



Studies of carbohydrates in eggs of *Aulocara elliotti* (Thomas) (Orthoptera, Acrididae) in relation to development, temperature, maternal age and crowding
by Kenneth Leon Quickenden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Entomology
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Abstract:

The qualitative and quantitative carbohydrate changes occurring in the egg of the grasshopper species *Aulocara elliotti* (Thomas) at various stages of development were investigated. Carbohydrate distribution, interconversions and temperature effects were considered. Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified by chromatographic means in 70% ethanolic extracts of eggs. Mannose was detected in early prediapause eggs but was not detected in diapause or post-diapause eggs. As mannose levels declined, mannitol accumulated but the quantity of free mannose was not sufficient to serve as the only precursor for mannitol synthesis. Neither trehalose nor "glycogen" levels declined as mannitol accumulated. Mannitol was temporally associated with diapause. Although mannitol synthesis does not depend on cold exposure, cold might have an elevating effect. Glycerol appeared to be an extremely variable moiety, was not associated with diapause and would not appear to be important to cold-hardiness. There appeared to be slight early and late utilization of glycogen but levels were higher in the definitive egg than in the newly laid egg. Trehalose was generally 91 ± 5% of the free neutral sugars in eggs, increased by two to three-fold during diapause and then declined following blastokinesis. Glycogen, trehalose and mannitol distributions in the egg are discussed in relation to membrane permeability and changes occurring at blastokinesis. Radioactive tracing indicated that glucose could be converted to trehalose and mannitol and that, as mannitol disappeared from the eggs, conversion of mannitol to trehalose and "glycogen" was possible even though these two compounds did not accumulate at that time.

The effects of maternal age and crowding on egg weight and trehalose and "glycogen" levels, at a time when embryonic development had not progressed beyond the blastoderm stage, were measured in eggs collected from adults reared at three densities throughout the fecund period. Glycogen levels in these eggs increased with maternal age as did egg weight. Parental density had no noticeable effect on glycogen content. The greater the density, the greater the amount of trehalose that was partitioned to eggs during the first two-thirds of the reproductive period. During the last half of the fecund period, trehalose decreased from 57.6 to 20.2 µg/egg in eggs obtained from adults reared at the highest density. This is probably due to the combined effect of crowding and maternal age. Maternal age and density effects on trehalose levels partitioned to eggs are discussed in relation to rate of development and a density-stress response mechanism which may be likened to that of vertebrates.

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DEVELOPMENT, TEMPERATURE, MATERNAL AGE AND CROWDING

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KENNETH LEON QUICKENDEN

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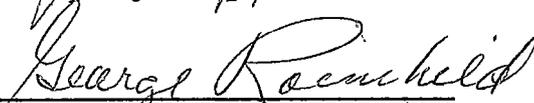
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Approved:


Head, Major Department


Chairman, Examining Committee


Graduate Dean

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ABSTRACT

The qualitative and quantitative carbohydrate changes occurring in the egg of the grasshopper species Aulocara ellioti (Thomas) at various stages of development were investigated. Carbohydrate distribution, interconversions and temperature effects were considered. Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified by chromatographic means in 70 % ethanolic extracts of eggs. Mannose was detected in early prediapause eggs but was not detected in diapause or post-diapause eggs. As mannose levels declined, mannitol accumulated but the quantity of free mannose was not sufficient to serve as the only precursor for mannitol synthesis. Neither trehalose nor "glycogen" levels declined as mannitol accumulated. Mannitol was temporally associated with diapause. Although mannitol synthesis does not depend on cold exposure, cold might have an elevating effect. Glycerol appeared to be an extremely variable moiety, was not associated with diapause and would not appear to be important to cold-hardiness. There appeared to be slight early and late utilization of glycogen but levels were higher in the definitive egg than in the newly laid egg. Trehalose was generally $91 \pm 5\%$ of the free neutral sugars in eggs, increased by two to three-fold during diapause and then declined following blastokinesis. Glycogen, trehalose and mannitol distributions in the egg are discussed in relation to membrane permeability and changes occurring at blastokinesis. Radioactive tracing indicated that glucose could be converted to trehalose and mannitol and that, as mannitol disappeared from the eggs, conversion of mannitol to trehalose and "glycogen" was possible even though these two compounds did not accumulate at that time.

The effects of maternal age and crowding on egg weight and trehalose and "glycogen" levels, at a time when embryonic development had not progressed beyond the blastoderm stage, were measured in eggs collected from adults reared at three densities throughout the fecund period. Glycogen levels in these eggs increased with maternal age as did egg weight. Parental density had no noticeable effect on glycogen content. The greater the density, the greater the amount of trehalose that was partitioned to eggs during the first two-thirds of the reproductive period. During the last half of the fecund period, trehalose decreased from 57.6 to 20.2 $\mu\text{g}/\text{egg}$ in eggs obtained from adults reared at the highest density. This is probably due to the combined effect of crowding and maternal age. Maternal age and density effects on trehalose levels partitioned to eggs are discussed in relation to rate of development and a density-stress response mechanism which may be likened to that of vertebrates.

INTRODUCTION

Biologists are often called upon to explain population fluctuations in animals. Dempster (1963) indicates that a combination of factors, external and internal, account for these fluctuations.

Richards (1961) states that the four most likely causes of insect mortality are external features. These are weather, parasites, predators and disease. While these external features are important, a reliance solely upon the external environment has failed to adequately explain many sudden changes in numbers.

Internal population regulation is a widespread phenomenon in the animal kingdom. Among the vertebrates it is theorized to operate through the pituitary-adrenal and/or the pituitary-gonadal axis in response to stress. Research involving mammals indicates that such a feature occurs in this class (e.g. Christian, 1950; Christian and Davis, 1964). Hane et al. (1966) have demonstrated that salmon show an increase in the levels of plasma 17-hydroxycorticosteroids, adrenal cortex hormones, in response to stresses of handling and migration. There is also decreased responsiveness to ACTH, a pituitary hormone, when injected at the end of migration. Flickenger (1966) recorded changes in gonad size in response to greater social conflict resulting from larger group size.

The mechanisms by which the internal factors integrate into the problem of population fluctuations are largely unknown in insects. Many responses to density and other stresses, however, have been observed. There are reports that crowding retards fecundity in some Acrididae (Norris, 1952) and induces changes in ovariole numbers in progeny

(Albrecht, et al. 1958). Maternal stress thus extended to the developing embryos. O'Brien and Wolfe (1964) state, without giving reference, that overcrowding is known to transform locusts from the solitary to gregarious form and influences the rate of yolk deposition in eggs.

Field studies of the grasshopper Aulocara ellioti (Thos.), which is an economic pest of rangelands (Pfadt, 1949; Anderson and Wright, 1952; Anderson, 1964), indicate that wide fluctuations in density occur in this species. Hastings and Pepper (1964) have shown that nymphs of different populations of A. ellioti vary in their ability to withstand the stresses of temperature extremes and starvation. One of their suggestions to explain such population dependent variation is that stresses imposed on the adult may be transmitted through the developing embryo to the nymph.

In an effort to understand the internal environment of developing embryos of A. ellioti and to study density or other stress effects, a multifaceted approach was begun at Montana State University. Van Horn (1963, 1966a, 1966b) studied the embryonic development noting wide variation with respect to rate of development and numbers of retarded embryos in different populations. She also found that density may change the developmental pattern of these embryos. In a population studied in 1966, Van Horn (1968) found that the density effect was modified by photoperiod such that the rate of embryonic development became successively greater for long day-one pair per cage, long day-two pairs per cage, short day-one pair per cage and short day-two pairs per cage.

There have been two attempts to establish the existence of biochem-

ical differences in eggs of A. ellioti from different populations and differences resulting from density effects. Svoboda (1964) and Svoboda et al. (1966) could detect no differences in lipids nor could Bunde (1965) or Bunde and Pepper (1968) find differences in amino acids of eggs from different populations of A. ellioti which could be attributed to density. Still, changes in the vigor of a population might be reflected in the biochemical constitution of the eggs. Vuillaume (1955) showed that crowded nymphs of the acridid Zonocerus variegatus had a higher fat content than isolated nymphs and Matthée (1945) found that the gregarious phase of several locusts and noctuids contained a higher percentage of fat than did the solitary phase.

There have been a number of studies directed at the embryonic developmental physiology of A. ellioti. Roemhild (1961, 1965a, 1965b, 1967, 1968b) has directed his attention principally toward egg compartmentation and the blastokinesis occurring at diapause termination. He has found that membrane structures divide the egg into compartments containing different physical and chemical properties. He believes that the integrity of these membranes is important to the maintenance of the diapause state and has found that their rupture at blastokinesis exposes the embryo to a very different pH (7.4 to 6.6). Laine (1966) investigated temperature effects on the embryonic oxygen consumption and Leopold (1967) conducted a histochemical study of ovarian development and vitellogenesis.

With the previously noted investigations and with the many hormonal

effects on carbohydrate metabolism observed in insects recently summarized in a review by Wyatt (1967), it is reasonable to expect that a density or other stress effect might be reflected in the carbohydrates found in eggs and embryos of A. ellioti. A study of the principal carbohydrates would contribute some needed basic information concerning the internal environment and developmental physiology of this species.

The disaccharide trehalose, which is the main blood sugar of insects, has been identified in an impressive list of lower plants and invertebrates. The importance of this sugar in such physiological events as molting, reproduction and flight has been well noted. Its occurrence in a number of life stages is tabulated in a recent biochemical review of sugars and polysaccharides in insects by Wyatt (1967). Reports of trehalose in insect eggs are more restricted but it probably occurs universally. Clegg and Filosa (1961) simply state that it occurs in eggs of Aedes aegypti (Diptera). In eggs of the oak silkworm, Antheraea pernyi, Egorova and Smolin (1962) found 0.026 % of the dry weight is trehalose. According to Dutrieu (1961b), newly laid eggs of Carausius morosus (Phasmida) lack trehalose but it then appears and increases from 2.5 to 3 % during the last month of embryonic life.

Glycogen has been reported to be present in oocytes of the orthopteran Tachycines (Radecka, 1962) and the dermapteran Anisolabis (Bonhag, 1956) but absent in oocytes of the cockroach Periplaneta (Kugler et al., 1956; Bonhag, 1959) and the hemipteran Oncopeltus (Bonhag, 1955). Leopold (1967) came to the conclusion that glycogen was absent in oocytes

of A. ellioti on the basis of several histochemical techniques. He decided that a major portion of the yolk was in the form of protein-carbohydrate complexes with significant amounts of acid and neutral mucopolysaccharides. However, his case may remain to be proven as it rested in part on the assumption that treatment with cereal amylase would digest glycogen and yield a negative Schiff's reaction. Whatever the nature of polysaccharides in eggs of A. ellioti, glycogen-like material was assayed at various developmental stages to determine its contribution to embryogenesis and will be referred to henceforth as "glycogen."

There are many factors which may influence the identity or amount of various carbohydrates found in insects. These include development, temperature and age and will be discussed in turn where they may have some application to this paper.

Changes of trehalose during embryogenesis have been followed in eggs of Bombyx mori (Dutrieu, 1961 a, b; Yamashita, 1965) and Melanoplus differentialis (Randall and Derr, 1965). Dutrieu reports that in the non-diapause eggs of bivoltine and tetravoltine strains of B. mori trehalose increases from a low level (1.5 % dry weight) to a maximum of 15 % of the dry weight at blastokinesis. She states that hibernating eggs are initially about the same in trehalose level. Yamashita found that the initial levels of about 4 mg/g wet weight in hibernated eggs were greater than the 0.5 mg/g occurring in non-diapause eggs. Randall and Derr noted infertile eggs of M. differentialis held 88 mg/100g fresh weight as trehalose and diapause samples reflected an initial level of 174 mg/100g.

After 4 and 14 days at 5°C they found levels of 196 and 166 mg/100g respectively. The trehalose levels had quadrupled by the time eggs were about to hatch. It was accordingly expected that this carbohydrate would occur in eggs of A. ellioti and that by tracing levels throughout their embryonic development and correlating results with previous studies some insight might be gained of its contribution as an energy and/or as a carbon source.

Although, as Agrell (1964) pointed out, fat metabolism predominates in most insect eggs, the importance of polysaccharides as an energy source has long been known. Rothstein (1952) cites at least six workers dating back to 1885 who observed a decrease in fat, glycogen and protein in post-diapause eggs of B. mori. Glycogen decreases to approximately 87% of initial levels. An early, more rapid decrease was noted by Moulinier (1957) and Chino (1957). Yamashita (1965) notes that a slightly higher level of glycogen was found in hibernated eggs than in non-diapause eggs and that glycogen in both decreased throughout development. Agrell cited four authors who noted that both fats and polysaccharides were steadily consumed during "embryogenesis of the silkworm and grasshopper."

According to a theory of sequence of metabolites used as an energy source during development, the order of utilization is carbohydrate, protein and fat (Needham, 1931, 1942). Along with those studies just noted, other studies of insect eggs seem to bear out an early reliance upon carbohydrate as an energy source. Rothstein (1952) reports that glycogen (when expressed as a percentage of the constant nitrogen content)

decreases from 58.4 % to 25.9 % in developing eggs of the Japanese beetle, Popillia japonica. He states that the loss occurs in two phases and that it furnishes the energy for early embryogenesis. Ludwig and Ramazzotto (1965) point out that fat furnishes the main source of energy for embryogenesis in most insects but in the yellow mealworm, Tenebrio molitor, glycogen is the main energy source. They state that the 51.5 % loss of glycogen (4.7 to 2.2 mg/100 eggs) occurred throughout the embryonic period and fat was not utilized until the last day. No evidence was found of protein utilization for energy in either the mealworm or the Japanese beetle.

As the diapause state is entered by eggs of Bombyx mori, there is an increase in cyanide resistance (Wolsky, 1949) and a depression of respiration rate concurrent with a rapid conversion of glycogen to sorbitol and glycerol (Chino, 1957, 1958, 1960). These polyols were then reconverted to glycogen at diapause termination. He advanced the explanation that accumulation of polyols in B. mori occurred as a result of the inactivation of the electron transport system, the hydrogen being transferred to unusual intermediates in this anaerobic condition. Harvey (1962) states, however, that this hypothesis "faces serious difficulties" and points out that Chino gives no evidence that such a block occurs. Wyatt (1962) came to the same conclusion as Harvey.

Melanoplus differentialis also shows increased cyanide resistance during embryonic diapause (Bodine and Boell, 1934) and a low rate of oxygen consumption (Bodine, 1929). Randall and Derr (1965), however,

report that the accumulation of sorbitol and glycerol does not occur in this species. The absence of glycerol is also a feature of hibernating eggs of Melanoplus bivittatus (Salt, 1957).

As pointed out by Roemhild (1965a), diapausing eggs of A. ellioti have a number of characteristics different from those of M. differentialis, including a requirement for low temperature in breaking diapause. Laine (1966) discovered that when A. ellioti entered diapause the respiration rate did not drop but continued at a fairly high level. Roemhild (1965b) noted that reducing materials were highest during the diapause period. Roemhild (1966) also found an increase in osmotic pressure coincident with diapause initiation. This month-long period of elevated levels occurred without any low temperature exposure. On the basis of thin layer chromatography in one solvent system, Roemhild tentatively attributed this temporary increase to glycerol production by the embryo. Unfortunately the levels declined before this could be confirmed in other solvent systems. In view of the unlike results obtained with B. mori and M. differentialis with respect to carbohydrate metabolism in diapause and the different character of diapause in A. ellioti, it is of interest to see if any polyhydric alcohols accumulate in this species.

Diapause occupies a major time interval in the embryonic development of A. ellioti. Van Horn (1963) reported that, during a prolonged cold storage of eggs, there was marked increase in the size of the embryonic fat body and a deposition of lipid. Svoboda (1964) and Svoboda et al. (1966) were unable to confirm this deposition by separately analyzed

lipid extractions of embryo and yolk. They state, however, that small... changes in lipid location may have gone undetected. The fat body, a tissue noted for glycogen accumulation, has been shown to be the major site of trehalose synthesis in the adult locust, Schistocerca gregaria (Candy and Kilby, 1961). In view of the above findings, the present study investigates the effect of the lengthy embryonic diapause of A. elliotti on carbohydrate levels in the egg.

Howe (1967) comments that many biochemical and physiological studies are conducted at uncontrolled temperatures even though temperature has been shown to influence the amounts of unsaturated fatty acids in the mosquito, Culex tarsalis and the proteolytic activity in Tenebrio and Tribolium beetles. Temperature changes are also known to influence some carbohydrate levels in insects. Somme (1966) showed that in larvae of the Mediterranean flour moth, Anagasta kuehniella, hemolymph levels of glycerol and glucose rose at low temperatures and trehalose increased at 0°C but decreased at -6°C. During prolonged storage of Hyalophora cecropia pupae at 5°C, there is an increase in trehalose (Wyatt and Kalf, 1957).

Temperature effects on carbohydrates have also been observed in insect eggs. Somme (1964) found that in eggs of the black willow aphid, Pterocomma smithia, glycerol was 5.5 % at 20°C but only 1.2 % if the eggs were stored at 5°C for one week. Yamashita (1965) states that, while there is little trehalose in newly laid diapause eggs of Bombyx mori, cold exposure favors its appearance. Dutrieu (1961b) reported that

trehalose levels in the "directly developing" eggs of B. mori were augmented by the action of cold. She also says that cold does not change trehalose levels in diapause eggs of B. mori which are newly laid, in prediapause or in early estivation. She also notes that cold has no action on trehalose levels in eggs of Carausus morosus (Phasmida).

The possibility then remained that any change observed during prolonged cold storage of diapausing eggs of A. ellioti could be due to either the temperature or the developmental state. The present study makes a brief attempt to distinguish between any temperature or developmentally induced changes in carbohydrate levels which might occur during this period.

Clark and Rockstein (1964) review papers that indicate that the time of oviposition may be important to the rate of development or viability of a number of insect eggs. For example, Richards and Kolderie (1957) noted that eggs of the milkweed bug, Oncopeltus fasciatus, weighed less and took longer to develop when laid very early or very late in the fecund period. Wellington (1965) suggests that an unequal partitioning of maternal food reserves in eggs of the tent caterpillar might account for the greater vigor in larvae from early egg masses as opposed to late laid eggs. Since carbohydrate is utilized first in the embryonic development, it is possible to hypothesize that there was more carbohydrate in the early laid eggs and that this may partially account for their more rapid development or the greater vigor of resultant larvae. Senescence in some insects is known to influence the amount of trehalose and glycogen available to adults for flight or in the fat body (Clark and Rockstein,

1964; Rockstein and Srivastava, 1967).

Van Horn (1966b) has observed a different phenomenon in eggs from a population of A. ellioti. Eggs laid the last two weeks of the laying season developed to a 30 day mean stage of 16.8, according to criteria established by Van Horn (1963, 1966a), while those laid during the first two weeks only reached a mean stage of 12.3 in 30 days. She stated that, although it seemed unlikely that the quantity of yolk alone could be responsible for more rapid development in late laid eggs, there was no data relating egg weight to maternal age. The present investigation seeks to discover if later laid eggs are indeed heavier and what effect aging has upon the maternally donated carbohydrate substrate in eggs collected from three different parental densities.

The study of the principal soluble carbohydrates found in Aulocara ellioti eggs presented here is intended to compliment previous studies of eggs of this species. The study was conducted in two phases. The first dealt with the qualitative and quantitative changes in carbohydrates during the development of the eggs and considers temperature effects; the second with the effects of maternal age and density as reflected in newly laid eggs.

METHODS AND MATERIALS

Experimental Design

During the first phase of this study, conducted in 1966-1967, whole eggs in a number of stages of development were analyzed qualitatively and quantitatively for glycogen and mono- and oligosaccharides. Since the distribution of substrates within the egg is important to development, yolk and embryo were also separately analyzed at each stage marked (*) below. The changes in carbohydrate constitution and distribution were traced under the times and conditions schematically represented as:

	Prediapause State					Diapause State			Post-diapause State						
Days of development in state when sample was taken:	1	3	7	15	25	45	25	40	175	1	2	7	14	20	
Morphological stage (Van Horn, 1966a):	1	5	10	18			19	19	19	20-22		23-24	25	26	27
				*	*				*	*		*			
Temperature at which eggs were maintained:	25°C					8-12°C			Group I: 8-12°C		Group II: 25°C		25°C		

The effect of temperature was further evaluated by analysis of a stage 19 sample after 139 days at 25°C. In an attempt to verify possible synthesis of trehalose and establish any possible interconversions, samples in each state were treated with radioactive glucose and late diapause samples with the appropriate radioactive polyol. Late diapause and post-diapause field-collected eggs were compared with known age eggs of the same stage.

During the second phase of this investigation, eggs were collected weekly throughout the reproductive period from grasshoppers maintained in the greenhouse at three different densities and the effects of maternal

age and density on egg weight and carbohydrate partitioned to the eggs were studied.

Biological Samples

Third and fourth instar nymphs were collected from a population of A. ellioti near Decker, Montana early in the summers of 1966 and 1967. Nymphs were reared under greenhouse conditions in cylindrical clear plastic-walled cages, 20.5 cm in diameter and 27.5 cm high, set on dirt-filled pans as described by Anderson and Hastings (1966). This population markedly decreased in abundance from 1966 to 1968. In 1966 the nymphs were reared and maintained in 47 cages at a density of 3 pairs per cage. On alternate days, the grasshoppers were provided with fresh cuttings of western wheatgrass, Agropyron smithii, which is one of their preferred food-plants (Pfadt, 1949; Anderson, 1964).

Egg pods were collected daily in 1966 by sifting dirt in the early evening hours when oviposition activity had ceased. The egg pods were then stored in plaster of paris blocks as described by Van Horn (1966a), at 25°C until needed. After a period of from 63 to 84 days, when the remaining embryos had entered diapause, the blocks were transferred to a refrigerator maintained at 8 to 12°C. Eggs were also field collected in the fall of 1966 and stored at this temperature. After at least 153 days of cold exposure, eggs that were not yet selected nor destined for longer cold exposure were replaced in the 25°C cabinet to initiate post diapause development. A number of embryos, however, began blastokinesis at the lower temperature. Egg pods and blocks were watered lightly on each

third day while at 25°C and about weekly while at 8-12°C.

Only eggs which had developed to the desired degree and appeared to be viable when removed from their pods were included in samples. In the samples which were 15 or 25 days old, a representative egg was selected from the center of each pod for staging. The staging criterion used in this study was that established by Van Horn (1966a). At all other ages investigated, the embryo either had not formed or was visible from the exterior and could be staged while immersed in water. Generally, each sample size was 100 eggs. Each whole egg sample was rinsed, surface dried, weighed, triturated for five minutes in distilled water at 5°C and freeze-dried to preserve it in that state. This latter procedure was made necessary due to the number of samples involved and is a recommended procedure for storing biological material (Hais and Macek, 1963; Burchfield and Storrs, 1962). Thirty-six whole egg samples were treated in the above fashion. The distribution of samples in each previously designated stage may be observed in the included tables.

Eight 100-egg samples were taken at stages previously indicated for separate analysis of yolk and embryo. The embryos were dissected from the remainder of the eggs under distilled water at 5°C. Three such operations were performed in a volume of 0.4-0.5 ml water. As little water as possible was removed with removal of embryos from the distilled water. The remaining egg parts and water constituted the yolk fraction. Both fractions were freeze-dried.

Five radioactive egg samples were prepared with the view of demon-

strating enzyme systems capable of converting glucose to other carbohydrates during prediapause, diapause and post-diapause but incubations are of such duration that results do not yield information on specific enzymic pathways followed. The method of introducing radioactive material was that used by Bunde (1965). Two 75-egg prediapause samples were collected from a parental density of three pairs per cage in 1967 and each was exposed to $0.75 \mu\text{c}$ of glucose-U- C^{14} in 0.75 ml water for 42 hours, following three days of desiccation over silica gel and calcium sulfate crystals. The prediapause samples were 25 and 45 days old at the initiation of this procedure and had developed to mean stages of 11.9 and 18.9 respectively. Two post-diapause samples were treated the same except one field collected sample of 100 eggs was exposed to $10 \mu\text{c}$ of radioactive glucose in 1.0 ml and one greenhouse collected sample of 80 eggs was exposed to $8 \mu\text{c}$ of radioactive glucose in 0.8 ml. The post-diapause samples were in stages 20 - 22 when incubated and ranged from 23 to 25 when processed. A 50-egg diapause sample was desiccated for six days, incubated for a like period in $2.5 \mu\text{c}$ of glucose-U- C^{14} in 0.5 ml water and processed the day following incubation. Carbohydrates were extracted from all other samples that had been exposed to radioactive glucose immediately after incubation and rinsing with distilled deionized water. The extraction technique is described in the next section of this paper.

After a desiccation period of three days, four samples, each composed of 34 field collected eggs that had received sufficient cold to break diapause, were exposed to $2.5 \mu\text{c}$ of D-mannitol-1- C^{14} in 280 μl of

water for 70 hours at 10°C. Carbohydrates were immediately extracted from one sample and the other samples were placed at 25°C for 1, 3 and 5 days prior to extraction. Nothing was known about the changing pattern of carbohydrates in postdiapause development of A. ellioti at the time this procedure was initiated. It was reasoned that if the polyhydric alcohols were reconverted to glycogen at diapause termination (as is the case in Bombyx mori) an accumulation of radioactivity would be observed. Since a similiar feature did not exist, exposure of A. ellioti eggs to the appropriate polyol at diapause termination only serves to demonstrate the existence of enzyme systems capable of converting mannitol to other carbohydrates.

Egg samples for the 1967-68 study of density and age effects were collected and treated in the same fashion as the whole egg samples taken for the study of carbohydrate changes occurring during development. Nymphs were maintained to adulthood at one, three and six pairs per cage, with 18 pairs at each density contributing eggs for study. High parental mortality and lowered egg production at the highest density made continuation of this experiment impossible after six weeks. Each of the 18 samples taken during this phase of the project was composed of 100 eggs except for those laid by the parental density of six pairs per cage during weeks 1, 5 and 6. Here the sample sizes were 70, 85 and 80 eggs respectively. Since eggs were less than seven days old when freeze-dried, development was thought not to have progressed beyond the blastoderm stage (Van Horn, 1966a).

Two hemolymph samples were obtained from females that had been used for the study of maternal age effects. These females had been reared at densities of one and six pairs per cage and had survived the sixth week of oviposition. The hemolymph quantities were approximately 40 and 35 μ l respectively. They were obtained by severing one leg near the thorax and, with gentle squeezing, drawing up the hemolymph that exuded from the wound with a 100 μ l syringe. These samples were also freeze-dried.

Carbohydrate Extraction

Two extracts were obtained from each freeze-dried sample in a fashion similar to that described by Kemp and Van Heijningen (1954) for separate analysis of glucose and glycogen. Each sample was extracted 3 times in 4 ml of 70 % aqueous ethanol. Following the initial 10 minute trituration, the homogenate was centrifuged for 8 minutes and the supernate removed with a pipette. The residue was twice resuspended in 70 % ethanol, centrifuged as above, and the supernatant liquid added to the first extract. The ethanol was removed in vacuo at 36°C on a Rinco rotary evaporator and the composite extract was deproteinated with Somogyi's reagent (2 volumes each of 0.3 N barium hydroxide and 5 % zinc sulfate) as recommended by Hais and Macek (1963). After filtration through sintered glass and rinsing the residue with 20 ml of distilled, deionized water, the volume was reduced in vacuo at 36°C to about 5 ml. The extract was clarified by successively passing it through a column containing 3 grams of Dowex 50 (H+) and a column containing 2 grams of

Dowex 1 (CO_3^-) prepared after Burchfield and Storrs (1962). The columns were fitted with glass joints and drip tips in order that the eluate from the Dowex 50 column might drip directly on the Dowex 1. The neutral sugars in the percolate were displaced through the columns with 100 ml of distilled, deionized water and the extract was again reduced in vacuo to a volume of about 2 ml. Following three rinses of the flask, the resultant volume of 6 to 7 ml was reduced under a stream of nitrogen to less than 0.5 ml and then brought up to a final volume of $1^{\pm}.01$ ml. In the case of those samples that had been exposed to radioactive mannitol, amino acids were eluted from the Dowex 50 column and organic acids from the Dowex 1 column after Burchfield and Storrs (1962).

The residue which remained after the 70 % alcohol extraction was defatted by suspending it in 5 ml of diethyl ether, centrifuging and decanting. The ether was evaporated from the residue at room temperature. Next, "glycogen" was extracted from the residue with 5 ml of 5 % trichloroacetic acid (TCA) in a boiling water bath for 30 minutes, centrifuged 8 minutes and the supernatant liquid placed on a column containing 1.0 g of Dowex 50 (H^+). The remaining residue was twice re-suspended in 5 ml of 5 % TCA, centrifuged and the supernatant liquid placed with the hot TCA extraction. The composite extract was not run through a Dowex 1 (CO_3^-) column since it was experimentally determined that loss of oyster glycogen occurred in such a column. The glycogen extract was concentrated to a final volume of $10^+.03$ ml under reduced pressure following a 75 ml rinse of the column with distilled, deionized H_2O .

Paper Chromatography

The concentrated 70 % ethanolic extract was subjected to qualitative analysis by chromatography on Whatman No. 1 filter paper. Resolution of components was performed by the descending technique at room temperature. The chromatographic cabinets were fashioned of wood and sealed with wax. Identification of the carbohydrates in A. ellioti eggs was begun by co-chromatography of the extract and standards in at least four solvent systems and noting similiar migration distances. In all, seven solvent systems were employed in the identification of sugars and polyhydric alcohols in the egg. These solvent systems were: ethyl acetate:pyridine:water (8:2:1 v/v), iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v), phenol:water (4:1 w/v), n-butanol:ethanol:water (4:1:5 v/v, upper phase) (all listed by Block et al., 1958), n-butanol:ethanol:water (4:1:1 v/v) (Hough and Jones, 1962), 88 % phenol:water (4:1 v/v) and n-butanol:acetic acid:water (4:1:1 v/v).

The positions of the sugars and polyols on chromatograms were detected by the alkaline silver nitrate method of Trevelyan et al. (1950). Selective visualization of different moieties and further characterization of them was performed by using more specific indicator sprays. The modified Fleury's reagent, for the detection of polyols, may be followed with p-anisidine and results in the formation of multi-colored spots when observed in daylight and under ultra-violet illumination (Lambou, 1956). The p-anisidine phosphate reagent, prepared as modified by Mukherjee & Srivastava (1952), gave differently colored spots for aldohexoses and

ketohexoses, aiding in identification. The ketohexose was also detected with 0.2 % w/v naphthoresorsinol in acetone:3 N phosphoric acid (5:1 v/v) (Block et al., 1958). This spray did not detect the aldohexoses or the polyhydric alcohols. The alpha-naphthol:phosphoric acid reagent described by Block et al. (1958) is more sensitive for ketose although aldose will react. When used on chromatographic separations in this study only the standard and egg ketoses were detected.

Gas-liquid Chromatography

The identity of neutral free moieties soluble in aqueous ethanol was further confirmed by gas-liquid chromatography of their respective O-trimethylsilyl ethers. Biological material used for preparation of these derivatives was that which remained after paper chromatographic identification and quantitative estimation. This material was freeze-dried and, as were the carbohydrate standards, stored over desiccating silica gel crystals and phosphorous pentoxide prior to preparation of the O-trimethylsilyl ethers. These derivatives were prepared by the method of Perry (1964) using pyridine which had been redistilled over potassium hydroxide and trimethylchlorosilane and hexamethyl disilizane (K & K Laboratories, Plainview, N.Y.) which were used as received.

The F and M Biomedical Gas Chromatograph model 400 equipped with a flame ionization detector was used for analysis. The trimethylsilylation reaction mixtures (0.5 - 1.0 μ l) were injected on U-shaped glass columns (1/8 in. I.D. x 4 ft.) which had been packed with either 3.8 % silicone gum rubber on Gas Chrom Q (100 x 120 mesh) or 5 % silicone gum rubber on

Gas Chrom Z (100 x 120 mesh). Helium was used as the carrier gas at a flow rate of 40 ml/min. The column was maintained at a temperature of 150°C to note retention times of hexoses and the hexitol and 210°C for trehalose characterization.

Quantitative Analysis

Colorimetric quantitative estimation of sugars and polyols in the 70 % ethanolic extract were made in conjunction with paper partition chromatography. The solvent system iso-propanol:pyridine:acetic acid: water (8:8:1:4 v/v) was chosen for use in developing chromatograms since there was good resolution of all compounds of interest and none was washed from the Whatman #1 paper employed in the 18 hour run required. Previous to development, the egg extract and standards were spotted parallel to the aliquot to be analyzed. Following development, the resolved spots were visualized on the guide strips by the alkaline silver nitrate method of Trevelyan et al. (1950). The compounds thus located were eluted with distilled, deionized water from strips parallel to the detected spots and filtered through sintered glass in preparation for colorimetric assay. Measurements of glycogen and the total free sugars (unfractionated 70 % ethanol soluble neutral carbohydrates with reducing or potentially reducing groups) were made without individual chromatographic resolution.

The above determinations were made using the phenol-sulfuric acid method of Dubois et al. (1956). The instrument used in taking readings was the Bausch and Lomb Spectronic 20 Colorimeter. Absorbancy readings,

which were made at a wavelength of 490 μ , were preferably read in the range of 0.250 to 0.450 where the third decimal place could be estimated. Standard curves were prepared for both glycogen and trehalose by plotting micrograms of known vs. absorbance at 490 μ . The estimates of total free sugars were made by reference to the standard trehalose curve since greater than 90 % of the absorbancy values were found to be attributable to this moiety. Blanks were prepared for standard curves, glycogen and trehalose determinations by substituting purified water for the sugar solution. Since trehalose in eggs or egg parts was measured following chromatographic separation, its blank was prepared by eluting a parallel strip of the chromatogram representing the same area. Measured aliquots of chromatographically isolated trehalose and samples of glycogen or total free sugars were analyzed in triplicate to minimize the danger of accidental contamination of cellulose lint. Further, each ethanolic extract was chromatographed three times to avoid errors resulting from missing a section of paper containing trehalose. This resulted in 9 absorbance readings for trehalose and 3 each for glycogen and total free sugar in each sample. The error observed with standards was less than 5%.

Where the hexitol existed in amounts greater than 5 μ g/egg, it was estimated after elution from the same chromatograms used to resolve the trehalose. The method used depends on a short periodate oxidation and assay of formaldehyde produced as described by Lambert and Neish (1950). The Spectronic 20 Colorimeter was used at a wavelength of 570 λ . Blanks and the standard curve were prepared similiary to those previously

described. Restricted amounts and sensitivity allowed only duplicate readings of absorbances for each of the replicated spots. Six observations of the amount of this polyol in each sample resulted. Observed error was again less than 5 %. Where less than 5 $\mu\text{g}/\text{egg}$ existed, estimates of amount were derived by comparing extract spot size and intensity on chromatograms with spotted standards of known quantity.

Glycerol was estimated in the same fashion as hexitol. Reference was made to the standard hexitol curve and multiplication of the μg of polyol so obtained by a conversion factor of 0.5055 to get μg glycerol present per egg. Where no other polyol was present, glycerol was determined directly on the extract. Lambert and Neish (1950) stress that error resulting from glucose present in equal concentration to glycerol amounts to only 2.5 to 5.0 % and no such concentration existed here. It was verified that trehalose in concentrations greater than are found in the egg did not interfere with polyol measurement since synthetic mixtures of polyol and trehalose were determined by this method of accuracies of 101 and 104 %.

Radio-assay

Sugars and polyols in the radioactive samples were subjected to paper chromatographic resolution in preparation for counting of activity and blanks for background counting were prepared in the same way that trehalose was quantitatively measured. Here, however, the phenol:water (4:1 v/v) solvent system was chosen for use since it completely separated the ketose, aldose, polyol and trehalose occurring in eggs of A. ellioti.

Following elution of radioactive compounds from chromatograms, aliquots were reduced under a stream of nitrogen to a volume of about one ml and 15 ml of scintillation fluid were added to the counting vials. The liquid scintillation medium was made up of 6.0 grams of PPO and 120 grams of naphthalene per liter of dioxane after Bush and Hansen (1965) modified as recommended by Howald (1966).

Aliquots were counted for a ten minute period with a model 6804 Nuclear Chicago liquid scintillation counter. In order to correct for different counting efficiencies, the observed counts per minute were adjusted to disintegrations per minute with the aid of a quenching curve prepared by Dr. G. Strobel, Botany and Microbiology Department, Montana State University.

RESULTS AND DISCUSSION

Qualitative Analysis

Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified in the neutral 70 % ethanolic extracts taken from eggs of A. ellioti. Corresponding R_{gl} values (range relative to glucose) were obtained by co-chromatography on paper of standards and egg extracts in at least four solvent systems (Table I). Five solvent systems were used in mannose characterization, six for glucose and fructose and seven for mannitol and trehalose.

Selective sprays were used in conjunction with paper chromatography to further aid in identification of the sugars and polyols. As indicated by Lambou (1956), standard and egg mannitol were both viewed as gray spots on heated chromatograms which had been sprayed with the modified Fleury reagent and, after overspraying with p-anisidine phosphate, they appeared as dull yellow spots in daylight and yellow under ultraviolet light. Under the above conditions, glycerol appeared as gray, white and lavender spots respectively; fructose spots were observed as tan, golden brown and gold; glucose as black, dull yellow and dull yellow; and mannose spots appeared as pale tan, pale tan and yellow. Trehalose was not detected following treatment of chromatograms with the Fleury reagent but made a fleeting pale pink appearance under daylight conditions when the p-anisidine was oversprayed. When chromatograms were only sprayed with the improved p-anisidine phosphate reagent of Mukherjee and Srivastava (1952), egg and standard mannose and glucose were viewed as light brown spots and fructose spots were yellow. Polyols were not detected with this

Table I

Rgl values from chromatograms of extracts of Aulocara ellioti eggs and of various sugars and polyhydric alcohols.

Extract or Standard	Solvent System*						
	BAW	EPW	IPAW	PW ₁	PW ₂	BEW ₁	BEW ₂
<u>A. ellioti</u> eggs	100	99	100	100	100	101	----
	29	24	67	69	53	16	16
	122	95	98	131	129	113	111
	125	121	109	118	----	----	----
	142	136	----	154	156	143	----
	378	266	129	307	----	----	----
	26**	23**	39	39	----	**	----
Glucose	100	100	100	100	100	100	100
Trehalose	29	24	67	69	53	16	16
Mannitol	122	94	98	132	129	113	111
Mannose	125	121	109	119	----	----	----
Fructose	141	136	109	153	156	142	----
Glycerol	378	266	129	307	----	----	----
Sorbitol	117	92	----	141	133	105	----
Sorbose	121	131	109	----	----	----	102
Ribose	197	239	----	----	----	----	----
Xylose	----	----	119	----	----	----	----
Galactose	----	90	----	----	----	----	----

* Solvent systems used were: BAW, n-Butanol:Acetic Acid:Water, 4:1:1 v/v; EPW, Ethyl Acetate:Pyridine:Water, 8:2:1 v/v; iso-Propanol:Pyridine:Acetic Acid:Water, 8:8:1:4 v/v; PW₁, Phenol:Water, 4:1 w/v; PW₂, 70 % Phenol; n-Butanol:Ethanol:Water, 4:1:5 v/v (upper phase), BEW₁; BEW₂, n-Butanol:Ethanol:Water, 4:1:1 v/v.

** Not completely resolved from trehalose in egg extract.

reagent when used alone. Fructose was visualized as a red spot when the naphthoresorcinol spray reagent outlined by Block et al. (1958) was used and as a blue-violet spot when the alpha-naphthol:phosphoric acid reagent described by Block et al. was used.

The identities of trehalose, mannitol and mannose were further confirmed by gas-liquid chromatography and comparison of retention times of the trimethylsilyl ether derivatives that had been prepared from neutral ethanolic egg extracts and carbohydrate standards (Table II).

Trehalose characterization was concluded by hydrolysis in sulfuric acid and paper chromatography of the neutralized hydrolyzate. Material in the egg, which had migrated on paper the same distance as standard trehalose in iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v), yielded only glucose when hydrolyzed in this acid. A one normal solution of sulfuric acid released only small amounts of glucose in 30 hours at room temperature (as judged by co-chromatography with standard glucose and trehalose in phenol:water (4:1 v/v), ethyl acetate:pyridine:water (8:2:1 v/v) and iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v)). The trehalose spot was much reduced in intensity and a prominent glucose spot was observed when the material was chromatographed in the above solvent systems following hydrolysis in boiling 3 N sulfuric acid for one hour.

Due to deproteination by Somogyi's reagent, which includes the use of barium hydroxide, production of fructose by the Lobry de Bruyn - van Ekenstein transformation is possible. Since it was a minor component in eggs, (always less than about 0.5 μg /egg, as judged by spot size and

Table II

Retention times of trimethyl silyl ethers of standard and egg carbohydrates.

a) Column of 3.8 % SE-30 on Gas Chrom Q.

TMS ethers prepared from:	Retention times (minutes)					
	at 210°C		at 205°C		at 150°C	
Diapause egg carbohydrates	0.65	8.7	0.65	9.2	7.6	
Trehalose	8.6		9.2			
Mannitol	0.65		0.65		7.6	
β -D-Glucose	0.70				9.6	
β -D-Fructose					4.3	

b) Column of 5 % SE-30 on Gas Chrom Z.

TMS ethers prepared from:	Retention times (minutes)					
	at 150°C			at 160°C		
Prediapause egg carbohydrates	3.8	5.8	8.8	10.2		
Diapause egg carbohydrates			6.8	10.2	5.5	
Mannose	3.4 & 3.8					
Mannitol			6.7		5.5	
Undetermined		X	X	X		

detection limits), no effort was made to confirm its true occurrence in eggs of A. ellioti. However, fructose was detected in hemolymph samples where Somogyi's reagent was not used. Therefore, it is believed that fructose naturally occurs in eggs of this species.

There was one unidentified spot, detected with AgNO_3 , that migrated less than trehalose in most solvent systems employed (Table I). This moiety could not be detected with the more selective sprays previously noted nor with the Morgan-Elson reagent for the detection of amino sugars (Waldi, 1965) and did not form a color complex when the phenol-sulfuric acid method of Dubois et al. (1956) was used. It was therefore judged not to contain an aldo or keto sugar, nor be a polyol or acetylated amino sugar and further characterization was not attempted.

In two hemolymph samples from females that had survived the sixth week of oviposition, only glucose, trehalose and a slight amount of fructose were identified. In a recent review, Wyatt (1967) notes that about 20 sugars were found in Locusta migratoria hemolymph and their occurrence was diet dependent. Mannose was not detected in the hemolymph of A. ellioti even though it was found in newly laid eggs in greater amounts than glucose.

Most of the carbohydrates identified in A. ellioti eggs have been found in eggs of other insects. The occurrence of trehalose and glucose in insect eggs is probably universal. No previous report was found of mannose in eggs. Somme (1964) has previously reported the presence of a polyhydric alcohol that was "probably mannitol" in eggs of the fall

cankerworm, Alsophila pometaria, and the black willow aphid, Pterocomma smithia, but no other reports of its occurrence in insect eggs could be located. Figure 1 and Table I distinguish between mannitol and sorbitol, which has been observed in a number of insects. Roemhild (1966) tentatively identified glycerol in A. ellioti eggs. Glycerol has been reported in eggs of a number of other insect species (Somme, 1964, 1965; Chino, 1958; Hanec, 1966) but has not been detected previously in any other acridid egg studied (Salt, 1957; Randall and Derr, 1965).

Quantitative Analyses of Developing Eggs

Changes in mannose and mannitol during development

During the development of A. ellioti eggs, the carbohydrates that were detected changed in quantity and identity. Mannose was present in week old eggs in quantities of up to about 7 $\mu\text{g}/\text{egg}$. The quantity of mannose present in eggs was markedly reduced between 15 and 25 days of development (figure 2) and was not detected after 45 days of development.

The amount of mannitol present in each egg sample during development is tabulated in Table III. Figure 3 exhibits the means and extremes of mannitol during development. As mannose disappears from the eggs, mannitol makes its appearance. The quantity of free mannose is not sufficient to serve as the only precursor for mannitol synthesis. However, mannose may be an intermediate and/or a component of the protein-carbohydrate complexes that Leopold (1967) found to be a major portion of yolk material deposited in A. ellioti oocytes. This view is supported by the observations that there is a striking increase in the free amino acid



Figure 1. Chromatograms of neutral carbohydrates in post-diapause *Aulocara ellioti* eggs showing resolution of mannitol and sorbitol. Chromatogram on left was developed for 30 hours in n-butanol:ethanol:water (4:1:5 v/v). Chromatogram on the right was developed for 41 hours in n-butanol:acetic acid:water (4:1:1 v/v). Detection of spot: alkaline silver nitrate. E: sample from diapause eggs; G: glucose; T: trehalose; M: mannitol; S: sorbitol; F: fructose. Quantities of extracts spotted represent 1/2 an egg.



Figure 2. Chromatogram of neutral carbohydrates in prediapause *Aulocara ellioti* eggs. The developing solvent was ethyl acetate:pyridine:water (8:2:1 v/v). Detection of spots: alkaline silver nitrate. E_A : sample from 15 day old eggs; E_B : sample from 25 day old eggs; E_C : sample from 45 day old eggs; A: upper spot is fructose, middle spot is glucose, lower spot is trehalose; B: upper spot is mannose, lower spot is glucose; C: mannitol. The trehalose spot was reduced in intensity along with background color, but spots were marked prior to treatment with ammonium hydroxide. Quantities of extracts spotted represent 1 1/2 eggs where age is 15 or 25 days and 1 egg where age is 45 days at 25°C.

Table III

Mannitol in Aulocara ellioti eggs laid in the summer of 1966.

Description of samples					Amount of mannitol		
Days 25°C	Days 8-12°C	Days 25°C	Stages	Mean stage	$\mu\text{g}/\text{egg}$	Average $\mu\text{g}/\text{egg}$	mg/100g
Greenhouse reared:							
(no mannitol was detected in 1, 3, 7 or 15 day old samples)							
25	0	0	8-12	10.2	4.0	3.3	65.1
25	0	0	8-12	10.2	4.0		
*25	0	0	8-12	10.2	1.8		
*25	0	0	8-12	10.2	3.2		
45	0	0	16-19	18	25.5	23.1	325.2
45	0	0	16-19	18	20.5		
*45	0	0	16-19	18	26.3		
*45	0	0	16-19	18	21.2		
74	25	0	19	19	27.9	28.8	402.0
69	25	0	19	19	29.7		
63	40	0	19	19	30.0	32.0	431.1
63	40	0	19	19	34.0		
139	0	0	19	19	18.8	18.8	290.7
76	174	0	19	19	23.1	23.6	253.4
75	176	0	19	19	23.2		
*80	174	0	19	19	24.6	24.7	229.1
84	170	0	20-22	20.6	23.9		
83	171	1	20-22	20.3	22.6		
*80	172	0	20-22	20.5	27.5		
83	170	0	23-24	23.1	23.1	27.4	237.4
81	173	1-3	23-24	23.2	28.0		
*69	154	2	23-24	23.1	31.0	7.4	76.5
76	179	7	25	25	7.6		
72	184	7	25	25	7.2		
68	191	14	26	26	2.0		
65	192	14	26	26	3.3	2.7	27.9
65	191	21	27	27	1.7	1.7	18.7
Field Collected:							
153	0		19	19	23.5	25.5	240.1
154	0		19	19	25.7		
155	0		19	19	27.5		
153	0		20-22	20.6	25.9	26.2	242.6
153	0		20-22	20.3	26.4		
153	0		23-24	23.2	25.4	24.0	218.9
154	0		23-24	23.1	22.7		
140	19		27	27	2.1	2.1	25.2

* Values not included in amount of mannitol relative to fresh egg weight.

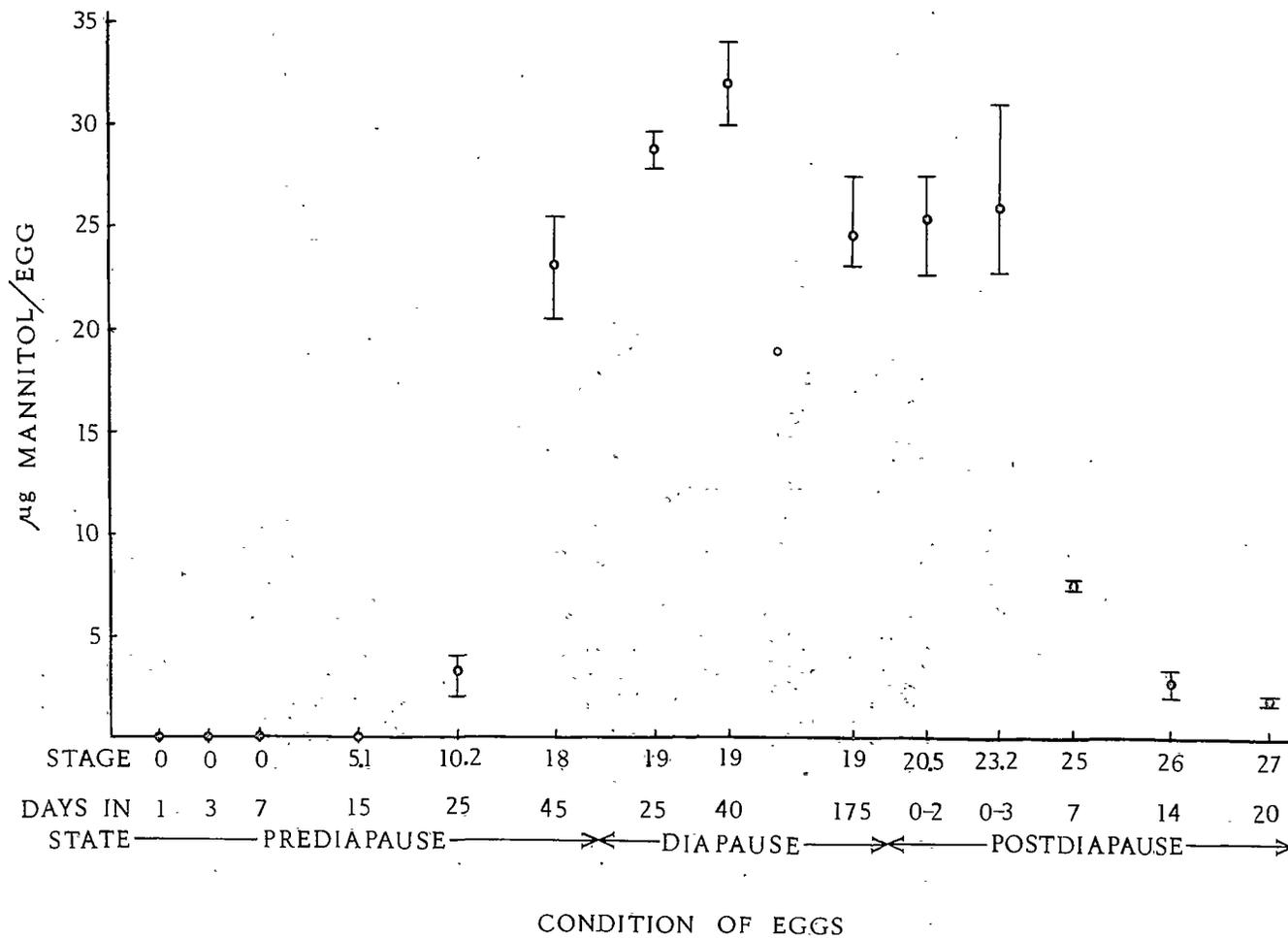


Figure 3. Mannitol in Aulocara elliotti eggs during development. ●, Greenhouse or field collected eggs; ○, Greenhouse diapause eggs with no cold exposure.

concentration from the prediapause to the diapause period (Bunde, 1965; Bunde and Pepper, 1968) and, as noted in Table V, the quantity of mannose increased during the first two weeks of egg development. Nothing is known about the identity of moieties in the protein-carbohydrate complexes in the yolk of this species but Lipke et al. (1965) found that mannose was the principal neutral sugar in all plasma glycoproteins of the cockroach and, as indicated by Chen (1966) and Brookes and Dejmál (1968), a number of workers have concluded that some blood proteins are incorporated into oocytes in insects.

Mannitol changes are associated, at least temporally, with diapause in A. ellioti. The first appearance of mannitol coincides with the time in embryonic development when the egg first becomes compartmented. In the diapause egg these compartments contain fluid with very different chemical and physical characteristics (Roemhild, 1967). Mannitol appearance also coincides approximately with the first embryonic stage that was found by Roemhild (1965a) to be able to survive cold, undergo diapause and still hatch. He also found that cold exposure for 45 - 50 days was sufficient to break diapause in at least one population. The highest average concentration of mannitol observed in this study (32 $\mu\text{g}/\text{egg}$ or 431 mg/100 g fresh egg weight) was in diapause eggs after 40 days of cold exposure (Table III, Figure 3). It may also be seen that the elevated levels of mannitol persist through diapause, blastokinesis and until the beginning of the more rapid mitotic activity that distinguishes between diapause and post-diapause development. Roemhild (1968a) determined that

this resumption of mitotic activity occurred at stage 24 (according to staging criteria of Van Horn, 1966a). After the rapid decline of mannitol occurring at this point, there is continued decrease in levels toward the definitive embryo.

As pointed out in the Introduction, cold exposure has been shown to influence levels of some polyhydric alcohols in insects. A brief attempt was made to evaluate this possibility in eggs of A. elliotti. It was found that cold exposure may result in elevated mannitol levels in eggs of this species but more samples and more and lower temperature levels are needed to confirm this effect and to more completely evaluate the role of cold. The sample with no cold exposure is positioned on figure 3, with respect to total egg age, between those receiving 40 and 175 days of cold. The sample with no cold exposure contained less mannitol per egg than any other diapause egg sample. For example, eggs which were 103 days old (including 40 days of cold exposure) contained $32 \pm 2 \mu\text{g}/\text{egg}$ and the 139 day old eggs with no cold exposure had only $18.8 \mu\text{g}/\text{egg}$. The difference persists when those eggs receiving no cold are compared with those cold exposed eggs of lesser age but disappears when compared with cold exposed eggs of more advanced age and when the amount of mannitol is evaluated on the basis of mg/100 g fresh egg weight (Table III). This is believed to be due to the somewhat dehydrated condition of the eggs receiving no cold exposure compared to those of advanced age (0.6469 g/100 eggs vs. 0.9830 g/100 eggs respectively).

Glycerol in eggs

A wide variation is noted between 100 egg samples in the same morphological stage of development (Figure 4) which is hard to explain. It was also observed by Roemhild (1966) that osmotic pressure of fluids in the various compartments of A. ellioti eggs varied a great deal. It does not appear possible that the differences in glycerol values could be due to the experimental technic or to bacterial contamination of the alcoholic extract. The differences observed in glycerol levels between replicated samples can not be accounted for by either the variation between samples in other carbohydrate components or any summation of these latter variations.

Due to extreme variation, there is no apparent pattern to the amount of glycerol observed in eggs of A. ellioti. As previously indicated, glycerol is accumulated during diapause in a number of insects and is generally lost only after diapause is broken. No such relationship was observed in this study as glycerol was also detected in pre- and post-diapause eggs. The month-long increase of osmotic pressure in diapausing eggs of A. ellioti observed by Roemhild (1966) in one population of this grasshopper and thought to be due to glycerol also failed to materialize the next year (Roemhild, 1968a).

It has been suggested that the relationship between glycerol and diapause was a coincidence of concurrent timing (Salt, 1961; Somme, 1965) and that glycerol in overwintering insects can increase the cold-hardiness by depressing the supercooling and freezing points (e.g. Salt, 1959,

1961; Somme, 1964; Hanec, 1966). However, the highest level of glycerol detected in any sample in this study (39.1 $\mu\text{g}/\text{egg}$ or 667 mg/100 g fresh egg weight) would depress the freezing point only 0.13°C and it does not, therefore, appear to be important in this respect in A. ellioti eggs.

Changes in glycogen during development

It will be recalled that glycogen served as the source for polyhydric alcohol synthesis in diapausing eggs of B. mori (Chino, 1958). Since there is a net increase in glycogen levels at the same time glycerol and mannitol increase in diapausing A. ellioti (Table IV, Figure 5), it is apparent that glycogen does not serve as the ultimate source of mannitol and glycerol in this species.

The overall contribution of glycogen as an energy source for embryogenesis of A. ellioti appears to be slight. First, levels are higher in the definitive egg than in the newly laid egg, which is in contrast to other insect eggs as pointed out in the Introduction. Second, the amount of glycogen in eggs of A. ellioti is slight in comparison to some insect eggs. For example, the highest concentration of glycogen noted in eggs in this study was about 3.5 mg/g (Table IV) whereas hibernated eggs of Bombyx mori may contain 40 mg/g (Yamashita, 1965).

Although there was an overall net synthesis of glycogen by eggs of A. ellioti, slight early and late utilization was noted (Figure 5). Loss of glycogen in other insect eggs has been observed to be either a steady decrease or an early and late decrease. Following the early use of glycogen by A. ellioti, eggs regained their initial levels during the

Table IV

Glycogen in A. ellioti eggs laid in the summer of 1966.

Description of samples			Amount of glycogen		
			Average		
			$\mu\text{g}/\text{egg}$	$\mu\text{g}/\text{egg}$	$\text{mg}/100\text{g}$
Greenhouse Reared Prediapause:					
Age (days at 25°C)	Stages	Mean stage			
1	0	0	23.5	} 20.7	329.1
1	0	0	17.9		
3	0	0	26.1	} 19.7	329.6
3	0	0	13.3		
7	0-1	0	24.2	} 19.3	312.6
7	0-1	0	14.4		
15	2-8	5.1	14.3	} 15.2	255.7
15	2-8	5.1	16.2		
25	8-12	10.2	15.7	} 15.2	252.3
25	8-12	10.2	15.2		
*25	8-12	10.2	14.7		
*25	8-12	10.2	15.4		
45	16-19	18	22.1	} 20.6	270.8
45	16-19	18	16.2		
*45	16-19	18	21.1		
*45	16-19	18	22.9		
Greenhouse Reared Diapause:					
Stage 19					
(Days at 25°C)	(Days at 8-12°C)				
74	25		18.3	} 20.2	281.7
69	25		22.1		
63	40		17.3	} 19.0	256.2
63	40		20.8		
139	0		17.4	17.4	269.3
76	174		22.4	} 26.9	255.6
75	176		24.3		
*80	174		28.3		
*61	176		32.7		
Field Collected Diapause					
	153		24.8	} 25.2	236.9
	154		25.7		
	155		25.1		

Table IV
(Continued)

Description of samples					Amount of glycogen		
					$\mu\text{g}/\text{egg}$	Average $\mu\text{g}/\text{egg}$	mg/100g
Greenhouse Reared Post-diapause:							
Days 25°C	Days 8-12°C	Days 25°C	Stages	Mean stage			
84	170	0	20-22	20.6	27.6	} 29.6	262.1
83	171	1	20-22	20.3	25.7		
*80	172	0	20-22	20.5	35.7		
83	170	0	23-24	23.1	36.3	} 35.6	326.6
81	173	1-3	23-24	23.2	34.0		
*69	154	2	23-24	23.1	36.5		
76	179	7	25	25	30.4	} 30.7	317.4
72	184	7	25	25	30.9		
68	191	14	26	26	34.2	} 31.8	303.9
65	192	14	26	26	29.3		
65	191	21	27	27	23.5		
Field Collected Post-diapause:							
	Days 8-12°C	Days 25°C	Stages	Mean stage			
	153	0	20-22	20.6	29.1	} 29.8	276.3
	153	0	20-22	20.3	30.5		
	153	0	23-24	23.2	37.3	} 38.5	350.2
	154	0	23-24	23.1	39.7		
	140	19	27	27	28.6	28.6	340.8

* Values for these samples are derived by summing separately analyzed yolk and embryo samples. Since weights were not available, these values were not used in calculating weight of glycogen relative to fresh egg weight.

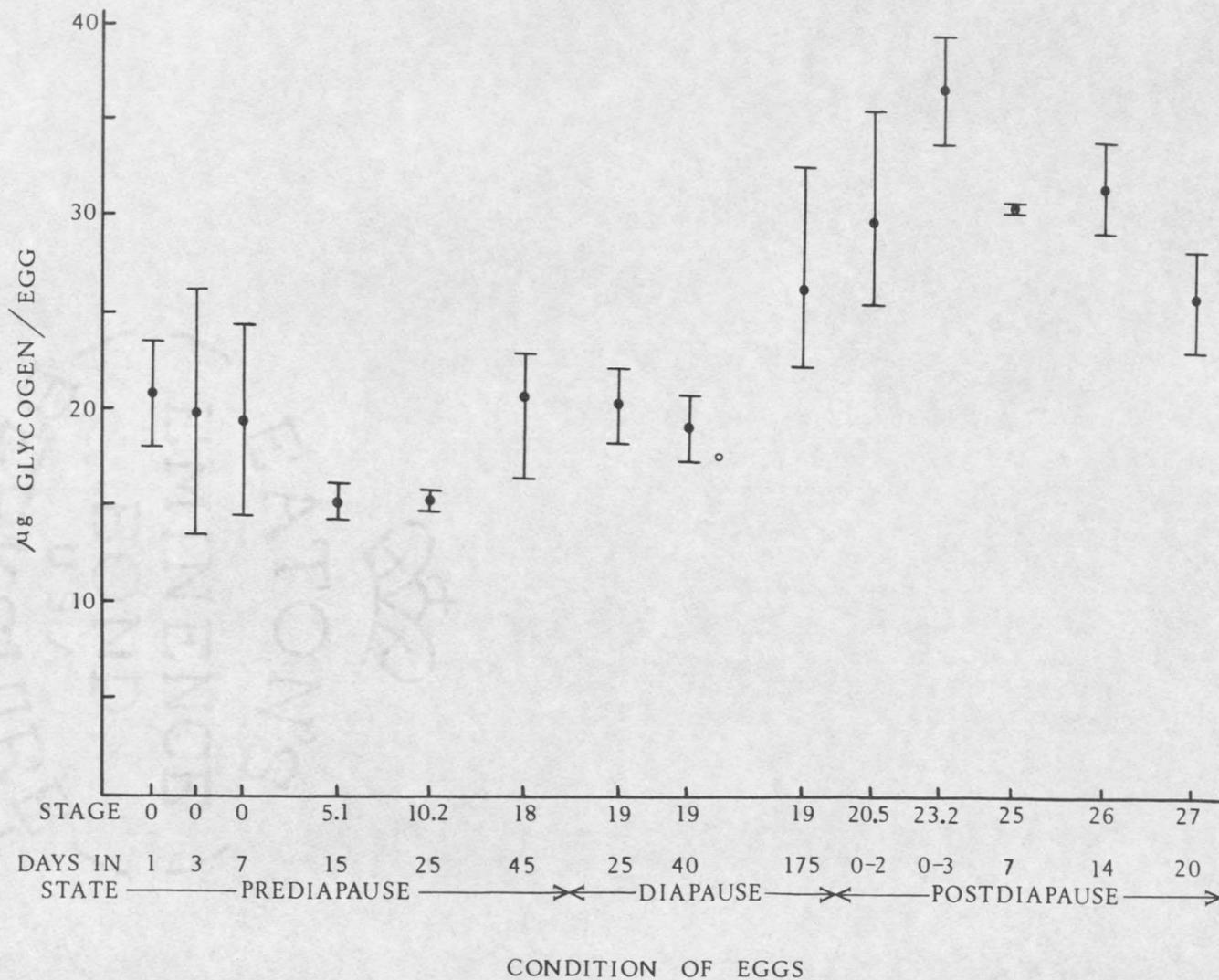


Figure 5. Glycogen in Aulocara ellioti eggs during development. ●, Greenhouse or field collected eggs; ○, greenhouse collected eggs with no cold exposure.

last 20 days of pre-diapause development and in later diapause development continue a slow synthesis. The mean glycogen level increased from 26.2 to 36.8 $\mu\text{g}/\text{egg}$ during blastokinesis. Since there was this rapid net gain of glycogen, it seems that it furnishes no energy for blastokinesis in A. ellioti. Rothstein (1952) found glycogen to be constant during this period in Popilia japonica. Similarly, Yamashita (1965) showed a slight decrease in hibernated eggs and a slight increase in non-diapause eggs of B. mori during blastokinesis.

Changes in trehalose levels during development

Trehalose levels would appear to increase slightly during pre-diapause development of A. ellioti (Table V, Figure 6). This is in general agreement with the report that trehalose levels double during prediapause development of Melanoplus differentialis (Randall and Derr, 1965). However, due to the effects of maternal age on trehalose partitioned to eggs of A. ellioti, which will be discussed later, it is not possible to state positively what the nature of changes in trehalose levels during prediapause development are.

During the prolonged diapause state of greenhouse collected A. ellioti embryos, the absolute amount of trehalose increased from a mean of about 40.9 to 100.1 $\mu\text{g}/\text{egg}$ (Tables V-VI, Figure 6). This increase occurred whether the eggs were held at 25°C or were transferred to 10°C at the completion of prediapause development. Randall and Derr (1965) did not find any significant change in the trehalose content during diapause of Melanoplus differentialis but a marked increase in post-diapause develop-

Table V

Trehalose and free reducing sugars in greenhouse collected prediapause A. ellioti eggs.

Description of samples			Amount of trehalose			Total free sugar	% Trehalose of total free sugar	Minor components
Days 25°C	Stages	Mean stage	µg/egg	Average µg/egg	mg/100g	µg/egg		µg/egg **
1	0		30.5			33.0		
1	0	0	35.5	33.0	497 ± 30	38.9	91.9 ± 0.7	2-3 mannose. <1 glucose.
3	0		32.6			37.5		
3	0	0	30.3	31.4	525 ± 20	34.0	88.1 ± 1.2	3-4 mannose. .5-1 glucose.
7	0-1		39.3			43.5		
7	0-1	0+	31.7	35.5	574 ± 62	39.1	85.8 ± 4.7	4-5 mannose. .5-1 glucose.
15	2-8		41.3			48.0		
15	2-8	5.1	33.3	37.3	627 ± 79	38.8	86.0 ± 0.2	≈5 mannose. ≈1.5 glucose.
25	8-12		36.5			40.4		
25	8-12		38.7			43.4		
*25	8-12	10.2	29.7	33.9	614 ± 26	33.8	89.6 ± 0.6	≈1.5 glucose.
*25	8-12		30.9			36.8		
45	16-19		43.1			50.4		
45	16-19		34.2			40.5		
*45	16-19	18	43.5	40.9	546 ± 21	51.0	85.7 ± 2.1	traces of mannose and fructose.
*45	16-19		43.0			49.0		4-5 glucose.

* Values were derived by summing separately analyzed yolk and embryo samples and, since egg weight was not taken, were not used in calculating mg trehalose per 100 g fresh egg weight.

** Quantities were estimated by spot size and intensity on chromatograms and the difference between total free sugar and trehalose. Total free sugar is that soluble in 70 % ethanol and reacting with the phenol-sulfuric acid method of Dubois et al. (1956).

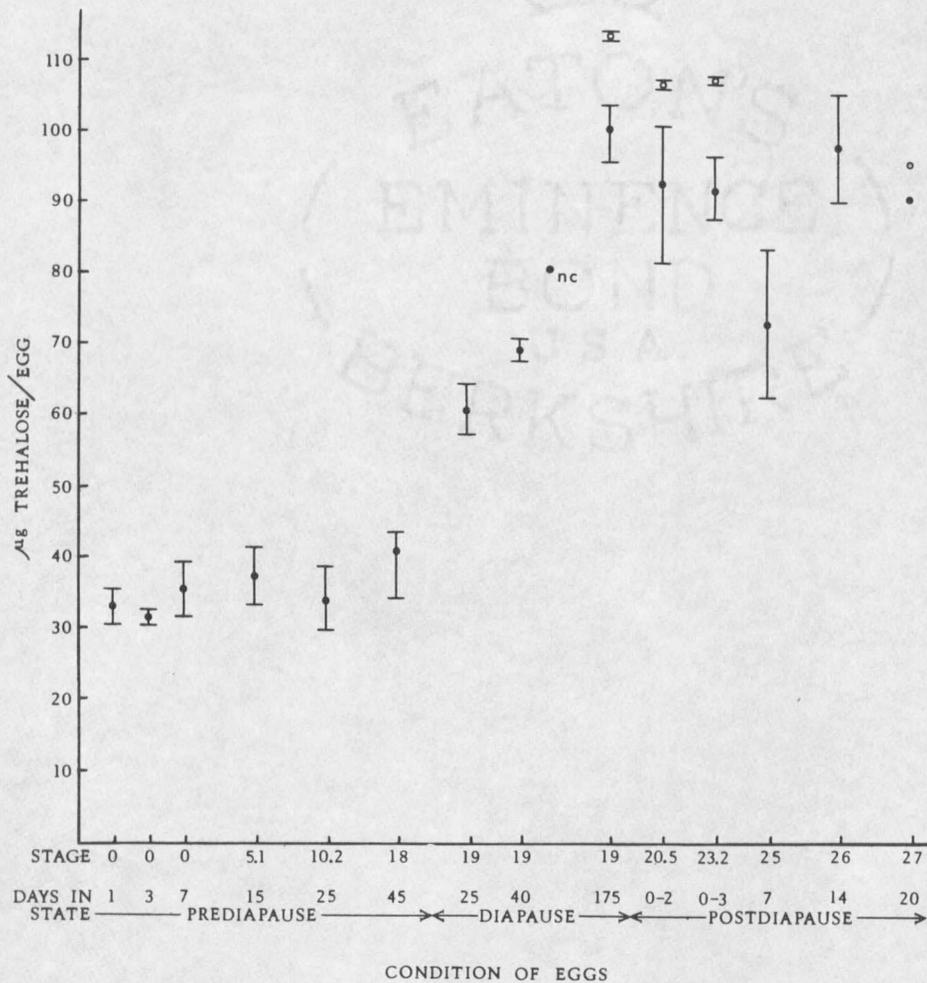


Figure 6. Trehalose in *Aulocara elliotti* eggs during development. ●, Greenhouse collected; ○, field collected; nc, no cold exposure.

Table VI

Trehalose and free reducing sugars in diapause A. ellioti eggs laid in 1966.

Description of samples		Amount of trehalose			Total free sugar	% trehalose of total free sugar
Days 25°C	Days 8-12°C	µg/egg	Average µg/egg	mg/100g	µg/egg	
Greenhouse collected:						
74	25	57.3	60.7	848 ± 54	71.5	81.4 ± 1.2
69	25	64.2			77.8	
63	40	67.5	69.0	930 ± 63	71.7	90.1 ± 4.1
63	40	70.6			82.1	
139	0	80.6	80.6	1246	81.8	98.5
76	174	103.5	100.1	1124 ± 23	107.3	96.4 ± 0.2
75	176	101.4			105.0	
*80	174	95.5			99.2	
Field collected:						
	153	112.7	113.4	1076 ± 11	121.4	92.7 ± 0.1
	154	114.1			123.2	
	**155	106.4	106.4	983	122.5	86.9

* This value was not used in calculating the weight of trehalose relative to fresh egg weight since the sample weight was not available. This value was derived by summing separately analyzed yolk and embryo fractions.

** Since this sample had some characteristics of stage 20 embryos (serosal connection to hydropile was broken and weight was greater than other diapause samples), it was not averaged with other late diapause samples.

ment. This may be reconciled by considering that the time interval checked in M. differentialis was only two weeks or that this is another reflection of species differences. In view of the findings that the fat body of A. ellioti increased markedly in size during prolonged embryonic diapause (Van Horn, 1963), the fat body serves as the principal site of trehalose synthesis in the adult locust (Candy and Kilby, 1961), and the present finding that there is a marked increase in trehalose levels during diapause (Figure 6) it appears that the embryonic fat body may serve the same function as in the adult.

Bunde (1965) and Bunde and Pepper (1968) indicate that the highest levels of free amino acids in eggs of A. ellioti occurred during diapause. This may be a reflection of cleavage of the protein-carbohydrate complexes in yolk and would seem to be a possible source of glucose for the trehalose synthesis that occurs during this period. The synthesis of trehalose may result from this hydrolysis coupled with a reduced requirement for energy during diapause.

Trehalose levels declined from a mean of 105.6 to 97.9 $\mu\text{g}/\text{egg}$ during blastokinesis (Tables VI - VII, Figure 6). However, this decrease of 7.7 $\mu\text{g}/\text{egg}$ is counterbalanced by a net gain of 10.6 $\mu\text{g}/\text{egg}$ in glycogen. As previously indicated, mannitol also remained constant during this period (Figure 3). Therefore, if oxidation of such reducing compounds as glucose furnishes energy for blastokinesis, as suggested by Ludwig (1956), it would appear to come from some other source than trehalose, glycogen or mannitol in A. ellioti. These results are not inconsistent with the

Table VII

Trehalose and free reducing sugars in post-diapause A. ellioti eggs laid in 1966.

Description of samples				Amount of trehalose			Total free sugar	% trehalose of total free sugar	
Days 25°C	Days 8-12°C	Days 25°C	Stages	Mean stage	$\mu\text{g}/\text{egg}$	Average $\mu\text{g}/\text{egg}$	mg/100g	$\mu\text{g}/\text{egg}$	
Greenhouse collected:									
84	170	0	20-22	20.6	81.5	92.5	871 \pm 73	93.6	
83	171	1	20-22	20.3	95.4			105.6	88.9 \pm 1.8
*80	172	0	20-22	20.5	100.5			109.3	
83	170	0	23-24	23.1	91.2	91.7	870 \pm 21	99.4	
81	173	1-3	23-24	23.2	96.1			103.3	91.4 \pm 2.1
*69	154	2	23-24	23.1	87.7			98.2	
76	179	7	25	25	83.2	72.9	754 \pm 111	88.1	
72	184	7	25	25	62.7			70.5	92.7 \pm 3.8
68	191	14	26	26	105.0	97.5	1015 \pm 30	110.0	
65	192	14	26	26	90.0			94.5	95.3 \pm 0.1
65	191	21	27	27	90.4			90.4	
Field collected:									
153	0	0	20-22	20.6	107.1	106.5	988 \pm 3	120.5	
153	0	0	20-22	20.3	105.9			110.7	92.1 \pm 4.3
153	0	0	23-24	23.2	106.4	107.1	975 \pm 16	116.0	
154	0	0	23-24	23.1	107.7			115.8	92.4 \pm 0.6
140	19	19	27	27	95.3	95.3	1136.7	108.9	

* Values for these samples were derived by summing separately analyzed yolk and embryo samples. Since weights were not available, these values were not used in calculating weight of trehalose relative to fresh egg weight.

Total free sugar is that soluble in 70 % ethanol and reacting with the phenol-sulfuric acid method of Dubois et al. (1956).

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hypothesis advanced by Roemhild (1968a) that this movement in A. ellioti may be accomplished by means of water pressure and serosal shrinkage.

Following blastokinesis, a new embryonic cuticle is deposited (Van Horn, 1968). Since there is a 70 % increase in embryo weight between diapause termination and cuticle deposition at stage 24 (Roemhild, 1968b), it appears that more carbohydrate is needed for chitin synthesis. Since there is a decrease in trehalose levels noted when post-diapause development has progressed to stage 25 (Table VII, Figure 6) and a sharp decrease in mannitol (Figure 3) it is possible that these moieties contribute carbons for chitin synthesis. It is also possible that mannitol and trehalose furnish some energy for post-diapause development but the considerable utilization of lipid, particularly triglyceride, that occurs during this time (Svoboda et al., 1966) indicates that fat is the chief energy source for post-diapause development of A. ellioti.

When greenhouse and field collected eggs were compared with respect to carbohydrate levels, sample values for glycogen, mannitol and glycerol overlapped (Tables III - IV, Figure 4). Trehalose levels in eight field collected egg samples were consistently about 10 % higher than any greenhouse collected egg samples at late diapause and three post-diapause stages of development (Tables VI - VII, Figure 6). These data are interpreted as some evidence that the parental greenhouse conditions used did not greatly alter the carbohydrate metabolism of developing eggs. However, the consistently higher trehalose levels detected in field collected eggs implies that the parental environmental conditions may have

the effect of altering the quantity of trehalose in eggs making up the next generation. This implication was further investigated during Phase II and is discussed in more detail later.

Distribution of carbohydrates in eggs

Andrewartha (1952) suggested that an "intractability" of food materials might lead to diapause and the cessation of growth and Wigglesworth (1956) suggested that membrane impermeability might be responsible for maintenance of the diapause state. Kaocharern (1958) disagrees with the "intractability" theory, finding a deposition of lipid in mesodermal tissue during embryonic diapause of Melanoplus differentialis. Van Horn (1963) also noted that lipid material was deposited in the embryonic fat body during diapause of A. ellioti. Roemhild (1967, 1968b) found that membranes are formed in eggs of A. ellioti at about 20 days of development and result in compartments which contain a differential distribution of Na⁺, K⁺, H⁺, amino acid, and protein. He notes that these membranes are obliterated at blastokinesis and the embryo goes from a pH of 7.4 to 6.6 and hydrates markedly. The present study has shown that trehalose is synthesized during diapause but, since previously discussed results have dealt with analyses conducted on whole egg samples, the site of synthesis was only suggested to be the embryonic fat body. This conclusion drew support from temporally associated morphological changes in the fat body and known functions of this organ in the adult locust. The present section reports and discusses data on the distribution of trehalose, mannitol and glycogen between the embryo proper and the remainder of the egg

after the membranes have partitioned the egg into compartments and until after they are ruptured by blastokinesis.

Figure 7 and table VIII show that the quantity of glycogen in the yolk-fluid-cuticle fraction remains relatively constant. The increase in the quantity of glycogen between stages 8 to 12 (mean = 10.2) and late diapause occurs in the embryo proper and is retained by it. The higher percentage of glycogen in the embryo that occurs during blastokinesis (stages 20 - 24) appears to result from transfer of glycogen from yolk or fluids to the embryo and from a rapid synthesis. These might result from more favorable temperature and pH conditions and easier transfer of materials from yolk to embryo made possible by destruction of enveloping membranes and by hydration of the embryo.

The increase in the quantity of trehalose that occurs during pre-diapause and diapause development is restricted less to the embryo than is glycogen. Mannitol is still restricted less to the embryo but its point of synthesis is not known (Figure 8 and Table VIII). If, as suggested, the fat body is a principal site of trehalose synthesis in eggs of A. ellioti, these results indicate that membranes of the egg are somewhat permeable to this carbohydrate. Due to this permeability, it was not possible to localize the increase in the quantity of trehalose within the embryo. It should, however, be pointed out that the yolk-fluid-cuticle fraction includes the embryonic hemolymph and much of the trehalose may be in this fluid. Rupture of membranes and hydration of the embryo during blastokinesis induce movement of trehalose into the

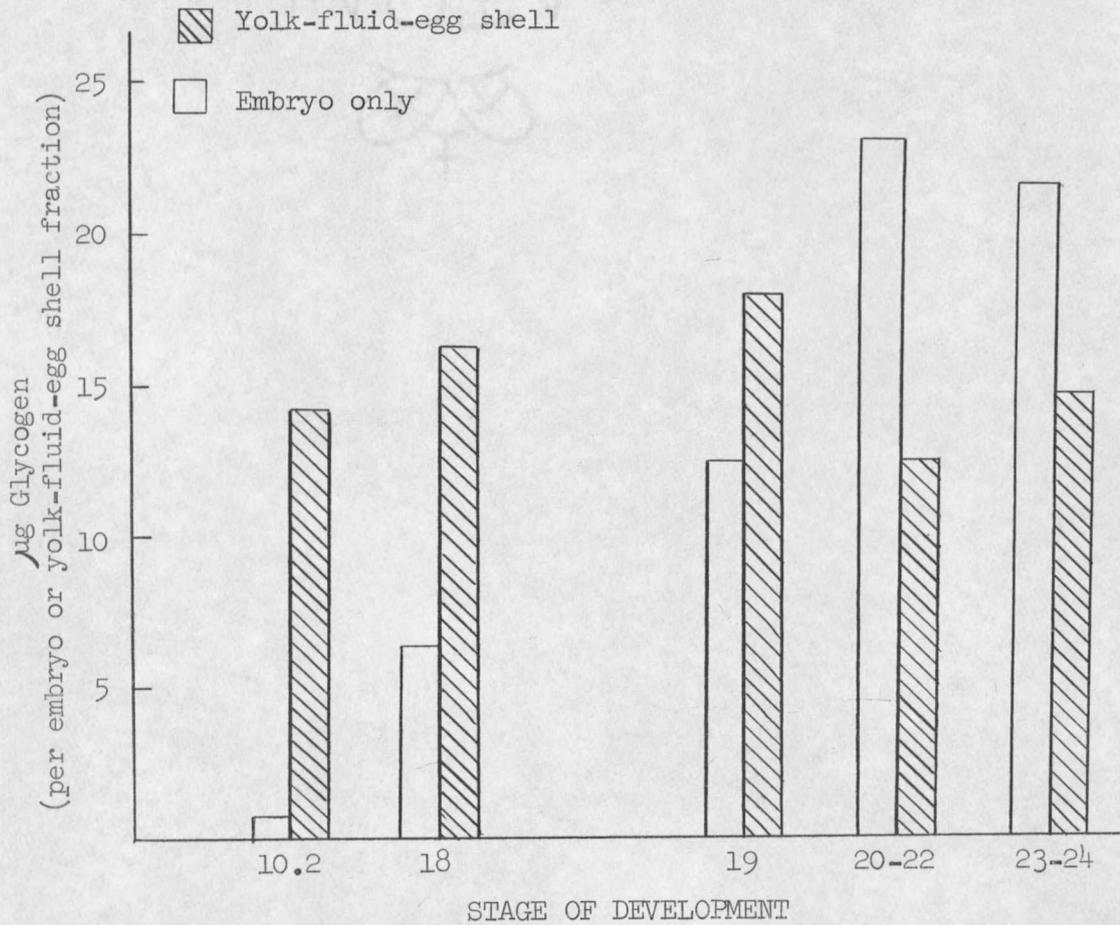


Figure 7. Distribution of glycogen in isolated embryos and the yolk-fluid-egg shell fraction of *A. ellioti* eggs. Glycogen values are expressed in micrograms per individual embryo or yolk-fluid-egg shell fraction and reflect the mean value of sample separations in each developmental stage noted.

Table VIII

Distribution of carbohydrates in embryo and yolk-fluid-egg shell fractions of A. ellioti eggs.

Description of samples* (Mean stage) Fraction	Glycogen per individual (μg) (sample)(stage)	Glycogen (% in fraction)	Trehalose per individual (μg) (sample)(stage)	Trehalose (% in fraction)	Mannitol per individual (μg) (sample)(stage)	Mannitol (% in fraction)
(stage 10.2)						
yolk A	13.6	14.2	28.3	28.9	1.5	88.0
B	14.7	94.7	29.4	95.1	2.9	2.2
embryo A	0.8	5.3	1.4	4.9	0.3	12.0
B	0.7		1.6	1.5	0.3	
(stage 18)						
yolk A	19.1	72.5	35.7	35.1	21.9	80.9
B	14.2		34.5	82.0	16.6	19.3
embryo A	5.6	27.5	6.8	18.0	4.4	19.1
B	6.9		8.5	7.7	4.6	
(stage 19)†						
yolk A	13.2	59.5	73.9	73.9	22.6	91.8
B	23.1			77.4	22.6	
embryo A	15.1	40.5	21.6	21.6	2.0	8.2
B	9.6		22.1	22.6	2.1	
(stage 20.5)						
yolk	12.5	35.0	59.8	59.8	25.0	90.8
embryo	23.2	65.0	40.7	40.7	2.5	9.2
(stage 23.1)						
yolk	14.8	40.6	59.4	59.4	27.8	90.2
embryo	21.7	59.4	28.3	28.3	3.1	9.8

* The fraction referred to as yolk also includes compartmental fluids, chorion and cuticle.

† Since some of the 70 % ethanol extract of late diapause yolk sample B was lost, only sample A was used to determine mean mannitol and trehalose quantities and percent in each fraction.

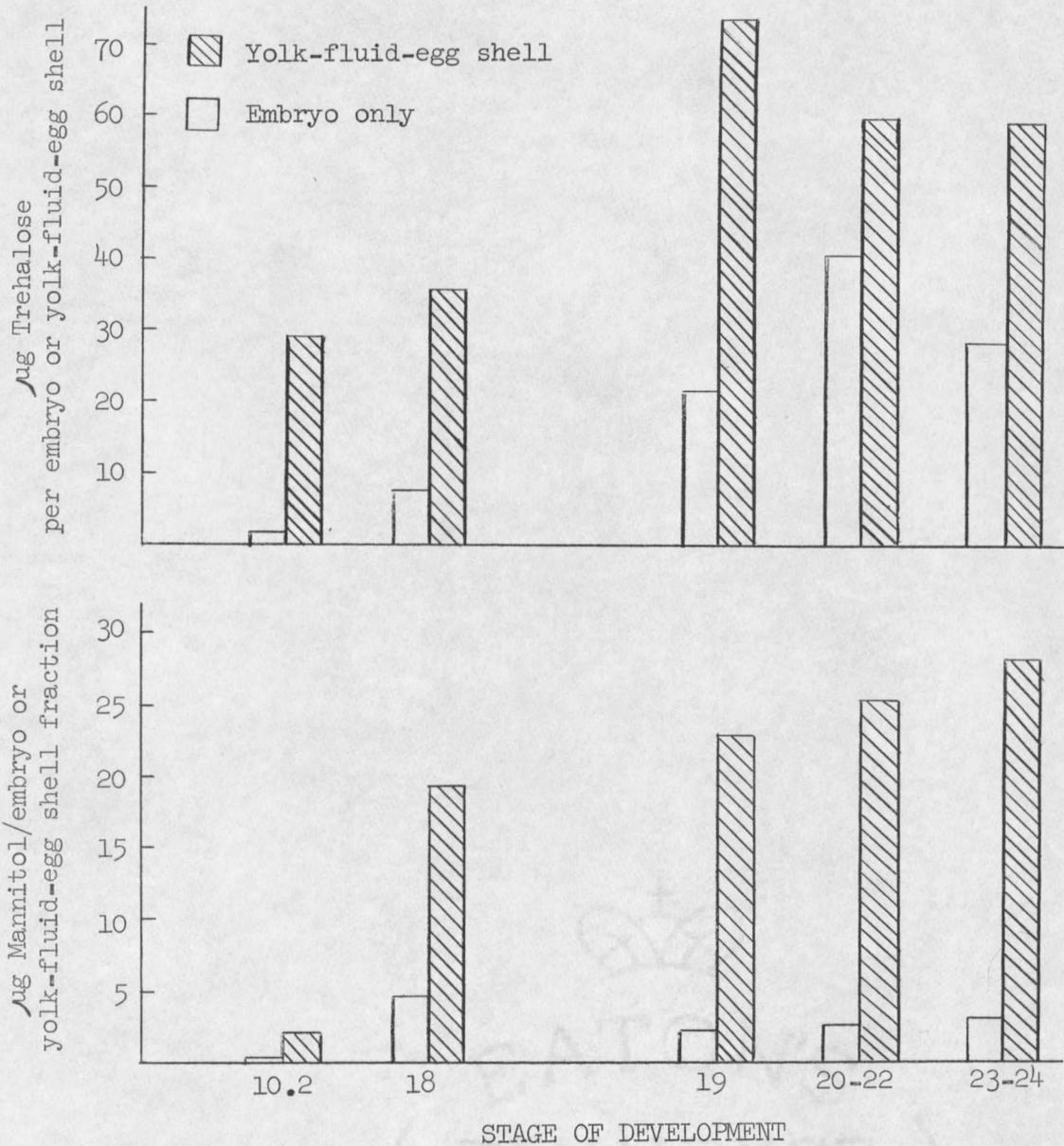


Figure 8. Distribution of trehalose and mannitol in isolated embryos and the yolk-fluid-egg shell fraction of *A. ellioti* eggs. Values shown reflect the mean value of sample separations in each developmental stage noted.

embryo. While it is still possible that membrane structures between the embryo and yolk are responsible for maintenance of the diapause state of A. ellioti (Roemhild, 1965b), the present study indicates that material for trehalose and glycogen synthesis can pass through these membranes, as trehalose may, and separation of food reserves from the embryo by membrane structures does not appear to be a main factor controlling diapause.

Radioactive tracing

Evaluation of the roles played by trehalose, mannitol and glycogen in development are based on net syntheses and degradations. It is, however, possible that even though little or no net change is observed there could be a rapid rate of turn over. Radioactive tracing was not performed in such a way as to estimate the rate of turn over of any moieties but is designed to show if such a phenomenon is possible by demonstrating the existence of enzyme systems.

D-glucose-U-C14 was absorbed by prediapause eggs of A. ellioti and the 70 % ethanol soluble, neutral extracts were subjected to quantitative paper chromatographic analysis using phenol:water (4:1 w/v) as the developing system. Although figure 3 shows a marked increase in the quantity of mannitol during prediapause development and figure 6 shows no marked change in the quantity of trehalose, the radioactivity was primarily located in trehalose (Table IX). The data may then be interpreted as an indication that there is a rapid synthesis and degradation of trehalose during this period or that glucose serves more efficiently as a precursor for trehalose synthesis than it does for mannitol synthesis.

Table IX

Distribution of radioactivity in neutral 70 % ethanolic extracts from eggs of A. ellioti following incubation with glucose-U-C¹⁴.

	Radioactivity (DPM/egg)	
	at 25 days age (mean stage=11.9)	at 45 days age (mean stage=18.9)
Prediapause eggs		
non-migrating	43.6	27.1
unidentified *	81.6	7.3
trehalose	434.0	93.4
glucose	16.5	12.0
mannitol	50.7	14.5
Diapause eggs	Radioactivity (DPM/egg)	
non-migrating	6.4	
unidentified *	3.9	
trehalose	15.0	
glucose	4.7	
mannitol	2.8	
Post-diapause eggs in stages 23 - 25 (incubated at 20 - 22)	Radioactivity (DPM/egg)	
	greenhouse collected	field collected (mean stage=24)
non-migrating	48.8	25.2
unidentified *	14.1	9.7
trehalose	874.3	844.0
glucose	138.4	60.1
mannitol	353.9	295.2

* The unidentified spot, detected with AgNO₃, was located between the base line and trehalose on paper chromatograms run in phenol:water (4:1 w/v).

There was less incorporation of glucose into other moieties as the eggs approached and entered diapause, possibly as the result of decreased permeability through the membrane structures and a dehydration of the embryo. The radioactivity was still mainly located in trehalose in the diapause and post-diapause samples but the latter also contained a substantial amount of radioactivity in the form of mannitol. Greater incorporation of glucose into trehalose and mannitol in the post-diapause samples may be partly due to the rupture of membranes at blastokinesis and to hydration and partly due to exposure of eggs to a greater quantity of radioactive glucose. Data in table IX indicates that enzyme systems for the conversion of glucose to mannitol and trehalose may still be active even though figures 3 and 6 show a decrease in the net amounts of mannitol and trehalose at the time of the post-diapause stages included in the radioactive samples.

It will be recalled that when embryos of Bombyx mori break diapause there is a net increase in the quantity of glycogen that corresponds to the decrease in glycerol and sorbitol (Chino, 1958) but when embryos of A. ellioti break diapause neither glycogen nor trehalose accumulate as mannitol is lost. The distribution of radioactivity in the 70 % ethanol soluble fractions and the succeeding TCA extraction from post-diapause eggs 0, 1, 3 and 5 days after exposure of diapause eggs to radioactive mannitol is noted in Table X. Although most of the radioactivity was observed to be in the ethanol soluble, neutral fraction at all times, there was some activity incorporated into polysaccharides, amino acids

Table X

Distribution of radioactivity in 70 % ethanol soluble extracts fractionated on ion exchange columns and in the TCA soluble extracts from post-diapause eggs of *A. ellioti* 0, 1, 3, and 5 days after incubation of diapause eggs with mannitol-1-C¹⁴.

Fraction	Radioactivity (DPM/egg)			
	at 0 days	at 1 day	at 3 days	at 5 days
neutral ethanol soluble components	345.7	335.1	72.0	221.3
amino acids	8.2	11.6	6.5	46.7
organic acids	10.8	1.1	0.5	3.1
polysaccharides	55.4	20.9	14.7	21.5

Table XI

Distribution of radioactivity in neutral 70 % ethanolic extracts from post-diapause eggs of *A. ellioti* 0, 1, 3, and 5 days after incubation of diapause eggs with mannitol-1-C¹⁴.

Egg component	Radioactivity (DPM/egg)			
	at 0 days	at 1 day	at 3 days	at 5 days
non-migrating	0	0	-0.8	1.4
unidentified spot*	-5.2	0	-2.4	-3.0
trehalose	1.0	5.8	13.0	50.7
glucose	-1.2	4.8	-2.1	2.0
mannitol	302.5	326.0	56.0	122.3
fructose area	-0.5	0	1.8	0

* The unidentified spot, detected with AgNO₃, was located between the base line and trehalose on paper chromatograms run in phenol:water (4:1 w/v).

and organic acids. Table XI shows that the radioactive label is still principally in mannitol in all samples but there is a greater transfer of label to trehalose with time. Although no specific enzyme sequence for polyol metabolism in eggs of A. ellioti is indicated by available data, it is felt that the degradation of mannitol proceeds principally through glucose or phosphorylated glucose due to the finding that trehalose and polysaccharides contain most of the transferred radioactive label that was observed. This would then support the suggestion that mannitol might furnish some of the carbon source needed for synthesis of embryonic chitin.

Maternal Age and Density Effects on Carbohydrate Partitioned to Eggs and on Egg Weight

Effects on egg weight

Table XII describes 1 to 7 day old eggs of A. ellioti laid throughout the fecund period by parental densities of 1, 3 and 6 pairs per cage. Eggs laid later in the season were about 10 % heavier on a dry weight basis than those laid earlier. The percentage of water appeared to be relatively constant. Fresh and freeze-dried weights of eggs collected from the highest parental density were greater than weights of eggs collected from lower densities during the first four weeks of the fecund period but during the last two weeks eggs from the highest parental density weighed less. Since the weight of eggs from the 3 pair per cage parental density was greater some weeks and less other weeks than those collected from single pairs, the effectiveness of this density in altering

Table XIII

Description of 1 to 7 day old eggs of A. ellioti laid throughout the fecund period by parental densities of 1, 3 and 6 pairs per cage.

Week Laid	1 pair per cage			3 pairs per cage			6 pairs per cage		
	# eggs in sample	Weight/100 eggs fresh wt.(g)	freeze-dried % Water	# eggs in sample	Weight/100 eggs fresh wt.(g)	freeze-dried % Water	# eggs in sample	Weight/100 eggs fresh wt.(g)	freeze-dried % Water
1	100	0.5572	0.2627 52.9	100	0.5572	0.2445 56.1	70	0.5858	0.2757 52.9
2	100	0.5946	0.2707 54.6	100	0.5894	0.2725 53.8	100	0.5986	0.2808 53.1
3	100	0.5916	0.2712 54.2	100	0.5911	0.2650 55.2	100	0.6081	0.2796 54.0
4	100	0.6034	0.2708 55.1	100	0.6100	0.2705 55.7	100	0.6399	0.2866 55.2
5	100	0.6350	0.2812 55.7	100	0.6464	0.2888 55.3	85	0.5780	0.2156 62.7
6	100	0.6382	0.2860 55.2	100	0.6520	0.2930 55.1	80	0.6314	0.2805 55.6

egg weight is not obvious. Figure 9, however, might indicate that the rate of increase in egg weight throughout the fecund period is influenced by the 3 pair per cage as well as the 6 pair per cage parental density. Judging from this figure, the increase in egg weights from different parental densities was in the order of 3 pairs per cage > 1 pair per cage > 6 pairs per cage. It then appears that the most severely crowded parents moderated increased egg weight through the fecund period and that less crowded parents had the opposite effect. Due to relatively small differences between the weight of eggs from more or less crowded parental conditions, the inexplicability of one much lower egg sample weight and, since only one sample replication was used, it is not certain whether these differences are real or only apparent.

Effects on carbohydrates partitioned to eggs

Trehalose, mannose, glucose and glycerol were identified in the neutral 70 % ethanolic extracts of newly laid eggs as previously indicated. Mannose was present in quantities from 5 - 7 $\mu\text{g}/\text{egg}$, glucose in quantities of only about 1 - 2 $\mu\text{g}/\text{egg}$ and the greatest amount of glycerol found was 4.4 $\mu\text{g}/\text{egg}$ (Table XIII). Glycerol was not detected on chromatograms during the first three weeks of the oviposition period but was found to be present in quantities of less than 1.1 $\mu\text{g}/\text{egg}$ using the colorimetric procedure described earlier. Trehalose comprised from 86.4 to 91.2 % of the free neutral sugar fraction for the first four weeks. During weeks five and six of the oviposition period, 81.2 to

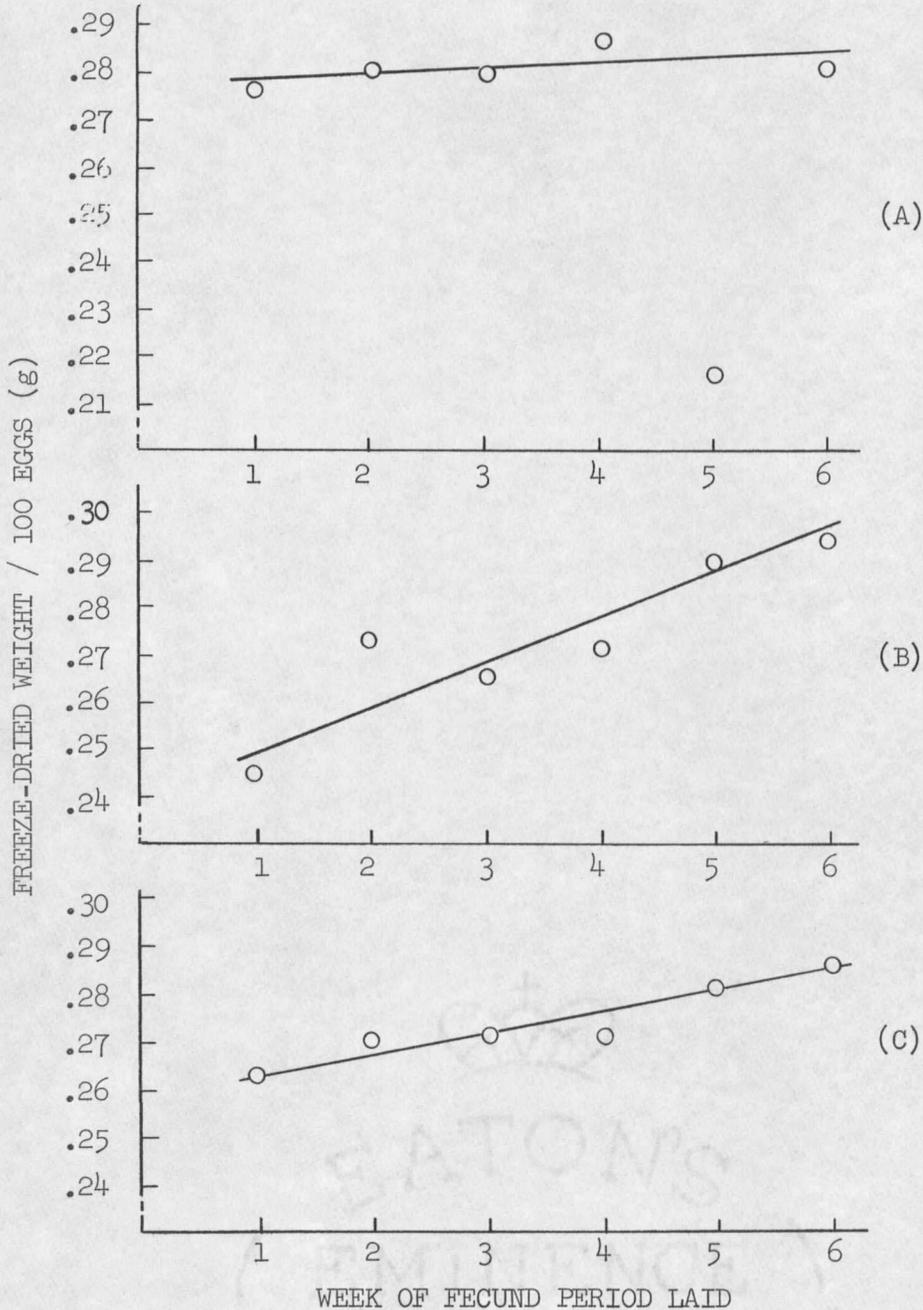


Figure 9. Weight of 1 to 7 day old *Aulocara elliotti* eggs laid throughout the fecund period at parental densities of (A) 6 pairs per cage, (B) 3 pairs per cage and (C) 1 pair per cage.

Table XIII

Carbohydrates in 1 to 7 day old *A. ellioti* eggs laid throughout the reproductive period.

Week Laid	1 pair per cage				3 pairs per cage				6 pairs per cage			
	Treha-lose	Total free sugar	Glyco-gen	Glycer-ol	Treha-lose	Total free sugar	Glyco-gen	Glycer-ol	Treha-lose	Total free sugar	Glyco-gen	Glycer-ol
1	38.5*	42.5	17.7	0.7	39.5	45.5	20.1	0.8	46.9	51.5	20.6	0.8
	6.90		3.17		7.09		3.60		8.00		3.51	
	1.46		0.67		1.62		0.82		1.70		0.75	
2	45.3	51.5	22.6	1.1	47.1	53.9	22.8	0.9	50.8	56.3	22.0	1.1
	7.62		3.80		7.99		3.87		8.49		3.68	
	1.68		0.84		1.73		0.84		1.81		0.78	
3	47.5	53.8	22.6	0.9	51.0	55.9	21.6	0.9	57.6	63.5	25.4	0.7
	8.02		3.82		8.63		3.66		9.46		4.17	
	1.75		0.83		1.93		0.82		2.06		0.91	
4	43.5	50.0	21.3	1.9	46.6	53.9	20.6	1.2	52.6	59.6	23.0	2.7
	7.20		3.53		7.63		3.38		8.22		3.60	
	1.61		0.79		1.72		0.76		1.84		0.80	
5	33.5	39.9	23.0	2.0	43.7	50.9	25.1	1.4	29.9	36.8	23.8	4.4
	5.28		3.62		6.76		3.88		5.18		4.13	
	1.19		0.82		1.51		0.87		1.39		1.11	
6	35.5	43.6	25.0	2.4	40.8	46.5	23.8	2.3	20.2	26.5	24.3	3.2
	5.57		3.92		6.26		3.65		3.21		3.85	
	1.24		0.88		1.39		0.81		0.72		0.87	

* Units for upper value of carbohydrates in each sample are $\mu\text{g}/\text{egg}$, middle values are in mg/g of fresh egg weight and lower values are in % of dry weight.

87.6 % of the free neutral sugar was trehalose at the lower densities. At the highest density during the sixth week, only 76.3 % of this sugar was trehalose.

It appears that later laid eggs contain more of the carbohydrate measured as glycogen (Figure 10). This, however, is no longer apparent when glycogen content is related to egg weight since eggs laid later in the season were heavier than those laid earlier (Table XIII).

Figure 11 shows that there is a maternal age effect since eggs laid during the first part of the oviposition period contain progressively more trehalose. After the third week of egg laying, the trehalose content then begins to lessen in all three of the densities. In some insects it has been shown that energy reserves decrease markedly with age (Clark and Rockstein, 1964; Rockstein and Srivastava, 1967). The lesser amounts of trehalose observed in eggs during the last half of the oviposition period may be a reflection of this depletion. On the other hand it has been shown that the corpora cardiaca (CC) contain a hormone capable of elevating hemolymph trehalose levels in several insects (Steele, 1961; Bowers and Friedman, 1963; Wiens and Gilbert, 1967; Friedman, 1967). It may be that the variability seen is due to change in titer of CC hormone(s).

It may be seen from Figure 11 that, for the first few weeks of the oviposition period, the trehalose level increases in the newly laid egg as the density of the parents increases. This relationship continues throughout the entire oviposition period at parental densities of 1 and 3

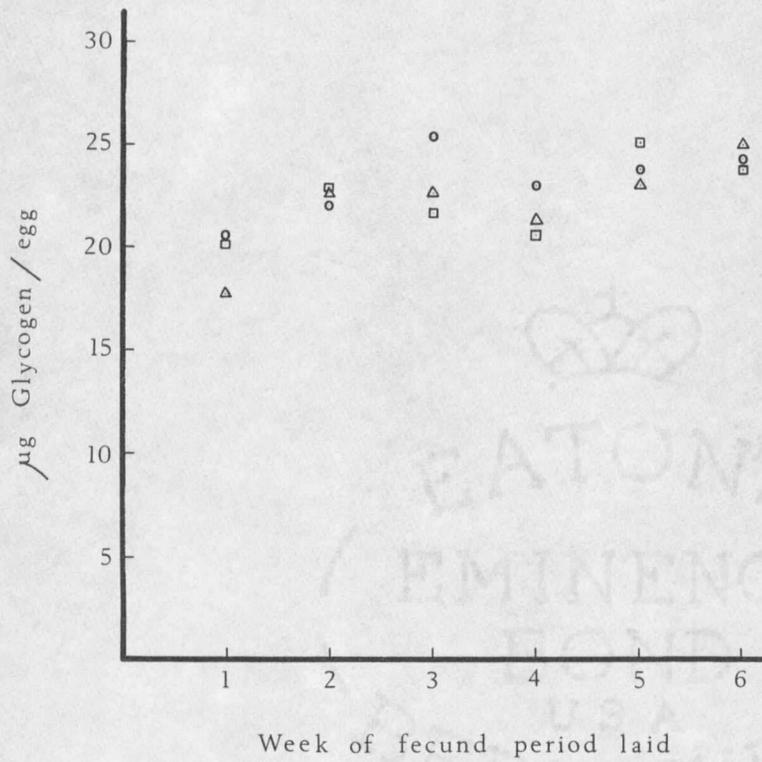


Figure 10. Glycogen in 1 to 7 day old *Aulocara elliotti* eggs laid throughout the fecund period. Eggs were collected from parental densities of 1 pair per cage, Δ ; 3 pairs per cage, \square ; 6 pairs per cage, \circ .

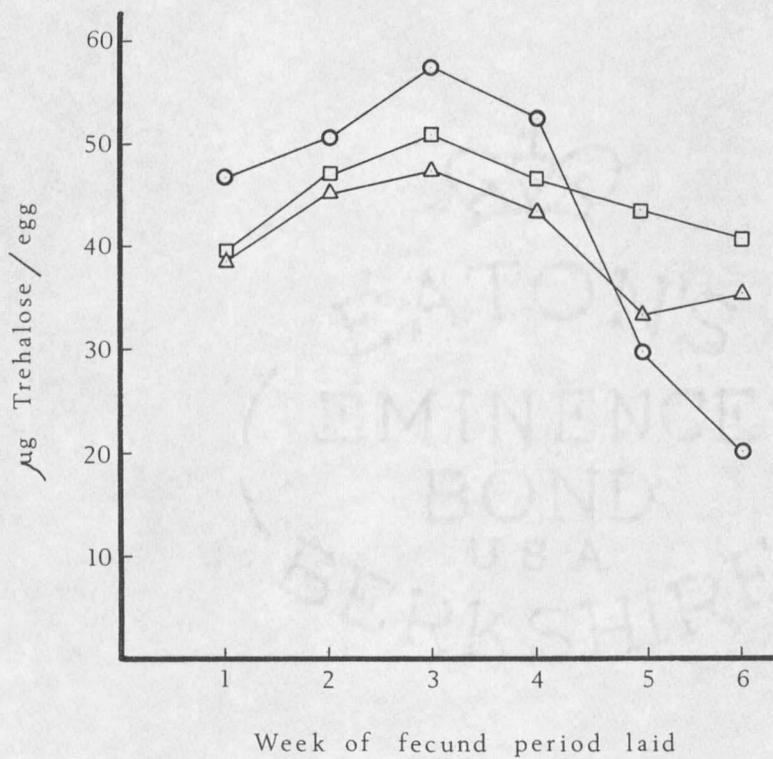


Figure 11. Trehalose in 1 to 7 day old Aulocara ellioti eggs laid throughout the fecund period. Eggs were collected from parental densities of 1 pair per cage, Δ ; 3 pairs per cage, \square ; 6 pairs per cage, \circ .

pairs per cage but does not continue after the fourth week at the 6 pair per cage density. It may be hypothesized that the elevated trehalose levels in eggs result from increased neurosecretion by the CC as a consequence of increased stimulation by a density stress. Hodgson and Geliday (1959) have reported that in the cockroach the CC exhibit a depletion of neurosecretory material following stresses of electrical shocks and forced hyperactivity. Highnam (1961) found that the CC of the desert locust released neurosecretory material in response to these stimuli and copulation.

The pattern of change in trehalose levels in grasshopper eggs at the highest density may be similar to the changes in blood glucose levels in vertebrates that occur during the general adaptation syndrome. This stress syndrome has been hypothesized by Christian (1950) to be involved in the internal control of mammalian population levels. The differences observed in trehalose levels of newly laid eggs at the highest density may be interpreted as evidence that a mechanism similar to that hypothesized for mammalian population control operates in insects. Ewing (1967) has also likened the death from stress observed in the roach, Nauphoeta cinerea, to mammalian deaths induced by the stress syndrome.

It might appear that the rapid decrease in trehalose partitioned to eggs taken from the highest parental density during the last half of the fecund period (9.46 to 3.21 mg/g fresh weight) is due to depletion of energy reserves of the female since high density causes increased

activity. This would seem unlikely, however, since there is no depletion of glycogen reserves partitioned to these eggs (Figure 10). It would seem more likely that the CC might fail to secrete trehalose mobilizing hormone or that the target tissue of the female might fail to respond to increased levels of this hormone. Failure of tissues involved in the stress syndrome of vertebrates to respond is known; wherein adrenal glands do not respond to the pituitary hormone, ACTH, following prolonged stress (Hane et al., 1966) or there is adrenal atrophy (Christian, 1953).

It may be expected that the different levels of trehalose partitioned to eggs throughout the oviposition period would be paralleled by hemolymph trehalose levels in the female. Two hemolymph samples from females that had been maintained at one and six pairs per cage and had survived the sixth week of the oviposition period were analyzed. Trehalose levels were obtained that generally corresponded to those in eggs laid that week. Mannose was not detected in hemolymph and glucose was the principal monosaccharide.

Trehalose levels partitioned to eggs paralleled the mean rate of prediapause development (Table XIV). Eggs collected from eleven single pairs per cage and used by Van Horn (1968) were staged after 30 days of development at 25°C. These grasshoppers were collected at the same time and place as those contributing eggs for the carbohydrate analyses and were maintained identically. No data are available for developmental rates at densities other than one pair per cage in these 1967 studies.

Table XIV

The effect of maternal age on the stage to which embryos of *A. elliotti* develop in 30 days at 25°C, and the trehalose content of eggs at the time of laying.

Staging data from Van Horn (1968) *	Week of oviposition period					
	1	2	3	4	5	6
No. embryos staged	101	98	127	95	97	37
Extremes in daily mean stages	7.0-12.0	11.7-14.0	13.0-14.4	12.1-13.6	11.0-12.4	9.0-11.8
Mean stage	10.8	12.5	13.4	12.5	11.5	10.5
Trehalose content μg trehalose/egg	38.5	45.3	47.5	43.5	33.5	35.5
mg trehalose/g fresh egg weight	6.90	7.62	8.02	7.20	5.28	5.57

* Eggs checked for developmental rate and those subjected to carbohydrate analysis were collected from parental densities of one pair per cage.

In a population studied in 1966, Van Horn (1968) found that the rate of embryonic development was greater when the parental generation was maintained at two pairs per cage. It appears, then, that trehalose levels as affected by either maternal age or density might have some effect on rate of embryonic development. There exists, however, the possibility that hormonal regulators may be differentially incorporated into eggs of A. ellioti and may be directly involved in the rate of development (Van Horn, 1966b). If this is true, data may indirectly indicate that these regulators come from the CC or are under control similar to trehalose.

Since there are no survival data on offspring of this species in relation to maternal age or density and it is not known if differential trehalose (or other energy source) levels persist through the definitive embryo, no conclusions can be drawn as to the significance of sugar level in eggs on population change. Differences in the amount of carbohydrate supplied by the female might be a possible mechanism to partially account for the differential vigor of offspring noted by Wellington (1965).

SUMMARY

The study was conducted in two phases. The first dealt with the qualitative and quantitative changes in carbohydrates during the development of Aulocara ellioti eggs and considered temperature effects; the second with the effects of maternal age and density as reflected in newly laid eggs. The principal findings and conclusions are:

- (1) Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified in neutral 70 % ethanolic extracts taken from eggs.
- (2) As mannose disappeared from the eggs during the last half of pre-diapause development, mannitol made its appearance. The quantity of free mannose was not sufficient to serve as the only precursor for mannitol synthesis but it may be an intermediate and/or a component of the protein-carbohydrate complexes in yolk. Neither trehalose nor glycogen seemed to be an ultimate precursor for mannitol synthesis.
- (3) Cold may have an elevating effect on mannitol but its accumulation does not depend on cold exposure.
- (4) Mannitol was temporally associated with diapause.
- (5) Glycerol appeared to be an extremely variable moiety in developing eggs and was not associated with diapause.
- (6) Neither glycerol nor mannitol levels were sufficiently high to indicate importance in cold-hardiness.
- (7) There appeared to be slight early and late utilization of glycogen but levels were higher in the definitive egg than in the newly laid egg, which is in contrast to the usual observation in insect eggs, and indicated little overall reliance on this moiety as an ultimate source of

energy.

(8) There is evidence that energy for blastokinesis in A. ellioti may not come from mannitol, trehalose or glycogen.

(9) Trehalose was generally $91 \pm 5\%$ of the free neutral sugar in all ethanolic extracts.

(10) The large increase in trehalose content during diapause, indicating that this is not a quiescent state with respect to carbohydrate metabolism in A. ellioti, is thought to result from continued hydrolysis of the protein-carbohydrate complexes in yolk coupled with reduced energy requirements during diapause.

(11) It appears that trehalose may be synthesized in the embryonic fat body.

(12) Trehalose and mannitol may serve as carbon sources for chitin synthesis and/or may serve as an energy source following resumption of increased mitotic activity.

(13) Membrane structures dividing the egg into compartments appear to be permeable to some but not all carbohydrate that was measured in developing eggs. If conclusions 10 and 11 are correct it appears that the membranes enveloping the embryo must retain permeability to material needed for trehalose synthesis during diapause.

(14) Higher percentages of the egg trehalose and glycogen were found in the embryo following rupture of membranes at blastokinesis and may be the result of more favorable temperature and pH conditions, hydration of the embryo and easier transfer of material from yolk to embryo as well as

conversion of some trehalose to glycogen.

(15) Conversion of glucose to mannitol and trehalose may occur at all times these moieties are detected in A. ellioti eggs. There is also a pathway for conversion of mannitol to trehalose and "glycogen" as the mannitol disappears from the eggs during post-diapause development even though neither of the two latter compounds accumulate at this time.

(16) Eggs laid later in the fecund period were found to be heavier and contained more glycogen than eggs laid earlier.

(17) Crowding at the parental densities imposed in this study had no noticeable effect on glycogen content in eggs.

(18) The greater the parental density, the greater the amount of trehalose that was partitioned to eggs during the first two-thirds of the fecund period. This effect is attributed to differential stimulation of the maternal brain-corpora cardiaca axis imposed by crowding.

(19) The trehalose content of newly laid eggs collected from three different parental densities throughout the fecund period followed an inverted U-shaped curve. This change is attributed to a parental age effect and may be due to change in titer of corpora cardiaca hormone(s) in the female.

(20) During the last half of the fecund period, the amount of trehalose partitioned to eggs decreased to about 35 % of peak levels in eggs obtained from the greatest parental density and was probably due to the combined effect of crowding and maternal age. Possibilities suggested to explain this observation were: (a) more severe depletion of maternal

food reserves at the highest density and/or (b) failure of the maternal brain-corpora cardiaca axis or failure of target tissues to respond.

(21) It is suggested that factor(s) responsible for prediapause growth rates and perhaps differentially incorporated in yolk may be under the same control as factor(s) responsible for trehalose mobilization and incorporation in yolk.

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