J. Exp. Biol. (1963), 40, 531–540
With 5 text-figures
Printed in Great Britain

OXYGEN REQUIREMENTS AND THE PHYSIOLOGICAL SUPPRESSION OF SUPERNUMERARY INSECT PARASITOIDS

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(*Received* 16 *April* 1963)

INTRODUCTION

There are many references, in the literature of biological control, to the physiological suppression of competing endoparasitic Hymenoptera. As a phenomenon it has received a variety of explanations, such as the secretion of some toxic substance by the surviving parasitoid (Timberlake, 1910, 1912), or of a cytolytic enzyme which destroys its competitors (Spencer, 1926; Thompson & Parker, 1930). None of these explanations, however, has been accompanied by any critical examination of its possible validity.

In an earlier paper of this series (Fisher, 1961*b*) the availability of oxygen to the host insect was shown to have an important effect on the outcome of competition between its parasitoids. The normal outcome of such competition is for the younger one to be suppressed and for the older to survive. This can be reversed by raising the oxygen content of the air surrounding the host to 33% or 50% by volume. Conversely, the effects of lowering the oxygen content of the atmosphere by the addition of nitrogen is to retard the development of both parasites, particularly the younger one. The development of single eggs or of young larvae is arrested and later they become invested with a spherical capsule of blood phagocytes in exactly the same manner as do younger larvae when suppressed in the presence of an older one.

Internal parasitoid larvae of the Ichneumonidae make no direct contact with the tracheal system of their hosts and obtain their oxygen directly from the host haemolymph in which they lie. There is no respiratory pigment and the haemolymph contains oxygen in simple solution in a concentration which is proportional to the partial pressure of oxygen in the atmosphere surrounding the caterpillar, and depends also upon the osmotic pressure of the haemolymph. It was therefore postulated that lack of oxygen in the host haemolymph due to the respiratory activity of the older parasite larva was responsible for the physiological suppression of the younger one. In order to test the validity of this hypothesis the relationship between oxygen tension of the atmosphere surrounding the host and that in its haemolymph has been studied, both before parasitization and during the course of parasite development. Secondly, the effects of varying oxygen tension in the host haemolymph on the behaviour and survival of the parasite larvae have been assessed during the period of early development when suppression normally occurs. Thirdly, the oxygen uptake of the host and parasite have been measured for the same period.

MATERIAL AND METHODS

The hymenopterous parasitoids used in this work are the ichneumon wasps *Nemeritis canescens* Grav. and *Horogenes chrysostictos* Gmelin (Ophioninae). Both species attack mature larvae of the moth *Ephestia kühniella* Hb. (= *sericarium* Scott) (Phycitidae). All three species are maintained in laboratory culture by methods already described (Fisher 1961 *a*). Since it has already been shown that the mechanism of physiological suppression is the same in both intra- and inter-specific competition between these two parasitoids (Fisher 1961 *b*) most of the work described here was carried out with *Nemeritis*, a parthenogenetic species which can be bred more easily and more abundantly in the laboratory than *Horogenes*.

Two methods for measuring the oxygen content of *Ephestia* blood were used. Roughton & Scholander's (1943) method relies on the extraction of dissolved gases from a 40μl. blood sample. Since a mature *Ephestia* caterpillar has only about 10 μl. of blood, pooled samples from five or more caterpillars were required for each determination.

Because of the difficulty of collecting reliable samples of sufficient volume for estimation by this method, and also because the oxygen content of the blood proved to be of the same order as that already in solution in the reagents used in the process of extraction, the accuracy of the results could not be relied upon. Consequently the oxygen estimation method of Krogh (1911) as modified by Pryor (1955) was adopted in later work and used for the majority of results recorded in this paper. In this technique a small bubble of air or a known gas mixture of oxygen and nitrogen is injected into the insect's body cavity. The gases in the bubble are allowed to equilibrate with those in solution in the blood for several hours before the insect is cut open under glycerine. The bubble is then removed in a glycerine-filled pipette to a bridge slide containing glycerine. The diameter of the bubble is then measured microscopically before and after the absorption of its contained oxygen with alkaline pyrogallol. The volume change can then readily be calculated. The method is only accurate within about 2% but it gives a quick indication of the percentage saturation with oxygen in individual insects. Controls were run with each set of readings, using either air or oxygen-nitrogen mixtures, bubbled into glycerine.

The respiration of *Nemeritis* larvae in the first, second and third instars was measured using the Cartesian diver respirometer (Holter, 1943). The parasitoid larvae were obtained by dissecting the host caterpillars in insect Ringer; they were then transferred to the bulb of the diver in a small drop of Ringer, the neck seals were placed in position and the oxygen uptake was measured over a period of 2–4 hr. at 20° C.

The oxygen consumption of healthy and parasitized *Ephestia* larvae was measured with the use of a standard Warburg respirometer.

Atmospheres for the study of parasite survival under varied conditions of oxygen tension were obtained by filling an aspirator of 12 l. capacity with various mixtures of oxygen and nitrogen. The gas mixture was humidified and equilibrated at the incubator temperature of 25° C. by being bubbled through a wash bottle of water before being passed, at a rate of 0·5 l. per hour, through 6 × 1 in. glass tubes containing the parasitized larvae. Gas exit from the tubes was provided by capillary valves.

EXPERIMENTS AND RESULTS

Oxygen content of Ephestia *blood*

Estimation of the dissolved oxygen content of *Ephestia* larval haemolymph by the Roughton & Scholander (1943) method gave a mean value $0{\cdot}39 \pm 0{\cdot}155$ ($N = 42$) vol. O_2 per 100 vol. haemolymph, and by the modified Krogh's method (Pryor, 1955) the haemolymph was found to be in equilibrium with an atmosphere containing $14{\cdot}0 \pm 1{\cdot}2$ % oxygen by volume. This is equivalent to a calculated solubility of 0·37 vol. O_2 %. By using the latter method the oxygen content of gas bubbles in equilibrium with the haemolymph of *Ephestia* caterpillars maintained in various oxygen/nitrogen

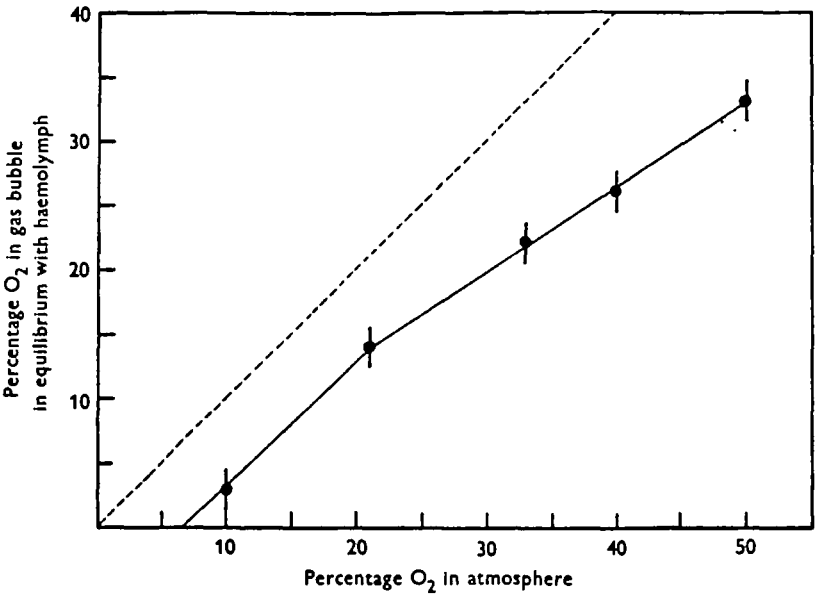

Fig. 1. Relationship between the oxygen tension of the atmosphere and that of gas bubbles equilibrated with the haemolymph of *Ephestia* larvae. Each point represents the mean of twenty readings together with their standard deviation.

Table 1. *The solubility of oxygen in haemolymph of* Ephestia

% oxygen in atmosphere	% oxygen in gas bubble equilibrated with haemolymph	Solubility of O_2 in 1·6 % NaCl at 20° C. in ml./l.	Calculated solubility of O_2 in haemolymph at 20° C. in ml./l.
5	•	1·375	—
10	3	2·75	0·825
21	14	5·61	3·74
33	22	8·81	5·86
40	26	10·65	6·92
50	33	13·35	8·81

• No oxygen detected.

mixtures, of range 2–50 % oxygen, was found to increase with the partial pressure of oxygen in the atmosphere surrounding the caterpillar (Fig. 1). Thus it is possible to use the partial pressure of oxygen in the atmosphere surrounding the host to alter the

oxygen tension in the host haemolymph. Furthermore, the actual oxygen content of the haemolymph at any given oxygen tension may be calculated from the freezing-point depression of 1·15° C. measured by Rouschal (1940) for the closely related species *E. elutella*, which is equivalent to a 1·6% solution of sodium chloride (Table 1) and the known solubility of oxygen (Prosser, 1950).

The relationship between parasite development and the oxygen content of the host haemolymph was studied by making a series of estimations at each larval instar of the parasite (Fig. 2). It was found that the oxygen content of the bubbles equilibrated with the host haemolymph falls with the progress of parasitism from the normal level of 14% to a mean minimum of 1·5% when the contained parasite larva reached the fifth and final larval instar. This stage of the parasitoid is polypneustic and breaks out of the host skin soon after moulting from the fourth instar.

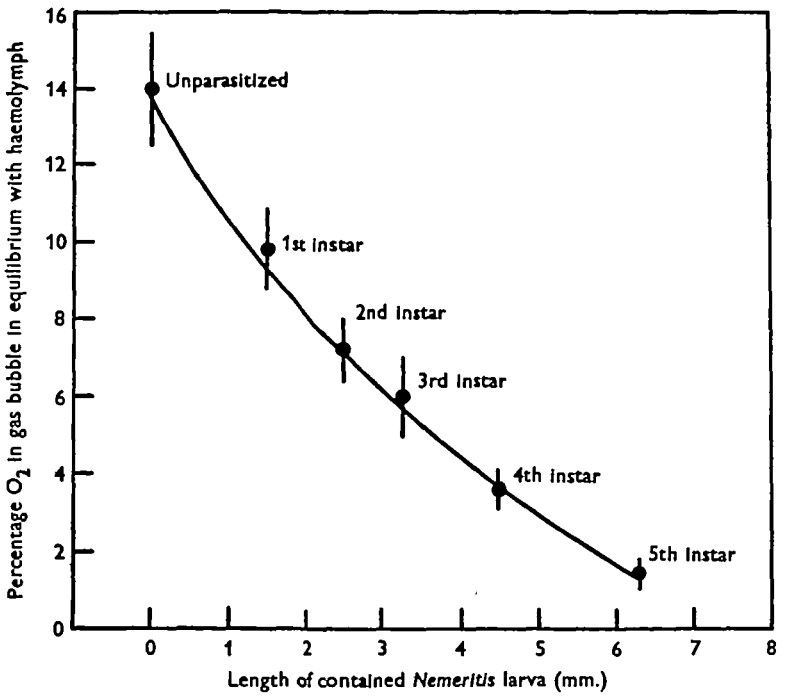

Fig. 2. Reduction in the oxygen tension in gas bubbles equilibrated with *Ephestia* haemolymph due to parasitism by *Nemeritis*. Hosts kept in normal air at 25° C.; mean of twenty observations at each point with standard deviations.

Oxygen consumption of Nemeritis *larvae*

The oxygen uptake of young *Nemeritis* larvae in the first, second and third instars were made with the Cartesian diver respirometer for comparison with the oxygen availability in the host haemolymph. The results (Fig. 3) show the rate of oxygen consumption in microlitres per hour at N.T.P. plotted against the body length of the individual parasite measured with an eyepiece micrometer. The minimum uptake measured was about 0·03–0·06 μl./hr. for young first-instar larvae, but this rose rapidly with age, increasing about eightfold by the end of the first instar.

Oxygen consumption of Ephestia *caterpillars*

The oxygen uptake of both healthy and parasitized *Ephestia* larvae of 25 mg. live weight was measured, primarily to give some indication of its rate in comparison with those of its parasites. However, since the respiratory rate varied with the activity of the caterpillar, some form of continuously recording respirometer would be necessary to give a complete picture of the host respiration during the course of parasite development. Nevertheless, the results obtained with the Warburg apparatus (Fig. 4) show that there is a gradual fall in the rate of oxygen consumption of the parasitized *Ephestia*, particularly as the contained parasite approaches maturity and the host becomes more and more lethargic. The oxygen consumption of healthy, unparasitized *Ephestia* is shown for comparison.

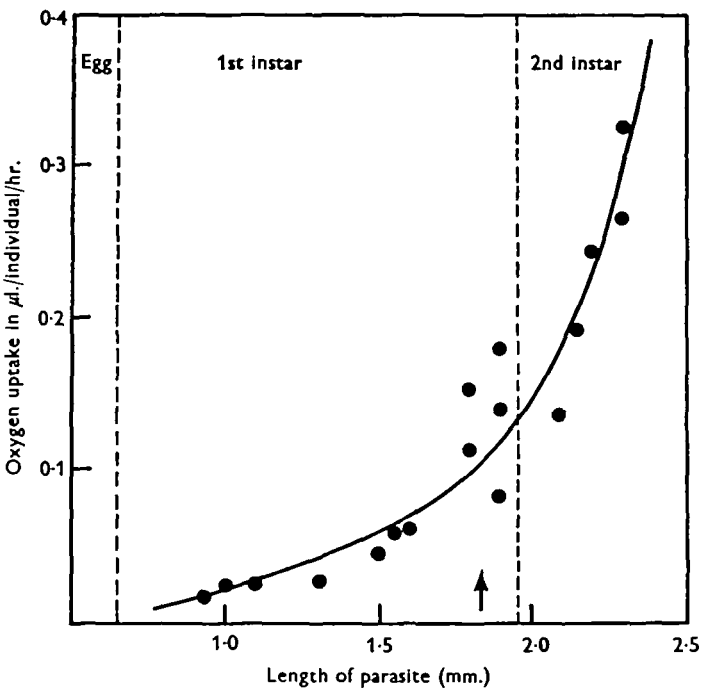

Fig. 3. The oxygen uptake of *Nemeritis* larvae in relation to body length in the first and second instars. The arrow shows the point at which the parasite first becomes capable of suppressing its younger competitors.

Survival of Nemeritis *in reduced oxygen tensions*

Since the host haemolymph has a capacity for oxygen which is dependent upon the partial pressure of oxygen in the insect's tracheae and the osmotic pressure of the haemolymph, any increase in the oxygen demands of the parasite due to its growth must absorb all the dissolved oxygen available in the haemolymph of the host and possibly produce an increase in the oxygen uptake into the haemolymph from the host's trachae. This follows from the observation that the oxygen consumption of newly hatched larvae of *Nemeritis* (0·03–0·06 µl./hr.) is roughly equal to the total oxygen content of the hosts' haemolymph at any given time (0·035 µl. per caterpillar of 10 µl.

total haemolymph volume). Because of the many difficulties which arise in attempting to culture internal parasitoids in artificial media outside their hosts, the effects on the parasitoid of lowering the oxygen tension of the host haemolymph have been observed *in vivo*. This was done by altering the partial pressure of oxygen in the atmosphere surrounding the host, since it has been shown that the oxygen content of the haemolymph varies directly with the partial pressure of oxygen in the atmosphere (Fig. 1). Experimental stocks of 100 singly parasitized *Ephestia* were kept at each partial pressure of oxygen. Ten caterpillars were removed and dissected each day from each stock for the first 10 days of their development in order to examine the condition of their contained parasites. Because of the necessity for dissection of the hosts the survival of individual parasites could not be followed.

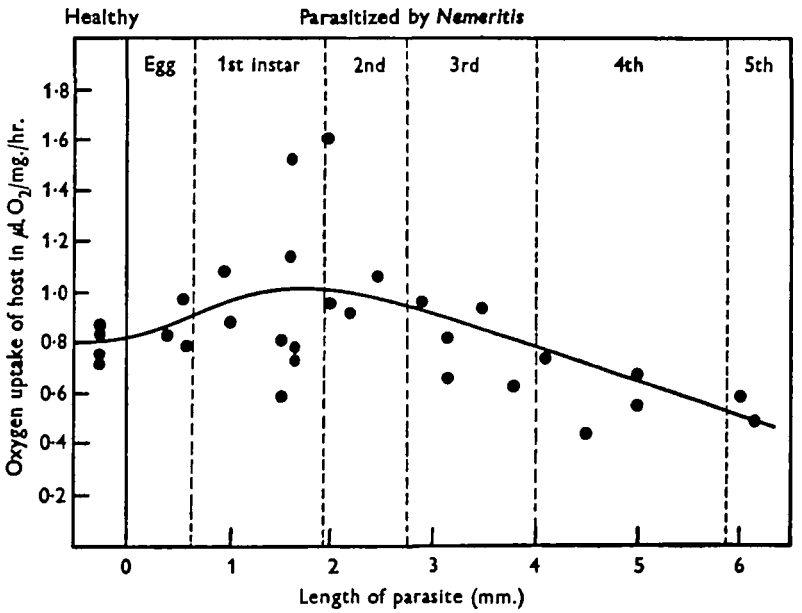

Fig. 4. Graph showing the oxygen uptake of *Ephestia* caterpillars in relation to parasitism by *Nemeritis*.

Humid gas mixtures containing either 2, 5, or 10% oxygen in different tests were passed through glass tubes containing the larvae. Growth curves obtained for *Nemeritis* at different oxygen tensions are compared with those obtained in normal air in Fig. 5. The lowering of the oxygen tension of the host haemolymph by this means affects the rate of parasite development, primarily by delaying the hatching of the egg, but a retardation of development in the first instar also occurs.

The condition of the parasitoids, particularly those reared in 5 and 2% oxygen, closely resembles that of physiologically suppressed larvae in cases of multiparasitism. Retarded first-instar larvae remain very small, curled up upon themselves and almost motionless. When they become moribund they are attacked by the host haemocytes in a manner exactly similar to that seen in cases of multiparasitic physiological suppression. Furthermore, there is a far greater mortality of eggs and young first-instar larvae in 2 and 5% O_2 than at 10 and 21% (air controls). Mortality of th

parasites in each day's sample is given in Table 2 and the total from each line represents the percentage mortality of 100 parasites observed in the 10-day period. In addition, it may be noted that the mortality due to oxygen lack in these tests is greater in the first 6 days than subsequently; from which it may be supposed that the late first-instar larva and those in subsequent instars are better able to survive low oxygen tension than the eggs and newly hatched larvae.

The differential survival of the instars with respect to very low oxygen tension was examined further by keeping *Nemeritis* eggs and larvae of all instars individually in glass cells of approximately 300 mm^3. capacity, completely filled with liquid paraffin and sealed, without including air bubbles, with a greased coverslip. The actual volume of oxygen dissolved in the paraffin was not measured, but in a qualitative test, drops

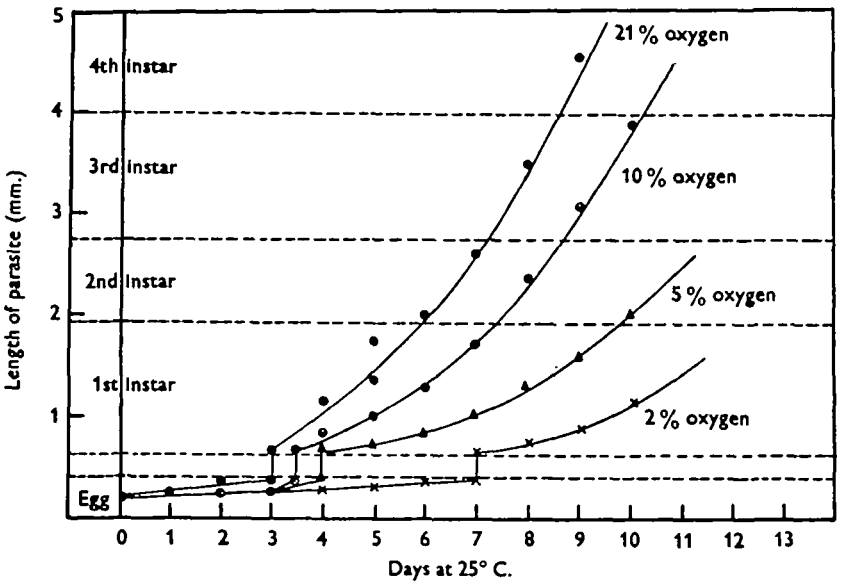

Fig. 5. The effect of lowering the atmospheric oxygen tension on the growth rate of *Nemeritis* eggs and larvae at 25° C.

Table 2. *The mortality of* Nemeritis *larvae during the first* 10 *days of development under conditions of reduced oxygen tension, at* 25° C.

Day ...	1	2	3	4	5	6	7	8	9	10	Total (% mortality)
21 % O_2 (controls)	1	—	1	2	1	2	2	3	2	2	16
10 % O_2	2	1	3	4	4	2	3	4	3	4	30
5 % O_2	1	2	4	3	6	6	4	5	4	6	41
2 % O_2	8	9	10	10	8	9	8	8	9	9	88

of alkaline pyrogallol injected anaerobically into the cells did not darken at all rapidly, though they eventually turned a pale straw colour after a period of 8 days. Since the parasite's spiracles remain closed until the final instar is reached the closed respiratory system of the larva is not impaired by immersion in liquid paraffin. Eggs and larvae

in the first to fourth instars were immersed individually in the glass cells and observed daily during incubation at 25° C.

The eggs showed no development after 24 hr. and by the same time all the newly hatched first-instar larvae (less than 1·0 mm. long) had also died. Late first-instar larvae and those in subsequent instars remained alive and were moving normally 4 days later. They continued to 'feed' so that by the fourth day their mid-guts were distended with globules of paraffin. One of the third-instar larvae had successfully moulted to the fourth instar. An apneustic fourth instar had also by this time moulted to the fifth instar whose spiracles are normally open for atmospheric respiration after it leaves the host. Liquid paraffin had entered the tracheal system and the larva was dead.

Evidently the eggs and newly hatched larvae have a low, but absolute, requirement for oxygen which must be satisfied if they are to survive, while mature first-instar larvae and those in subsequent instars, with the exception of the last, are apparently able to survive and feed in very low oxygen tensions.

DISCUSSION

In an earlier paper (Fisher, 1961*b*) the availability of oxygen to competing parasitoids was shown to be important in controlling the outcome of multiparasitic competition. An hypothesis of asphyxiation was put forward as a possible explanation of the physiological suppression of supernumerary insect parasitoids. The experiments described here were carried out to ascertain whether the respiratory activity of the relevant stages of the parasite larvae and the oxygen content of the host haemolymph would support this hypothesis.

The oxygen content of the haemolymph of unparasitized *Ephestia* caterpillars kept in air, though low compared with blood that contains a respiratory pigment, is in fact about 66% saturated with oxygen and approaches the value for the solution of oxygen in an equivalent salt solution (0·56 vol. % for a 1·6 solution of sodium chloride). It is apparent that the haemolymph of *Ephestia* carries no more oxygen than physical solution will permit. This is in accordance with other determinations made on the haemolymph of lepidopterous larvae (Adler, 1917; Babers, 1938; Pryor, 1955).

Measurement of oxygen tensions in the haemolymph over a range of partial pressures of oxygen show that, in atmospheres containing from 10 to 50% of O_2 by vol. the dissolved oxygen content varies in direct proportion to its partial pressure in the atmosphere surrounding the insect. Thus, by keeping the hosts in various oxygen/nitrogen mixtures, it was possible to examine the effects of oxygen tension on the behaviour and survival of the parasite. When this was done it became immediately apparent that lowering the oxygen tension of the haemolymph causes a considerable delay in the time taken for the egg to hatch and also retards the subsequent larval development. In cases of extreme retardation, particularly in 2% oxygen, many of the eggs and larvae become surrounded by the host's haemocytes as in true physiological suppression.

It was noticeable, however, that the retarding effects of reduced oxygen tension decrease with the increasing age of the parasite larva, especially after the first instar is completed. This differential tolerance was confirmed by testing the survival of all

instars in sealed glass cells of liquid paraffin in which the oxygen tension is very low. Eggs and newly hatched larvae cannot survive in liquid paraffin, whereas the first and subsequent instars can do so. Evidently the former have a very low, but absolute, requirement for oxygen which must be satisfied by the host if they are to survive. Since the oxygen content of the host haemolymph does in fact decrease with the progress of parasitism, this would be an advantageous adaptation on the part of the endoparasite, which, through its own development, progressively destroys its host.

Since the total dissolved oxygen content of normal host haemolymph at any given time is of the order of 0·04 μl. and the minimum measured oxygen uptake per hour of a young first-instar *Nemeritis* larva is about equal to this figure (0·03–0·06 μl./hr.) it is highly probable that the parasitoid is utilizing all the dissolved oxygen available to it. Once the parasite larva has established itself in the haemolymph of its host and is respiring oxygen at a rate in excess of the total oxygen content of the haemolymph, its own respiration becomes limited by the rate at which oxygen can diffuse into the blood from the host's tracheae. The progressive lowering of oxygen tension in the host haemolymph observed during the development of a parasite (Fig. 2) suggests that this rate of diffusion is the limiting factor.

The initial observation that the host cannot support more than one parasite unless the oxygen content of its haemolymph is raised artificially supports this explanation (Fisher, 1961*b*). The observations that superparasitism often results in a prolongation of the pre-imaginal development of the survivor (Salt, 1934; Simmonds, 1943) and that suppressed larvae recover when transferred to fresh and hitherto unparasitized hosts (Simmonds, 1943; Fisher, 1961*b*) are also in accordance with an interpretation of this kind. Clearly the availability of oxygen in the haemolymph of the host is important to the survival of the parasite and the evidence presented here strongly favours asphyxiation as the mechanism of physiological suppression.

SUMMARY

1. The oxygen content of the haemolymph of mature larvae of *Ephestia kühniella* has been measured in healthy individuals and in those parasitized by the ichneumonid *Nemeritis canescens*. In the latter the oxygen content decreases with the progress of parasitism.

2. The respiratory rate of first, second- and third-instar larvae of *Nemeritis* has been measured and found to increase rapidly at the end of the first instar, at about the time at which the phenomenon of physiological suppression appears.

3. The partial pressure of oxygen in the gas mixture surrounding the *Ephestia* larva affects the oxygen content of its haemolymph proportionally and so was used to alter the availability of oxygen to the parasite *in vivo*.

4. The survival of *Nemeritis* larvae increases with the availability of oxygen in the haemolymph of its host.

5. The capacity for survival under conditions of low oxygen tension is minimal for eggs and newly hatched larvae of *Nemeritis*, but rapidly increases with age.

6. The hypothesis of physiological suppression of supernumerary parasitoid larvae by asphyxiation is discussed with respect to these observations and is held to be supported by them.

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