

PROLINE POWERS PRE-FLIGHT WARM-UP IN THE AFRICAN FRUIT BEETLE *PACHNODA SINUATA* (CETONIINAE)

LUTZ AUERSWALD, PETER SCHNEIDER* AND GERD GÄDE†

Zoology Department, University of Cape Town, Rondebosch 7701, South Africa

*Present address: Zoologie III, Universität Heidelberg, Im Neuenheimer Feld 504, D-69120 Heidelberg, Germany

†Author for correspondence (e-mail: ggade@botzoo.uct.ac.za)

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Summary

We investigated thoracic temperatures (T_{th}) during different activities of the endothermic fruit beetle *Pachnoda sinuata* and analysed which energy substrates are used for the pre-flight warm-up of its flight muscles.

Pachnoda sinuata elevates its T_{th} prior to take-off either by basking in the sun or by warming endothermically to a narrow range around 34 °C. During lift-generating tethered flight at low ambient temperatures ($T_a=25$ °C), T_{th} of *P. sinuata* decreases steadily until it reaches 28 °C, which is not sufficiently high to sustain flight. T_{th} remains stable during lift-generating tethered flight at high T_a (31 °C). Wingbeat frequency (f_w) is dependent on T_{th} : when T_{th} declines, f_w decreases in a linear manner.

The proline concentrations in the haemolymph and flight muscles decrease during warm-up. In contrast, the carbohydrate levels in the haemolymph and flight muscles are not affected by the warm-up process, while the

glycogen level of the flight muscles declines significantly during the first 10 s of lift-generating tethered flight. This suggests that the energy for endothermic warm-up is produced solely by the oxidation of proline.

Measurements of the respiratory quotient (RQ) confirmed that *P. sinuata* uses a combination of carbohydrates and proline to power its flight. At rest and during lift-generating tethered flight, the RQ is approximately 0.9. During warm-up, the RQ is significantly lower at 0.82, which is close to the theoretical value of 0.8 for the partial oxidation of proline. The rate of oxygen consumption during endothermic warm-up is 45 % of that during lift-generating tethered flight

Key words: insect flight, energy metabolism, proline, pre-flight warm-up, respiratory quotient, metabolite, African fruit beetle, *Pachnoda sinuata*.

Introduction

Many insects require thoracic temperatures (T_{th}) to fly that are well above ambient temperature (T_a) and, therefore, need to raise their body temperature prior to flight either passively by basking in the sun or by endothermic warm-up (see Heinrich, 1993). Large beetles are able to elevate their T_{th} endothermically. For example, in dung beetles of different genera, T_{th} can reach 38–42 °C, and this is maintained even during terrestrial activity (Bartholomew and Casey, 1977; Heinrich and Bartholomew, 1979). Male rain beetles of the genus *Plecoma* fly with a T_{th} of approximately 38 °C, even in snowstorms, to find the flightless females (Morgan, 1987). In flower scarabs, such as *Cotinus texana* (Chappell, 1984) and *Pachnoda sinuata* (Heinrich and McClain, 1986), T_{th} is elevated endothermically to more than 35 °C before flight.

In many insects, endothermic heat production is achieved by muscle contraction (shivering) with or without wing beats (see Heinrich, 1993). Other authors suggest that heat can also be generated by non-shivering thermogenesis. This involves substrate cycling of, for example, fructose 6-phosphate and fructose 1,6-diphosphate by the enzymes

phosphofructokinase and fructose diphosphatase (Newsholme *et al.* 1972).

In *Manduca sexta*, it was demonstrated that carbohydrates are used to power pre-flight warm-up (Joos, 1987). Otherwise, little is known about the fuels involved in generating heat during this process. We therefore wanted to investigate this problem for the African fruit beetle *Pachnoda sinuata*, which uses proline and carbohydrates to power flight (Zebe and Gäde, 1993; Lopata and Gäde, 1994) and elevates its T_{th} endothermically prior to flight (Heinrich and McClain, 1986). Moreover, we also investigated how T_{th} influences wingbeat frequency as a parameter of flight performance.

Materials and methods

Insects

Male fruit beetles of the species *Pachnoda sinuata flaviventris* (Gory and Percheron) were caught in the vicinity of Cape Town, South Africa, and were maintained as outlined previously (Zebe and Gäde, 1993). Animals in all experiments had a body mass of 1 ± 0.18 g.

Flight and warm-up experiments

Beetles were kept in a transparent plastic container prior to experimentation. Only beetles which were absolutely quiescent in the container, i.e. did not walk around or open the elytra, were used. Experiments were performed at 24–25 °C in a laboratory close to a window which was exposed to bright sunlight or at 31 °C in a constant-temperature room. The beetle was attached *via* the prothorax to a bent insect pin using dental wax. The pin (mass 180 mg) could move inside a piece of metal tube (diameter 2 mm) in such a way that it was possible to detect whether the animal produced lift as during natural flight (Fig. 1). This apparatus was held in a wooden clamp mounted on a stand. When the top of the insect pin moved up and down, the insect was considered to be producing lift. In addition, this method allowed the insect to turn on its vertical and horizontal axes (Fig. 1). A frontal laminar air current of 1.6 m s^{-1} was created by placing a fan (27.5 W) at one end of a rectangular metal tunnel (12 cm×12 cm×50 cm). Air was forced through a 1 cm thick grid made up of 64 squares (13 mm×13 mm) which was placed inside the tunnel 10 cm from the opening opposite to the fan. Laminar flow was confirmed by passing smoke through the system.

After the beetle had been attached to the pin, a 1 µl haemolymph sample was taken from the intersegmental membrane between the head and prothorax for determination of metabolite concentrations (see below). After attachment to the stand, the beetle was provided with a styrofoam ball for tarsal contact. The beetle remained quiescent until it started to warm up. As judged by T_{th} , which in eight beetles (measured using a thermocouple; see below) rose from 25.9 ± 0.2 °C to 34.5 ± 2.0 °C (mean \pm s.d.) at take-off, the warm-up period took

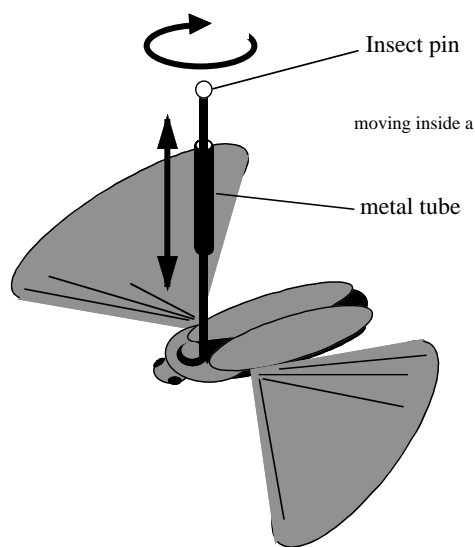


Fig. 1. To detect lift-generating flight, the beetle was attached *via* the prothorax to an insect pin using dental wax. The insect pin was mounted in such a way that it could move inside a piece of metal tube. This allowed the beetle to move on the vertical and horizontal axes as indicated by the arrows. The piece of metal tube was held in a clamp (according to Schneider, 1989).

83 ± 23 s. Most of the beetles had a pre-warm-up T_{th} slightly above T_a , probably as a result of slow movement prior to experimentation. In most of the beetles, the beginning of warm-up was indicated by pumping movements of the abdomen, after which the head also started to move back and forth in rhythm with the abdomen. Thereafter, the beetle started to walk on the styrofoam ball for a few seconds. When it tried to open the elytra to take off, the beetle plus attached pin was taken off the clamp, a 1 µl haemolymph sample was taken and the beetle was replaced. This procedure took no longer than 15–20 s. The beetle was then allowed to fly while producing lift for 10 s. Animals that did not start flying voluntarily or flew without producing lift were excluded from the experiment. A third 1 µl haemolymph sample was taken immediately after the flight period. For metabolite determination in the flight muscles, resting beetles, beetles that had completed warm-up and those that had warmed up and flown for 10 s were dissected. The head, prothorax, legs and wings were cut off, and the gut was subsequently removed. The flight muscle tissue was wrapped in aluminium foil and immediately frozen in liquid nitrogen. The whole procedure took no more than 1 min. Preparation of samples and metabolite determinations were performed as described below.

Haemolymph

Haemolymph (1 µl) was either blown immediately into 100 µl of concentrated H_2SO_4 for the determination of total lipid concentration (Zöllner and Kirsch, 1962) or carbohydrate levels (Spik and Montreuil, 1964), or pipetted into 60 µl of 80 % acetonitrile for amino acid analysis (see below).

Tissue samples

Perchloric acid extracts from frozen tissues were prepared according to Zebe and Gäde (1993).

Glycogen levels

Glycogen was extracted as described in Zebe and Gäde (1993) and analysed using the modified anthrone method (Spik and Montreuil, 1964) with glucose as a standard.

Proline and alanine levels

Derivatisation of extracts with dansyl chloride and determination of proline and alanine levels by high-performance liquid chromatography (HPLC) were carried out as described previously (Zebe and Gäde, 1993).

Wingbeat frequency and thoracic temperature

The wingbeat frequency (f_w) during flight was determined using an infrared light barrier which was placed at 90° to the plane of the wing beat. The set-up for flight was the same as described above except that the insect pin was in a fixed position, so that the beetle could not generate lift. The ensuing impulses were recorded using an electronic counter and read every 20 s. In the same experiment, T_{th} was determined by introducing a copper–constantan thermocouple (accuracy 0.1 °C, mounted in a syringe needle) ventrally through a pre-

drilled hole into the metathorax and the temperature was read at 20 s intervals. Measurements of f_w and T_{th} continued for 6 min. During measurements of T_{th} in the field, made at a farm 30 km northeast of Cape Town, South Africa, the thermocouple was inserted into the flight muscles immediately after capture. T_a was measured in the shade using the same thermocouple.

Determination of respiratory quotient

An open flow-through respirometry system was used. Changes in the oxygen concentration in the system were determined using an S-3A analyser (Applied Electrochemistry Inc., Sunnyvale, CA, USA) fitted with an N-22M oxygen sensor and an R-1 flow control. Changes in CO_2 concentration were measured using a 225 MK3 infrared gas analyser (ADC, UK). The flow rate of the whole system was 250 ml min^{-1} for resting animals, 500 ml min^{-1} for warm-up and 700 ml min^{-1} for lift-generating tethered flight. The chambers had the following volumes: rest, 10 ml; warm-up, 50 ml; lift-generating tethered flight, 144 ml. Thus, the chamber air was renewed in 2.4, 6.0 or 12.3 s, respectively. These small chamber volumes and the high flow rates ensured a quick response of the instruments so that corrections for gas mixing could be omitted. Flow rate was measured using a bubble flowmeter before and immediately after each experiment. The oxygen analyser was connected downstream from the pump, followed by the CO_2 analyser. Air was drawn through the system. Before air reached the chamber, it flowed through a container filled with Carbosorb (BDH), to remove atmospheric CO_2 , and was dried by means of a plastic syringe filled with silica gel. A narrow plastic pipe, 10 cm long and containing silica gel, was attached behind the chamber so that moisture generated by the beetles could be removed. The oxygen analyser was calibrated with atmospheric air, and the zero value was checked regularly with an oxygen-free gas mixture (CO_2+N_2). The carbon dioxide analyser was set to zero by filling the reference cell with CO_2 -free air or 350, 750 or 1450 p.p.m. CO_2 (in N_2 , oxygen-free) and calibrated with 350, 750, 1450 or 2100 p.p.m. CO_2 (in N_2 , oxygen-free), respectively. All gas mixtures were from Afrox (R.S.A). Oxygen and carbon dioxide content were measured to the nearest 0.001 %. The delay between the two analysers was 5–10 s.

Measurements were taken only after the system had equilibrated following insertion of an animal into the chamber. Data from animals that became active immediately upon being placed in the chamber were discarded. The incoming air current in the chamber was directed towards the beetle's head. For measurement of the resting value, eight animals were used at a time, while single animals were investigated in all other experiments. Prior to flight, they were kept in the chamber in the dark. Body masses were determined immediately after each measurement. These data, together with the measured flow rate, were used to calculate rates of oxygen consumption per gram per hour at standard temperature and pressure (STP).

For separate measurements of rates of oxygen consumption, the carbon dioxide analyser was omitted and the flow rate was set to 250 ml min^{-1} .

All respiration experiments were performed in a controlled-temperature room at 28°C .

Statistical analyses

All data are presented as means \pm S.D. Significance levels of differences were determined using Student's *t*-tests or, where appropriate, paired *t*-tests.

Results

Body temperature and tethered flight performance

Wingbeat frequency f_w was measured at T_a values of 25 and 31°C . The highest wingbeat frequencies were recorded at 25°C immediately after the onset of tethered flight; after 20 s, f_w decreased significantly with decreasing T_{th} (Fig. 2A). At $T_a=31^\circ\text{C}$, f_w and T_{th} remained constant during the entire 6 min of flight and no significant relationship was found between them (Fig. 2B). Because smaller beetles have a relatively larger heat loss than larger individuals (Bartholomew and Heinrich, 1978), data from two beetles of the same body mass are shown in Fig. 3. At a T_a of 25°C , the beetle warmed up to 37°C and started flying voluntarily. During the first minute of flight, T_{th} did not increase further, but instead began to decrease (Fig. 3B). Although a T_{th} of 31°C is sufficiently high for take-off, at a T_a of 31°C the beetle also warmed up to approximately 36°C at take-off (Fig. 3B). Therefore, the flight temperature for *P. sinuata* at which heat loss and heat production are approximately equal should be between the values of T_a investigated.

The T_{th} of *P. sinuata* was measured in the field and in the laboratory during different types of activity. Beetles resting in the shade (underneath leaves) had a T_{th} of $24.6\pm0.9^\circ\text{C}$ ($N=5$), close to T_a (25°C), while T_{th} of beetles resting in bright sunlight was several degrees higher at $31.1\pm1.6^\circ\text{C}$ ($N=6$). The latter beetles were able to take off for flight without any visible warm-up or preparation such as those in the cockchafer *Melolontha melolontha* (Schneider, 1980). T_{th} was $33.8\pm1.9^\circ\text{C}$ ($N=6$) in beetles after endothermic warm-up (tethered) in the laboratory in conditions similar to those in the field ($T_a=25^\circ\text{C}$; exposed to sunlight). Beetles that were walking slowly (in the field, not exposed to bright sunlight, $T_a=25^\circ\text{C}$) had a lower T_{th} of $27.4\pm1.5^\circ\text{C}$ ($N=5$). Some beetles which were resting on leaves or flowers (in the field, not exposed to direct sunlight, $T_a=25^\circ\text{C}$) were forced to fly without allowing them to warm up by dropping them from a height of 1 m. For those that opened their wings and tried to fly, T_{th} was $27.9\pm1.5^\circ\text{C}$ ($N=7$). We also measured T_{th} of beetles which had just arrived at a plant after a flight of unknown duration. We either caught the individuals in the air before landing or picked them from the plant within a few seconds after landing. For these beetles, T_{th} was $32.1\pm1.1^\circ\text{C}$ ($N=7$). Our results indicate that a T_{th} below approximately 28°C is not sufficient for flight in *P. sinuata*.

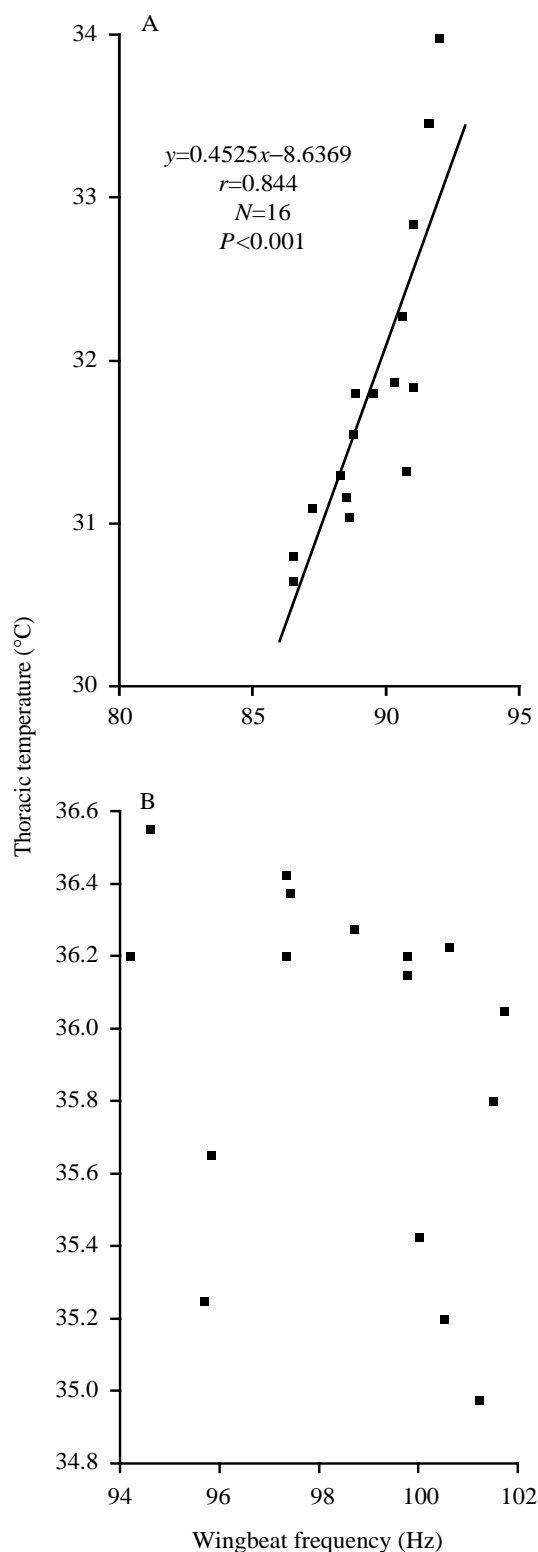


Fig. 2. Relationship between thoracic temperature and wingbeat frequency of *Pachnoda sinuata*, flying at an ambient temperature of 25 °C (A) and 31 °C (B). Data points are means of 3–6 individuals. The correlation was calculated using least-squares regression.

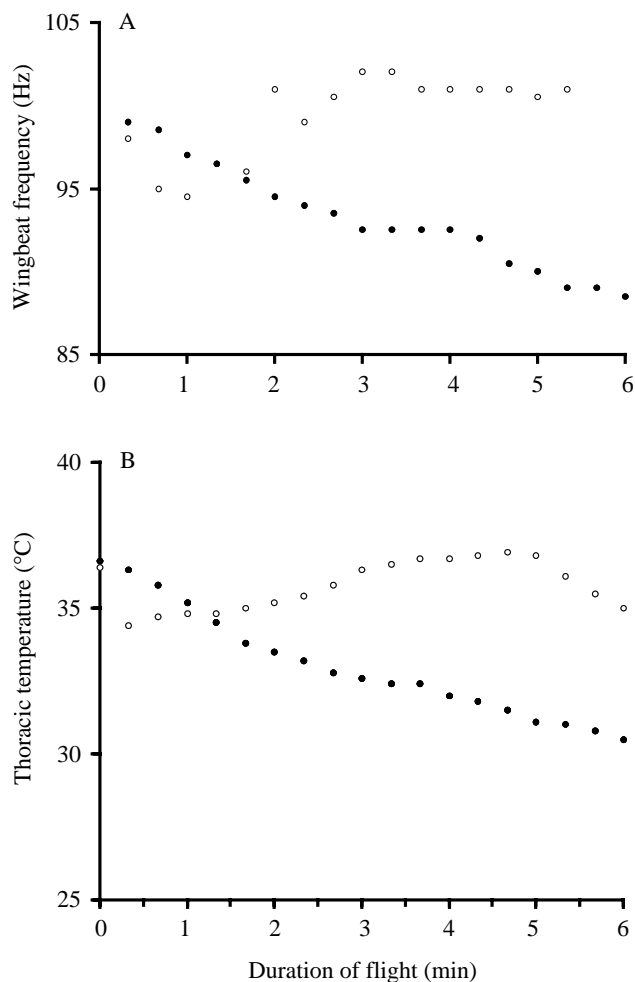


Fig. 3. Wingbeat frequency (A) and body temperature (B) during tethered flight of *Pachnoda sinuata* at ambient temperatures of 25 °C (filled circles) and 31 °C (open circles). The figure shows results for a different representative individual (body mass 1 g) for each ambient temperature.

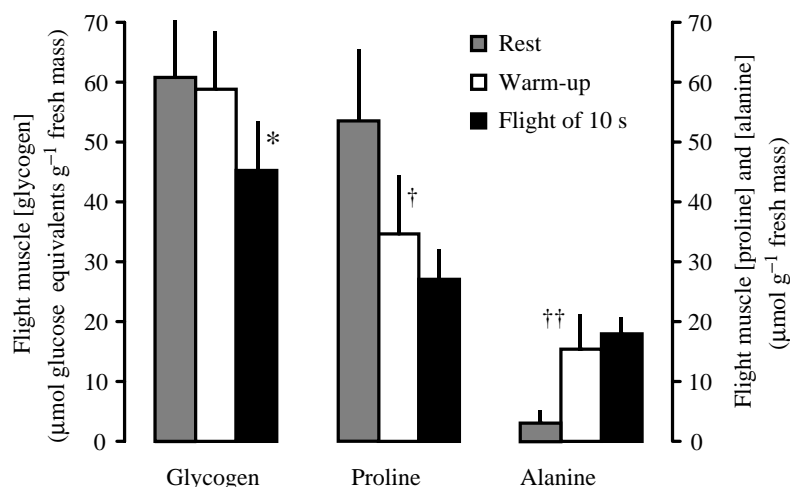
Metabolic fuels during warm-up

As can be seen from Fig. 4, the proline concentration in the flight muscles decreased significantly during warm-up from 53.5 ± 12.1 to $34.7 \pm 9.2 \mu\text{mol g}^{-1}$ fresh mass and remained at this level ($27.1 \pm 4.9 \mu\text{mol g}^{-1}$) during 10 s of lift-generating tethered flight. The alanine concentration in the flight muscles, however, increased significantly from 3.1 ± 1.8 to $15.4 \pm 5.6 \mu\text{mol g}^{-1}$ during warm-up and remained at this level ($18.0 \pm 2.6 \mu\text{mol g}^{-1}$) during 10 s of flight.

The glycogen concentration in the flight muscles was not affected by warm-up, remaining at $58.8 \pm 9.3 \mu\text{mol glucose equivalents g}^{-1}$ compared with $60.8 \pm 9.1 \mu\text{mol g}^{-1}$ at rest. The glycogen level decreased significantly, however, during the following 10 s of flight to $45.3 \pm 8.1 \mu\text{mol glucose equivalents g}^{-1}$ (Fig. 4).

Haemolymph samples were taken at rest, after warm-up and after 10 s of subsequent tethered flight from ten beetles for

Fig. 4. Concentrations of glycogen, proline and alanine expressed per gram fresh mass in flight muscle tissue of *Pachnoda sinuata* at rest, after warm-up and after 10 s of tethered flight following warm-up. Values are means + s.d. ($N=6$). Significance levels of warm-up value compared with resting value, $\dagger P<0.02$, $\dagger\dagger P<0.001$; flight value compared with warm-up value, $*P<0.05$ (Student's t -test).



determination of haemolymph proline, alanine, carbohydrate and lipid concentrations. Although the variability of the resting values was high, there was a significant reduction in proline concentration during warm-up; there was a tendency for proline levels to decrease during the subsequent 10 s of tethered flight, although this was not significant. All individuals showed a significant increase in alanine levels during warm-up and a further significant increase during 10 s of tethered flight (Fig. 5).

Concentrations of total carbohydrates in the haemolymph did not change significantly during warm-up and the subsequent 10 s of tethered flight. Total carbohydrate concentration for 24 beetles was 13.0 ± 5.4 , 13.5 ± 5.6 and 14.3 ± 5.3 mg ml^{-1} , respectively. Haemolymph lipid levels did not change either, staying at approximately 10 mg ml^{-1} .

Metabolite stores in the fat body are not mobilised during warm-up (Heinrich and McClain, 1986); therefore, we did not measure the consumption of metabolites in this tissue during warm-up.

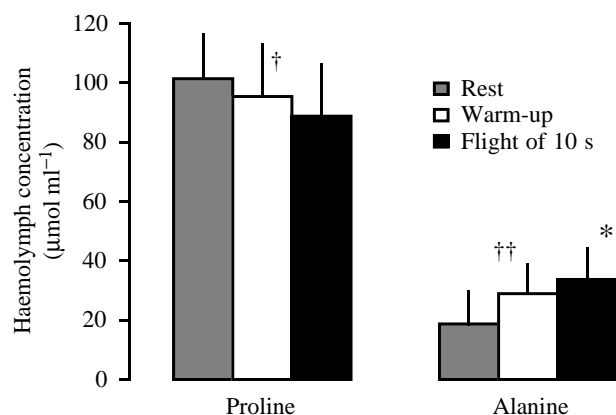


Fig. 5. Concentrations of proline and alanine in the haemolymph of *Pachnoda sinuata* during rest, warm-up and 10 s of lift-generating flight. Values are means + s.d., $N=10$. Significance levels of warm-up value compared with resting value, $\dagger P<0.05$, $\dagger\dagger P<0.001$; flight value compared with warm-up value, $*P<0.005$ (paired t -test).

Oxygen consumption

The rate of oxygen consumption of resting *P. sinuata* was 1.3 ± 0.4 $\text{ml g}^{-1} \text{h}^{-1}$ ($N=8$), while during warm-up a maximum value of 46.8 ± 7.0 $\text{ml g}^{-1} \text{h}^{-1}$ ($N=8$) was measured. Oxygen consumption rates were highest after 2 min of lift-generating tethered flight at 104.4 ± 7.9 $\text{ml g}^{-1} \text{h}^{-1}$ ($N=8$).

Respiratory quotient

The resting RQ of *P. sinuata* was 0.89 ± 0.05 ($N=10$), and similar values were measured during lift-generating tethered flight lasting 2 min (0.90 ± 0.01 ; $N=6$). In contrast, a significantly ($P<0.01$) lower value of 0.82 ± 0.05 ($N=10$) was determined during pre-flight warm-up.

Discussion

In the present study, we have found that the African fruit beetle *Pachnoda sinuata* starts flight activity voluntarily when a T_{th} of at least 34°C is reached. It was also found that the beetle stops flying when T_{th} drops below approximately 28°C . Although a T_{th} of 31°C is sufficient for flight, *P. sinuata* elevates its T_{th} a few degrees above this value prior to flight. This is probably a compensatory mechanism to allow for heat loss to occur when T_{a} is low (Figs 2, 3). Like the closely related protea beetle *Trichostetha fascicularis* (Nicolson and Louw, 1980), *P. sinuata* is unable to retain the heat generated during flight at low T_{a} . From our data, we can conclude that the T_{a} at which heat generation and heat loss during flight are equal is approximately 30°C . An elevated T_{th} is necessary to maintain flight performance. As in the cockchafer *Melolontha melolontha* (Schneider, 1980), wingbeat frequency, and thus flight performance, is closely related to T_{th} : with a decrease in T_{th} , wingbeat frequency declines (Figs 2, 3). This is probably a result of the properties of the enzymes involved in muscle contraction. Barnett *et al.* (1975) found temperature optima for *P. sinuata* flight muscle phosphorylase and actomyosin ATPase of approximately 40°C , below which activities declined steeply.

In contrast to certain dung beetles, which maintain a high

body temperature during terrestrial activity (Bartholomew and Casey, 1977; Bartholomew and Heinrich, 1978; Heinrich and Bartholomew, 1979), *P. sinuata* elevates its T_{th} only for flight. Even then, basking is preferred to endothermic warm-up (Heinrich and McClain, 1986). This discrepancy between the potential to elevate T_{th} and the maintained low body temperature during non-flight activity was described as 'laziness' (hypothermia) by the latter authors.

It is likely that the beetles avoid metabolic warm-up in the field when the high energy cost for this process is considered. In our experiments, the maximum rate of oxygen consumption, and thus metabolic rate during warm-up, reaches 45 % of that during flight.

How do the beetles meet this high energy demand? This study provides unequivocal evidence that proline supplies most (if not all) of the energy required for the endothermic elevation of T_{th} during warm-up. While no changes were found in haemolymph lipid and carbohydrate levels, proline concentration decreased sharply and the concentration of alanine, the end product of the partial oxidation of proline, increased concomitantly. In addition, glycogen stores in the flight muscles seemed to be unaffected by warm-up. These data are supported by measurements of RQ. During flight as well as at rest, RQ was approximately 0.9, confirming previous results showing that *P. sinuata* uses a combination of proline and carbohydrates to power its flight (Zebe and Gäde, 1993; Lopata and Gäde, 1994). During warm-up, however, the RQ was significantly lower at 0.82, which is close to the theoretical value of 0.8 calculated for the partial oxidation of proline (see review by Bursell, 1981).

Little is known about metabolic fuels for warm-up in other insects. Joos (1987) found that carbohydrates are the energy source during warm-up in the endothermic sphinx moth *Manduca sexta*, which otherwise uses predominantly lipids during flight (Ziegler and Schulz, 1986). The high metabolic rates observed during endothermic warm-up in the present study support the suggestion of Heinrich and McClain (1986) that endothermic heat production in *P. sinuata* is achieved by flight muscle activity (shivering). The participation of glycolytic substrate cycles, and thus non-shivering thermogenesis, as proposed for *Bombus* spp. (Newsholme *et al.* 1972) and *Acherontia atropos* (Surholt and Newsholme, 1983) seems unlikely. The activities of key enzymes of these cycles in flight muscle tissue of specimens of the genus *Pachnoda* were found to be very low (Mitchell *et al.* 1977; Surholt and Newsholme, 1981).

The importance of proline as a flight substrate in certain insects has been demonstrated previously. Its quantitative role, however, varies quite substantially. In the blowfly *Phormia regina*, for example, proline serves in small amounts as a 'sparker' of the Krebs cycle at take-off (Sacktor and Wormser-Shavit, 1966; Sacktor and Childress, 1967), while it is a major fuel in combination with carbohydrates in several beetle species (see, for example, Weeda *et al.* 1979; Zebe and Gäde, 1993; Auerswald and Gäde, 1995). In the tsetse fly *Glossina morsitans* (see Bursell, 1981) and, more recently, in certain

dung beetles (Gäde, 1997a,b), proline has been shown to be the sole energy source during flight. In the present study, we show that proline also plays a role during endothermic pre-flight warm-up and is probably the only substrate for this process in *P. sinuata*.

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