emergence of 2 hoppers at day 12 of incubation from the stock of eggs incubated and not used for metabolic measurement (Fig. 34). Field 1 and 2 eggs had been laid 4 days prior to the beginning of the experiment, while field 3 eggs had been in diapause for 35 days previously.

On extraction from the egg pods, field collected eggs were more turgid and heavier compared with laboratory diapause eggs (Table 7). The mean mass of individual laboratory reared and field collected eggs was significantly different ($t_{0.05, 38}$ = 3.8, P = 0.0004) although their ranges overlapped (Table 7). The stages of embryonic development in laboratory and field diapause eggs were similar (Table 8 & 9).

The metabolic rate of field collected eggs not placed on moist soil was used as a control to compare with field and laboratory eggs. Eggs maintained a low stable metabolic rate with an average of 0.61 ± 0.48 µLCO_2/mg/h (Table 10, Fig. 35). On dissection the embryos were found to be in anatrepsis.

TABLE 10. Mean metabolic rate of dry field eggs [all embryos were at stage 28, according to Matthée's (1951) classification].

Time (Days)	**Mean metabolic rate (µLCO_2/mg/h)**	**Sample of embryo picture**
0	0.33	
3	0.37	
5	0.4	
7	1.4	
10	1.5	
12	0.46	
14	0.57	

17	0.67	
19	0.23	
21	1.2	
25	0.04	
27	0.16	

Laboratory reared eggs had a significantly higher metabolic rate than either field or control eggs ($F_{0.05;\ 44} = 37.4$, $P = 0.329$). No differences were found in the metabolic rate of field and control eggs. The range in the metabolic rate of laboratory diapause, field diapause and control eggs was (0.6 - 2.8), (0.1 - 1.3) and (0.04 - 1.5) μLCO_2/mg/h, respectively.

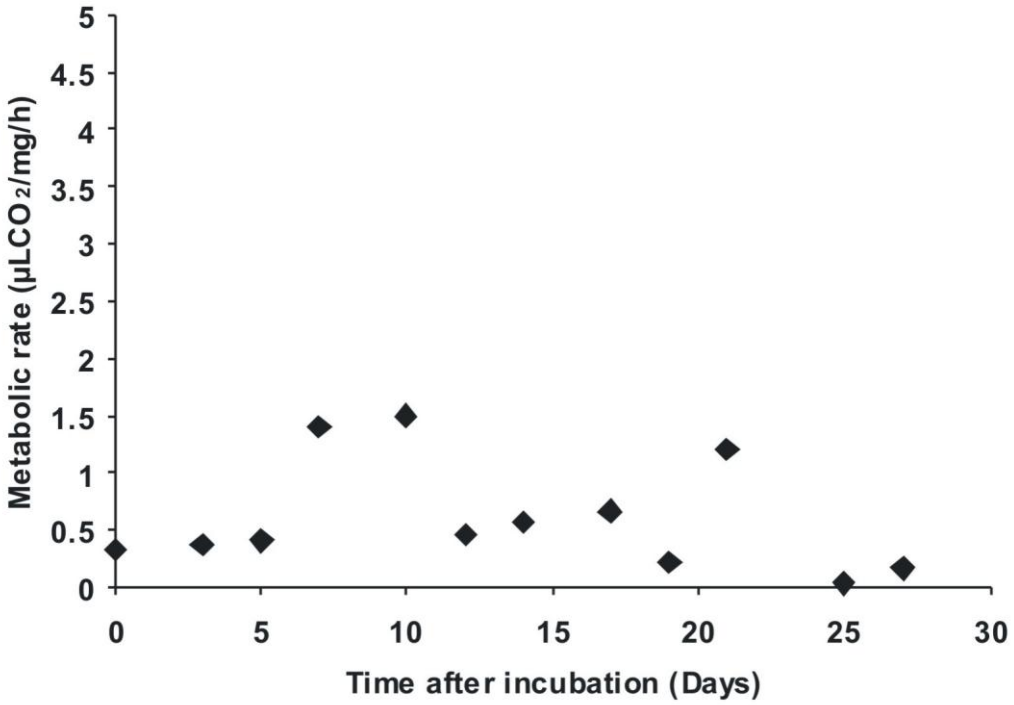


Figure 35. Metabolic rate of dry eggs collected from De Aar and kept on dry soil.

The embryonic stages of laboratory reared and field collected diapause eggs were all in late anatrepsis (stages 28 - 29). The mean basal metabolic rate appeared to be higher for laboratory reared eggs compared with dry eggs (Fig. 35). Most of the

laboratory reared eggs had hatched while still in the egg pods prior to the beginning of the experiment. Very few field collected diapause eggs were stimulated to resume development.

3.7 Metabolic rate of quiescent eggs

Quiescent eggs can be obtained in 2 ways. They can be obtained from freshly laid eggs that have been kept dry from time of laying for 45 days. Under such circumstances diapause is broken in 95 % of these eggs resulting in these eggs entering quiescence (Matthée, 1951). Secondly, after catatrepsis has occurred non-diapause eggs will enter quiescence under dry conditions (Matthée, 1951). The original sample size of quiescent eggs was small (section 3.5; Table 6C) and therefore the metabolic rate measurements were obtained from 3 new batches of eggs in which quiescence has been induced (section 2.5, Fig. 8)

TABLE 11. Mean metabolic rate of normal developing eggs that were induced into quiescence (as outlined in Fig. 8).

Days after removal from egg pods	**Mean metabolic rate (µLCO_2/mg/h)**		
	Batch 1	**Batch 2**	**Batch 3**
2	1.9	2.8	3
5	4.6	4.6	3.6
7	3.3	3.8	2.5
9	5.1	2.8	5.3
11	4	1.4	4.9
15		5.3	
17		6.4	

The average metabolic rate at day 2 of the experiment was 2.6 µLCO_2/mg/h (Table 11). Non-diapause eggs after 2 days of incubation had an average metabolic rate of 3.5 µLCO_2/mg/h (section 3.5, Table 6A). These values were not significantly different ($t_{0.05, 7}$ = 0.05, P=0.1). There was an initial increase in the metabolic rate to an average 3.4 µLCO_2/mg/h at day 2 and 5 in all 3 egg batches (Fig. 32). This increase was equivalent to that of normal developing eggs (section 3.5, Fig. 32). Desiccating the eggs for 2 and 4 days at day 7 and 9 resulted in a slight average increase in metabolic rate by 0.4 µLCO_2/mg/h (Fig. 36).

On rehydration of batch 2 eggs at day 11 to prevent death, the metabolic rate increased to an average 5.9 µLCO_2/mg/h (Fig. 36). This was a 2 - fold increase in the metabolic rate which is similar to non-diapause eggs about to hatch. In batch 2 eggs quiescence had been broken and the embryos were on their way to hatching. Some of the eggs in batch 2 had embryos which occupied the whole egg and this was an

indication that they were alive and about to hatch. However by this stage there were no eggs available to continue with the experiment to observe hatching.

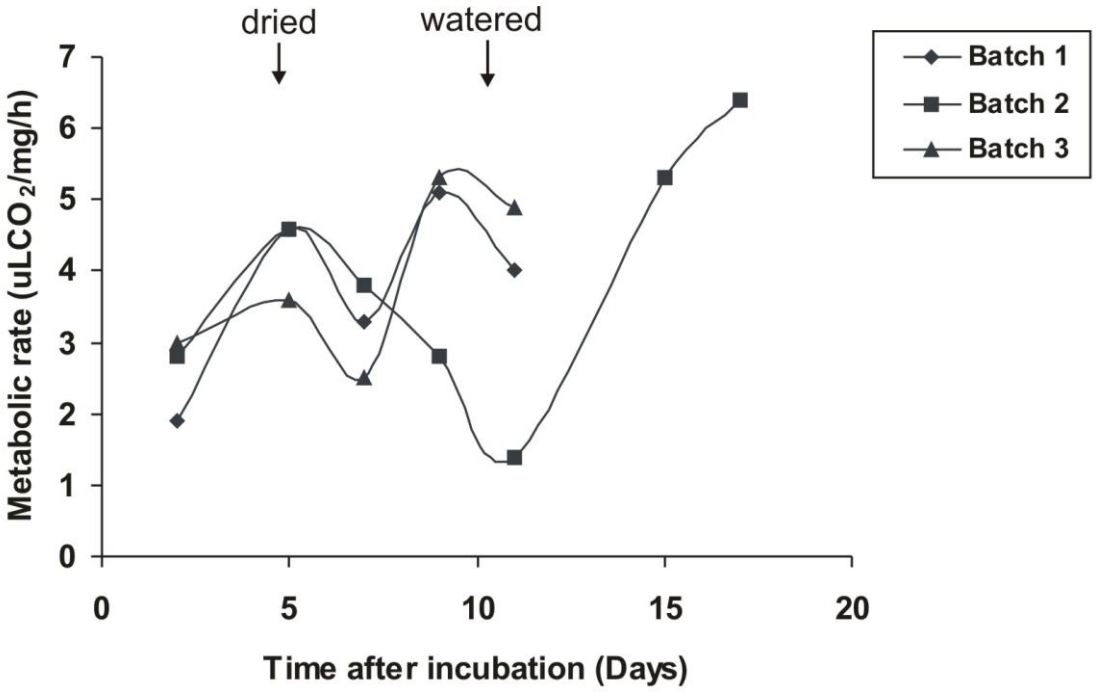


Figure 36. Metabolic rate of quiescent eggs in 3 different batches. There were no eggs left after 11 days in batches 1 and 3.

Eggs in quiescence were also found after 15 and 18 days of incubation with an average of 0.17 µLCO_2/mg/h (section 3.5, Table 6C). The category of these eggs was known but it was unknown how long they had been in quiescence. This low value of the metabolic rate could be due to a longer period of quiescence. Batch 2 eggs that were rehydrated after day 11 and 15 of incubation (Fig. 36) had a relatively higher mean metabolic rate compared to these quiescent eggs (section 3.5, Table 6C).

3.8 The metabolic rate of dead eggs

The recorded metabolic rate of diapause and quiescent eggs was lower than expected. In order to provide a control, eggs were killed by boiling them. These are detailed results from section 3.5 Fig. 32.

TABLE 12. Mean metabolic rate of dead eggs (n=20).

Time (days)	**Metabolic rate (μLCO_2/mg/h)**
0	0.05
2	0.16
5	0.3
7	0.51
8	0.37
12	0.42

The lowest metabolic rate recorded on dead eggs was 0.05 μLCO_2/mg/h at day 0 (Table 12). Dead eggs are susceptible to fungal infection and any of the eggs observed to have fungus were discarded.

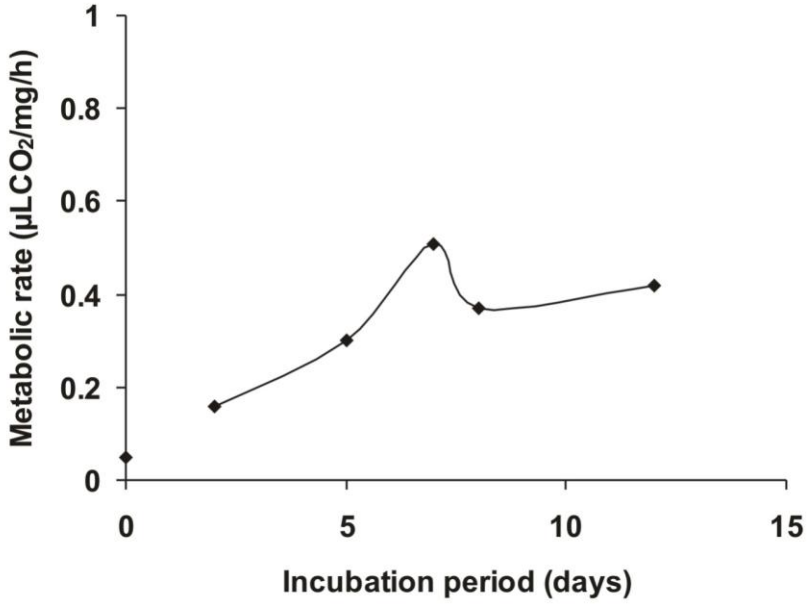


Figure 37. Metabolic rate of dead eggs.

There was a steady increase in the metabolic rate from day 2 to day 7 (Fig. 37). This was followed by a drop in the metabolic rate after day 7 and a steady increase in the metabolic rate until day 12 of incubation. This steady increase was probably due to microorganisms inside the egg. The eggs were covered with fungus after day 12 and therefore the experiment was terminated.

3.9 The effect of dehydration and hydration on egg metabolism

In the field eggs are subjected to dry and wet conditions. Eggs need to absorb a certain amount of water for catatrepsis to be reached. If adequate water is absorbed it is believed that development will continue even in dry conditions (Matthée, 1951). Eggs were placed under alternate dry and wet conditions to examine the effect on their metabolic rate. Table 13 shows the metabolic rate of samples from a batch of eggs that were alternately dried and hydrated.

TABLE 13. Mean metabolic rate of dehydrated and hydrated eggs (n=70).

Time (days)	**MR (dry) µLCO_2/mg/h**	**MR (hydrated) µLCO_2/mg/h**
0	1.84	
After 48h		2.7
2	2.81	
After 48h		3.2
5	3.7	
After 48h		4.7
7	0.86	
After 48h		0.62
9	3.1	
After 48h		9.6
12	2.4	
After 48h		5

There was variation in the metabolic rate recorded during the early stages of alternate drying and hydration (Table 13, Fig. 38). A steady increase in the metabolic rate after the eggs were moistened was observed from day 0 to day 6 (Fig. 38), showing that development is occurring despite periodic desiccation of eggs.

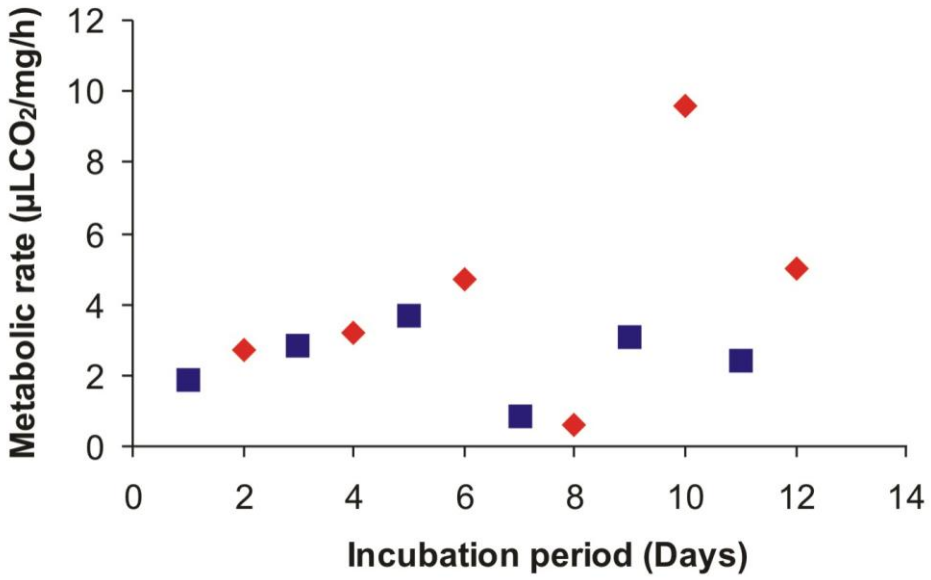


Figure 38. Metabolic rate from dehydration and rehydration experiment. Dry eggs (■) and hydrated eggs (♦).

The steady increase in the metabolic rate was followed by a drop after day 7 and large variation from day 9 to 13 (Fig. 38). Metabolic rate decreased in eggs that were dehydrated and increased in most eggs when hydrated (Fig. 38). Constant handling stresses the eggs and yet they managed to cope with differences in moisture for 12 days. None of the eggs hatched during the experiment.

CHAPTER 4 DISCUSSION

This work has contributed to the understanding of aspects that promote the survival of *Locustana* in the field. These include the importance of diapause, non-diapause and quiescent eggs, egg survival under extreme desiccation conditions, alternate drying and hydration of the eggs and egg structure and physiology.

4.1 Separating diapause and non-diapause eggs

It is important to separate diapause from non-diapause eggs for future experiments. It is difficult to extract eggs from the egg pod particularly newly laid eggs. Freshly laid eggs are difficult to work with as they are soft and puncture easily. Dry older eggs are fragile and tend to shatter. Consequently a large number of eggs were damaged during the extraction process and were discarded.

Matthée (1951) used 3 days of hydration to separate eggs by inspection, with non-diapause eggs becoming turgid. Females often lay egg pods with mixed egg types, particularly those reared in the laboratory. From our experience the separation of eggs cannot be reliably determined visually. In this study it was assumed that mass gain by the eggs equals water absorbed. Eggs were individually weighed to check progress. Initially 24 hours was used to try and separate diapause and non-diapause eggs as eggs start absorbing water within 24 hours (Matthée, 1951).

In the current experiment some eggs absorbed water and increased in mass while others stayed the same and did not absorb much water. The experiment showed that 24 hours was not enough time for accurate differentiation between egg types because there was insufficient mass gain of all the non-diapause eggs. These results confirm Matthée's (1951) suggestion that at least 48 hours was needed for better discrimination of eggs. Eggs had a substantial mean mass increase of 2.1 mg after 48

hours as opposed to 1 mg after 24 hours of incubation on moist soil. Thus the assumption was verified that non-diapause eggs took up water more rapidly than diapause eggs which absorbed little if any.

In this experiment egg weighing was continued for 12 days so that eggs could be followed to see whether those that absorbed water went on to hatch. Eggs that increased in mass went on to hatch, while eggs that did not absorb much water remained alive and did not hatch. Similar observations have been reported in eggs of *Melanoplus differentialis* (Thompson & Bodine, 1936; Slifer (1938), *Gryllulus commodus* (Browning, 1952), *Chorthippus brunneus* (Moriarty, 1969a) and *Zonocerus variegatus* (Chapman & Page, 1978). *Melanoplus* eggs initially showed slow water absorption which then increased rapidly and later stabilized due to the development of egg membranes (Chapman & Page, 1978). It can be concluded that the increase in egg mass after 48 hours is the key to separating diapause from non-diapause eggs.

4.2 Desiccation of eggs

In this study *Locustana* eggs desiccated over 30 day period lost water slowly until they reached a calculated value of 34 % of the original mass and thereafter no further mass loss occurred. Saacks (2005) desiccated 2 separate batches of eggs for 10 and 14 days, respectively. The lower limit of those eggs was calculated to be between 50 - 70 % relative water content (Saacks, 2005). We kept individual eggs out of pods under extreme conditions on dry soil in a desiccator at 15 % relative humidity. Eggs shrank and collapsed on desiccation but swelled again becoming turgid on hydration. In *C. brunneus* eggs were still viable after 14 days of desiccation (Moriarty, 1969b) when

hatching occurred as eggs remained in diapause. A similar trend to that of *Locustana* eggs was observed in the cricket, *G. commodus*, and in the beetle, *Acanthoscelides obtectus*. Water loss in eggs of *G. commodus* desiccated at 20.9 ºC showed a slow decrease in the rate of water loss in the first few days and subsequently stabilized (Browning, 1952). A similar trend was also observed in fertilized eggs of the beetle *A. obtectus* kept dry at 25 ºC and 5 % RH for 7 days (Biemont *et al.*, 1981). The rate of water loss in fertilized eggs of *A. obtectus* was slower compared to that of unfertilized mature eggs. The degree of resistance to desiccation in *A. obtectus* was not dependent on the age of the eggs. The greatest quantity of water loss was found in freshly laid eggs and 4 day old eggs (Biemont *et al.*, 1981). There is a steady decrease in water loss until death in insects (Hadley, 1994) and in mites (Yoder *et al*., 2006).

In this study done on *Locustana*, eggs showed a rapid water loss during the first few days after which they stabilized in agreement with Saacks (2005). Matthée (1951) desiccated eggs for 2 days at different relative humidities ranging from 0 to 80 %. He found an inversely proportional relationship between the rate of water loss and the relative humidity. *Locustana* eggs consist of 66 % water. In this study it was found that eggs can lose almost all the water and this agrees with Matthée (1951).

Eggs may enter quiescence as a mechanism of maintaining the cell integrity under stressful conditions of water loss (Matthée, 1951). In *Locustana* eggs the secondary wax layer and the yellow cuticle protect the egg against desiccation (Matthée, 1951). In *M. differentialis* the resistance of diapause eggs to desiccation was found to decrease with the age of the embryo (Thompson & Bodine, 1936). This study has improved on Matthée's (1951) work, (he desiccated eggs for 2 days only) in that t has shown that *Locustana* eggs can survive extreme desiccating conditions for 30 days. This is made even more pertinent by the fact that field conditions are not as

demanding as those to which the eggs were presently subjected. *Locustana* eggs in the field are very resistant to desiccation, altering survival through long periods of drought resulting in the build-up of egg beds in the field which contributes to their success. Long term survival of field *Locustana* eggs has been reported to be 3½ years (Smit, 1939) and 3 years in the laboratory (pers com. R. Price). Our field collected eggs have been kept in plastic tubs under dry conditions in the laboratory at a RH of 30 % and 22 ºC and given a sprinkle of water every 4 months for 1½ years to date.

4.3 Changes in the hydropyle structure after hydration

In this study the general structure of hydropyle cells corresponded to Matthée's (1951) report with the exception of chromatin granules which were found to be evenly dispersed inside the nuclei during hydration. Hydropyle cells were shown to increase in size up to late anatrepsis which corresponded with similar studies done by Saacks (2005). Hydropyle cells at days 5 and 6 remained squamous while other hydropyle cells increased in size and became cuboidal due to water absorption. Matthée (1951) did not report on morphometric measurements of hydropyle cells during development. In the present study measurements showed a general increase in hydropyle cell nuclear area and cell height as water was being absorbed. The cells later decreased in size as they were degenerating and dying as embryo development proceeded in non-diapause eggs.

There were also dark toluidine blue patches near the nucleus, only observed in hydropyle cells of hydrated non-diapause eggs. The significance of these patches is not known. Toluidine blue has an affinity for acidic material such as DNA and RNA. There is possibly an increase in the rough endoplasmic reticulum in these patches. Dark granules were found near the periphery of the nucleus and could be related to

secretion as they were associated with rough endoplasmic reticulum. Wax filaments described by Slifer & Sekhon (1963) in *M. differentialis* were not observed in the cuticle possibly because the sections were not thin enough. Wax filaments give rise to wax layers (Locke, 1965) which are responsible for water loss prevention (Matthée, 1951). Matthée (1951) found a secondary wax layer which is important in preventing water loss.

On dissection of day 5 eggs that were in anatrepsis it would seem that the single layer of serosal cells had collapsed on top of the hydropyle cells resulting in the formation of a double layer of cells. Serosal folding observed in anatrepsis at day 5 therefore seems to be as a result of a dissection artefact. After catatrepsis this would have occurred naturally forming a second layer, a result of breaking of the serosal strand first, prior to catatrepsis which agrees with Matthée (1951).

Matthée (1951) reported that the hydropyle cells were responsible for absorbing water into the egg. However Slifer & Sekhon (1963) opposed the view and reported that the epicuticle of the hydropyle area allows for water absorption due to its semi-permeable nature with increased foldings in the cuticle. Cuticle foldings were not found in this study. Saacks (2005) found small microvilli near the cuticle and this disagrees with Slifer and Sekhon (1963) who reported that they were absent. For evidence of water transport one would expect hydropyle cells to show morphological structure similar to the insect Malpighian tubules namely numerous microvilli, lots of mitochondria and basal infoldings of the cell membranes (Nation, 2002). The cell membrane infoldings increase the surface area for absorption. In the present study light and electron micrographs showed intercellular spaces and vacuoles, membrane infoldings, deep lateral infoldings below the endocuticle confirming the results of Saacks, (2005) and mitochondria associated with rough endoplasmic reticulum.

Saacks (2005) also reported relatively deeply folded microvilli on the apical hydropyle cell membrane. Saacks (2005) also reported the presence of septate junctions between hydropyle cells. These junctions prevent inward and outward movement of material via intercellular spaces. They allow communication between cells and control water movement (Nation, 2002). The presence of membrane channels indicates the regions through which water was transported. This process requires energy. The present study supports Matthée's (1951) idea that hydropyle cells are responsible for water absorption in *Locustana*.

The mechanism of water absorption has sparked a debate amongst some authors. According to Matthée (1951) passive diffusion occurs during the early stages of water absorption before any stretching of the egg membranes occur. As water enters through diffusion, the egg becomes turgid. Once the egg becomes turgid, water can no longer enter through diffusion. The membranes stretch without rupture as a result of active water intake. Matthée (1951) suggested that both passive and active mechanisms of water absorption occur in *Locustana* eggs. On the basis of other insect eggs, Hinton (1981a) suggested that water absorption is an active process which occurs along with aerobic respiration, for example, in *Melanoplus* (Thompson & Bodine, 1936) and in *Locustana* eggs (Matthée, 1951). Matthée (1951) showed that *Locustana* eggs can absorb water actively from a hypertonic glucose solution.

Water absorption in *M. differentialis* diapause eggs is prevented by the presence of a dark homogenous layer reported by Slifer & Sekhon (1963). Matthée (1951) found a protein layer in *Locustana* eggs. Both studies reported that the boundary layer observed soon disappeared allowing water absorption to occur Matthée (1951), Slifer & Sekhon, (1963). We did not monitor the boundary layer in this study.

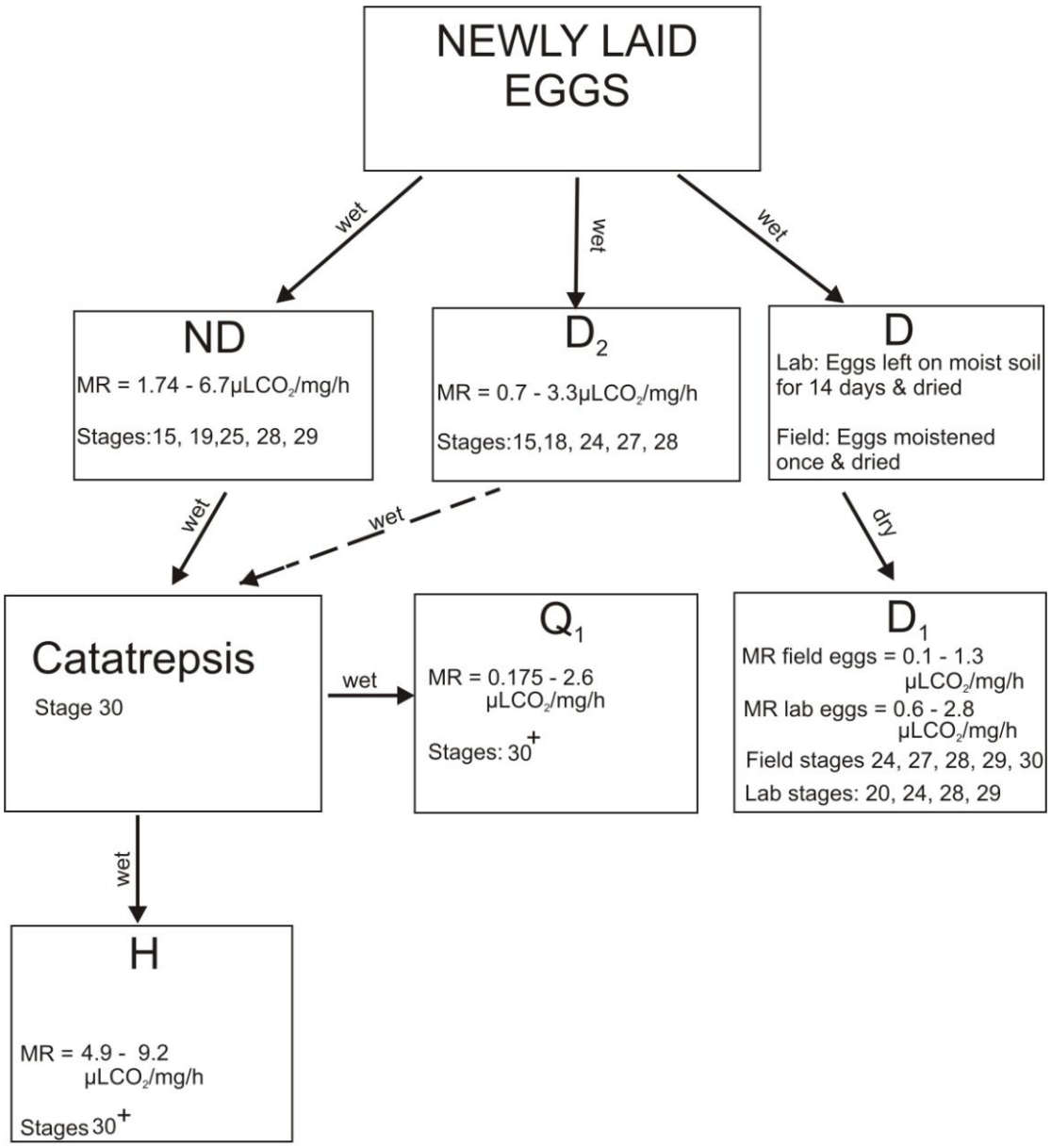
In this study it was shown that non-diapause eggs have immediate water uptake while diapause eggs have limited or no water absorption. Structures namely toluidine blue patches and dark granules not previously reported in *Locustana* and morphological structures supporting evidence of active rather than passive water uptake have been presented.

There may be concern that the fixation of dehydrated eggs was not completely satisfactory and this could lead to the distortion of some organelles and the formation of small vacuoles and spaces. This could lead to the misinterpretation of results. These dry eggs may start absorbing water from the water - based fixative resulting in the swelling of the hydropyle cells. Fixation using the freeze drying method could solve this problem but this was unavailable. In this study day 0 eggs were very different to those of later stages of development showing very little vacuole formation and while the fixation process was not perfect it allowed us to observe structural changes as development occurred.

4.4 Metabolic rate of eggs correlated with embryonic development

This study has linked the egg metabolic rate with stages of embryo development. This was done in order to understand diapause and non-diapause embryo development. Stages of development were found to be the same as observed by Matthée (1951). Due to the low metabolic rates recorded and the time constraints, the system could not detect adequate readings in the time that the eggs were still alive. Thus we could not measure individual eggs and a group of 3 eggs gave us reliable and repeatable results. In most cases eggs were in similar stages of development and those that differed widely were discarded. Some of the variability found could be explained by this.

Both diapause and non-diapause eggs may be laid by the female in the same egg pod and one cannot assume that the embryos will develop at the same rate. The metabolic rate of laboratory reared and field collected eggs were also investigated. Other investigators such as Bodine (1929), Thompson and Bodine (1936), Roemhild (1965), Hartley (1971), Modder (1978) and Woods & Singer (2001) looked at the metabolic rate in various insect eggs without linking it to embryo development. Field collected diapause eggs had a metabolic rate of 0.14 $mLCO_2$ /egg/h in *Z. variegatus* (Modder, 1979) and 0.21 $mLCO_2$ /egg/h in *M. differentialis* (Modder, 1978). In this study field and laboratory reared *Locustana* eggs had a metabolic rate of 0.00728 and 0.0209 $mLCO_2$ /egg/h, respectively (Fig. 39). These metabolic rate values were relatively lower compared with those of *Z. variegatus* and *M. differentialis* eggs. *Zonocerus variegatus* and *M. differentialis* eggs are of similar size with a mean dry weight of 3.6 mg (Modder, 1978) compared with 4.3 mg mean dry weight in *L. pardalina*. This partially explains the relatively lower metabolic rate in *Locustana* eggs. *M. differentialis* eggs had a metabolic rate of 0.28 $mLCO_2$ /egg/h prior to the onset of diapause (Modder, 1979). In the grasshopper, *Homorocoryphus* laboratory reared eggs had a metabolic rate of 0.00154 $mLCO_2$ /egg/h just prior to hatching (Hartley, 1971) which is lower than that recorded in *Locustana* eggs (Fig. 39), however, Hartley (1971) did not mention the weights of the *Homorocoryphus* eggs. *Locustana* diapause eggs have a relatively lower metabolic rate than that of other locust eggs.



Key:

MR - metabolic rate
ND - non-diapause
D1 & D2 - first & second diapause (according to Matthée, 1951)
Q1 - first quiescence
Catatrepsis - embryo rotation
H - Pre - hatching

Figure 39. Diagram of developmental pathways of *Locustana pardalina*, including stages from Matthée (1951) and metabolic rates from this study.

An illustration of the pathway of diapause and non-diapause eggs as observed in this study of *Locustana* is demonstrated in Fig. 39 (refer to pg 11, Fig 3). The

metabolic rate was low in all stages initially. A large variation was found in the metabolic rate at day 6 of incubation. This seems to be the point at which some of the embryos in anatrepsis enter diapause and others continue development. This confirms Matthée's (1951) work that newly laid non-diapause eggs enter diapause or enter catatrepsis between day 5 and 6 of development. Some hydropyles sectioned at this stage showed signs of water absorption and cells increased to cuboidal shape while other hydropyles did not absorb water and their cells remained squamous in shape.

In this study non-diapause eggs developed quickly after day 6 with high metabolic rates and hatching occurring at day 10 (Fig. 39) at 30±0.5 ºC. The metabolic rates had an almost exponential increase after day 8 of incubation with embryos differentiating rapidly. This trend of increased metabolic rate as development progressed was also observed in non-diapause eggs of *Aulocara elliotti* (Roemhild, 1965), *Eupholidoptera smyrnensis* (Ingrisch, 1987), *Z. variegatus* (Modder, 1978), and *M. differentialis* (Thompson & Bodine, 1936). In *Locustana* it seems that catatrepsis is the trigger to increase metabolic rate and differentiation (Fig. 39).

Quiescence only occurs during catatrepsis. Eggs can enter in and out of quiescence. When eggs enter quiescence they have a similar metabolic rate to that of diapause eggs. However, the longer they are in quiescence, the lower the metabolic rate. A metabolic rate of 0.175 µLCO_2 /mg/h was recorded on quiescent eggs (Fig. 39). This was the lowest metabolic rate recorded on live eggs.

The average metabolic rate of dead eggs was investigated to ensure that both diapause and quiescent eggs were still alive during the recording process. Dead eggs had a significantly lower metabolic rate.

Water absorption in *Locustana* eggs was shown to be dependent on aerobic respiration (Matthée, 1951). Water absorption allows the oxidation of nutrients such as yolk for the building up of new tissue and results in the production of carbon dioxide and energy (Nation, 2002). Cellular activity increases resulting in the expansion and growth of cells and this may explain the marked increase in the metabolic rate of non-diapause eggs. Development is initiated by increased water absorption which is associated with increased metabolic rate (Modder, 1978). The increased metabolic rate during non-diapause enables the embryo to develop until hatching occurs. Thompson and Bodine (1936) investigated the rate of desiccation and the stages of development in *M. differentialis*. They found that more developed and larger embryos had greater resistance to desiccation. The rate of oxygen consumption also decreased during desiccation.

On dissection diapause embryos were found to be in late stages of anatrepsis ranging from stage 15 to 30 Matthée's classification (1951). These are stages IIIb and IVc according to Wardhaugh (1978). The metabolic rate of diapause eggs varied within a narrow range compared with that of non-diapause eggs (Tables 6A & 6B, Fig. 39). This agrees with Lees (1955) who found that arrested growth was closely linked with a decrease in the respiration intensity. Reduction in metabolic rates was reported in diapause eggs of *Eupholidoptera smyrnensis* (Ingrisch, 1987) and *Z. variegatus* (Modder, 1978). This is the only study that has looked briefly at quiescence. A decrease in the metabolic rate of diapause and quiescent eggs slows down the rate at which accumulated reserves are used by the insect (Canzano, 2006). It is possible that the cellular respiration is kept at a minimum due to a decline in the metabolic rate during diapause and growth arrest (Yamashita and Hasegawa, 1985). Yamashita and Hasegawa (1985) also suggested that this decline in the metabolic rate

would help conserve carbohydrates and lipids. Reduced metabolic rate during diapause and quiescence is important for the conservation of energy during unfavourable environmental conditions (Nation, 2002).

Nevertheless diapause and quiescent eggs need energy for maintenance which is provided from yolk reserves (Canzano, 2006). These low energy levels during diapause allow for growth arrest to persist over long periods before diapause termination (Koštál, 2006), which is important for long term survival in the field. Egg beds in the field have been observed to persist for at least 3 years (Lounsbury, 1910, Faure, 1932 & Smit, 1939). Hoppers hatching from eggs under laboratory conditions after 3 years were very small in size (R. Price, pers comm.). This illustrates that a proportion of reserves have been used up to maintain the embryo during diapause and quiescence rather than contribute to growth.

4.5 Metabolic rate of laboratory reared and field collected diapause eggs

Fortuitously laying females were found in the field and eggs were collected for metabolic rate measurements. This provided an opportunity to validate laboratory data. Field collected eggs were a mixture of diapause and non-diapause types. The majority of the field collected eggs were in diapause because only 2 hoppers emerged at day 12 after incubation. From these metabolic studies, it was confirmed that older females and short day length favour the laying of diapause eggs. Diapause field eggs showed a more stable metabolic rate than laboratory diapause eggs. Development seems to become less synchronized in eggs of solitary locusts and in those eggs reared under laboratory conditions.

Field eggs were more turgid and heavier compared with laboratory diapause eggs. This is because it is difficult to replicate ideal field conditions in the laboratory. All the stages of embryonic development in both laboratory and field diapause eggs were similar, although the basal metabolic rate appeared to be higher for laboratory reared eggs (Fig. 39) which is difficult to explain. Both laboratory and field diapause eggs maintained a low stable metabolic rate. To show that results were repeatable, eggs were kept in 3 separate batches.

Laboratory diapause eggs remained in diapause for 61 days before hatching compared to 40 days reported by Matthée (1951). This is by no means exhaustive in terms of experimentation and diapause may last much longer. It is worth further experimentation. The hatching after 61 days confirms that eggs were alive and healthy during the experiment. Metabolic rate is the key indicator of continued diapause once 6 days of development has passed as a low stable value was maintained.

Alternate drying and hydration of embryos during early stages gave a variation in the recorded metabolic rate. Initially there was a steady increase in the metabolic rate despite desiccation. This could have been as a result of continued development assisted by the effectiveness of barriers against water loss. Towards the end of the experiment after 30 days, eggs could not withstand the dry conditions. However they showed adaptability by stopping development, lowering their metabolic rate and still maintaining the embryo. Individual unprotected dechorionated eggs survived dry conditions and continued development. This alternate drying and hydration is unlikely to happen so rapidly in the field.

In conclusion, the environment poses a threat to developmental success of *Locustana*. For this reason the female may produce a mixture of both non-diapause and diapause eggs in the same egg pod. There is an abundance of energy and

continued water uptake in non-diapause eggs until hatching. Diapause eggs on the contrary absorb little water, if any, and conserve the energy associated with the eggs stored during the prediapause state. This allows the desiccation resistant diapause eggs to accumulate in the soil over a number of years without hatching. Such eggs may prolong their dormancy by entering into quiescence. This has the effect of standardizing development bringing all embryos into synchrony. As soon as diapause and quiescence is broken and after periods of sufficient rainfall, these eggs would hatch simultaneously resulting in swarming. This contributes to the success of *Locustana* in harsh environments.

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APPENDIX 1. Rate of water loss over time.

Time (day)	**Average (mf/mi)**	**Log (mf/mi)**
0	1	0
2	0.656364	-0.18286
5	0.540274	-0.26739
7	0.504015	-0.29756
9	0.47132	-0.32668
12	0.441608	-0.35496
14	0.433038	-0.36347
17	0.437647	-0.35888
19	0.402357	-0.39539
21	0.396498	-0.40176
23	0.388029	-0.41114
26	0.380561	-0.41958
30	0.368725	-0.4333

APPENDIX 2. The dry weight of eggs.

	Day 0	**Day 3**	**Day 4**	**Day 6**
	15.9	4.4	4.4	4.4
	14.0	4.5	4.5	4.6
	13.4	3.9	3.9	3.9
	13.9	4.3	4.3	4.4
	11.0	4.3	4.4	4.4
	15.1	4.2	4.2	4.1
	11.8	4.2	4.1	4.1
	14.8	4.6	4.6	4.6
	11.2	4.2	4.2	4.2
	11.4	4.3	4.2	4.3
	9.9	4.1	4.1	4.1
	14.5	4.5	4.6	4.6
	16.0	4.4	4.3	4.3
	13.8	4.6	4.6	4.6
	15.6	4.4	4.3	4.4
	15.3	4.3	4.3	4.3
	10.4	3.8	3.8	3.8
	8.4	3.9	3.9	3.9
	10.8	4.3	4.2	4.1
	9.5	4.8	4.8	4.8
Average	12.8	4.3	4.3	4.3
Range	3.81- 4.78	0.250166	0.260818	0.267449
Mean	4.28			
SD	0.26			

APPENDIX 3. The average mass of field collected diapause eggs, laboratory diapause eggs and the control.

	Laboratory	**Field**	**Control (dry)**
	9.2	10.0	10.4
	8.4	12.2	10.4
	9.7	13.8	10.6
	9.3	17.7	10.0
	9.4	15.5	12.8
	12.5	14.6	11.3
	9.6	13.1	14.7
	9.9	13.3	13.6
	12.1	12.5	12.2
	13.2	11.4	16.9
	10.6	11.1	15.2
	11.5	11.5	12.6
	10.1	12.1	
	9.7	20.1	
	8.6	19.5	
	11.2	15.1	
	9.8	15.4	
	12.5	8.6	
	13.2	12.5	
	14.0	14.3	
	14.2	19.5	
	14.7	11.9	
	9.4	12.0	
	12.9	12.5	
	10.7	11.4	
	8.9		
	11.6		
	12.2		
	10.5		
	12.4		
Mean	11.1	13.7	12.6
SD	1.8	3.0	2.2
Range	8.4 - 14.8	8.6 - 20.2	10 - 16.9

APPENDIX 4. Average metabolic rate of non-diapause eggs.

Time (day)	**Mean metabolic rate (μLCO_2/mg/h)**
0	2.02
1	2.52
2	3.52
5	1.74
6	5.32
7	1.90
8	6.70
9	4.90
10	9.20
Average	4.20

APPENDIX 5. Metabolic rate of laboratory non-diapause, diapause, quiescent and dead eggs.

A. Non-diapause.

Time (day)	**Metabolic rate (μLCO_2/mg/h)**
0	3.18
0	3.9
0	1.91
0	1.4
0	1.6
0	1.04
0	0.14
0	2.1
0	2.4
0	2.6
0	1.94
1	1
1	2.2
1	2.3
1	3.2
1	3.88
2	4.1
2	1.7
2	3.19
2	3.2
2	3.79
2	3.3
2	5.33
5	1.9
5	1.4
5	1.96
5	1.7
6	6.9
6	3.19
6	5
6	8.7
6	2.8
8	6.7
9	4.9
10	9.2
Av	3.25
Range	0.14 - 9.2
SD	2.07

B. Diapause with embryos.

Time (Day)	**Metabolic rate (μLCO_2/mg/h)**
8	2.3
8	0.153
9	0.69
10	1.12
12	1.73
12	2.8
13	0.55
15	1.42
20	3.3
23	1.63
26	0.59
26	1.92
30	0.54
Av	1.441769
Range	0.54 - 3.3
SD	0.959411

C. Sterile eggs.

Time (Day)	**Metabolic rate (μLCO_2 /mg/h)**
6	5.5
6	1.96
6	2
6	0.152
7	1.25
8	0.153
8	1.5
9	0.21
9	1.8
10	1.32
12	1.35
14	1.27
16	0.75
Av	1.478077
SD	1.371549
Range	0.152-1.96

D. Quiescent eggs.

Time (day)	**Metabolic rate (μLCO_2 /mg/h)**
15	0.175
18	0.157
Av	0.166

E. Dead eggs.

Time (day)	**Metabolic rate (μLCO_2 /mg/h)**
0	0.05
0	0.21
0	0.2
1	0.87
2	0.16
2	0.52
5	0.3
6	0.644
7	0.51
8	0.37
12	0.42
Av	0.39
Range	0.05-0.87
SD	0.23