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Hypoxia and anoxia in insects: microcalorimetric studies on two species (*Locusta migratoria* and *Manduca sexta*) showing different degrees of anoxia tolerance [☆]

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Abstract

Microcalorimetry was used to study the effects of graded hypoxia and anoxia on two species of insects that differ in their tolerance of anoxia. Locusts (*Locusta migratoria*) can survive an atmosphere of pure nitrogen for not more than 4 h (at room temperature), whereas hawk moths (*Manduca sexta*) can recover from more than 24 h of anoxia.

To produce graded hypoxia, air and pure nitrogen were mixed and this mixture was passed through the cells of a twin calorimeter equipped with circulation cells. A gas flow containing 2% or more of oxygen had no significant effect on behaviour (as observed in parallel experiments using transparent cells) or heat flow rate. If oxygen content was reduced to 1% or less the effects of oxygen lack became conspicuous; at a "critical oxygen concentration" (between 2 and 1% oxygen) the animals became agitated; they hyperventilated and showed escape movements, which were followed by a loss of body posture and complete immobility within a few minutes.

This behaviour was reflected by a distinct peak in heat flow rate followed by a precipitous decrease in heat flow below the normoxic rate. During graded hypoxia, the heat flow rate approached a new value which was correlated with the degree of hypoxia. Under strict anoxia, 5.3 ± 1.1% and 3.6 ± 1.8% of the normoxic heat flow rates were reached by *Locusta* and *Manduca*, respectively. Thus the two insect species reacted similarly with respect to behaviour and metabolic rate (as indicated by heat flow) to both graded hypoxia and anoxia. A striking difference between the species was seen, however, in the rate of heat flow during recovery from hypoxia or anoxia. Readmittance of air after an anoxic or hypoxic interval led to a rapid increase in heat flow above the normoxic rate, but the amount of "excess heat

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production" during recovery was much higher in *Locusta* than in *Manduca*. The difference in the amount of "excess heat production" during posthypoxic recovery could not be accounted for by differences in behaviour (muscular activity) or by the effect of ambient temperature.

Keywords: Anoxia; Hypoxia; *Locusta*; *Manduca*; Microcalorimetry

1. Introduction

Insects are a highly successful class of animals. Having conquered all land habitats, insects are only rivalled in their dominating role in the biosphere by higher vertebrates, birds and mammals. The insects' success is based on several aspects of their biology, not least their remarkable physiological capacities (for a review see [1,2]). Insect flight is a case in point because it requires most acute senses, rapid information processing, and strong and fast muscles that are fuelled by means of a precisely regulated and very active catabolism of substrates. Owing to their very efficient respiratory system (oxygen is conveyed in gaseous form directly to the tissues via an elaborate system of air-filled tubes, the tracheae), energy metabolism during flight is based entirely on aerobic pathways. Even during maximum power output, such as in flight at maximum speed, ATP is produced aerobically. In fact, flying insects can sustain aerobically the highest metabolic rates encountered in the animal kingdom (for reviews see [1,3,4]). Metabolic rates at rest (standard metabolic rates) are also relatively high in insects.

Insects thus show a type of metabolism that would commonly be regarded as being incompatible with a high tolerance of anoxia, i.e. the capacity to survive an extended interval of oxygen lack. This hypothesis holds good for higher vertebrates, because they have high metabolic rates and have been found unable to recover if subjected to anoxia for more than a few minutes (5–8 min in man, even less in small mammals or birds). Organs that are particularly dependent on aerobic ATP production, such as the brain and heart, have proved very vulnerable to hypoxia/anoxia (for reviews and references see [5,6]).

The situation is strikingly different in adult insects, even though they are specialized in aerobic ATP production, have high standard metabolic rates and can sustain higher metabolic rates during work (e.g. flight) than higher vertebrates (for review and references see [1,7,8]). Yet all insects studied in this respect are much more tolerant to anoxia than higher vertebrates, although different insect species may differ markedly in their degree of tolerance to anoxia (for review see [9]). The migratory locust *Locusta migratoria*, for instance, can survive a maximum of 4 h of anoxia (at room temperature), whereas hawk moths (*Manduca sexta*) will recover from more than 24 h of anoxia. A comparison of these two species might therefore shed light on those metabolic and physiological properties that confer upon animals a high tolerance of anoxia.

In animals tolerant of anoxia, a marked depression of metabolism (i.e. decrease in metabolic rate) has been observed during anoxic intervals. It has been proposed that this capacity is crucial for the survival of extended periods of hypoxia/anoxia (for review see [2,6,7,10]). In order to investigate whether this correlation is valid also in insects, we have studied the effect of graded hypoxia (down to 0.2% oxygen) and of strict anoxia on the rate of heat flow as an index of metabolic rate. Direct calorimetry is the most convenient and accurate method for estimating metabolic rate when oxygen is a limiting factor. The effect of ambient temperature on the rate of heat flow was also studied during normoxia and anoxia.

2. Materials and methods

2.1. Animals

Males of the migratory locust *Locusta migratoria* (Saltatoria, Acrididae) 20–25 days after their final ecdysis were used throughout the study. The animals were reared in the department at ≈ 30°C with a photoperiod of 16 hours light : 8 hours dark. They were fed on fresh grass and bamboo leaves, supplemented with bran and dry dog food (for references see [11]).

The hawk moths *Manduca sexta* (Lepidoptera, Sphingidae) were reared from eggs on an artificial diet (for references see [12]). After eclosion from the pupa, the moths were kept in cages at ≈ 25°C (16 h light : 8 h dark) where they could fly and mate. Adult moths have ample fuel stores, accumulated during the larval stage. Under field conditions they would feed very little, so they were not provided with food or water during their 7–8 day adult period. One day old female moths were used in the experiments.

2.2. Microcalorimetry

A Calvet MS 80 twin calorimeter from Setaram (Lyon, France), equipped with circulation cells of 100 ml volume, was used. The calorimeter was calibrated by means of "Joule cells", and the sensitivity was $\approx 53\ \mu\mathrm{V\ mW}^{-1}$. In order to maintain well defined conditions in the calorimeter cells, a flow of gas was passed through the cells at a constant rate of $500 \pm 5\ \mathrm{ml\ h}^{-1}$ per cell. The gas flow was maintained using concentric tubes entering the calorimeter cell through its lid. The inner tube (for the incoming gas) protruded into the lower third of the cell and the gas left through the outer tube at the top of the cell. The gas flow rate was continuously measured using flow meters of high precision (MF-200 from Setaram) and, if necessary, the rate was readjusted by means of microvalves (Millimite 1315G6Y/MM, Hoke, MA, USA).

Normoxic conditions were obtained using synthetic air (Messer Griesheim, Frankfurt/Main), a mixture of 20% oxygen and 80% nitrogen. Hypoxia was produced by mixing pure nitrogen and synthetic air by means of gas mixing pumps (Digamix SA18 and SA27, Wösthoff, Bochum). In order to prevent evaporation from the experimental animals, the gas was moistened before it entered the calorimeter by passing it through gas washing bottles kept in a water bath set at the same temperature as the calorimeter (Thermomix 1441, Braun, Melsungen).

The calorimeter signals were recorded and processed by a computer using software provided by Rosenberg Elektronik (Nieder-Olm) and Multigraf from Weka-Software (Frankfurt/Main).

2.3. *Experiments*

Experiments were performed on single insects. An insect was weighed and placed head first into one cell (experimental cell) of the calorimeter, while the other cell served as a reference. At the bottom of the cell was a plastic grid to which the insect could hold on with its forelegs while the hindlegs, abdomen and wings touched the walls of the cell. Transparent plastic cells of identical proportions were used in parallel experiments to observe the behavioural responses during hypoxia/anoxia and during posthypoxic recovery.

3. Results

3.1. *Behavioural effects of graded hypoxia*

The insects were subjected to graded hypoxia in transparent cells to observe their behaviour during hypoxia and posthypoxic recovery. Behaviour was found to be very similar in both species. Lowering the oxygen level to 2% did not noticeably affect the insects. Both locusts and moths kept quiet, maintained their body posture in the cells but were still able to move. When we decreased the oxygen content in the cells further by passing through the calorimeter a stream of gas containing 1% of oxygen, the insects remained unaffected for some time but then became agitated; they showed hyperventilation (abdominal pumping) followed by escape movements, and a few minutes later lost their mobility and remained motionless. These effects were very similar to those observed on exposure to anoxia. Thus, between 2 and 1% of oxygen a "critical concentration" is reached, at which body coordination and physiological functions are rapidly lost.

3.2. *Heat flow rates during graded hypoxia*

The insects were subjected to graded hypoxia as described in the Methods section and the rate of heat flow was monitored calorimetrically. No significant change in heat flow was observed when the oxygen concentration was lowered to 2%. However, passing gas that contained 1% or less of oxygen through the calorimeter had a conspicuous effect. When a critical oxygen concentration below 2% was reached, a sudden increase in heat flow rate was observed, which was followed by a precipitous drop to a level below the normoxic rate (see Figs. 1-3). This peak in heat flow rate coincided with the brief period of agitation, and obviously reflects the concomitant muscular activity (see above). At 1% oxygen the heat flow rate was reduced to 59% of the normoxic value in *Locusta* and to 66% in *Manduca*. Heat flow rates were further reduced as the degree of hypoxia increased, reaching in both

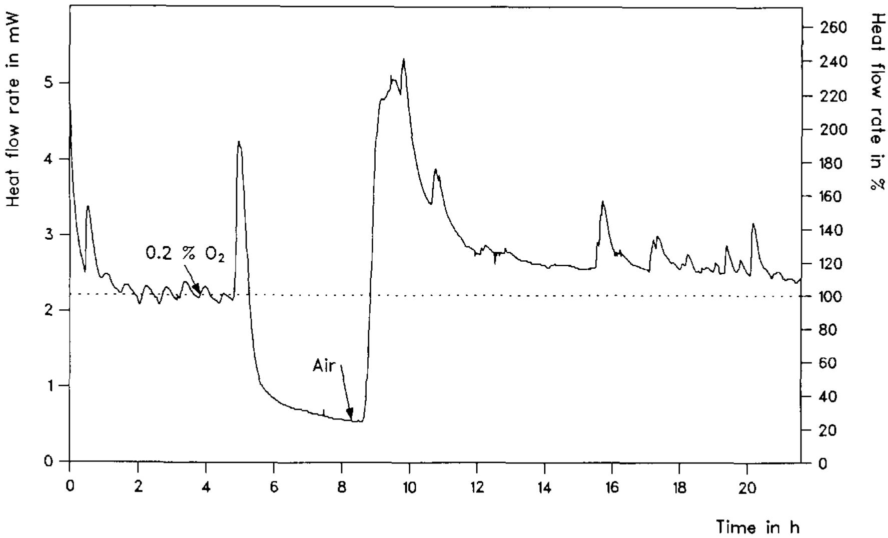

Fig. 1. Heat flow rate of a locust (1.40 g body weight) during normoxia, hypoxia and posthypoxic recovery at 20.3°C. Hypoxia was brought about by passing a stream of gas containing 0.2% oxygen through the calorimeter (start indicated by an arrow). The gas was produced by mixing "artificial air" and pure nitrogen gas. Gas flow rate through each calorimeter cell (of 100 ml volume) was 500 ml h^{-1}. After 4.5 h, the gas flow was changed back to artificial air. At a "critical oxygen content" the insect would get agitated (see text); its hyperventilation and escape movements produced a peak in heat flow, which was followed by a precipitous decrease to below 25% of the normoxic value. On readmission of air the heat flow rate increased rapidly to a rate more than twice the normoxic rate. The posthypoxic heat flow rate was maintained at this peak value for ≈ 1 h, then declined, but did not reach the normoxic rate during the following 12 h of the experiment.

insects 20–25% of the respective normoxic value. Thus, also with respect to heat flow rate, the response to graded hypoxia seemed not to differ significantly in the two species. Two typical experiments with graded hypoxia are given in Figs. 1 and 2.

3.3. *Heat flow rates during recovery from graded hypoxia*

Readmittance of air after graded hypoxia brought about a rapid increase in heat flow rate in both insect species. However, the magnitude of the increase during the posthypoxic period in relation to the normoxic rate was strikingly different in locusts and moths. The data shown in Figs. 1 and 2 are characteristic of these differences. After a hypoxic period brought about by passing 0.2% oxygen through the calorimeter for 4.5 h, a locust produced heat initially at a rate more than twice the normoxic rate. This rate decreased with time, but did not return to the normoxic rate during a 12 h period.

In contrast, *Manduca* returned to the normoxic rate of heat production within 2 h after air had been readmitted. Only a small and transient increase in heat flow rate above the normoxic rate was observed during posthypoxic recovery; the peak value was 30–40% above the normoxic rate.

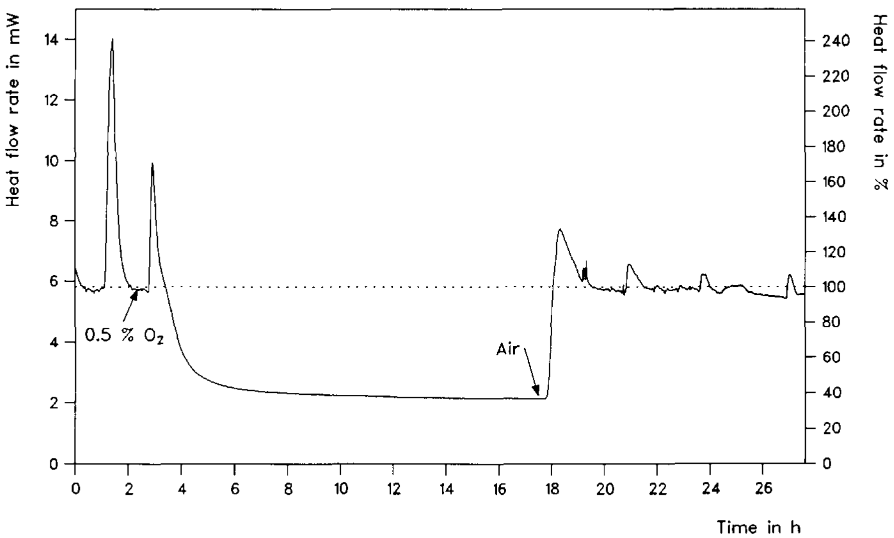

Fig. 2. Heat flow rate of a hawk moth (1.82 g body weight) during normoxia, hypoxia and posthypoxic recovery at 20.3°C. Hypoxia was brought about by passing a stream of gas containing 0.5% oxygen through the calorimeter cells. After 15 h the gas flow was changed to air again. The peak in heat flow during normoxia is due to movements of the moth, and the peak after the gas mixture was changed reflects the hypoxic "escape response" (see Fig. 1 and text). During hypoxia the heat flow rate reached $\approx 40\%$ of the normoxic value. Changing the gas flow back to artificial air resulted in a rapid increase in heat flow, but the rate was only slightly and transiently increased above the normoxic value (for details see text).

Although the *Manduca* were subjected to hypoxia for a much longer period (15 h) than the *Locusta* (4.5 h), their "excess heat production" during posthypoxic recovery was much smaller than that for *Locusta*. The differences between the species were consistently observed in graded hypoxia, and are hence characteristic not of the degree of hypoxia but of the species. The larger amount of excess heat produced by *Locusta* during recovery cannot be accounted for by a higher degree of muscular activity. After an anoxic or hypoxic interval both locust and moth remained motionless during the first phase of recovery ($\approx$ 30 min after anoxia or severe hypoxia) when the difference in heat production was largest. Muscular activity is supposed to produce distinct peaks of activity, as can be seen during normoxia and in the later phases of recovery (see Figs. 1–3).

3.4. *Effects of anoxia at different temperatures*

Locusta and *Manduca* responded similarly when subjected to oxygen lack, but they differed markedly during posthypoxic recovery. The locust produced much more "excess heat" than the moth. This pattern was the same irrespective of whether the insects were subjected to graded hypoxia (see above) or strict anoxia (see [8]). These experiments were performed at 20.3°C, which was significantly

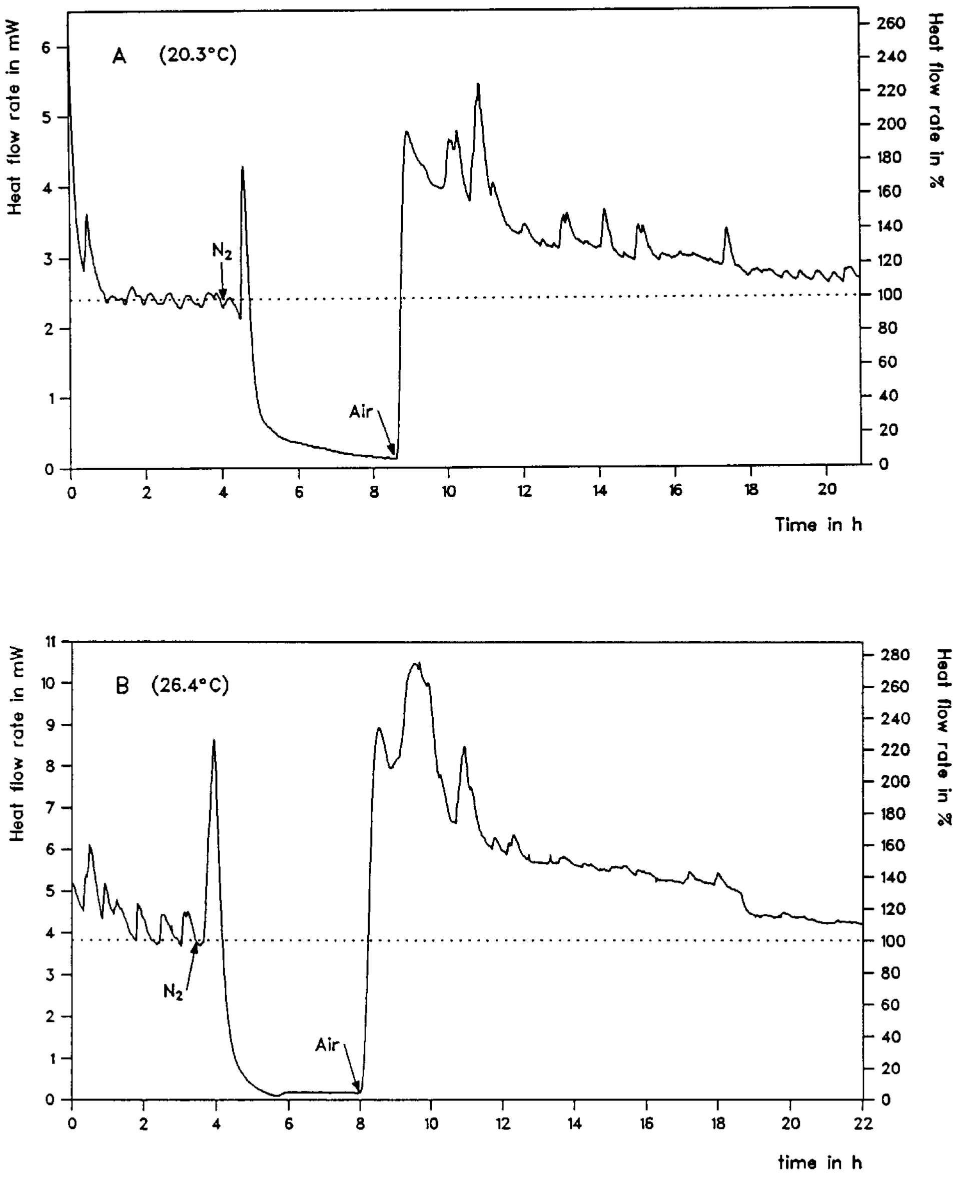

Fig. 3. Heat flow rate of locusts during normoxia, hypoxia, anoxia and postanoxic recovery at 20.3°C (A) and 26.4°C (B). Anoxia was brought about by passing a stream of pure nitrogen through the calorimeter cells (start indicated by an arrow) for 4.5 h, then the gas passing through the cells was changed back to "artificial air". At 26.4°C the anoxia-induced decrease in heat flow rate was steeper than at 20.3°C. The production of "excess heat" during postanoxic recovery at 26.4°C appears more pronounced than at 20.3°C (for details see text). Body weight of the locusts was 1.50 g in (A) and 1.44 g in (B).

lower than the temperature at which the insects were reared. In order to find out what effect temperature had on heat flow during hypoxia/anoxia and on postanoxic recovery, the experiments were repeated at 26.4°C.

Under normoxic conditions both insects showed a higher degree of spontaneous movement and a higher standard metabolic rate at 26.4°C compared with 20.3°C. Although the response to the passage of pure nitrogen instead of air through the calorimeter was similar at both temperatures, the decrease in heat flow rate at the higher temperature was slightly steeper in *Manduca* and significantly steeper in *Locusta*. The minimum heat flow rates (relative to the normoxic rates) were not significantly different at the two temperatures; at 26.4°C they were $(5.2 \pm 3.1)\%$ $(n = 6)$ in *Locusta* and $(4.4 \pm 1.5)\%$ $(n = 5)$ in *Manduca*.

The "excess heat" produced during postanoxic recovery in *Locusta* was even more marked at 26.4°C than at 20.3°C (a typical experiment is shown in Fig. 3), whereas only a small amount of "excess heat" was observed at both temperatures in *Manduca* (data not shown). The pronounced differences in the amount of "excess heat production" between *Locusta* and *Manduca* were similar at the two ambient temperatures.

4. Discussion

Organisms use ATP as a source of free energy to drive physiological processes. For the synthesis of ATP, oxygen is required by most animals. One reason for this is that aerobic metabolism, i.e. the oxidation of substrates with oxygen as final electron acceptor, is much more efficient in terms of ATP production than anaerobic metabolism. If oxygen is wanting, vertebrates and insects are left with glycolysis as virtually the only pathway for synthesis of ATP (for review see [7,10,13]), but glycolysis yields only 2 moles of ATP per mole of glucose compared with 36–38 moles if oxygen can be utilized. It is therefore no surprise to find highly mobile and active animals particularly dependent on an adequate supply of oxygen. Adult insects and higher vertebrates are in this category, and their response to lack of oxygen is dramatic. Breathing pure nitrogen instead of air will result in rapid loss of physiological functions in insects and higher vertebrates (for review and references see [5,7,9,14]).

The situation is different with respect to graded hypoxia. In this study both insect species appeared unaffected by an atmosphere containing as little as 2% of oxygen, a degree of hypoxia that humans could survive for no more than a few minutes. The capacity to make use of relatively small concentrations of oxygen has been reported for other insects such as cockroaches [15] and hornets [16]; it demonstrates the great efficiency of the respiratory system in insects.

Even more impressive is the ability of insects to recover completely from prolonged periods of anoxia or severe hypoxia. All insects appear much more tolerant of anoxia than mammals, yet different insect species vary greatly in this respect (for review see [9]). It is not known which properties of cell structure or metabolism are decisive for the capacity of animals to recover from prolonged anoxia. Animals tolerant of anoxia have been observed to suspend physiological functions during periods of anoxia, thus decreasing their metabolic rate, with the consequences that energy expenditure is reduced and the accumulation of toxic anaerobic end products is limited.

The two insects studied in this work differ markedly in their tolerance of anoxia, yet the effects of hypoxia/anoxia on metabolic rate were similar in both species. Effects of ambient temperature could also not account for the different degrees of anoxia tolerance.

The most striking differences between *Locusta* and *Manduca* were seen during posthypoxic or postanoxic recovery. *Manduca*, which is much more tolerant to oxygen lack than *Locusta*, produced significantly less "excess heat". There is no indication that this difference could be due to different degrees of muscular activity, as both insects remained immobile during the initial phase of postanoxic recovery when the differences in heat production in the two species were most conspicuous. The data suggest energy metabolism in *Locusta* to be less efficient during postanoxic recovery than it is in *Manduca*. The mechanisms causing this effect are unknown and the object of further studies.

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