PHOTOPERIODIC INDUCTION OF PUPAL DIAPAUSE IN THE FLESH-FLY *Sarcophaga argyrostoma*

by

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To My Family,

for their love and encouragement

throughout my studies.

I hereby declare that this thesis was composed by myself, and that the work it contains is my own.

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ABSTRACT

1. Sarcophaga argyrostoma has a temperature modified photoperiodic response curve typical of a long day species. In the present study both the intrauterine embryos and larvae were exposed to the same photoperiod and temperature, resulting in an increase in the incidence of pupal diapause and the value of the critical daylength when compared to curves in which only the larval stage had experienced inductive photoperiods.
2. S. argyrostoma is thought to possess a temperature compensated photoperiodic 'counter' which adds up successive short days (or long nights), the final response being a consequence of the number of diapause inductive cycles that the insect experiences during the restricted photoperiodically sensitive period (Saunders, 1971). Gibbs's (1975) model for the photoperiodic counter was tested by altering the length of the sensitive period using methods other than temperature. Manipulations such as prevention of larviposition or starvation of early larval instars tended to increase the length of the larval period and resulted in an increase in the incidence of diapause. On the otherhand, crowding larvae during the feeding phase or the premature extraction of larvae from the food decreased the

length of the larval period and resulted in a decrease in diapause incidence. Unexpectedly, an increase in the post feeding phase, caused by crowding larvae during this stage, was associated with a decrease in diapause incidence. Photoperiodic sensitivity was shown to decline from a maximum during the late embryonic stage through to the mature feeding larvae and ceased shortly after the onset of the wandering stage. These results are discussed in relation to Gibbs's model, and possible refinements to the model are suggested.

3. Two artificially selected strains were produced from a stock culture of S. argyrostoma by breeding from the first and last individuals to form puparia; these strains were named 'fast' and 'slow' respectively. The behaviour of the strains and hybrids produced by interstrain crosses were investigated under a range of photoperiods and temperatures, the results suggesting that both fast and slow strains were highly polygenic. The strains were found to differ only in the duration of the post feeding stage; selection had not affected the duration of any other fraction of the life cycle, although the critical photoperiod of the fast strain appeared to have shortened by 1 hour compared to

that of stock and slow. In conditions of low temperature and short daylengths puparium formation in the 'fast' strain was highly synchronised whilst that of the 'slow' strain was characterised by a long negative skew. Since 'fast' and 'slow' produced individuals with widely differing larval periods, they provided a useful tool to investigate the summation of photoperiodic cycles in S. argyrostoma, and data from the behaviour of the strains in diapause-inductive cycles was consistant with the 'refined' version of Gibbs's model.

4. Low temperature is not a prerequisite for diapause termination in S. argyrostoma; spontaneous termination of diapause can occur at constant temperatures of 20°C or 25°C. In this study, it was demonstrated that diapause termination itself is temperature independant. The rate of diapause termination was also similar in both sexes, and was independant of both the larval (diapause inductive) photoperiod and the degree of diapause in the group from which the diapause pupae were drawn. Results from preliminary experiments suggested that male and female flies possess different thresholds for diapause induction.

5. Pupal diapause in S. argyrostoma is a result of the inactivation of the brain-prothoracic gland system, ultimately resulting in the absence of the peak of ecdysone usually associated with pharate adult development. The levels of ecdysteroids in the haemolymph of diapause destined and non-diapause destined larvae and prepupae were measured, using a radioimmunoassay technique. The results failed to support the hypothesis that the temporary cessation of endocrine activity was a result of a negative feedback system involving ecdysteroids and the PTTH neurosecretory cells.

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GENERAL INTRODUCTION

The annual climatic variation of the seasons is most marked in regions of high latitudes. This variation may produce periods of adverse environmental conditions (cold winters and arid summers) which are unfavourable for the continuous development of some animals, particularly poikilotherms. In such regions the possession of a definite dormant stage within the lifecycle of a species is an important adaptation for survival.

Dormancy may occur in the form of simple quiescence or as a diapause. The term diapause (= rest; interruption of work) was first used by Wheeler (1869) to describe a specific stage in the embryogenesis of a species of grasshopper. Henneguy (1904) redefined diapause as a condition of arrested growth in any development stage of an organism, thus including quiescence in his classification. In 1929, Shelford restricted the term 'diapause' to cases in which development was arrested 'spontaneously' and used the term quiescence for cases where the interruption of growth was in direct response to unfavourable conditions. A similar classification of types of dormancy was proposed by Robaud (1930) who used the terms 'diapause vrai' (true diapause) and 'pseudo diapause' (quiescence). Today quiescence can

be defined as a passive state of dormancy which is a direct or immediate response to adverse physical conditions such as cold or desiccation, and is terminated as soon as these conditions are removed. For example, the chironomid Polypedilum vanderplanki breeds in ephemeral aquatic habitats; in the dry season, the larvae became totally dehydrated but can remain viable for many years in this state and will recover immediately on immersion in water (Hinton, 1951).

Diapause is an active state that is induced by factors which themselves are not adverse but signal the approach of unfavourable weather conditions. Diapause is often terminated long after the adverse weather conditions have disappeared, i.e., diapause is controlled endogenously rather than exogenously and is regulated by internal physiological events.

Diapause was originally divided into two categories: obligatory diapause and faculatative diapause. The former type was considered to occur in univoltine species, in which every individual in every generation apparently underwent diapause as part of it's life history. In some examples, the start of diapause induction was considered to be genetically determined and completely independant of environmental conditions e.g., the spruce budworm Choristoneura fumiferana (Harvey, 1957). Facultative diapause, on

the other hand, was usually found in multivoltine species in which the onset of diapause was directly controlled by environmental conditions. Thus there was the potential for one or more generations each year to undergo continuous development and bypass the diapause stage, e.g., the flesh fly Sarcophaga argyrostoma (Fraenkel and Hsiao, 1968a).

Muller (1970) reclassified the above diapause classes, an English translation of this classification being given by Thiele (1973). Muller, as before, differentiated quiescence from diapause and then divided diapause into three classes: parapause, eudiapause and oligopause. Parapause was equivalent to obligatory diapause which is described above. The terms eudiapause and oligopause divided the facultative type of diapause into two classes. In species with an eudiapause, the diapause is induced by one factor (e.g. photoperiod) but can be terminated by changes in the level of a different factor (e.g., temperature). This type of diapause is found in Sarcophaga argyrostoma. In oligopause, induction and termination are under the control of the same factor e.g. photoperiod. However, the division of diapause into eudiapause and oligopause may not be valid, since, on further investigation temperature has been found to influence diapause termination in many insects in which termination had been considered to be

entirely under photoperiodic control, e.g. Antheraea pernyi (Mansigh and Smallman, 1971). The factors influencing diapause termination are discussed in more detail on page 41 in this thesis.

STAGE SPECIFICITY OF DIAPAUSE

Diapause can occur at almost any developmental stage of an insect. In the egg, larval, nymph or pupal instar, it takes the form of an arrest of development. If in the adult, there is usually a reproductive diapause, i.e., a failure of the reproductive system to develop fully. However, within each species diapause is usually restricted to a specific stage, e.g. Sarcophaga argyrostoma will only diapause as a pupa, never as a larva or adult (Fraenkel and Hsiao, 1968a). Saunders (1982) gives examples of over three hundred insect species in which diapause has been demonstrated, and lists the developmental stage at which diapause occurs. The stage of diapause correlates poorly with taxonomic groupings at family level and above. However, within the Lepidoptera there are few examples of egg diapause e.g. Bombyx mori (Kogure, 1933) or adult diapause e.g. Danus plexippus (Herman, 1973), the majority of Lepidopterans diapausing either as larvae or pupae. Those that diapause as mature larvae include Ostrinia

nubilalis (Beck and Hanec, 1960), Diatraea saccharalis (Chippendale and Reddy, 1973), Pectinophora gossypielle (Adkisson et al. 1963) and Ephestia elutella (Bell, 1976). Examples of pupal diapause include Pieris brassicae (Way et al. 1949) Manduca sexta (Rabb, 1966), Antheraea pernyi (Williams and Adkisson, 1964) and Mamestra brassicae (Masaki, 1968).

Within the order Diptera, the few examples of egg diapause are mainly found in the genus Aedes, e.g. Aedes togoi (Vinogradova, 1965). However there are large numbers of dipteran species which diapause as larvae, Lucilia species (Cragg and Cole, 1955; Ring, 1967), Calliphora vicina (Vinogradova and Zinovjeva, 1972), Wyeomyia smithii (Bradshaw and Lounibos, 1972), Tipula pagana (Butterfield, 1976) and Drosophila deflexa (Basden, 1954), or as adults: Anopheles freeborni (Depner and Harwood, 1966), the majority of the Drosophilidae (Lumme, 1977), Phormia regina (Stoffolano et al., 1974). There are fewer reported examples of pupal diapause, but it is still quite common; e.g. it is known in one species of Drosophila, D. alpina (Lumme, 1977), in the horn fly, Lyperosia irritans (Depner, 1962), the cabbage fly Delia brassicae (Read, 1969) and in all species of Sarcophagidae which have been investigated (Fraenkel and Hsiao 1968a; Denlinger, 1971, 1979; Ohtaki and Takahashi 1972; Roberts and Warren 1975;

Vinogradova, 1976).

In the orders Hymenoptera, Homoptera, Heteroptera, Orthoptera, Odonata and Neuroptera there are examples of every type of diapause within each order. Insect species which have been extensively investigated include the aphid Megoura viciae (egg diapause) (Lees, 1959), Pyrrhocoris apterus (adult diapause) (Hodek, 1968) and Nasonia vitripennis (larval diapause) (Saunders, 1965a).

The order Coleoptera is an exception: nearly all species diapause as adults. Examples include the Colorado potato beetle Leptinotarsa decemlineata (deWilde, 1954), the carabid Pterostichus nigrita (Thiele, 1971) and the weevils Hypera brunneipennis (Madubunyi and Koehler, 1970), Hylobius pales and Pachylobius picivorus (Taylor et al., 1975). There are very few examples of larval diapause among the beetles and no known examples of diapause in the pupa or the egg.

Although diapause is usually stage specific within a species there are exceptions, e.g., the spruce budworm Choristoneura fumiferana, can enter diapause as a first instar larva or as a fourth instar larva (Harvey, 196), the mosquito Wyeomyia smithii as a third or fourth instar larva (Lounibos and Bradshaw, 1975), and the pine moth Dendrolimus pini in almost any larval stage (Geispitz, 1965).

Most individuals will only enter diapause once within a lifecycle. Again, however, there are exceptions. In some long-lived species, e.g., the pine moth Dendrolimus pini (Geispitz, 1965), most individuals diapause twice at different stages in their two year life cycle. In the alfalfa weevil Hypera brunneipennis some females lived for over three years and underwent three successive diapauses (Madubunyi and Koehler, 1970; Litsinger and Apple, 1973). An insect may also diapause several times at the same stage. Larvae of the wheat stem sawfly Cephus cinctus, enter an obligatory summer diapause, but if the temperature is too high, the larvae will enter a second facultative diapause after termination of the first. This is an example of a species which has both an obligatory and facultative diapause (Villocorta et al., 1972).

PHYSIOLOGICAL ADAPTATIONS OF THE DIAPAUSE STATE

It is well known that the energy requirements for growth are much greater than requirements for tissue maintenance - an insect in diapause will automatically have a lower metabolic rate than a developing insect of a comparable stage. Survival during the months of diapause necessitates economical utilisation of food reserves, hence it is important to have as low a

metabolic rate as possible and to have a large fat reserve.

An increase in the fat reserves of diapause destined insects have been reported many times; e.g. in larvae of Ostrinia nubilatis (Beck and Hanec, 1960), Pectinophora gossyiella (Squire, 1940; Bull and Adkisson, 1962; Adkisson et al., 1963), Diatraea grandiosella (Chippendale and Reddy, 1972), Lucilia sericata (Mellanby, 1938) and Gasterophilus intestinalis (Levenbook, 1951). An increase in fat has also been reported in the adult diapause of the weevil Hypera brunneipennis (Manubunnyi and Koehler, 1970) and the mosquito Culiseta momata (Barnard and Mulla, 1970).

The low metabolic rate can be measured by oxygen consumption. Low rates of consumption are most strikingly seen in pupal diapause, since the pupa has neither the cost of locomotion nor feeding which occurs in larval or adult diapauses. Squire (1936) reported that the respiration rate of diapausing pupae of Papilo archisiades dropped to almost zero. Likewise, diapausing larvae of the pink bollworm Pectinophora gossypiella have a slower heart beat and consume oxygen at one sixth of the rate of non-diapausing larvae (Adkisson et al., 1963). Denlinger et al. (1972) carried out a more quantitative investigation into the oxygen consumption of Sarcophaga argyrostoma.

The oxygen uptake of a pupa in diapause is 10% of the minimum oxygen consumption seen in a non-diapause pupa just before adult differentiation starts (10-20 mm/g/h compared with 140-150 mm/g/h). They also reported infradian cycles of oxygen consumption: in Sarcophaga argyrostoma, peaks occurred every three to five days, in S. bullata every five to ten days, and in S. crassipalpis the cycles peaked every four to six days. In the latter the periodicity of the oxygen cycles changed during the course of diapause, becoming further apart as diapause progressed and then closer together nearer the onset of diapause termination. The authors postulated that these rhythms might reflect a pattern of a particular method of energy utilisation. For example, metabolic end products might not be utilised continually and then accumulated to critical levels before being oxidised. A lower respiration rate was also reported in the larval diapause of Ostrinia nubilalis; here the oxygen consumption of larvae in diapause was only 35% of that of non-diapausing animals (Beck and Hanec, 1960). Unlike Sarcophaga species, however, they found no evidence of cyclic oxygen uptake in O. nubilalis: respiration was continuous. Reduced levels of respiration during adult diapause were also reported in the weevils Hylobius pales and Pachylonius picivorus (Taylor et al., 1975).

The rate of respiration is clearly not static during diapause and an insect can respond to an emergency, e.g., wound repair. Deliberate injury to a diapausing pupa of S. argyrostoma resulted in a gradual increase in respiration which remained elevated until diapause development was completed (Denlinger et al., 1972). Beck and Hanec (1960) similarly injured the diapausing larvae of Ostrinia nubilalis; this also resulted in an increase in the metabolic rate and like S. argyrostoma, the rate remained elevated until diapause termination. This demonstrated that in both species there was a safety margin of potentially useable reserves.

To summarise: an insect in diapause possesses both a high food reserve and a low metabolic rate.

COLD HARDINESS AND DIAPAUSE

Induction of diapause does not necessarily result in the induction of cold tolerance in the majority of temperate zone insects. Hanec and Beck (1960) defined 'cold hardiness' as the ability to survive prolonged exposure to subzero temperatures. They found that diapause larvae of Ostrinia nubilalis collected from the field in August and September showed little resistance to cold, whereas those collected in November had dramatically raised levels of cold hardiness. The authors concluded that diapause may

serve as a period during which the insect undergoes a physiological cold hardening, but neither the conditions that induce diapause nor diapause itself produce the phenomenon.

In a wide range of insects and mites, several authors have found that cold hardiness and the ability to survive supercooling are facilitated by an increase in glycerol levels in the haemolymph. Glycerol acts as an antifreeze. The enzyme that converts glycogen to glycerol is activated by low temperatures in Bombyx mori (Zieger and Wyatt, 1975). Young and Block (1980) investigated cold tolerance in the mite Alaskozetes antarcticus. They found an inverse linear relationship between glycerol concentration and supercooling ability, likewise, low temperature or desiccation stimulated glycerol production. A similar relationship between glycerol and cold hardiness has been reported in an Alaskan carabid beetle Pterostichus brevicornis (Baust and Muller, 1970) and the moth Anagasta kuehniella (Somme, 1966).

Duman (1979) found factors in the haemolymph of several overwintering beetle species which caused thermal hysteresis (= difference between freezing and melting points). The possession of these thermal hysteresis factors (T.H.F.) is yet another adaptation to low temperature. In the beetle Dendroides

canadensis, seasonal changes in day length were responsible for controlling the levels of T.H.F. in the haemolymph. Larvae collected in early autumn and transferred to LD8:16, 20°C, showed nearly a three fold increase in thermal hysteresis. Larvae kept in LD16:8 did not differ from those obtained on the day of collection. Using resonance experiments (see p 32) the authors demonstrated that the photoperiodic timing mechanism was responsible for the increased T.H.F. levels in autumn and that this was somehow causally related to the circadian system (Horwath and Duman, 1982). Low temperature or short photoperiods also induced T.H.F. in the haemolymph of Tenebrio molitor larvae (Patterson and Duman, 1978).

In several species, cold hardiness, i.e., glycerol production, seems to be related directly to diapause. In mature larvae of the rice stem borer Chilo suppressalis glycerol production was stimulated in non diapausing larvae at low temperatures (10°C). However, the levels of glycerol in the haemolymph of diapausing larvae under short day conditions at 25°C was much higher than non diapausing larvae under long day photoperiods at low temperatures. Once diapause was terminated by exposure to low temperatures, glycerol concentration in the haemolymph decreased (Tsumukii and Kanehisa, 1980). Likewise, glycerol levels increased immediately without a period of

chilling in diapausing eggs of Bombyx mori (Chino, 1957), and development of cold hardiness appeared to be part of the general diapause physiology in mature larvae of the woolly bear Isia isabella (Mansingh and Smallman, 1972).

The ability to survive supercooling in some species may be a consequence of diapause physiology. Many insects in diapause have a decreased water content and this partial desiccation is associated with survival at very low temperatures. For example, the water content of larvae of the viceroy butterfly Limenitis archippus, decreased from 80% to 50% (Frankos and Platt, 1976). Similar decreases were found in Diatraea grandiosella (Chippendale and Reddy, 1972) and Ostrinia nubilalis (Beck and Hanec, 1960).

Salt (1953) hypothesised that in some species, cold hardiness might be a consequence of not feeding during diapause. He found that non feeding premoult or freshly moulted larvae of Agrotis orthogonia and Ephestia kuhniella survived supercooling more successfully than feeding larvae - probably because food remnants in the gut made insects more susceptible to freezing. However, this view was not supported by Beck and Hanec (1960) since, early, but non feeding diapausing larvae of Ostrinia nubilalis were not cold hardy.

In summary, in the majority of insect species examined, the relationship between cold hardiness and diapause is not causal, but results in the concurrent timing of the two processes.

ECOLOGICAL ADAPTATION OF THE DIAPAUSE STATE

Diapause is an adaptation that enables an insect species to persist in regions where seasons of adverse climatic conditions occur. In addition to this role, diapause may also act to synchronise individual life cycles within the population, thereby increasing the probability of mating, and synchronising feeding or reproduction to the availability of food plants and suitable oviposition sites.

Danilevskii (1965) emphasised the role of diapause in the regulation of a lifecycle, and outlined how this might occur, using a hypothetical example of a multivoltine insect with a winter diapause: The rate of development of non diapausing individuals during the spring and summer would inevitably be different due to variation in the climate of microhabitats. This asynchronisation would increase with successive generations so that by the end of summer season, the species would be found in all stages of the life cycle. The switch from continuous development to diapause would occur in

advance of adverse weather conditions, and as the photoperiod decreased, more and more insects within the population would enter diapause, so that by autumn the majority of the population would become dormant. Once the temperature fell below the threshold for active development, diapause development or reactivation would be initiated (see p.41). The proportion of completely reactivated insects would then increase gradually during the winter. However, these individuals would not be able to complete development, i.e., pupate, or feed or start adult differentiation until the temperature rose above the developmental threshold; development would then proceed at a uniform rate.

Denlinger (1971) studied the seasonal phenology of pupal diapause in the flesh-fly Sarcophaga bullata, illustrating the regulatory role of diapause in nature. Adult flies emerged in May and were kept in an outdoor insectary. In S. bullata, the critical photoperiod (see p. 18) is between 13-14 hours of light per 24 hour cycle, which at 40^{o}N is reached by the beginning of September. In July, Denlinger observed a 2% incidence of pupal diapause in progeny from these flies, this rose to 20% in August and by September virtually all progeny had entered diapause. By transferring diapausing pupae from the outside insectary to a room kept at 25^{o}C, Denlinger found that

diapausing pupae formed in September completed diapause development by the beginning of January. Cold temperatures during January, February and March, however, prevented further development. Thus the first signs of adult differentiation were delayed until early April. The median date of emergency (May 11th) was the same for all pupae regardless of when they had entered diapause. Thus although the timing of the completion of diapause development varied, the suppression of adult development by temperature provided the synchronisation mechanism for uniform development of pupae of various ages.

Denlinger (1971) also transferred laboratory bred flies with no diapause history to the outside insectary in the middle of April. These flies survived and produced progeny with a high incidence of diapause, induced by an interaction of the still diapause inductive short photoperiod and low temperatures. However, this problem does not occur in nature: Henrich and Denlinger (1982) have since demonstrated that there is a maternal inhibition of diapause in S. bullata, the progency of the over-wintering generation thus avoiding diapause even under short daylengths and low temperatures. This problem is re-examined for Sarcophaga argyrostoma in this thesis (Chapter IV).

PHOTOPERIODIC REGULATION OF DIAPAUSE

The length of day or night changes systematically with the time of year and latitude. Extreme seasonal fluctuations in photoperiod are found at high latitudes, whilst, on or close to the equator, the photoperiod remains close to LD12:12 at all times of year. In the regions of high latitudes, photoperiod is a precise and reliable indicator of the seasons and has many advantages over the highly variable seasonal changes in temperature, humidity and food resources.

Light and temperature regimes vary in opposite direction with latitude. With increasing latitudes, summer daylength increases, whilst mean summer temperatures decline. In addition, Bradshaw (1976) found the length of the growing season, which he arbitrarily defined as the mean number of frost-free days, decreases with increasing latitudes. Thus in temperate regions, the summer provides less time for active growth and development. This will restrict the number of generations a year and will necessitate an earlier onset of diapause compared to southern populations. However, nearer the equator, the number of generations per year may be limited by summer droughts. Thus a species may possess both a winter and summer diapause, if it has a large enough range of

distribution. For example, Mamestra brassicae has a winter diapause in the north, but further south, in the sub-tropics, the species has a summer diapause (Masaki, 1980).

The daylength that marks the transition from continuous development to diapause has been called the critical photoperiod. This can be obtained experimentally by exposing groups of insects to a range of photoperiods (holding other factors such as temperature and food constant), and plotting diapause incidence as a function of photoperiod. Such a plot is called a Photoperiodic Response Curve (PPRC). The point on the curve where the diapause incidence rises to 50% is called the critical daylength. The critical daylength is thus the median of a population of individual critical daylengths, and the Photoperiodic Response Curve is a population response.

The shape of the Photoperiodic Response Curve is dependent on whether the species has a winter or summer diapause. A summer active species with a winter diapause will possess a 'long day' response: i.e., continuous development occurs at long photoperiods whilst diapause occurs when daylength falls below the critical (Figure 1). Short day PPRCs on the other hand, are found in those species which aestivate during the summer months and develop and reproduce during the short days of autumn or winter. There are

some species which exhibit PPRCs intermediate between these two types. For example, those species with both a winter and a summer diapause will usually possess two critical photoperiods for diapause induction, e.g., Mamestra brassicae (Masaki, 1980).

The PPRC is usually obtained by subjecting insects to photoperiods ranging from constant light (LL) to constant darkness (DD). Thus a large portion of the PPRC is the insect's response to photoperiods never experienced in nature. The ecologically important part of the PPRC is the region around the critical photoperiod (Danilevskii, 1965; Danilevskii, 1971). This region of the PPRC has been subjected to natural selection such as geographical location, temperature and nutritional factors.

GEOGRAPHICAL LOCATION

The latitudinal effect on the length of the growing season has already been described. In addition, a rise in altitude also results in a reduction of the growing season. The effect of latitude on critical daylength has been investigated in a number of species. Much of the early investigation was reported in Russian: however Danilevskii et al. (1971) have reviewed this early work in English. Their principal finding was that the critical daylengths, at constant temperature, in the mite

Tetranychus urticae and Lepidopterans Acronycta rumicis, Mamestra brassicae, Pieris rapae, and Dendrolimus pini, all vary with latitude. These and other examples led Danilevskii et al. (1970) to propose the empirical rule that the critical day-length in genetic clines lengthens 1 hour to 1 hour 30 minute per 5 degree rise of latitude northwards.

Bradshaw (1976) carried out a thorough investigation of the effect of altitude, latitude and longitude on the critical daylength of the mosquito, Wyeomyia smithii. The larvae of this species develop within the water-filled leaves of the pitcher plant Sarracenia purpurea, and have a facultative winter diapause. Bradshaw collected 22 populations over a range of 19 degrees of latitude, 20 degrees of longitude and 1200 metres altitude. Within these ranges the critical photoperiod was closely correlated with latitude and altitude but not with longitude. A change of 1 degree of latitude and a rise of 142 metres in altitude were equivalent. The effect of latitude was more important however, since it accounted for 80.5% of the variation, whilst altitude accounted for only 15.5%. He also found a strong correlation between the length of the 'growing season' (see p.17) and the critical photoperiod.

Jordan and Bradshaw (1978) also found geographical variation in the photoperiodic response

of the western tree hole mosquito Aedes sierrensis, a species which diapauses in the 4th larval instar. In addition to an increase of 1 hour in the critical photoperiod per 4.8 degrees increase in latitude, the authors found that short day photoperiods became more effective with increasing latitudes: the incidence of diapause increased from 35% at 33 degrees N to 100% at 44 degrees N.

Eertmoed (1978) studied the photoperiodic responses of 15 strains of the psocid Peripsocus quadrifasciatus ranging from latitudes of 31 degrees N to 48 degrees N and altitudes from 9 to 488 metres. This species has an embryonic diapause. He found that the latitudinal and altitudinal effects on the position of the critical daylength within populations were similar to those found by Bradshaw (1976) (i.e., an increase of 15 minutes in the critical photoperiod per 1 degree increase in latitude or 275 metres increase in altitude). The latitudinal effect was more important than that of altitude, the former accounting for 86% of the variation and the latter only 4%.

Similar geographical variation in critical day-length of the induction of the adult reproductive diapause in Drosophila littoralis was found by Lumme and Oikarinen (1977). Lumme (1982) expressed the opinion that this continuous latitudinal cline in critical daylengths was the result of 2 factors which

worked together to stabilise the selection of critical daylengths in any particular area. These were: 1. any individual possessing too long a critical photoperiod for the area would enter diapause whilst conditions were still favourable for reproduction, resulting in reduced 'fitness', and 2. the possession of too short a critical photoperiod would delay the onset of diapause and might result in the death of that individual, and any progeny, in autumn frosts. Taylor (1980) concluded that the 'optimal' switching time precedes the onset of winter by a constant amount of time equal to the age of first reproduction plus the time to produce one generation plus the difference between the sensitive stage and the diapausing stage.

The critical photoperiod or photoperiodic response curve for a population of insects can be modified by a number of other factors - particularly temperature and to a lesser extent diet. Thus all experiments demonstrating geographical effects of the photoperiodic response have been carried out under constant conditions of diet and temperature.

EFFECTS OF DIET ON THE PHOTOPERIODIC RESPONSE

The quality of diet may influence the photoperiodic response. Under short day conditions, the incidence of diapause in larvae of the pink bollworm Pectinophora gossypiella increased with the fat

content of the diet (Adkisson, 1961; Bull and Adkisson, 1962). Thus, in the field, a greater number of diapausing larvae were found in mature cotton bolls with high fat content than in immature green bolls with lower fat content (Bull and Adkisson, 1962). Likewise, in the boll weevil Anthonomus grandis, a higher incidence of diapause occurred in adults feeding on mature as opposed to immature cotton, and the incidence of diapause in the mite Panonychus uimi increased when the mites were fed on senescent leaves (Lees, 1955).

In the wasp, Nasonia vitripennis, which parasitises pupae and pharate adults of Cyclorraphous Diptera, the host species offered to the female for feeding and oviposition influences the incidence of diapause in her progeny. For example, when female wasps were given pupae of Calliphora vicina or Phormia terraenovae, the incidence of diapause in the resulting 4th instar larvae was 30.6%, whilst if pupae of Sarcophaga argyrostoma were offered as hosts, the diapause incidence increased to 64.7%. This effect is maternal, and probably due to the qualitative differences associated with the haemolymph of the different species of host on which the wasps feed (Saunders, 1971). Vinogradova and Zinovjeva (1972) looked at the photoperiodic responses of Alysia manducata, a braconid parasite of blowfly pupae,

in relation to that of its host, Parasarcophaga similis. They showed that although photoperiod played a leading role in diapause induction the physiological state of the host influenced the proportion of parasites entering diapause: when the diapause incidence in the host pupae was 0% the incidence of diapause in the parasite was 15%. However, if 50% of the host pupae were in diapause, the incidence of diapause in the parasite rose to 43%.

TEMPERATURE EFFECTS OF DIAPAUSE INDUCTION

The effect of temperature on photoperiodic responses is highly complex and variable, depending on whether it is experienced as a temperature cycle (= thermoperiod), or as a constant temperature.

In some insects, 'short day' thermoperiods themselves are capable of inducing diapause in the absence of light, e.g. Nasonia vitripennis (Saunders, 1973); on the other hand, thermoperiods in addition to photoperiod can enhance the effect of short day-lengths on the diapause response. This type of response to temperature cycles was found in Diatraea grandiosella (Chippendale et al., 1976). Maximum diapause incidence occurred when the low phase of a $30^{o}C$:$23^{o}C$ thermoperiod coincided with a scotophil (dark phase) of between 10 to 14 hours duration (long

night). Similar thermoperiodic effects have been found in Pectinophora gossypiella (Bull and Adkisson, 1960; Adkisson et al., 1963), Ostrinia nubilalis (Beck, 1962) and Anthonomus grandis (Cobb and Bass, 1960; Lloyd et al., 1967).

Constant temperature can have two effects: a) shift the critical value, and b) change the degree of the diapause response. Danilevskii et al. (1971) cite four examples in which critical daylength varies with temperature. In Leptinotarsa decemlineata and Chloridea obsoleta the critical daylength increased by 2 hours for each 5°C fall in temperature; and in Acronycta rumicis there was a 1.25h increase in the critical value for a similar decrease. The most extreme example was found in Leucoma salicis in which the critical daylength increased from 12 hours to 19 hours when the temperature decreased from 23°C to 18°C. In contrast, the critical photoperiod in Manduca sexta increased by only 15 minutes with a decrease from 30°C to 26°C (Bell et al., 1975), and Ostrinia nubilalis showed an increase of only 30 minutes over a temperature range of 29°C to 19°C (Beck and Hanec, 1960); these examples are often cited as examples of the temperature compensation of the critical value. The critical photoperiod is also temperature compensated over a wide range of temperatures in Pieris brassicae (Danilevskii, 1965).

The critical photoperiod of Sarcophaca argyrostoma also remains constant up to 18°C; above this temperature the diapause response is too low to measure a critical photoperiod (Saunders, 1971).

In insects with a winter type of diapause, the maximum diapause incidence in laboratory conditions occurs under short days and low temperatures. In strong short days, an increase in temperature can lower the diapause response whereas a decrease in temperature can raise it. For example, in larval cultures of Sarcophaga argyrostoma held at LD10:14 all pupae entered diapause at 15°C and 18°C, only 70% were in diapause at 20°C, whilst at 25°C the diapause incidence was reduced to 10% (Saunders, 1971). A similar decrease in the diapause response with increasing temperature occurred in Drosophila littoralis (Lumme et al., 1974) and high temperature completely nullified the photoperiodic control of diapause induction in the Saurniids, Antheraea pernyi and Antheraea polyphemus (Mansingh and Smallman, 1971).

In many insects, the diapause response remains saturated over a wide range of temperatures, e.g., the incidence of pupal diapause in the cabbage white butterfly Pieris brassicae remained high from 12°C to 26°C, but at 29°C the photoperiodic response disappeared (Danilevskii, 1965). Likewise, the incidence of diapause in the mosquito Aedes atropalous

was only affected by extremely high temperatures: up to 27°C and under short day photoperiods, there was a 100% incidence of diapause, but at 32°C there was a sharp decline in the response (Anderson, 1968). In Ostrina nubilalis, 100% diapause occurred at photoperiods ranging from LD 9:15 to LD15.5:8.5 at 20°C. 'Temperature compensation' of the diapause response only occurred in photoperiods between LD10.5:13.5 to LD13.5:10.5; outside this range, the incidence of diapause was inversely proportional to the rearing temperature. For example, at LD15:9 and LD9:15 at 29°C, the diapause incidence was 24.2% and 11.3% respectively, whilst at the same temperature the incidence of diapause was still saturated under the photoperiod of LD13:11 (Beck and Hanec, 1960).

Temperature may also affect the rate of diapause reactivation: this is reviewed on pp. 41-44.

In summary, although photoperiod is clearly the main environmental factor for the induction of facultative diapause, the response can be modified by less reliable factors such as temperature and diet.

TIME MEASUREMENT IN INSECT PHOTOPERIODISM

The critical daylength effecting diapause induction is often very sharply defined, i.e., the photoperiodic response curve is very steep around the

critical daylength and a small change in the photoperiod of only 15 minutes can effect a developmental switch from non diapause to diapause (Lees, 1963; Williams and Adkisson, 1964; Eertmoed, 1978). This implies the existence of some kind of mechanism that can accurately measure day or night length. One of the central questions in the study of photoperiodism is how this time measurement is accomplished.

Many models have been devised to explain photoperiodic time measurement. These fall into two basic categories: (1) hour glass or interval timer, and (2) those involving one or more circadian oscillators.

The hour glass mechanism was favoured by early workers who envisaged a temperature compensated interval timer that could be started by dusk and stopped by dawn, or vice versa, an essential factor or substance being synthesised or accumulated during one component of the light cycle and degraded or removed during the other phase. The amount of the substance accumulated would be compared to some internal threshold corresponding to a critical night length. For example, if the threshold concentration had not been reached by dawn, the light cycle might be 'read' as a short night (= long day), whereas if the threshold concentration was exceeded the light might be read as a long night (= short day). Hour glass types of clocks have been implicated in time

measurement in the aphid, Megoura viciae (Lees, 1966) and within many Lepidoptera e.g., Ostrinia nubilalis (Bowen and Skopik, 1976). An hour glass mechanism can only execute one cycle of time measurement and would require to be reset by another period of illumination.

The second type of model was originally proposed by Bunning in 1936, but was not seriously considered as an alternative to the hour glass in the entomological literature until 1960 when Bunning suggested that daily (i.e., circadian) rhythms might be causally involved in photoperiodic induction. He envisaged a model in which the 24 hour light cycle was divided into approximately two half cycles differing in their sensitivity to light, one half he termed photophilic (light loving) and the other scotophilic (dark loving). Under a long day photoperiodic regime the light component of the cycle would extend into the scotophil and would be 'perceived' as a long day, whereas in a short day, light would be restricted to the photophil.

A more refined version of Bunning's hypothesis, known as the external coincidence model, was devised by Pittendrigh and coworkers (Pittendrigh, 1960; Pittendrigh and Minis, 1964). This model was based on a study of the phenomenon of entrainment of the pupal eclosion rhythm in Drosophila pseudoobscura (Pittendrigh, 1966) and differed from that of Bunning's

in that the entire light sensitive scotophase was replaced by a temporally restricted photoperiodic inducible phase, denoted by $\emptyset_i$. The original model suggested that a single circadian oscillator was involved in photoperiodic induction and that the photoinducible phase ($\emptyset_i$) occurred at a specific phase or point in the oscillation. The natural period (τ) of the endogenous oscillator became entrained to the period of the environmental light cycle (T), so that the oscillator formed a steady-state phase relationship to the light cycle. Under long day photoperiodic regimes $\emptyset_i$ would be illuminated but under short days $\emptyset_i$ would fall in the dark. Therefore light, in this model, has two roles: (1) entrainment (entrainment can be defined as the synchronistion of a biological oscillation to that of a driving environmental light cycle) and (2) photoperiodic induction.

The essential feature of the external coincidence model is the coincidence of a single endogenous circadian rhythm with the environmental light cycle. An alternative model to this might involve two or more internal circadian oscillators, where photoperiodic induction would depend on the interaction between these oscillatory components. In this type of model light has only one role, that of entrainment. In 1966, Tyshchenko put forward such a 'two-

oscillatory' model. He proposed the existence of two 'unequivalent' oscillators termed A and B. The A oscillator was assumed to be phase set by dawn and the B oscillator phase set by dusk. Under short day photoperiodic regimes (e.g. LD12:12) there would be no temporal overlap between the active phases of the oscillators and diapause induction would occur. However, under light regimes where there was some temporal coincidence between the oscillators active phases, e.g. long day (LD16:8) or ultra short day-lengths, this would promote continuous development.

Pittendrigh (1960, 1972) also put forward the idea that photoperiodic induction in insects might be a function of the interaction between the entrained steady states of many oscillators. He proposed that the mutual phase relationships between the oscillators would probably have an effect on the physiology of the insect. Therefore, a change in photoperiod would change the phase relationships between oscillators and, perhaps only under certain photoperiods, would the critical phase points of two oscillators coincide and effect photoperiodic induction. Pittendrigh called this the Theory of Internal Coincidence, and in this model, like Tyshchenko's, light has only one role - that of entrainment.

Time measurement in Sarcophaga argyrostoma has

been extensively studied by Saunders (1973, 1976, 1978). The experiments used to investigate this phenomenon regarded the insect as a 'black box', cultures being exposed to a wide variety of normal and abnormal light cycles, and the 'output' of the clock measured as diapause incidence. There are several experimental regimes that differentiate an hour glass from an oscillator type of clock. In a resonance or T experiment, the light component of the cycle is kept constant and the dark period is systematically extended to give light cycles between T=16 and 72 hours or more. In the extended dark phase an oscillator would repeat itself with circadian periodicity, whereas an hour glass would only measure one time period as it would require to be reset by another light pulse. In the former, therefore, diapause incidence would be expected to vary in a circadian fashion with increasing T, whereas in the hour glass, diapause would be maximal once the critical night length was exceeded. Saunders (1973b) applied a resonance experiment to Sarcophaga argyrostoma. He used regimes in which the light component of the cycle was either 4, 8, 12, 14 or 16 hours in light cycles with T varying from 16 hours to 72 hours. He plotted the proportion of each experimental culture entering diapause against the length of the driving cycle (T), and found that

the maximum amount of diapause occurred approximately 24 hours apart, at T=24, 48 and 72 hours, thereby demonstrating an endogenous circadian component in the photoperiodic clock. The resonance experiment also revealed an additional complication in photoperiodic induction in S. argyrostoma. The second peak of diapause induction occurred at T=48 when the light component of the cycles was less than 12 hours, but occurred successively later at T=50, 52, and 56 hours when the light component was 14, 16 and 20 hours respectively. Saunders concluded that the oscillator controlling photoperiodic induction must be 'damped out' in photoperiods longer than 12 hours and then restarted at dusk, so that in these longer photoperiods the oscillator is phase set in a similar manner to the oscillator controlling pupal eclosion in Drosophila pseudoobscura (Pittendrigh, 1966). Thus, the clock, although oscillatory exhibits hour glass properties when measuring night length. The resonance experiment also emphasised the importance of night length in photoperiodic induction in S. argyrostoma. For example, when the light component was held at 16 hours (equivalent to a long day, when T=24) high levels of diapause were observed once the dark period became greater than the critical value (9.5 hours).

Saunders (1975b) also used 'night interruption'

experiments to demonstrate the oscillatory nature of the clock in S. argyrostoma. In this type of experiment the light component of the cycle (T=24 hours or longer) is kept constant and the dark component scanned with an additional pulse of light. Theoretically, if the clock complies with the external coincidence model, diapause should be averted when the scanning pulse coincides with $\emptyset_i$. In 24 hour cycles, Saunders (1975b) found that the photoinducible phase occurred approximately 9.5 hours after dusk (i.e. at the end of the critical night). In cycles with an extended night (e.g. T=48 or 72 hours) however, $\emptyset_i$ was seen to occur with circadian rhythmicity (Saunders, 1976). At that time it was concluded that much of the data for diapause induction in S. argyrostoma was consistent with the external coincidence model.

Although the aforementioned experiments can differentiate between an hour glass and an oscillatory clock, they cannot distinguish between the internal and the external forms of coincidence, since results from resonance experiments can also be explained in terms of the internal alternative (Pittendrigh, 1972). Maximum diapause induction occurs at approximately T=24, 48 and 72 hours whilst minimum induction occurs at T=36 and 60 hours. If photoperiodic induction relies on the phase relationship between two internal

oscillators, it is important that the natural period τ of both oscillators can entrain to the period of the light cycle T. Inductive maxima in resonance experiments occur at values of T which are approximately $n\times\tau$, when circadian organisation will be nearly normal, whereas the minima occur at approximately $n\times\tau + \tau/2$ when entrainment might be disrupted. The results merely justify the conclusion that the amount of induction is a function of the entrained steady state of the circadian organisation.

One of the main differences between the internal and external coincidence models is the role of light: in the external coincidence model light acts to entrain the internal oscillator(s) to the environmental light cycle and is also involved in photoperiodic induction, whereas in the internal coincidence model light is only involved in entrainment. An appropriate test to differentiate between the two theories is to use another zeiteber apart from light to entrain the internal oscillators. Saunders (1973, 1978b) used temperature cycles as a zeiteber in constant darkness, using a parasitic wasp Nasonia vitripennis and the flesh-fly Sarcophaga argyrostoma. The results showed that thermoperiod simulated the effects of photoperiod in diapause induction in N. vitripennis, but not in S. argyrostoma. These results supported Saunders's view that time measurement in

N. vitripennis is effected by some sort of internal coincidence clock, but that the external coincidence model remained appropriate for S. argyrostoma.

The external coincidence model in its simplest form cannot explain the low incidence of diapause which occurs when S. argyrostoma is subjected to ultra short photoperiods (less than 6 hours) or to constant darkness: in both regimes the external coincidence model predicts 100% diapause induction since $\emptyset_i$ would not be illuminated. Bearing this and other previously described results in mind, Saunders (1982) has concluded that S. argyrostoma may possess a clock whose properties, although conforming to the external coincidence model, contains a multioscillator circadian component that measures night length as if it were an hour glass once the photoperiod exceeds 12 hours, but shows its circadian properties in extended dark periods.

Time measurement in insects is clearly a very complex and diverse subject, since the few species which have been extensively investigated seem to achieve time measurement in very different ways: Saunders (1982) hypothesised that a continuous spectrum of clock mechanisms has evolved, but, '... how similar or how different these mechanisms really are, and whether the diversity represents convergence or a common ancestory, however, are

questions reserved for the future'.

THE PHOTOPERIODIC SENSITIVE PERIOD

The environmental factors responsible for induction of facultative diapause are commonly photoperiod and temperature (see pp.17-24). However, insects are not sensitive to photoperiod throughout their whole life cycles: the sensitive period is often restricted to a particular stage of development. To identify these stages, most experimental protocols have involved the reciprocal transfer of developing insects between non-diapause and diapause determining conditions.

In some species, the sensitive period and the resulting diapause occur during the same developmental stage, e.g. diapause in the eggs of the mosquito Aedes triseriatus is directly induced by the short day photoperiods experienced by the eggs (Clay and Venard, 1972), and the imaginal diapause of both Pyrrhocoris apterus and Leptinotarsa decemlineata is induced by the photoperiod experienced as young adults, although the photoperiod experienced by the nymphs or larvae can modify the diapause response if adults are exposed to intermediate photoperiods near the critical value (Hodek, 1977). In long-lived adults of the latter species, which have already reproduced, short days can induce diapause, and the insects can over-

winter a second time (Ushatinskaya, 1961). Likewise, the imaginal diapause in the red locust Nomadacris septemfasciata is induced by short daylength experienced as adults, but influenced by the photoperiods seen by the nymphal stages (Norris, 1965).

In other insect species, the sensitive period directly precedes the diapausing stage, e.g., the early larval instars of Grapholitha molesta (Dickson, 1949), Aedes triseriatus (Clay and Venard, 1972), Ostrinia nubilalis (Beck and Hanec, 1960) and Pectinophora gossypiella (Bell and Adkisson, 1964) are photoperiodically sensitive, and diapause supervenes in the mature larvae. Likewise, the larval stages, particularly the later instars, are sensitive in Antheraea pernyi (Tanaka, 1950), Diataraxia oleracea (Way and Hopkins, 1950), Manduca sexta (Rabb, 1966) and Pieris brassicae (Claret, 1966a, b); in these species, however, diapause occurs in the pupal stage.

The sensitive stage and the resulting diapause may be separated by one or more intervening instars. Usually both stages occur during the life cycle of the particular insect, but in some cases the diapause may occur in the next generation. There are many examples of the former type, e.g. the flesh-flies (Sarcophagidae) which all enter diapause in the pupal stage. Diapause induction will only occur in Sarcophaga crassipalpis if the intrauterine embryos

experience short day photoperiods during the last 1 to 2 days of embryonic life. Maximum diapause occurs if short day photoperiods are also given for the first 2 days after larviposition, but short day photoperiods given throughout larval development (but not to the embryos) are themselves insufficient to induce diapause (Denlinger, 1971). Other species of flesh flies e.g. Sarcophaga argyrostoma (Saunders, 1971), Sarcophaga bullata (Denlinger, 1972), Tricholioprocta impatiens (Roberts and Warren, 1975), Parasarcophaga similis and Boettcherisca septendrionalis (Vinogradova, 1976), however, are capable of entering diapause if the larvae but not the embryos are exposed to short days, even though the embryos are photoperiodically sensitive. Different species, therefore show a different degree of larval sensitivity.

Maternal induction of diapause is an example in which the sensitive period and the resulting diapause occur in succeeding generations. In Aedes atropalpus, for example, the 4th instar larvae and subsequent developmental stages of the maternal generation are sensitive to photoperiod. If these stages experience short day photoperiods, the adult females will deposit diapausing eggs, whereas exposure to long days causes eggs to hatch without an arrest. This is strictly a maternal effect (Kappus and Venard,

1967; Anderson, 1968). Likewise, the production of diapausing eggs in the psocid Peripscocus quadrifasciatus was a response to conditions that prevailed during the later maternal nymphal stages and early adult life. However, the photoperiod experienced by the nymphs does not fix the adult response, young adults experiencing a reversed photophase will alter their response.

The larval diapauses of Calliphora vicina (Vinogradova and Zinovjeva, 1972) Lucilia caesar (Ring, 1967) and Lucilia sericata (Fraser and Smith, 1963) are all under maternal control, although in C. vicina, this influence is only revealed at temperatures of 15°C or below. Similarly, the larval diapause of the wasp Nasonia vitripennis is controlled by the photoperiods the adult females experience (Saunders, 1965). The induction of pupal diapause in the horn fly Lyperosia irritans, is also controlled by the interaction between the photoperiod that the adult flies are exposed to and the response of the immature stages to low temperatures (Wright, 1970).

The most extreme example of separation between the sensitive and diapausing stages is seen in the silkworm, Bombyx mori. In this species embryonic diapause is determined during the late embryonic and early larval stages of the generation before. Therefore, although, diapause in B. mori occurs

during the autumn and winter, it is induced by the long days and high temperatures of summer, experienced by the maternal generation (Kogure, 1933).

ENVIRONMENTAL FACTORS THAT AFFECT DIAPAUSE DEVELOPMENT

The state of diapause is dynamic rather than static; insects in diapause are continually modulating their physiological state, e.g. in diapausing pupae of Ostrinia nubilalis (Beck and Hanec, 1960) or Sarcophaga bullata (Denlinger et al., 1972) the rate of oxygen uptake immediately increases in response to deliberate injury. Insects in larval or adult diapause are frequently capable of co-ordinated behaviour such as feeding or flight. Diapausing larvae of Aedes triseriatus are still active and capable of feeding, although at a lower rate than developing larvae (Clay and Venard, 1972). In some species, the endocrine system remains active throughout diapause: the southwestern cornborer Diatraea grandiosella, for example, can undergo stationary larval moults during diapause as a final instar (Chippendale and Reddy, 1972).

The physiology of insects in diapause also shows progressive changes as diapause proceeds. Andrewartha (1952) used the term 'diapause development' to describe these physiological events that occur in

preparation for the active resumption of morphogenesis. Many factors such as photoperiod and temperature can influence the rate of diapause development and thus constitute the major diapause maintaining environmental factors. Therefore in most species these factors regulate the diapause development that leads to diapause termination.

In some insect species diapause is induced and maintained by photoperiod (= oligopause see p. 3), in other species photoperiod plays no part in diapause development (= eudiapause). Examples of the former include the larval diapauses of Chironomus tentans (Engelmann and Shappiro, 1965), and Pectinophora gossypiella (Bell and Adkisson, 1964) and the pupal diapauses of Antheraea pernyi, Antheraea Polyphemus and Hyaiophora cecropia (Williams and Adkisson, 1964) which are all induced and maintained by short day photoperiods and terminated on exposure to long day conditions. In the latter species, the photoperiodic control was nullified on exposure to high temperatures of 30°C and above (Mansigh and Smallman, 1972). Diapause development in the corn borer Ostrinia nubilalis occurred at all photoperiods without chilling (McLeod and Beck, 1963), but the rate of diapause development under long day photoperiods was 5 times that under short daylengths (Beck and Alexander, 1964). Likewise, in the bug, Pyrrhocoris

apterus induction and termination of adult reproductive diapause is under photoperiodic control at 25°C. However, 12 to 16 weeks chilling at 4°C can override this photoperiodic control of termination - eggs are laid at all photoperiods on return to favourable temperatures (Saunders, 1983).

In many insects, a period of chilling can promote diapause development or alter the insect's response to photoperiod. For example, in the larval diapause of the treehole mosquito Toxorhychites rutilus chilling has a triple role: (1). it promotes response to progressively shorter daylengths thereby decreasing the critical photoperiod for termination, (2). it accelerates the response to long daylengths thereby decreasing the depth of diapause, and (3) prolonged exposure to chilling itself can eventually terminate diapause directly, regardless of photoperiod. The interaction between chilling and photoperiod prevents premature development during a warm winter since unchilled larvae rely on photoperiod to maintain diapause (Bradshaw and Holzapfel, 1977).

As previously mentioned, in some species the termination of diapause is independant of photoperiod. For example, in Ephestia elutella the most efficient completion of diapause development during larval diapause occurred after a period of low temperature (5 to 10°C) followed by a sustained period at a

higher temperature (25°C) (Bell, 1983). Likewise, in the flesh-flies Saarcophaga argyrostoma and Sarcophaga bullata, diapause termination occurs after a suitable period of chilling, but will occur 'spontaneously' at higher constant temperatures (Fraenkel and Hsiao, 1968a). Diapause development in Sarcophaga argyrostoma is described in more detail in Chapter IV.

ENDOCRINOLOGY OF DIAPAUSE

The general hypothesis that diapause is directly caused by failure to secrete specific hormones was first suggested by Wigglesworth (1934). Support for this idea came from later work by Williams (1946, 1947, 1952) working on the pupal diapause in the moth Hyalophora (= Platysamia) cecropia. Using implantation and parabiosis experiments, Williams showed that diapause resulted from the failure of the brain to supply a non-species specific factor (now known to be prothoracotropic hormone, PTTH), which activated the prothoracic glands to produce the moulting hormone. This form of endocrine control, i.e., inactivation of the brain-prothoracic gland system has been postulated in the larval diapauses of Cephus cinctus (Church, 1955), Ostrinia nubilalis (Cloutier et al., 1962; Beck and Bean, 1980) and Lucilia caesar (Fraser, 1960) and in the pupal diapauses of Mamestra configurata (Bodnaryk, 1977), Mimas tiliae (Highnam,

1958) and the flesh-fly Sarcophaga argyrostoma (Fraenkel and Hsiao, 1968b).

At first sight the simple hormonal-failure theory does not adequately explain the larval diapauses of several lepidopterans. In Diatraea grandiosella, for example, diapause is not caused by a lack of ecdysone, but by high titres of juvenile hormones (JHI, II, III) in the blood throughout the duration of diapause (Yin and Chippendale, 1973; Bergot and Schooley, 1976). Likewise, the larval diapauses in the rice stem borer Chilo suppressalis (Yagi and Fukaya, 1974) and the codling moth Laspeyresia pomonella (Sieber and Benz, 1977) are induced by high levels of JH in the final larval instar, although, in the latter species, JH is not necessary for diapause maintenance. Takeda (1978) also demonstrated that during the early stages of the prepupal diapause of Monema flavens, the corpora allata were actively releasing JH whereas the neurosecretory cells within the brain appeared to be inactive. An explanation for the role of JH in the regulation of larval diapause was put forward by Nijhout and Williams (1974). They found that high levels of JH in the haemolymph of final instar larvae of Manduca sexta inhibited the secretion of PTTH. Once JH disappeared from the blood, however, PTTH was released and the larvae pupated. The authors postulated that high JH titres were thus causally involved in the

larval diapauses of at least some species of Lepidoptera.

Juvenile hormones are also implicated in the control of imaginal diapause. Joly (1945) found that ablation of the corpora allata in Dytiscus marginalis resulted in ovarian regression. Likewise, surgical removal of the corpora allata in Leptinotarsa decemlineata had the same effect on the ovaries as short day treatment of the adult females (DeWilde and DeBoer, 1961). However, whereas following allatectomy, diapause was easily reversed by the implantation of two active corpora allata (CA) similar implantations did not terminate normal short day induced diapause, i.e., diapause is not just a simple inactivation of the CA. Similarly, inactivity of the corpora allata is thought to result in reproductive diapause in the locust Anacridium aegyptium (Gieldiay, 1969), the mosquito Cuiex pipens (Spielman, 1974) and the butterfly Danaus plexippus (Herman, 1973). The long day reproductive diapause in the male carabid beetle Pterostichus nigrita is also regulated by JH; short daylengths activate the corpora allata.

Hormonal regulation of egg diapause contrasts with that of the post-embryonic diapauses which are all consistant with the hormonal failure theory of Wigglesworth (1934). In the silk moth Bombyx mori embryonic diapause is determined by the presence of a

'diapause hormone' (DH) produced by the neurosecretory cells of the suboesphageal ganglion of moths reared from eggs and young larvae exposed to long days (Hasegawa, 1957). The production of DH is controlled by the brain and the target organ is the ovary (Kobayashi and Ishitaya, 1964). Evidence suggests that the hormone is incorporated in the ovarian egg and imposes diapause on the embryo after oviposition. A similar endocrine mechanism has been proposed for the tussock moth Orgyia antigua (Kind, 1965). Evidence that the pupal diapause in Phalaenoides glycinae may be induced by a 'diapause hormone' produced by the suboesophageal ganglion was provided by Andrewartha et al., (1974). It was not possible to prove that the hormone directly induced diapause since injury increased diapause intensity in this species. However, if the suboesphageal ganglion from a moth kept in diapause inducing conditions was transferred to the silkworm Bombyx mori, diapausing eggs were subsequently produced. However, extracts with DH activity have also been isolated from the cockroach Periplaneta americana, and diapause is absent in this species. It is possible therefore, that 'diapause hormone' in P. glycinae has no role in pupal diapause.

A diapause inducing hormone in short day insects has been sought in many species without success, e.g.

Sarcophaga argyrostoma (Gielbultowicz and Saunders, 1983). For nearly all species with a post-embryonic diapause, the doubtful exception being P. glycinae, Wigglesworth's theory of hormonal failure remains appropriate, in spite of the fact that the regulatory schemes controlling post-embryonic diapause have been shown to be highly diverse, e.g. the hormonal control of larval diapause within the Order Lepidoptera. One common factor in all cases is that diapause ultimately results from the inactivation of neurosecretory cells within the brain.

The endocrine control of pupal diapause of Sarcophaga argyrostoma, particularly emphasising the possible role of ecdysone, is discussed in more detail in Chapter V.

PHOTOPERIODIC SUMMATION

The previous sections have described the role of photoperiod in the induction of diapause, the possible ways in which insects measure night or day length, how photoperiodic sensitivity is restricted to a particular developmental stage within each species, and the hormonal control of diapause. One of the more obscure problems in diapause induction is how the environmental light signals become transduced in the brain to affect the neuro-hormonal output which

controls the final outcome, diapause or non diapause development.

In the mosquito Chaoborus americanus, one long day in the presence of food terminates diapause in a significant proportion of the population (Bradshaw, 1967). However, this is unusual, since most insect species require a number of light cycles for diapause induction or termination, implying that the photoperiodic information must be accumulated or 'summed' in some way.

The final outcome of the photoperiodic response is a consequence of the number of photoperiodic cycles the insect experiences during the restricted sensitive period. Many authors have asked: Is there a required number of photoperiodic cycles necessary to induce or terminate diapause?

The proportion of pupae terminating diapause in Antherae pernyi is related to the number of long day cycles experienced. Williams and Adkisson (1964), for example, found that 20% of unchilled pupae terminated diapause after 2 weeks at LD16:8, but 50% and 100% terminated diapause after 4 and 8 weeks respectively. Likewise, in Ostrinia nubilalis there was a cumulative effect of long days on diapause development. If pupae experienced 10 days of long day cycles, then 10 days of short day cycles, followed by a return to long day photoperiods, diapause termination was

delayed by exactly 10 days. This showed that the effect of the first 10 long days was not lost and that short days did not reverse or cancel the diapause development that had already been accomplished (Beck and Alexander, 1964).

The effect of short day cycles on the induction of pupal diapause in Barathra (= Mamestra) brassicae was studied by Goryshin and Tyshenko (1973). The effect of long day or short day cycles in induction or prevention of diapause was estimated by the number of photoperiodic cycles that produced a final diapause incidence of 50% (critical point). They found that a minimum of 14 short day photoperiods produced the critical amount of diapause. They also observed that short day cycles were equally effective during any stage of the larval sensitive period and that short day information was accumulated and stored even if short day and long day photoperiods were alternated in different ways.

Several authors have observed that the number of light cycles required to induce a photoperiodic response is temperature-compensated. Goryshin and Tyshchenko (1970) and Tyshchenko et al. (1972), working with pupal diapause in the moth Acronycta rumicis, looked at the effect of a single, abrupt change in the length of day (i.e. long to short, or short to long) on the accumulation process. Both

short day or long day effects on the photoperiodic response were reversible; however, the earlier the change, the more complete the effect of the new photoperiod. In a strain of A. rumicis from Belgorod (50°N), the reversion of the reaction was found to occur with equal success whether the short daylengths were replaced by long photoperiods, or vice versa. The accumulated photoperiodic 'information' was finally utilised at the pupal stage, the last signal being active irrespective of previous photoperiodic conditions. They found that 11 terminal long or short day periods were required to produce 50% diapause among the pupae i.e., long and short photoperiods were equally effective in this strain of A. rumicis. Furthermore, the number of such cycles required were the same throughout a range of temperatures from 18°C to 26°C. The authors postulated the existance of a photoperiodic counter, in which a 'critical day number' was accumulated in a temperature-compensated fashion. Using a different strain of A. rumicis from the Black Sea Coast (43°N), Tyschenko et al. (1972) found that long and short daylengths were not equally effective: the critical day number for long days was only 6 or 7, whereas, for short daylengths, it was 16 or 18. In both strains of A. rumicis, both long and short daylengths were accumulated, unlike M. brassicae (Goryshin and

Tyshchenko, 1973) in which only short days were accumulated; however in both species the accumulation was temperature-compensated.

Earlier work by Saunders (1966a and b) on the parasitic wasp Nasonia vitripennis provided convincing evidence for the existence of a photoperiodic counter. Larval diapause in this species is controlled by the photoperiod experienced by the maternal generation (see p.40). Females incubated in short day lengths (14 hours or less) at 18°C, switched to producing diapause progeny after an average of 7.9 to 9.6 days. In long days, however, the switch to producing diapause progeny, if it occurred, was delayed until near the end of imaginal life. The timing of the 'switch' in 'strong' short daylengths was compensated over a range of temperatures (15°C to 30°C), requiring between 5 to 8 cycles to produce diapause destined progeny throughout this range. Saunders used the term 'Required Day Number' (RDN) which he defined as the number of calendar days or photoperiodic cycles (T= 24) required to raise the proportion of larvae in that days batch to 50%; this is equivalent to the 'critical day number' of Tyshcheko et al. (1972).

The flesh-fly Sarcophaga argyrostoma is also suitable for the analysis of the cumulative effects of photoperiod. Studies in this species by Saunders

(1971, 1972 and 1975) have produced data comparable to that of N. vitripennis. These results and others are reviewed extensively in the introduction to Chapter II.

INTERACTION OF THE PHOTOPERIODIC COUNTER AND ENVIRONMENTAL VARIABLES

As described above, the number of photoperiodic cycles required to induce diapause is temperature-compensated, and sensitivity to photoperiod is restricted to a particular stage of development. It follows that any factors such as temperature, diet or even photoperiod which alter the duration of the sensitive period will also affect the final diapause response. As previously described (p. 26), the effects of short daylength and low temperature complement each other, resulting in an increase in diapause. This can be interpretated in a number of species as an interaction between the counter and developmental rate. For example, in Aedes triseriatus, the incidence of diapause increases if the developmental rate of young larvae is retarded by temperature or an inadequate diet. Under these conditions, the larvae experience a sufficient number of short day photoperiods to induce diapause (Clay and Venard, 1972). Likewise in the mosquito Aedes atropalpus, prolongation of the 4th instar by starvation allowed the larvae to accumulate more than

the RDN of short day cycles (7), thereby inducing the resultant adults to deposit diapausing eggs at temperatures as high as 28°C (Beach, 1978).

A more thorough investigation of the effect of temperature and diet on the Required Day Number (RDN) (i.e. the photoperiodic counter) was carried out by Saunders (1966a and b) on Nasonia vitripennis. As already described, the RDN (5 to 8) in this species was largely independant of temperature (Q = 1.04). However, temperature directly affected the rate of oviposition and the length of reproductive life. Thus wasps kept at LD12:12, 30°C started to oviposit after 3 to 5 days and lived on average 11.5 days, whilst wasps kept at 15°C under the same photoperiodic condition had a very protracted lifespan (about 34 days) and a slow rate of oviposition, the peak occurring around day 14. The interaction between the temperature-compensated mechanism accumulating light cycles resulted in higher incidences of diapause at low temperatures e.g. the diapause incidence at 15°C was 90.9%, at 20°C and 25°C it decreased to 71.2% and 61.0% respectively, and dropped to 27.4% at 30°C (Saunders, 1966a).

Further evidence supporting the existence of the photoperiodic counter was given by Beach (1978) working on three geographically distinct populations of the mosquito Aedes atropalpus. This species is

photoperiodically sensitive during the 4th larval instar and the pupa of the maternal generation, and the resulting adult female deposits diapausing or developing eggs. Beach found that the critical daylength for diapause induction increased with increasing latitude (see p. 20) but remained constant with increasing temperature. However, the diapause response at different temperatures varied for each population: the incidence of diapause in the strain from Ontario, Canada (45°N) remained saturated at all temperatures tested (20°C to 28°C) whilst that in the population from Georgia, U.S.A. (34°N) dropped from 100% at 22°C to 65% at 24°C and in those from El Salvador (14°N) the diapause response was only 68.4% at 22°C dropping from 95.4% at 20°C. He then used transfer experiments (i.e., long day to short day conditions, or vice-versa) during the 4th instar and pupal stage to determine the RDN, which he found to be different in all three population: the Canadian strain had a RDN of 4, and those from Georgia and El Salvador had RDN of 7 and 10 respectively. Beach interpretated these results thus: the strain from Canada had a very low RDN (4), this caused the strain to enter diapause under short daylengths even up to temperatures of 28°C since the sensitive period even at very high temperatures was longer than 4 days. This was appropriate for an area where the transition

from summer to winter was rapid and closely correlated with photoperiod. In the most southerly strain from El Salvador, with a RDN of 10, however, the population although capable of entering diapause rarely did so, because in the prevailing temperatures, adult mosquitoes eclosed before seeing the RDN of light cycles. Likewise, the population from Georgia, having an intermediate RDN, deposited developing eggs at high temperatures so that in the field if the onset of short daylengths coincided with unseasonably warm weather, further breeding could occur. Beach concluded that the observed differences in the RDN were the product of natural selection and of real ecological significance. He also observed that if precocious females emerged before experiencing the RDN, they produced non-diapausing eggs.

The aim of this study was primarily, to investigate the photoperiodic summation of long night cycles in the flesh fly Sarcophaga argyrostoma, by manipulating the duration of the larval (sensitive) period using methods other than temperature. Experiments were designed to test a simple hypothesis derived from the photoperiodic counter model proposed by Gibbs (1975): shortening the sensitive period, thereby reducing the number of long nights experienced, should lower the incidence of diapause, whereas lengthening the sensitive period should increase it. In addition, the following were

also studied: 1) The importance of the intrauterine stage in the photoperiodic induction of diapause; 2) The genetic control of the length of the post feeding stage; 3) The factors controlling the duration of diapause and 4) the ecdysteroid levels in the haemolymph of diapause and non-diapause destined mature larvae and early prepupae.

GENERAL MATERIALS AND METHODS

The experiments described in this thesis were carried out using a strain of *Sarcophaga argyrostoma* (Robineau-Desvoidy), maintained in the Zoology Department, Edinburgh, since 1961; before this, its history is unknown.

Light and Temperature Conditions

Stock Cultures

Stock cultures were kept in rooms at 25°C ± 1°C. The temperature was controlled by thermostats. Illumination was by overhead fluorescent bulbs, controlled by Venner time switches.

Experimental Cultures

Most experiments were carried out in walk-in constant temperature rooms. Temperature control was accurate to ± 0.5°C. Cultures were placed in light proof wooden boxes within the constant temperature room. Each box was fitted with a fluorescent light source (Philips, 4W) and water-jacketted to counteract excess temperature fluctuation. Light cycles were controlled by Venner time switches.

Stock Cultures

Adult flies were kept in breeding cages (60 x 50 x 45 cm) constructed from a metal frame on

a solid base. Each cage was covered with a tube of fine white gauze knotted at each end. The bottom of each cage was lined with white paper. The flies were provided with a continuous supply of granulated sugar (in a dish) and water (in a glass jar upturned on a petridish containing a cotton wool pad). Fresh meat (beef muscle) was supplied daily to provide both a food source for the flies and to function as a larviposition site. These stock cages were kept at 25°C ± 1°C in either continuous light (LL) or in long nights (LD12:12), the former to provide non-diapausing individuals and the latter a supply of diapausing pupae.

Larvae from the stock cages were bred at 25°C ± 1°C, LL. Cultures were established by transferring pieces of meat carrying larvae deposited over the previous 24 hours to 3 Tupperware dishes (13 x 18 x 8 cm) containing about 600 ml of a dried milk-yeast-agar synthetic medium (see Appendix I). Each culture contained 1000-1500 larvae, progeny of a number of different females from the stock population. The larval culture dishes were placed inside metal biscuit tins (21 x 21 x 19 cm) containing about 2.5 cm of finely sieved sawdust and covered with a fine gauze lid. Whilst in the medium, the larvae fed and moulted twice. After 5-6 days, the mature 3rd instar larvae crawled from the culture dish into

the sawdust and formed puparia after a short (1 to 3 days) wandering period. Newly formed prepupae (Fraenkel and Bhaskaran, 1973) were then sieved from the sawdust and transferred to a tin (20 x 10 x 7 cm) covered with a black cloth* and allowed to dry out at room temperature for two days. The pupae were then divided between as many jars as there were cages at that time, the jars then sealed with paper towelling secured with elastic bands, and kept in constant darkness at 22°C until the first adults emerged. The jars were then transferred to the stock cages, and the emerging adults allowed to intermix with the existing stock. (See pp. 64 and 65 for a summary of the lifecycle of Sarcophaga argyrostoma.)

Experimental Cultures

Except when otherwise stated, experimental cultures were set up by placing the required number of larvae on a 50 g piece of beef muscle using a pair of fine tweezers. The meat was then transferred to a white tupperware dish (25 x 8 x 8 cm) containing 250 ml of synthetic medium. This was placed inside

*Cultures were covered with gauze or black cloth to protect them from the wasp Nasonia vitripennis (Hymenoptera, Pteromalidae) which parasitises the intrapuparial stages of Cyclorrhaphous Diptera and is endemic in the Edinburgh laboratory.

a white plastic 'mousetray' (30 x 12 x 12 cm) containing about 1.5 cm of finely sieved sawdust. The cultures were then transferred to conditions described in the protocol for each experiment. After larvae became post-feeding, newly-formed prepupae were collected daily unless otherwise stated and placed in open petridishes and kept in the same conditions as during development for a further three days. The pupae were then transferred to constant darkness in an incubator maintained at 22°C. After 21 days, the pupae were scored for diapause or non-diapause development using the criteria described below.

CRITERIA FOR THE DETERMINATION OF NON-DIAPAUSE AND DIAPAUSE DEVELOPMENT

Pupae of Sarcophaga argyrostoma usually entered diapause after the head had become evaginated, before the pupal-adult apolysis and before the developing antennae became visible (Fraenkel and Hsiao, 1968).

Newly formed pupae were given sufficient time to allow adult differentiation to take place in non-diapausing flies. The puparia were then opened to reveal the state of development inside. This was achieved, without injury, by removing the anterior 'cap' of the puparium along the preformed lines, at the level of the larval anterior spiracles, which

crack during eclosion. Although, Fraenkel and Hsiao (1968) state that the first sign of the absence or termination of diapause was the appearance of the white antennal discs beneath the transparent head cuticle, this was very difficult to see. Therefore, in this thesis, the development of red pigments in the eyes and dark coloured bristles on the head were the criteria used for non-diapause development. Pupae, of the same age, which showed no signs of adult differentiation were assumed to be in diapause (see Fig.8, p.67). Dead insects were recognisable by the discoloration or lack of a definite structure of the pupa inside the puparium.

Results are expressed as 'total diapause' (total number of diapausing pupae divided by the total number of live pupae, times 100 per cent).

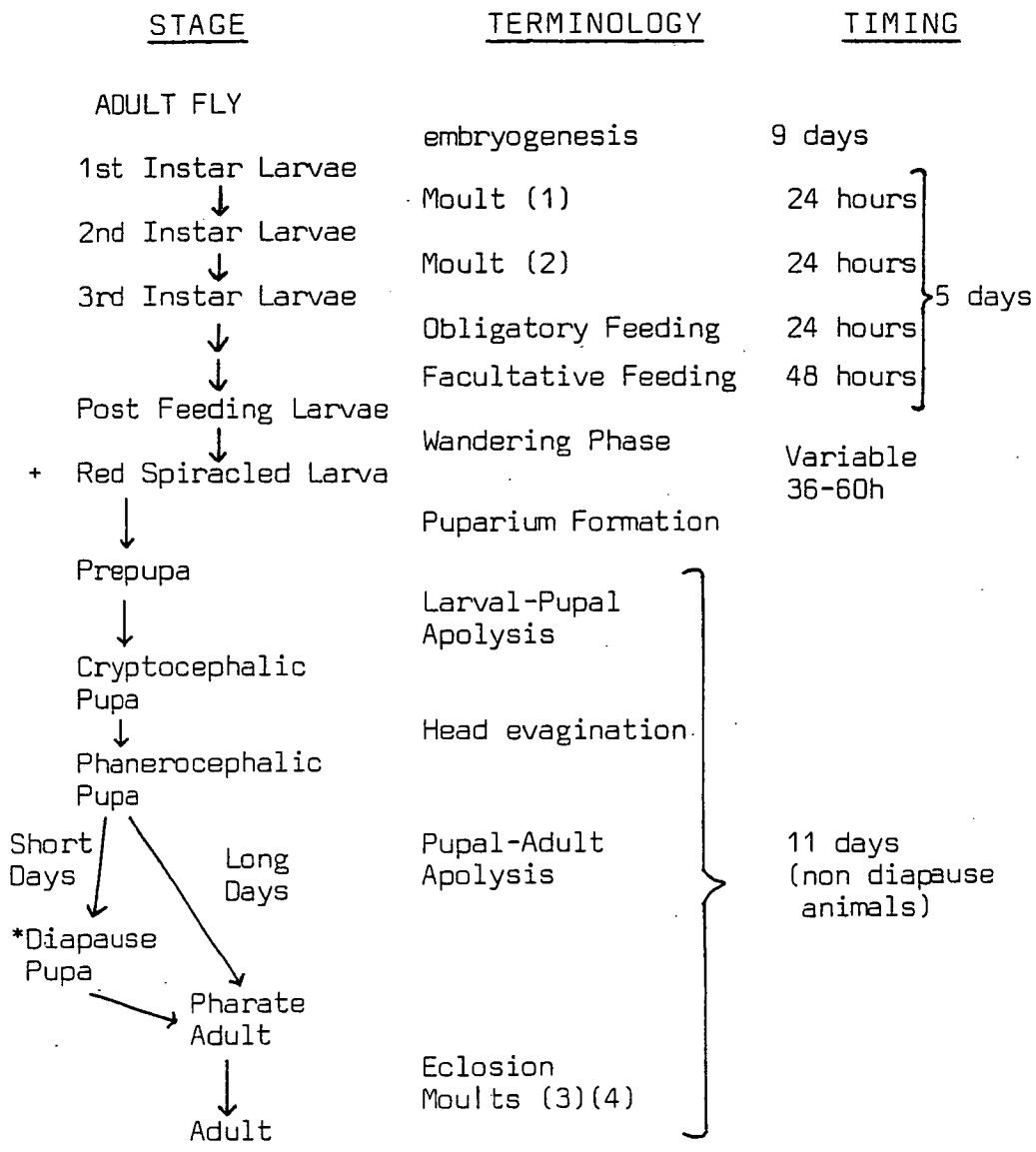
AUTOGENY IN SARCOPHAGA ARGYROSTOMA

Denlinger (1971a) reported that S. argyrostoma was capable of egg maturation in the absence of protein. To determine whether this was also the case in the Edinburgh strain, adults from three hundred non-diapausing pupae derived from adult stock kept at LL 25°C ± 1°C were allowed to emerge within two small adult cages kept at LL, 25°C ± 1°C. The remaining empty puparia were removed immediately. Both cages were supplied with granulated sugar and water, but

only one cage was given beef muscle from day 4 onwards.

Nine days after eclosion, larvae were deposited in the cage which had been provided with meat. The cage containing flies which had received no meat was maintained for 21 days. During this time, no larvae were found anywhere within the cage. Therefore, the Edinburgh strain of Sarcophaga argyrostoma is anautogenous.

LIFE CYCLE OF *SARCOPHAGA ARGYROSTOMA* AT 25°C



*At 25°C pupae terminate diapause after 90-120 days.

+In *Sarcophaga* larvae that are due to pupariate within 2-3h, the space between the posterior spiracles (peritreme) begins to tan, and this tanning spreads gradually into the region around the spiracles. This stage is called the red spiracled larva. (Sivasubramanian *et al.*, 1974).

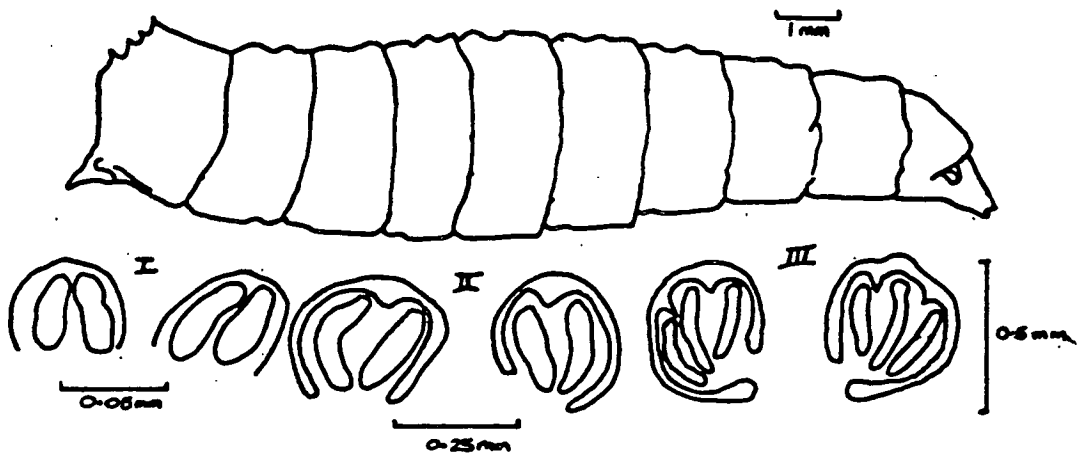


FIGURE A.

Larva of S. argyrostoma. Figure depicts posterior spiracle development during the three larval instars.

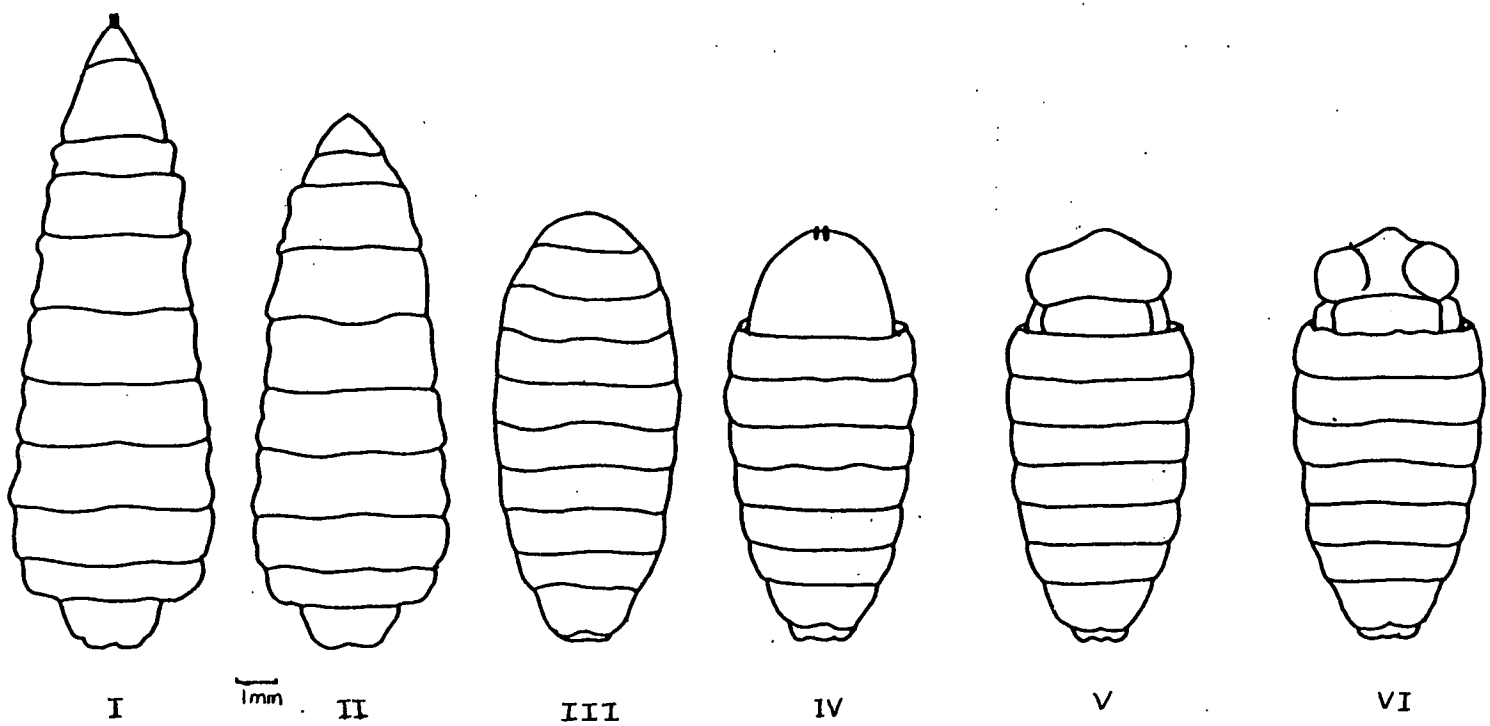


FIGURE B : Puparium Formation in S. argyrostoma (I-III). Larval-pupal moult occurs inside the puparium forming the cryptocephalic pupa (IV). Evagination of the head forming the phaerocephalic pupa (= diapause stage) (V). Adult differentiation (VI).

C H A P T E R I

PHOTOPERIODIC INDUCTION OF PUPAL DIAPAUSE IN SARCOPHAGA ARGYROSTOMA

CHAPTER I

PHOTOPERIODIC INDUCTION OF PUPAL DIAPAUSE IN SARCOPHAGA ARGYROSTOMA

Pupal diapause in Sarcophaga argyrostoma was first reported by Roubaud in 1922; but no details of the conditions under which diapause arose were given. The phenomenon of pupal diapause in this species was accidentally rediscovered by Fraenkel during the winter of 1963, with the result that a short note by Fraenkel and Hsiao, describing induction of diapause by short days was published in 1966 and more detailed papers describing the morphological and endocrinological aspects of the diapause in 1968. A more thorough investigation of the photoperiodic induction of diapause in S. argyrostoma was carried out by Saunders (1971). He cultured larvae in a full range of photoperiods at temperatures of 15, 20 and 25°C. The results demonstrated that S. argyrostoma possessed a temperature-modified photoperiodic response typical of a long-day species, with a well defined critical daylength of 13.5 to 14 hours of light per day. In these experiments, the photoperiodic response curves (PPRCs) were constructed using progeny of adults kept at LD18:6, 25°C; therefore, the photoperiodic sensitivity of the intrauterine stages was not taken into account.

Embryonic sensitivity to photoperiod was demonstrated in Sarcophaga crassipalpis by Denlinger (1970 and 1971), and in Sarcophaga argyrostoma and Sarcophaga bullata in 1972 (Denlinger 1972). Denlinger found that no diapause occurred if only the larvae of S. argyrostoma experienced short-day photoperiods at 25°C. However, if the intrauterine stages were also exposed to short daylengths, 31.8% of the pupae entered diapause.

The intrauterine embryos received the photoperiodic signals directly through the integument of the female's abdomen, i.e., there was no maternal influence on diapause determination (Denlinger, 1971). However, in Sarcophaga bullata, flies that had experienced pupal diapause produced progeny that would not enter diapause even when reared under diapause inductive conditions. This was shown to be a maternal effect, transmitted prior to embryogenesis, probably during oogenesis (Henrich and Denlinger, 1982). An experiment was performed to determine whether this phenomenon exists in the 'Edinburgh' strain of S. argyrostoma.

For technical reasons it has proved convenient in this laboratory to establish experimental larval cultures using larvae deposited by the stock female flies maintained at 25 ± 1°C, and either LL or LD12:12. This procedure has given rise to a number

of PPRCs with different combinations of embryonic and larval exposure to photoperiod and temperature. A more "realistic" PPRC has also been obtained by exposing pregnant adults and feeding larvae to the same experimental temperature and photoperiods (Fig. 3). The variety of experimental approaches, however, has produced a 'family' of PPRCs which underline, amongst other things, the differential photoperiodic sensitivity of embryos and larvae, the results of which are described in this section. A summary of the protocols used in the construction of the different photoperiodic curves is given in Table 1 p. 71.

MATERIALS AND METHODS

Early Embryonic Sensitivity to Photoperiod

Newly deposited larvae from adult stock cages, kept in constant light (LL), 25°C ± 1°C, were reared under the same conditions (using the procedure for culturing stock described on p.59). After the start of puparium formation, (7 days later), prepupae which had formed over a 24 hour interval were collected and divided into nine groups of three hundred. Each group was then placed in a small insect cage and kept at LL, 25°C ± 1°C. This procedure was adopted to provide a synchronised population of non-diapausing insects.

TABLE 1

SUMMARY OF PROTOCOLS USED IN THE CONSTRUCTION OF THE PHOTOPERIODIC RESPONSE CURVES

Curve No.	ADULTS		LARVAE	
	Temp. ^{o}C	Photoperiod (Hours)	Temp. ^{o}C	Photoperiod (Hours)
I	25 ± 1	LL	17 ± 0.5	0, 2, 4, 6, 8, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24,
II	25 ± 1	LL	17 ± 0.5	8, 10, 12, 12.5, 13, 13.5, 14, 14.5, 15, 15.5, 16.
III	25 ± 1	12:12	20 ± 0.5	12, 13.5, 13, 13.5, 14, 14.5, 15, 15.5.
IV	25 ± 1	LL	20 ± 0.5	0, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18, 20, 22, 24.
V	20 ± 0.5	LL	20 ± 0.5	"
VI	20 ± 0.5	0, 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 18, 20, 22, 24.	20 ± 0.5	"
VII	25 ± $1^{o}C$	0, ½, 2, 4, 6, 8, 13, 14½, 16.	25 ± $1^{o}C$	0, ½, 2, 4, 6, 8, 13, 14½, 16.

At the start of eclosion (= day 0), one cage was immediately transferred to LD12:12, 25°C ± 1°C and, thereafter, one cage was similarly transferred on days 1, 2, 3, 4, 5, 6 and 7. One further cage was maintained in LL, 25°C ± 1°C until larviposition (day 9). The larvae were then transferred to LD12:12 25°C ± 1°C. In this way different groups of adult flies experienced a change from LL (non diapause inductive) to LD12:12 (diapause inductive) conditions at different stages of their ovarian development and pregnancy intrauterine development.

Two larval cultures were established for each adult cage: the first, from the first 250 larvae deposited, and the second from larvae deposited during the following 24 hours. All cultures were kept at LD12:12 ± 25°C throughout their development. Once the larvae left the food, each culture was divided into two groups at different densities: either 100 larvae per mousetray (12.5 x 30 x 12.5 cm) containing 2.5 cm of dry sieved sawdust or 2 larvae per plastic jar (3.5 cm diameter x 6 cm height) containing 1 cm of dry sawdust. This was done to avoid any density effects on the timing of puparium formation (see p. 170). Prepupae were collected daily and maintained at LD12:12, 25°C ± 1°C for 14 days, then scored for diapause or non diapause development (as described on p. 61).

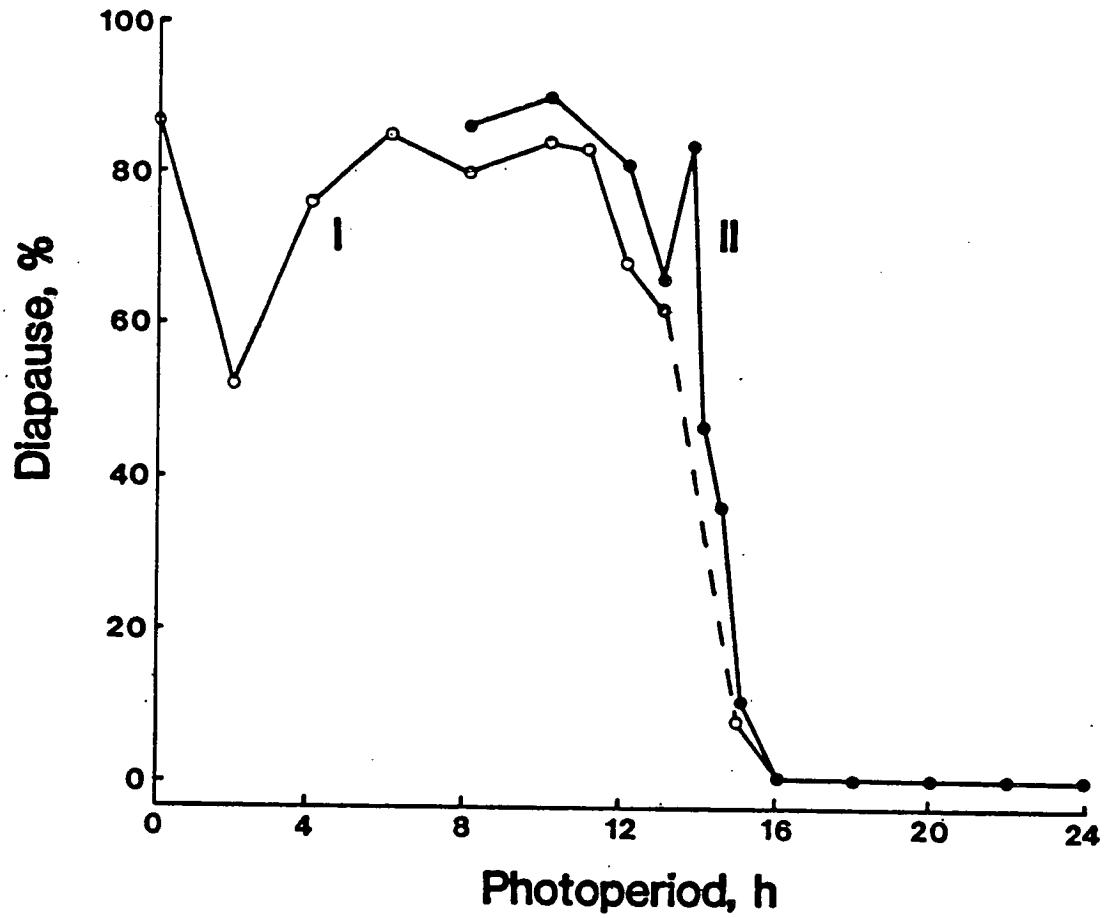


FIG. 1. The effect of photoperiod on the induction of diapause in S. argyrostoma
Adults at LL, 25 ± 1°C; larvae transferred to photoperiods at 17 ± 0.5°C. I (o-o) and II (●-●) are replicate experiments.

formation and the diapause incidence for each culture. The median of puparium formation decreased with increasing photoperiod and the range of days over which puparia were formed also decreased.

Tables 5a and 5b and Figure 2 show the effect on the photoperiodic response curve of exposing the interuterine stages to LD12:12, 25 ± 1°C (Curve III) as opposed to LL 25°C, (Curve IV), before transferring larvae to a range of photoperiods between 12 to 16 hours of light per day at 20 ± 0.5°C. The diapause response became 'saturated' (i.e. approximately 100%) and the critical photoperiod was over 15.5 hours in cultures in which the intrauterine stages had experienced diapause-inductive cycles, whereas the diapause incidence was negligible in cultures in which embryos had experienced constant light. This result underlines the importance of the photo-periodically sensitive intrauterine stage.

Figure 3 and Table 6 illustrate the increase in diapause incidence that occurred when the intrauterine stages experienced the same photoperiod as the developing larval instars, as opposed to constant light as embryos. This was achieved by keeping adult flies in cages within the wooden boxes described on p 58 . Unfortunately, the number of flies that survived to produce larvae was low, possibly due to the very high humidity that built up

TABLE 3

The effect of photoperiod on the timing of prepupal formation and on the incidence of diapause in S. argyrostoma (PPRC I). Adults at LL, 25 ± 1°C, larvae at 17 ± 0.5°C.

Photoperiod (h)	Number of Prepupae Formed	Median and Range of Pupariation (days)	Diapause %
0(DD)	195	$17,^{14-24}$	87
2	200	$16,^{13-22}$	52.2
4	273	$17,^{13-21}$	76.2
6	265	$18,^{13-23}$	85.0
8	210	$16,^{18-19}$	80.0
10	408	$16,^{12-22}$	84.0
11	310	$16,^{12-22}$	83.0
12	491	$16,^{12-22}$	68.0
13	403	$15,^{12-19}$	62.0
14	FAILED	-	-
15	315	$12,^{11-14}$	8.4
16	453	$10,^{10-13}$	0.4
18	304	$11,^{11-13}$	0
20	279	$11,^{11-13}$	0
22	488	$11,^{11-13}$	0
24(LL)	272	$12,^{11-13}$	0

TABLE 4

The effect of photoperiod on the timing of prepupal formation and on the incidence of diapause in S. argyrostroma (PPRC II). Adults at LL, 25 ± 1°C, Larvae at 17 ± 0.5°C.

Photoperiod (h)	Number of Prepupae Formed	Median and Range of Pupariation (days)	Diapause %
8	283	$22,^{14-33}$	86.9
10	185	$22,^{15-30}$	90.0
12	191	$18,^{14-27}$	81.4
12.5	261	$19,^{14-27}$	73.0
13	252	$17,^{13-26}$	66.5
13.5	231	$22,^{14-31}$	84.5
14	212	$17,^{14-25}$	47.0
14.5	210	$17,^{13-24}$	37.4
15	218	$16,^{14-20}$	10.9
15.5	265	$15,^{13-21}$	3.8
16	299	$15,^{13-18}$	0.6
18	208	$16,^{14-19}$	0

TABLE 5a

The timing of prepupal formation and the incidence of pupal diapause for *S. argyrostoma* larvae deposited by adults kept in LD12:12, 25 ± 1°C and kept in a range of photoperiods at 20 ± 0.5°C during larval development (PPRC 111).

Photoperiod (h)	Number of Prepupae Formed	Median and Range of Pupariation (days)	Diapause %
12	203	16, 12-20	97.5
12.5	153	15, 11-18	98.0
13	394	16, 11-22	96.9
13.5	224	15, 11-19	95.4
14	248	15, 13-23	97.9
14.5	161	15, 12-18	98.0
15	157	15, 12-18	93.2
15.5	153	14, 10-17	66.0

TABLE 5b

The timing of pupariation and the incidence of pupal diapause in S. argyrostroma larvae which experienced LL, 25 ± 0.5°C. (PPRC IV).

Photoperiod (h)	Number of Prepupae Formed	Median and Range of Pupariation (days)	Diapause %
0	260	12, 10-15	0
2	260	12, 10-16	12.9
4	189	11, 10-14	6.9
6	309	11, 10-14	8.7
8	320	11, 10-14	23.3
10	203	10, 10-11	1.0
12	338	10, 10-11	4.2
13	444	11, 10-13	0
14	186	10, 10-12	0
15	371	11, 10-13	0.3
16	166	10, 10-13	0
18	237	11, 10-13	0
20	327	11, 10-14	0.3
22	291	12, 10-13	0
24	207	12, 10-14	0

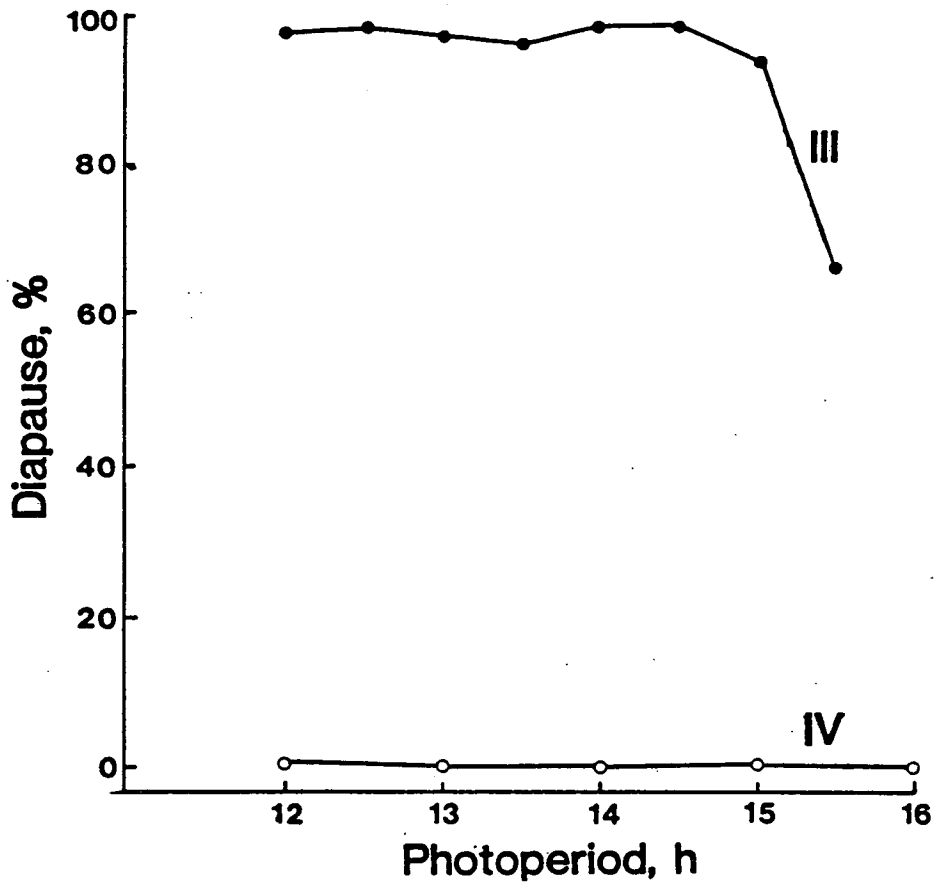


FIG. 2. The effect on the PPRC of S. argyrostoma of exposing the intrauterine stages to LD12:12, 25 ± 1°C (Curve III ●-●) as opposed to LL, 25 ± 1°C (Curve IV o-o), before transferring larvae to photoperiods at 20 ± 0.05°C.

within the boxes, and the protracted embryogenesis (about 20 days at 20°C compared with 9 days at 25°C). In several photoperiods, females produced very few viable progeny, and consequently, in some photoperiods, the number of larvae per culture is unusually low. Under these conditions, the diapause response was saturated at 20 ± 0.5°C and the critical photoperiod lay between 14 and 15 hours. This experiment was repeated, but adult mortality was again high and small numbers of larvae were produced at only 2 photoperiods, LD12:12 and DD. Ninety percent of individuals which had experienced LD12:12 throughout development entered pupal diapause (n = 41), whereas, 3 out of 10 larvae which experienced continuous darkness (DD) entered diapause. Although this number was ridiculously small, the result demonstrated that diapause induction could occur in DD at 20 ± 0.5°C. The proportion of pupae entering diapause in short daylengths was very low (less than 20%) in cultures when intrauterine stages experience LL 20 ± 0.5°C (Curve IV) or LL 25 ± 1°C (Curve V) (Fig. 3).

A similar experiment, exposing adults and larvae to the same photoperiods but at a higher temperature (25 ± 1°C) was performed. The results, (Table 7, PPRC VII) also demonstrated the importance of the intrauterine embryonic stage: although the diapause

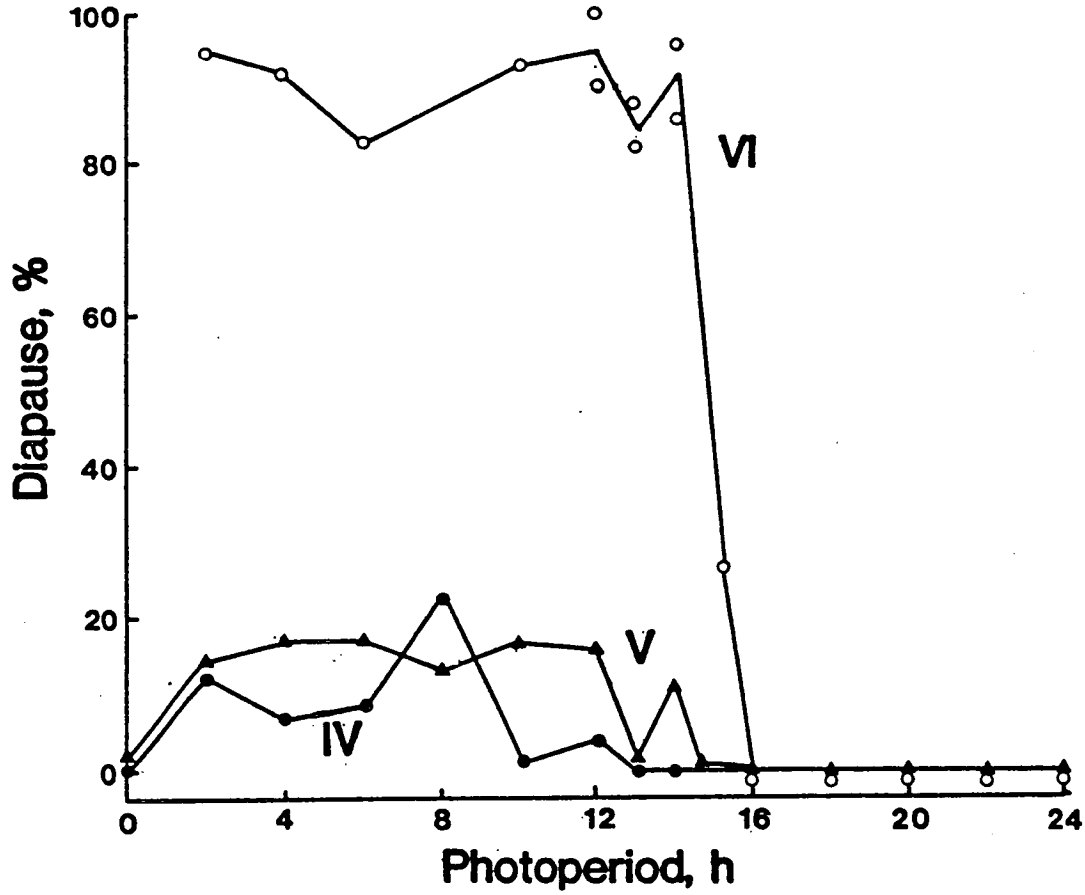


FIG. 3. The effect on the PPRC of S. argyrostoma of exposing the intrauterine stage to the same photoperiod as the developing larval instars (Curve VI o-o, adults and larvae at 20 ± 0.5°C) as opposed to LL before transfer to photoperiods after larviposition (Curve IV ●-● adults 25 ± 1°C, larvae 20 ± 0.5°C; Curve V ▲-▲ adults and larvae 20 ± 0.5°C).

TABLE 6

The effect of intrauterine exposure to the same photoperiod as developing larvae (PPRC VI) on the photoperiodic induction of pupal diapause in S. argyrostoma, as opposed to constant light (LL), before transfer to photoperiods after larviposition (PPRC V). All at 20 ± 0.5°C.

Photoperiod (h)	Adults, LL		Adults, Photoperiods	
	Number of Prepupae Formed	Diapause %	Number of Prepupae Formed	Diapause %
0	250	2.2	-	-
2	144	14.9	23	95
4	125	17.6	286	90.6
6	103	17.7	57	83.0
8	130	13.4	-	-
10	152	17.0	118	93
12	130	16.1	26	100
13	148	1.4	410	82.4
13	-	-	403	88.1
14	139	11.5	39	86.0
14	-	-	123	96.0
14.5	128	1.7	-	-
15	161	6.6	39	27.5
16	148	0	101	0
18	158	0	-	-
20	146	0	-	-
22	189	0	38	0
24	175	0	58	0

incidence was unsaturated under these regimes, larvae experiencing LL 25 ± 1°C as embryos before transfer to short day photoperiod, produced no diapausing pupae (Table 8). The results also showed that a light cycle of LD 0.5:23.5 was capable of inducing a similar photoperiodic response as LD6:18, but no diapause occurred in DD, 25 ± 1°C.

Figure 4 depicts the photoperiodic response curves of larvae which experienced the same photoperiod throughout embryonic and larval development at 20 ± 0.5°C (Curve VI) and 25 ± 1°C (Curve VII) and demonstrates that temperature can modify the photoperiodic response by reducing the overall diapause incidence: the diapause incidence at 20°C at 6:18 and 13:11 being 83% and 85.3% whereas under the same photoperiods at 25°C being 14.7% and 51.7% respectively. The critical photoperiod lay between 14.5 and 15 h at 20°C, but the incidence of diapause was too low at 25 ± 1°C to determine a critical photoperiod.

A comparison of Curves I (Fig. 1) and IV (Fig. 3) also illustrates a similar effect of temperature on the photoperiodic response. Again, an increase in temperature resulted in a decrease in the level of diapause.

TABLE 7

The incidence of pupal diapause in S. argyrostoma larvae which experienced the same photoperiod and temperature (25 ± 1°C) as intrauterine embryos and larval instars (PPRC VII).

Photoperiod (h)	Number of Prepupae Formed	Diapause %
0	381	0
0	218	0
0.5	193	22.3
0.5	98	14.4
2	255	19.6
2	153	19.6
4	122	10.6
4	222	9.6
6	40	Not used
6	211	14.7
8	182	28.7
8	289	37.3
13	196	39.2
13	175	64.2
14.5	83	34.1
14.5	159	36.6
16	161	0
16	53	0

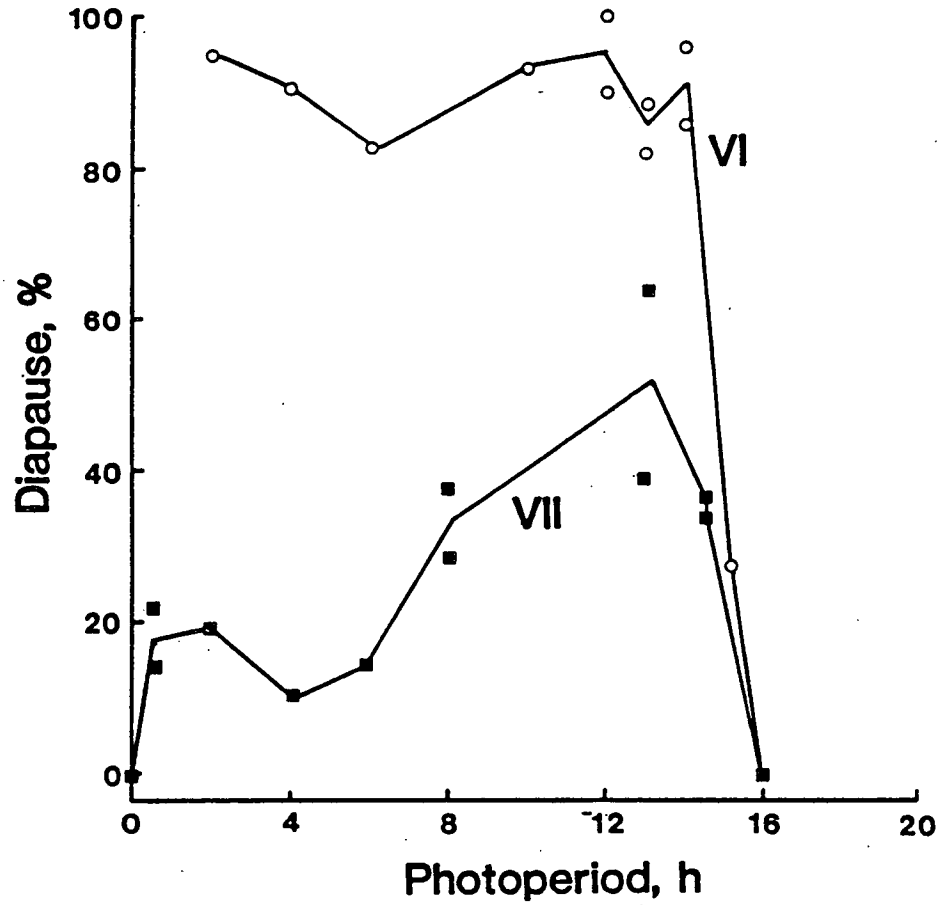


FIG. 4. The effect of constant temperature on the incidence of pupal diapause; adults and larvae maintained in same photoperiod throughout development, at either 20 ± 0.5°C (Curve VI o-o) or 25 ± 1°C (Curve VII ■-■).

Early Embryonic Sensitivity to Photoperiod

Adult flies were kept at LL 25 ± 1°C and transferred to LD12:12, 25 ± 1°C at sequentially later stages of ovarian and embryonic development. Larvae produced by these flies were kept at LD12:12, 25 ± 1°C, throughout development. Further details of the experiment are given on p. 70 .

The rate of embryonic development in short days was slower than that under constant light (Table 8), flies maintained at LD12:12, 25 ± 1°C since eclosion, depositing larvae on day 12, whilst those adults that experienced only constant light, producing larvae 9 days after eclosion. Flies which were transferred to LD12:12 after 1, 2, 3 and 4 days in LL, all produced larvae on the same day, but in differing numbers, a greater number of larvae being produced by flies which had experienced more LL cycles. For example, adults which experienced 3 days at LL produced more progeny than adults which had experienced only 2 days.

Table 9 and Figure 5 illustrate the importance of the intrauterine stage in the induction of pupal diapause in Sarcophaga argyrostoma. All cultures became post feeding after 6 days, and with one exception, the median of puparium-formation occurred about 3 days later, therefore larvae

TABLE 8

The effect of the number of LD12:12 cycles experienced by adults on the rate of embryogenesis at 25 ± 1°C.

Number of days in LL 25 ± 1°C	Number of LD12:12 cycles experienced	Days from eclosion to Larviposition
0	12	12
1	10	11
2	9	11
3	8	11
4	7	11
5	5	10
6	3	9·5
7	2	9
9	0	9

TABLE 9

The incidence of pupal diapause in cultures transferred from LL to LD12:12, on sequentially later days of ovarian and intrauterine embryonic development. Adults and larvae at 25 ± 1°C.

No. of Days in LL	No. of LD12:12 cycles experienced before larviposition	Larvae per mousetray	Diapause %	Total No. Larvae Density 2/Jar	Diapause %
0	12	98	63	137	88
	12	90	75.9	124	79
1	10	88	60.5	145	86
	11	69	85	130	87
2	9	97	84	144	93
	10	27	84	120	95
3	8	97	79	125	91
	9	46	79	121	93
4	7	111	83	124	84
	8	-	-	136	89
5	5	102	85	138	93
	6	101	78	164	89
6	3	83	89	130	90
	4	95	87	140	93

TABLE 9 (Contd.)

No. of Days in LL	No. of LD12:12 cycles experienced before Larviposition	Larvae per mousetray	Diapause %	Total No. Larvae Density 2/Jar	Diapause %
7	2	99	78	141	81
	3	91	94	141	99
9	0	95	0	110	0
	0	51	0	126	0

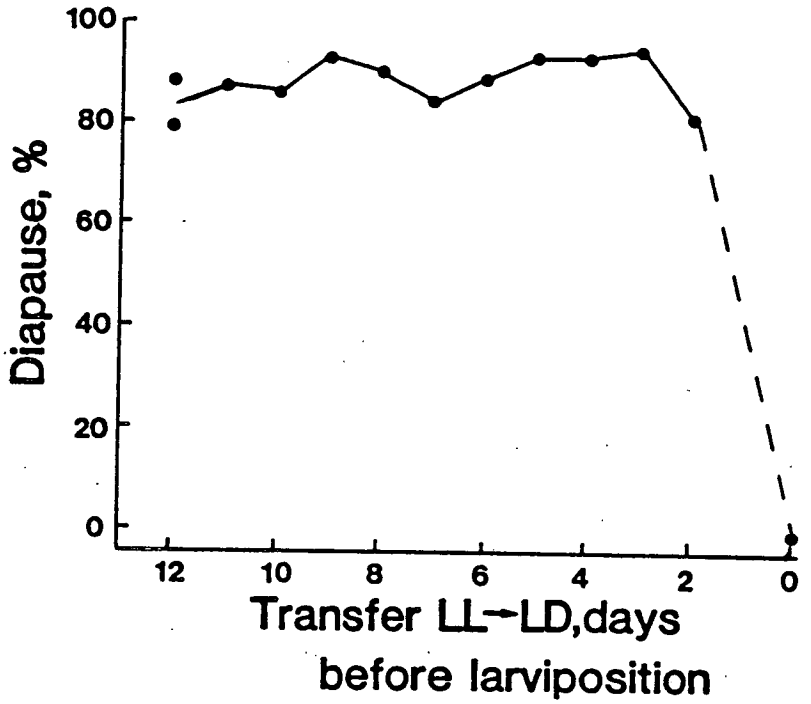


FIG. 5. The incidence of pupal diapause in cultures of S. argyrostoma, transferred from LL, 25 ± 1°C to LD 12:12, 25 ± 1°C at sequentially later stages of ovarian and embryonic development. Deposited larvae were maintained in LD 12.12, 25 ± 1°C throughout development.

experienced on average an additional 9 LD12:12 cycles between larviposition and puparium-formation. The median of puparium formation in progeny from adults kept in LL occurred 2 days after larvae became postfeeding, thus these larvae experienced on average 8 LD12:12 cycles.

Eight LD12:12 cycles experienced during larval development at 25 $\pm$ 1^{o}C were insufficient to induce diapause. However an additional 2 cycles of LD12:12 experienced just before larviposition plus 9 inductive cycles during larval development raised the diapause incidence to 80%. If the intrauterine embryos experienced three LD12:12 cycles, and LD12:12 cycle were given throughout larval development, the diapause response became saturated.

MATERNAL INFLUENCE ON THE INCIDENCE OF DIAPAUSE AMONG THE 1st GENERATION PROGENY OF ADULTS WITH A HISTORY OF DIAPAUSE

Adult flies with and without a history of pupal diapause were produced by methods given on p.73, and progeny from these flies were reared in a range of strongly diapause-inductive conditions. A summary of the experimental protocol is illustrated in Table 2 and the results of the experiment displayed in Table 10.

TABLE 10

The incidence of pupal diapause in progeny of individuals with no diapause history (N) and individuals with a diapause history (D).

Adults 25°C LD12:12	Larvae Photo-period	Larvae Temperature °C	Number of Prepupae	Diapause Incidence %
N	2:22	17 ± 0.5	157	94.3
N	4:20	"	165	95.1
N	8:16	"	162	93.8
N	10:14	"	158	94.3
*N	12:12	"	222	94.1
N	13:11	"	172	94.2
N	14:10	"	199	97.0
N	14:5:9.5	"	181	92.3
D	12:12	17 ± 0.5	575	94.8
D	12:12	"	435	97.1
*D	12:12	"	182	90.6
D	12:12	25 ± 1.	162	99.4
D	12:12	25 ± 1.	322	89.1

X^2 on cultures marked with asterisk = 1.3, df = 1
$P > 0.05$ N.S.

Under all the experimental conditions used, the diapause response in all cultures was close to saturation. A X^2 test was performed on the proportion of individuals entering diapause in cultures which had experienced identical conditions during embryonic and larval development and differed only in the diapause history of the parental generation, $X^2 = 1.3$, df = 1 $p > 0.05$. Thus, there was no significant difference in the proportion of individuals entering diapause in progeny of parents with or without diapause histories.

DISCUSSION

GENERAL FEATURES OF THE PHOTOPERIODIC RESPONSE

The results from this section are consistent with those reported by Saunders (1971): Sarcophaga argyrostoma possesses a temperature modified photoperiodic response, typical of a long day species, where in wild long day photoperiods promote continuous development and short day lengths combined with low temperatures tend to induce diapause (Fig. 1). Thus at short days, an increase in the larval breeding temperature produces a decrease in the overall photoperiodic response whereas a decrease in temperature results in an increase (e.g. Fig. 4).

Saunders (1971), using the Edinburgh strain of S. argyrostoma, raised larvae, deposited by adults kept in LD16:8 25°C, in short day photoperiods at a range of temperatures. A comparison of the levels of diapause obtained then and those reported in this thesis, indicate that the degree of the photoperiodic response in the Edinburgh strain has diminished. In 1971 for example the incidence of diapause in larvae bred at 20°C in strong short day photoperiods (9-13 h of light/24) ranged from about 75% to 85%, whereas, in an equivalent experiment (Table 5b PPRC IV), performed in 1982, the incidence of pupal diapause over the same range of photoperiods was negligible (0-4.2%). In short, the same degree of photoperiodic response achieved in 1971 at 20°C could only be produced in 1982 by lowering the larval breeding temperature to 17°C (Fig. 1). This type of reduction in diapause incidence within a stock culture over generations of breeding is not unknown. Adkisson (1966) for example, found that in the pink bollworm, Pectinophora gossypiella, over 23 generations with no selection pressures, there was a 25% decrease in diapause incidence. Likewise, House (1967) reported a decreasing occurrence of diapause in the fly Pseudosarcophaga affinis through successive laboratory reared generations. In contrast, however, the present results for

S. argyrostoma (Fig. 1) demonstrate that the critical daylength has remained remarkably constant throughout the 12 year period, the value being close to 13.5 to 14 h light per day. This indicates, perhaps that the time measuring properties of the system have remained unchanged, even through diapause incidence in short days has declined.

The photoperiodic response curves and the values for the critical daylength for the Edinburgh strain of Sarcophaga argyrostoma have previously been derived from regimes in which the intrauterine stage experienced non-diapause inductive photoperiods (LD18:6 or LL) before larval exposure to a range of inductive daylengths. Present data (Fig. 2 and 3), however, now demonstrate the importance of the intrauterine embryo in diapause induction, confirming earlier observations (Denlinger, 1972). For example, under short day conditions at larval breeding temperatures of 20 and 25°C and prior embryonic exposure to LL, diapause induction is low, but if intrauterine embryos experience LD12:12 or the same short photoperiod as the developing larvae, the incidence of pupal diapause is close to saturation. In addition to causing a quantitative change in the incidence of diapause, embryonic exposure to short day photoperiods also affects the position of the critical daylength. For example, if embryos

experienced LL, the critical photoperiod lies between 13.5 and 14 h (Fig. 1, Curves I and II), whereas if both embryos and feeding larvae experience the same inductive photoperiod and temperature, the critical daylength becomes 14.5 to 15 h (Fig. 3, Curve VI). A further increase in the critical photoperiod to a value greater than 15.5 h (Fig. 2, Curve III) may then be achieved by exposing pregnant females to LD12:12 cycles before transferring the newly deposited larvae to a range of photoperiods at 20 ± 0.5°C. Since Saunders (1971) found that the 'position' of the critical photoperiod was temperature compensated, the above changes in the critical photoperiod are not due to differences in the larval breeding temperature. One possible explanation of these results may be derived from the original model for photoperiodic summation (Saunders, 1971), which proposed that during the photoperiodic sensitive period (the entire larval period) the larvae 'add up' successive short day cycles and enter diapause if a sufficient number of such cycles (Required Day Number, RDN) have been seen before sensitivity ends. The RDN was found to be almost temperature compensated (Q_{10} = 1.44), but varied with the larval breeding photoperiod: at 20°C and LD10:14 the RDN was 13-14 but at daylengths close to the critical photoperiod e.g. 10:14 it was 16-17. At still longer photoperiods, it was suggested

that the RDN would be so large that, at 20°C, none of the larvae would experience the RDN before the end of the sensitive period. For S. argyrostoma at 20°C, the RDN at LD14:10 (16-17) was slightly greater than the number of light cycles experienced by only the developing larvae. However, the additional embryonic exposure to 14:10 supplied in the present work would serve to increase the number of light cycles experienced to a value greater than the RDN at that photoperiod and, consequently, account for the increase in diapause from 0% to 96% (Table 6). A similar relationship between RDN and photoperiod had earlier been observed for Nasonia vitripennis (Saunders, 1966a). This explanation may be over-simplified, however, since data shown in Chapter II of this thesis present two additional complications to the simple cumulative model: (1) that photoperiodic sensitivity declines from embryo to mature larvae, and (2) that photoperiod and/or diapause-commitment itself extends the length of larval life and may thus allow further accumulation of inductive photo-periods before the end of the sensitive period. These complications are discussed overleaf.

DELAY OF PUPARIATION IN DIAPAUSE - COMMITTED LARVAE

Denlinger (1972) and Saunders (1972) working with Sarcophaga bullata and Sarcophaga argyrostoma, respectively, reported that larvae with a history of exposure to long days developed more rapidly than those with a history of short days. Data in this section (Tables 3 and 4) and results for rate of puparium formation of stock, fast and slow strains of S. argyrostoma (Section III Table 40) are consistent with this observation. There is no significant difference in the rate of larval development during the feeding stage in long day (18:6) and short day (12:12) individuals at 25 $\pm$ 0.5°C (Richard, D.S. and Saunders, D.S. pers. comm.). Therefore, any difference in the duration of larval development must lie in the length of the post feeding (wandering) stage. Denlinger (1972) concluded that the delay in puparium formation seen in a short day individual was a result of the larva's commitment to diapause, i.e., the delay in pupariation was a result of the altered endocrinological state of the diapause-committed larva. On the other hand, Saunders (1972) regarded the protracted larval development in short day conditions to be a result of a direct photoperiodic effect: at 17°C the duration of the larval period being on average 12.8 days at 16:10, 13.9 days at

14:10 and 16.0 days at LD12:12. The R.D.N. (Required Day Number) at LD12:12 was sufficiently low so that as a consequence of the protracted larval development at that photoperiod, the larvae experienced more than enough inductive light cycles and consequently entered diapause. Short days (long nights) induced both pupal diapause and protracted development and the synergistic effect of both resulted in a heightened diapause response and presumably, a sharpened critical daylength. Further evidence supporting the hypothesis that development was independently programmed by photoperiod was put forward by Saunders (1976): non-diapause destined larvae bred in 10:14 (n=920) formed prepupae on average 2 days later than non-diapause destined larvae bred at 14:10 (n=639). However, the difference in the size of the cultures, and therefore the post feeding density might provide a possible alternative explanation to account for the differential timing of puparium formation, since high density during the post feeding stage is known to delay pupariation (section II, pp. 170, Fig. 18). Data taken from the photoperiodic curves in this thesis, moreover (Table 11), show that the wandering period in non-diapause destined larvae bred in short day photoperiods was not significantly different to that of non-diapause destined larvae of a similar culture

TABLE 11

The length of the larval period (days) of non-diapause destined larvae of S. argyrostoma, adults and larvae maintained in various combinations of photoperiod and temperature.

Photoperiod and Temperature				No. Prepupae Formed	% Diapause	Days to Puparium Formation Mean ± 1SD
Adults		Larvae				
LL	20°C	10:14	20°C	203	1	10.0 ± 0.2
LL	20°C	18:6	20°C	237	0	10.5 ± 0.5
LL	20°C	13:10	20°C	148	1.4	10.2 ± 0.86
LL	20°C	16:8	20°C	148	0	10.9 ± 0.68
LL	25°C	12:12	20°C	338	4.2	10.3 ± 0.52
LL	25°C	20:4	20°C	327	0	11.6 ± 0.77
12:12	25°C	12.5:11.5	20°C	153	98	14.5 ± 1.2
12:12	25°C	14.5:9.5	20°C	161	98	15.7 ± 1.2
12:12	25°C	15:9	20°C	157	93.2	16.2 ± 1.4

size, bred in long day photoperiods. Slightly weaker evidence supporting the hypothesis that it is the larval commitment to diapause that causes the delay in pupariation is provided in cultures where prior embryonic exposure to LD12:12 before transfer to photoperiods resulted in a saturated incidence of diapause at LD12.5:11.5, LD14.5:9.5 and LD15:9. Again, the rates of pupariation at all photoperiods were similar (Table 5a and Table 11). Although an unequivocal discrimination has not been made between these opposing views and, indeed, both are possibly valid, the new evidence seems to support the proposition that the delay in puparium formation in short days animals only occurs when the majority of larvae are committed to enter diapause. As pointed out by Denlinger (1972), this delay may be an adaptive advantage to a diapause destined larva, allowing more time for it to find a suitable place to hibernate.

C H A P T E R II

THE PHOTOPERIODIC COUNTER

THE PHOTOPERIODIC COUNTER

INTRODUCTION

In many insect species, the final photoperiodic response has been shown to be a consequence of the number of photoperiodic light cycles that the insect experiences during the restricted photoperiodically sensitive period. This summation of photoperiodic cycles has been found to be temperature-compensated in a number of species, e.g. Acronycta rumicis (Goryshin and Tyshchenko, 1970); Mamestra brassicae (Goryshin and Tyshchenko, 1973) and Nasonia vitripennis (Saunders, 1966). These authors concluded that photoperiodic summation was achieved by means of a temperature-compensated photoperiodic "counter". These results and other evidence supporting the existence of the counter are described in detail in the General Introduction (p.48-53).

Saunders (1966), working with the wasp Nasonia vitripennis, introduced the concept of the Required Day Number (RDN) which he defined as the number of photoperiodic cycles required to raise the proportion of female wasps producing diapause progeny to 50% (see pp. 54). The flesh-fly Sarcophaga argyrostoma is also suitable for the analysis of the cumulative effects of photoperiod, and work by Saunders (1971, 1982) has produced data comparable to

that from Nasonia vitripennis. Saunders kept larvae of Sarcophaga argyrostoma under short day, diapause-inductive, light cycles (LD10:14), at a range of constant temperatures (16, 18, 20, 22, 24 and 26°C). When the incidence of pupal diapause in each group was plotted against the number of light cycles seen, a 'family' of curves, all showing the same upward trend was produced. In addition, the temperature coefficient for the number of light-dark cycles needed to raise the proportion of diapausing pupae in one day's collection to 50% was close to unity (Q_{10} = 1.4).* These results provided compelling evidence that S. argyrostoma possessed a temperature-compensated photoperiodic counter which adds up successive short days or long nights. In temperatures at which the diapause response was not saturated (22 and 24°C), the curves showing the daily rate of puparium formation were bimodal; developing individuals tended to be found in the first peak and diapausing individuals in the second. Since a batch of larvae form puparia over a range of days, and the intrapuparial stages are insensitive to photoperiod (Saunders, 1971) it follows that those larvae which form puparia first experience fewer light cycles than those which form puparia later.

*Gibbs (1975) demonstrated that a temperature step up or down within 24 hours of puparium formation can alter the final diapause incidence in a culture. If this result is taken into account the Q_{10} in this experiment may be recalculated as 1.04 (Saunders, 1976).

As described before, the incidence of pupal diapause has been shown to rise if the larvae experience increasing numbers of short day (= long night) cycles. Saunders also showed that the rate of larval development was temperature dependent (the temperature coefficient (Q_{10}) for the rate of larval development (16-26°C) was 2.7). Bearing these results in mind Saunders therefore proposed that the induction of diapause resulted from an interaction between a temperature-dependent process (larval development) and a temperature-compensated photoperiodic counter: at high temperatures (e.g. 26°C) and short day conditions, the rate of larval development was fast (= short sensitive period) and larvae did not experience a sufficient number of short day cycles to induce diapause. At intermediate temperatures (22 and 24°C) larvae which formed puparia first did not see enough inductive light cycles to induce diapause, whereas larvae which formed puparia later saw more than the RDN and became dormant. At lower temperatures, when larval development was protracted, larvae experienced more than the RDN and consequently entered diapause (Saunders, 1971).

Gibbs (1975), also working with Sarcophaga argyrostoma, found that the incidence of pupal diapause could be modified by a temperature shift

within 24 hours of puparium formation, i.e., after the loss of photoperiodic sensitivity. For example when puparia formed at 16°C were transferred to 22, 24, 26 or 28°C (temperature steps-up), a decrease in the incidence of pupal diapause was observed, the degree of the effect increasing with the size of the temperature step. A temperature step down (22 to 16°C), on the other hand, resulted in an increase in the number of pupae entering diapause. He suggested that temperature can alter the incidence of diapause in S. argyrostoma by directly affecting the neuroendocrine system, as well as indirectly by modifying the length of the sensitive period.

To account for these findings, and those of Saunders (1971), Gibbs proposed a model for the photoperiodic counter in which the diapause-inducing effects of successive short day (or long-night) cycles were summed and stored in the form of a 'diapause titre' during the sensitive period (larval stage). The developmental fate of a given individual was determined by a comparison of this 'diapause titre' with an internal threshold value: if the 'titre' exceeded this threshold, the individual would enter diapause, whereas if the titre was below the threshold, the pupa would not enter diapause. Gibbs's model incorporated three assumptions: (1) There was a distribution of values

of thresholds amongst individuals, and that the same distribution of thresholds was present in individuals which formed prepupae early as well as those which formed puparia later; (2) the threshold of each individual was invariant with time; and (3) the rate of increase in 'diapause titre' per short day was constant over time and identical for all individuals in the population. The model is illustrated in Fig. 6. Gibbs envisaged that a temperature step-up might lower the 'diapause titre' below the internal threshold and a step-down might increase it. He also found that when pupae were transferred from 16°C to 28°C individuals which had previously experienced 30 short day cycles were relatively more resistant to a change in diapause incidence than those individuals which had experienced only 21 short days. This implied that the diapause titre was higher in the former group, i.e., the higher the diapause titre in relation to the threshold, the more difficult it was to lower it below the threshold using a temperature step up.

Gibbs's model of the photoperiodic counter may be tested by altering the sensitive period using methods other than temperature, thereby manipulating the number of diapause inductive cycles which larvae experience. The simple hypothesis is that an increase in the length of the photoperiodically

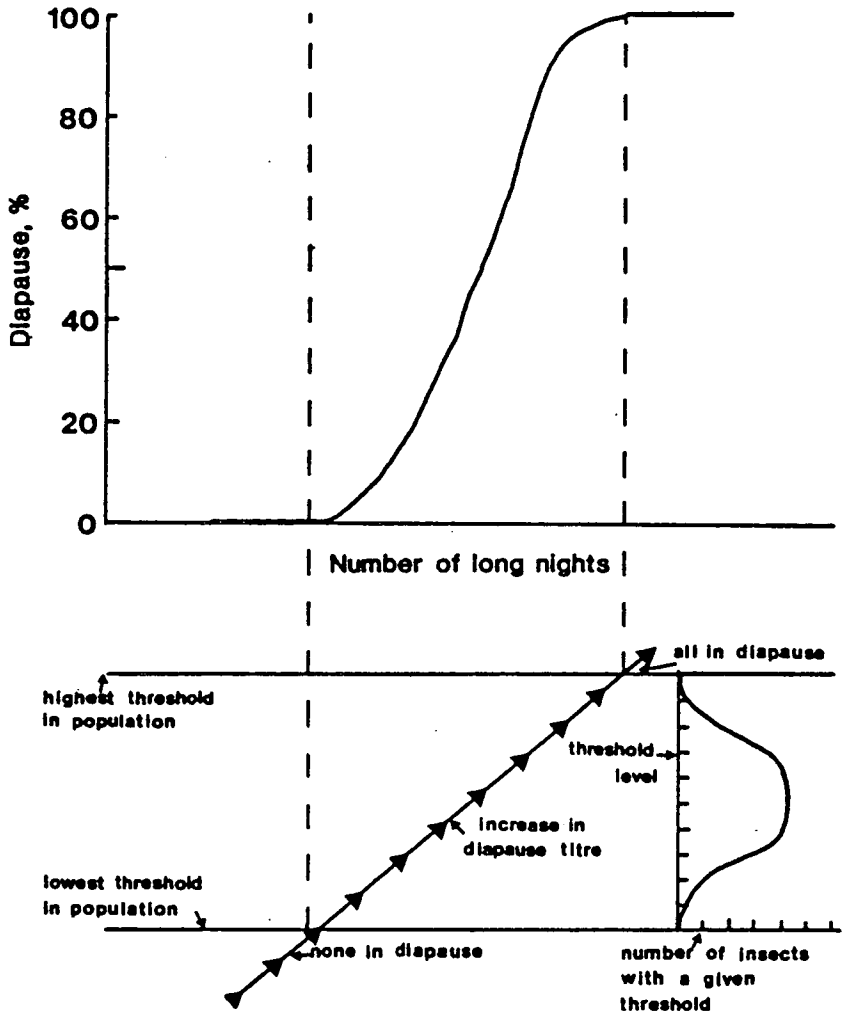


FIG. 6. Theoretical model to account for the summation of long nights by the photoperiodic counter. Upper panel: the increase in pupal diapause with an increase in the number of long nights experienced during the larval sensitive period. Lower panel: the theoretical increase in the 'diapause titre' with increasing number of long nights, against the presumed lowest and highest individual larval thresholds in the population. As more and more larvae cross their particular internal thresholds a greater proportion of the population enter diapause. At right: the theoretical distribution of diapause thresholds in the population. (From Gibbs, 1975).

sensitive larval period would result in an increase in the level of diapause, whereas a decrease in the length of the larval stage, would produce a subsequent decrease in the incidence of diapause. Preliminary experiments of this type have been attempted before (Saunders, 1975; Droop, 1975), but this section presents new and more extensive data.

The duration of the larval stage was experimentally manipulated by a number of methods; these are described on pp.112-121, in this thesis, and in the outline given below.

Treatment	Projected (Theoretical) Effect on the Duration of Larval Development and hence the sensitive period, based on Gibbs's simple model	
1) Wax treatment of adult flies to prevent larviposition	Increase	because it increases the number of ante-natal photo-periods
2a) Starvation of larvae still in the obligatory feeding phase (i.e. 1st, 2nd and early 3rd instar larvae)	Increase	because it lengthens the feeding phase.
2b) Starvation of larvae in the faculative feeding phase (late 3rd instar larvae):	Decrease	because it induces premature puparium formation.
3) Premature Extraction of 3rd instar larvae	Decrease	"

Treatment	Projected (Theoretical) Effect on the Duration of Larval Development and hence the sensitive period, based on Gibbs's simple model	
4) Crowding during the feeding phase:	Decrease	because it induces premature puparium formation.
5) Crowding during the wandering phase	Increase	because it delays puparium formation.
6) Wet Treatment During the wandering phase to prevent puparium formation	Increase	"

The above experiments were designed to test the validity of Gibbs's model in its broadest sense.

The following three questions were also asked:

1) Are short day (long night) cycles equivalent during the life cycle, i.e., is one long night experienced during the intrauterine stage equivalent to a long night seen later in development?
2) When does photoperiodic sensitivity end?
3) Is the 'diapause titre' subject to decay after the end of larval photoperiodic sensitivity?

MATERIALS AND METHODS

MANIPULATION OF THE SENSITIVE PERIOD: THE USE OF WAX TO DELAY LARVIPOSITION

Larvae taken from adult stock cages kept at LL 25 ± 1°C were cultured in the same conditions. Newly formed prepupae were divided into two equal groups, then placed in glass jars, which were sealed with paper, and incubated in constant darkness (DD) at 22°C. To obtain synchronised colonies, each jar was placed inside a small 'insect' cage for a period of 24 hours to collect emerging adults. Twenty four hours later, each of the jars was then transferred to another cage, and this procedure was repeated until all the flies had emerged. The two cages containing the largest numbers of flies were kept at LL 25 ± 1°C; the rest were discarded. The adult flies were then provided with water and sugar ad libitum and with meat from day 4 onwards. On day 9 both cages produced a small number of larvae; these were discarded. A fresh piece of meat was then placed in one of the cages (control). Individual female flies were removed from the other cage by hand, and a small amount of molten ester wax was painted on to the tip of the abdomen, effectively preventing normal larviposition. The 'wax treated' flies were then placed inside a new cage and provided with sugar, water and meat. After twenty

four hours the meat containing newly deposited larvae was removed from the control cage, and three groups of about 300 larvae were set up for culture, and then transferred to LD12:12, 16.5°C ± 0.5°C. At the same time, the cage containing the 'waxed' females was also transferred to LD12:12, 16.5°C ± 0.5°C. After 48 hours, the 'waxed' females were sacrificed by cutting off the tip of the abdomen and expressing the intrauterine larvae onto a 50 gram piece of meat. It was not possible to count individual larvae at this stage, since they were very small and tightly clumped, but larvae from thirteen abdomens were used in each culture. Three cultures were set up in this manner and kept at LD12:12 16.5°C ± 0.5°C. Newly formed prepupae were then collected daily, and kept under the same conditions for four days, before transfer to an incubator at 22°C in constant darkness for a further fourteen days. Pupae were then scored for diapause or non diapause development using the criteria described on p. 61.

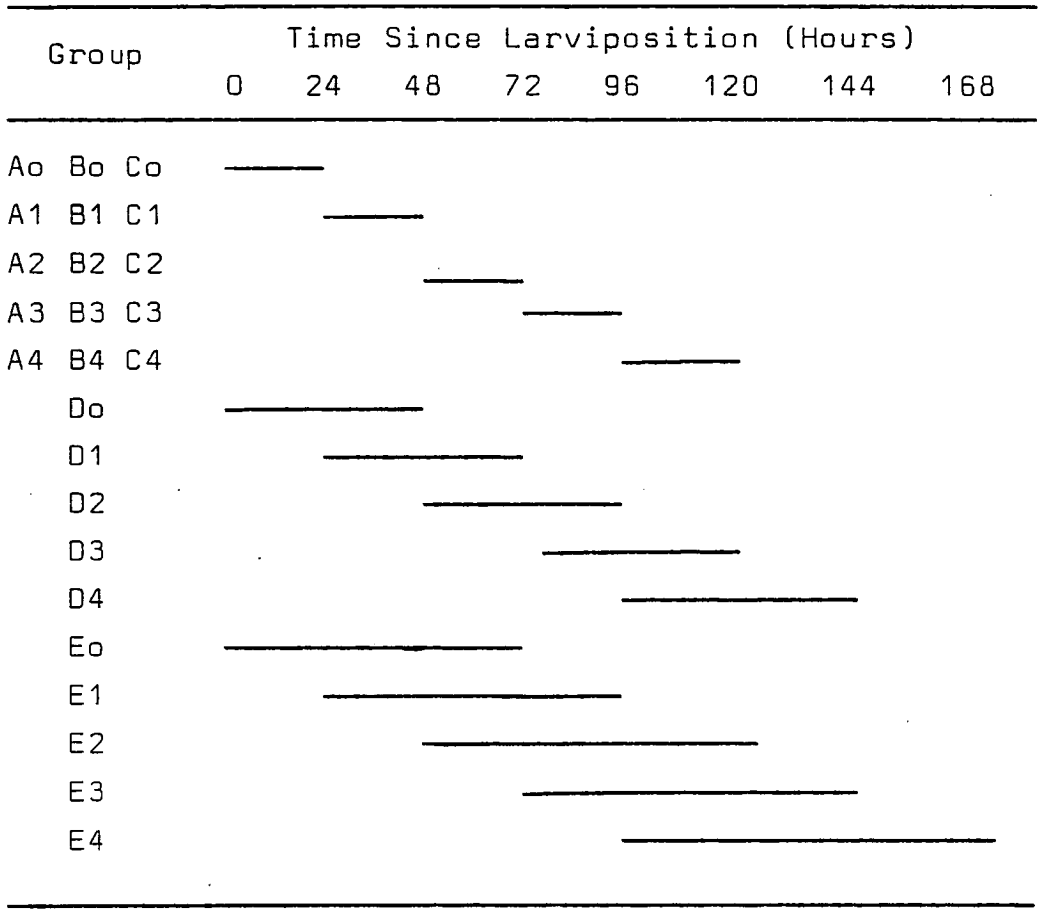
MANIPULATION OF THE SENSITIVE PERIOD: DELAY BY STARVATION

Meat carrying larvae deposited over the previous 24 hours was removed from adult stock cages kept at LL, 25°C. Larvae from each cage were pooled, and cultures of 250 larvae set up as described on [illegible] p.60 . The cultures were then transferred to LD12:12, 17°C ± 0.5°C. On day 1, 18 cultures were set up in this manner and a further 12 cultures set up on day 2. Different groups of larvae were then starved for either 24, 48 or 72 hours at different times during their developmental period (see p. 115). Three control cultures were set up on day 1 and two on day 2; these cultures were left to develop without interference.

To starve cultures, larvae were removed from the food source and, with the exception of newly deposited larvae, rinsed in water at 17°C and placed on damp filter paper (Whatman 5.5 cm 6 qualitative) inside a 1 lb glass kilner jar. Newly deposited larvae (groups A_o, B_o, C_o, D_o, E_o), on the other hand, were counted directly on to the damp filter paper. The jars were then closed with fine cotton gauze held in place by an elastic band. To prevent the desiccation of young larvae, the jar was then placed inside a 2 lb glass kilner jar containing 1"

MANIPULATION OF THE SENSITIVE PERIOD:

DELAY BY STARVATION



——— denotes period of starvation.

Groups A, D and E were set up on day 1, groups B and C (replicates of group A) on day 2.

of saturated KCl solution. (A saturated solution of 1 part by weight KCl:2 parts by weight H_2O gave a RH of 80% (Solomon, 1951)). First and second instar larvae are especially susceptible to desiccation, therefore the 2 lb kilner jars were sealed with air tight lids in all groups $A_{(o)}$-$E_{(o)}$ and A_1-E_1. In all other groups, the jar was not sealed to avoid asphyxiation of the larger 3rd instar larvae. After the period of starvation, the larvae were returned to the same tray of medium from which they had been removed. Early instars A-$E_{(o)}$ and $A_{(1)}$-$E_{(1)}$ were placed on a fresh 50 g piece of meat before being returned to the medium.

Cultures were examined daily to monitor the number of larvae which had left the medium in the previous 24 hours. Newly formed puparia were collected daily and placed in open petri dishes within the light tight boxes. After three days, each group was weighed, then transferred to a sealed plastic tube (3.5 cm diam.) and incubated at 22°C for 10 days. Pupae were subsequently scored for their non diapause or diapause status.

PREMATURE EXTRACTION

MATERIALS - METHODS

Meat carrying larvae deposited over the previous twenty four hours was removed from each of the adult stock cages kept at LL, 25°C. The larvae from each cage were pooled, then cultures were established by counting 250 larvae on to a 50 g piece of meat which was placed on 250 ml of synthetic medium in a tupperware dish. Cultures were transferred to LD12:12 17°C. Nine of these cultures were set up daily, for five consecutive days (see p.118); an additional two cultures were set up on the first day to monitor the timing of larval moults.

1. Timing of larval moults

On days 0, 1, 2 and 3 of cultures established on day 1, and on day 2 of cultures established on day 2, 3, 4 and 5, approximately 200 larvae were killed in 95% alcohol and examined under a binocular microscope. First and second instars were distinguished by the presence of anterior spiracles on the 2nd segment of the latter. Second and third instar larvae were distinguished by the number of slits in the posterior spiracles: two in the former and three in the latter. (Figure A, page 66).

The ratio of third instar larvae: second instar larvae provided an indication of the number of

larvae in other cultures which would be in the facultative feeding phase within the next 24 hours and therefore be competent to form puparia when extracted on day 3 of the culture.

Culture	Day of Extraction
1	Killed on Day 2
2	3
3	4
4	5
5	6
6	7
7	8
8	9
9	Control

Larvae were extracted from the culture medium on days 3, 4, 5, 6, 7, 8 and 9. Larvae in the control culture were left undisturbed. Extraction was carried out by scraping medium and larvae on to a fine mesh metal sieve, and the medium surrounding the larvae was gently washed away using water at $17^{o}C$. Using forceps, larvae were then placed in a mouse tray containing ½" of finely sieved sawdust. Larvae started to leave the medium on their own accord from day 7 of culture, therefore the number

of larvae already in the sawdust in cultures which were extracted on day 7 onwards was recorded.

Newly formed puparia were collected daily, placed in open 9 cm diam. plastic petri dishes to dry out, and then kept at DD 17°C. After three days, each group of puparia was weighed collectively, then transferred to a sealed 3.5 cm diameter plastic tube and incubated at DD 22°C for a further 10 days. The pupae were then scored for non diapause or diapause development as before (p. 61).

STARVATION AND PREMATURE EXTRACTION AT LD12:12 25°C

Meat carrying larvae deposited over the previous 24 hours was removed from the adult stock cage kept at LD12:12 25°C. Groups of 250 larvae were counted on to a 50 g piece of meat, then placed in a tupperware dish containing 250 ml of medium. The larvae were cultured at LD12:12 25°C. Groups of larvae were set up in this manner over consecutive days and were used either in the starvation or premature extraction experiments (see Tables 15-20). Starvations and extractions were performed using the techniques given on pp. 114 and 118. The two groups of larvae starved on day 0 were

combined on return to media (see Table 15). Once the larvae became post-feeding, the cultures were examined every 12 hours, newly formed puparia being collected and maintained at 25°C for 14 days, before being scored for diapause or non diapause development.

CROWDING DURING THE POST-FEEDING PERIOD

A large culture (about 200 larvae) was set up from the adult stock cage kept at LD12:12, 25 ± 1°C, by placing the meat containing the newly deposited larvae into a large tupperware dish (18 x 13 x 8 cm) containing 600 ml of synthetic medium. The dish was then placed inside a fine white gauze cover. Extra medium was added when necessary to ensure an excess of food throughout development. The entire experiment was performed at LD12:12, 25 ± 1°C, unless otherwise stated. When larvae started to leave the food, the culture (medium and larvae) was placed in a fine sieve and washed gently with water at 25°C to remove the medium. The larvae were then dried on paper towelling and transferred to containers at the different densities and conditions described below.

a. Containers: Glass Jars 11 cm high x 5 cm diameter

Plastic Jars 6 cm high x 3·5 cm diameter

b. Densities: 1, 2, 5, 10, 20, 25, 50, 75 and 100 larva/larvae per jar.

c. Conditions: Three flat teaspoons or one flat teaspoon of sawdust per glass jar or plastic jar, respectively. The finely sieved sawdust was either dry or supersaturated with water.

The jars containing dry sawdust were not sealed. In experiments using wet sawdust, however, the jars were sealed to prevent the sawdust from drying out. The jars were examined twice daily at 9 a.m. and 5 p.m., newly formed puparia being collected and kept at LD12:12, 25 $\pm$ 1^{o}C for a further 14 days, then scored for diapause or non diapause development.

RESULTS

MANIPULATION OF THE SENSITIVE PERIOD: THE USE OF WAX TO PREVENT LARVIPOSITION

The duration of the embryonic or intrauterine stage was prolonged by 48 hours, by preventing larviposition, using wax to block the ovipositor of the pregnant female. The delay resulted in a significant increase in the incidence of pupal diapause (X^2. 17.4, $p < 0.01$). The duration of the post feeding (wandering stage) was also increased, which resulted in a delay in the median of puparium formation (Table 12). Therefore, the increase in

TABLE 12

Lengthening the duration of larval development, by delaying larviposition by 48 hours. Adults LL, 25 ± 1°C, Larvae LD12:12, 16.5 ± 0.5°C.

Treatment	No. of Prepupae Formed	Time in Medium (Days)	Wandering Period (Days)	Pupariation Median (Days)	Diapause %
Non Wax 1	361	10	7.8	17.8	77.0
Non Wax 2	391	10	7.0	17.0	82.1
*Non Wax 3	331	10	+ 9.8	19.8	83.0
Wax 4	215	9 (+2)	16.6	27.6	96.5
*Wax 5	171	9 (+2)	+ 10.0	21.0	98.1
Wax 6	244	9 (+2)	17.3	28.3	97.6
Wax 7	195	9 (+2)	13.7	24.7	99.4

X^2 (Yates Correction Factor) carried out on the non wax culture with the highest diapause incidence and the wax culture with the lowest diapause incidence (cultures 3 and 5) $X^2 = 17.4$, $p < 0.01$.

diapause incidence may be due to two factors: (1) The additional two cycles of LD12:12 the embryos experienced whilst still in the maternal uterus; or (2) the additional number of LD12:12 cycles seen during the longer post feeding period. However, it is possible to exclude factor 2 by comparing cultures 3 and 5 (Table 12). The median of pupariation in culture 3 (non wax) occurred 19.8 days after larviposition, and 83% of the pupae entered diapause, whereas in culture 5 (wax), the median of pupariation was 21.0 days but 98.1% of the pupae were in diapause. A X^2 test was carried out on the proportion of pupae entering diapause in the two cultures (X^2, 21.4, $p < 0.01$). In these cultures at least, the two extra 12:12 cycles experienced by the intrauterine embryos resulted in a highly significant increase in diapause incidence.

The level of diapause incidence in control cultures was unsaturated but very high ($\sim$80%), possibly because the temperature of the constant temperature room cycled around 16.5 $\pm$ 0.5°C instead of 17°C $\pm$ 0.5°C as described in the starvation and premature extraction experiments, thus preventing a direct comparison of the slopes of the regression lines. The diapause incidences in the experimental cultures were highly saturated, i.e., $\sim$96%. It is possible that if the experiment was repeated at a

slightly higher temperature, where the diapause incidence in the controls was lower, the extreme sensitivity of the intrauterine larvae may not be masked. This was attempted but unfortunately the mortality rate in the waxed females was too high and subsequently too few viable larvae were produced.

MANIPULATION OF THE LENGTH OF THE FEEDING STAGES: STARVATION

To increase the duration of the larval feeding stage and hence the sensitive period, groups of larvae were starved for 1, 2 or 3 days commencing 0, 1, 2, 3 or 4 days after larviposition (see pp. 114-116 for details of methods used). Experiments using these techniques were performed on larvae which had been deposited by adults kept at LL, 25 ± 1°C and then transferred to LD12:12, 17 ± 0.5°C. Cultures A, D and E were from larvae deposited on the same day, cultures B and C from larvae deposited 24 hours later.

The number of days larvae spent in the food medium, i.e., excluding the period of starvation) are shown in Table 13a and 13b. Larvae which had been starved for one day during the early part of development, e.g., day 0, day 1 and day 2 (replicate A) and day 0 (replicates B and C) spent 8 days in the medium whereas if larvae were starved for one day on day 3 (replicate A) and days 1 and 3 (replicates B and

TABLE 13a.

The effect of the timing and duration of the starvation period on the duration of the feeding phase in S. argyrostoma larvae (% larvae leaving media per day) Adults LL, 25 ± 1°C Larvae LD12:12, 17 ± 0.5°C. A, D and C are groups of sibling larvae. Median day of pupariation underlined * denotes bimodality.

Duration of Starvation (Days)	Start of Starvation (Days)	Number of Days Spent in Medium									n
		3	4	5	6	7	8	9	10	11	
1	0(A)					9.9	80.9	9.1			121
1	1						52.0	44.9	3.1		98
1	2			0.7	2.0	24.4	68.0	4.8			147
1	3				17.4	44.5	31.2	7.3	4.1		218
2	1(D)		1.9	1.9	3.8	43.3	39.6	9.4			106
2	2		1.2	5.3	0.6	22.3	46.4	18.2	5.9		170
*2	3		46.0	0.6	-	19.9	31.0	2.5			161
3	2(E)	1.2	0.6	9.8	8.6	77.0	2.9				174
*3	3	48.0	3.4	-	-	-	17.8	30.7			179
(1)	Control A					64.4	24.8	15.2			177
(2)	Control D				1.0	2.9	29.9	42.6	10.8	12.7	204
(3)	Control E				0.5	9.1	39.0	37.6	12.7	1.0	197

TABLE 13b

The effect of the timing of a one day starvation period on the duration of the feeding phase in S. argyrostoma larvae (% larvae leaving media per day). Adults LL, 25 ± 1°C. Larvae LD12:12, 17 ± 0.5°C. B and C are groups of sibling larvae. Median day of pupariation underlined. *Denote bimodally.

Start of Starvation (Days)	Number of Days Spent in Medium									n
	3	4	5	6	7	8	9	10	11	
0(B)					4.0	86.0	10.0			150
1			0.9	-	76.9	16.2	6.0			117
*2		28.4	4.2	0.5	7.4	30.2	24.7	4.7		215
3		1.5	0.5	5.1	85.1	7.7				195
*4		51.7	1.7	0.6	14.8	30.7	0.6			176
0(C)					2.4	85.5	12.1			165
1			0.8	7.6	57.6	33.3	0.8			132
*2		9.0	1.7	-	15.7	38.2	29.2	6.2		178
3			0.9	1.4	85.2	11.4				210
*4			17.3	2.2	1.3	73.3	5.8			225
Control (B)				1.1	14.8	70.4	10.0	3.7		189
Control (C)				0.5	1.0	4.5	65.3	19.6	9.0	199

C), the number of days spent in the medium was reduced to 7. Cultures starved for 2 or 3 days fed on average between 7 and 8 days. The average time spent in the food by larvae in control cultures was between 8 to 9 days with one exception. In the culture designated control A, the majority of larvae left the food prematurely on day 7, although there was no obvious reason why this should have occurred. In all the afore-mentioned cultures, the larvae resumed feeding when replaced in the food after starvation. However, in several of the cultures a proportion of the larvae did not feed when returned, and left the food prematurely, resulting in a bimodal distribution of time spent in culture for these groups. This bimodal distribution was clearly seen when larvae were starved for 2 and 3 days on day 3 (Table 13a) and slightly less clearly when larvae were starved for 1 day on days 2 and 4 (Table 13b). It is probable that these larvae had reached a critical point in their development to allow puparium formation to occur and thus had entered the facultative feeding phase, whereas the larvae which fed on being returned to the medium were still in the obligatory feeding phase (Shaaya and Levenbrook, 1982).

The cumulative rates of puparium formation are depicted in Fig. 7; the median times of puparium formation and the diapause incidence for each culture

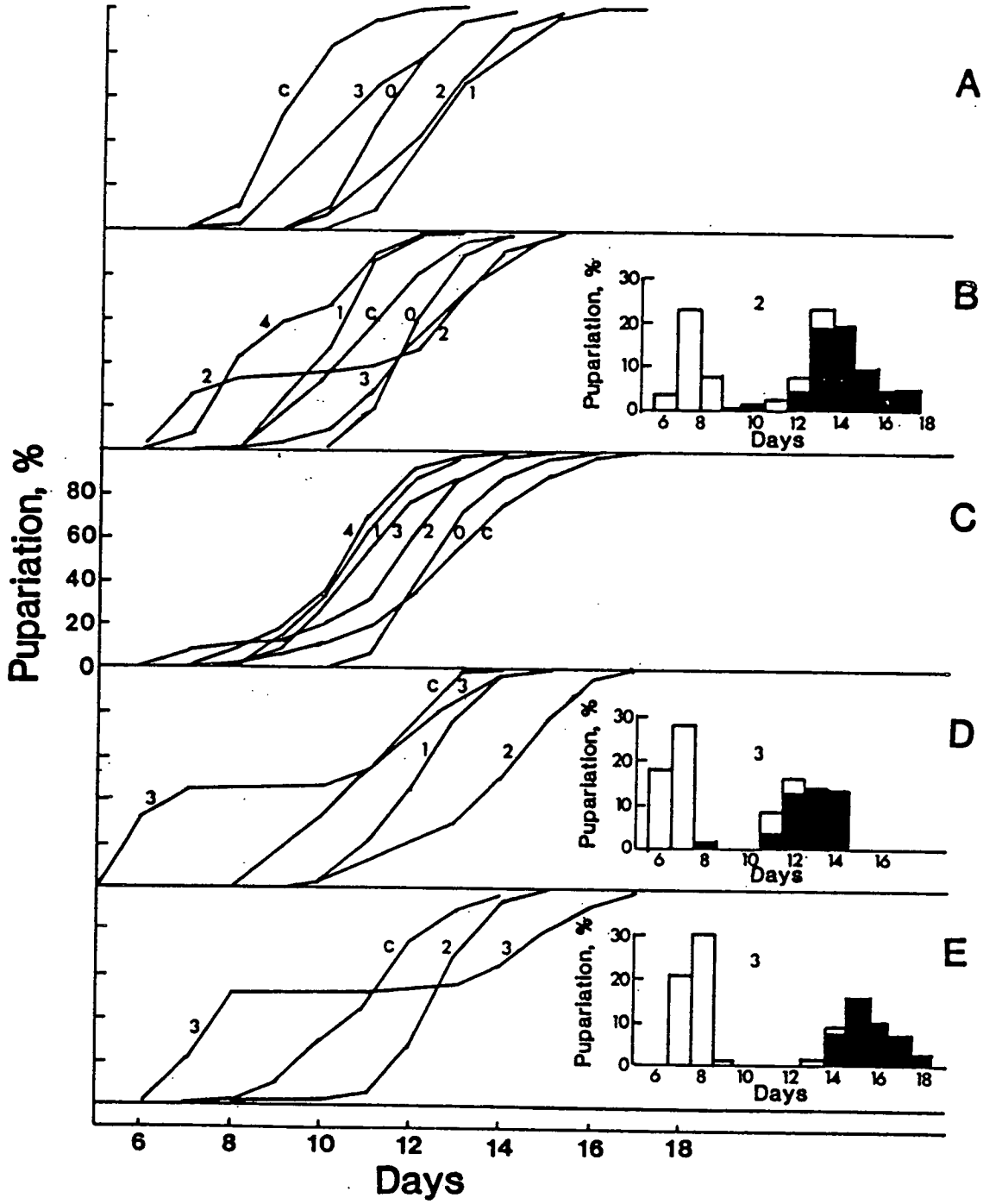


FIG. 7. Main Figure: Lengthening the duration of the larval period by starvation. Groups (A, D and E) and Groups (B and C) are sibling cultures. Numbers on curves denote start of the starvation period, c is the control culture. A, B and C were starved for 1 day, D and E for 2 and 3 days respectively. Adults LL, 25 ± 1°C, larvae LD 12:12, 17 ± 0.5°C.
Inset: the bimodal distribution of puparium formation of cultures in which larvae were starved during the transition from the obligatory to the facultative feeding phase.

are displayed in Tables 14a and 14b. The median of puparium formation was variable in both groups of controls, e.g., 9.0, 10.75 and 11.0 days and 10.5 and 12.75 days, therefore it was not valid to make quantitative comparisons between the experimental results and the controls. However, in the first group (Table 14a, Fig. 7,A,D and E) puparium formation was delayed in all but one culture (larvae starved for 1 day starting on day 3) if larvae were still in the obligatory phase when returned to the medium. This delay in puparium formation was greatest in cultures in which larvae had been starved for 2 or 3 days. In the second group (Tables 14b, Figure 7 B and C) the effects of one day of starvation during different points of larval development were highly variable: the average times of puparium formation of larvae starved on day 0 and day 3 were 11.75 and 12.5 days and 12.0 and 11.0 days, whilst larvae starved on day 1 and day 4 pupariated on average after 10 or 10.5 days and 8.5 or 10.5 days respectively. The bimodal distribution of time spent in media, described above was also reflected in the rate of puparium formation: larvae which failed to feed when returned to the medium formed puparia prematurely. In these cultures the distribution of puparium formation split into 2 clear peaks, the medians of which were over seven days apart (Figure 7

TABLE 14a

Lengthening the duration of the larval period in S. argyrostoma larvae by starvation. Adults LL, 25 ± 1°C, Larvae LD12:12, 17 ± 0.5°C. A, D and E are groups of sibling larvae.

Duration of Starvation (Days)	Start of Starvation (Days After Larviposition)	Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %	Meanweight of Puparia (mg)
1	0 A	122	11.0	32.7	95.2
1	1	98	12.5	53.7	116.7
1	2	144	12.0	64.8	110.4
1	3	207	10.5	33	95.7
2	1 D	96	12.0	54	90.3
2	2	152	14.0	83.5	85.9
* 2	3	75/87	5.7 12.9 bimodal	1·3/74.7	52.3/110.6
3	2 E	137	12.5	76.0	71.1
* 3	3	93/82	7.2 14.7 bimodal	0/90.9	46.1/112.3
1	Control A	177	9.0	4.0	91.1
2	Control D	195	10.75	8.9	100.6
3	Control E	197	11.0	48.1	100.0

TABLE 14b

Lengthening the duration of the larval period in S. argyrostoma by a one day period of starvation. Adults LL, 25 ± 1°C, Larvae LD12:12, 17 ± 0.5°C. B and C are groups of sibling larvae.

Start of Starvation (Days)	Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %	Mean Weight of puparia (mg)
0 (B)	146	11.75	32.7	110.4
1	114	10.0	20.9	106.7
* 2	72/147	6.3 13.0	2.8/77.8	45.4/93.6
3	195	12.0	73.0	104.3
4	170	8.5	15.5	78.5/118.3
0 (C)	157	12.5	60.6	119.4
1	127	10.5	42.8	92.5
2	177	11.5	52.1	40.4/86.2
3	208	11.0	49.2	111.2
4	214	10.5	32.2	84.8/117.6
Control (B)	184	10.5	40.9	97.4
Control (C)	199	12.75	72.6	92.8

insets. B2, D3, E3). The separation of the two peaks was clearest in cultures starved for 2 or 3 days on day 3 (D3 and E8) and for one day starting on day 2 (B2). The bimodal distribution of feeding times in cultures C_2, B_4 and C_4 was not reflected in the rates of puparium formation for these cultures. The weights of puparia formed in the first few days were much lower than those of puparia formed later, and the number of lighter puparia was similar to the number of larvae that left the medium prematurely. However since there was no clear separation, i.e., puparium-formation was continuous in these cultures, no separate pupariation medians or diapause incidences were calculated. The mean weights of puparia displayed in Table 14 show that larvae which fed when returned to the medium, with one exception (larvae starved for 3 days on day 2), reached weights equivalent to those of controls (more than 90 mg). The pupae which formed prematurely from larvae starved on day 2 for 1 day or day 3 for either 2 or 3 days, however, weighed only 40-52 mg, this weight being similar to the weights of pupae which had been prematurely extracted on day 3 of culture (pp.142-143).

There was a positive correlation ($r = +0.87$; $p < 0.001$) between the median of pupariation and the diapause incidence in a culture (Fig. 8). This line

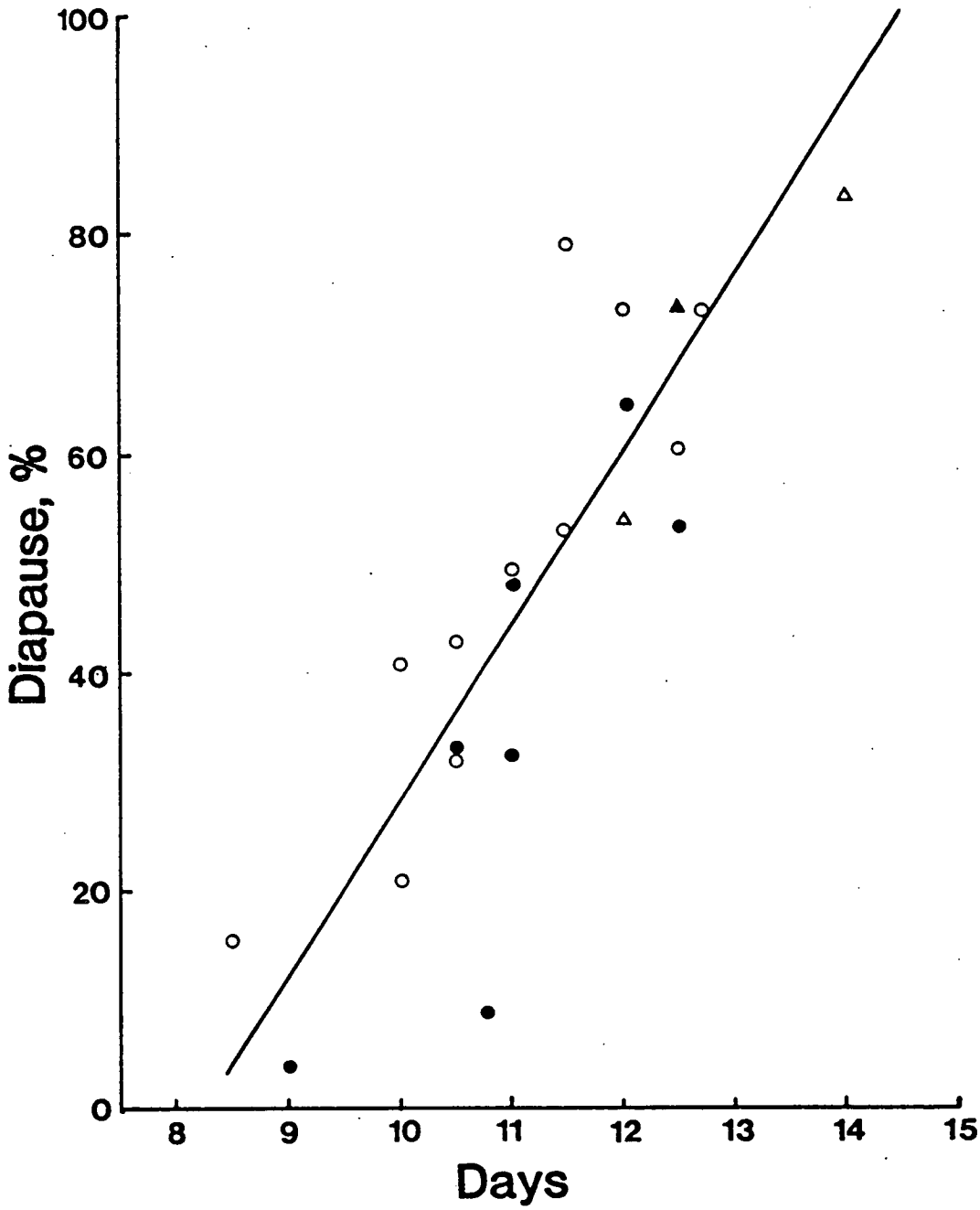


FIG. 8. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. Groups A (●) D (△) and E (▲) are cultures of sibling larvae starved for periods of 1, 2 and 3 days respectively. Groups B and C (o) are replicate cultures of sibling larvae starved for 1 day only. Calculated regression: $y = 15.7x - 129$ ($r = 0.87$, $P < 0.001$).

was calculated using results from all the groups except those with a bimodal distribution of puparium formation. (There was a stronger correlation if the groups were separated into larvae from the same day, e.g. groups ADE, $r = 0.91$, $p < 0.001$; Groups B,C $r = 0.9$, $p < 0.001$). This relationship between duration of larval life (larviposition to puparium formation) and increased diapause incidence was shown in cultures with clearly separated bimodal distributions of puparium formation (Table 14 cultures marked with an asterisk Inset Fig. 7). Individuals in the first peak had very low or no incidence of diapause whilst individuals which formed puparia later, in the 2nd peak, had high diapause incidences, e.g. 1.3/74.7% 0/90.9% and 2.8/77.8%.

A similar experiment was performed on larvae deposited by adults kept in LD12:12 25 $\pm$ 1°C; and subsequently maintained under the same conditions throughout development (see p.119 for method). The results of this experiment are shown in Table 15. The total time spent feeding was the same for both starved and control cultures, and the final diapause incidences and medians of puparium formation were very similar, ranging from 10.2-11.0 days and 72.2 to 87.7% diapause. Thus, under these conditions, starvation of larvae at different times of the life

TABLE 15

The effect of a one day period of starvation on the duration of the larval feeding phase, timing of pupariation and subsequent diapause incidence. Adults and Larvae LD12:12, 25 ± 1°C.

Start of Starvation (days from Larviposition)	Time in Media Excluding 1 Day Starvation	Number	Median Pupariation	Diapause %
0	6	117	10.5	87.7
1	6	122	10.6	72.2
1	6	99	10.2	78.0
2	6	200	10.3	84.2
2	6	186	11.2	82.6
Control	6	237	11.0	81.7

cycle failed to affect the total length of larval development. This experiment was repeated, however, as some of the puparia were "ill treated" on collection which affected the final diapause incidence. The results of this experiment are described on pp.146-152, Table 22 and illustrated in Fig.12.

MANIPULATION OF THE LENGTH OF THE FEEDING STAGES: PREMATURE EXTRACTION

To decrease the length of the larval feeding stage, larvae were removed manually from the food medium commencing 3, 4, 5, 6, 7 and 8 days after larviposition (see p.117 for detail of methods used). These experiments were carried out at 17 ± 0.5°C LD12:12 on larvae previously deposited by females kept in LL, 25 ± 1°C. Replicates of each premature extraction experiment were set up over 5 consecutive days. A culture was sacrificed on days 2 and 3 after larviposition to determine when the larval moults took place at 17 ± 0.5°C (Table 16): the majority of larvae moulted to the 3rd instar by day 2. Therefore, premature extraction was started from day 3 onwards. In addition one culture from each of the five replicates was killed on day 2, to check that the majority of larvae had reached 3rd instar, and therefore the sibling cultures would be competent to

TABLE 16

Timing of Larval Instar Moults of S. argyrostoma at 17 ± 0.5°C, LD12:12 (% larvae)

Instar	Days After Larviposition			
	0	1	2	3
1st	92.8	20.4	0	0
2nd	7.2	79.6	37	1.6
3rd	0	0	63	98.4
n	208	137	173	251

TABLE 17

The Proportion (%) of larvae of S. argyrostoma in 2nd or 3rd instars on day 2 for each replicate.

Instar	Replicate				
	1	2	3	4	5
2nd	37.0	8.3	17.0	22.9	14.3
3rd	63.0	91.7	83.0	77.1	85.7
n	173	229	218	240	210

form prepupae when extracted on day 3 (see Table 17). These results also gave a comparison of the relative age structure between replicate cultures. A X^2 test was carried out on the proportion of 2nd instar:3rd instar larvae in each culture. No difference was found between cultures 3, 4 and 5. However, there were significantly more 2nd:3rd instar larvae in culture 1 ($p < 0.001$), and culture 2 contained more 3rd instar larvae ($p < 0.01$). This implied that larvae on day 1 were slightly younger, whereas larvae on day 2 were slightly older when cultured on days 3, 4 and 5.

Post feeding larvae were observed in the cultures from day 7 onwards, therefore the proportion of larvae which had already left the medium before extraction took place and the rate at which larvae left the control cultures were recorded (Table 18). The extraction day (to the nearest 0.5 day) was calculated when necessary from cumulative frequency curves. The mean weight of puparia, three days after pupariation, is shown in Table 19. Larvae become competent to form puparia from day 3 of culture onwards, although a proportion of larvae in each culture extracted on day 3 failed to pupate (1. 17.7% 2. 5.6% 3. 24% 4. 6% 5. 3.4%) possibly because they were still in the obligatory feeding phase. As expected, the mean weight of puparia increased from

TABLE 18

The proportion (%) of post feeding larvae in each culture from day 7 onwards.

Culture/ (extraction day)	Days After Larviposition 7	8	9	Total Number Larvae
1 (7)	4.6			219
(8)	-	73.7		171
(9)	-	7.0	53.5	144
C	-	5.6	80.8	177
2 (7)	12.2			172
(8)	16.3	70.1		184
(9)	19.7	50.0	29.8	188
C	23.8	38.9	36.8	193
3 (7)	31.1			212
(8)	63.0	30.8		227
(9)	34.7	58.7	4.9	225
C	70.7	28.9	0.9	225
4 (7)	71.9			242
C	48.5	37.1	9.7	237
5 (7)	5.6			213
(8)	43.0	42.2		230
(9)	36.6	53.3	5.3	227
C	22.0	84.0		200

On day 6, 37.4% of Larvae in culture 4(6) had become postfeeding.

35.4-53.4 mg on day 3 to about 90.1-110.2 mg on day 7 when larvae started to become post feeding in most cultures. Control cultures from replicate 1 remained in the medium for 8.5 to 9 days, about 1.5-2 days longer than the other replicate control cultures, possibly because replicate 1 larvae were slightly younger at the start of the experiment.

The premature extraction of larvae from their food, 3 to 7 days after larviposition, caused larvae to form prepupae prematurely; an example is shown in Fig. 9. The medians of pupariation, given in Table 19, increased systematically with time spent feeding. Thus, the premature extraction technique produced cultures with a wide range of larval periods. When the median of pupariation of a culture (i.e., mean length of larval development) was plotted against the diapause incidence (Fig. 10) there was a clear positive linear relationship ($r = +0.94$ $p < 0.001$).

A similar premature extraction experiment was carried out at LD12:12 25 $\pm$ 1°C using larvae from females also kept at 25 $\pm$ 1°C, LD12:12, the results of which are shown in Table 20. As before, premature extraction of larvae 2-4 days after larviposition caused a systematic change in the mean length of larval development, as measured by the median of pupariation, and again a positive linear relationship between the length of larval development and the

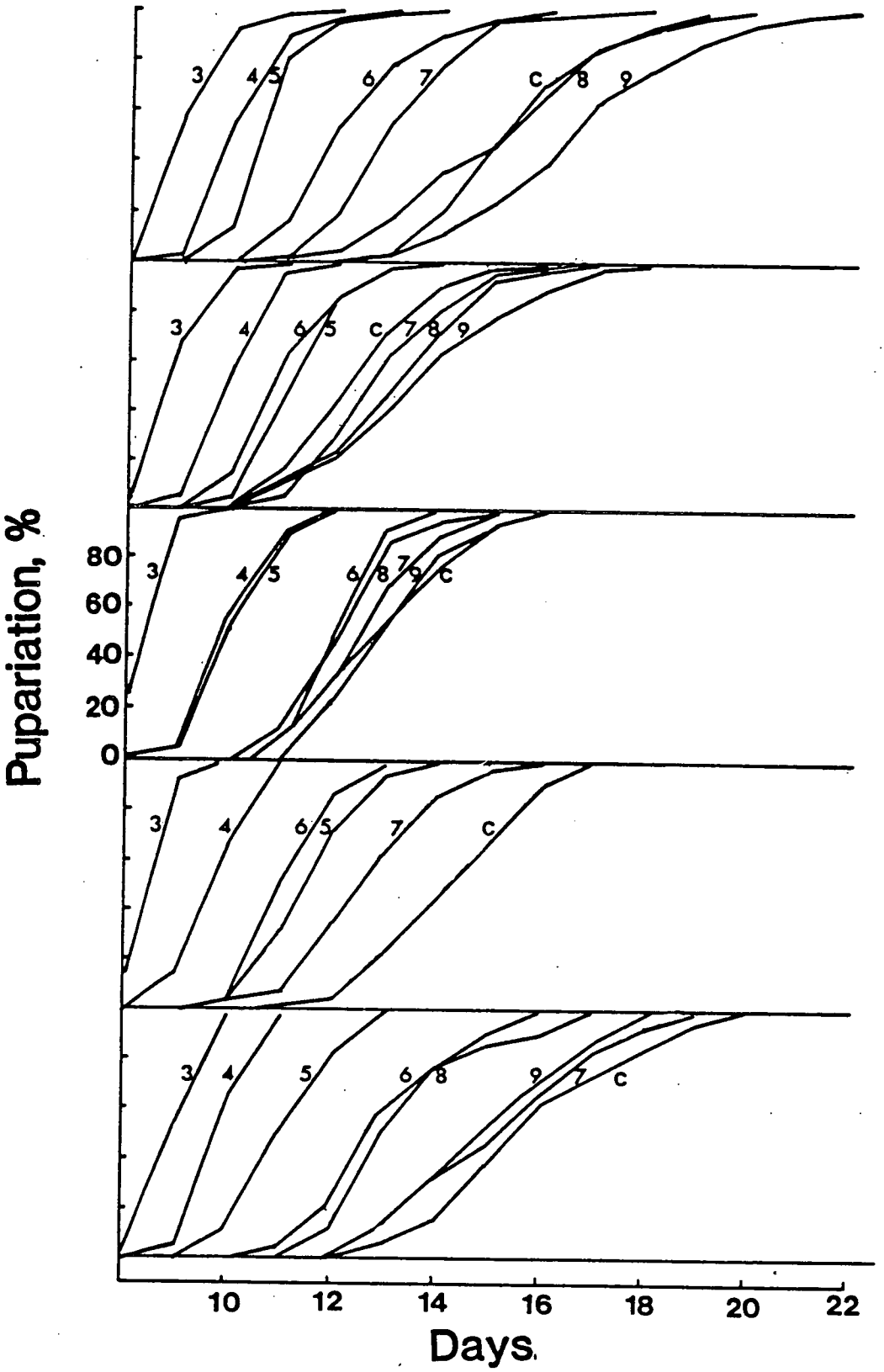


FIG. 9. Shortening the larval period of S. argyrostoma by the premature extraction of larvae from the food medium. The numbers beside the curves denote day of extraction, relative to day of larviposition. c = control culture. 1-5 are replicate experiments. Adults LL, 25 ± 1°C, Larvae LD12:12, 17 ± 0.5°C.

TABLE 19

Shortening the larval period by the premature extraction of larvae from the food medium. Adults LL, 25 ± 1°C, Larvae LD12:12,17 ± 0.5°C. 1-5 are replicate experiments.

	Extraction Day	Mean Weight of Pupae (mg)	Number	Median Pupariation	Diapause %
1	3	35.4	141	9.5	24.5
	4	46.0	195	10.0	23.0
	5	65.4	279	11.5	30.7
	6	110.5	158	12.0	53.3
	7	98.5	219	13.0	59.5
	*8	118,3	171	15.0	78.0
	*9	127.4	144	16.5	80.0
	*8½	114.0	177	15.0	67.2
2	3	53.4	269	8.5	17.4
	4	76.7	211	10	25.1
	5	95.7	228	11	32.4
	6	114.7	201	10.5	36.3
	7	110.2	172	12.5	63.3
	*7½	106.2	184	13	60.7
	*7½	117.5	188	13.5	64.2
	*7½	117.5	193	12	54.7
3	3	58.1	196	8.5	14.9
	4	72.4	249	10	43.1
	5	99.4	225	10	29.5
	6	111.9	229	12	43.6
	7	90.1	212	13	45.0
	7	88.8	227	12	29.0
	7	104.6	225	13	62.8
	6½-7	95.2	225	13	49.3
4	3	49.2	201	8.5	20.0
	4	78.2	223	9.5	29.0
	5	94.9	229	11.5	46.9
	6	102.8	270	11	36.4
	6½	91.9	242	12.5	60.4
	7	89.9	237	14	61.4

TABLE 19 (Contd.)

Extraction Day	Mean Weight of Pupae (mg)	Number	Median Pupariation	Diapause %
3	70.4	205	9	21.8
4	71.6	219	10	26.5
5	109.2	207	11	52.0
6	95.6	259	12.5	58.4
7	94.9	213	15	66.0
7	113.3	230	13	55.6
7	103.9	227	15	75.3
7½	98.9	200	15.5	75.6

$r = 0.94$, 35 df, $p < 0.001$.
$a = -52.6$, $b = 8.3$
i.e. $y = 8.3x - 52.6$

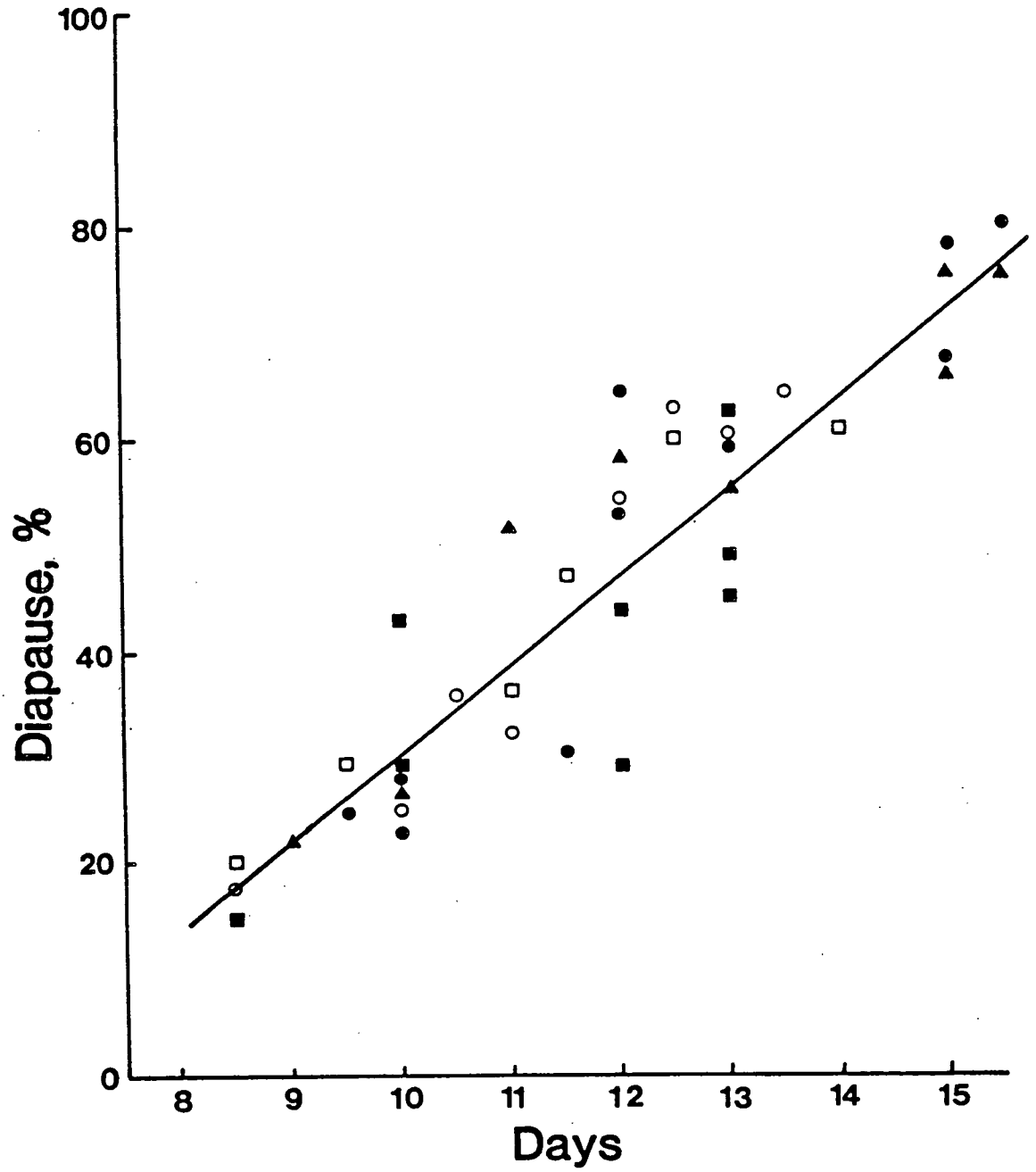


FIG. 10. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. Groups 1-5 are replicate cultures, 1 (●), 2 (o), 3 (■), 4 (□) and 5 (▲). Calculated regression $y = 8.3x - 52.6$, $r = 0.94$, $p < 0.001$. Adults LL, 25 ± 1°C, Larvae, LD12:12, 17 ± 0.5°C. (Premature Extraction)

TABLE 20

Shortening the larval period by the premature extraction of larvae from the food medium. Adults and larvae LD12:12, 25 ± 1°C.

Extraction Day	No. Larvae	Median Pupariation	% Diapause
2	125	5.8	38.9
3	212	8.4	28.5
4	121	8.8	42.6
C	240	12.6	64.4
2	234	6.4	35.0
3	184	8.8	42.5
4	181	11.0	77.4
C	204	13.3	82.1

final diapause incidence was recorded ($r = 0.88$ $p < 0.01$) (Fig. 11).

The experiment described above was a replicate of a larger experiment performed under the same conditions. This experiment was set up using larvae deposited over five days and contained both starvation and premature extraction treatments. Unfortunately, due to a misunderstanding, newly formed puparia were placed in airtight plastic jars (3.5 cm diam. x 6 cm high) for a 3-5 day period.* If there were more than about five prepupae per jar, the puparia were unable to loose water and consequently become waterlogged and warm to touch. This had a dramatic consequence on the diapause incidence, causing an effect similar to the temperature step up effect reported by Gibbs (1975). In these pupae, diapause incidence was significantly reduced, an example of which is shown in Table 21. Columns III and IV on day 12 and 12.5 clearly show the reversal of diapause. On day 12, for example, the proportion of diapausing pupae per day's collection was 66.7% and 87.5% respectively, whereas, in a normal culture, the proportion of pupae in diapause increased in daily collections. In the waterlogged cultures, however, the proportion of prepupae entering diapause fell

*I was unable to collect puparia over a 3½ day period. They were collected by a volunteer.

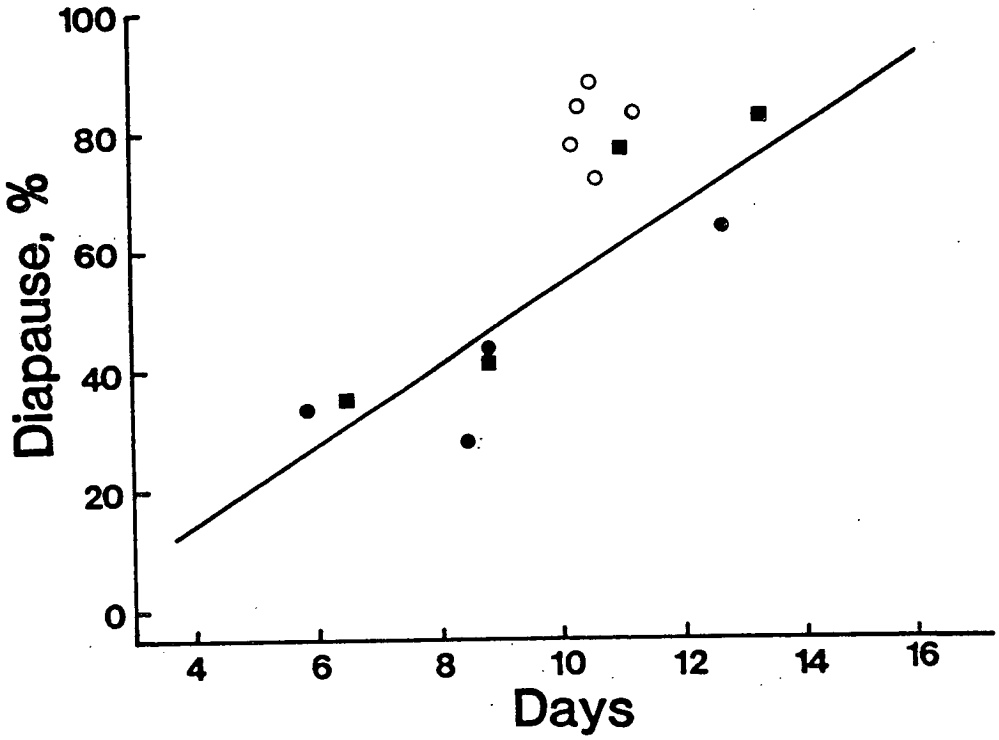


FIG. 11. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. (●) and (■) are results from replicate experiments. Calculated regression $y = 6.7x - 12.5$, $r = 0.88$, $p < 0.01$. (○) are results from the starvation experiment performed on sibling cultures (Table 15). Adults and larvae LD12:12, 25 ± 1°C.

TABLE 21

Reversal of pupal diapause in diapause destined, newly formed prepupae of S. argyrostoma, by preventing normal 'water loss'. ═ is the point at which prepupae were allowed to become waterlogged. Columns I and II are controls.

	I				II				III				IV			
	1. Starved Day 0				2. Control				3. Control				1. Starved Day 2			
Days From Larviposition	Total	Develop	Diapause	Dead	Total	Develop	Diapause	Dead	Total	Develop	Diapause	Dead	Total	Develop	Diapause	Dead
8					3	0	3	0								
8.5					5	2	2	1								
9					8	6	2	0								
9.5	4	2	2	0	38	9	25	4								
10	4	1	2	1	36	6	19	11					1	0	1	0
10.5	35	4	14	17	31	5	25	1	10	5	3	2	4	3	0	1
11	32	0	15	17	12	2	8	2	8	0	1	7	7	1	6	0
11.5	31	3	27	1	6	3	3	0	24	17	7	0	23	6	16	1
12	10	0	8	2	4	0	4	0	25	8	16	1	25	3	21	1
12.5	1	0	1	0	1	0	1	0	33	24	0	9	32	20	6	6
13									28	13	13	2	37	5	28	4
13.5									36	28	7	1	29	1	28	0
14									29	0	0	29	10	0	8	2
14.5									12	1	10	1	2	1	1	0
15									5	1	4	0				
15.5									1	0	1	0				
Total	117	10	69	38	144	33	92	19	212	98	62	52	170	40	115	15
% Diapause		88.5				73.6				38.8				74.2		

respectively to 0% and 23%. Thus it was probable that any prepupae collected during the 3 day period would have been affected in this manner, and the final diapause incidence in those cultures would not reflect the duration of the larval period. Therefore the percentage of pupae affected in this manner in each culture was calculated (Table 22), and only those cultures in which less than 10% of the puparia had been affected (marked with asterisk) were used to calculate a regression equation between the duration of larval period (median pupariation) and final diapause incidence (Figure 12). Again, there was a highly significant positive correlation ($r = + 0.94$, $p < 0.001$): the starvation of larvae for one day resulted in an increase in the duration of the larval period, whereas the premature extraction of larvae from their food resulted in a decrease.

MANIPULATION OF THE SENSITIVE PERIOD: CROWDING EFFECTS DURING LARVAL DEVELOPMENT

A preliminary experiment was designed to determine whether density during larval development affected the length of the larval period and hence diapause incidence. Larvae deposited by adults kept in 25 $\pm$ 1°C in either LL or LD12:12 were cultured under different densities at 18 $\pm$ 0.5°C, LD12:12. The results of this experiment are displayed in

TABLE 22

The effect of a one day period of starvation, or premature extraction of S. argyrostoma larvae from the food medium, on the duration of the larval period and subsequent diapause incidence. The majority of newly formed prepupae were accidentally overcrowded, the proportion of prepupae in each culture affected in this way (% prepupae affected) is shown below; only results marked with * were used in the regression calculation. In the Treatment column, 10S represents larvae from replicate 1, starved for 1 day commencing 0 days after larviposition and 32E represents larvae from replicate 3 extracted from the food 2 days after larviposition.

Treatment	Time in Media	N	Median Pupariation	Diapause %	% Prepupae Affected
10S	6	117	10.7	88.5	* 0.9
11S	7	49	10.4	94.7	* 0
12S	7	170	12,35	74.2	64.8
13S	6	190	11.8	72.7	44.4
1C	6	212	12.6	38.8	68.1
20S	7	40	12.0	85.7	*small nos no effect
2C	6	144	9.8	73.6	* 7.7
31S	6	55	10.4	82.6	*small nos no effect
32S	6	146	11.3	62.9	93.9
32E	2	128	5.8	19.2	* 0
33E	3	213	8.3	44.7	* 4.2
34E	4	215	10.0	53.0	50.2
3C	6	205	11.2	60.3	75.5
41S	7	88	10.8	46.2	100
42S	6	142	11.6	34.9	97.9
42C	2	31	5.8	37.0	0
43E	3	204	8.9	18.8	47
44E	4	237	10.3	27.5	88.6
4C	6	178	11.0	38.8	95.5
52E	2	138	6.3	20.2	* 0
53E	3	152	8.0	33.3	52.4
54E	4	143	9.2	14.7	90.2
5C	6	154	9.9	18.8	99.3

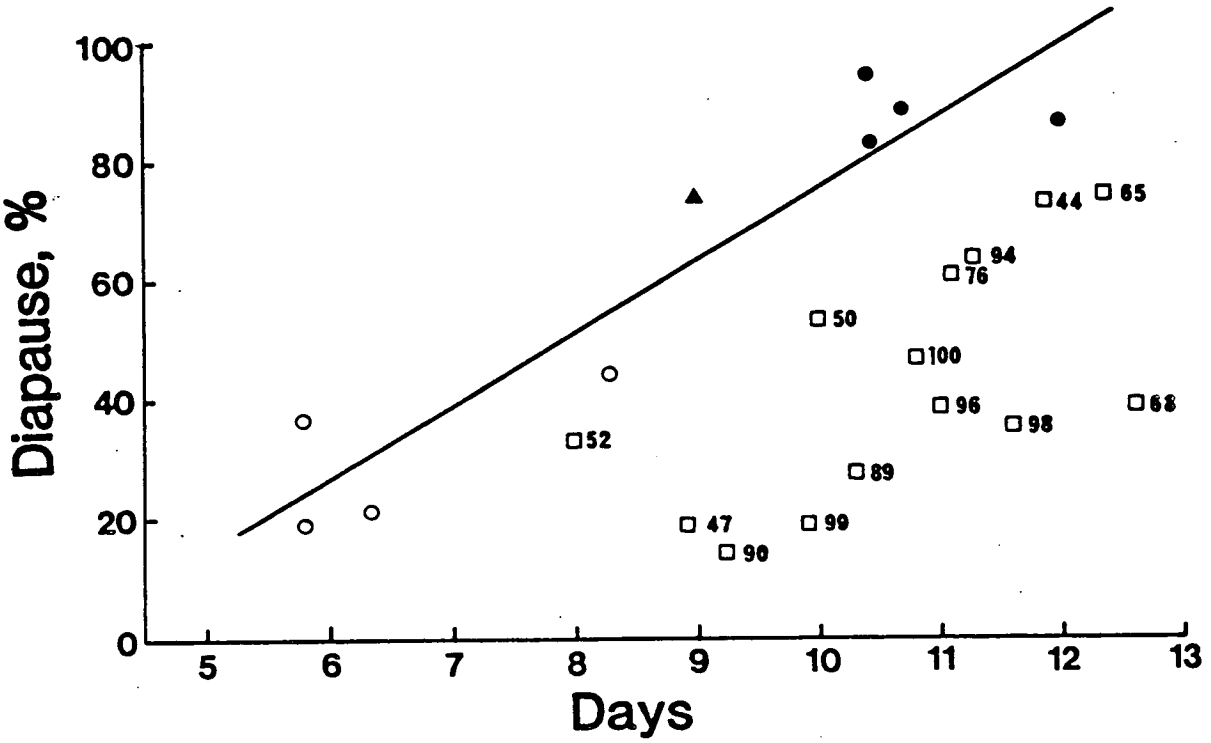


FIG. 12. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. (o) premature extraction (●) starvation (▲) control. Calculated regression $y = 12.2x - 46.7$, $r = 0.94$, $p < 0.001$.

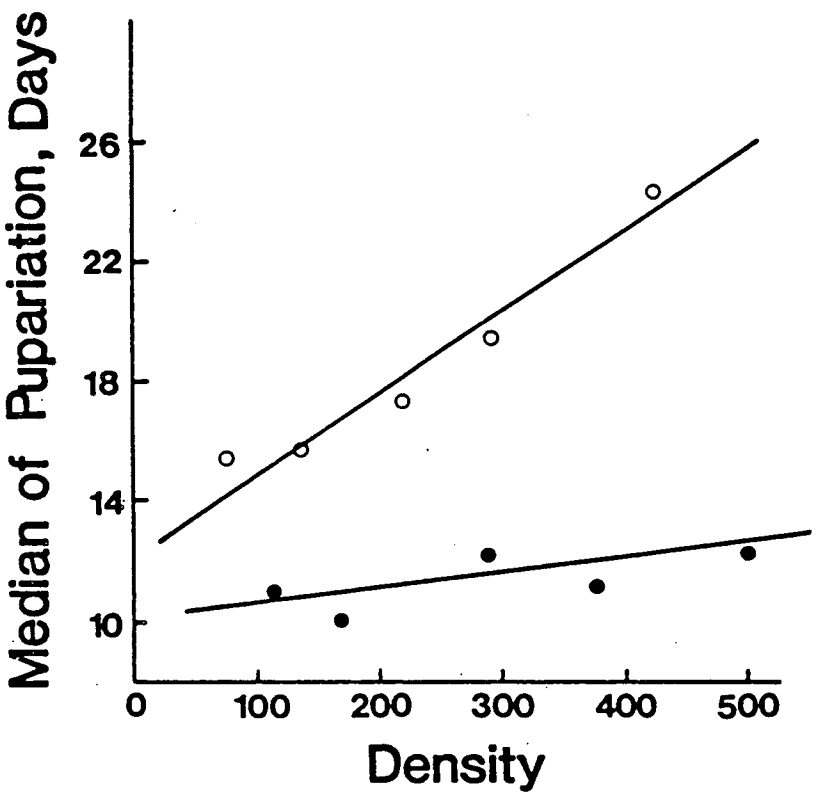
(□) cultures affected by overcrowding newly formed prepupae, numbers denote percentage of prepupae per culture affected in this manner. These cultures were not included in the regression calculation.

Table 23. There was a clear positive linear relationship between the density of larvae during development and the average length of the larval period (measured by the median of pupariation), ($r = 0.97$ $p < 0.01$ (Fig. 13)). In larvae which experienced LD12:12 as embryos, the diapause incidence at all densities was saturated (i.e., 94% or more of the pupae entered diapause). There was no significant linear relationship between density and the length of larval period in larvae deposited by adults kept in LL ($r = 0.719$, $p > 0.10$), although the results showed the same trend as before: the length of the larval period increased with increasing density. Under these conditions, the diapause response was unsaturated, and showed a trend to increase with increasing duration of the larval period; ($r = 0.84$, $df = 3$, $0.1 > p > 0.05$). Larvae which experienced LD12:12 as intrauterine embryos formed puparia later than larvae which experienced LL (e.g. the median of pupariation ranged from 10.9 to 12.3 days for LL larvae and 15.4-24.4 days for larvae from LD12:12 (Table 23)). The time spent in the medium was similar for both types of larvae, therefore the difference in the length of the larval period lay in the length of wandering (post feeding period). Thus, a high incidence of diapause in a culture was associated with a longer wandering period. The

TABLE 23

The effect of larval density on the duration of the larval period and subsequent incidence of pupal diapause in S. argyrostoma. Larvae LD12:12, 18 ± 0.5°C.

Adults	Estimated Larval Density	Number of Prepupae	Median of Pupariation (Days)	Diapause %
LL	100	107	10.9	7.0
LL	200	164	10.0	8.0
LL	300	288	12.2	25.0
LL	400	373	11.2	25.0
LL	500	481	12.3	28.0
LD12:12	100	74	15.4	98.5
LD12:12	200	135	15.8	94.5
LD12:12	300	220	17.3	95.3
LD12:12	400	290	19.4	96.6
LD12:12	500	421	24.4	98.4



which experiment ?

FIG. 13. The increase in the duration of the larval period plotted as a function of the increase in larval density. Larvae at LD12:12, 18 ± 0.5°C. (o), Adults. LD12:12, 25 ± 1°C. Calculated regression: $y = 0.03x + 12.5$, $r = 0.97$, $p < 0.01$, (●) Adults LL, 25 ± 1°C. Calculated regression: $y = 0.05x + 10.04$, $r = 0.72$, $p > 0.1$.

implications and possible explanations of this result are discussed on p.100 .

The results from the preliminary experiment indicated that crowding might be a useful tool to manipulate the duration of the larval period and hence the sensitive period. Larvae from adults kept at LL, 25 ± 1°C and bred at 18 ± 0.5°C, yielded only 7-25% pupal diapause. To achieve a slightly higher diapause incidence the larvae in the following experiment were bred at 17 ± 0.5°C.

A more extensive study on the effect of density on the duration of larval development was performed using larvae deposited by adults kept in LL, 25 ± 1°C, using approximate larval densities of 2, 5, 10, 25, 100, 300, 500, 700 and 1500 per culture. The duration of the feeding phase decreased with increasing larval densities; very low density cultures (2-25) feeding for 10 to 11 days and forming very heavy pupae weighing, on average, 160 to 179 mg, whilst overcrowded cultures of over 1,000 larvae leaving the medium after 5 days and forming small pupae weighing about 50 mg. (Regression calculation, larval density vs feeding phase duration $r = 0.701$, $0.05 > p > 0.02$). Although overcrowded cultures spent less time in the medium than low density cultures the duration of the wandering phase was very protracted in the former (Table 24): the cultures containing

TABLE 24

The effect of larval density on the duration of the larval feeding phase and post feeding phase, the mean pupal weight and incidence of pupal diapause in S. argyrostoma. For lower feeding densities the number in brackets denotes number of replicates. Adults LL, 25 ± 1°C, Larvae LD12:12, 17 ± 0.5°C.

Feeding Density	Duration of Feeding Phase (Days)	Mean Pupal Weight (mg)	Wandering Phase (Days)	Median of Pupariation (Days)	Diapause %
2(9)	11	179	5	16	50
5(9)	11	174	5	16	68
25(3)	9	166	3.5	12.5	14
90	7	158	5.0	12.0	15
90	7	137	See Table 25		17.7
227	8	108	4.0	1.2	11.3
315	6	75	6.5	12.5	25
440	6	73	See Table 25		19.6
401	6	84	6.5	12.5	20.3
1151	5	51	10	15	40

TABLE 25

The effect of larval post feeding density on the duration of the wandering phase and subsequent incidence of diapause in S. argyrostoma.

Feeding Density	Post Feeding Density	Wandering Phase (Days)	Median of Pupariation	Diapause %
90	430	5.5	12.5	17.7
440	440	7	12.9	19.6
440	85	6	11.7	27.0

1151 larvae wandering on average for 10 days before puparium formation, whilst low density cultures (e.g. 90 per culture) forming prepupae about 5 days after leaving the larval medium. There was a positive linear relationship between the median of pupariation (i.e., duration of larval life) and the incidence of diapause in each culture ($r = 0.95$ $p < 0.001$, Fig. 14).

To determine whether the length of the wandering phase was predetermined by the density experienced during the feeding phase, 100 larvae were removed from a culture which had contained 440 post feeding larvae, and placed in a separate mousetray. Ninety-one larvae which had experienced a feeding density of about 100 were then marked with green acetone paint and placed into the culture from which the other larvae had been removed. The results of this experiment are shown in Table 25. The wandering period of 5.5 days of the 90 larvae placed in the culture containing 340 larvae, was significantly longer than the 5 day wandering period of larvae at a post feeding density of 90 (Table 24) (median test, $X^2 = 10.9$ $p < 0.001$ on the final medians of pupariation). Likewise the 85 larvae originally from a culture containing 440 larvae formed puparia over a day earlier than individuals remaining in the culture (median test $X^2 = 46.4$ $p < 0.001$).

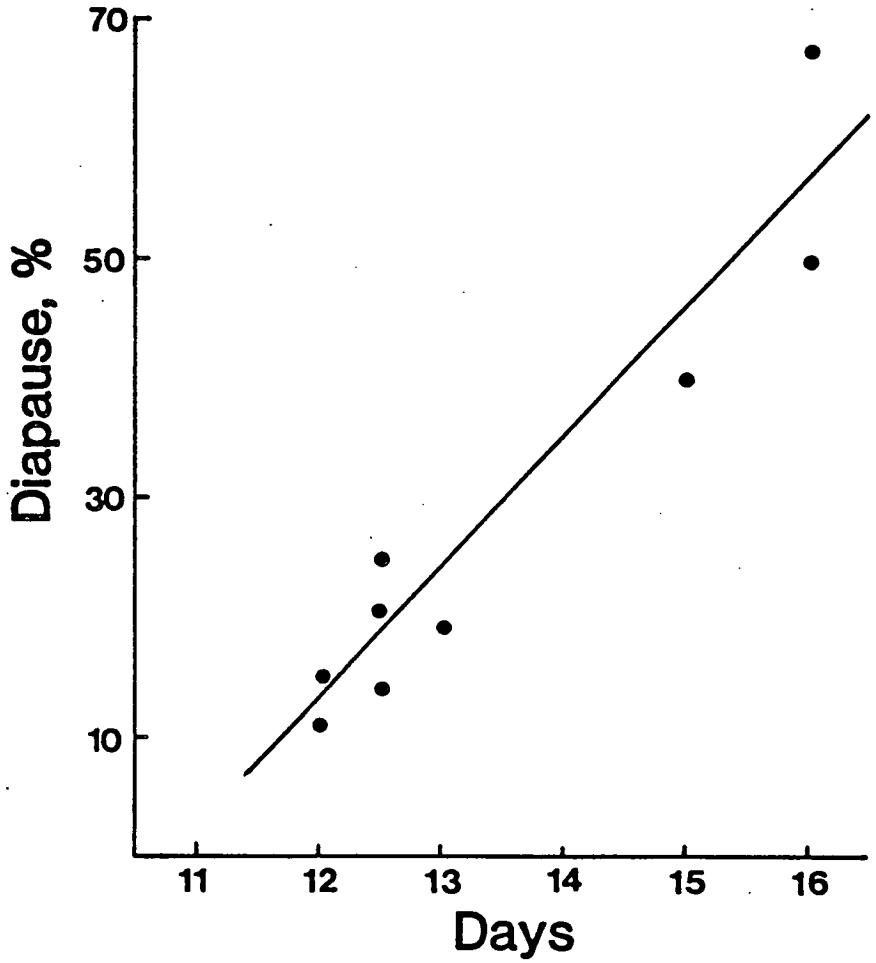


FIG. 14. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. Calculated regression: $y = 10.85x - 116.8$. $r = 0.95$ $p < 0.001$. (Larval density varied)

Since the above result implied that crowding during the wandering phase delayed puparium formation, this phenomenon was investigated further. Larvae deposited by adults kept in LL, 25 ± 1°C were bred at densities of 100, 300, 500, 700 and 1000 at LD12:12, 17 ± 0.5°C, and three replicates (A, B, C) were set up on three consecutive days. When larvae in each culture became post feeding, they were transferred to densities of 100, 200 or 400 larvae per mousetray to avoid the modifying effect of crowding during the post feeding stage (p.167). The actual density of larvae during the feeding phase was estimated by the number of post feeding larvae. The number of larvae per culture influenced the duration of the feeding phase (Table 26, $r = 0.935$, $p < 0.01$), cultures containing 100 larvae feeding for 9 days, whereas cultures containing about 750 larvae ceasing to feed after 6 days. Probable explanations are that larvae at higher densities consumed the limited food material more rapidly, or left the medium earlier because it became polluted by excretory products, or perhaps the raised temperature within the crowded cultures increased the rate of development. Pupal weights from the previous experiment (Table 24) implied that larvae in overcrowded cultures consumed less food than those in control cultures, entered the post

TABLE 26

The effect of larval feeding density on the duration of the larval feeding phase (a, b and c are replicates). Adults LL, 25 ± 1°C, Larvae 17 ± 0.5°C.

Estimated Density		No. of Post Feeding Larvae	Feeding Phase (Days)
100	a	91	9
100	b	98	9
100	c	109	8
300	a	253	8
300	b	275	7
300	c	207	8
500	a	471	7
500	b	415	7
500	c	450	7
700	a	623	7
700	b	510	7
700	c	683	6
1000	a	772	6
1000	b	746	6
1000	c	753	6

TABLE 27A

The effect of larval density on the duration of the larval period and subsequent incidence of pupal diapause in S. argyrostoma. Adults LL, 25 ± 1°C, Larvae 17 ± 0.5°C. A, B and C are replicate experiments.

Density during Feeding Phase	Density during Post Feeding Phase	Wandering Phase (Days)	Median of Pupariation	Diapause %
100	93	3.7	12.7	32.2
300	99	4.2	12.2	39.1
300	141	4.6	12.6	39.1
500	99	3.7	10.7	6.1
500	181	3.9	10.9	10.5
700	98	3.5	10.5	16.3
700	197	4.2	11.2	27.6
700	328	4.5	11.5	19.4
1000	98	4.3	10.3	23.2
1000	191	4.7	10.7	19.0
1000	401	5.7	11.7	23.9

TABLE 27B

The effect of larval density on the duration of the larval period and subsequent incidence of pupal diapause in S. argyrostoma. Adults LL, 25 ± 1°C, Larvae 17 ± 0.5°C. A, B and C are replicate experiments.

Density during Feeding Phase	Density During Post Feeding Phase	Wandering Phase (Days)	Median of Pupariation	Diapause %
100	95	2.7	11.7	26.3
300	92	3.9	10.9	14.6
300	157	4.4	11.4	21.2
500	90	4.4	11.4	43.2
500	195	4.8	11.8	40.1
700	89	4.4	11.4	31.0
700	197	5.5	12.5	40.6
700	185	4.7	11.7	31.8
1000	85	4.6	10.5	12.9
1000	195	5.3	11.3	24.5
1000	353	5.4	11.4	13.7

TABLE 27C

The effect of larval density on the duration of the larval period and subsequent incidence of pupal diapause in S. argyrostoma. Adults LL, 25 ± 1°C, Larvae 17 ± 0.5°C. A, B and C are replicate experiments.

Density during Feeding Phase	Density during Post Feeding Phase	Wandering Phase (Days)	Median of Pupariation	Diapause %
100	105	4.6	12.6	20.0
300	93	3.9	11.9	26.1
500	96	4.0	11.0	12.8
500	171	6.0	13.0	18.0
700	92	4.7	10.7	13.0
700	192	4.7	10.7	12.0
700	306	5.0	11.0	19.4
1000	96	4.3	10.3	26.9
1000	191	4.7	10.7	12.0
1000	353	5.1	11.1	22.0

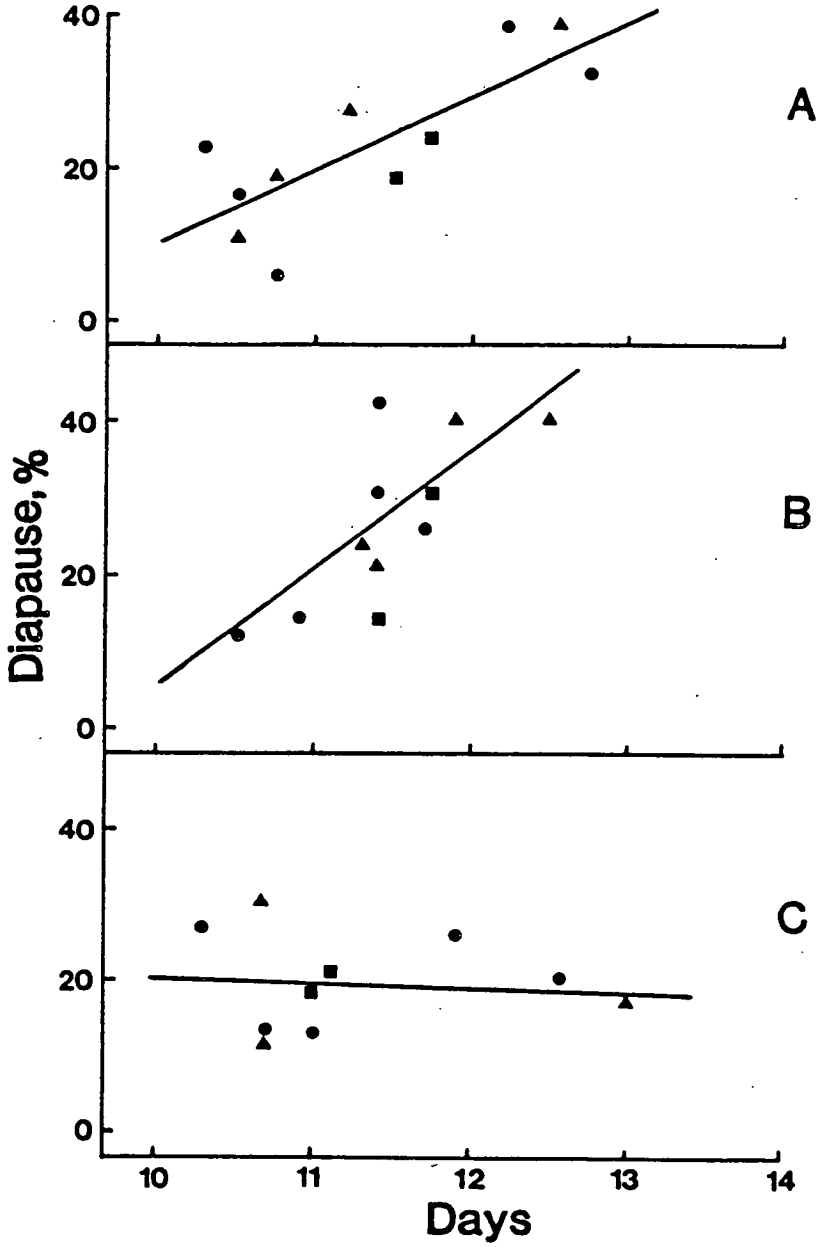


FIG. 15. The increase in the incidence of pupal diapause plotted as a function of the increase in the length of the larval period. A B and C are replicate experiments. Calculated regressions: A, $y = 9.8x - 88.0$, $r = 0.77$, $0.01 > p > 0.001$; B, $y = 15.3x - 147.0$, $r = 0.77$, $0.01 > p > 0.001$; C, $y = 22.9x - 0.3$, $r = -0.04$, $p > 0.05$. Post feeding densities (larvae/culture): ●100, ▲200 and ■400.

feeding stage earlier and consequently formed premature and undersized prepupae. The different densities during the feeding and post feeding stages produced a variety of different pupariation times (Tables 27A B and C). Each replicate was analysed separately. In replicates A and B there was a highly significant positive linear relationship between the duration of the larval period (measured by the median of pupariation) and the incidence of pupal diapause ($r = 0.77$ $p < 0.01$). However in replicate C there was no such relationship ($r = 0.04$ $p > 0.5$) (Fig. 15). There is no obvious explanation why the results from this replicate should be so different.

MANIPULATION OF THE SENSITIVE PERIOD: CROWDING DURING THE POST FEEDING PHASE

The previous experiments described how crowding larvae during the feeding phase could be used as a method to alter the length of the larval period. The effect of crowding during the post feeding stage of larvae maintained throughout their development (including embryogenesis) at LD12:12, 25 $\pm$ 1°C was investigated by transferring the post feeding larvae to different densities in a range of containers (see pp.120+121). LD12:12, 25 $\pm$ 1°C was chosen to provide an 'unsaturated' final incidence of diapause.

Experiments I and II were carried out in glass jars (5 cm diameter by 11 cm high); the densities used and the consequent effect on the timing of pupariation and diapause incidence are given in Table 28a. Figure 16 demonstrates a significant positive linear relationship between the density of the post feeding larvae and the median time of puparium formation: solitary larvae formed puparia on average 7.8 or 7.65 days after larviposition, whereas with increasing larval density puparium formation was delayed so that it occurred 10.9 or 11.5 days after larviposition at 100 larvae per jar. The systematic increase in the duration of the larval period was associated with a subsequent decrease in diapause incidence (Fig. 17, Curves I and II). A further set of experiments (III-V) was carried out under the same photoperiod and temperature described for Experiments I and II. However, in these experiments, larvae were placed in smaller plastic jars (3.5 cm diameter by 6 cm high) so that the densities used in experiments I and II were not equivalent to those used in Experiments III, IV and V. Again, however, there was a clear positive linear relationship between post feeding density and the timing of puparium formation, the median of pupation increasing with an increasing density (Table 28b) (Fig. 18). In all replicates (Figs. 17 and 19)

TABLE 28a

The effect of Larval Post-feeding Density on the timing of puparium formation and subsequent incidence of pupal diapause in S. argyrostoma. Adults and Larvae LD12:12, 25 ± 1°C.

	Density (Larvae per glass jar)	Total Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %
I	1	97	7.8	96
	5	125	84.5	93
	10	120	8.6	86
	25	149	8.75	79
	50	148	9.0	82
	75	146	9.1	72
	100	193	10.9	79
II	1	84	7.65	87.8
	5	59	8.1	84.0
	25	146	9.3	59.0
	50	151	10.25	52.7
	75	153	10.75	48
	100	201	11.5	48.4

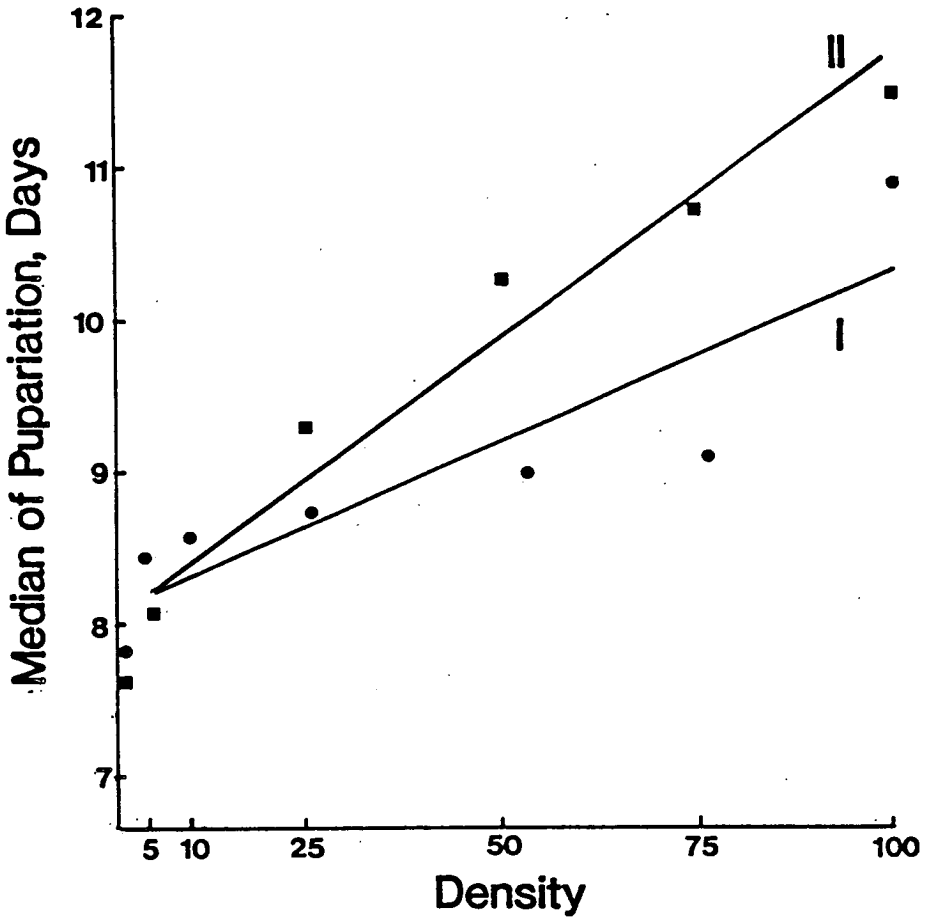


FIG. 16. The increase in the duration of the post feeding (wandering stage) plotted as a function of the increase in larval post feeding density. I (●) and II (■) are replicate experiments. Calculated regressions:
(I) $r = 0.9$, $y = 0.023x + 8.1$, $0.01 > p > 0.001$
(II) $r = 0.98$, $y = 0.037x + 8.0$, $p < 0.001$.

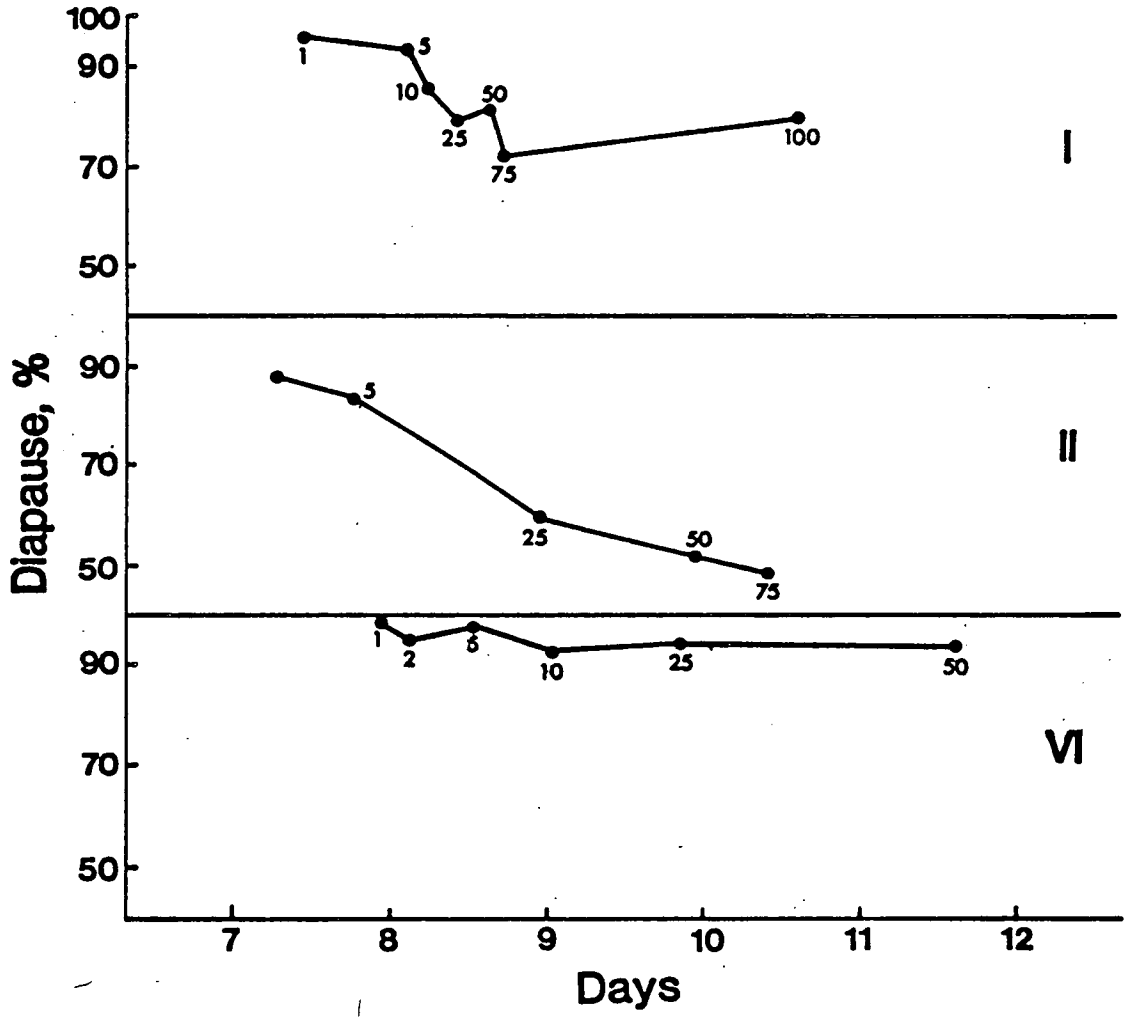


FIG. 17. The reduction in the incidence of pupal diapause associated with the increase in the duration of the wandering phase resulting from increased larval post feeding densities. I and II at 25 ± 1°C VI at 23 ± 1°C. Numbers on curve denote number of larvae per glass jar. (see text).

TABLE 28b

The effect of larval post feeding density on the timing of puparium formation and subsequent incidence of pupal diapause in S. argyrostoma. Adults and Larvae, LD12:12, 25 ± 1°C.

	Density (larvae per plastic jar)	Total Number of Prepupae Formed	Median of Pupariation (Days)	Diapause Incidence %
III	1	164	7.75	93
	5	117	8.35	82.8
	10	108	8.5	77
	25	130	9.5	66
IV	1	192	7.5	92
	10	117	8.5	64
	25	143	9.0	67
	50	146	9.75	64.7
	75	143	10.5	70
V	1	130	7.35	88.4
	10	122	8.75	42.5
	25	142	9.7	52.1
	50	142	10.45	40.0
	100	193	11.5	40.2

POSTFEEDING DENSITY vs MEDIAN OF PUPARIATION

III $r = 0.9824$ $y = 0.068x + 7.8285$ $df = 2$ $0.02 > p > 0.01$
IV $r = 0.9716$ $y = 0.036x + 7.8605$ $df = 3$ $0.01 > p > 0.001$
V $r = 0.9267$ $y = 0.038x + 8.1404$ $df = 3$ $0.05 > p > 0.02$

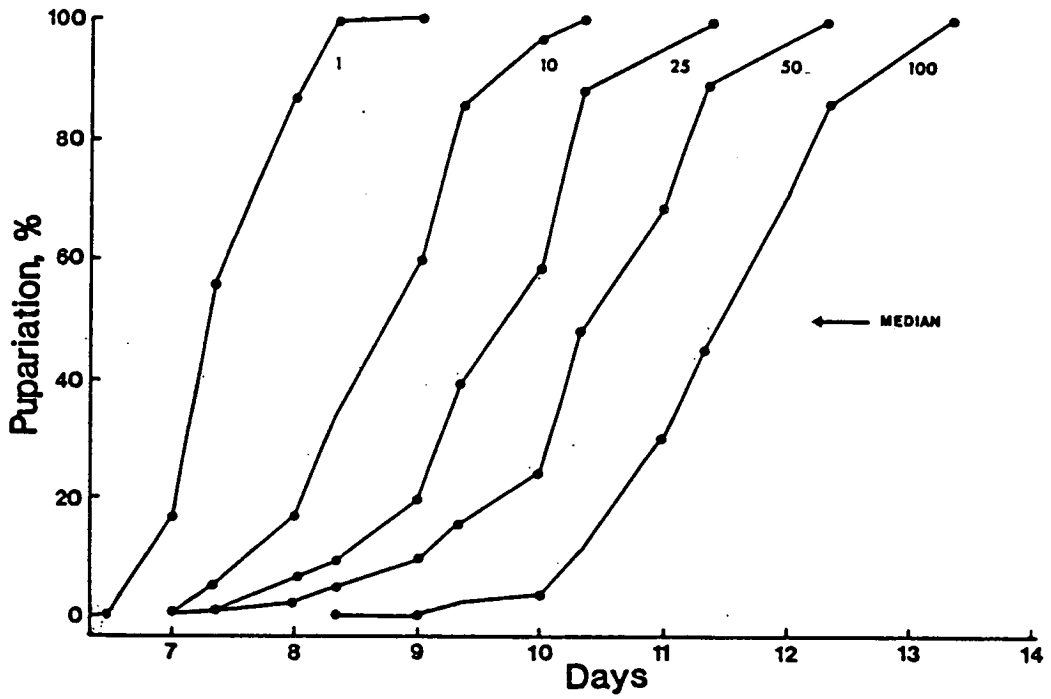


FIG. 18. The effect of larval post feeding density on the duration of the wandering phase (measured by the rate of puparium formation). Numbers on curves denote number of larvae per plastic jar (see text). (V)(Table 28b).

(Curves I-V), the diapause incidence initially fell with an increase in the wandering phase then reached a plateau where a further increase in the duration of the larval period did not result in a change in the diapause incidence. Thus crowding during the post feeding phase increased the duration of the wandering phase but produced a subsequent fall in diapause incidence despite an increase in the number of diapause-inducing light cycles experienced before puparium formation. This result, therefore, is unlike that reported for crowding during the feeding phase.

Similar density experiments (VI and VII) were carried out at LD12:12 but a slightly lower temperature (23 $\pm$ 1^{o}C). At this lower temperature the timing of puparium formation also increased systematically with increasing density (Table 29a, $r = 0.996$ $y = 0.08X + 8.3$ $p < 0.001$, Table 29b, $r = 0.954$, $y = 0.07x + 7.6$ 0.02 $p < 0.01$). Unlike the experiments at 25 $\pm$ 1^{o}C, however, in experim-ent VI there was generally no subsequent fall in the incidence of diapause, despite an 8.3 day delay in the average time of puparium formation of larvae kept at a density of 100 per jar (Fig. 17, Curve VI). In experiment VII there was a slight initial fall in the incidence of diapause from 100% diapause in solitary larvae to about 88% in crowded larvae (Fig. 19, Curve VII).

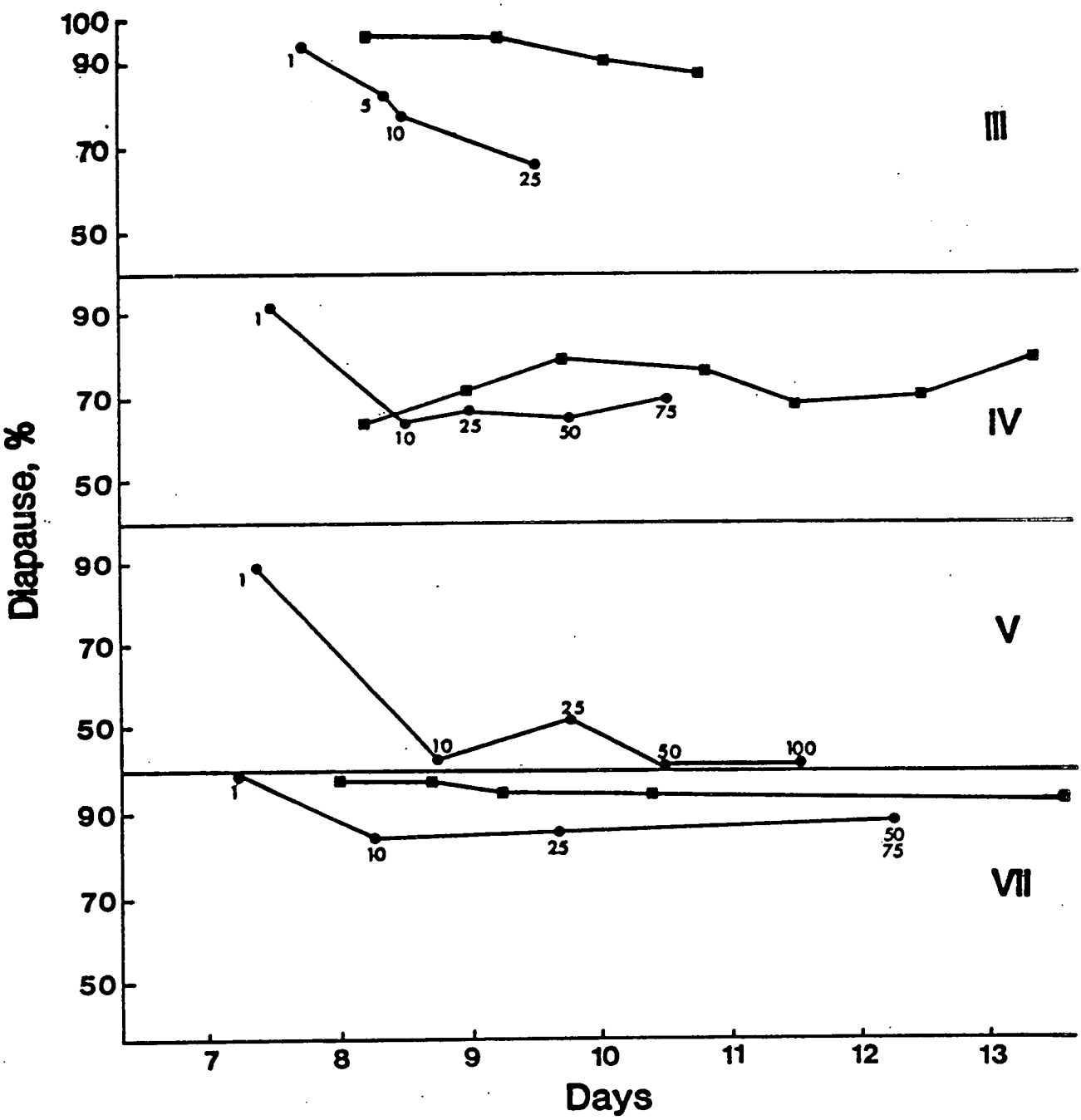


FIG. 19. (●-●) The reduction in the incidence of pupal diapause associated with the increase in the duration of the wandering period, resulting from increased larval post feeding densities. Numbers on curve denote number of larvae per plastic jar. (see text).
(■-■) The incidence of pupal diapause plotted as a function of the increase in the duration of the wandering period, in cultures of post feeding larvae placed in wet sawdust for 1 to 8 days (see text) before transfer to dry sawdust.
III, IV and V at 25 ± 1°C, VII at 23 ± 1°C.

TABLE 29a

The effect of larval post feeding density on the timing of puparium formation and subsequent incidence of pupal diapause in S. argyrostoma. Adults and Larvae LD12:12, 23 ± 1°C.

	Density (Larvae per glass jar)	Total Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %
	1	99	8.3	98
	2	97	8.45	95
	5	118	8.85	98
VI	10	120	9.35	93
	25	148	10.15	94
	50	143	11.9	93.5
	75	153	14.25	94.8
	100	191	16.8	93.4

TABLE 29b

The effect of larval post feeding density on the timing of puparium formation and subsequent incidence of pupal diapause in S. argyrostoma. Adults and Larvae LD12:12, 23 ± 1°C.

	Density (Larvae per plastic jar)	Total Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %
	1	109	7.25	100
VII	10	107	8.3	83.5
	25	125	9.65	86.0
	50	98	12.35	87.6
	75	147	12.35	88.0

WET TREATMENT AS A METHOD TO INCREASE THE LENGTH OF THE WANDERING PHASE

Ohtaki (1966) and Saunders (1975) reported that puparium formation in Sarcophaga species was delayed if post feeding larvae were kept in wet conditions. Therefore, solitary, post feeding larvae were kept in plastic jars containing sawdust saturated with water for different numbers of days (Table 30), before being transferred to plastic jars containing dry sawdust (see p.121). These experiments were carried out in conjunction with the density experiments; the solitary larvae, placed in dry sawdust immediately on reaching the post feeding phase (Tables 28 and 29) acting as controls for both series of experiments. Any prepupae which formed in the wet sawdust were collected. The results of these experiments are shown in Table 30 and Figure 19, curves III, IV and VII. The diapause incidence in individuals which had formed in the wet sawdust (W) was very low compared to that of the dry controls. However, this was probably not a reflection of the increase in the median of pupariation within this group, but a response similar to that described on p. 146 when the newly formed prepupae were over-crowded immediately after collection. The majority of larvae were unable to form puparia whilst in the wet, therefore wet treatment of post feeding larvae

TABLE 30

The effect on the timing of puparium formation and diapause incidence of keeping solitary post feeding larvae in wet conditions for up to eight days (W1-W8) before transfer to dry conditions. (D) dry control (W) wet control I, II, III, IV and VII are sibling cultures of density experiments described in Tables 28 and 29.

EXPERIMENT	TREATMENT	TOTAL NUMBER OF PREPUPAE FORMED	MEDIAN OF PUPARIATION (DAYS)	DIAPAUSE %
I	W	51	9.0	67
25 ± 1°C	D	97	7.8	96
II	W	72	10.5	0
25 ± 1°C	D	84	7.65	88
III	W1	48	8.25	96
	W2	49	9.25	96
25 ± 1°C	W3	48	10.0	91
	W4	50	10.75	87
	W	52	14.0	30
	D	164	7.75	93
IV	W1	49	8.25	65
	W2	48	9.0	72
	W3	50	9.75	80
	W4	46	10.75	77
25 ± 1°C	W5	48	11.5	69
	W6	44	12.5	71
	W7	46	13.5	80
	W8	42	14.5	70
	W	45	13.0	10
	D	192	7.5	92
VII	W1	104	8.0	98
	2	94	8.7	98
23 ± 1°C	3	92	9.25	95
	4	93	10.25	95
	8	61	13.8	93
	D	109	7.5	100

produced an increase in the length of the wandering phase, delaying puparium formation by the time spent in the wet sawdust, plus about 1.5 to 2.5 days after transfer to dry conditions. Figure 20a shows the distribution of puparium formation in groups from experiment III. In the dry control, the median of pupariation occurred 2.75 days after transfer to dry sawdust and puparium formation occurred over a range of 7-10 days. The time between transfer to dry sawdust and the median of pupariation, and the range of days over which puparium formation took place, decreased with an increase in the time spent in the wet sawdust e.g. 92% of larvae kept in wet sawdust for 4 days formed puparia within a 24 hour period and the median of pupariation occurred 1.75 days after transfer.

In all cultures, a delay in puparium formation produced by wet treatment resulted in a higher incidence of diapause than an equivalent delay produced by crowding larvae. For example, in experiment III (Fig. 19), concerning groups kept dry at a density of 5 or 25 larvae per jar, the median of pupariation occurred 8.35 and 9.5 days after larviposition and the incidence of diapause was 82.8% abd 66%, respectively. On the other hand, equivalent delays in pupariation produced by keeping solitary larvae in wet sawdust for 1 and 3 days were

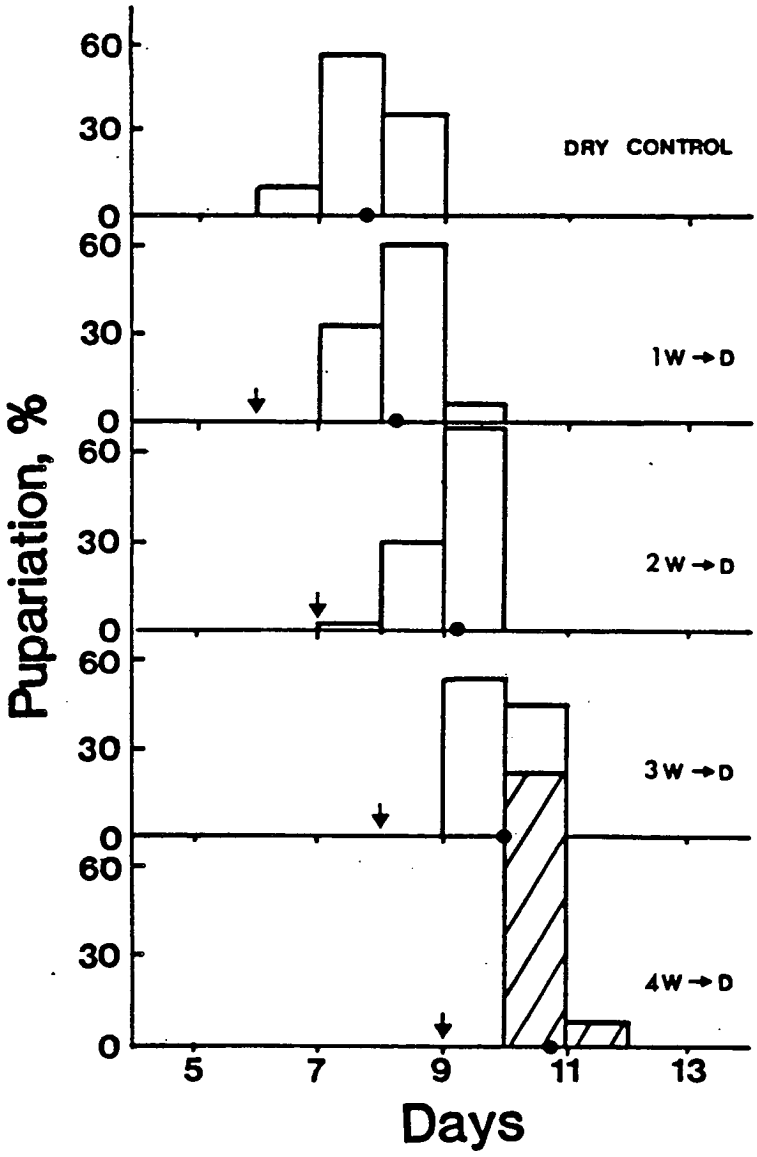


FIG. 20a. The delay in puparium formation in groups of post feeding larvae placed singly in jars containing wet sawdust for 0 (dry control) 1, 2, 3 or 4 days before transfer to dry sawdust. Adults and larvae LD12:12, 25 ± 1°C. (↓) time of transfer from wet to dry sawdust. (●) median time of pupariation in each culture. W(wet) and D(dry) sawdust.

8.25 and 10.0 days after larviposition, and the incidence of diapause was 96% and 91%. A similar result was obtained when the experiment was carried out at 23 $\pm$ 1^{o}C (exp. VII), although, in culture IV, the reduction in the incidence of diapause in 'wet' treated larvae was very variable, ranging from 65% (1 day wet treatment) to 80% (3 or 7 days wet treatment). The following three experiments were then performed to determine whether larvae were sensitive to crowding during wet treatment. The combinations of the various treatments, the median of pupariation and the incidence of diapause are given in Table 31. A median test was used to test for differences in the rates of pupariation. With one exception (V, IW→ ID, IOW→ ID), there was no significant difference in the rate of puparium formation in larvae which experienced different densities during their 'wet' treatment (Fig. 20b). This is in contrast to the behaviour of larvae under different post feeding densities in dry conditions (Fig. 18). Therefore larvae do not appear to react to crowded conditions whilst wet but do so during dry treatment. A possible explanation for this phenomenon is put forward on p. 198.

TABLE 31

The effect of larval density and wet conditions during the post feeding stage of S. argyrostoma on the timing of puparium formation and diapause incidence. Larvae were placed in wet sawdust for 2 days before transfer to dry sawdust. V and VII are sibling cultures of density experiments described in Tables 28 and 29. Results from Median Test to determine differences in rate of puparium formation: (NS) $p > 0.05$, (*) $0.05 > p > 0.01$ (**) $0.01 > p > 0.001$ (***) $p < 0.001$.

Replicate	Treatment		Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %	Median Test Result	
	Wet Density	Dry Density					
VII	1	1	94	8.7	98	0.48 NS	55.1 ***
23 ± 1°C	10	1	70	8.6	98.5		
	1	10	108	9.65	96	0.004 NS	66.3 ***
	10	10	79	9.65	91		
	-	1	106	7.5	100		
	-	10	104	8.3	83.5		
VIII	1	1	73	8.6	79.4	0.21 NS	0.59 NS
	10	1	30	8.65	72.0		
25 ± 1°C	20	1	32	8.7	79.3		0.001
	1	10	86	9.7	55	0.66 NS	NS
	10	10	43	9.9	51.3		7.3 **
	1	20	71	10.45	61.4	1.5 NS	
	20	20	36	10.75	55.6		
	-	1	127	7.7	86.5		
	-	10	249	8.65	43.9		
	-	20	216	9.3	37.7		

TABLE 31 (Contd.)

Replicate	Treatment		Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %	Median Test Result	
	Wet Density	Dry Density					
V 25 ± 1°C	1	1	99	8.95	62.5	4.6 *	9.6 **
	10	1	99	9.2	84.3		
	1	10	102	10.05	32.3	0.04 NS	
	10	10	109	10.1	51.8		
	-	1	129	7.8	89.1		
	-	10	122	8.75	42.5		

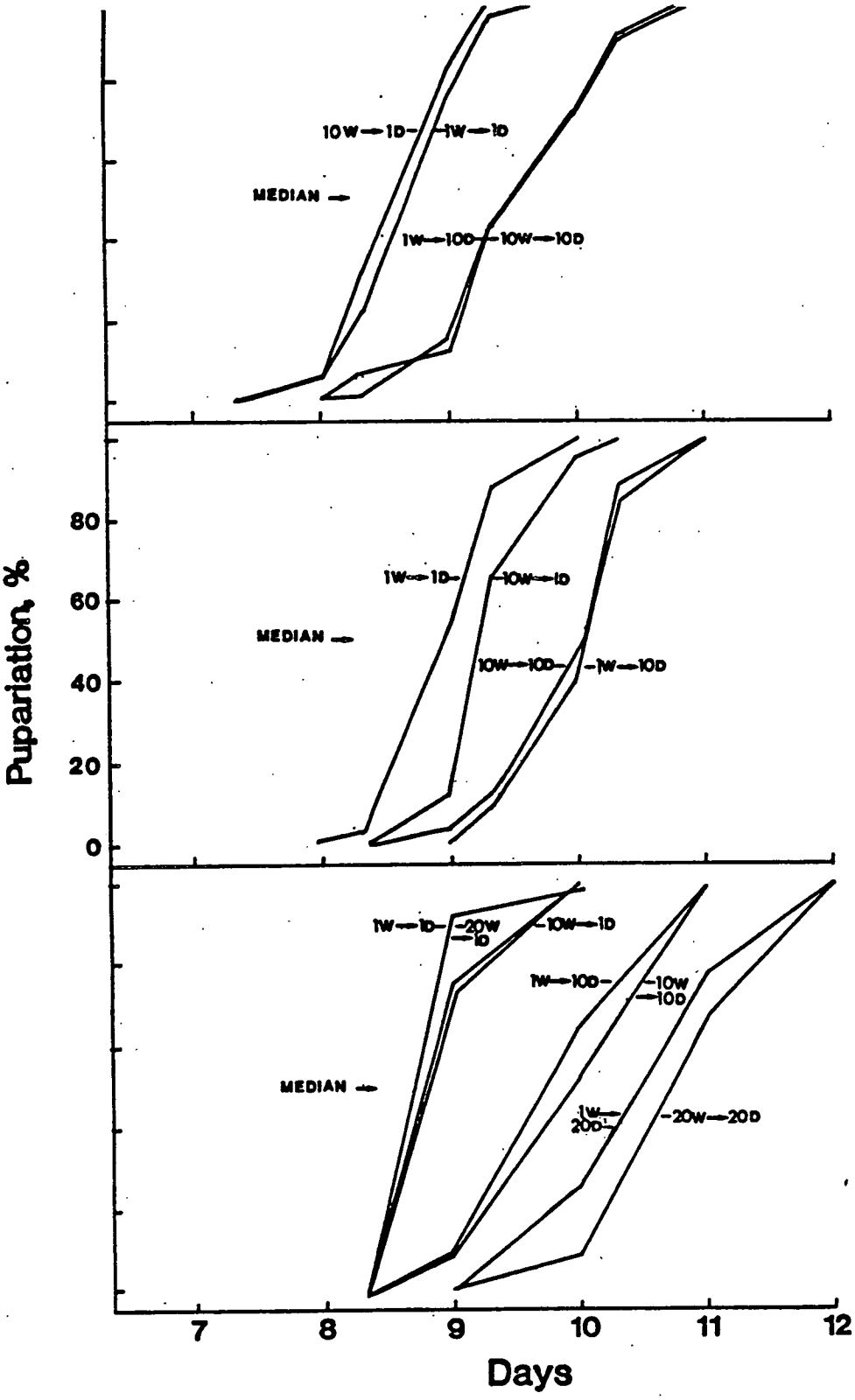


FIG. 20b. The effect of larval density and wet conditions during the post feeding stage of S. argyrostoma on the timing of puparium formation. Larvae were placed in the wet sawdust (W) for 2 day before transfer to dry sawdust (D). Numbers denote larval density. Adults and larvae at LD12:12, 25 ± 1°C. Data from 3 replicate experiments.

THE CROWDING EFFECT: PHYSICAL OR CHEMICAL?

To determine whether the crowding effect of delayed puparium formation was caused by either an airborne pheromone or a contact pheromone or by some other factor such as nervous inhibition by propriocepters, the following experiments were carried out. Approximately 2000 post feeding larvae were placed in a plastic box (30 x 20 x 8 cm) containing ½ inch of finely sieved sawdust and covered with a gauze lid. A single larva was then placed in each of 25 plastic jars (6 cm high 3.5 cm diam.) and the jars placed open end down on the gauze lid above the overcrowded culture. Control larvae were placed in a similar manner above a plastic box containing sawdust only. There was no significant difference in the rate of puparium formation or diapause incidence between the two groups of larvae (Table 32), i.e., the delay in puparium formation in crowded cultures was not due to an airborne pheromone. To test whether the delay was due to a contact pheromone, 100 larvae were removed from a newly post feeding culture and kept in wet conditions in a sealed 1 lb kilner jar. The remaining larvae were placed in an airtight plastic box containing ½ inch dry sawdust. The larva were removed from the kilner jar after 24 hours and placed at a density of 1 larva per jar in plastic jars containing ½ inch

TABLE 32

The rate of puparium formation in (E) solitary post feeding larvae which 'shared air' with a crowded culture of postfeeding larvae and (C) solitary post-feeding larvae which 'shared air' with fresh sawdust only. Adults and larvae LD12:12, 25 ± 1°C.

Treatment	Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %
E	25	7.2	84.0
C	22	6.95	81.8

Median Test on cumulative rates of pupariation, $X^2 = 1.2$, $p > 0.05$ X^2 on proportion pupae entering diapause, $X^2 = 0.001$, $p > 0.05$.

TABLE 33

The rate of puparium formation in (E) solitary larvae placed in sawdust from a crowded culture of postfeeding larvae and (C) solitary postfeeding larvae placed in fresh sawdust.

Treatment	Number of Prepupae Formed	Median of Pupariation (Days)	Diapause %
E	47	8.25	63.8
C	56	8.5	64.3

Median Test on cumulative rates of pupariation, $X^2 = 0.23$, $p > 0.05$. X^2 on proportion of pupae entering diapause, $X^2 = 0.25$, $p > 0.05$.

of sawdust taken from the crowded culture. Control larvae were placed in jars containing fresh sawdust. There was no difference in either the rate of pupariation or in the proportion of pupae entering diapause, (Table 33) i.e., the crowding effect is not due to a contact pheromone.

To test whether the crowding effect could be mimicked by continuous contact, individual larvae were placed inside clear plastic tubing (10 cm long x 0.5 cm internal diameter), blocked at both ends by a piece of gauze. The larvae were capable of forward and backward movements but were constantly touching the walls of the plastic tubing. Solitary larvae in glass jars (11 cm x 5 cm diameter) containing no sawdust acted as a control. Results showed that contact on all sides resulted in a significant delay in the timing of puparium formation (Table 34). A similar experiment was carried out in which larvae were placed head first inside a clear plastic 1.5 ml test tube which tapered from an internal diameter of 9 mm to 4 mm; the tubes were then stored upright. Control larvae were kept in plastic tubes (6 cm x 3.5 cm diameter) in which they were free to move in all directions. The result of this experiment was similar to that above; larvae prevented from moving freely showed a delay in puparium formation ($p < 0.001$), (Table 35). In order to mimic the constant movement (jostling) of

TABLE 34

The rate of puparium formation in solitary post feeding larvae (E) in narrow clear plastic tubing and (C) in plastic jars. Adults and Larvae LD12:12, 25 ± 1°C.

Treatment	No. of Prepupae Formed	Median of Pupariation (Days)	Diapause %
E	36	9.9	88
C	97	7.8	96%

No overlap of cumulative rate of pupariation. X^2 on proportion of pupae entering diapause, $X^2 = 1.4$ $p > 0.05$.

TABLE 35

The rate of puparium formation in solitary post feeding larvae (D) in narrow test tubes (S) in shaking flat bottomed flasks and (C) plastic jars. Adults and larvae LD12:12, 23 ± 1°C.

Treatment	No. of Prepupae Formed	Median of Pupariation (Days)	Diapause %
D	39	10.15	99
S	114	8.65	97
C	95	8.2	97

Median Test on cumulative rates of pupariation
a) D/C, $X^2 = 106.2$, $p < 0.001$
b) S/C, $X^2 = 1.74$ $p > 0.05$.

an overcrowded culture, solitary larvae were placed in glass flat bottomed flasks (3.5 cm diam.) mounted on a shaking water bath and agitated at 100 movements per minute. Control larvae were kept solitarily in plastic tubes (3.5 cm diam). Agitating solitary larvae resulted in a slight and insignificant delay in the median of pupariation (Table 35). These experiments were performed at 23 ± 1°C, and although the confined larvae showed delayed puparium formation, at this temperature, the diapause incidence remained saturated in all groups (Table 35). Therefore the decrease in diapause incidence associated with delayed puparium formation in crowded conditions was not recorded.

DISCUSSION

Gibb's threshold distribution model for diapause induction in Sarcophaga argyrostoma was based on the hypothesis that the diapause inducing effects of short day/long night cycles were summed, during the sensitive period, by a temperature-compensated photoperiodic counter (Saunders, 1971), and stored in the form of a 'diapause titre'. The developmental fate of an individual was then determined by a comparison of this titre with an internal threshold: if the titre was below the threshold, the individual would not enter diapause, whereas, if the titre exceeded the threshold, the individual would enter diapause (Gibbs, 1975).

Gibbs assumed that the rate of increase in the diapause titre was consistent per short day cycle through the entire sensitive period. However, results from experiments on S. argyrostoma by Saunders (1980) suggested that the photoperiodic responsive period declined in sensitivity from the intrauterine embryos to puparium formation. The results also implied that mature larvae were practically insensitive to photoperiod, although earlier experiments (Saunders 1971) had suggested that photoperiodic sensitivity extended into the wandering phase. These data suggest that one short day/long night cycle experienced during the intrauterine stage might not be equivalent to a long night seen later in development. The results presented in this Chapter show that manipulation of the length of the feeding stages has a very different effect on the final diapause incidence than manipulation of the length of the post feeding stage, these experiments will be discussed separately.

MANIPULATION OF THE FEEDING STAGES

The feeding stage of the 3rd larval instar of S. argyrostoma can be divided into two phases: an earlier obligatory phase followed by a facultative feeding stage. At 26°C, 3-10 hours of feeding

after the last larval ecdysis were sufficient for larvae to enter the facultative phase. (Zdarek and Slama, 1972). In the Edinburgh strain of S. argyrostoma, the moult from the 2nd to the 3rd instar usually occurred between 2 and 3 days after larviposition in larvae bred at LD12:12, 17°C. When larvae bred under these conditions were starved during the 1st and 2nd instar stages and the early part of the obligatory phase, they survived from 1 to 3 days, depending on the timing of the starvation period, with no obvious further growth and development. On return to the food, these larvae immediately resumed feeding and eventually pupariated, forming puparia of similar weights to those formed by unstarved (control) larvae. In contrast, larvae of Calliphora vicina, starved whilst in the obligatory feeding phase, did not attain the full weight of normal unstarved larvae (Shaaya and Levenbrook, 1982). Although Zdarek and Slama (1972) stated that no growth and development occurred in S. argyrostoma during a period of starvation, the results from this study, in which larvae from sibling cultures were starved for a range of days, commencing 3 days after larviposition, suggest that some growth may have occurred: larvae starved for 1 day were still in the obligatory phase on return to the food, whereas

a proportion of larvae in cultures starved for either 2 or 3 days had already entered the facultative feeding phase and failed to feed when returned to the medium. At a larval rearing temperature of 17°C, the transition from the obligatory to the facultative feeding phase occurred about four days after larviposition; larvae starved for a 24 hour period commencing on day 4 failed to feed when returned to the medium. Likewise after a similar period of starvation, commencing on day 2, a proportion of the larval culture became post-feeding only 2 days after being returned to the medium. It is possible that the "trauma" of starvation had caused these larvae to leave the food at the start of the facultative feeding phase.

One effect of starvation was to produce cultures with a range of medians of pupariation since when larvae were starved during the obligatory phase, puparium formation was delayed for a period similar to the duration of starvation. Results showed a highly significant positive linear relationship between the median of pupariation and the final diapause incidence in the culture. This relationship was most obvious within cultures showing a bimodal pattern of pupariation. For example, the incidence of diapause was negligible within

individuals in the first peak which had formed prepupae having only experienced 6 or 7 cycles of LD12:12, whilst individuals in the 2nd peak, which formed prepupae some 7 days later, had a very high diapause incidence. However, it should be noted that larvae within these cultures had been previously deposited over a 24 hour period by flies kept at LL, 25°C. Those larvae deposited earlier could be 'older' on transfer to LD12:12, 17°C than those larvae deposited later. It is possible, therefore, that after a period of starvation, the 'older' larvae might be in the group that had entered the facultative feeding phase, and formed prepupae prematurely. These larvae would have experienced more LL, 25°C during the highly sensitive early 1st instar than larvae deposited later, and this experience, and the curtailed larval period, might act synergistically to produce a very low incidence of diapause.

Premature extraction of 3rd instar larvae which had entered the facultative phase, specifically affected the older instars which are thought to be less photoperiodically sensitive (Saunders, 1980). Likewise, crowding larvae during the feeding phase mainly affected the 3rd instar larvae. Both treatments resulted in the premature formation of light-weight puparia and a wide range in the timing

of puparium formation and, as with results from the starvation experiment, there was a positive linear relationship between the length of the larval period (as measured by the median of pupariation) and the final diapause incidence. The slope of the regression line produced by starving the young feeding larvae is slightly, but not significantly, steeper than the slope of the regression line produced when larvae were manipulated in the later feeding stage (analysis of covariance $p\ 0.1 > p > 0.5$) suggesting that short days/long nights have a greater effect on younger larvae. This agrees with Saunders's (1980) suggestion that photoperiodic sensitivity declines as larval development proceeds.

The results from experiments in which the duration of the larval feeding stage was manipulated are thus consistant with the simple hypothesis derived from Gibbs's model, in which a reduction in the number of diapause inductive cycles experienced by curtailing the length of the sensitive period would result in a decrease in the incidence of diapause, and an increase in the length of the sensitive period would result in a subsequent increase in diapause. However, Gibbs's assumption that the rate of increase in the 'diapause titre' per short day was constant with time should be modified to take into account the differential

sensitivity of the larval instars.

MANIPULATION OF THE POST FEEDING STAGES

The observation that crowding during the post feeding stages could affect the subsequent timing of puparium formation in the Edinburgh strain of S. argyrostoma was first observed by J.M. Giebultowicz (unpublished work, 1980). In this present study it was demonstrated that there was a clear and positive linear relationship between the density of the post feeding larvae and the delay in the average time of puparium formation, and that the delay was a result of tactile stimulation rather than a chemical one. The density effect can be mimicked by shaking solitary larvae placed within round bottomed flasks on an oscillating platform shaker (Denlinger, 1981; Saunders, pers. comm.) and by forcing solitary larvae into narrow plastic tubing. A similar delay in pupation has been reported in space-deprived larvae of the wax moth, Galleria mellonella (Sehnal and Edwards, 1969; Alexander, 1970; Pipa, 1971). In Galleria it was suggested that sensory input, presumably via mechano-receptors, inhibits the neurosecretion of factors necessary for pupation, although the mechanism is unknown. External stimulation was also found to delay the release of ecdysone, and hence puparium

formation, in the blue bottle Calliphora vicina (Berreur et al., 1978), although whether external stimulation directly affected ecdysone release or perhaps acted via the brain, inhibiting the neurosecretion of PTTH, is not known.

The larvae of S. argyrostoma will not form prepupae when in contact with water. This phenomenon was first reported in Sarcophaga peregrina by Ohtaki (1966). The cause of this delay was neither the direct action of water on the cuticle nor interference with respiration, since pupariation took place in wet conditions if the larvae had been previously exposed to dry conditions for sufficient time for the hormones associated with puparium formation to be released (Ohtaki, 1966). When post feeding larvae were ligatured behind the brain and ring gland and then kept in wet conditions, the hind portion 'pupariated' after an injection of ecdysone (Ohtaki 1966; Zdarek and Fraenkel, 1970). Ohtaki concluded that retardation of ecdysone release was the final reason for delayed pupariation. However, Roberts (pers. comm), working with Sarcophaga bullata, has shown that 'wet treatment' does not inhibit ecdysone release from the ring glands: water in which post feeding larvae had been placed, was found to contain 20-hydroxyecdysone, the level of which increased at a steady rate during

the four day period of the experiment. Moreover, the ring glands of post feeding larvae which had experienced wet treatment were more active than ring glands of 'dry' larvae of an equivalent age, although 'dry maggots' had more haemolymph hormone than 'wet maggots'. This suggested that the rate of excretion and/or possibly deactivation of 20-hydroxyecdysone was very efficient in 'wet maggots'. Rapid deactivation of ecdysone was demonstrated in mature larvae of S. peregrina by Ohtaki, Milkman and Williams (1968), the half life of injected ecdysone being less than 1 hour at 25°C. These authors concluded that ecdysone in fly larvae is in a highly dynamic state and acts by the accumulation of "covert effects" within the target organs. These "covert effects" were shown to undergo spacial and temporal summation to a critical threshold, finally discharging the overt developmental response, e.g. pupariation. The "covert effects" were shown to be subject to decay if their temporal accumulation was interrupted, short of the discharge level, by transferring the larvae to wet conditions, suggesting a certain instability of these covert effects (Ohtaki et al., 1968; Zdarek and Fraenkel, 1970). However, it should be pointed out that the authors' conclusions rest on the following premises: Mature larvae do not

release ecdysone when in contact with water, and the water treatment stops further release of ecdysone even after this release has begun. Furthermore, since recent work by Roberts, described on pp.195-196, has shown that ecdysone secretion does occur in wet conditions and the haemolymph levels of 20-hydroxyecdysone are very low, it is possible that most of the hormone is inactivated before uptake by the target organs. Therefore very little summation of the covert effects of the hormone will take place, and since the covert effects are known to be subject to decay, the accumulation will not reach discharge level and pupariation will not occur in the wet. Roberts's data, therefore, do not invalidate the results. In the present study, wet treatment has been shown to synchronise puparium formation within groups of larvae kept in wet conditions, the degree of synchronisation increasing with the increased period of wet treatment. Indeed, many workers have kept post feeding larvae in wet conditions to provide a synchronised population for experimental purposes (Ohtaki 1966; Ohtaki, Milkman and Williams 1968; Zdarek and Fraenkel, 1970). Roberts (pers. comm.) found that the levels of 20-hydroxyecdysone in the haemolymph of prepupae 0-24 hours after pupariation, which had previously experienced wet conditions for 48 h and 144 h

respectively, required 53% and 37% of the moulting hormone of dry control animals. Therefore, some accumulation of the covert effects of ecdysone must have occurred during wet treatment and thus 'less' ecdysone was required to initiate pupariation on return to dry conditions. Larvae of S. argyrostoma are insensitive to crowding effects during wet treatment - possibly the response to wet treatment i.e. ecdysone release and rapid excretion and deactivation (Roberts, pers. comm), overrides the response to crowding i.e., inhibition of ecdysone release (Berreur et al.). Inhibition or delay of pupariation by moisture and/or crowding allows the larva to find more favourable situations in which to pupariate (Berreur et al., 1976; Ohtaki, 1966).

Gibbs (1975) assumed that the photoperiodically sensitive phase in S. argyrostoma extended from the intrauterine embryo to puparium formation, and his model for the photoperiodic counter implies that any treatment which prolonged the larval period in diapause inductive conditions should result in an increase in the incidence of diapause: extra short day/long night cycles would 'raise' the 'diapause titre'. However, in this study, an increase in the duration of the post feeding stage, produced by crowding, was associated with a decrease in diapause incidence, even though the larvae

experienced extra long night or inductive cycles. This decrease was observed only when the incidence of diapause in solitary (control) larvae was unsaturated. It is possible that soon after the wandering behaviour commences, photoperiodic sensitivity ceases (Saunders, 1980) and no further 'diapause titre' is produced or stored. During a protracted wandering period the diapause titre may decay. If this titre then falls below the internal threshold for diapause induction the previously diapause committed larva will not now enter diapause as a pupa. This situation would only occur in conditions of unsaturated diapause, in individuals whose titres were originally close to their internal threshold. In conditions of saturated diapause, on the other hand, the titre would be too far above the threshold for this reduction in diapause to occur. The results from experiments testing the photoperiodic response of the 'fast' and 'slow' strains derived from a stock culture of S. argyrostoma are consistant with this hypothesis (see p.253).

Evidence for this idea that the 'diapause titre' might be unstable was provided by Gibbs (1975). He found that in S. argyrostoma a temperature shift (upwards) within 25 hours of pupariation was capable of reversing a commitment to diapause. Furthermore,

the diapause incidence finally achieved in a given group was a function of both the size of the temperature step up and of the number of short day photoperiods experienced during earlier larval life. Gibbs suggested that "the difficulty in inducing an individual to cross its threshold was in effect a measure of its distance from that threshold". The present study demonstrated that if newly formed pupae were allowed to become water-logged, or pupariation took place in wet sawdust, previously diapause destined individuals failed to enter diapause. Presumably, as with temperature shifts, this 'physiological shock' may have had some effect on the stored 'photoperiodic information' or perhaps may have resulted in the release of PTTH stored in the neurosecretory endings (Fraser, 1960; Kono 1973), thus overriding the diapause commitment.

In conclusion, the results presented in this thesis are consistent with the hypothesis that S. argyrostoma possesses a temperature compensated photoperiodic counter which adds up successive short days or long nights, the final photoperiodic response being a consequence of the number of light cycles that the insect experiences during the restricted photoperiodically sensitive period. Gibbs's model for the photoperiodic counter remains

valid for summation of photoperiodic cycles by S. argyrostoma with the following refinements: (1) All three larval instars are not equally sensitive to photoperiod, sensitivity declining from a maximum during the late intrauterine embryo stage through to the mature feeding larva, and ceasing shortly after the onset of the wandering stage. Since the response to photoperiod is not constant throughout development, as assumed by Gibbs, this may be reflected in the change in the 'diapause titre', possibly more 'diapause titre' being 'accumulated' early in embryonic and larval development per long night cycle than later. (2) The results from this study also suggest that the stored 'diapause titre' might be unstable. After photoperiodic sensitivity ends it is subject to decay and, if the titre subsequently falls below the 'threshold' for diapause induction, the individuals original commitment to diapause will be reversed.

There is direct evidence supporting the idea of a temperature compensated photoperiodic counter (see pp.48-53). However, concrete evidence for an accumulated 'diapause titre' is lacking. The possible interactions between time measurement (the clock), the photoperiodic counter (summation) and

the subsequent programming of the cerebral neuroendocrine system is discussed on pp. 324-332.

C H A P T E R III

SELECTION FOR EARLY AND LATE PUPARIATION IN SARCOPHAGA ARGYROSTOMA

CHAPTER III

SELECTION FOR EARLY AND LATE PUPARIATION IN SARCOPHAGA ARGYROSTOMA

The genetics of the photoperiodic response have been investigated in a variety of insect species, and many investigations have suggested that both diapause incidence and its duration are heritable traits. For example, a series of crosses between strains of the moth Acronycta rumicis, with differing critical photoperiods, indicated that the genetic control of the photoperiodic response was polygenic (Danilenskii, 1965), and recent work by Lumme and coworkers, on the reproductive diapause of Drosophila littoralis, has demonstrated that a single genetic unit was responsible for the continuous latitudinal cline in the critical daylength (Lumme, 1982).

Many studies have used strains with high and low diapause incidences, utilizing these lines to evaluate genetic differences in the diapause response by interstrain crossing. McLeod (1978), using two strains of the European cornborer Ostrinia nubilalis, found that the time to pupation in non-diapausing conditions was maternally determined, whereas the actual level and duration of the diapause response was influenced more by the male parent. Kurahashi and Ohtaki (1977) carried out hybridisation

between non-diapausing (tropical) and diapausing (temperate) races of Sarcophaga peregrina. In diapause inductive photoperiods, the original "saturated" parental diapause response of the temperate race was reduced to about 60% in the hybrid crosses. Likewise, the temperate critical daylength of 14 to 15 hours decreased to between 11 and 12 hours. The authors proposed the existence of anti diapause and diapause alleles and suggested that the physiological state of the progeny was determined by a combination of such alleles and the environmental conditions. A similar study, using high and low diapausing lines derived through selection and subsequent inbreeding from an original laboratory strain of Sarcophaga bullata, was carried out by Henrich and Denlinger (1984). Their results revealed that the ability to enter diapause depended greatly upon "heritable factors", although the patterns of inheritance were not additive. Moreover, the authors concluded that a relatively small number of loci were likely to control the diapause response in this species.

Henrich and Denlinger (1982) selected a strain of Sarcophaga bullata in which puparium formation (at LD15:9, 25^{o}C) occurred about 1 day later than in the unselected stock. They found that the genetic factors that influenced the length of

larval development also affected the developmental rate in other stages of the life cycle. Moreover, the selected individuals were more likely to enter diapause and remained in diapause for longer than the unselected individuals.

Early in the present study it was assumed that the genetic variability of the Edinburgh strain of Sarcophaga argyrostoma would be low after at least 20 years maintenance in the laboratory. However, the large amount of variation in the timing of puparium formation (see p. 211) suggested that this might not be the case. For this reason flies were bred from the first and the last larvae to form puparia, for 13 consecutive generations to give two strains, 'fast and slow' with differing wandering periods; these were subsequently maintained in the laboratory without further selection. It became clear that the 'fast' and 'slow' strains could produce individuals with widely differing larval periods, and might provide a useful tool to investigate the summation of photoperiodic cycles in S. argyrostoma, and provide information about the genetics of the photoperiodic response. It was also hoped that the data produced from the strains would be consistent with results described earlier in this thesis, in which the length of the larval period had been artificially manipulated using methods other than temperature.

ARTIFICIAL SELECTION OF STOCK TO OBTAIN TWO STRAINS

MATERIALS AND METHODS

Larvae were deposited over a 6 hour period by adult stock kept at LL, 25 ± 1°C. The first 115 and last 54 individuals to form prepupae were placed in jars and labelled 'fast' (F) and 'slow' (S) respectively and kept in DD, 22°C. Upon eclosion, each jar was placed in a small insect cage at LL, 25 ± 1°C, thus adults (from each strain) were massmated. The first progeny (~ 200 to 400 larvae) from each cage were cultured at LL, 25 ± 1°C and newly formed prepupae collected either once or twice daily. The first 100 puparia from the 'fast' strain were retained and used to breed the next generation, likewise the last 100 puparia from the progeny of the 'slow' strain provided adult stock for the next generation. This selection procedure was repeated for 13 generations, in later generations, cultures of 300 larvae were used to monitor the time of pupariation of the two strains. Unfortunately, the majority of the progeny of generation 7 failed to eclose, therefore generation 8 were the progeny of about 30 flies.

Interstrain crosses (F x S and S x F, female designated on the left) were made by massmating females of one strain with the males of the other. This was achieved by allowing flies from each strain

to emerge into separate cages. Within 1 day of eclosion, the flies were removed from the cage, sexed, and placed in a separate appropriate cage to allow the reciprocal mass mating at LL 25 ± 1°C. Each cage contained about 200 flies of each sex. Progeny were either cultured at LL, 25 ± 1°C and the newly formed prepupae collected twice daily and used to set up the interstrain crosses described below, or the progeny were used to construct the photoperiodic response curves at 20 ± 0.5°C and 17 ± 0.5°C described on pages 236 and 240.

Pupae from the F x S hybrid were reciprocally massmated with the 'S' strain using the procedure described above. The cages were kept at either LD12:12, 25 ± 1°C. Likewise an F_2 generation was produced by allowing progeny from the S x F hybrid to interbreed at random at LD12:12 25 ± 1°C. The progeny from these crosses were used to construct the Photoperiodic Response Curves described on page 242.

Newly formed puparia from all the experiments carried out at 17 ± 0.5°C or 20 ± 0.5°C were collected daily and maintained in DD, 20°C for 2 weeks, then scored for diapause development or death.

SOURCE OF VARIATION IN FAST AND SLOW STRAINS

Two cultures of 300 larvae deposited over the previous 24 hours were taken from both fast and slow adult cages kept at LL, 25°C. The larval cultures were also kept at LL, 25°C. Newly formed puparia were collected twice daily. The puparia from each culture were divided into approximately three equal groups based on the timing of puparium formation and labelled beginning (B) middle (M) or end (E) respectively, and stored at LL, 25°C. On eclosion, each jar was placed in a small insect cage provided with sugar, water and meat, and kept at LL, 25°C. The first 250 larvae from each cage to be deposited over a period of 24 hours were cultured at LL, 25°C. Newly formed puparia were collected daily.

SELECTION FOR FAST AND SLOW PUPARIATION

Results

In the previous chapter, it was shown that under conditions of photoperiod and temperature which produce an unsaturated photoperiodic response, a decrease in the length of the larval period was correlated with a decrease in the incidence of diapause, and vice versa. A simple hypothesis

derived from this result, might be: if by selection it were possible to produce strains with a shorter or longer larval period, this would result in a decrease or an increase, respectively, in the incidence of pupal diapause. It was not possible to select for time spent in the food medium, as larvae tended to leave the medium at 25 ± 1°C over a 12 hour period. However, in stock cultures, puparium formation usually occurred over a range of 3-4 days, therefore there was potential for the selection of individuals with differing wandering periods. This was carried out as follows: Larvae deposited by adult stock over a period of 6 hours were cultured at LL, 25 ± 1°C. The first 115 and the last 54 prepupae formed out of a total of 1519 individuals were selected and raised to adulthood and designated 'fast' (F) and 'slow' (S) respectively. The adults were then mass-mated and their progeny cultured (see pp.206 for details). The two strains were found to diverge immediately, (Table 36, Figure 21): in the "Fast" strain, the median of pupariation was 6.25 days, whereas the median of pupariation in the "slow" strain was 9.1 days. The strains remained separated throughout the 13 generations of selection.

There was no difference between the two strains, in the time taken for adult differentiation

TABLE 36

The Medians of puparium formation in 'fast' and 'slow' selected strains of S. argyrostoma over 16 generations. Adults and Larvae LL, 25 ± 1°C except when temperature in insectory dropped to 23 ± 1°C (*).

Generation	Fast		Slow		
	Number	Median	Number	Median	
1	523	6.2	466	9.1	
2	633	6	419	8.6	
3	886	6.3	468	9.3	
4	306	7.6	129	9.5	*
5	257	7.5	416	10.5	*
6	413	6.5	527	11.7	
7	326	7.2	364	11.3	
8	472	7.2	472	9.5	
9	158	6.2	254	8.2	
10	323	6.5	363	10.2	
11	256	7.5	287	9.3	
12	301	7.0	132	9.7	
13	338	7.3	Not recorded		
14	338	8.5	152	11.9	*
15	229	8.7	201	13.3	*
16	293	8.5	461	12.6	*

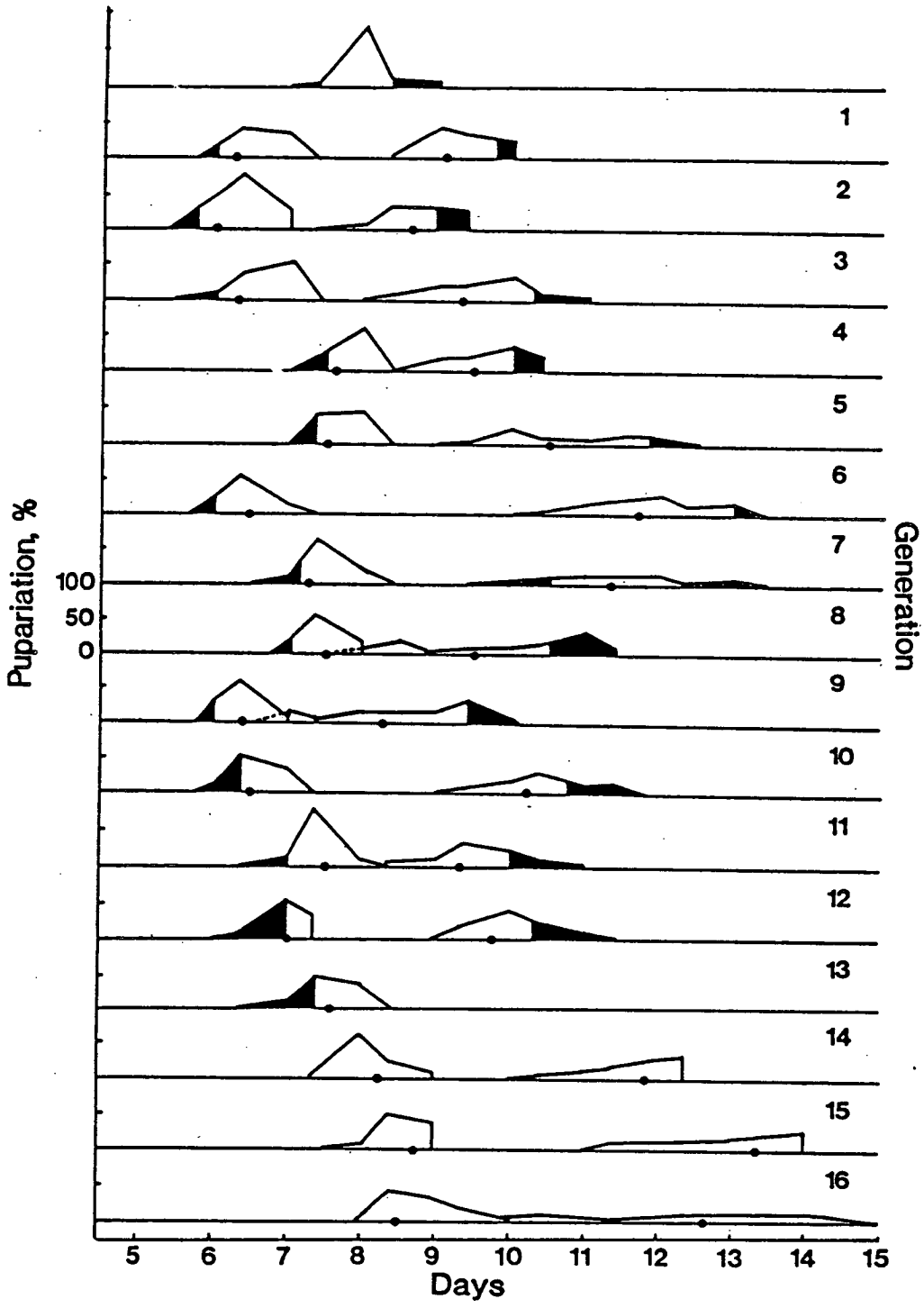


FIG. 21. The distribution of puparium formation (daily percentage puparium formation) over 16 generations in 'fast' and 'slow' strains selected from a stock culture of S. argyrostoma. Shaded areas denote individuals used to provide adult stock for next generation (●) Median of Pupariation. Adults and larvae at LL, 25 ± 1°C. (Generations 4, 5, 14, 15 and 16 at 23 ± 1°C).

(puparium formation to eclosion) and in the time for embryogenesis (eclosion to larviposition) (Table 37). (The latter measurement is more variable since it was not a measure of the time taken to produce the first progeny, but the time elapsing before at least 250 larvae were produced within a 12 hour period). Larvae of both strains also fed for the same period: 5-6 days. Thus selection for a shorter or longer larval life did not result in a change in the duration in any fraction of the life cycle other than the length of the post feeding (wandering) stage.

The timing of puparium formation in both strains is presented in Fig. 21. In the 'fast' strain, puparium formation occurred over a range of about 36 hours. Although the median of pupariation varied throughout the 13 generations (Table 36), this was probably due to uncontrollable environmental variation e.g., the ambient temperature of the insectary was variable occasionally dropping from 25 to 23^{o}C in colder weather.

In order to determine whether the distribution of puparium-formation was due to extrinsic rather than intrinsic variation, two cultures derived from adults of generation 7 were bred at LL, 25 $\pm$ 1^{o}C. The resulting prepupae were then divided into approximately 3 equal groups based on the timing of

TABLE 37

Comparison of the time taken for adult differentiation and embryogenesis between the selected 'fast' and 'slow' strains of S. argyrostoma. Adults and Larvae LL, 25 ± 1°C (Generation 5 at 23 ± 1°C).

Generation	Puparium Formation to Eclosion (Days)		Eclosion to Larviposition (Days)	
	Fast	Slow	Fast	Slow
1	11	11	10	9
2	11	12	10	9
3	11/12	11	9	10
4	11	12	10	10
5	13	11	12	10
6	11	11	10	10
7	11/12	12	10	10
8	13	N/K	9	N/K
9	12	11	10	10
10	12	12	11	10
11	13	12	9	11
12	12	12	10	11
13	11	12	11	11

pupariation, and designated 'beginning' (B), 'middle' (M) and 'end' (E). Adults from each group were then allowed to mass-mate and their progeny cultured (see p.208 for more detail). All cultures fed for 5-6 days. The medians of pupariation of the parental cultures were 7.2 (n=472) and 6.6 (n=243) days, the 0.6 day difference between these values possibly being due to a crowding effect on pupariation (see p.167).

The medians and ranges of pupariation of the progeny were not significantly different (Table 38 Fig. 22); therefore, the "fast" strain is possibly monogenic or, if it is polygenic, the effect of the alleles is masked because the prepupal collection was not frequent enough to reveal any subtle fluctuations.

A similar experiment was carried out using the slow strain (see p.208 for details); the results are displayed in Table 39 and Fig. 23. Unlike the results described for 'fast' the 'beginning' component of the 'slow' strain formed prepupae earlier than all other cultures: puparium formation took place over a range of 8-11 days and the medians of pupariation occurred 9.9 and 9.6 days, respectively, after larviposition. In the first cultures, there was also a 1 day difference in the median of pupariation of the 'middle' and the 'end' components,

TABLE 38

Comparison of time taken for adult differentiation and embryogenesis, and the timing and range of pupariation of intrastrain crosses derived from the selected 'fast' strain of S. argyrostoma. Adults and Larvae at LL, 25 ± 1°C.

Strain	Pupariation to Eclosion (days)	Eclosion to Larviposition (days)	Median of Pupariation (days)	Range of Pupariation	Number of Prepupae Formed
Parent	13	9	7.2	7-8	472
Beginning	12/13	13	6.5	6-9	220
Middle	12/13	12	6.5	6-8	253
End	12/13	11	6.6	6-8	235
Parent	13	9	6.6	6-8	243
Beginning	12/13	13	6.4	6-8	237
Middle	12/13	11	6.5	6-8	201

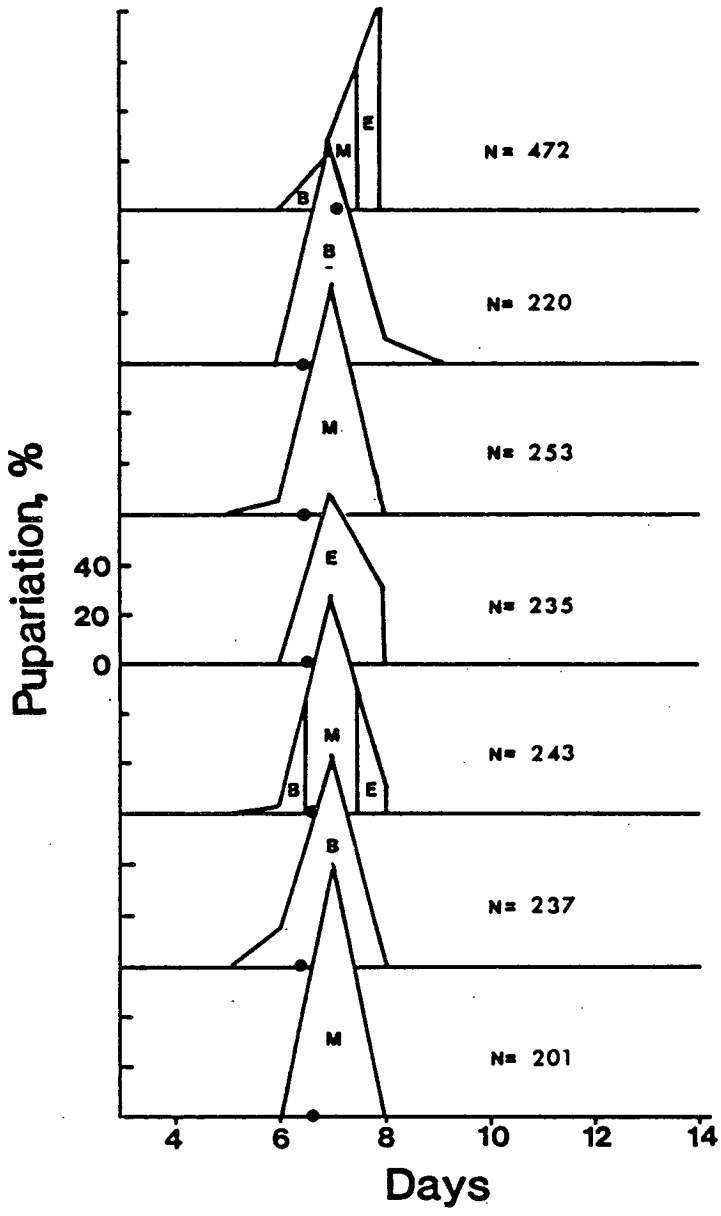


FIG. 22. The distribution of puparium formation in intra strain crosses derived from the selected 'fast' strain of S. argyrostoma. Numbers beside polygons denote total number of prepupae formed, (●) median of pupariation, (B) beginning (M) middle and (E) end puparia of parental fast stock used to provide adults of next generation. Adults and larvae LL, 25 ± 1°C.

TABLE 39

Comparison of time taken for adult differentiation and embryogenesis, and the timing and range of pupariation of intrastrain crosses derived from the selected 'slow' strain of S. argyrostoma. Adults and larvae at LL, 25 ± 1°C.

Strain	Pupariation to Eclosion (Days)	Eclosion to Larviposition (Days)	Median of Pupariation (Days)	Range of Puparium Formation (Days)	Number of Prepupae
Parent	12/13	10	11.3	7-14	364
Beginning	12	9/10	9.9	8-11	169
Middle	12/13	9/10	11.1	9-13	323
End	12	12/13	12.1	9-15	351
Parent	12/13	11	12.1	7-14	245
Beginning	12	12/13	9.6	8-11	263
Middle	12/13	11	11.6	9-13	230
End	12	11	11.2	9-13	233
End	12	12/13	11.6	10-13	255

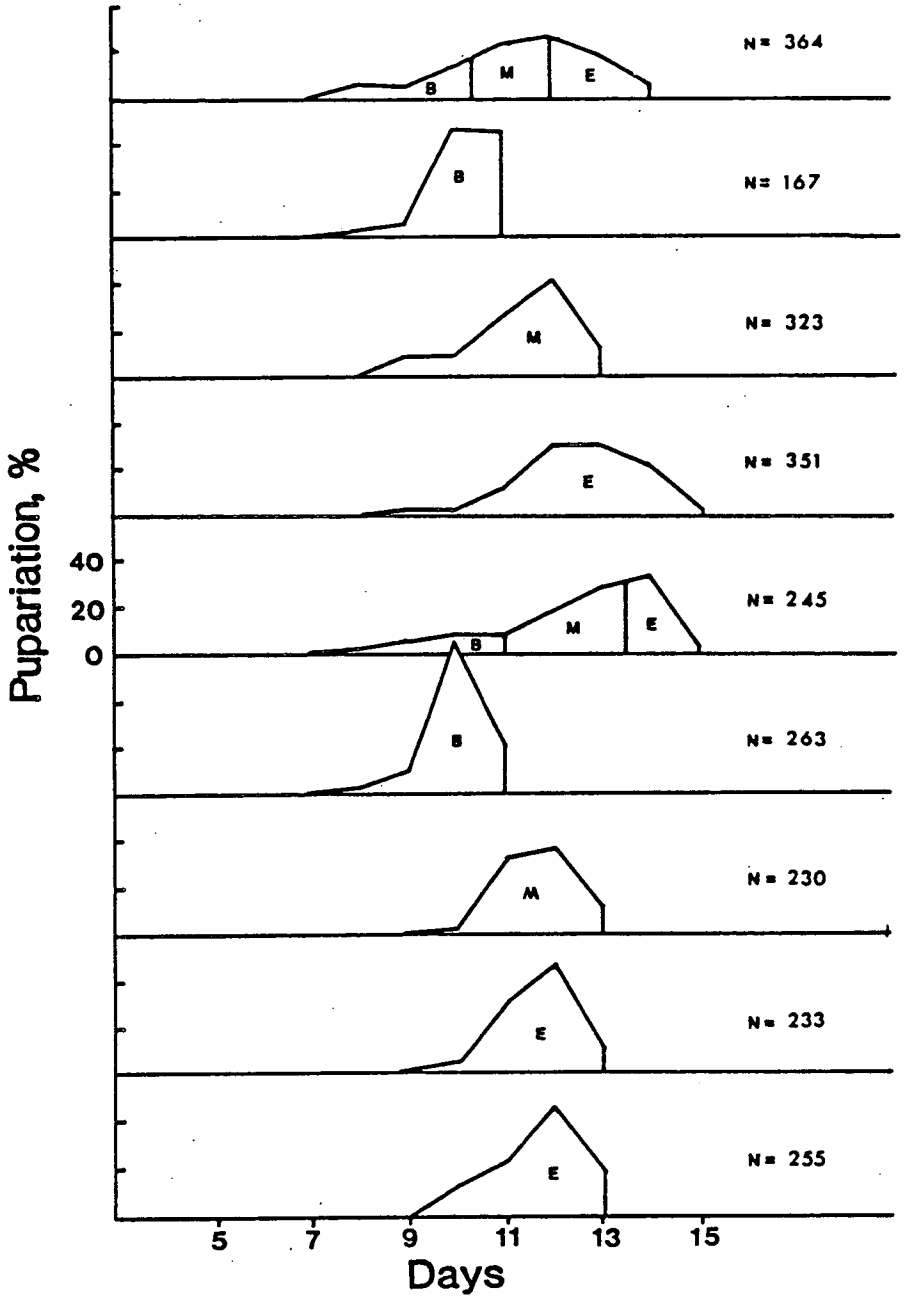


FIG. 23. The distribution of puparium formation in the intrastrain crosses derived from the selected 'slow' strain of S. argyrostoma. Numbers beside polygons denote total number of prepupae formed, (●) median of pupariation, (B) beginning (M) middle and (E) end of puparia of parental 'slow' stock used to provide adults of next generation. Adults and larvae at LL, 25 ± 1°C.

although this result was not found in the second cultures. These results suggest that the 'slow' strain is polygenic. This conclusion is further supported by an examination of the rate of puparium formation of the 'slow' strain over successive generations of selection (Table 36, Fig. 21), in which the median of pupariation of the 'slow' strain was observed to 'drift' between generations 1 and 7, and again, after the population 'crash' (generation 7), between generation 8 and 16.

The behaviour of the "fast" and "slow" strains was also examined at a lower temperature (17± 0·5°C) and in a range of photoperiods from LD8:16 to LD16:8. The 'stock' strain was also tested for comparison. All cultures fed on average for 8-9 days. The median and range of pupariation times are presented in Table 40 and displayed as polygons in Fig. 24. (The photoperiodic response of the strains is presented on pp.234- of this thesis). At all photoperiods tested, the 'fast' strain pupariated earlier and over a narrower range of time than either the 'stock' or the 'slow' strains. There was also considerable overlap between 'stock' and 'slow', however, although the median of pupariation of 'stock' was less than that of 'slow' with the exception of two photoperiods, 12.5 and 14.5 h. Puparium-formation in the 'slow' strain was also

TABLE 40

The timing of puparium formation in three strains of S. argyrostoma, Fast, Stock and Slow, in a range of larval photoperiods at 17 ± 0.5°C. Adults at LL, 25 ± 1°C.

Photoperiod (h)	FAST			STOCK			SLOW		
	Pupariation		n	Pupariation		n	Pupariation		n
	Median (Days)	Range (Days)		Median (Days)	Range (Days)		Median (Days)	Range (Days)	
8	13.5	12-17	216	21.4	14-33	283	22.7	15-41	178
10	13.2	11-17	199	21.5	15-30	185	23.4	14-44	441
12	13.2	12-17	167	17.8	14-27	191	20.1	14-32	165
12.5	14.8	12-19	176	18.2	14-27	261	15.8	13-33	130
13	13.2	12-17	204	16.9	14-27	252	19.5	14-37	264
13.5	13.3	11-17	154	21.7	14-31	231	27.7	14-41	231
14	13.7	10-16	219	16.6	14-25	212	19.8	14-41	192
14.5	12.0	10-15	239	16.5	14-25	210	14.8	13-28	215
15	12.5	10-15	250	15.6	14-20	218	18.2	13-35	196
15.5	12.7	11-14	183	14.9	14-21	265	15.6	13-22	276
16	11.1	10-13	232	14.9	14-21	299	15.9	14-23	350
18	11.7	11-14	346	15.5	14-19	209	16.9	13-26	228

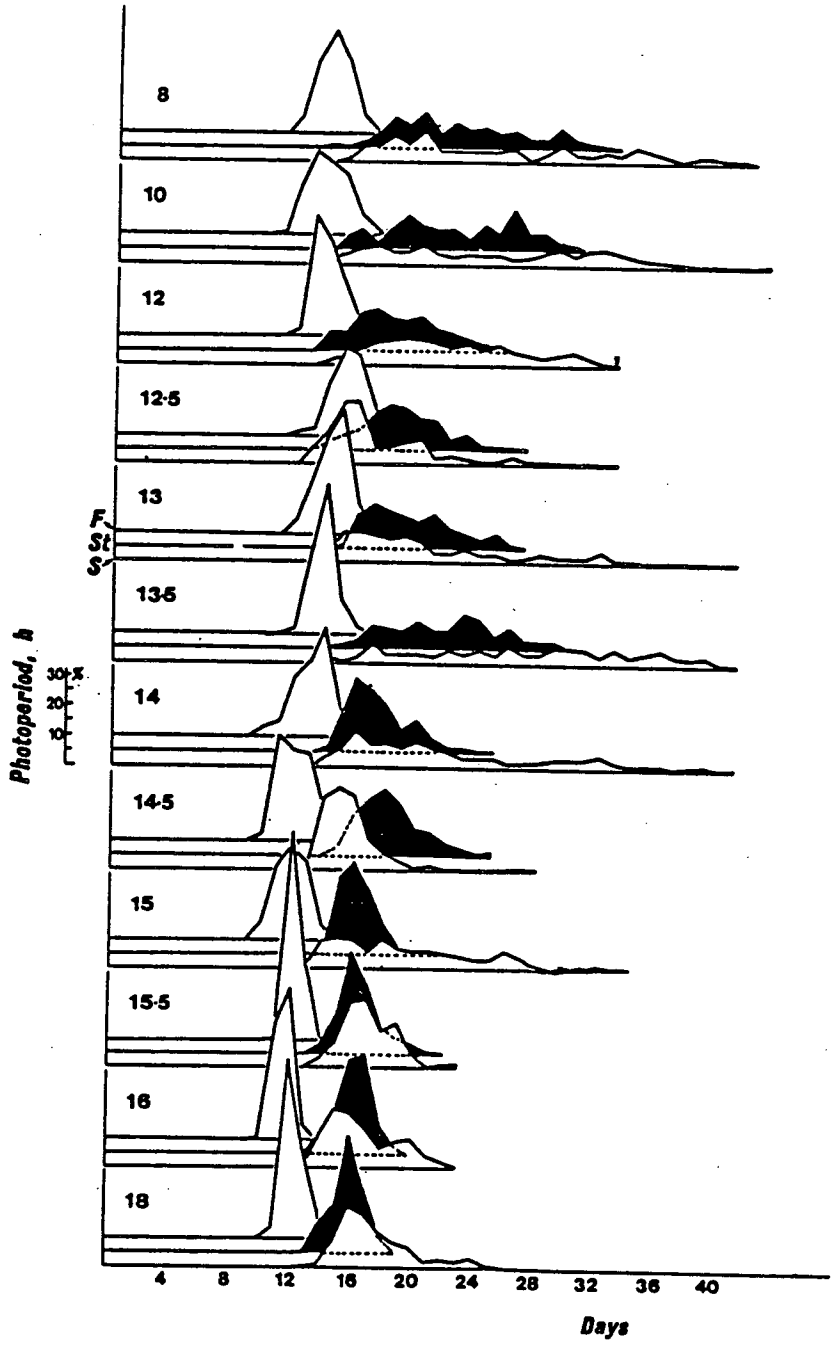


FIG. 24. The distribution of puparium formation in the selected strains (F) 'fast' and (S) 'slow' and the (St) 'stock' strain of S. argyrostoma in a range of larval photoperiods at 17 ± 0.5°C. Adults at LL, 25 ± 1°C.

characterised by a long negative skew which produced a very wide range of pupariation times; for example at LD10:14 pupariation continued from day 14 to 43. At LD12:12 the medians of pupariation of the 'fast' and 'slow' strains were 13.5 and 22.7 days respectively with ranges of 12-17 and 15-41 days.

There was also a systematic shortening of the larval period of all strains with increasing photoperiod and an associated reduction in the overall ranges of pupariation. This result was consistent with those for stock described on p.71 .

INTERSTRAIN CROSSES

Interstrain crosses (F x S and S x F, female designated left) were made by mass-mating females of one strain with the males of the other. Similarly, an F_2 hybrid was produced by allowing progeny of the F x S hybrid to interbreed, and a backcross with the 'slow' (S) strain was achieved using the female progeny of the F x S hybrid, (F x S) x S. The behaviour of these hybrids, the parental lines, 'fast' and 'slow' and 'stock', in a range of photoperiods at 20 $\pm$ 0.5^{o}C was then determined. All adult flies were kept in LD12:12,25 $\pm$ 1^{o}C. In this experiment, the mean and standard deviations of puparium formation, in addition to the median, were calculated for each culture (Tables 41 and 42). All

TABLE 41

The timing (median) of pupariation in seven strains of S. argyrostoma in a range of larval photoperiods at 20 ± 0.5°C. Adults at LD12:12, 25 ± 1°C. ('fast', F, 'slow',S, in hybrids females designated left.)

Photoperiod (h)		Fast	F_1 (FxS)	F_1 (SxF)	Slow	Stock	F_2 ((FxS) x (FxS))	BC.S ((FxS) xS)
12	Median	9.2	16.3	17.1	32.5	16.9	13.6	14.8
	No.	248	360	272	131	203	242	329
12.5	Median	9.7	13.7	15.4	30.2	14.1	14.9	15.8
	No.	239	218	222	162	153	217	218
13	Median	10.5	14.5	17.2	32.2	15.5	14.1	14.0
	No.	274	395	261	154	394	158	144
13.5	Median	9.8	14.6	14.6	31.6	14.6	14.7	15.7
	No.	253	211	321	164	224	154	150
14	Median	9.9	15.9	15.2	30.8	15.0	14.3	15.2
	No.	158	238	339	246	248	167	163
14.5	Median	10.2	14.6	-	-	13.8	12.3	16.2
	No.	237	199	-	-	161	217	174

TABLE 41 (Contd.)

Photoperiod (h)		Fast	F_1 (FxS)	F_1 (SxF)	Slow	Stock	F_2 ((FxS) x (FxS))	BC.S ((FxS) xS)
14.75	Median	-	-	13.9	29.1	-	-	-
	No.	-	-	340	224	-	-	-
15	Median	8.4	13.5	-	-	14.3	10.6	15.0
	No.	293	326	-	-	157	167	254
15.5	Median	9.0	13.0	12.2	24.8	12.7	10.4	13.8
	No.	249	318	212	128	153	241	175

TABLE 42

The mean ± 1 standard deviation of the timing of puparium formation in 7 strains of S. argyrostoma in a range of larval photoperiods at 20 + 0.5°C, Adults at LD12:12, 25 ± 1°C. ('fast', F, 'slow', S, in hybrids females designated left).

Photoperiod (h)	MEAN ± 1 STANDARD DEVIATION (DAYS)					
	FAST	SLOW	F_1(FxS)	F_1(SxF)	$F_{2_{x(FxS)}}^{((FxS)}$	BC.S$^{((FxS)}_{xS)}$
12	9.8 ± 0.5	32.8 ± 4.9	16.7 ± 1.3	17.5 ± 2.1	14.0 ± 1.6	14.2 ± 2.1
12.5	10.1 ± 0.5	29.6 ± 5.0	14.1 ± 1.1	15.1 ± 1.5	14.8 ± 2.5	16.0 ± 2.6
13	11.0 ± 0.6	31.3 ± 4.5	14.9 ± 1.4	16.9 ± 2.1	14.3 ± 2.3	14.1 ± 1.9
13.5	10.3 ± 0.7	29.7 ± 6.1	14.8 ± 1.9	15.1 ± 1.9	15.2 ± 2.0	15.9 ± 2.1
14	10.4 ± 0.5	29.1 ± 4.9	15.9 ± 1.7	15.6 ± 1.6	14.7 ± 1.9	15.7 ± 2.3
14.5	10.4 ± 0.6	-	15.1 ± 2.1	-	12.7 ± 1.8	16.1 ± 2.1
14.75	-	25.6 ± 4.0	-	14.3 ± 1.3	-	-
15.0	8.9 ± 0.5	-	13.0 ± 1.3	-	11.4 ± 1.9	14.8 ± 1.8
15.5	9.6 ± 0.3	28.7 ± 4.1	12.3 ± 1.2	13.5 ± 1.2	11.1 ± 1.2	12.6 ± 1.3

cultures fed on average for 6-7 days. As before, individuals from the 'fast' strain formed prepupae very much sooner and over a narrower range than those of the 'slow' strain in which puparium-formation was very delayed and protracted (Fig. 25). Puparium formation in the reciprocal F_1 hybrids occurred between the "fast" and "slow" strains, a comparison of the medians and means of pupariation of the reciprocal F_1 hybrids indicating that although there was variation, it was random and not systematic; therefore there was no maternal or paternal effect. The simplest genetic model that could be used to test the results of these crosses assumed additive inheritance with no dominance: the F_1 hybrid should therefore have possessed a median of pupariation, intermediate to that of the parental lines. Using this logic, an expected median of pupariation was calculated for each photo-period, thus:

$$\text{Median } (F_1) = \frac{\text{median}_{(F)} + \text{median}_{(S)}}{2}$$

The percentage of larvae which had already formed prepupae by the expected median day of pupariation was also calculated (Table 43). At all photo-periods tested, the observed median of pupariation differed significantly from that of the expected ($p < 0.001$) that is, more than 90% of the larvae had

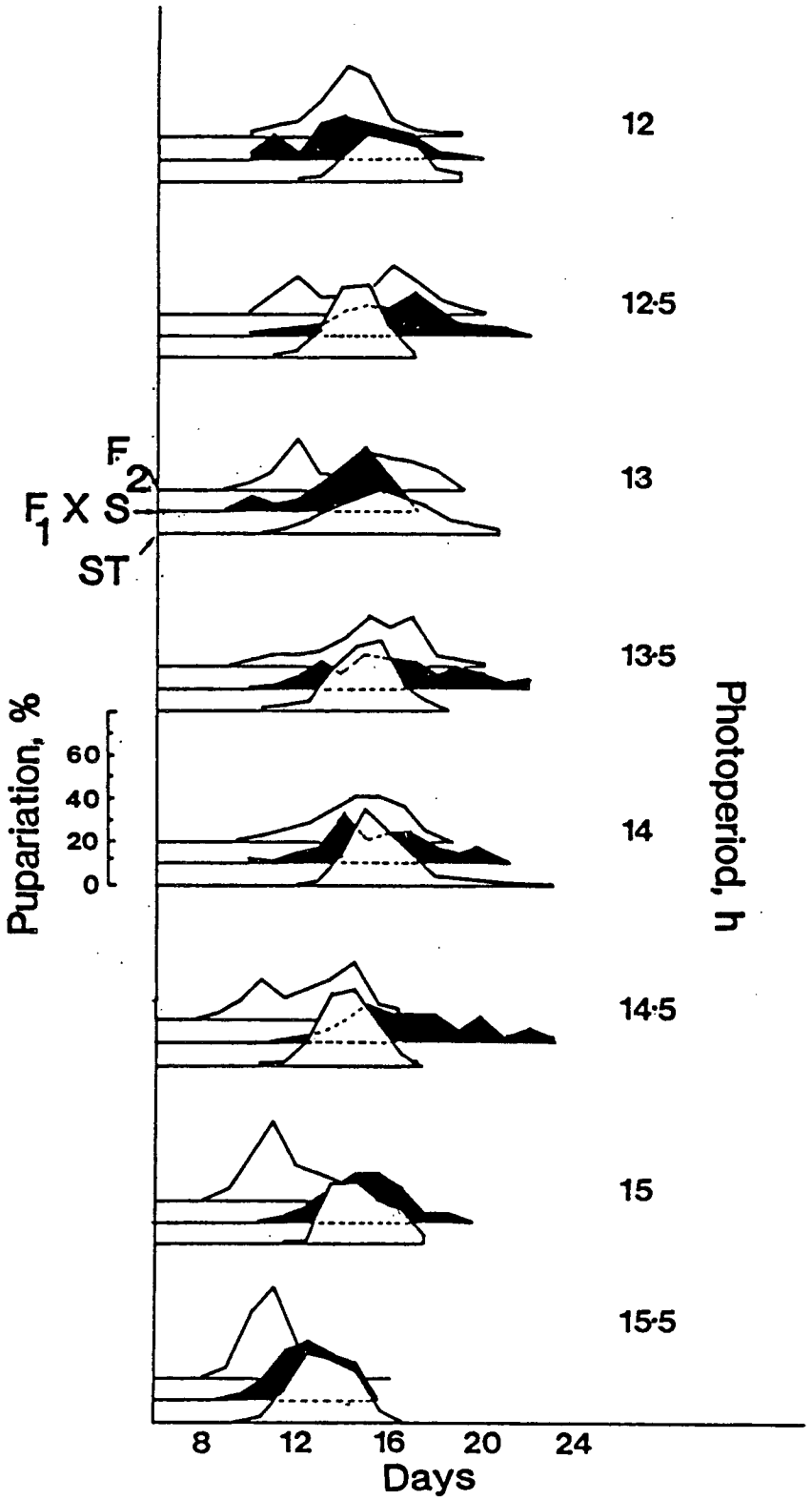


FIG. 25. The distribution of puparium formation in the selected strains (F) 'fast' and (S) 'slow' of S. argyrostoma and their reciprocal hybrids, in a range of larval photoperiods at 20 ± 0.5°C. Adults at LD12:12, 25 ± 1°C. Polygons from top to bottom of figure at each photoperiod are: 'fast', 'fast' x 'slow', 'slow' x 'fast', and slow. In hybrids, female designated left.

TABLE 43

The expected medians of pupariation of the F_1 reciprocal hybrids (FxS and SxF, female designated left) calculated from the observed parental medians of pupariation: (F) 'Fast', (S) 'Slow'. Adults at LD12:12 25 ± 1°C, Larvae at photoperiods, 20 ± 0.5°C.

Photoperiod (h)	Expected Median (Days)	% of Individuals which had Formed Puparia by the Expected Median of Pupariation	
		FxS	SxF
12	20.9	99.5	98.2
12.5	20.0	100	99.5
13	21.4	100	94.3*
13.5	20.7	99.5	97.3
14	20.4	100	100
15.5	16.9	100	100

Null hypothesis (NH): There is no difference in the number of individuals that have formed prepupae by the expected and observed median days of pupariation.

e.g. SxF at LD13:11*

	exp	OBS
Puparia	246	131
Larvae	15	129

$X^2 = 122.7$

$\therefore$ reject NH, there is a highly significant difference $p < 0.001$.

formed prepupae by the expected median day. For this reason, the model assuming additive inheritance with no dominance was rejected in favour of a hypothesis in which the inheritance of diapause amongst the two lines was additive, and the 'slow' trait was incompletely dominant. Using this hypothesis the expected median of pupariation (M) of the backcross (BC.S) and the F_2 was calculated using the following equations:

$$M_{B.C.} = \frac{M_{F_1} + M_S}{2} \quad \text{and} \quad M_{F2} = \frac{M_F}{4} + \frac{M_{F_1}}{2} + \frac{M_S}{4}$$

The median value for the F_1 was calculated using an average of the medians for the reciprocal F_1 hybrids given in Table 41. The expected values of the medians of pupariation and the actual proportion of individuals which had formed prepupae by that time are presented in Table 44. Again there is a highly significant difference between the expected and observed medians of pupariation for both the F_2 and BC_S crosses for all photoperiods tested. Therefore this approach to the genetic analysis of the strains was not valid.

The immediate segregation of the two strains ('fast' and 'slow') after one generation of selection implied that very few genes might be involved. However, the results from the F_2 and backcrosses conflict with this idea. If very few

TABLE 44

The expected medians of pupariation of the F_2 (FxS)x (FxS) and Backcross with 'slow'((FxS)xS) (female designated left) calculated from the observed parental and F_1 hybrid medians of pupariation (F) 'fast', (S) 'slow'. Adults at LD12:12, 25 ± 1°C, Larvae at photoperiods, 20 ± 0.5°C.

Photo-Period (h)	F_2		BC(S)	
	Expected Median of Pupariation	Proportion of Individual which had formed puparia	Expected Median of Pupariation	Proportion of Individual which had formed puparia
12	18.7	97.9	24.6	100
12.5	17.2	87.0*	22.3	100
13	18.6	98.7	24.0	100
13.5	17.5	93.1	23.1	100
14	18.0	100	23.2	100
15.5	14.8	99.6	18.7	100

Null Hypothesis (NH): There is no difference in the number of individuals that have formed prepupae by the expected and observed days of pupariation.

e.g. F_2, LD12.5:11.5*

	EXP.	OBS.
Puparia	189	109
Larvae	28	108

$X^2 = 66.8$

$\therefore$ reject NH, there is a highly significant difference $p < 0.001$.

genes are involved, the F_2 should produce individuals which exhibit properties closer to the parental extremes, i.e., there should be a large amount of variation compared to that of the F_1. Table 42 shows the mean $\pm$ 1SD of the timing of puparium formation in the strains and hybrids. The values of the standard deviations of the 'fast' and 'slow' strains emphasise the difference in the ranges of the distribution. The standard deviations of the F_1 crosses lay between those of the parental strains. The standard deviations or variances of the F_2 and the Backcross with the slow strain were of the same order of that of the F_1. This implied that there was a large number of genes involved and that 'fast' and 'slow' must be polygenic. It must be stressed however that since the data are not normally distributed, (see Figs. 25 and 26), it was not technically correct to carry out parametric statistical tests. Nevertheless, the means and medians of the F_1 were closer to that of the fast parent than that of the slow, this implying that the 'fast' strain contains an allele/alleles that is/are dominant over the 'slow'. The medians and means of the F_2, in the majority of photoperiods tested, were also greater than that of the F_1 (Fig. 25), and this implied theoretically, that the 'slow' component contained an allele/alleles which

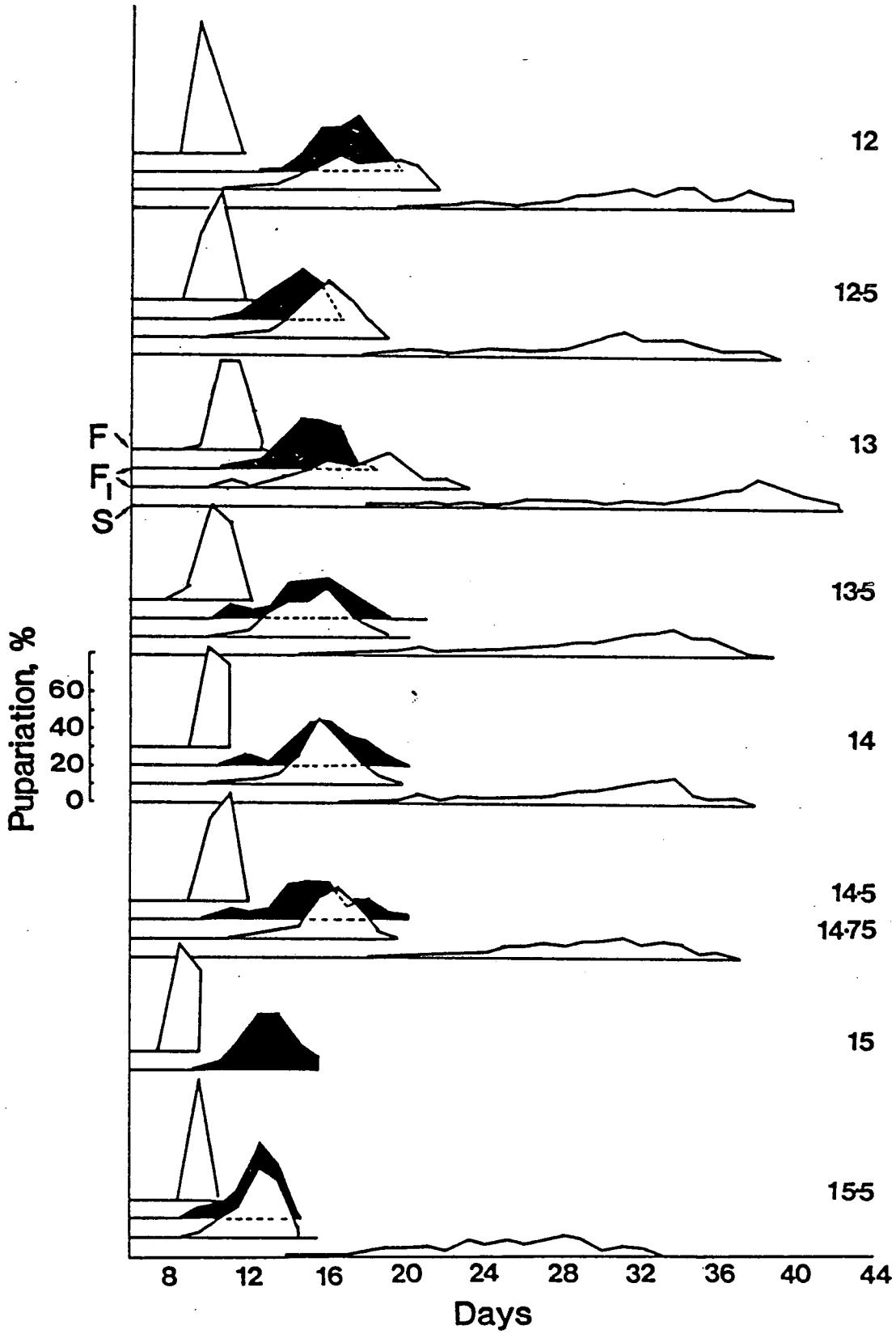


FIG. 26. The distribution of puparium formation in stock and two hybrid strains of S. argyrostoma in a range of larval photoperiods at 20 ± 0.5°C. Adults at LD12:12, 25 ± 1°C: (F_2 ('fast' × 'slow') × ('fast' × 'slow'), (S_1, 'slow' and (St), stock. In hybrids female designated left.

were dominant over the 'fast'. However, it should be stressed that the F_2 and Backcross was carried out on a different occasion to the F_1 and parental crosses, and that the former crosses have a lower incidence of diapause (~ 80%) to that of the latter (~ 90%)(see p. 245). It has been demonstrated that a high incidence of diapause in a culture is associated with a long larval period (Saunders, 1971; Denlinger, 1972), although whether this is cause or effect is not known. Therefore it is possible that the shorter larval period shown by the F_2 is a consequence of a lower incidence of diapause, and that the differences between the F_1 and F_2 were not 'real' genetically. Further evidence that many genes are involved is provided by the fact that the small standard deviation of the Backcross 'slow' also shows none of the extreme features of the 'slow' parental strain. It should also be noted that the distribution of the F_1 hybrid curves are regular in outline whereas those of the F_2 and Backcross are irregular fluctuating peaks, and in several cases the F_2 (at 12.5, 13 and 14.5) shows bimodality. This also suggests that many genes must be involved.

In conclusion, the complicated behaviour of the strains and hybrids made a thorough genetic analysis of the result impracticable. However the results

suggested that the length of the wandering (post-feeding) stage was under genetic control, that both 'fast' and 'slow' strains were probably polygenic and that 'fast' was partially dominant over 'slow'.

PHOTOPERIODIC RESPONSE OF THE SELECTED STRAINS

RESULTS

The main objective in the selection of the 'Fast' and 'Slow' strains was to produce individuals with widely differing larval periods. As previously shown the 'Fast' and 'Slow' strains differed only in the length of the post feeding stage. To test what effect selection has had on the photoperiodic response curve and the degree of diapause response, newly deposited larvae of the two selected strains, and stock, were raised in a range of photoperiods at 17 $\pm$ 0.5^{o}C (see p.219 for more detail). The rates of puparium formation under these regimes have been described before (pp 219-222). The photoperiodic response of the strains is given in Table 45 and displayed in Fig. 27. Larvae from all strains experienced LL, 25 $\pm$ 1^{o}C as embryos, before transfer to photoperiods at 17 $\pm$ 0.5^{o}C, these conditions being insufficient to produce a saturated diapause. Therefore, under strong shortday larval photoperiods, (8-12 h per day) the diapause incidence in stock was

TABLE 45

The effect of photoperiod on the incidence of diapause in 'fast', 'slow' and 'stock' strains of *S. argyrostoma*. Adults at LL, 25 ± 1°C, larvae transferred to photoperiods at 17 ± 0.5°C.

	Diapause Incidence %		
Photoperiod	Fast	Stock	Slow
8	47.9	86.9	43.2
10	70.6	90.0	63.9
12	52.5	81.4	56.9
12·5	43.0	72.9	63.2
13	33.0	66.5	48.5
13·5	12.7	84.5	58.7
14	8.5	47.0	43.9
14·5	6.8	37.4	12.1
15	2.4	10.9	15.3
15·5	0	3.8	0.7
16	0	0.6	0.3
18	0	0	0

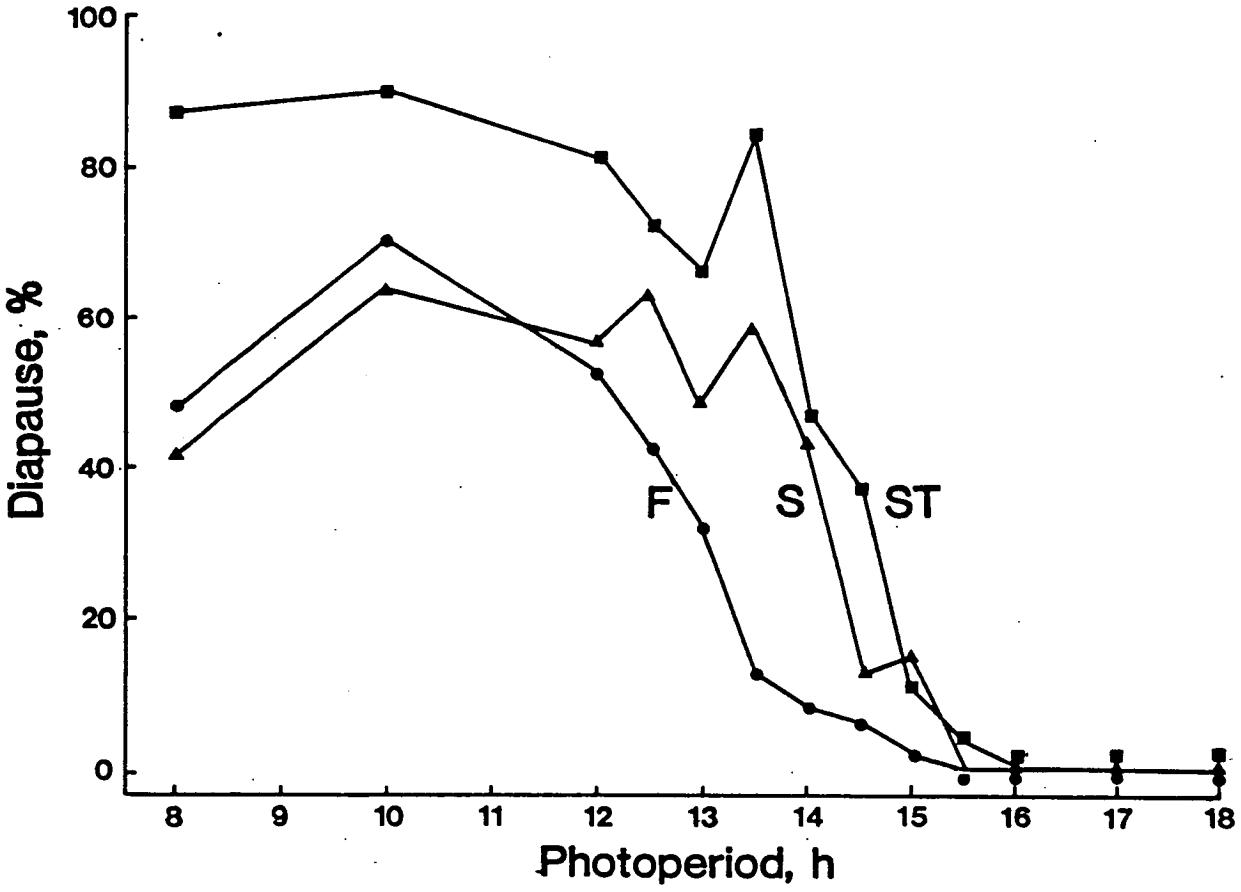


FIG. 27. The effect of photoperiod on the incidence of pupal diapause in the selected strains, 'Fast (●) and slow (▲) and the stock strain (■) of S. argyrostoma. Adults at LL, 25 ± 1°C, larvae transferred to photoperiods at 17 ± 0.5°C.

between 80-90%, but in the selected strains, the incidence of pupal diapause over this range was reduced to 40-70%. The critical photoperiod for stock appeared to be between 14 and 14:5 hours. The selected strains, however, appeared to possess different critical photoperiods, although it was not possible to calculate the true critical value because the incidence of diapause was too low in both strains. However, the incidence of diapause in the 'fast' strain dropped from 33% at 13:11 to 12.7% at 13.5:10.5, whereas the diapause incidence of the 'slow' strain remained above 40% even at 14:10 and then dropped to 12.1% at 14.5:9.5. The critical daylength for 'slow' is therefore judged to be greater than that for 'fast' in the present investigation, although that for 'slow' may be close to that for 'stock'.

An attempt was made to repeat the above experiment with the addition of the F_1 (F x S) hybrid. However, survival of both adult F_1 and 'slow' type flies was very poor and consequently very few larvae were produced and it was not possible to construct a complete photoperiodic response curve for these strains. The results of this experiment are given in Tables 46 and 47. As before all cultures fed on average for 8-9 days, so the differences in the medians and ranges of pupariation of the strains were

TABLE 46

The effect of photoperiod on the timing of puparium formation in 'fast', 'slow' and 'stock' strains of S. argyrostoma and the F_1 hybrid (Fast x Slow). Adults at LL, 25 ± 1°C, larvae transferred to photoperiods at 17 ± 0.5°C.

Photoperiod	FAST			STOCK			F_1 (FxS)			SLOW		
	Median	Range	No.	Median	Range	No.	Median	Range	No.	Median	Range	No.
12	11.6	11-14	306	17.3	12-23	253				30.8	21-41	164
12.5	11.0	11-13	127	18.5	13-26	256				35.6	18-43	209
13	11.4	11-13	173	17.0	14-23	286	14.2	12-24	181			
13.5	10.6	10-13	122	18.3	13-28	342	15.3	12-23	273			
14	10.8	10-13	170	15.8	12-22	307	13.0	12-21	228			
14.5	11.6	11-14	176	17.7	13-23	250	15.4	12-18	285			
15	11.4	11-14	347	17.0	13-21	289						
15.5	11.6	11-13	368	15.3	13-18	291				20.8	17-30	196

TABLE 47

The effect of photoperiod on the incidence of diapause in 'fast', 'slow' and 'stock' strains of S. argyrostoma and the F_1 hybrid (fast x slow). Adults at LL, 25 ± 1°C, larvae transferred to photoperiods at 17 ± 0.5°C.

PHOTOPERIOD	DIAPAUSE INCIDENCE %			
	FAST	STOCK	F_1	SLOW
12	13.0	85.2	-	71.6
12.5	12.1	83.8	-	83.7
13	13.9	60.3	64	-
13.5	4.2	84.0	63.2	-
14	4.8	61.3	29.2	-
14.5	1.5	53.2	29.2	-
15	6.3	39.6	-	-
15.5	0	0.7	-	21.6

entirely due to different post feeding times. As before, the diapause incidence of 'stock' in strong short days was unsaturated (~ 80%), and the critical photoperiod was about 14.5:9.5 (Table 47). The diapause incidence in the 'fast' strain was very low (13% at LD12:12) and it is not possible to calculate a critical photoperiod. The degree of the photoperiodic response of the 'slow' strain was much stronger than in the previous experiment. For example, under LD12:12 and LD12.5:11.5 the diapause incidence was 72% and 83.7%, respectively. At the other photoperiod tested (15.5:8.5) however, the diapause incidence was 21.6%, and the incidence of pupal diapause in both 'fast' and 'stock' at this photoperiod was negligible. The incidence of diapause in the F_1 was tested over a range of 4 photoperiods: at 13:11 and 13.5:10.5 the incidence of diapause was slightly lower than that of stock (~ 60%) and had dropped to 29.2% at 14:10; again, however, it was not possible to calculate a critical photoperiod.

To determine whether selection had affected the critical photoperiod, it was necessary to increase the incidence of diapause under strong short days. Since it was not feasible to reduce the rearing temperature of the larvae any further, especially in the case of the slow strain in which puparium

formation already took on average about 30 days (at 17°C), the adult flies of all strains tested ('Fast', 'Slow', 'Stock', reciprocal F_1, F_2 and backcross to the 'Slow' strain) were kept under short day conditions until they deposited larvae. The progeny were then bred in a range of photoperiods at 20 ± 0.5°C. Since all individuals had experienced diapause inductive cycles as intrauterine embryos, diapause incidence was much higher, but the photoperiodic response curves produced (Fig. 28) were not comparable to those described previously (Fig. 27) because the latter were exposed to LL as embryos. Under these temperature and photoperiodic regimes, diapause incidence in both selected strains was saturated. The critical daylength of the 'Fast' strain lay between 14.5 h and 15.0 h, whereas the critical photoperiod of both 'Stock' and 'Slow' was about 15.5 h. The critical photoperiods of the F_1 hybrids was about 15 h, and was thus intermediate between the parental strains, although Fig. 28b illustrates that the response was closer to that of the 'Fast' parent. This result was similar to that described on p.216 for the genetic control of the timing of puparium formation for which the 'Fast' strain was thought to be partially dominant over 'Slow'. The Photoperiodic Response Curves of the F_2 and Backcross with the

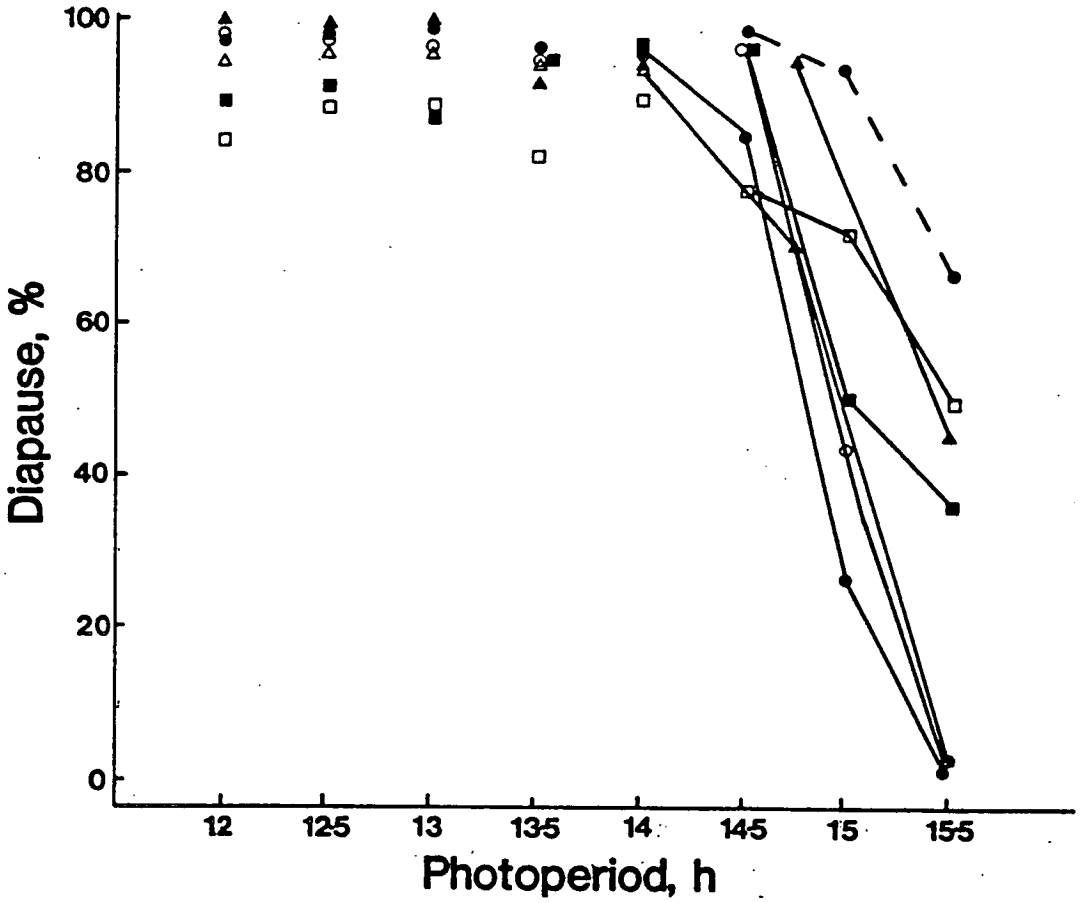


FIG. 28a. The effect of photoperiod on the incidence of pupal diapause in the strains, fast (●) slow (▲) and stock (●---●) and the hybrids F_1(F x S)(○), F_1(S x F)(△), BC.S((F x S)(■) and F_2((F x S) x (F x S))(□). Adults at LD12:12, 25 ± 1°C, larvae transferred to photoperiods at 20 ± 0.5°C.

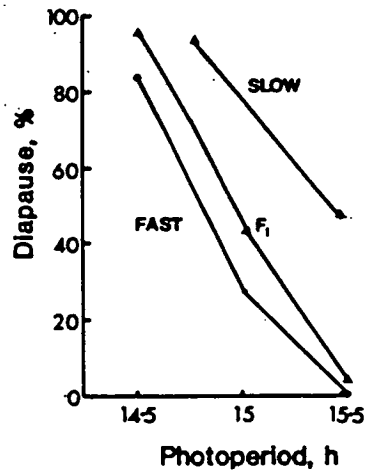


FIG. 28b. Enlargement of the region around the critical photoperiod in Fig. 28a, depicting the diapause response of 'fast' and 'slow' strains and the combined response of the F_1 hybrids.

'slow' were constructed on a different occasion from those of the other strains and crosses. The diapause response was unsaturated in both these cultures, although the cause of this was unknown, since the temperature and photoperiodic conditions during embryogenesis and larval development were identical to those experienced by the other crosses. Therefore, the critical photoperiods derived for these crosses were possibly not comparable to those of the other hybrids. However the F_2 and Bc.S. have more diapause at longer photoperiods, for example 15.5 h, than either the Fast or reciprocal F_1 crosses (Table 48), implying that the former hybrids may contain allelles for diapause induction at longer photoperiods.

The results from these experiments suggest that selection for a strain with a shortened wandering period has also reduced the critical photoperiod; selection for a protracted wandering period however, has produced no obvious change in the critical photoperiod compared to that of stock. Under temperature and photoperiod regimes that produce an unsaturated diapause response in the stock culture, both the 'fast' and 'slow' strains have similarly reduced photoperiodic responses, although the reduction in diapause in each strain may have been caused by different factors (see p. 253).

TABLE 48

The effect of photoperiod on the incidence of diapause in 'fast', 'slow' and 'stock' strains of *S. argyrostoma*, and the hybrids F_1(FxS), F_1(SxF), F_2((FxS) x (FxS)).and BC.S. ((FxS) x S). Adults LD12:12, 25 ± 1°C Larvae transferred to photoperiods at 20 ± 0.5°C.

Photo-period	DIAPAUSE INCIDENCE, %						
	FAST	F_1 (♀F x ♂S)	F_1 (♀S x ♂F)	SLOW	STOCK	F_2	BCS.
12	96.7	97.1	94.7	100	97.5	84.1	89.4
12.5	97.9	97.2	95.9	98.2	98.0	88.4	91.5
13	98.8	96.4	96.1	100	96.9	89.2	87.2
13.5	96.0	94.8	95.3	92.2	95.4	82.1	94.5
14	96.8	96.2	93.5	94.6	97.9	89.7	96.2
14.5	84.7	95.9	-	-	98.0	78.2	96.1
14.75	-	-	71.0	94.8	-	-	-
15	27.2	43.9	-	-	93.2	71.3	50.0
15.5	0.8	3.8	0.5	45.1	66	50.4	36.3

THE EFFECT OF CROWDING ON POST FEEDING LARVAE OF THE FAST AND SLOW STRAINS

The following experiment was performed to determine whether a) the protracted wandering period associated with the 'slow' strain was a result of an increased sensitivity to crowding (see pp.161- for the effect of post feeding density on the wandering period in stock) and b) if a mixed culture of post feeding larvae from 'fast' and 'slow' strains would segregate into distinct peaks of timing of puparium formation.

Post feeding larvae, from cultures of 'fast' and 'slow' strains previously bred at LL, 20 ± 0.5°C at a feeding density of 300 larvae per culture, and derived from adults at LL, 25 ± 1°C, were transferred to densities of 1, 100 or 200 larvae per culture. In addition, 100 larvae of each strain were placed within one container; a spot of blue paint (Humbrol Enamel) was placed dorsally on the middle segment of each 'fast' larvae and a spot of yellow paint was similarly applied to 'slow' larvae. The median of pupariation of each culture, derived from daily collections of puparia, is given in Table 41a. The density of the post feeding larvae in both fast and slow strains affected the timing of puparium formation in a manner similar to that described for stock (see p.167), i.e., an increase in the post

TABLE 49a

The effect of post feeding density of the timing of puparium formation in larvae of the 'fast' and 'slow' strains of S. argyrostoma. Adults at LL, 25 ± 1°C, Larvae at LL, 20 ± 0.5°C.

Strain	Density of Post Feeding Larvae	Median of Pupariation (Days)
Fast	1/Jar	7.7
Slow	1/Jar	8.4
Fast	100/mousetray	8.7
Slow	100/mousetray	10.5
Fast	200/mousetray	9.2
Slow	200/mousetray	10.5
Fast	100 mousetray	8.5
Slow	100 mousetray	9.4

TABLE 49b

The effect on the timing of puparium formation of painting post feeding larvae of the slow strain kept at a post feeding density of 100 larvae per mouse tray. Adults and larvae LL, 25 ± 1°C.

(Humbrol) Enamel	Median of Pupariation (Days)
None	7.55
Blue	7.65
Yellow	7.55

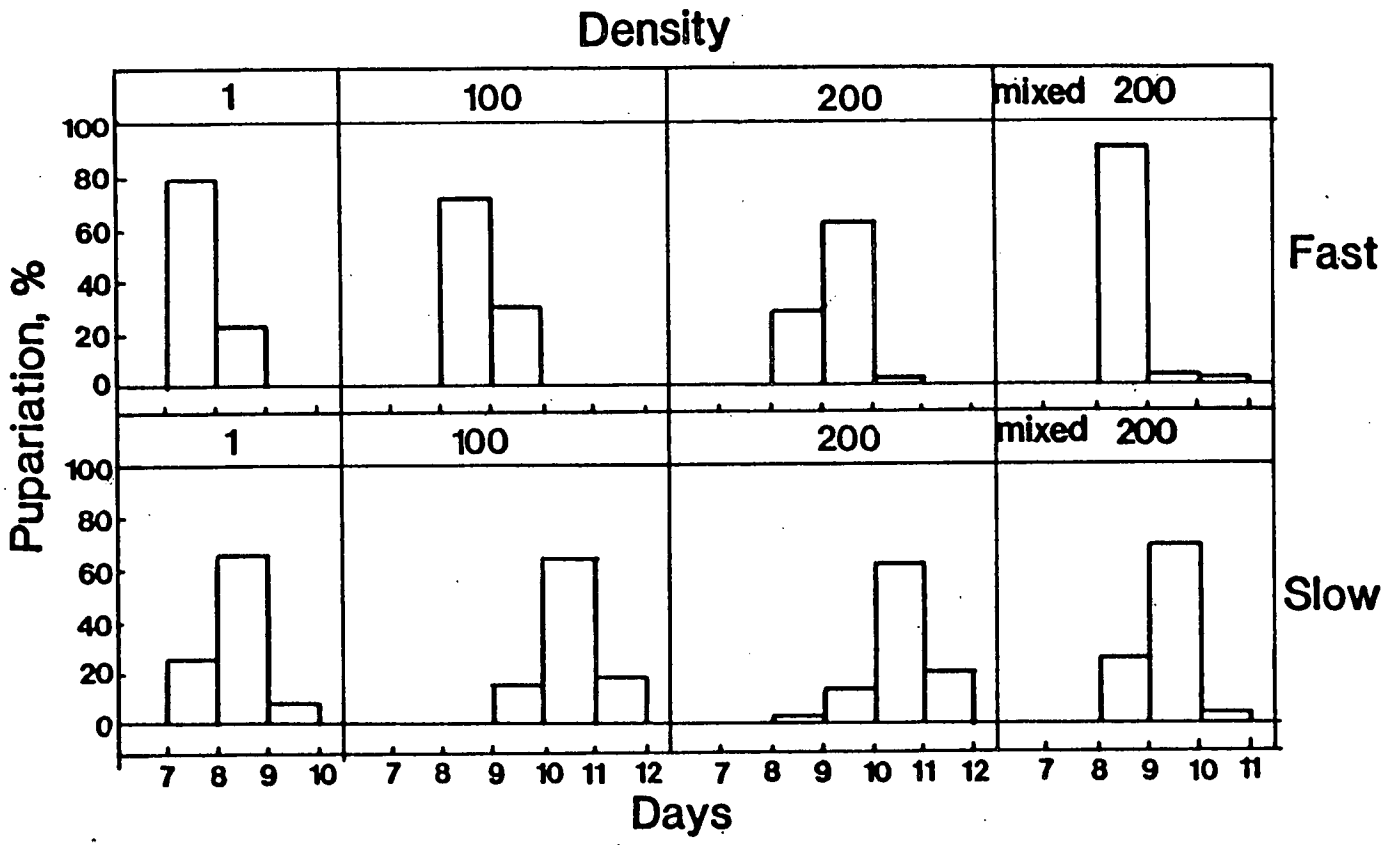


FIG. 29. The distribution of puparium formation in larvae of the 'fast', and 'slow' strains of S. argyrostoma in different post feeding densities: 1,100, 200 or a mixed culture of 100 larvae of each strain. Adults at LL, 25 ± 1°C, Larvae at LL, 20 ± 0.5°C.

feeding density produced an increase in the length of the wandering period (Fig 29). There was a 0.7, 1.8 and 1.3 day difference in the medians of pupariation between the fast and slow strains at densities of 1, 100 and 200 larvae per culture respectively. This result suggested that crowding during the wandering stage had a stronger delaying effect on puparium formation in the slow strain than in the fast strain. In the mixed culture containing post feeding larvae of both strains, the medians of pupariation for larvae of the fast and slow strains were 8.55 and 9.4 days respectively. This experiment was conducted 5 generations after the selection pressure had been relaxed in both strains, results for puparium formation rate in the slow strain (Table 49b) suggested that the strain had become faster (median of pupariation = 7.6 days). Although the post feeding larvae were kept at the lower density of 100 larvae per mousetray, during selection, the median of puparium formation had varied between 8.6 and 11.7 at 25 $\pm$ 1^{o}C at densities of 250-300 larvae.

DISCUSSION

Artificial selection from the first and last individuals to form puparia within a stock culture of S. argyrostoma, produced two strains, labelled

'fast' and 'slow' respectively, which diverged within one generation and remained separated over 13 generations of selection. In non diapause inductive conditions, the selected strains differed only in the duration of the wandering period, all other fractions of the life cycle being of similar duration to that of stock. This is in contrast to the strain of Sarcophaga bullata produced by Henrich and Denlinger (1982) in which selection for greater duration of the larval period also increased the duration of other stages of the life cycle.

Puparium formation in the 'fast' strain was highly synchronised, the variance around the mean of pupariation being smaller than either stock or 'slow' strains, agreeing with the expectation that genetic variability and thus phenotypic variance should decrease as a result of selection. In contrast, however, the variance of the slow strain was greater than that of 'stock'. This contradictory result was also observed in the 'late pupariating' strain produced by Henrich and Denlinger (1982) who suggested that if the frequency of alleles that tended to delay pupariation was initially low, then selection for late pupariation might increase heterozygosity at those loci and increase the variance. However, results from intra strain crosses in the present study have suggested that the

'slow' strain is highly polygenic, therefore their explanation may not be applicable in this case. Alternatively, it is possible that over the generations, the selection procedure may have 'removed' slightly faster partially dominant alleles allowing phenotypic expression of the remaining 'slow' alleles, resulting in an increase in the range of pupariation. The behaviour of the strains and the interstrain crosses in a range of photoperiods and temperatures revealed that the duration of the wandering stage was dependant mainly on heritable factors, but could be greatly influenced by the environmental conditions that the developing larvae experienced. Although diapause inductive conditions are known to influence the duration of the wandering period in Sarcophaga species (Saunders, 1971; Denlinger 1972), the slow strain was particularly sensitive; the wandering period of the slow strain individuals becoming highly protracted in short day conditions, the pupariation curve being characterised by a long negative skew, which was reduced only when the breeding photoperiod approached the critical daylength. In the present study, the density of the post feeding larvae has been shown to affect the duration of the wandering period of 'stock' (pp. 161), and again individuals from the slow strain

appeared to be more susceptible to this effect than individuals from the fast strain. Thus, a combination of diapause inductive breeding conditions, high post feeding density, and 'slow' genes, will act synergistically to produce highly protracted wandering periods.

Selection for the duration of the wandering period in non diapause-inductive conditions, resulted in both a qualitative and quantitative change in the photoperiodic response curve: strong short day larval photoperiods which produced between 80 to 90% diapause incidence in stock, induced only 40-70% pupal diapause in fast and slow strains. In contrast, Henrich and Denlinger (1982) found that individuals of the selected "late pupariation" strain were more likely to enter diapause, and suggested that "the relationship between the developmental rate and diapause traits may stem from the pleiotropic effects of genes associated with late pupariation or from one or more genes associated with late pupariation being closely linked to genes that affect diapause". However, the selected strains in this study appear to differ only in the length of the wandering period, therefore the change in diapause incidence may be alternatively explained using the modified version of Gibbs's model for photoperiodic summation,

described in this thesis (p. 201). Results from Saunders (1971) indicate that in environmental conditions which produce an unsaturated incidence of pupal diapause, all or at least some portion of the wandering period is very important in photoperiod induction. Therefore, if photoperiodic summation occurs in at least the beginning of the wandering period, in conditions producing an unsaturated (80 to 90%) incidence of diapause in the stock culture, individuals in the fast strain would experience fewer diapause inductive cycles during the shortened wandering period and consequently the final diapause incidence would be lower than that of stock. In contrast, during the highly protracted wandering period, individuals of the slow strain might have originally experienced enough short day photoperiods to increase the 'diapause' titre to above the internal threshold, but, eventually, the diapause titre would begin to 'drain away', so that by puparium-formation, the titres may have dropped below the internal threshold and consequently the individual will not enter diapause. Therefore the delay in puparium formation in the slow strain is equivalent to the delay in puparium formation produced by crowding post feeding larvae of the stock strain (see pp. 171—), in its effect on the final incidence of diapause.

The shapes of the photoperiodic response curves produced by exposing the intrauterine embryos of fast, slow and interstrain crosses to diapause inductive cycles before transfer to a range of photoperiods suggest that artificial selection for a shortened wandering period also reduced the value of the critical photoperiod by about 1 hour, compared to that of stock. Selection for a longer wandering period did not affect the position of the critical photoperiod. Since the value for the critical daylength for the reciprocal hybrids was closer to the 'Fast' parent than the 'Slow' parent, (a result similar to that described for the genetic control of the timing of puparium formation), the 'Fast' strain may contain one or more alleles which are partially dominant over the slow allele/ alleles. These results indicate that the laboratory strain of S. argyrostoma still contains genetic variation with respect to critical daylength. The existence of latitudinal clines in the critical day-length has been reported in several insect species, e.g. Drosophila littoralis (Lumme 1982) Wyeomyia smithii (Bradshaw 1976) and Peripsocus quadrifasciatus (Ertmoed, 1978) (see Gen. Intro., pp.20-22). Such potential for genetic variation has allowed the species to colonise a wide range of latitudes.

C H A P T E R IV

DIAPAUSE DEVELOPMENT AND TERMINATION

DIAPAUSE DEVELOPMENT AND TERMINATION

INTRODUCTION

Diapause development and termination in insects were discussed in general on pp.41-44 of this thesis. Diapause development, defined as the physiological events that occur in preparation for resumption of active morphogenesis, was shown to be influenced in many insects by photoperiod and/or temperature. In the flesh fly Sarcophaga argyrostoma, however, termination of pupal diapause was found to be independant of photoperiod (Denlinger, 1972). This was considered ecologically appropriate by the author since pupae of Sarcophaga are normally buried under the surface of the soil. Fraenkel and Hsiao (1968a) found that termination of diapause in S. argyrostoma occurred spontaneously at high temperatures: the duration of diapause of pupae previously bred at LD8:16, 18°C, was dependant on the temperature at which the diapausing pupae were maintained. For example, pupae kept at 18°C eclosed after a period of 120 days or more, whilst pupae kept at 23 and 29°C emerged after 90-100 and 29-55 days respectively. A similar result was obtained by Denlinger (1972), working with S. argyrostoma, S. bullata and S. crassipalpis, who showed that diapausing pupae kept at 17°C throughout their development, broke

diapause spontaneously after an average of 227 days, whilst pupae transferred to 25°C emerged on average after only 92 days. He also found that when pupae bred at 17°C were transferred to 25°C at different times throughout the duration of the diapause, diapause termination occurred earlier under this combination of temperatures than under constant exposure to either temperature.

Robaud (1922) working with experimental groups of one found that chilling rather than mechanical or chemical treatments reduced the duration of diapause. Fraenkel and Hsiao (1968a) also found that chilling at 6°C for 10, 27 or 69 days before transfer to 18°C or 23°C had a positive effect on the termination of diapause in S. argyrostoma. For example, if pupae were maintained at 23°C throughout diapause, diapause termination (measured by adult eclosion) occurred after 106-127 days, whereas pupae which were transferred to 6°C for either 27 or 69 days terminated diapause 100 and 58 days after being returned to 23°C. A similar result was observed in pupae which were chilled at 6°C and then kept at 18°C.

A more thorough investigation of diapause termination in Sarcophaga argyrostoma, was designed to answer the questions listed below,

1. What period of chilling is required to complete diapause development?

2. Is there a "depth" of diapause: Does the duration of diapause vary in individuals which were bred at different photoperiods and temperatures?

3. Is there a sex difference in the duration of diapause?

SEX AND DIAPAUSE INCIDENCE

Denlinger (1972) reported that males of Sarcophaga crassipalpis showed a greater tendancy to enter diapause than females. For example, there was a 1:1 ratio of males to females at 0 and 100% diapause, but in conditions of partial diapause, such as 10%, 87% of individuals entering diapause were male, whereas in conditions of 90% diapause, only 25% of the non diapausing individuals were male. A similar result was found in a field study of Sarcophaga bullata. In August when only 20% of the population had entered diapause, 79% of the individuals were male. However, by September, when 99% of the population were in diapause the proportion of males had decreased to 45% (Denlinger, 1972b). Denlinger (1981), working on S. crassipalpis, found that although males and females survived the diapause period equally well, diapause had a deleterious effect on female reproductive success: females that had overwintered in pupal diapause produced only a quarter of the fertile eggs produced by females which had not experienced diapause. Diapause did not affect male reproductive success however. Commenting upon these

sex differences in post diapause reproductive success, Denlinger (1982) suggested that since the fertility of a diapausing female drops in proportion to diapause duration, avoiding early diapause in the field allowed the female to realise her maximum reproductive output, but also enhanced the reproductive output of her diapausing female progeny by shortening their period of diapause. For males there was no selection pressure to minimise the diapause period and this is reflected in the lower environmental threshold of diapause observed in males.

To determine whether a skewed sex ratio also occurred in the Edinburgh strain of S. argyrostoma, the ratio of male:female flies in several of the experiments described in this thesis was recorded.

MATERIALS AND METHODS

THE EFFECT OF PROLONGED CHILLING ON DIAPAUSE TERMINATION

Larvae deposited by adults kept at LD12:12, 25 ± 1°C, were cultured at LD12:12, 20 ± 0.5°C. Newly formed prepupae were collected and transferred to DD, 20 ± 0.5°C to allow any non-diapausing individuals to eclose. Thirty-eight days after larviposition, the remaining pupae were transferred to DD, 4°C, and chilled for 14, 28, 42, 53, 66 or 82 days. On removal from 4°C, groups of about 100 pupae were

transferred either to DD, 20 ± 0.5°C or to DD, 25 ± 1°C. Each group of pupae was examined every 7 days, and the number of flies which had eclosed during this period was recorded.

The above experiment was repeated using diapausing pupae bred in an identical manner. Groups of about 200 pupae were chilled (4°C) for 22, 35, 51 or 68 days before being transferred to DD 20 ± 0.5°C or 25 ± 1°C. In addition, a group of pupae was transferred directly to either 20 ± 0.5 or 25 ± 1°C without a period of chilling. The pupae were examined weekly for signs of adult development.

DIAPAUSE DEVELOPMENT

Larvae deposited over the previous 24 hours by adults kept at LD12:12, 25 ± 1°C, were bred at 17 ± 0.5°C under a range of short day photoperiods (2, 4, 8, 10, 12, 13, 14 and 14.5h light per 24h cycle). A further eight cultures of larvae were taken from 'stock' kept at LD12:12,25 ± 1°C, three of which were transferred to LD12:12, 20 ± 0.5°C, the remaining cultures were bred at LD12:12, 25 ± 1°C. The post-feeding larvae in cultures at 17°C and 20°C were allowed to disperse into mouse-trays containing 1.5 cm of sawdust, whereas the post-feeding larvae formed at 25°C were transferred to plastic jars, (3.5 cm diam. x 6 cm height) containing 1.5 cm of sawdust, at varying

densities of 1, 5, 10, 25 and 50 larvae per jar. Newly formed prepupae were collected and kept at the same temperature at which they had been bred until any non-diapausing individuals had eclosed. The remaining diapausing pupae were placed in glass jars, transferred to DD, 25 ± 1°C, and then examined weekly for signs of diapause termination. Thereafter, the number and sex of flies emerging in each jar was recorded daily.

RESULTS

THE EFFECT OF CHILLING ON DIAPAUSE TERMINATION

Diapausing pupae, previously bred at LD12:12, 20 ± 0.5°C were chilled for various periods at 4°C, commencing 38 days after larviposition. After chilling, the pupae were transferred to either 20 ± 0.5°C or 25 ± 1°C (see p.259). The timing of diapause termination (measured by adult eclosion) for each group is presented in Tables 50a and b. The pupae were examined weekly; therefore, the inspections were not performed at close enough intervals to warrant the calculation of means and standard deviations, so only the ranges of eclosion, accurate to 7 days, were calculated. The results from the replicate experiments 1 and 2 showed that chilling at 4°C decreased the duration of diapause once the pupae were returned

TABLE 50a

The effect of chilling on termination of pupal diapause in S. argyrostoma. Adults at LD12:12, 25 ± 1°C, Larvae at LD12:12, 20 ± 0.5°C. After chilling at 4°C, DD pupae were transferred to either 20 ± 0.5°C or 25 ± 1°C. DD. Tables 50a and b are replicate experiments.

No. of Days at 4°C	Transferred to 20 ± 0.5°C			Transferred to 25 ± 1°C			Total Duration of Diapause at 25°C including period of chilling
	n	Range of Eclosion ±7 Days After Transfer	% Mortality	n	Range of Eclosion ±7 Days After Transfer	% Mortality	
14	254	122-136	0	171	87-111	4.1	101-125
28	238	108-122	0	248	73-97	4.8	101-125
53	267	48-79	6.0	234	27-57	0	80-110
66	274	35-66	2.2	238	35-44	4.2	101-110
82	214	28-43	3.3	208	19-30	0	101-112

TABLE 50b

The effect of chilling on termination of pupal diapause in S. argyrostoma. Adults at LD12:12, 25 ± 1°C, Larvae at LD12:12, 20 ± 0.5°C. After chilling at 4°C, DD. pupae were transferred to either 20 ± 0.5°C or 25 ± 1°C. DD. Tables 50a and b are replicate experiments.

No. of Days at 4°C	Transferred to 20 ± 0.5°C			Transferred to 25 ± 1°C			Total Duration of Diapause at 25°C including period of chilling
	n	Range of Eclosion ±7 Days After Transfer	% Mortality	n	Range of Eclosion ±7 Days After Transfer	% Mortality	
0	200	119-N/K*	0	183	94-119	32.8	94-119
22	200	97-N/K	0	200	72-97	0	94-119
35	200	70-N/K	0	263	35-59	16.0	70-94
51	200	54-N/K	0	237	19-43	17.3	70-94
68	232	26-51	12.9	161	9-26	0	77-97

*N/K - not known, but greater than 119 days, and less than 147 days.

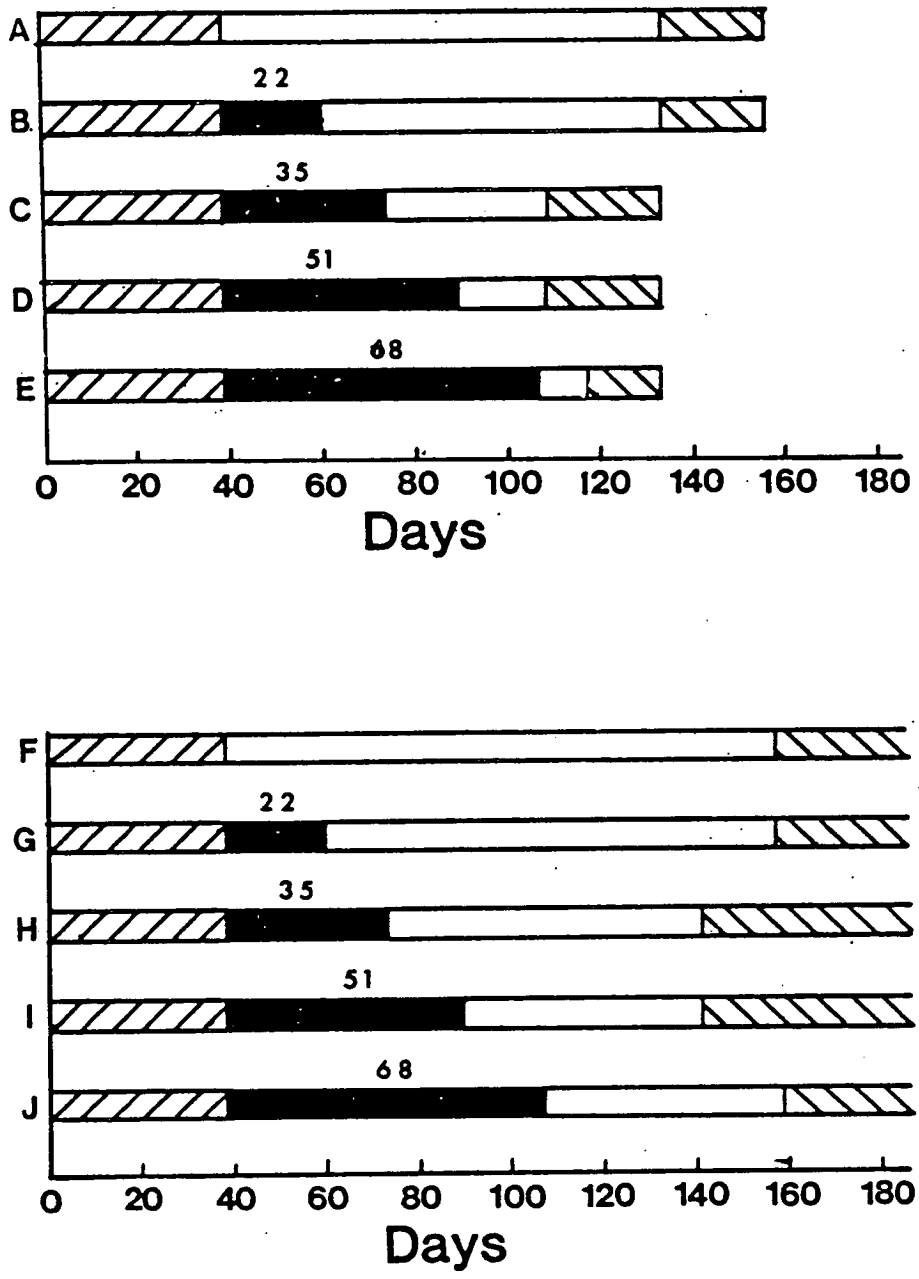


FIG. 30. The effect of chilling (■)(numbers denote period of chilling) on the termination of diapause in S. argyrostoma, measured by adult eclosion (▨). Adults at LD12:12, 25 ± 1°C, larvae at LD12:12 20 ± 0.5°C (▨). A-E, pupae transferred to 25 ± 1°C, DD, F-J pupae transferred to 20 ± 0.5°C, DD (▭) after the period of chilling at 4°C.

to a higher temperature. Table 50b shows that larvae transferred from LD12:12, 20 ± 0.5°C to DD, 25 ± 1°C, without a period of chilling, terminated diapause 94-119 days after transfer, whereas chilling larvae for 22 days reduced the duration of diapause to between 72 and 97 days and chilling for 35 or 68 days reduced the duration still further to 35-59 and 9-26 days respectively (Fig. 31a). However, it should be noted that the total duration of diapause, inclusive of the period of chilling was similar in unchilled and chilled pupae (Fig. 31b). The duration of diapause was greater when pupae were transferred to 20 ± 0.5°C, DD as opposed to 25 ± 1°C, DD.

The expected developmental time of a non-diapausing individual from the phanerocephalic pupa (the stage at which diapause normally occurred) to adult eclosion was about 9 days in DD, at 25 ± 1°C (pp. 65). Eclosion at this temperature usually took place over a range of 2-3 days. Therefore, a proportion of pupae chilled for 68 days (Table 50b) must have completed diapause development since they were competent to initiate immediate adult development on return to a higher temperature. When pupae were transferred directly from LD12:12, 20 ± 0.5°C to DD 25 ± 1°C, spontaneous termination of diapause occurred between 94-119 days, i.e. a range of 24 days. A similar range of adult eclosion was

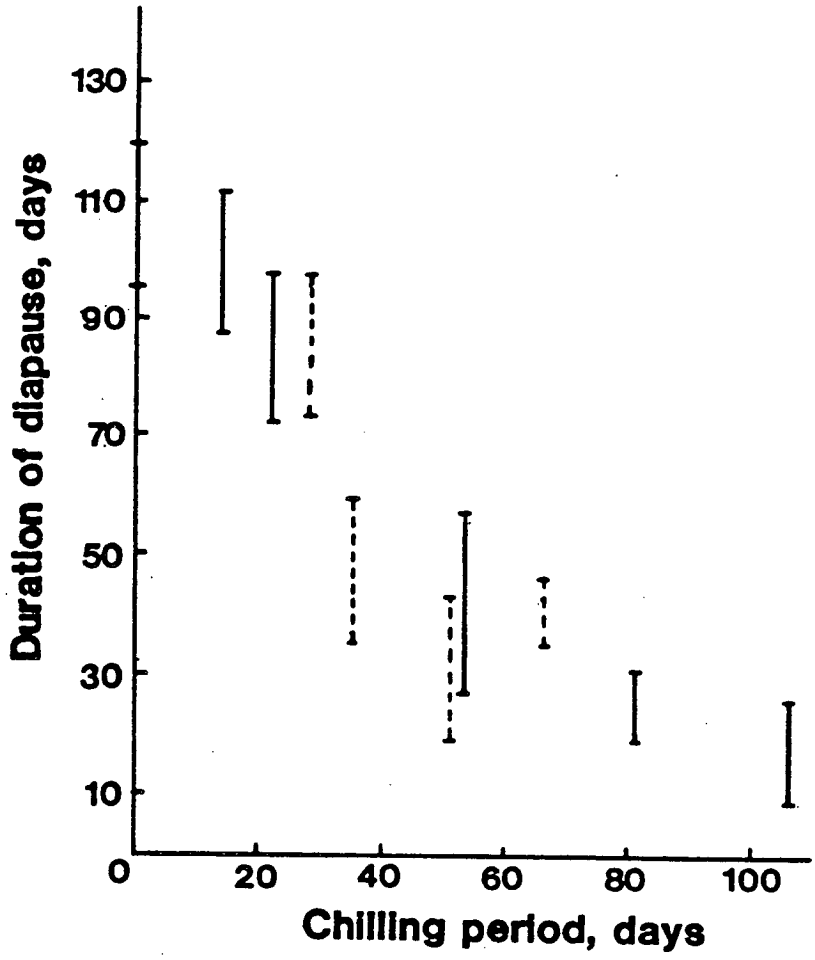


FIG. 31a. The duration (range) of diapause after transfer to 25 ± 1°C, DD in S. argyrostoma, plotted as a function of the increase in the chilling period. (⊢—⊣) replicate a (Table 50a) (⊢---⊣) replicate b (Table 50b). Adults at LD12:12, 25 ± 1°C, larvae at LD12:12, 20 ± 0.5°C, pupae transferred to 25 ± 1°C, DD after the period of chilling at 4°C.

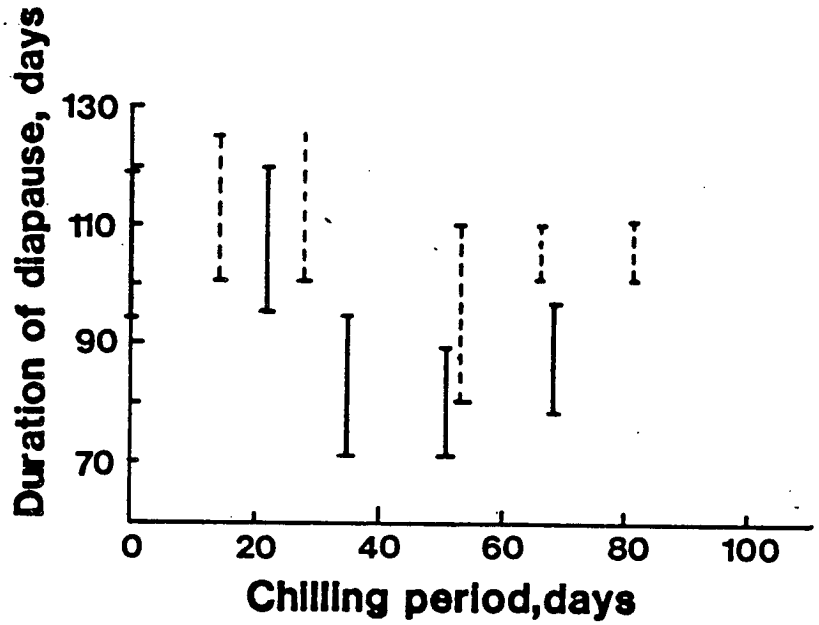


FIG. 31b. The total duration (range) of diapause (inclusive of the chilling period) plotted as a function of the increase in the chilling period. (⊢—⊣) replicate a, (⊢- - -⊣) replicate b. For breeding conditions see Fig. 31a.

seen when pupae were chilled for periods of up to 53 days. With periods of chilling longer than this, the range of eclosion was reduced to 11-17 days. Even allowing for the weekly inspections, this order of range was much greater than the range of adult eclosion seen in non diapausing flies at a similar temperature. Possibly if pupae had been chilled for a longer period, allowing all individuals in the group to complete diapause development, the range of eclosion might have been similar to that of non-diapausing flies.

A more thorough investigation of the spontaneous termination of diapause, without a period of chilling, was undertaken as follows:

To determine whether there was a difference in the duration of diapause between the sexes, three cultures of diapausing pupae, previously bred at LD12:12 20 ± 0.5°C, were transferred to DD, 25 ± 1°C, 34 days after composition. Under these rearing conditions, the diapause incidence was close to saturation (Table 51a). The pupae were examined daily for diapause termination (adult eclosion) and the sex of the newly eclosed fly recorded. One hundred and forty days after transfer to 25°C, any remaining pupae were opened to determine the proportion of each replicate which were either dead or still in diapause. Dead pupae were divided into

TABLE 51a

Incidence of pupal diapause in groups of S. argyrostoma derived from adults kept at LD12:12, 25 ± 1°C and bred at LD12:12, 20 ± 0.5°C. A, B and C are replicate cultures of sibling larvae.

Replicate	No. of Prepupae	% Diapause	No. of non-diapause individuals ♂	♀
A	180	94.0	10	1
B	186	93.5	7	5
C	174	96.0	3	4

TABLE 51b

Spontaneous termination of pupal diapause in S. argyrostoma. Pupae maintained at 25 ± 1°C, DD for 140 days. For breeding conditions see Table 51a.

Replicate	Total No. of Diapausing Pupae	% Successful Eclosion	% Diapause Termination	% Remaining in Diapause	% Mortality
A	169	81.6	89.9	0	10.0
B	174	79.8	97.1	0.6	2.9
C	167	88.6	97.6	1.8	0.6

TABLE 51c

Timing of diapause termination (measured by adult eclosion) in male and female flies. For breeding conditions see Table 51a.

Replicate	Total No.	Mean ± 1sd (Days)	No. ♂	Mean ± 1sd (Days)	No. ♀	Mean ± 1sd
A	138	95.9 ± 7.1	49	95.2 ± 7.6	89	95.9 ± 6.7
B	139	95.4 ± 7.2	66	95.6 ± 7.9	73	95.2 ± 6.5
C	148	95.5 ± 6.8	65	95.5 ± 7.0	83	96.8 ± 6.2

two categories: those which had died as pupae (pupal mortality) and those which had terminated diapause but had failed to eclose (Table 51b). The rate of reactivation was high, ranging from 89.9% to 96.6%, the proportion of flies which failed to eclose ranged from 8.3% to 17.3%, and pupal mortality was low. The mean and standard deviation of adult eclosion was calculated as a whole and for both sexes, for each culture (Table 51c, Fig. 32). There was no difference in the timing of eclosion between the replicate cultures (Analysis of Variance, $F = 0.2$, d.f. = 2, 423, $p > 0.05$). There was also no significant difference in the duration of diapause between males and females (Analysis of variance, replicate A, $F = 0.3$ df 1, 136, replicate B, $F = 0.1$, d.f.=1, 137 and replicate C $F = 1.05$, d.f.=1, 146, $p > 0.05$). The mean value of adult eclosion ranged from 95.4 to 95.9 days with a standard deviation of about 7 days.

THE EFFECT OF THE REARING PHOTOPERIOD ON THE DURATION OF DIAPAUSE

Diapausing pupae previously bred under a range of short day photoperiods at 17 ± 0.5°C were transferred to DD, 25 ± 1°C, 42 days after larviposition (see pp.259 for details). Under these breeding conditions, the incidence of pupal diapause was greater than 90% (Table 52a). The pupae were examined daily for adult

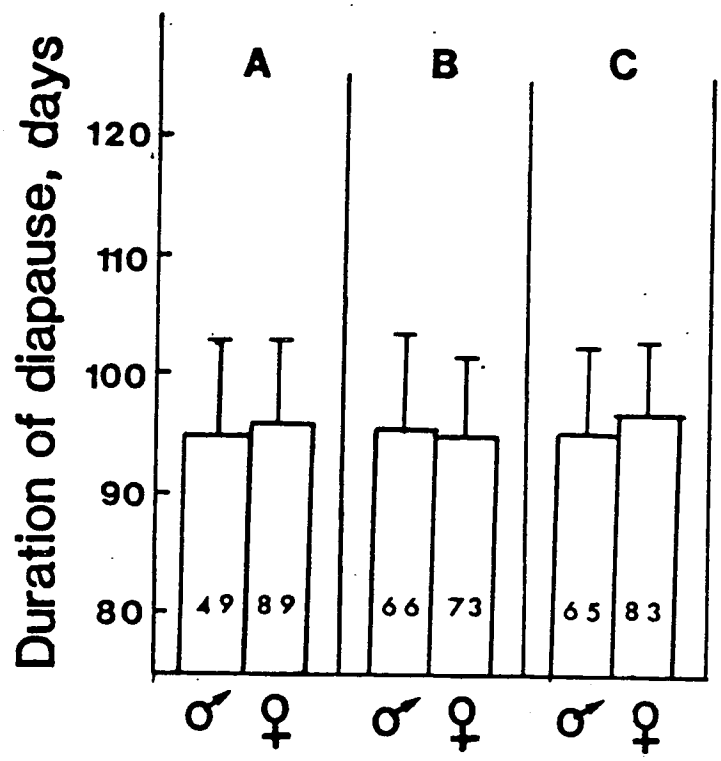


FIG. 32. Spontaneous termination of diapause (mean ± 1SD) in groups of pupae maintained at 25 ± 1°C, DD. Adults at LD12:12, 25 ± 1°C, larvae at LD12:12, 20 ± 0.5°C. Timing of eclosion measured in days after transfer to 25 ± 1°C, DD. A, B and C are replicate cultures of sibling larvae. Numbers in columns denote number of pupae in each group.

TABLE 52a

Incidence of pupal diapause in sibling groups of S. argyrostoma derived from adults kept at LD12:12, 25 ± 1°C and bred at 17 ± 0.5°C in the photoperiods given below.

Photoperiod h	No. of Prepupae	% Diapause	No. of non-diapausing individuals ♂	♀
2	157	94.3	3	6
4	165	95.1	4	4
8	162	93.8	4	6
10	158	94.3	5	4
12	222	94.1	7	6
13	172	94.2	3	7
14	199	97.0	1	5
14.5	181	92.3	7	7

TABLE 52b

Spontaneous termination of pupal diapause in S. argyrostoma. Pupae maintained at 25 ± 1°C, DD for 140 days. For breeding conditions see Table 52a.

Photoperiod h	No. of Diapausing Pupae	% Successful Eclosion	% Diapause Termination	% Remaining in Diapause	% Pupal Mortality
2	146	90.4	93.1	0	6.8
4	157	79.0	80.2	1.9	19.4
8	156	70.5	71.8	3.8	24.3
10	149	71.4	75.3	3.2	21.4
12	209	78.5	81.3	0	18.7
13	162	83.9	90.7	1.2	6.7
14	193	92.0	92.2	1.03	6.7
14.5	167	92.2	94.6	0.6	4.8

TABLE 52c

Timing of diapause termination (measured by adult eclosion) in pupae maintained at 25 ± 1°C, DD for 140 days. For breeding conditions see Table 52a.

Photoperiod h	Total Number Flies ♂	♀	Eclosion, Days After Transfer to DD, 25°C Mean ± 1sd
2	65	67	82.4 ± 6.4
4	56	68	82.7 ± 6.6
8	53	57	81.8 ± 6.7
10	52	58	86.5 ± 8.0
12	92	72	83.5 ± 6.3
13	62	72	85.7 ± 7.5
14	72	100	82.5 ± 7.1
14.5	79	79	85.4 ± 7.4

eclosion (Tables 52b and c). The proportion of individuals that successfully terminated diapause was variable, ranging from 70.5% to 92.2%, the low % of diapause termination (~70%) in some cultures being due to a high pupal mortality as opposed to a failure to eclose. Figure 33 displays the mean ± 1 standard deviation of adult eclosion for each photoperiod plotted as a bar diagram. The means of adult eclosion ranged from 81.8 to 86.5 days. A one-way analysis of variance was performed, giving $F = 6.96$, $df=7, 908$, $p < 0.01$; thus, there was a highly significant difference in the duration of diapause in pupae bred under different photoperiods. However, the variation seemed to be at random, the means of eclosion for photoperiods of 10, 13 and 14.5h being 86.5, 85.7 and 85.4 days respectively, whereas, the means of eclosion for the remaining photoperiods were lower ranging from 81.8 to 83.5 days. This random variation in the results could have produced a significant F value, although not, perhaps, being biologically meaningful.

IS THERE A 'DEPTH' OF DIAPAUSE?

An attempt was made to determine whether the duration of diapause in groups of pupae from cultures in which the proportion of individuals entering pupal diapause differed, the rationale behind this

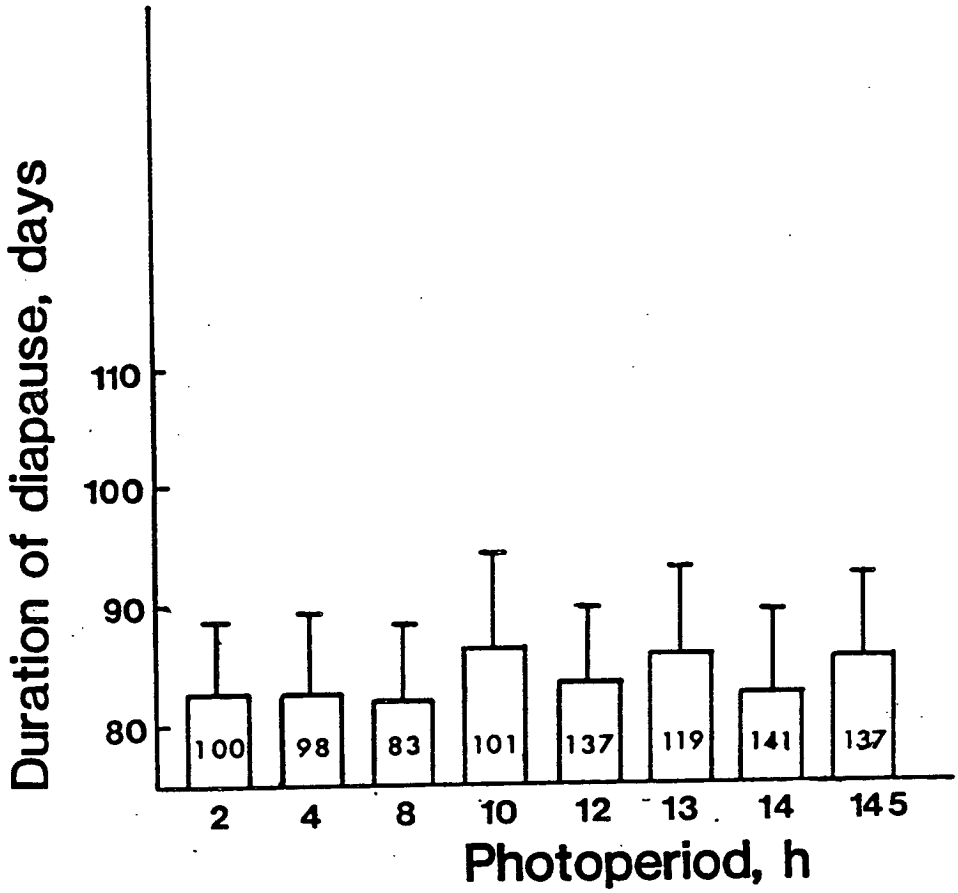


FIG. 33. Spontaneous termination of diapause (mean ± 1SD) after transfer to 25 ± 1°C, DD in sibling groups of pupae previously bred in a range of photoperiods at 17 ± 0.5°C. Adults LD12:12, 25 ± 1°C. Numbers in column denote number of pupae in each group.

investigation being that diapause pupae from a group with a higher incidence of diapause might be 'deeper' in diapause and consequently take longer to recover from that state. To answer this question, larvae previously deposited by adult 'stock' kept at LD12:12, 25 ± 1°C were bred under these same conditions. Since crowding during the wandering phase (pp.166-) can produce a change in the incidence of diapause, newly post-feeding larvae were transferred, at different densities, to plastic jars containing dry sawdust (see pp.120-121). After the eclosion of the non-diapausing individuals (21 days), the remaining diapause pupae were transferred to DD 25 ± 1°C. Unfortunately the range of diapause incidences in the cultures produced by this treatment was small, ranging from 78.5 to 98.4% (Table 53a). The pupae were examined daily for signs of diapause termination (adult eclosion). The proportion of individuals which terminated diapause ranged from 83.3 to 91.9%. However, since the rate of successful eclosion decreased with the increasing density that the post feeding larvae had experienced, this was probably a result of the increased wandering period of larvae kept at high densities, which may have reduced stored energy reserves necessary for successful diapause termination. For example, larvae at a density of 1 per jar pupariated 8.5-9.5 days after larviposition

TABLE 53a

Incidence of pupal diapause in groups of sibling larvae of S. argyrostoma maintained in varying post feeding densities. Adults and larvae at LD12:12, 25 ± 1°C.

Density	No. of Replicates	Number Prepupae Formed	% Diapause	No. Developing ♂	♀	N/K
1	205	202	98.4	0	2	-
5	50	244	89.3	12	14	-
10	12	103	81.5	8	11	-
25	8	205	78.5	16	27	1
50	4	189	80.4	9	19	9

TABLE 53b

Spontaneous termination of pupal diapause in S. argyrostoma. Pupae maintained at 25 ± 1°C, DD for 140 days. For breeding conditions see Table 53a.

Density	No. of Diapausing Pupae	% Successful Eclosion	% Diapause Termination	% Remaining in Diapause	% Pupal Mortality
1	185	79.4	91.9	1.1	7.0
5	211	62.6	85.8	3.3	14.2
10	84	72.6	89.3	3.6	7.2
25	157	60.5	87.9	5.1	7.0
50	152	43.5	83.3	4.6	9.9

TABLE 53c

Timing of diapause termination (measured by adult eclosion) in daily collections of prepupae previously maintained in varying densities. Time of pupariation is equivalent to the larval period. For breeding conditions see Table 53a.

Density	Time of Pupariation (Days)	Total n	No. developing individuals ♂	No. developing individuals ♀	Eclosion Time Mean ± 1sd (Days)	One Way Analyses of Variance
1	8.5	78	69	78	94.0 ± 7.6	F = 1.04
	9	41			92.7 ± 8.5	df,3,173
	9.5	28			91.6 ± 7.6	p > 0.5
5	8.5	24	61	71	95.0 ± 6.7	
	9	26			93.3 ± 9.3	F = 0.9
	9.5	32			96.2 ± 6.0	df 4,122
	10	33			95.3 ± 5.2	p > 0.5
	10.5	12			93.2 ± 4.0	
10	9	15	33	28	100.5 ± 4.5	F = 0.99
	9.5	18			96.8 ± 5.5	df 3,69
	10	19			97.9 ± 5.0	p > 0.5
25	10	14	54	41	95.0 ± 5.1	
	10.5	17			92.0 ± 6.9	F = 1.02
	11	17			94.1 ± 8.1	df 4,67
	11.5	14			94.9 ± 6.5	p > 0.5
	12	10			98.9 ± 4.0	
50	15.5	17	33	35	95.1 ± 6.4	F = 4.3
	16	12			90.7 ± 5.3	df 2,35
	16.5	9			98.7 ± 5.3	p < 0.5*

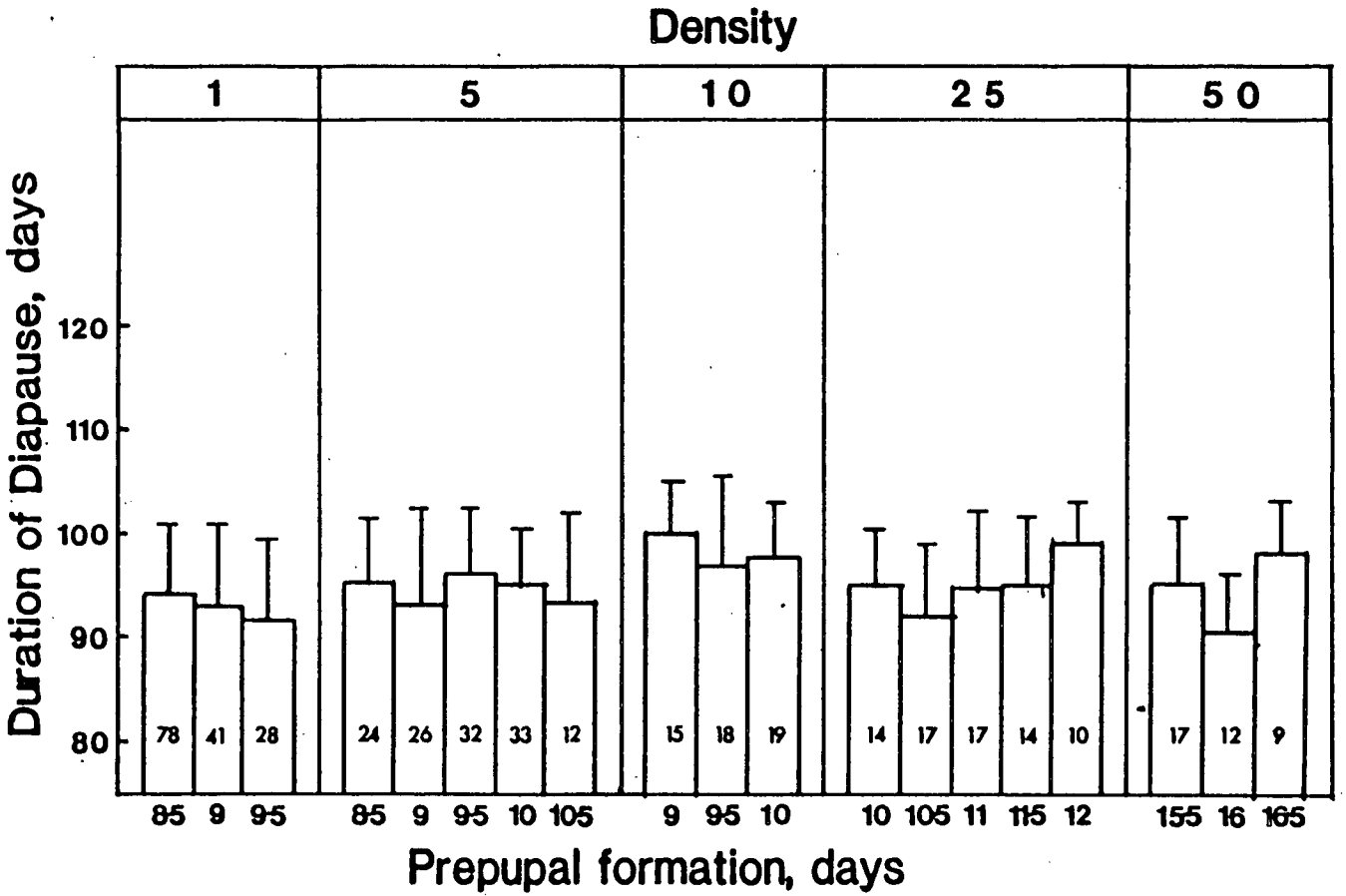


FIG. 34. Spontaneous termination of diapause (mean ± 1SD) after transfer to 25 ± 1°C, DD, in sibling groups of pupae previously kept in a range of post feeding densities at LD12:12 25 ± 1°C. Adults at LD12:12, 25 ± 1°C. Prepupal formation is a measure of the length of the larval period. Numbers in columns denote number of pupae in each group.

and 79.4% of the diapausing pupae successfully terminated diapause, whereas larvae kept at a density of 50/J formed prepupae after 15.5-16.5 days and only 43.5% successfully terminated diapause. The mean and standard deviation of eclosion was calculated for each daily collection where $n > 7$. A one-way Analysis of Variance was then performed on the data. There was no significant difference in the rate of diapause termination within groups at each density, although the means of each daily collection were variable within each group; e.g. at a density of 25/J the means of each daily collection ranged from 92.0 to 98.9 days. However, there was no significant difference in the mean duration of diapause in pupae from cultures kept at different densities, ($F = 1.2$ $df = 4, 14$ $p > 0.5$). In conclusion, although the incidence of diapause in these cultures varied from 78.5-98.4%, the results suggested that the duration of diapause was independent of the degree of diapause in the group from which they were drawn.

DIAPAUSE DURATION IN THE SELECTED STRAINS

Larvae deposited by adults of the 'fast', 'stock' and 'slow' stains and F_1 crosses and kept at LL, 25°C were cultured under a range of photoperiods at 17 ± 0.5°C (pp. 219-). Four days after puparium formation, the pupae were transferred to LD12:12,

25 ± 1°C. The number of developing individuals was recorded when adult eclosion occurred 10 days later, the results of which are given in Table 47. The remaining pupae were assumed to be in diapause, and were examined daily for signs of diapause termination (adult eclosion). The numbers and sex of flies emerging each day were recorded and the means and standard deviation of adult eclosion were calculated for each group (Table 54). Unfortunately, survival of the 'slow' strain during diapause was very low (28.5 and 0%), reflecting the poor state of the pupae. At photoperiods of 12h and 12.5h, 54.9% and 82.3% of the 'slow' pupae died within 14 days. It is possible that the very protracted larval wandering period experienced by these individuals had consumed much of the stored energy reserves necessary for successful adult differentiation and eclosion. Therefore it was not possible to calculate a value for the rate of diapause termination for this strain.

The results of diapause termination in the 'fast' strain bred at photoperiods of 13 and 13.5h were combined to produce a larger 'n' for statistical analysis, and a one-way analysis of variance was performed on the data. There was a significant variation within the stock cultures ($F = 5.02$, $df = 4, 608$, $p < 0.01$), whereas, there was no significant difference in the rate of diapause termination within

TABLE 54

Spontaneous termination of pupal diapause in 'fast', 'stock', F_1 and 'slow' strains of S. argyrostoma. Adults at LL, 25 ± 1°C, Larvae bred at 17 ± 0.5°C in the photoperiods given below. Pupae maintained at LD12:12, 25 ± 1°C.

Strain	Photoperiod (h)	Total Number of developing individuals	Eclosion Time Mean ± 1sd (Days)	% Reactivation+ by day 140
Fast	13/13.5*	27	100.6 ± 8.2	89.2
Stock	12	129	107.8 ± 6.6	60.2
"	12.5	130	106.6 ± 6.5	61.0
"	13 *	96	108.5 ± 7.6	55.8
"	13.5	146	110.1 ± 8.0	52.0
"	14	109	106.9 ± 7.2	59.2
F_1	13 *	96	104.2 ± 6.4	68.0
"	13.5	132	102.1 ± 5.9	90.4
"	13.5	170	104.2 ± 6.0	80.6
"	14	43	102.1 ± 6.7	65.1
Slow	12	16	-	28.5
	12.5	31	-	0.0

+The remaining pupae were moribund.

the F_1 cross ($F = 0.83$ df = 3, 439, $p > 0.5$). A one-way analysis of variance was also carried out between 'fast', 'stock' and F_1 which had been bred at LD13.5:11.5. Although there was a highly significant difference ($F = 17.2$, df=2, 216 $p < 0.01$), the data were not convincing evidence that the variation in the timing of eclosion was biologically meaningful, since the number of individuals within the 'fast' strain was so small and there was no information on diapause duration in the 'slow' strain.

THE SEX RATIO IN NON DIAPAUSE AND DIAPAUSE DESTINED FLIES

The influence of sex on diapause induction was investigated by comparing the sex ratio in groups of flies in which there was an unsaturated incidence of pupal diapause. The pupae used were part of three experiments described elsewhere in this thesis.

Groups of pupae with different diapause incidence were produced by placing post feeding larvae at different densities in jars containing dry sawdust or by keeping the larvae under wet conditions (see pp.166-169). Table 55 shows the sex ratio within the non-diapausing individuals. To determine the sex ratio in a completely non diapausing culture, larvae from stock adults kept at LL, 25 ± 1°C were bred at LD12:12 25 ± 1°C: the sex ratio did not deviate significantly

from 1:1. The sex ratio of the total non-diapausing individuals deviated significantly from a 1:1 ratio (X^2 = 21.9 $p<0.001$)(Table 55): of a total of 563 non-diapausing flies, 484 individuals emerged successfully, of these 65% were females. Unfortunately, it was not possible to sex the 79 individuals which failed to emerge from their puparia. By inference, therefore, the sex ratio of the diapausing flies was biased towards the males although this was not directly determined. Results from this preliminary experiment suggested that female flies have a higher threshold for diapause induction than males.

To confirm this observation it was clearly essential to calculate the <u>total</u> sex ratio within the culture, including both non-diapausing and diapausing individuals. To do this the diapausing pupae were reactivated by removing the anterior end of the puparia and applying 5 μl of cyclohexane to the head of the pupa (Denlinger, Campbell and Bradfield, 1980). Results are shown in Table 56a. Unfortunately, the rate of successful termination was low in many of the cultures, and a high proportion of individuals which managed to eclose, failed to form normal flies. Therefore, the sex ratios in the 'diapausing' flies were not representative of the true ratio and it was not possible to analyse these results further.

TABLE 55

The sex ratio of the non-diapausing flies, within sibling cultures with a range of diapause incidences. Data taken from Tables 28b and 31 - the effect of post feeding density and wet conditions on the timing of puparium formation. (puparium formation occurred in dry conditions.)

Density	Diapause %	Non-Diapausing Individuals ♂	♀	N/K*	Ratio of Male:Female
1/J	89.1	-	15	-	-
10/J	42.5	23	38	8	1:1.7
25/J	52.1	19	34	4	1:1.8
50/J	40.0	30	45	9	1:1.5
100/J	40.2	26	78	16	1:3.0
1D 10	64.5	7	10	-	1:1.4
10D 1	74.7	10	17	1	1:1.7
IW ID	62.5	9	20	7	1:2.2
10W 1D	84.3	3	11	1	1:3.7
1W 10D	32.3	23	25	15	1:1.1
10W 10D	51.8	19	22	11	1:1.2
TOTALS		169	315	79	1:1.9
Non Diapausing Control	0	136	137	-	1:1.01

*N/K - these individuals failed to emerge successfully from their puparia.

TABLE 56a

The sex ratio of non-diapausing and diapausing flies within sibling cultures with a range of diapause incidences. Data from Tables 28b and 30 - the effect of post feeding density and wet conditions on the timing of puparium formation. (Puparium formation occurred in dry conditions).

% Diapause in Culture		Non-Diapausing Individuals ♂	♀	N/K	Diapausing Individuals ♂	♀	N/K	% Successful Termination	Ratio Male:Female (Non-Diapausing)
1/J	93	4	7	0	25	49	38	73.6	1:1.75
5/J	82.8	4	16	0	10	23	5	39.6	1:4
10/J	77	8	17	0	20	21	17	70.7	1:2.12
25/J	66	17	21	5	15	22	14	61.4	1:1.24
W D1	96	0	2	0	10	5	7	47.9	-
2	96	0	2	0	7	8	8	47.9	-
3	91	1	3	0	15	8	9	74.4	1:3
4	81.5	1	5	0	9	9	17	83.3	1:5

However, the sex ratio in the total number of non-diapausing individuals was again significantly different from a 1:1 ratio (X^2 = 6.1, $0.01 < p < 0.05$), containing a total of 35 males and 73 females (Ratio 1:2.1). Table 56b shows the sex ratios of non-diapausing individuals which formed puparia whilst in wet sawdust. In contrast to the results from the crowded larvae (Table 56a), the sex ratio did not deviate significantly from 1:1 (X^2 = 0.01, $p > 0.05$), and was significantly different from that of the crowded larvae (X^2 = 6.2, $0.01 < p < 0.05$).

In an attempt to increase the proportion of individuals which terminated diapause successfully, pupae previously bred in a range of photoperiods at 20 ± 0.5°C were allowed to terminate diapause spontaneously at LD12:12 25 ± 1°C. [illegible] the proportion of the population that successfully terminated diapause was too low to estimate the sex ratio of the developing flies (50-60%), with the exception of two cultures in which the rate of termination was above 90% (Table 57). These cultures (marked with asterisk) were analysed fully; in both there was a 1:1 sex ratio (diapausing + non diapausing), (X^2 = 0.14 and 0.1, $p > 0.05$). The expected numbers of males and females in the diapausing or non-diapausing groups were calculated as follows: The diapause incidence in the 'fast'

TABLE 56b

The sex ratio of non-diapausing and diapausing flies within sibling cultures. Data from Table 30, as described in Table 56a. (Puparium formation occurred in wet conditions.)

% Diapause in Culture	Non-Diapausing Individuals ♂	♀	N/K	Diapausing Individuals ♂	♀	N/K	% Successful Termination	Ratio Male:Female (Non Diapause)
D→W(1) 29	14	12	3	6	2	-	93.1	1:0.86
D→W(2) 77	8	6	0	5	2	3	77.8	1:0.75
Wet Control 30	16	17	0	11	5	4	57.1	1:1.06

TABLE 57

The sex ratio of non-diapausing and diapausing flies in cultures with a range of diapause incidences. Data from Table 47 - The effect of photoperiod on the incidence of diapause in strains of S. argyrostoma.

Strain (Photoperiod) h	Diapause %	Non-Diapausing Individuals ♂	♀	% Termination	Diapausing Individuals ♂	♀	Total Numbers ♂	♀	Ratio Males:Females (Non-Diapausing)
Fast /13	13.9	63	87	91.3	18 (1:0.16)	3	81 (1:1.11)	90	*1:1.38
13.5	4.2	45	69	80.0	4	0	-		1:1.53
14	4.8	72	77	37.5	3	0	-		1:1.07
F_1 13.5	61.0	30	65	90.4	85 (1:0.51)	43	115 (1:0.94)	108	*1:2.17
13.5	65.5	56	75	80.6	111	59	-		1:1.34
14	29.2	66	87	65.1	29	15	-		1:1.32
Stock/13	60.3	30	87	59.2	78	59	-		1:2.9
13.5	84.0	23	31	52.0	61	63	-		1:1.35
12.5	83.8	12	27	61.0	76	66	-		1:2.25
12	85.2	10	22	60.2	76	33	-		1:2.20

TABLE 57 (Contd.)

Calculation of Expected sex ratios - I^2 test

NH No difference between the expected and observed proportion of each sex entering diapause.

1. FAST* ♂

	Exp.	OBS
Diap	11	18
Non Diap	70	63

$X^2 = 1.5$, $p > 0.05$

FAST* ♀

	Exp.	OBS
Diap	12.5	3
Non Diap	77.5	87

$X^2 = 5.1$, $p < 0.05$

2. *F_1 ♂

	Exp.	OBS
Diap	70	85
Non Diap	45	30

$X^2 = 3.9$, $p = 0.05$

*F_1 ♀

	Exp.	OBS
Diap	66	43
Non Diap	42	65

$X^2 = 9.0$, $p < 0.01$.

culture was 13.9% and there was a total of 81 available males and 90 available females; therefore 0.139 x 81 males and 0.139 x 90 females would be expected to enter diapause. A X^2 test was used to test for a difference between the observed and expected ratios. In both cultures, the proportion of females which had entered diapause was significantly lower than expected ($X^2 = 5.1$ $p < 0.05$, $X^2 = 9.0$ $p < 0.01$). There was an opposite but not significant trend for more males to enter diapause than expected ($X^2 = 1.5$ $p > 0.05$, $X^2 = 3.9$ $p = 0.05$). Again, the total sex ratio of non diapausing individuals was significantly different from a 1:1 ratio (407♂♂:627♀♀, $X^2 = 23.2$, $p < 0.001$).

These results suggest that male and female flies possess different thresholds for diapause induction. However, it should be pointed out that a more extensive investigation in which there is a high proportion of successful diapause termination would undoubtedly provide more convincing evidence.

DISCUSSION

Results from this section demonstrate that low temperatures (chilling) are not a prerequisite for diapause termination in S. argyrostoma, spontaneous termination of diapause occurring at constant temperatures of 20 ± 0.5°C and 25 ± 1°C. This result is therefore in accordance with those reported by Fraenkel and Hsiao (1968a) and Denlinger (1972). The latter author reported that diapause duration in flesh flies is temperature dependant; if diapausing pupae of S. argyrostoma were maintained at 17°C, diapause termination (recognised by the appearance of antennal spots, not adult eclosion) occurred after a mean of 227.4 days, whereas the mean duration of diapause at 25°C was only 91.7 days (Denlinger, 1972 1974, 1979).

Diapause termination in the Edinburgh strain of S. argyrostoma does not seem to be so strongly temperature dependant: adult eclosion occurred in unchilled diapausing pupae kept at a constant temperature of either 20 ± 0.5°C or 25 ± 1°C after 119-147 days and 94-119 days respectively. Adult morphogenesis (from the time from the cryptocephalic pupa to adult eclosion) which is included in these figures is highly temperature dependant, and is estimated at about 20 days at LL, 20°C and about 9 days

at LL, 25°C.

Fraenkel and Hsiao (1968a) reported that chilling at 6°C can significantly shorten diapause. In the present study, if pupae were kept at 4°C for more than 53 days, an increasing number of individuals initiated adult development immediately on return to 25°C (a developmental permissive temperature), suggesting that in some individuals diapause development (Andrewartha, 1952) had been completed at 4°C. However, if the total duration of diapause i.e., the chilling period at 4°C and at either 20°C or 25°C are taken together, rather than just the duration of diapause after the chilling period, it would seem that the duration of diapause is roughly the same in the chilled and unchilled pupae. This result was in fact reported by Fraenkel and Hsiao (1968a) but they concluded that "it was difficult to visualize a mechanism of temperature compensation by which exposure to 6°C would be equivalent to exposure to 23°C as regards spontaneous termination of diapause". A similar result was reported by Fraser and Smith (1963). Working with Lucilia caesar which diapauses as a mature 3rd instar larvae, they found that the longer the diapausing larvae were kept at 4°C, the greater the proportion which formed puparia on return to 25°C, but, 'unfortunately those larvae which terminated diapuase might well have done so at 25°C'. At 4°C, the

diapause break might similarly occur in different individuals at different times but development of these non-diapausing larvae was held up until they were returned to a higher temperature. In this manner, low temperatures can act to synchronise development within a population as shown by Danilevskii (1965), see pp. 14-15 of General Introduction. The chilling experiments undertaken in this thesis were performed only at 4°C. Ohtaki and Takashi (1972) showed that in Sarcophaga peregrina, diapause development was completed within 8 weeks of chilling, and suggested that temperatures of between 5-8°C gave a slightly higher rate of reactivation than 10°C. In retrospect, a more extensive investigation on the effect of temperature, using a greater range of temperatures, might have provided a clearer picture. Denlinger (1981b) has suggested that termination of diapause in Sarcophaga is a two part process: completion of a temperature insensitive phase is followed by a temperature-sensitive phase in which the pupae respond immediately to high temperatures. The results in this section seem to support this hypothesis, although the present results suggest that diapause termination itself is temperature independant, and that adult differentiation itself is the temperature dependant phase.

The duration of diapause in flesh flies is not dependent on the photoperiods experienced during diapause (Denlinger, 1972, Ohtaki and Takahashi, 1972). Results from this study have demonstrated that the length of the larval short day breeding photoperiod did not affect the rate of spontaneous termination of diapause (at LD12:12, 25 ± 1°C). Likewise, the duration of diapause is independent of the degree of diapause in the group from which they were drawn, i.e. there is no obvious "depth of diapause" in relation to Gibbs's Threshold Model. It would seem therefore that the duration of diapause is independant of how 'high' the diapause titre is above the 'internal threshold', or, once above the threshold, diapause is an "all-or-nothing" response. There is also no difference in the duration of pupal diapause within the sexes.

Henrich et Denlinger (1982) however, found that selection for late pupariation in S. bullata affected the diapause incidence and increased the duration of diapause, providing the first experimental evidence that diapause incidence and duration are related, diapause termination occurring 20 days later in the late strain in relation to the stock. Unfortunately in the 'slow' strain of S. argyrostoma, described in this thesis (Chapter IV), the wandering period was very protracted and successful eclosions after diapause termination were rare, probably because the protracted period of

larval wandering consumed most of the larval reserves.

Denlinger (1971c, 1972) found that the sex ratio of flesh-flies overwintering in pupal diapause was heavily biased towards the males. In this study, also, the proportion of females which entered diapause at photoperiods close to the critical was significantly lower than expected. However, there was not a significant trend for more males to enter diapause. At this point, the results therefore suggest that males and females possess different thresholds for diapause induction; the females, perhaps, avoiding diapause onset. Since female flesh flies that had overwintered in pupal diapause produced less than a quarter of the fertile eggs produced by females which had never experienced diapause, the fertility rate dropping sharply as the length of pupal diapause increased, Denlinger suggested that the timing of diapause inception was a 'trade off' between high fertility and environmental security. Males emerging from diapausing pupae however, appear to be as reproductively fit as males that have not experienced diapause (Denlinger 1981) and no such selection pressure operates on this sex. These arguments may also apply to the observed skewed sex ratios observed in this study for S. argyrostoma.

C H A P T E R V

ECDYSTEROID TITERS DURING LATE LARVAL AND EARLY PREPUPAL DEVELOPMENT

CHAPTER V

ECDYSTEROID TITRES DURING LATE LARVAL AND EARLY PREPUPAL DEVELOPMENT

The observation that pupal diapause in the moth Hyalophora cecropia was a result of the failure of the brain to activate the prothoracic glands was reported by Williams in 1946 (see General Introduction, p.44). Fraenkel and Hsiao (1968b) postulated that pupal diapause in Sarcophaga argyrostoma also resulted from the inactivation of the brain-prothoracic gland system. They showed that if mature non-diapausing larvae were ligatured after the 'critical period' the hind part formed a puparium and developed up to and including the pupal moult. Development then stopped immediately after the formation of the phanerocephalic pupa, at a state of differentiation identical to that of a diapausing pupa. Earlier work by Shaaya and Karlson (1965) on the blow fly Calliphora erythrocephala had demonstrated two main periods of ecdysone release; the first occurring shortly before the prepupal stage and inducing puparium formation and the larval-pupal apolysis, and the second commencing at the beginning of the phanerocephalic pupal stage and controlling adult differentiation. Fraenkel and Hsiao (1968b) therefore concluded that diapause in S. argyrostoma occurred during the 'trough' between the two peaks of

ecdysone release and resulted from the absence of the second. This conclusion was confirmed by Ohtaki and Takahashi (1972) and Walker and Denlinger (1980) who constructed ecdysone profiles from the past feeding larval stage to the pupa for both non-diapause and diapause destined individuals of S. peregrina and S. crassipalpis respectively. The second peak of ecdysone responsible for adult development, was absent in the diapausing pupae of both species.

THE ROLE OF HORMONES IN DIAPAUSE INDUCTION

Several workers have looked for differences in hormonal activity and cyclic nucleotide levels between flesh-flies destined for pupal diapause and flies destined for continuous development. Denlinger (1981) reviewed work by Gnagey and Denlinger (in press) on the levels of cyclic nucleotides cyclic AMP (cAMP) and cyclic GMP (cGMP). In pupariating S. crassipalpis, they found that the levels of cAMP within the brain and ring gland of non-diapause destined prepupae, were higher than in diapause destined individuals. The levels of cGMP, however, remained the same in both groups. An injection of cholera toxin (a stimulant of adenylate cyclase) into diapause destined larvae, prior to pupariation, boosted the levels of cAMP to non diapause levels (Gnagey and Denlinger (in press) and averted pupal diapause (Denlinger 1976). Since the

prothoracicotropic neurosecretion (PTTH) from the brain is thought to use cAMP as a 'secondary messenger' in the stimulation of ecdysone secretion from the prothoracic glands (Vedeckis et al., 1976), Denlinger (1981) concluded that the elevation of cAMP in non-diapause destined flies represented the stimulation of the prothoracic glands prior to the synthesis and release of ecdysone associated with adult differentiation. Walker and Denlinger (1980) have also shown that prepupae programmed for continuous pupal development showed a lower JH activity in whole body homogenates than diapause destined flies.

As previously mentioned, Ontaki and Takahashi (1972) measured the ecdysteroid content of diapause and non-diapausing flies from the post feeding larva to the pupal stage, although the two titres were measured under different temperatures, 20°C and 25°C respectively. At puparium formation, the level of ecdysteroids were similar in both groups. However, by 12 hours after pupariation, the level of ecdysteroids in diapause destined prepupae was nearly twice that of non-diapause destined individuals despite the fact that the latter were at 25°C. Twenty four hours after prepupal formation both titres were again the same.

The above results prompted the hypothesis (Giebultowicz, pers. comm; Saunders and Bradley, 1984) that the absence of the second peak of ecdysteroids in diapause destined flies might be a result of a negative feedback inhibition of ecdysone production possibly by an elevated titre of circulating 20-hydroxyecdysone. Evidence has been published in some insect species that suggests there is a positive feedback relationship between the moulting hormone and the prothoracic glands e.g. Philosamia cynthia (Siew and Gilbert, 1971) and Mamestra brassicae (Agui and Hiruma, 1977) whilst in other species a negative feedback relationship has been demonstrated e.g. Hyalophora cecropia (Siew and Gilbert, 1971), Rhodnius prolixus (Steel, 1973, 1975) and Pieris brassicae (Beydon and Lafont, 1983). Presumably positive feedback may occur when hormone production by the PGS needs to be amplified (Siew and Gilbert, 1971) whereas negative feedback may occur to cut back PG activity in the intermoult cycle (Steel, 1975).

In all previous studies of ecdysteroid titres in diapause and non-diapause destined individuals of Sarcophaga, the titres were measured using a Sarcophaga (Ohtaki and Takahashi, 1972) or Musca (Walker and Denlinger, 1978) bioassay. In these assays, the mature larval abdomen is isolated by ligation just

prior to the release of ecdysone from the prothoracic glands. If no sign of pupariation occurs in the hind part, these individuals are used as 'standard test abdomens', where the frequency of pupariation is a function of the amount of injected active material derived from whole body homogenates from the sample animal. This assay measures the levels of hormonally active substances in the samples and will also include their biogenetic precursors (Koolman, 1980). The development of a quick and accurate radioimmunoassay for ecdysteroids (Borst and O'Connor, 1972) was one of the major recent advances in insect physiology (Richards, 1981) and has made possible detailed analyses of moulting hormone titres in Sarcophaga bullata from the time of larviposition to adult eclosion (Wentworth et al., 1981), again using whole body homogenates. In the present study a radio-immunoassay was used to measure ecdysteroid titres in the haemolymph only of diapause and non-diapause destined mature larvae and prepupae of S. argyrostoma. A brief description of the theory of a radioimmuno-assay is given in the appendix (p. I), but essentially, it is a competitive inhibition assay and depends on the ability of an unlabelled antigen to inhibit the binding of a labelled antigen when limited amounts of specific antibody are present (Parker, 1976). The results of the assay are expressed in 20-

hydroxyecdysone equivalents since the antibody used recognises both ecdysone and 20-hydroxyecdysone and their close metabolic relatives.

MATERIALS AND METHODS

1. LIVING MATERIAL

a. Non Diapausing Stock

Larvae deposited by adults of the 'fast' strain kept at LL, 25 ± 1°C were reared under long day photoperiods of LL, LD18:6 or LD16:8 at 20 ± 0.5°C. These conditions produced non-diapausing individuals.

b. Diapausing Stock

Larvae deposited by adults of the 'fast' strain kept at LD12:12, 25 ± 1°C were cultured under short day photoperiods of LD12:12 at 20 ± 0.5°C; diapause incidence in these cultures was saturated.

Staging

Once larvae became post feeding, they left the medium and crawled into the surrounding sawdust. To synchronise the culture, the larvae were examined either every 30 (Assay 6) or 60 minutes (Assays 1-5) for the appearance of red spiracled larvae (see p.65). The formation of the white prepupal stage some 3 hours after the start of the red spiracle stage provided an ideal time for the precise

synchronisation of the population. Therefore all samples taken from puparia are aged in relation to the time of white puparium formation.

Collection of Blood Samples

A fine needle was used to puncture the cuticle of both larvae and prepupae. In assays 1-4 the puncture was made anteriorly on the 2nd segment. In assays 5 and 6 the puncture was made close to the tubercles surrounding the posterior spiracles. The larva or prepupa was then gently squeezed to express the haemolymph onto either a silconised glass slide (assays 1-4) or aluminium foil (assays 5 and 6). A 5 µl sample of the haemolymph was then taken using a Drummond disposable micro-pipette and expelled into 200 µl of 70% aqueous methanol (at 4°C) in a 1.5 ml plastic microcentrifuge tube (Sarstedt). Samples were then stored at -20°C until required.

1. Standards Solution for Standard's Curve

A Stock solution, containing 50 ng 20-hydroxyecdysone was used to construct the standard curve for each assay. The stock solution was prepared by dissolving 1 mg of 20-hydroxyecdysone (Rohto Pharmaceutical Co. Ltd. Osaka, Japan) in 1 ml of Analar 100% ethanol, and making the volume up to 10 ml using a further 9 ml of 100% Analar ethanol. The OD of the solution was measured using a

spectrophotometer.

The concentration of the stock solution was then calculated using the equation:-

$$\text{conc (moles/L)} = \frac{\text{absorbance (243 nm)}}{\text{extinction coefficient (10300)}}$$

The concentration in moles/litre was then converted to ng/ml. and the original solution diluted using 100% ethanol to give 25 ml of stock at a concentration of 50 ng/ml (see Appendix, p.2). This Stock was stored at 4°C in two 20 ml McCartney bottles.

b. Construction of the Standard Curve

Concentration (ng)	µl of Stock Solution
0	0
0.05	1
0.1	2
0.25	5
0.5	10
1	20
2	40
2.5	50
3	60
3.5	70
4	80
6	120

An example of a standard curve is given in the Appendix p.7.

^{3}H-Ecdysone Stock Solution

The NEN ^{3}H ecdysone stock was divided into 20 µl aliquots, placed into glass tubes, then sealed with a glass blow torch and kept at 4°C. A fresh stock solution was prepared for each assay as follows: The tip of one of the glass tubes was snapped off using a file, and a small amount of borate buffer (~ 100 µl) was added to the ^{3}H ecdysone in the vial. Using a pasteur pipette, the solution was then removed from the tube and pipetted into 10 ml of borate buffer solution* (see Appendix) in a McCartney bottle. The solution was vortexed thoroughly.

This solution gave 2000-3000 cpm/25 µl. To verify this, a 25 µl sample was pipetted into a 1.5 ml Eppendorf tube containing 25 µl distilled H_2O, the tube vortexed, and 0.5 ml of scintillant (Aquasol II) added. The tube was then thoroughly vortexed again, and the sample counted in a scintillation counter for 10 mins.

Antiserum Stock Solution

a) Ammonium Sulphate Assay

A 25 ml antiserum stock was prepared as follows:

22.5 ml borate buffer

2.5 ml Rabbit Serum

5 µl Antiserum (Horn II stored 1:1 Ab: Glycerol) stored at -20°C.

This was kept in two 12.5 ml McCartney bottles at $4^{o}C$.

b) Protein A Assay

A 20 ml antiserum stock was made:

20 ml borate buffer

10 mg BSA

8 μl Antiserum (Horn II)

This was stored at $4^{o}C$ in a bijoux bottle. The control serum stock was used to determine background radioactivity and the level of non specific binding.

Control Serum Stock

1. $(NH_4)_2\ SO_4$ Assay

5.0 ml total volume

a. 4.5 ml Borate Buffer

b. 0.5 ml Rabbit Serum

Stored at $4^{o}C$.

2. Protein A Assay

5 ml total volume

a. 5 ml borate buffer

b. 2.5 mg BSA

Stored at $4^{o}C$.

Saturated Ammonium Sulphate Stock

Five hundred ml of borate buffer was heated to $50^{o}C$. Ammonium sulphate was then added until the saturation point was reached. The solution was then

transferred to 4°C and allowed to crystallize. The resulting supernant was used as the 100% saturated ammonium sulphate stock solution.

50% Saturated Ammonium Sulphate

A 300 ml solution was prepared using equal volumes of borate buffer and the 100% saturated ammonium sulphate stock solution. This was stored at 4°C.

The assay and final scintillation counting was carried out in siliconised plastic, 1.5 ml micro-centrifuge tubes (Sarstedt).

ASSAY PROTOCOL (Ammonium Sulphate Termination)

DAY 1

1. A standard curve using 20-hydroxyecdysone was constructed (see p. 303).
2. Unknown samples were centrifuged at 11,500g for two minutes. 100 µl of the supernatant of each sample was pipetted into a microcentrifuge tube.
3. The samples and standards were evaporated to dryness under vacuum.
4. 100 µl of ^{3}H ecdysone stock solution was added to each tube, then vortexed.
5. 100 µl of Antiserum stock solution (as prepared for Ammonium Sulphate termination) was added to all but two tubes; 100 µl of control antiserum was added to these.

6. Each tube was vortexed thoroughly before being incubated overnight at $4^{o}C$.

DAY 2

7. The assay was terminated by adding 200 μl of 100% saturated ammonium sulphate to each tube. Each tube was vortexed thoroughly immediately after adding the ammonium sulphate.
8. The tubes were left to stand at $4^{o}C$ for 20 minutes.
9. The tubes were centrifuged at 11,600 g for 10 minutes.
10. The supernatant was aspirated using a pasteur pipette drawn out to a fine point.
11. 400 μl of 50% saturated ammonium sulphate was added to each tube. The tubes were then vortexed to resuspend the pellet.

12/13/14 Repeat of stages 8, 9 and 10.

15. 25 μl of distilled H_2O was added, and each tube was vortexed to redissolve the pellet.
16. 1.2 ml of scintillant (Aquasol II New England Nuclear) was added to each tube and thoroughly vortexed.
17. The tubes were then placed in glass scintillation vials and left for 6 hours at r.t., before being counted for 10 minutes on a scintillation counter.

Calculations

1. The mean background value for the control tubes was subtracted from each sample and standard.
2. A Standard Curve was plotted on semilog paper. (ordinate, % bound arithmetic scale; abscissa, ng 20-hydroxyecdysone equivalents, log scale).
3. The 20-hydroxyecdysone equivalent for the unknowns was calculated from the standard curve equation.

RIA. Protocol. (Protein A termination)

DAY 1

Steps 1-4 as in RIA protocol for Ammonium Sulphate.

5. 100 µl of Protein A Antiserum stock solution was added to all but two tubes; 100 µl of control antiserum was added to these two tubes.
6. Each tube was vortexed thoroughly before being incubated overnight at $4^{o}C$.

DAY 2

7. The assay was terminated by adding 20 µl of Protein A emulsion to every tube.
8. Each tube was vortexed thoroughly and then left to stand at $4^{o}C$ for 15 minutes.
9. The tubes were centrifuged at 11,600 g for 10 minutes.

10. The supernatant was removed using a drawn out pasteur pipette.
11. 50 μl of distilled water was added to each tube, and vortexed to redissolve the pellet.
12. 0.5 ml of scintillant was added to every tube. The tubes were vortexed and left to stand for 6 hours.

Counting and calculations were carried out as before.

RESULTS

The level of ecdysteroids in haemolymph samples drawn from diapause and non-diapause destined larvae and prepupae were determined using a radio-immunoassay in which ammonium sulphate was used to precipitate the bound antigen-antibody complex (see p 306 for method). The means $\pm$ 1 standard errors of the ecdysteroid titres in red spiracled larvae and white prepupae are given in Table 58. There was no significant difference between the ecdysteroid titre of diapause and non-diapause white prepupae (Assay 1, $F=1.16$; Assay 2, $F=1.95$; Assay 3, $F=2.7$; Assay 4, $F=2.92$, $P > 0.05$) or redspiracled larvae (Assay 1, $F=4.95$ $p > 0.05$).

A more rapid radioimmunoassay can be achieved by using an emulsion of Staphytococcus aureus cells (Protein A) in place of ammonium sulphate (see p. 308. for protocol). These different assay methods

TABLE 58

Mean $\pm$ 1se of Ecdysteroid Equivalents (ng) per 1 of Haemolymph in Diapause and Non-Diapause Destined Red Spiracled Larvae (RSL) and White Prepupae (WPO)

Assay No.	NON-DIAPAUSE				DIAPAUSE			
	RSL		WPO		RSL		WPO	
		$\bar{x} \pm$ 1se		$\bar{x} \pm$ 1se		$\bar{x} \pm$ 1se		$\bar{x} \pm$ 1se
1	3	1.1 $\pm$ 0.06	3	1.2 $\pm$ 0.09	3	1.3 $\pm$ 0.09	3	1.1 $\pm$ 0.04
2			17	1.0 $\pm$ 0.07			17	0.8 $\pm$ 0.03
3			6	1.3 $\pm$ 0.05			6	1.1 $\pm$ 0.08
4			11	1.3 $\pm$ 0.09			12	1.0 $\pm$ 0.04

produced similar equivalent results when performed on haemolymph samples of the tobacco hornworm, Manduca sexta,* taken on days 1-10 of the fifth larval instar. (See Table 59a). The efficiencies of the methods were similar, being 20.3% for the assay using ammonium sulphate and 23.7% for the assay involving protein A. (Table 59b). Ammonium sulphate formed an insoluble precipitate in the scintillation fluid, even at concentrations as low as 2 µl of 50% ammonium sulphate per 500 µl of scintillant. On the other hand no such precipitate formed in the scintillant during assays in which Protein A was used. It was therefore decided to adopt Protein A for termination because this more rapid procedure allowed a greater number of samples to be assayed with no loss in efficiency.

An experiment was carried out to determine the concentrations of Protein A and the anti-ecdysteroid antibody which would achieve a 30-40% binding in tubes containing no competing unlabelled ecdysone (see Appendix p. 4). A combination of 20 µl of Protein A and 8 µl of Antibody per 20 ml of borate buffer produced a 37.3% binding. Therefore these concentrations were used in assay 5 and 6.

The results of the preliminary assay (Table 60

*These experiments were carried out by kind permission of Professor L. Gilbert in the Department of Biology, University of North Carolina at Chapel Hill.

TABLE 59a

A comparison of the levels of ecdysteroid equivalents in the haemolymph of Manduca sexta taken on days 1-10 of the fifth larval instar, using either Ammonium sulphate or Protein A to precipitate the bound Antigen-Antibody complex.

Day	ASSAY TYPE		Sample Size 1
	Ammonium Sulphate + Rabbit Serum	Protein A	
1	4.97	17.0	40
2	24	6.9	40
3	8.5	27.0	40
4	15.6	6.9	40
5	68	76.2	20
6	185	201.4	20
7	1291	1819.3	2
8	698	762.4	5
9	246	447.2	10
10	236	309.0	10

TABLE 59b

A comparison of the efficiency of the Assay Techniques compared in Table 59a; efficiency measured by % recovery of number of counts, in tubes containing no competing unlabelled ecdysone.

Assay	Maximum % bound	r coefficient of standard curves
Ammonium Sulphate + rabbit serum	20.3	0.976
Protein A	23.7	0.991

Fig. 35) showed that the titres of ecdysteroids in the haemolymph of diapause-destined larvae and prepupae were significantly lower than those of non-diapausing individuals of the same physiological age. The levels of ecdysteroids in the haemolymph of individuals of both groups increased approximately two fold within 5 hours, from mean values of 0.83 and 0.58 ng ecdysteroids per µl of haemolymph of non diapause and diapause destined red spiracled larvae to mean values of 1.80 and 1.08 ng ecdysteroids per µl of haemolymph respectively 3 hours after puparium formation.

To improve the accuracy of staging, newly formed red spiracled larvae were collected at 30 minute intervals instead of the 60 minute collections used in previous assays. Two hours after this initial collection, the larvae were examined every 15 minutes for the assumption of the newly formed and immobile white prepupal stage. All ecdysteroid titres during the prepupal development were then plotted as a function of time after this, although the degree of synchronisation obviously decreased with the increasing age of the prepupae. The results of the experiment are given in Table 61 and Figure 36. In non-diapausing (short night) individuals, ecdysteroid levels were high (1.20 ng) at the beginning of the redspiracled larval stage. During the next 2 hours,

TABLE 60

Mean ± 1 SE of 20-hydroxyecdysone equivalents (ng) per µl of haemolymph in diapause and non diapause destined larvae and prepupae of various ages: red spiracled larvae (rsl), numbers in brackets denote age (hours) in relation to the time of white puparium formation (wp). (*) $0.05 > p > 0.01$ (**) $0.01 > p > 0.001$.

Stage	Mean ± 1 SE				F Ratio	
	n	Non-Diapause	n	Diapause		
RSL	13	0.83 ± 0.05	14	0.58 ± 0.05	12.7	**
WP(0)	17	0.96 ± 0.14	18	0.64 ± 0.06	4.7	*
WP(1)	8	1.28 ± 0.22	6	0.68 ± 0.09	4.9	*
WP(2)	5	1.40 ± 0.16	6	0.92 ± 0.13	5.6	*
WP(3)	5	1.80 ± 0.17	6	1.08 ± 0.14	12.5	**

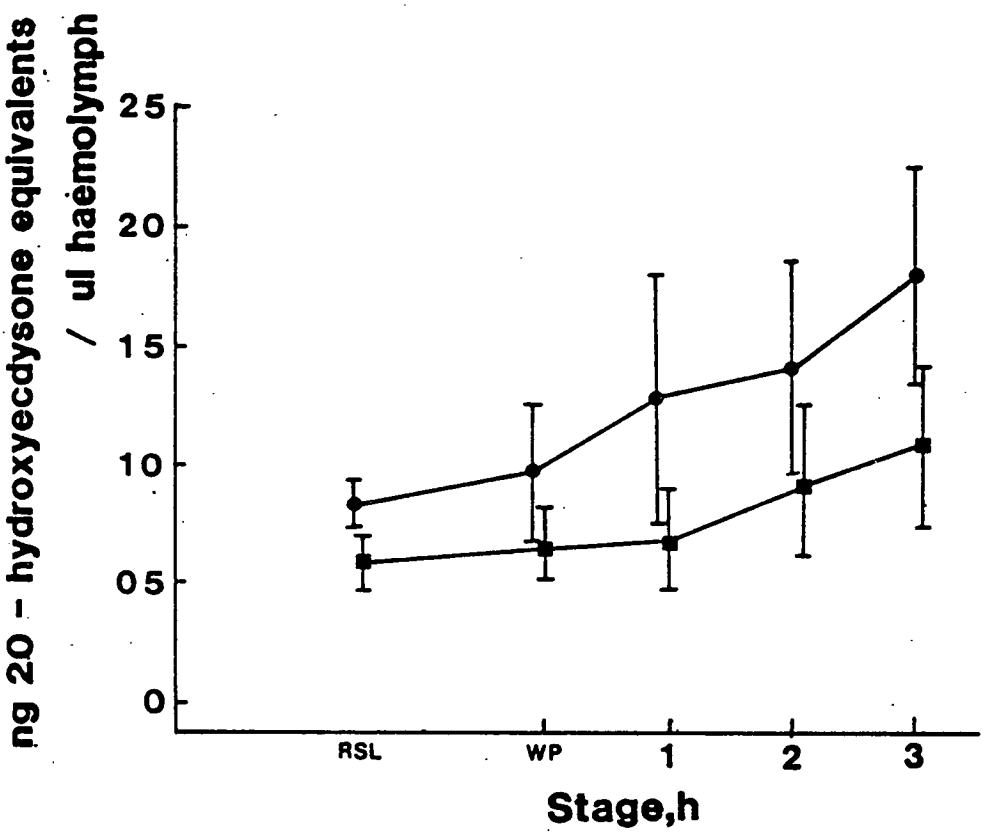


FIG. 35. Ecdysteroid titres during the red spiracled larval (rsl) and prepupal stages of development. (wp), white prepupal stage. The bars represent 95% confidence limits of the mean values. Larvae and prepupae were bred at 20 ± 0.5°C in photoperiods of either LD16:8 (●) non diapause or LD12:12 (■) diapause.

however, the titre fell to a mean of 1.07 ng, although this decrease was not significant ($F=3.7$, df. 1, 11, $p > 0.05$). Ecdysteroid levels then increased to 1.28 ng at the white prepupal stage and then remained within the range of 1.34-1.45 ng for a further 6 hours. There was then a significant increase ($F=12.1$, df. 1,12 $p < 0.01$) to 1.68 ng at 8 h, reaching a peak of 1.84 ng at 20 h. In diapause-destined (long night) larvae, the ecdysteroid levels also fell during the redspiracled stage, from 0.92-0.79 ng, although the decrease was again not significant ($F=2.11$, df 1,11 $p > 0.05$). The levels then increased to 1.02 ng/μl of haemolymph at the white prepupal stage and remained at this level for a further 6 hours, after which the ecdysteroid titres rose gradually to reach a significant peak of 154 ng 16 hours after white prepupa formation.

At all stages, ecdysteroid titres in the non-diapausing larvae or prepupae were higher than those in diapause destined individuals of a comparable physiological age. However, the differences were only highly significant ($p < 0.01$) between 2 and 8 hours after the formation of the white prepupa (Table 61). The possible significance of this consistent difference is described on p.320 .

TABLE 61

Mean ± 1 SE of 20-hydroxyecdysone equivalents (ng) per µl of haemolymph in diapause and non-diapause destined larvae and prepupae of various ages; red spiracled larvae (RSL), white puparium (WP) and numbers in brackets denote age (hours) in relation to the time of either RSL or WP formation. (O) $p > 0.05$, (*) $0.05 > p > 0.01$ (**) $0.01 > p > 0.01$ (***) $p < 0.001$.

Stage (hours)	Mean ± 1 SE Non Diapause n		Diapause n		F Value	
RSL(0)	7	1.20 ± 0.10	7	0.92 ± 0.06	5.97	*
(1)	6	0.90 ± 0.13	6	0.84 ± 0.09	0.17	o
(2)	7	1.07 ± 0.07	7	0.79 ± 0.08	7.21	*
WP(0)	6	1.28 ± 0.10	6	1.02 ± 0.16	2.27	o
(1)	7	0.93 ± 0.05	7	0.99 ± 0.10	0.32	o
(2)	7	1.39 ± 0.11	7	0.95 ± 0.06	11.71	**
(3)	5	1.41 ± 0.07	7	0.98 ± 0.06	20.12	**
(4)	7	1.45 ± 0.08	6	1.03 ± 0.05	21.92	***
(6)	7	1.34 ± 0.06	7	1.02 ± 0.09	9.67	**
(8)	7	1.68 ± 0.08	7	1.23 ± 0.09	14.25	**
(12)	7	1.64 ± 0.10	7	1.34 ± 0.12	3.57	o
(16)	6	1.76 ± 0.13	7	1.54 ± 0.10	1.91	o
(20)	6	1.82 ± 0.06	7	1.39 ± 0.09	14.4	**
(24)	7	1.42 ± 0.12	7	1.32 ± 0.06	0.61	o

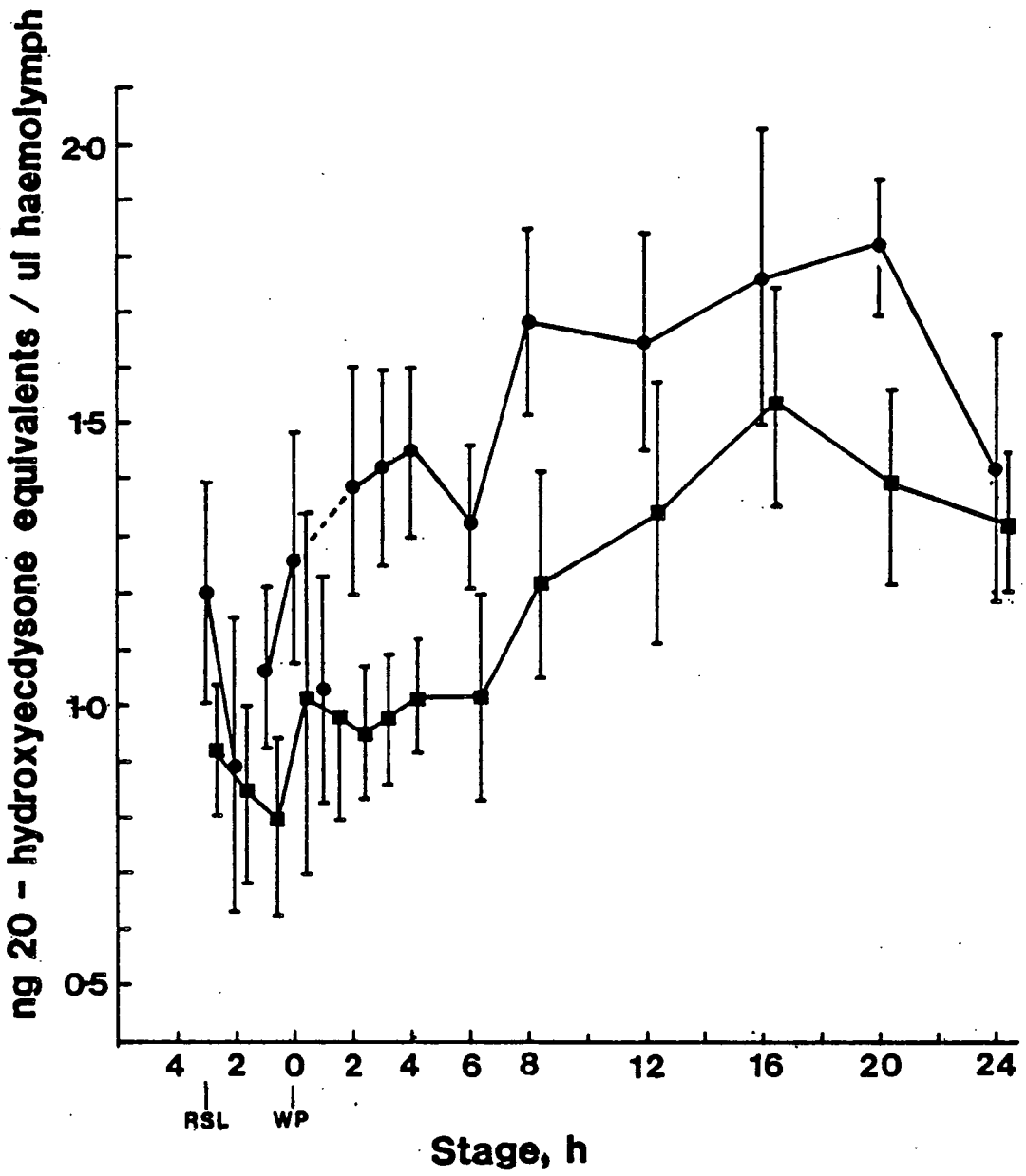


FIG. 36. Ecdysteroid titres during the red spiracled larval (rsl) and prepupal stages of development. (wp), white prepupal stage. The bars represent 95% confidence limits of the mean values. Larvae and prepupae were bred at 20 ± 0.5°C in photoperiod of either LD16:8 (●) non diapause or LD12:12 (■) diapause.

DISCUSSION

Results from this section demonstrate that the ecdysteroid titres in diapause destined prepupae of S. argyrostoma, shortly after puparium formation, are consistantly and significantly lower than titres in non-diapause destined individuals of the same age. This may reflect a real difference in the regulation of ecdysteroid levels between the two groups which is important in diapause induction; alternatively, however it could be an artefact of the analytical method (p. 321) or a result of the different duration of the post feeding stage. For example, the longer wandering phase seen in diapause destined larvae (see p. 100) may have had an effect on ecdysteroid titres at pupariation, similar to that resulting from wet treatment, which also delays puparium formation (see p. 195): Roberts (pers. comm.) demonstrated that the haemolymph ecdysteroid levels of prepupae maintained under wet conditions for 144 hours were only one third of the haemolymph titres seen in dry controls. A possible explanation for this phenomenon is given on pp. 195-198.

These data contrast sharply with similar work carried out by Ohtaki and Takahashi (1972) on Sarcophaga peregrona. These authors found, as in the present study, that at puparium formation the ecdysteroid titres in non-diapause and diapause

destined prepupae were similar. However, 12 hours after pupariation, the titre in the latter group was nearly twice that of the former. In a similar study, using S. crassipalpis, Walker and Denlinger (1980) found that the ecdysteroid titres around the time of pupariation in prepupae programmed for continuous development were slightly lower than the titres in prepupae destined for diapause, although the authors did not comment on these differences.

There are several reasons why the results from the present study may differ from those of Ohtaki and Takahashi (1972) and Walker and Denlinger (1980):-

1. In both of the published accounts, the ecdysteroid titres were measured using a bioassay (see p.299) whereas the present study used RIA. Koolman (1980) using Calliphora vicinae found that the levels of ecdysteroid measured by an RIA were three times that measured by a bioassay over the same developmental period. In addition, the profile produced by the RIA failed to reproduce a sharp peak of ecdysteroids, at pupariation, seen in data produced by the bioassay. The bioassay will have measured only biologically active material, whereas the RIA will have additionally detected immuno-genetical substances, e.g. ecdysteroids metabolites.

2. Ecdysteroid titres in the present study were assayed from haemolymph samples whereas both the

other studies used whole body homogenate extracts. Koolman (1980) found that haemolymph and whole body homogenates produced similar results, although the values were somewhat higher in haemolymph assays.

3. The Horn II antiserum used in this study has an affinity for ecdysone four times that for 20-hydroxyecdysone. This would amplify any differences in apparent ecdysteroid levels where ecdysone forms a significant fraction. Thus the differences seen in the ecdysteroid titres in the haemolymph may be due to a slight difference in the ratio of ecdysone to 20-hydroxyecdysone in diapause and non-diapause destined prepupae.

A comparable study of haemolymph ecdysteroid titres during development in S. bullata was carried out by RIA using an antiserum with approximately equal affinities for ecdysone and 20-hydroxyecdysone (Roberts et al., in press). They showed that haemolymph ecdysteroid titres during the red spiracled larval stage and the white prepupal stage ranged from 0.15 to 0.4 ng/µl whereas in this study the ecdysteroid titre ranged from 1.2 to 1.3 ng per µl of haemolymph during the equivalent developmental period. These apparent differences in ecdysteroid levels may be due in part to the differing affinities of the Horn II antiserum (this study) and the antiserum used by Roberts et al. (in press). For

ecdysone and 20-hydroxyecdysone respectively. Actual concentrations of ecdysone and 20-hydroxyecdysone at pupariation could be obtained by High Performance Liquid Chromatography (HPLC) (Lafont et al., 1980).

The results obtained in the present work do not support the hypothesis that the absence of the 2nd ecdysteroid peak in diapause destined prepupae of S. argyrostoma (Ohtaki and Takahashi, 1972) results from a negative feedback of the moulting hormone on the prothoracic glands. The preliminary nature of this study however does not justify any further discussion of these data in relation to other reported studies, and much more work on the relative ecdysteroid titres of diapause and non-diapause programmed S. argyrostoma needs to be done before a more realistic and testable hypothesis can be proposed.

A DISCUSSION OF THE PHOTOPERIODIC AND HORMONAL CONTROL OF DIAPAUSE

The minimal requirements for a photoperiodic response such as diapause induction must be a photoreceptor system to distinguish light from dark, a 'clock' to measure day or night length and an 'effector system' to regulate the appropriate humoral control mechanisms (Saunders, 1976). In addition, it is necessary to introduce the concept of a counter mechanism, since larvae of S. argyrostoma require a sequence of long night cycles for diapause induction to occur, and the number of such cycles required is independent of temperature (Saunders, 1971).

In a recent review, Saunders (1981) has suggested that

> "all photoperiodic responses encompass the same central problems: 1) how is day length or night length measured by the photoperiodic clock; 2) how is this qualitative 'information' accumulated during the insect's sensitive period; 3) what is the nature of this information and its storage; and 4) how is this information 'translated' into the neuroendocrine mechanisms controlling development?"

The formal properties of the 'clock' have been extensively investigated by Saunders and are reviewed on pp.27-37 of this thesis. At this stage, the external coincidence model remains the most appropriate for time measurement in S. argyrostoma. The model suggests that the circadian system is reset to a particular phase at the end of the light portion of a

cycle, and differentiates a short night from a long night by whether a photoinducible phase ($Ø_i$), which occurs 9.5 h after the onset of darkness, is or is not illuminated by light.

The formal properties of the photoperiodic counter have also been investigated by Saunders (1971 and 1975), and extensively so in the present study. A modified form of Gibbs's (1975) model for the photoperiodic counter has also been shown to be valid for photoperiodic summation in S. argyrostoma (see p. 201). Briefly, the model suggests that a 'diapause titre' is produced within the sensitive larval brain during each inductive long night, and this accumulates within the brain to a particular level, depending on the number of long nights seen during the sensitive stage. The level or titre is then compared to the individual's internal threshold for diapause induction. Only the effect of long night photoperiods on diapause induction has been investigated in this study. Several studies (Saunders, 1971, 1980; and Chester, unpublished), however, have suggested that embryos and larvae may 'add up' both long and short nights to a threshold. These experiments were performed by transferring groups of larvae from short to long nights, or from long to short nights, on sequential days of larval development. Since a particular number of short night cycles was

found to be capable of reversing the decision to enter diapause of potentially diapause committed larvae, Saunders (1980) suggested that this result "clearly showed that larvae 'add up' long nights and short nights to some sort of threshold before diapause or non-diapause programming is complete". However, the interpretation of such transfer experiments is difficult because of the differential photoperiodic sensitivity throughout larval development. An alternative hypothesis, in which 'short night' cycles play a more passive or neutral role might be as follows. Saunders (1981) has suggested that when $\emptyset_i$ <u>is not</u> illuminated 'diapause titre' is synthesised/released and possibly accumulated. It is possible that when $\emptyset_i$ <u>is</u> illuminated the diapause titre may not be released. If this 'titre' is unstable, as suggested in this present study, it may start to decay during a sequence of short nights, and fall below the individual's internal threshold for diapause induction, thus reversing the original commitment to diapause. The results presented in this manner support the interpretation that diapause is actively induced by long night lengths, and that continuous development must be regarded as the primitive state. Indeed, Denlinger has suggested that photoperiodic induction of diapause is an acquired characteristic, which has

enabled flesh-flies to spread from the tropics to temperate regions. Evidence in favour of this is that potential for diapause is widely observed among tropical flesh flies, although diapause is induced by cool temperatures and not by photoperiod (Denlinger, 1979).

Concrete evidence for an accumulated 'diapause titre' is lacking, although circadian changes in the structure of neurosecretory cells of certain parts of the neurosecretory system have been observed (Rensing, 1964; Rensing et al., 1965; Cymborowski and Dutkowski, 1969). Several studies have looked for neurophysiological differences between diapause and non-diapause destined individuals. Gainer (1972) monitored the activity of several neurosecretory cells in the mollusc Otala lactea. During diapause, one specific neuron was found to have a changed membrane input, resistance and resting potential which appeared to be due to a relatively selective increase in potassium permeability. In addition, the synthesis of a particular protein by the neuron, produced in non-diapausing individuals, was inhibited. Kono (1973), using light and electron microscopy, studied the neurosecretory control of pupal diapause in Pieris rapae. He found that in diapausing individuals large aggregates of neurosecretory granules were packed in the cytoplasm of specific

neurosecretory cells and found evidence of inclusion bodies, thereby demonstrating cytoplasmic breakdown of the stored granules. In contrast, in non-diapausing individuals, the neurosecretory material was released from the axon terminals just after the larval-pupal ecdysis and was thought to initiate adult development. Kono suggested that differences in the daylengths experienced during larval development had influenced the activity of the neurosecretory cells before pupation. Taking these results into account, Saunders (1981) proposed the following endocrine model for the photoperiodic counter: a neuronal photoperiodic clock causes daily circadian patterns of electrical activity which bring about circadian depolarisation of the membranes at the axon terminals causing release of the neurosecretory material. Such release is greater in short nights than in long nights resulting in a more rapid larval development in short nights. He suggested that the net result would be a daily stepwise accumulation of granules in the neurosecretory cells of long night larvae (i.e., the granules are equivalent to the 'diapause titre'), and envisaged a negative feedback inhibition of the neuronal clock, with an above threshold titre of granules shutting off the clock's electrical firing and axon depolarisation, completely inhibiting neurosecretory release.

Saunders suggested that the neurosecretory material may be PTTH. Both Fraser (1960) and Kono (1975) have demonstrated the inhibition of PTTH release within diapausing individuals. However, Saunders and Richards (pers comm.) have demonstrated that there is no difference in the rate of larval development during the feeding stage in long night and short night individuals. The duration of the wandering phase is greater in diapause destined larvae than in individuals programmed for continuous development, but this may be a consequence of the 'diapause state' rather than a result of the photoperiod experienced during earlier larval life (see p.100). It is possible that the changes in the firing patterns and rates of neurosecretion within neurons are also a consequence of the already programmed 'diapause state'. Diapause is a genetically determined state of suppressed development, since many characteristics of diapause can be inherited (Kurahashi and Ohtaki, 1976; Henrich and Denlinger, 1983). Ultimately, it is possible that the decision to enter diapause may be taken as the "switching off" of continuous developmental genes and the "switching on" of diapause genes although this does not provide an explanation for the more "down stream" physiological mechanism directly controlling diapause induction.

The implantation of a 'short night' brain from

wandering larvae of S. argyrostoma into a long night recipient resulted in the majority of individuals failing to enter diapause, whereas the implantation of 'long night' brains into 'short night' recipients failed to induce diapause. This result seems to exclude the possibility of a circulating "diapause hormone" (Giebultowicz and Saunders, 1983), but does not exclude the possibility of "diapause titre" accumulating within the brain in long night cycles. It is possible therefore that the titre in relation to the internal threshold of diapause induction may control the 'switch' in developmental programs.

It seems probable that pupal diapause within the flesh flies results from a failure of the neurosecretory cells within a 'long night' brain to release PTTH. It is therefore necessary to understand the physiology of release and retention of neurosecretory material from cell axons. Within the lepidoptera, it has been shown that in newly diapause-reactivated pupae of Hyalophora cecropia and Antheraea pernyi, a spike of c. AMP activity immediately precedes the ultra-structural changes within the median neurosecretory cells which are thought to be associated with the transport of neurosecretory vesicles (Rasenick et al. 1976, 1978). These changes, ultimately result in the release of PTTH associated with the initiation of adult development. However, Gnagey and Denlinger

(in press) failed to detect a rise in cyclic AMP in the brain-ring gland complex of Sarcophaga crassipalpsis prior to the resumption of adult differentiation.

The temporary cessation of endocrine activity in diapausing individuals might be a result of negative feedback (see p.299). Although results in this study seem to exclude ecdysteroids as the feedback agent, it is possible that the regulation of PTTH release could involve juvenile hormones: Walker and Denlinger (1980) for example, using a bioassay, have shown that diapause destined prepupae of Sarcophaga crassipalpis have a higher JH than non diapause destined prepupae of a similar age. Likewise, M.F. Bowen (cited in Saunders and Bradley, 1984) observed different timing profiles of in vitro JH III (but not JH I) by the corpora allata of short and long-day fifth instar larvae of the moth Manduca sexta. Bowen et al. (1984) also found that in addition to the inhibition of the release of PTTH, in diapause destined individuals, the prothoracic glands in vitro became refractory to PTTH stimulation within one day of pupation. It is possible that this may also occur in Sarcophaga argyrostoma and an in vitro study similar to Bowen et al. (1984) would be useful. Circumstantial evidence in support of this was reported by Gibbs (1975). Diapause commitment

in S. argyrostoma can be reversed by a 'temperature step up' only within 25 hours of pupariation. If a temperature 'shock' given 25 hours after pupariation still causes the release of the stored PTTH within the neurosecretory cells but, the prothoracic glands are no longer receptive to stimulation, diapause will still occur.

However, such endocrine events are likely to be 'downstream' phenomena and the initial 'decision' to enter diapause is almost certain to be taken within the brain. Future studies must be centred on possible neurophysiological differences within the long day or short day brain if the mechanism of photoperiodic summation is to be elucidated.

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APPENDIX

RECIPE FOR SYNTHETIC MEDIUM

Medium was made up in 4 l quantities.

INGREDIENTS

250g dried milk powder (Millac, instant spray dried separated milk with added vegetable fat and vitamins A, C and D).

30g Dried yeast (Distillers Company (Yeast) Ltd. Autolysed Yeast Granules).

20g Davis Agar (Mackay and Lynn Ltd.).

4l Tap water.

1 plastic basin, 1 aluminium saucepan, 1 wooden spoon.

METHOD

1. Mix dried milk and yeast in plastic basin.
2. Add enough water to make smooth paste.
3. Place agar in saucepan, mix to a smooth paste with water.
4. Add milk and yeast mixture to the agar paste and mix.
5. Add the remaining water.
6. Bring to the boil stirring constantly.
7. Pour the medium into dishes, leave to cool then store at 4 ^{o}C.

APPENDIX

THE RADIOIMMUNOASSAY (RIA) TECHNIQUE

(PARKER, 1976)

The radioimmunoassay performed in the present study uses a labelled antigen (^{3}H Ecdysone) and depends on the unlabelled antigen (Ag) inhibiting the binding of a labelled antigen (Ag*) when limited amounts of specific antibody (Ab) are present - it is thus a simple competition in which Ag reduces the amount of free Ab, thereby decreasing the availability of Ab to Ag*. In the assay Ag and Ag* are incubated in the presence and absence of samples containing Ab. After approaching equilibrium the free Ag* and the antibody bound Ag* are separated and determined by radioactive counting. The antigen concentration in the unknown is measured by comparing the reduction of Ag* binding produced by Ag in the sample, to that of a standard curve obtained by adding graded known amounts of Ag to the assay system (see standard curve, p.7).

APPENDIX

1. RIA Calculation for standard solution

1. 1 mg ecdysone — 1 ml ethanol

1:10 dilution

9 ml ethanol* OD solution = 2.85.

Conc. (moles/L) = $\frac{2.85}{10300}$ = 2.77×10^{-4} moles/liter

1 mole ecdysterone weights 480 g.

∴ original solution contains 1.33 x 1g/ml of ecdysterone

Require 25 ml of 50 ng/ml conc.

∴ Take 9.4 µl solution — 25 ml analar ethanol.

2. Borate Buffer Solution

6.184 g Boric Acid (0.1 m)

9.536 g Borax (Sodium tetraborate)(0.1 m)

4.383 g NaCl (0.075m)

Dissolve in IL of distilled water. Bring pH 8.4

Store at $4^{o}C$.

Calculation for ^{3}H Ecdysone Solution

Original Stock concentration = 50 Ci/0.5 ml.

(1mCi = 3.7×10^{7} dps)

∴ 50 Ci = 1.85×10^{6} dps/0.5 ml

assume 25% counting efficiency

= 4.6×10^{5} cps

= 2.78×10^{7} cpm/0.5 ml

∴ 20 µl = 1.1×10^{6} cpm

THE USE OF PROTEIN A AS A METHOD TO PRECIPITATE THE BOUND ANTIGEN-ANTIBODY COMPLEX

The aim of this experiment was to determine the concentrations of Protein A and the antibody (Horn II) which would achieve 30-40% maximum binding in an RIA assay.

Materials and Methods

The antibody solutions were tested at 5 concentrations, 0.01, 0.02, 0.03, 0.04 and 0.05 (Table A). Each antibody concentration was in turn tested against four concentrations of protein A, 15, 20, 25 and 30 µl. Each Antibody:Protein A pairing was replicated 3 times.

Table A. Preparation of Antibody Concentrations

CONCENTRATION		I	II	III	IV	V
Borate - Buffer	(ml)	20	20	20	20	20
BSA*	(mg)	10	10	10	10	10
Antibody	(µl)	4	8	12	16	20

The concentration of the stock used was 1990 cpm/ 25 µl. The protocol for the assay procedure is described on pp. 308-309.

*Bovine Serum Albumin.

RESULTS

Protein A (µl) \ Ab conc.	0.01	0.02	0.03	0.04	0.05
15	30.9	34.8	39.8	47.1	49.6
20	28.0	37.3	39.8	43.4	38.4
25	27.5	38.2	38.7	46.5	40.1
30	25.8	35.6	40.3	45.1	50.6

The results show that with the concentrations of protein A used, the binding was independent of the amounts of protein A. However, binding increased with increasing concentration of Antibody. The most suitable combination which achieved a binding % of 30-40% with maximum conservation of the antibody stock was 0.02. A concentration of 20 µl protein A per jar was used since this was the amount already used in RIA's carried out in the Chapel Hill laboratory.

A standard curve was constructed as described on pp.303. Each concentration of ecdysone was run in duplicate, thus the % bound for each concentration was a mean of the two values. In all samples the background value was subtracted, this was usually about 100 cpm. The 100% bound, i.e. no competing ecdysone was calculated on a mean of 5 replicates. The values obtained for the standard curve are given in Table C.

Table C. Standard Curve

ng Ecdysterone	% bound
0	100
0.25	78.05
0.5	66.5
1.0	51.25
2.0	35.9
2.5	28.95
2.75	25.3
3.0	24.4
3.5	21.7
4	18.7
6	18.1

The standard curve was plotted on semilog paper (ordinate % bound, arithmetic scale, abscissa ng 20-hydroxyecdysone equivalent (log)).

The equation of the best fitting line was calculated, (the correlation coefficient (r) = 0.995, $p < 0.001$)(Fig.37). The value of each sample was then calculated from the equation:

$$\log \text{ 20-hydroxyecdysone equivalent } (x) = -\left(\frac{\% \text{ bound } (y) - 99.1}{49.2}\right)$$

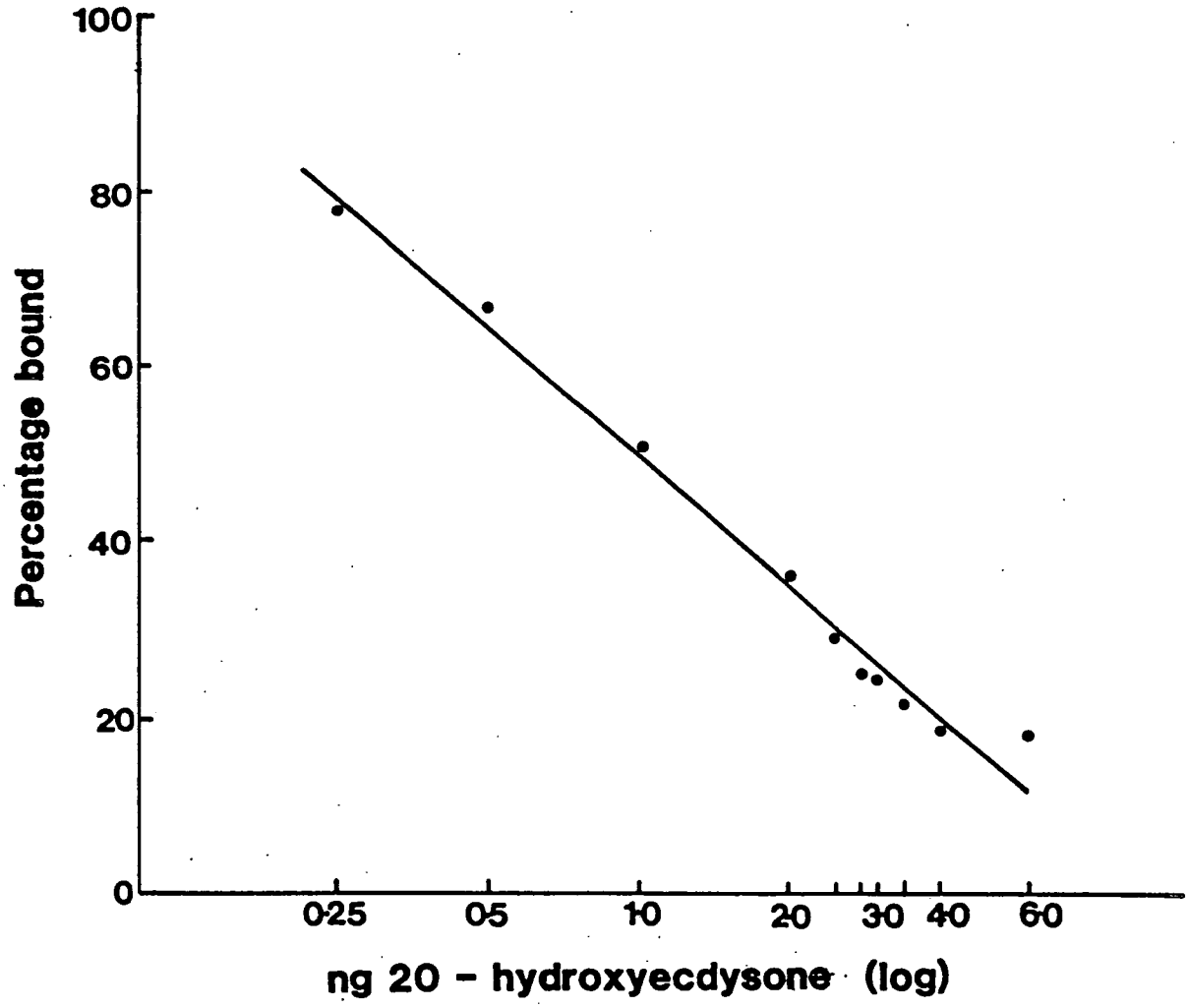

FIG. 37. Standard curve for radioimmunoassay. Ordinate: % bound (arithmetic scale); abscissa ng 20-hydroxyecdysone equivalents (log scale), $r = -0.995$, $p < 0.001$.

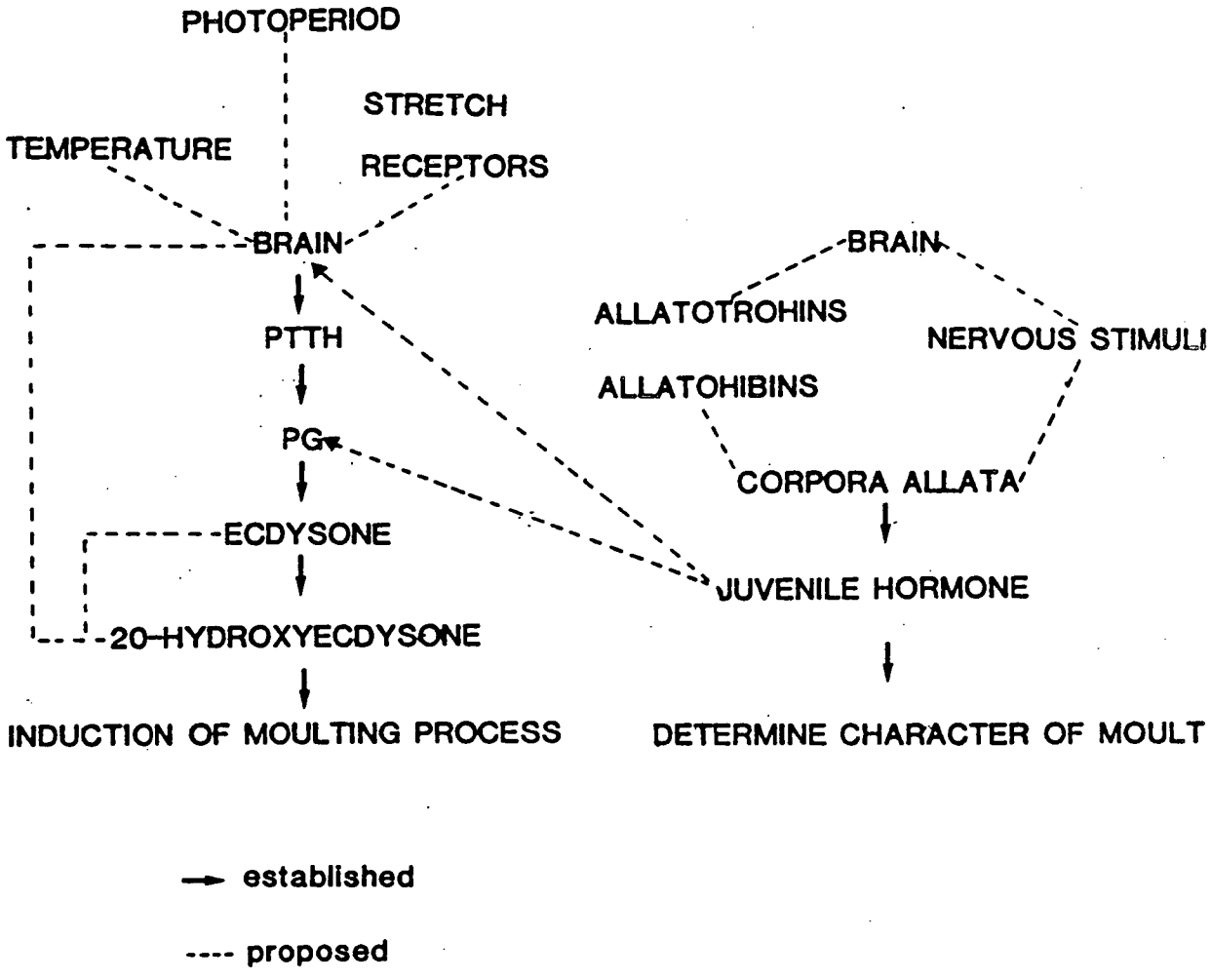

FIG. 38. The established and proposed interactions between insect developmental hormones, and the factors that affect their secretion (Gilbert and Goodman 1982)).