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Hypermetabolic Conversion of Plant Oil into Water: Endothermic Biochemical Process Stimulated by Juvenile Hormone in the European Firebug, *Pyrrhocoris apterus* L.

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ABSTRACT: The physiological and biochemical mechanisms that enable insects to feed on dry food to secure enough water for larval growth were investigated. The study was carried out with a plethora of physiological methods, ranging from the simple volumetric determination of O_2 consumption and water intake to more advanced methods such as scanning microrespirography and thermovision imaging of insect's body temperature. The experiments were done on the European firebug, *Pyrrhocoris apterus*, which feeds exclusively on dry linden seeds. In order to survive, it needs to drink water or suck a sap from plants occasionally. It was found that the young larval instars compensate the occasional water deficiency by the increased production of metabolic water. The juvenile hormone (JH)-dependent production of metabolic water, which was previously found in other species consuming dry food, was achieved in *P. apterus* by total metabolic combustion of the dietary lipid (neutral seed oil). The water-producing, hypermetabolic larvae were heated from inside by endothermic energy released from the uncoupling of oxidation from oxidative phosphorylation. The "warm", hypermetabolic larvae burning the dietary oil into CO_2 and water showed the increased rates of respiratory metabolism. Microrespirographic recording of these larvae revealed the ratio of the respiratory quotient (RQ, CO_2/O_2) of 0.7, which indicated the breakdown of a pure triglyceride. The warm hypermetabolic larvae could be easily spotted and distinguished from the "cold" larvae on the screen of a thermovision camera. The last instar larvae lacking the JH were always only cold. They metabolized a carbohydrate substrate exclusively (RQ = 1.0), while the dietary lipid was stored in the fat body. In comparison with the hypermetabolic larvae of some other species fed on dry food, which exhibited the highest rates of O_2 consumption ever recorded in a living organism (10–20 mL O_2/g per hour), the metabolic difference between the warm and cold larvae of *P. apterus* was only some 30% (not a reported 10-fold difference), which was presumably due to their ability to drink. We conclude that a very important, though still largely neglected, epigenetic biochemical role of insect JH depends on switchover between the utilization of dietary lipid (+JH; production of metabolic water) and carbohydrate (–JH; lipid storage in the fat body). The hypermetabolic water supply in insects fed on dry food, which is associated with enormous rates of O_2 consumption, liberates endothermic energy that heats the body and potentially influences the insect thermoregulation. A possibility that the JH-dependent lipolytic hormone stimulates the total metabolic breakdown of nutritional lipids may be absolutely different from the currently known adipokinetic peptides that have been emphasized.

KEYWORDS: hypermetabolism, O_2 consumption, respiratory quotient (CO_2/O_2), uncoupling of oxidation, endothermic energy, "warm" and "cold" larvae, juvenile hormone, JH, *Pyrrhocoris apterus*

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Introduction

The effects of hormones on respiratory metabolism of insects were extensively studied some 60 years ago. The most important article on the effects of juvenile hormone (JH) on O_2 consumption was published by Thomsen.[1] She found that allatectomy of female adults of *Calliphora* inhibited ovarian growth and decreased the rate of O_2 consumption. Conversely, the reimplantation of active corpora allata stimulated ovarian growth and increased the respiratory metabolism. These effects of JH were confirmed in adult females of cockroaches (*Leucophaea*[2]), locusts (*Locusta*[3]), and hemipterans (*Pyrrhocoris*[4]). It was generally assumed that JH could have a direct effect on the total body metabolism of insects.[2]

Novák and Novák and Sláma[5–8] investigated at that time the metabolic effects of insect hormones, using a model system represented by a hemipteran insect, the European firebug, *Pyrrhocoris apterus* L. They used the whole arsenal of endocrinological techniques, including allatectomy, removal of corpora cardiaca, removal of neurosecretory cells of the brain, and castration or implantation of active corpora allata. They confirmed the metabolic stimulation by JH in the adult females of *P. apterus*,[4,9] although there was no effect of JH on the metabolism of males.[10] During the larval stage, the metabolic effects of JH were strongly dependent on feeding and food utilization. Developmental and metabolic differences between the presence or absence of JH were manifested

by the appearance of characteristic, larval–larval (+JH) or larval–adult (–JH) metabolic cycles.[7,8,11,12] In all stages, however, the metabolic effects of hormones were dependent on the feedback responses from the ingestion and utilization of food. In larvae and pupae of the greater wax moth, *Galleria mellonella*,[13] endocrinological analysis also confirmed that JH had no effects on metabolism when applied to the nonfeeding, larval or pupal stages. Evidently, the effects of JH on insect metabolism were indirect, being closely dependent on the nutritional feedback.[4,13–15]

The theory of indirect action of JH on metabolism[15] was challenged by Sláma and Hodková[16] when they discovered an enormous, JH-dependent rise in respiratory metabolism (hypermetabolism) in the larvae of a carpet beetle, *Dermestes vulpinus F.* The treated larvae, which had arrested development for several weeks, started to feed voraciously and increased the rates of O_2 consumption and CO_2 output more than 10-fold (from 800 µL of O_2/g per hour to 10,000 or 20,000 µL O_2/g per hour). This large stimulation of respiratory metabolism by JH, which was called hypermetabolism, showed the highest rates of O_2 consumption ever recorded in a living organism.[16]

Further studies on hypermetabolism in *D. vulpinus* confirmed the record rates of respiratory metabolism induced by JH in a larval stage with the completely suspended development.[17] The treated larvae consumed more food (dry calf viscera); however, the hypermetabolic responses appeared only when the food contained a lipid. The selective advantage of hypermetabolism could thus depend on the oxidation of fatty acids to provide water for larval growth. The abnormally high O_2 consumption associated with the liberation of endothermic energy suggested a possibility of uncoupling of oxidation from oxidative phosphorylation.[17] According to Němec,[18] hypermetabolic larvae with uncoupled oxidation contained substantially increased amounts of hemolymph and water.

Retrospective analysis of the abovementioned data[17,18] suggested that the hypermetabolic responses to JH might be observed previously but were overlooked in other insects fed on dry food. We discovered, for instance, that the last instar larvae of *G. mellonella* ingested more food and exhibited the hypermetabolic rates of O_2 consumption (10,000 µL of O_2/g per hour) after implantation of active corpora allata.[13] A recent progress in the study of hypermetabolism was achieved by Sláma and Lukáš,[19] who invented to use a thermovision camera for the identification of the hypermetabolic specimens with increased body temperature. Insects are relatively small poikilothermic animals, and it is commonly believed that the increased temperature of their body should be quickly equilibrated with the environment.[20,21] In the previous study,[19] we found that even a relatively small insect body can stay warmer for several degree Celsius, provided that it is heated from inside by metabolically released endothermic energy.[19] It was reasonably concluded that hypermetabolism may open new avenues into the study of insect thermoregulation.

The abovementioned hypermetabolic responses to JH were obtained with larvae of *D. vulpinus and G. mellonella*, which feed on dry food and do not drink water.[19] In this article, we investigated hypermetabolic responses in an hemipteran insect, *P. apterus*, which feeds on dry linden seeds but differs from the abovementioned hypermetabolic species by its ability to drink water from the environment. The great advantage of *P. apterus* in hormonal research depends on the availability of previously accumulated data.[15] In this study, we took the advantage of the previous data on respiratory metabolism of *P. apterus*[4,7,10,11,15,17,22] and combined these data with the most recent results obtained by means of the advanced techniques of thermovision imaging and scanning microrespirography.[9,19,22–24]

Materials and Methods

Larvae and adults of *P. apterus* L. were reared under the long-day photoperiodic conditions (16-hour light: eight-hour dark) on dry linden seeds, with the cotton-plugged vials containing water. The rearing methods were described in a number of previous publications.[15] The methods related to the performance of allatectomy and corpus allatum transplantations were described by Novák and Sláma,[7] Novák et al,[8] and Sláma.[11] For topical treatments of JH analogs, we used the nontoxic, synthetically prepared, methyl ester of 7,11-dichloro-3,7,11-trimethyl-2-dodecenic acid (dichlorofarnesoate) with a standard, ID-50 unit of JH activity 0.001 µg/larva (compound no. T-30, as listed in the study by Sláma et al[15]). Indirect applications of the compound were made by treatments of the seeds with 0.1 mg of the indicated juvenoid per centimeter square of the ground surface (linden seeds).

The 50-year-old measurements of O_2 consumption were made by the conventional Warburg respirometric methods, described in detail in several publications.[4,7,10,11,25] In this work, we use the scanning microrespirographic technique that enables individual monitoring of O_2 consumption and CO_2 output in very small insects. Continuous monitoring of subnanoliter amounts of O_2 consumption per minute by microrespirography has been described in several insect species.[22–24]

A brief characteristic of the scanning respirographic method shows that it is in principle a constant volume and constant temperature respirometric technique, which is based on a common physical law of Boyle–Marriott. Essential technical feature of the method is the precisely stabilized, thermostated internal compartment, hosting a susceptible, semiconductor differential strain-gage transducer. The mechanical changes of internal pressure, caused in the respiratory vessel by O_2 consumption or CO_2 output of the organism, are converted into the corresponding changes in the electrical, 5-kHz, alternating feeding current of the electronic transducer. Finally, after amplification and decoding of the electrical signals, the resulting direct current output voltage has been recorded on PC. The more detailed description of the scanning microrespirographic

method has been previously published.[19,22–24] In this work, we used the DI-148 hardware and software package of DATAQ Instruments.

The methods related to the measurements of water metabolism in larvae of *P. apterus* were described earlier by Sláma and Žďárek.[26] They were based on the ability of the bug to locate and find almost immediately the source of water. In a dry breeding cage, for example, the larvae easily spotted and used to drink from a narrow orifice of a thin, calibrated capillary. The amount of water imbibed by the bugs, corrected for water evaporation in a control capillary, was used for the determination of the daily water intake ($n = 10$). The amounts of daily excreted water was determined according to the surface area deposited by the drops of urine on the stained, calibrated filter paper.[26]

The method used for monitoring the temperature of the normal and hypermetabolic specimens of *P. apterus* was previously described by Sláma and Lukáš.[19] In this work, we used the forward looking infrared (FLIR) B 360 thermovision camera with a 100-µm close-up arrangement; the view field is 32 × 24 mm. The QuickReport software of the FLIR camera was used for the detailed analysis of temperature gradients on thermovision images. Note that the right side of each thermovision image contains the calibrated temperature/color gradients. The breeding of larvae as well as the thermovision recordings were made at a consistent 27°C. The exact calibration of the temperature range of the camera to show the corresponding colors was made before each recording of insects. Small variations in the background colors could not be completely eliminated such as the bottom of the Petri dishes adhering to the metallic support, for example. Thermographic images #3 to #7 have been selected from a large number of measurements. The differences in larval temperature were evaluated visually based on the comparisons of multiple images stored in our database. Occasionally, there were some hypermetabolic specimens of third or fourth larval instars with the body temperature higher than 2°C (as shown in Fig. 3). For the purpose of this more or less initial work, however, we present only the definitely warm and cold specimens.

Results

The effect of JH on respiratory metabolism of *P. apterus*. Figure 1 shows a comprehensive list of the data published on the effects of JH on the respiratory metabolism of *P. apterus*. The results were constructed from extensive results on the effects of JH on O_2 consumption, published during the past 50 years. They show two basic types of respiratory cycles, determined by the presence or absence of JH. The first type shows the JH-conditioned, five-day, larval–larval respiratory cycles, found spontaneously in the first to fourth young larval instars. The second type of respiratory cycle, represented by the seven-day, larval–adult O_2

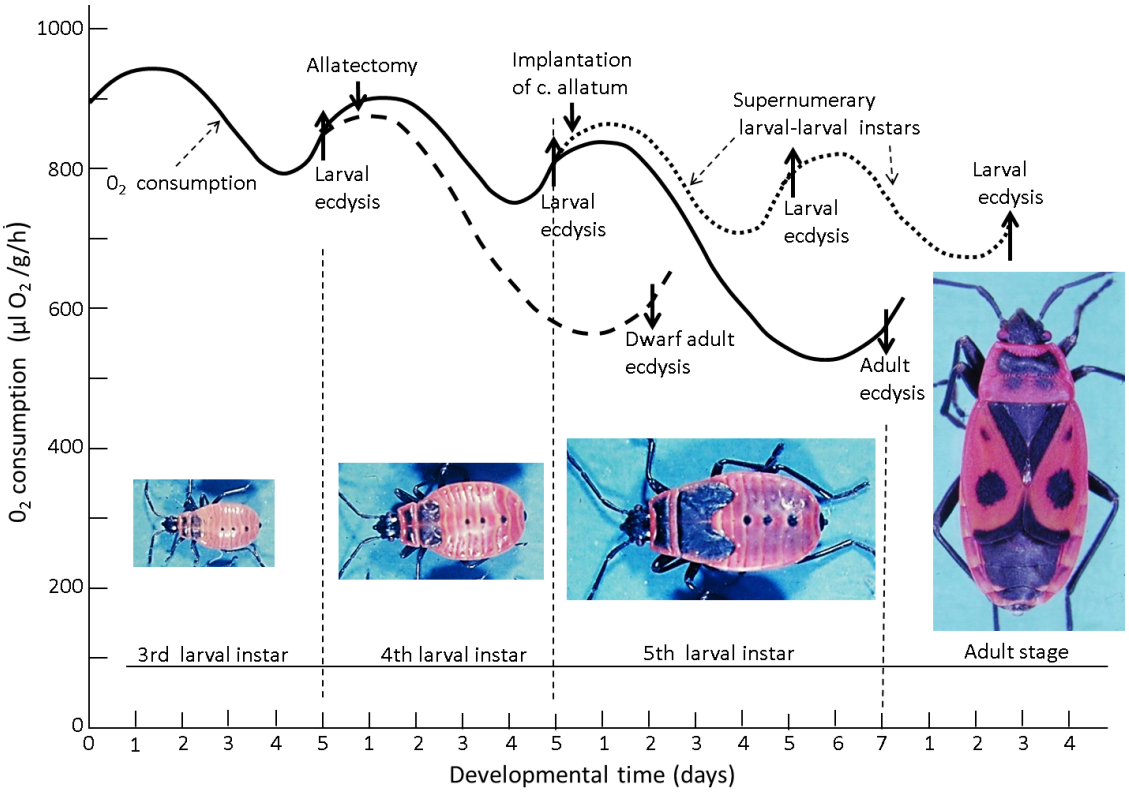

Figure 1. Changes in O_2 consumption during the development of normal larvae (full line), allatectomized prothetelic fourth instar larvae (broken line), and supernumerary larval instars induced by implantation of active corpora allata (dotted line), in the European firebug, *Pyrrhocoris apterus* L. The values are based on the data obtained from the studies by Novák and Sláma,[7] Novák et al,[8] and Sláma.[4,11,25]

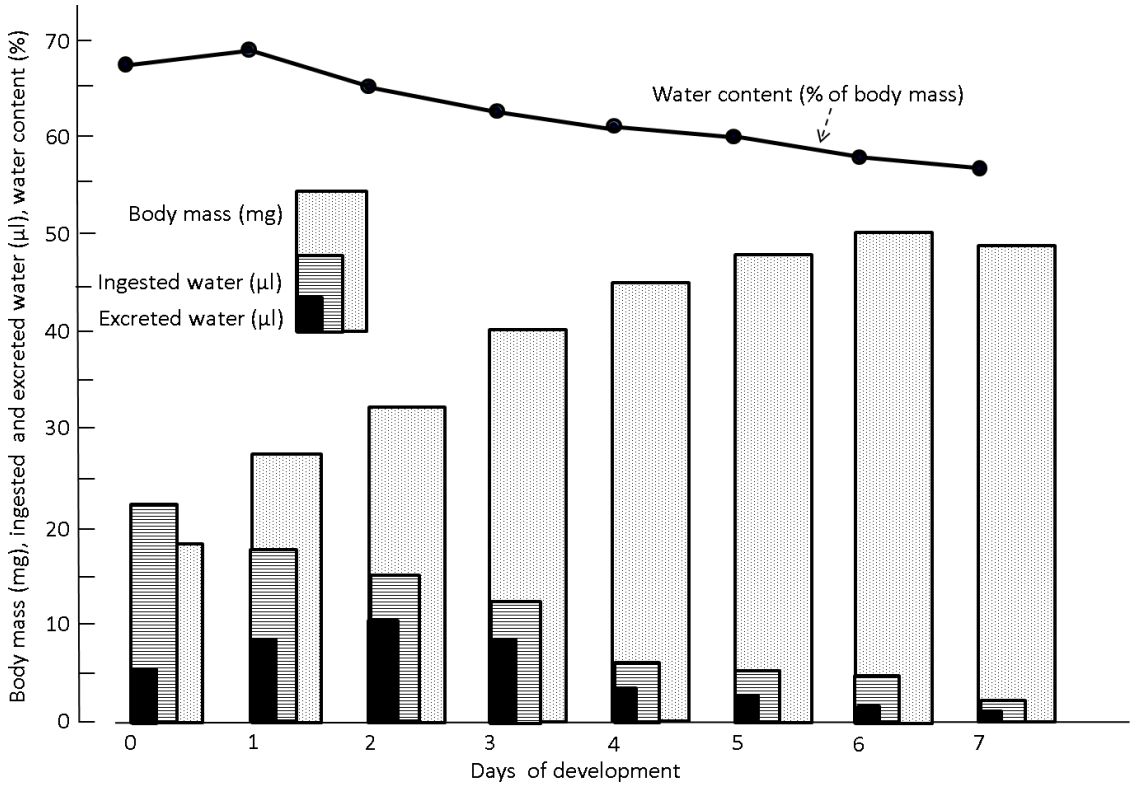

Figure 2. Changes in water content, body mass, and water imbibed and excreted during the development of the last (fifth) larval instar of *Pyrrhocoris apterus* L. (from Novák and Sláma,[7] Janda,[59] Janda,[60] Janda and Sláma,[61] and mainly from Sláma and Žďárek[26]).

consumption cycles, occurs spontaneously in the last, fifth larval instar, which does not contain JH. Experimentally, as shown in Figure 1, the seven-day metamorphosis cycle can be prematurely induced in the fourth, penultimate larval instar by allatectomy (broken line). Conversely, the supernumerary, five-day larval–larval respiratory cycles were installed in the last larval instar by the implantation of active corpora allata or by exogenous treatment with the JH analogs (dotted line in Fig. 1).

The data in Figure 1 also show that the presence or absence of JH has a relatively small effect on the course of O_2 consumption during the initial feeding period of each larval instar. The most profound difference in respiratory metabolism can be found during the nonfeeding, second-hand period of the larval–adult respiratory cycle, which is analogous to the common, U-shaped metamorphosis course known from the pupae of endopterygote insects.[25] The relatively small difference in respiratory metabolism between the five-day (+JH) and the seven-day (–JH) respiratory cycles (O_2 consumption between 800 and 900 µL/g per hour in Fig. 1) shows that the larvae of *P. apterus* do not exhibit the extraordinarily high, hypermetabolic responses to JH. In contrast to our expectations, these results were completely different from the results obtained in other species consuming dry food, where JH causes a 10-fold increase in O_2 consumption.[16] The absence of clear hypermetabolic responses to JH in *P. apterus* suggests that the bugs can get water without being obliged to burn down the dietary lipids.

Water metabolism in the last instar larvae of *P. apterus*. With respect to water metabolism, we used the extensive data provided earlier by Sláma and Žďárek,[26] which have been reproduced in Figure 2. They show several important features, as follows: (1) during the initial feeding period, the larvae contained 68% water in the body, which successively decreased down to 56% of water before adult emergence (upper curve in Fig. 2); (2) after ecdysis, a larva imbibed in average 23 µL of water, which was more than the whole larval body; and (3) the daily proportions of water excreted as urine followed basically a similar course with the ingested water. In conclusion, the results shown in Figure 2 clearly show that the larvae of *P. apterus* need to drink water every day. Due to this, these insects are not obliged to burn down and sacrifice all dietary lipids in order to get water, because they can drink. At the beginning of the feeding, the bug penetrates the shell by the proboscis and injects the saliva containing the digestive enzymes into the seed. The partly digested and emulsified nutrients are sucked back into the alimentary canal. The relatively large salivary glands need water in order to make the saliva. Observations in the laboratory showed that a successful colony of *P. apterus* always required the presence of drinking water.

The results shown in Figure 2 suggest that, under certain circumstances, *P. apterus* can get water by drinking, without

being dependent on the hypermetabolic water supply from the conversion of nutrients. The selective developmental advantage of this system depends on saving and storage of as much nutrients as possible, instead of burning them down for water. A question arises, however, how can the bugs survive if they could not get enough drinking water (drought, absence of suitable plants)? This emergency situation could easily occur among the fastest growing, smallest young larval instars. In the complete absence of water, the bugs show very high mortality. Survival of the limited number of individuals depends on occasional cannibalism (sucking hemolymph from other larvae).

Hypermetabolic responses to JH revealed by thermovision camera. The foregoing study[19] revealed that the water-producing, hypermetabolic larvae had a considerably higher body temperature. This prompted us to use the FLIR thermovision camera again to study the body temperature of *P. apterus*. Observations performed on the largest last instar larvae of *P. apterus*, however, showed no signs of the hypermetabolic, water-producing larvae having an increased body temperature (Fig. 3). The situation was completely different when we looked at the small juvenile larvae of the first to fourth larval instars. In this article, we performed more than 68 individual screening experiments and always encountered a mixture of "warm" larvae, interspersed with the "lukewarm" and, completely "cold" larvae maintaining the environmental temperature. Figure 4 shows an example of one such thermovision image. It indicates that, in contrast to the permanently "cold" last instar larvae, the younger larval instars show some "warm" specimens heated from inside the body by an endothermic metabolic process. In other words, Figure 4 shows a selected thermovision image with two warmer third instar larvae (22.8 and 23.1°C, yellow color) that are different from the temperature of the linden seeds (22.0°C, orange color)

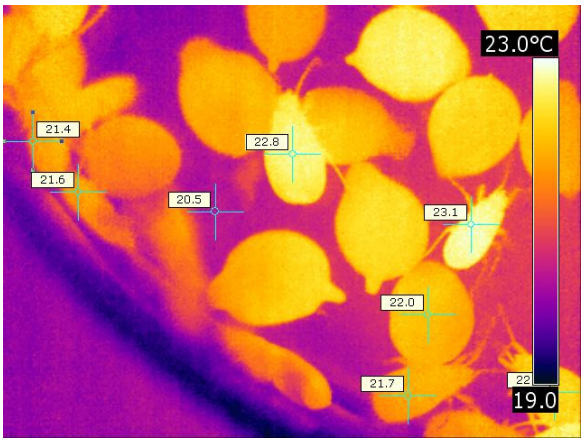

Figure 4. A close-up image obtained by forward looking infrared thermovision camera, showing the "warm" and "cold" body temperature of the relatively small, third instar larvae of *Pyrrhocoris apterus* (round objects are dry linden seeds, blue background is the glass Petri dish; breeding and experimental temperature, 27°C).

and the blue background color (21°C) of the glass Petri dish. A few "cold" third instar larvae (21.4, 21.6, and 21.7°C) can also be seen on the same image. We have more of similar thermovision images in our database, awaiting evaluation.

The abovementioned findings, which show that a relatively small larva of *P. apterus* (size 1–3 mm) can maintain a different, elevated body temperature, are quite intriguing. Until now, everybody assumed that such a small, poikilothermic animal would rapidly dissipate heat and assume the environmental temperature. In spite of these theoretical calculations, our practical measurements show that a number of miniature, third instar larvae of *P. apterus* can be heated from inside at 2–4°C above the environment. At the same moment, another larva of the same size can stay "cold" (Fig. 5). Someone might suggest that the "warm" larvae could perhaps be the

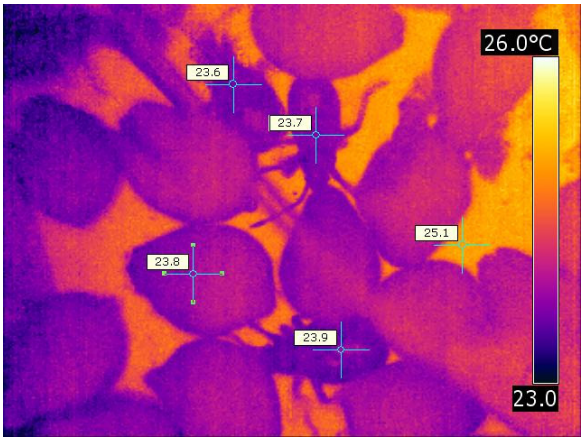

Figure 3. Thermovision image showing the "cold", last (fifth) instar larvae of *Pyrrhocoris apterus*. Note that the differential distribution of temperature gradients has been converted by forward looking infrared thermovision camera into the corresponding color scale (shown at the right side). The last instar larvae lack the juvenile hormone; their body temperature does not differ from the environment.

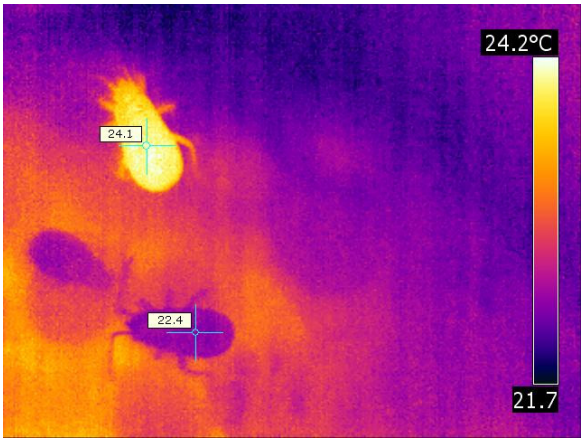

Figure 5. Thermovision image of third instar larvae of *Pyrrhocoris apterus*, showing relatively large differences in their body temperature. The tentatively hypermetabolic larva in upper left corner was substantially warmer (24.1°C) than the two larvae on the bottom (22.4°C; recorded at room temperature).

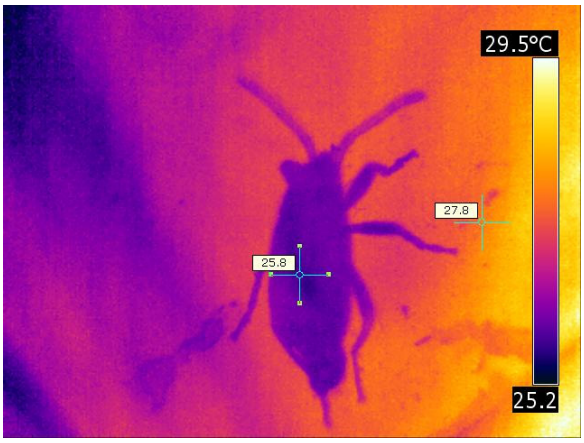

Figure 6. Thermovision image of a male *Pyrrhocoris apterus*. Occasionally, a male was colder than the surrounding environment (27°C).

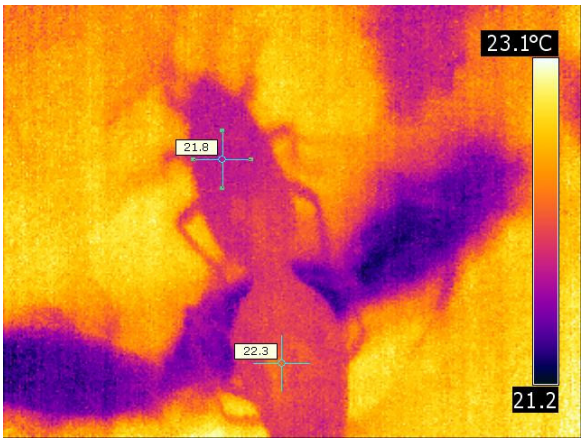

Figure 7. Thermovision image of a mating pair of the adult *Pyrrhocoris apterus*; the female body was slightly warmer (22.3°C) than that of the male (21.8°C at room temperature).

fast running ones, because muscular activity can also increase the body temperature, as given in the "Discussion" section. In our experiments, however, both the "warm" and "cold" larvae used to run wildly across the screen. We suspect that *P. apterus* can sense the infrared (IR) radiation of the camera and tries to get out of the focus area. We are convinced that the "warm", juvenile larvae of the bug have been heated by the same endothermic metabolic process similar to that of the hypermetabolic larvae of other species.

There has been a clear-cut correlation between the appearance of "warm", young instar larvae (active corpora allata = +JH) and "cold" last instar larvae (inactive corpora allata = no JH). However, the presence or absence of JH alone may not be pivotal, because of a physiological feedback mechanism dependent on feeding. For example, all adult males of *P. apterus* have a "cold" body (Fig. 6, illustration), in spite of the fact that they get JH from the active corpora allata. The males of *P. apterus* exhibited only the low environmental temperature on all thermovision images. They did not grow, fed only sporadically, and exhibited always the lowest rates of O_2 consumption throughout all of their life (400 µL O_2/g per hour).[4,10–12] Adult females, on the other hand, performed intensive, JH-stimulated reproduction cycles of feeding, ovarian growth, and oviposition. These five-day reproduction cycles were associated with the corresponding up and down cycles in respiratory metabolism.[4,27] The extensive thermovision screenings revealed a mixture of the "warm" and "cold" females, depending on the given stage of the reproduction cycle. Figure 7 shows a selected sample of a thermovision image taken with the mating pair of *P. apterus*. The image shows that the female partner was warmer than the male. We are convinced that this and other thermovision pictures may provide new insights into thermoregulation and reproduction physiology.

Scanning microrespirography. Thermovision imaging has revealed a new and important physiological features that can be briefly summarized as follows: (1) the hypermetabolic-like, "warm" specimens were found among the young larval instars and adult females, which both contained JH, ingested food, and grew larger in size; (2) the last instar larvae, which positively contain no JH (inactive corpora allata), also used to feed and grow in size, although their body remained "cold"; and (3) adult males, irrespective of the active (mating males) or inactive (diapausing males) corpora allata, ingested food only sporadically and were always "cold". Evidently, there are several physiological factors responsible for the release of endothermic energy as heat: (a) the presence of JH; (b) ingestion and utilization of food; and (c) water deficiency. Thermovision images perfectly identified specimens with the elevated body temperature, but they did not indicate the nature of the nutritional substrate (whether carbohydrate or lipid) that used to be metabolized. This valuable information could be provided by scanning respirography.

An example of the common scanning microrespirographic record, taken with an adult male of *P. apterus*, is shown in Figure 8. For those who are not familiar with the technique, we can explain that there are basically two types of records: (a) O_2 consumption in the presence of CO_2 absorbent (see the five scanning episodes in the upper trace of Fig. 8) and (b) differential recording without the CO_2 absorbent, which shows the balance between the consumed O_2 and the simultaneously produced CO_2 (see the two scanning episodes of CO_2–O_2 trace in the bottom of Fig. 8). The traces of the consumed or released gas have been offset within the measuring range by an electronic zeroing mechanism. This arrangement operated by opening the pressure valve for a second, whenever the output voltage reached a limit of the preset value.

The upper trace record in Figure 8 reveals a regular consumption of 500 nL O_2/min (= 500 µL of O_2/g per hour expressed per unit of mass). The lower, differential trace record in the absence of CO_2 absorbent (+CO_2–O_2) shows the rate of 170 nL/min, which indicates that this male simultaneously

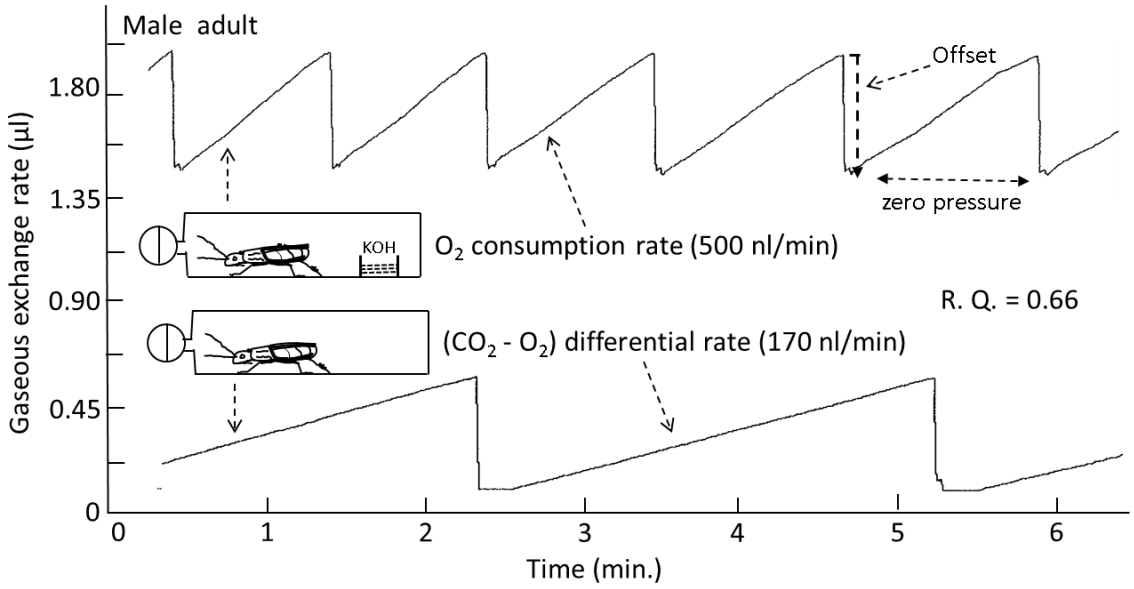

Figure 8. The scanning respirographic record obtained with an adult male of *Pyrrhocoris apterus* (60 mg body mass, 27°C). Upper record shows the five scanning episodes of O_2 consumption recorded in the presence of CO_2 absorbent (1% KOH). The cumulative record was electronically offset to zero in approximately one-minute scanning intervals. The rate of O_2 consumption shows 500 nL O_2/min, which is equivalent to standard 500 µL O_2/g per hour. The lower record, without the CO_2 absorbent, shows the differential (+CO_2–O_2) ratio of O_2 consumption (170 nL/min) decreased for the simultaneously released CO_2. The values indicate the release of 330 nL CO_2/min (= 330 µL CO_2/g per hour); the ratio of RQ (CO_2/O_2) is 0.66, indicating the metabolism of a lipid substrate.

released 330 nL CO_2/min. The respiratory quotient (RQ, CO_2/O_2) was 0.66, ie, close to the stoichiometrically determined breakdown of an ideal triglyceride (0.7).

Figure 9 shows a similar set of respirographic records taken with the last instar larva of *P. apterus*. The recordings show that the larva consumed 630 nL of O_2/min (= 1080 µL O_2/g per hour). The differential respirographic record on the bottom of Figure 9 shows equilibrium between the rates of O_2 consumption and CO_2 output. The differential (CO_2–O_2) trace indicates no change, and the RQ value of CO_2/O_2 = 1.

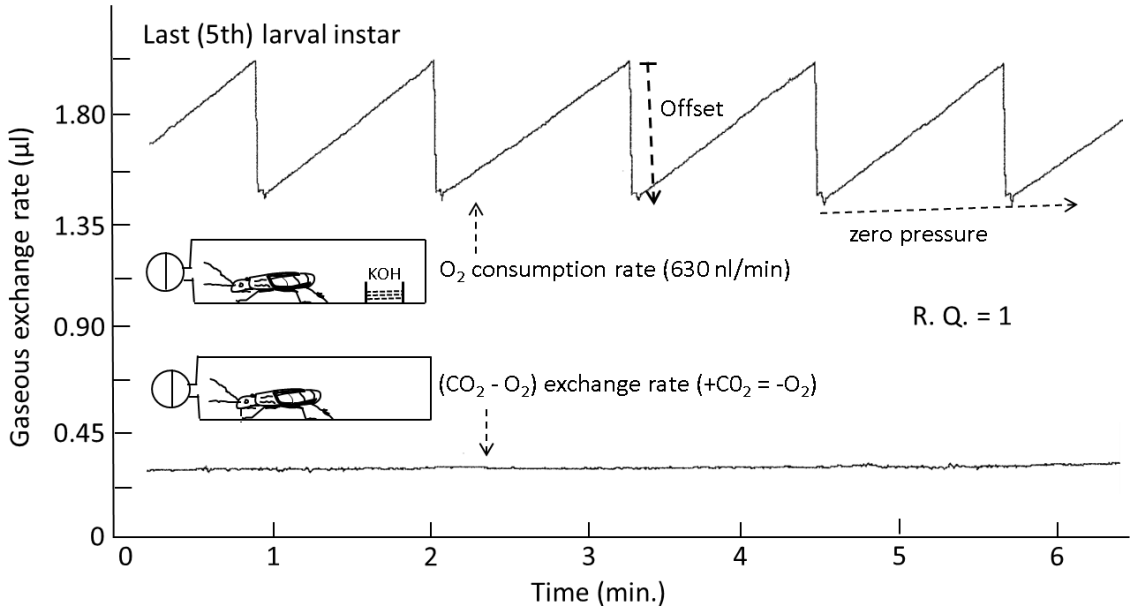

Figure 9. Similar scanning respirographic record as in Figure 8, taken with the last instar larva of *Pyrrhocoris apterus*, 35 mg body mass. The upper record shows a six-minute measuring interval with five scanning episodes of O_2 consumption (630 nL/min = 1080 µL O_2/g per hour). The lower differential record (+CO_2–O_2) shows equilibrium between the volumes of O_2 consumed and CO_2 released, indicating the ratio of RQ = 1 and a purely carbohydrate metabolism.

This feature is a prerequisite for the metabolism of an ideal carbohydrate.

The effect of JH on lipid metabolism. The respirographic recordings of adult males (Fig. 8) and last instar larvae (Fig. 9) always gave consistent results when repeated. In other words, the males always metabolized a lipid (RQ = 0.7), while the last instar larvae constantly metabolized a carbohydrate (RQ = 1.0). Unlike in this case, however, the recordings of RQ with the young, juvenile larvae (first to fourth instars) indicated a mixture of specimens with both lipid and carbohydrate metabolism. The problem prompted us to carry out additional respirographic measurements on a larger scale (n = 10–12). Thermovision technique was used to segregate the "warm" and "cold" larvae and measure them in separate groups.

The results of these studies have been summarized in Figure 10. They show that: (1) the "warm" larvae of the third instar (Fig. 10A) had remarkably higher average rate of O_2 consumption (1100 µL O_2/g per hour) in comparison with the "cold" larvae (850 µL O_2/g per hour, Fig. 10B) and (2) the bars indicating standard error of the mean (SEM) values indicate a larger variation in the group (Fig. 10A), presumably due to differences associated with feeding. The average RQ of the "warm" larvae indicated predominantly lipid metabolism (0.74 ± 0.1), while the "cold" larvae (Fig. 10B) metabolized predominantly carbohydrate (RQ = 0.96 ± 0.04). The relatively small SEM variations of RQ clearly document that the "warm" larvae metabolized lipid, whereas the "cold" larvae utilized the carbohydrate metabolic substrate and tentatively stored the lipid in the fat body. Curiously enough, the average output of CO_2 was virtually the same in both groups (814 µL CO_2/g per hour for the "warm" group versus 816 µL CO_2/g per hour for the "cold" group). The physiological implication of the results in Figure 10A and B indicates that the juvenile larvae of *P. apterus* metabolize both a lipid and a carbohydrate, depending on the extant physiological conditions (eg, the presence or absence of JH and the availability of food and water).

The results in Figure 10C and D show the effects of JH on the respiratory metabolism of the last larval instars of *P. apterus*. The normal last instar larvae, with the inactive corpora allata (Fig. 10C) metabolized almost exclusively the dietary carbohydrate (RQ = 1 ± 0.02) and stored the lipid resources for metamorphosis. The results presented in Figure 10D reveal the crucial role of JH in reinstallation of the larval–larval type of growth, ie, reinduction of lipid metabolism (RQ = 0.82 ± 0.12) associated with the development of the giant, supernumerary larval stage. In other words, the treatment with JH analogs reinstalled the previous juvenile metabolic conditions (like in Fig. 10A), characterized by a metabolic switchover from pure carbohydrate metabolism (Fig. 10C; RQ = 0.99 ± 0.01) into predominantly lipid metabolism (Fig. 10D; RQ = 0.82 ± 0.12). As far as the adult males are concerned (Fig. 10E; RQ = 0.7 ± 0.02) they exhibit a special case of the previously described,[10] hormonally independent respiratory metabolism based purely on the utilization of

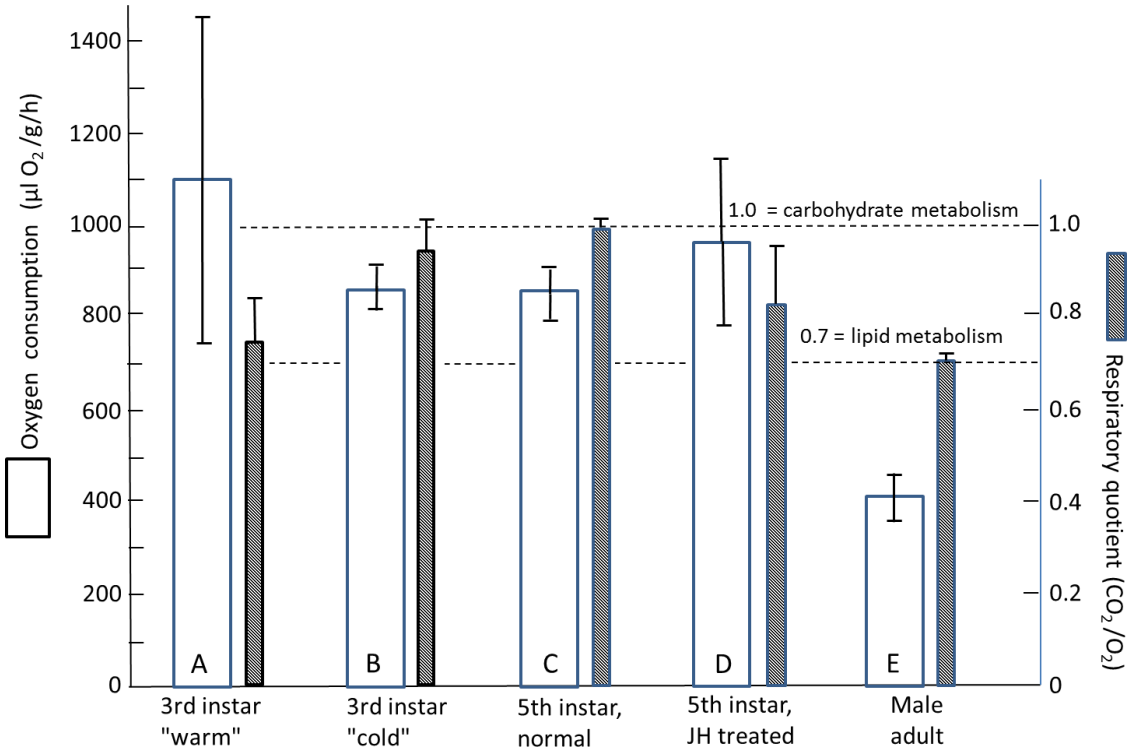

Figure 10. Average values of O_2 consumption and RQ, constructed from 10 to 12 individual respirographic recordings in each experimental group of *Pyrrhocoris apterus* (3× repeated measurements on four separate respirographic channels). The juvenile-hormone-treated last larval instars molted into the supernumerary larval instars. The bars indicate averages from 10 to 12 individual recordings; vertical lines indicate standard error of the mean.

a lipid substrate (according to the previous measurements,[4] the females of *P. apterus* show extremely variable values of O_2 consumption, which required additional statistical evaluation).

It is essential to point out, finally, that the respirographic records of *P. apterus* occasionally exhibit large, discontinuous outbursts of CO_2 when measured in dry air (not shown in the figures). Similar CO_2 bursts occur in a number of other insect species.[24] The problem of discontinuous CO_2 emissions has not been mentioned in this article, because it is not directly related to the present topic.

Discussion

The nature of hypermetabolic responses to JH. Insects are predominantly aerobic organisms. Their metabolic activity can be best characterized by the rate of O_2 consumption, conventionally expressed in microliters of O_2 consumed per gram of living mass per hour. The diapausing insects or insect eggs consume 10–50 µL of O_2/g per hour at room temperature; the growing larvae and pupae usually take 400–1000 µL, while the adult insect stages consume as much as 800–2000 µL O_2/g per hour.[4,22–25,27] The highest rates of O_2 consumption (5000 µL O_2/g per hour) were recorded in adult *Drosophila* during flight.[15] In comparison with the warm blooded vertebrates, the poikilothermic insects are real champions in O_2 consumption, using 100- to 500-fold more oxygen per unit of mass at room temperature. For example, the human body consumes only about 40 µL of O_2/g per hour, at 37°C.[15,28,29]

According to the respirometric data listed earlier, the "hypermetabolic" rates of O_2 consumption reported by Sláma and Hodková[16] are quite exceptional. The last instar larvae of these insects that feed on dry food (*D. vulpinus*, Coleoptera; *G. mellonella*, Lepidoptera) regularly consume 800–1000 µL of O_2/g per hour. When implanted with the active corpora allata or treated with the analogs of JH; however, the larvae voraciously started to feed and produced large piles of feces. Their "hypermetabolic" rates of O_2 consumption reached higher values of 10,000 or 20,000 µL O_2/g per hour. This was considered as the highest metabolic rate ever recorded in a living organism on this planet.[17] Further analysis of the hypermetabolic responses to JH revealed that a larva of *D. vulpinus*, which consumed 10,000 µL of O_2/g per hour, burned down 5 mg/h of the dietary lipid (triglyceride), expired 7250 µL of CO_2 per hour and produced 4.25 mg/h of metabolic water. This shows that the hypermetabolic larva yielded from dry food as much as 10% of water per day.[17] The delipidated food did not produce the hypermetabolic response, and the lipids stored in the fat body remained also untouched.

Initially, we expected that *P. apterus*, because it feeds on dry linden seeds, should also exhibit the hypermetabolic symptoms of converting dry food into water. The results shown in Figure 1 revealed, to our great disappointment, that the treatment of the last larval instar with JH analogs had a relatively small impact on the rate of O_2 consumption (Fig. 10C and D). According to the data in Figure 1, the initial rates of O_2 consumption in each larval instar follow a more or less common course, close to 1000 µL of O_2/g per hour. Implantations of the active corpora allata or treatments of the last instar larvae of *P. apterus* with the JH analogs increased the respiratory rate only for some 30% (Fig. 10D), not 10-fold as shown in *D. vulpinus*.[16] The relatively small effect of JH on the respiratory metabolism of *P. apterus* provided an indirect presumptive evidence that the seed-feeding species obtained water from other sources than the hypermetabolic combustion of the dietary lipid. The problem was clarified by the previous data of Sláma and Žďárek,[26] who found that the bugs used to drink water from the environment whenever it was possible (Fig. 2) or obtained water by sucking a sap from available plants.[30]

The question emerged, however, if the bugs could develop and survive without the drinking water. The breeding experience from the laboratory[5] showed that the bugs could survive on dry seeds only when they had access to vials containing drinking water. In the complete absence of water, the amount of "warm", hypermetabolic larvae slightly increased, but due to the devastating mortality the measurements were inconclusive (presumably, the larvae cannot grow after burning down all dietary lipid).

The developmental period in *P. apterus* that is most vulnerable to thirsting is the period of the small, fastest growing, juvenile first to fourth larval instars. We found by thermovision that these small juvenile larvae, which contained plenty of endogenous JH, exhibited a mixture of the "warm" larvae with elevated body temperature and the "cold" larvae maintaining the environmental temperature. The "warm" larvae as we have already stated (Figs. 4 and 5), had increased respiratory metabolism (1100 µL O_2/g per hour, ±350) and relatively low RQ close to 0.7, ratio, ±0.1, indicating the metabolism of a lipid (neutral seed oil; Fig. 10A). The "cold" larvae of the same size had relatively lower O_2 consumption (850 µL O_2/g per hour), with the RQ ratio of 0.95 indicating the carbohydrate metabolism (starch, glycogen). We have reasonably assumed that the "cold" larvae managed to imbibe enough drinking water. In this case, they could store the dietary lipid in the fat body as a reserve material for further growth, instead of burning it down for water. The metabolic system of the last instar larvae of *P. apterus*, which had inactive corpora allata and no endogenous JH (Fig. 10C), utilized only the dietary carbohydrate (RQ = 0.99 ± 0.01) irrespective of the low or high water content.

The normal respiratory metabolism versus hypermetabolism. The survey of literature shows that the usual range of O_2 consumption in larvae of endopterygote insects is 200–800 µL of O_2/g per hour.[4,17,23,25,28,29] Exopterygote insects like the cockroaches, locusts, or hemipterans fit into the range of 500–1000 (Fig. 1), while insects of relatively small body size, like aphids[22] or termites,[24] show the rates of O_2 consumption over 1000 µL O_2/g per hour. Insects belong to the "coldblooded" animals whose body temperature is

usually equilibrated with environmental temperature, except for a few instances of endothermy in the flying moths[20,21] and honeybees.[31] The textbooks of biochemistry[32] propose that the high endothermic energy liberated from oxidative metabolism becomes conserved by the process of oxidative phosphorylation, which is used to store the energy in the form of macroergic phosphate bonds (AMP → ADP → ATP).

Due to the relatively small size of insects, the determination of the body temperature represented a real technical challenge,[20] before the availability of the thermovision techniques. In our previous study,[19] we used thermovision techniques for the demonstration of enormous hypermetabolic rates in O_2 consumption, associated with the emanation of excessive endothermic energy, dissipated from the larval body as heat. The "warm", hypermetabolic larvae of *G. mellonella*, which had substantially elevated body temperature (up to 10°C increase), could be spotted on the screen of a thermovision camera. The current study extends our previous observations[19] for the relatively small and morphologically different larvae of the exopterygote insects (*P. apterus*).

During the past three decades, the main stream study of insect respiration was mostly concerned with the commercially available, very practical flow-through IR analyzers, recording the output of CO_2 in insects subjected to a constant flow of the scrubbed air.[33] The results of the commercial flow-through systems are useful for the recording of continuous or discontinuous output of CO_2; however, they do not permit a more detailed respirometric analysis. Our respirographic data, which record the differential rates of consumed O_2 and released CO_2 (Figs. 8–10), document that the larvae of *P. apterus* need JH in order to metabolize the dietary lipids. As has been already indicated, the onset of metamorphosis manifested by the absence of JH in the last larval instar changes lipid metabolism into the selective utilization of carbohydrate as the metabolic substrate (RQ = 1.0). In spite of the fact that the larvae of *P. apterus* can get water by drinking, the metabolic results found in this species are in good agreement with the JH-stimulated hypermetabolic combustion of the dietary lipid in other species, *D. vulpinus*[16,17] and *G. mellonella*[19] fed on dry food.

The existence of "warm" and "cold" third instar larvae in *P. apterus* shows certain important physiological implications: (1) there is a physiological homeostatic mechanism preventing the unnecessary combustion of the dietary lipid, when a larva received enough water by drinking; (2) since all third instar larvae contain the active corpora allata,[5] existence of the "warm" and "cold" larvae provides indirect evidence for a feedback mechanism, which can modify the action of the centrally produced JH from the periphery (feeding, drinking, actual water content). The existence of a peripheral feedback response may perhaps explain the virtual absence of hypermetabolic responses in the caterpillars of phytophagous noctuid moths, which get plenty of water from leaves. These caterpillars grow much bigger when treated with JH analogs. In contrast to larvae fed on dry food, however, the phytophagous larvae do not show the respiratory symptoms of hypermetabolism.[34]

In contrast to the abovementioned phytophagous insects, larvae of the stored product insects exhibit strong hypermetabolic responses, because they need water for larval growth. The larvae of *Tribolium castaneum*, for instance, have a variable number of larval instars. Determination of the ultimate, larval–pupal metamorphosis instar without JH depends on the amount of water that was accumulated during the previous, JH-stimulated larval–larval instars. The treatment of *T. castaneum* larvae with JH analogs led to several-fold repeated formation of the giant, supernumerary instars containing increased amounts of hemolymph and water.[19]

The direct or indirect metabolic effects of JH. Before the discovery of hypermetabolism in *D.vulpinus*,[16] it was generally believed that the effects of JH on insect metabolism were indirect, being only an outcome of anatomical and physiological changes induced by the hormone.[6,9,13,15,27,35] The effects of JH on O_2 consumption in *P. apterus* (Fig. 1) are superficially also in favor of the indirect metabolic alternative. These respiratory changes are closely linked with altered developmental changes. The pattern of metamorphosis can be characterized by the well-known U-shaped course[25] of pupal O_2 consumption, which is located here during the second-half period of the last larval instar (Fig. 1). The hypermetabolic effects of JH in the last larval instar of *D. vulpinus*, reported by Sláma and Hodková,[16] were quite exceptional. JH caused enormous hypermetabolic responses in respiratory metabolism under conditions of completely arrested development for several weeks.[16,17]

A strong metabolic effect of JH without a developmental change suggests epigenetic action of the hormone without the restructuralization of the DNA molecule. This recognition represents a challenge to the previous endocrinological conclusions about the mode of JH action based on: (a) induction of mitotic divisions among the cells;[5,14,36–38] (b) induction of an isometric tissue growth;[15,39] (c) stimulation of RNA synthesis;[40–43] (d) changes in the activity of esterase enzymes;[44] (e) induction of the peripheral *Met* gene;[42,45] JH Met receptor;[46] and (f) certain other developmental features.[47]

The most recent study by Jindra et al[46] neglects all the previously known physiological knowledge of JH action and explains its mode of action in a new way. They claim that there is a bHLH-PAS protein Met intracellular receptor for JH. The binding of JH to Met triggers dimerization of Met with its partner protein Tai, and the resulting complex induces transcription of target genes. This simple, JH-activated pathway is believed to be responsible for maintaining the juvenile status during the early postembryonic development when larvae/nymphs lack the competence to metamorphose.[46] This novel interpretation of JH action can be true as well as absolutely false, because there is no comparison with earlier JH data. We have no idea whether this interpretation of JH action could help to explain the hypermetabolic responses to JH.

The statement about the competence of larvae/nymphs to metamorphose is not true. There exists a well-based evidence that the competence of insect cells to metamorphose is present already as early as in the deep embryonic period, during the embryonic stage of blastokinesis, not in the larval/nymphal postembryonic period.[6,27,48,49]

Thermoregulation based on uncoupling of oxidation from phosphorylation. The velocity of heat dissipation from the insect body depends on the total body mass. The relatively large, hypermetabolic larvae of *G. mellonella* (270 mg) could be more than 10°C warmer[19] in comparison with 2°C found in the 100-times smaller hypermetabolic larvae of *P. apterus* (Figs. 4 and 5). Based on our previous findings in *D. vulpinus*[16] and *G. mellonella*,[19] we assume that the production of heat in the hypermetabolic larvae was not the main physiological reason of hypermetabolism. It was a secondary by-product attributed to the main process, which was the production of metabolic water. A possibility cannot be excluded, however, that the endogenous production of heat might develop as a specific thermal adaptation during evolution. The secretion of JH in insects is closely linked with the actively feeding stages. The hormone is never secreted in a nonfeeding stage.[15] It is also well known[9,27,35] that insect hormones produced by the central neuroendocrine system exert multiple biochemical and physiological functions through their subordinated endocrine glands of the second category.[6,9,14,29] In this connection, we ask a question, whether the hypermetabolic combustion of the dietary lipid was the direct action of JH, or whether it was mediated by a hormone of the subordinated endocrine gland of the second category.

According to Sláma and Lukáš,[19] the uncoupling of oxidation from oxidative phosphorylation, which is the main biochemical process attributed to the production of metabolic water, has not been previously included among general functions of insect adipokinetic hormones (AKHs) and JH. We anticipate that it might be a physiological disaster, when the JH-induced, total metabolic breakdown of the dietary lipid would proceed in a larva obtaining enough water in the food. A theory has been created on a well-based evidence[19] that the hypermetabolic responses to JH were mediated by a hitherto unknown, lipolytic superhormone secreted from the JH-subordinated prothoracic gland.

After the discovery of hypermetabolic responses to JH,[16] Chefurka[50] suggested that the analogs of insect JH could be regarded as a new class of uncouplers of oxidation from oxidative phosphorylation. The conclusion was corroborated by Sláma and Kryspin-Sørensen,[17] who confirmed that the hypermetabolic responses to JH in *D. vulpinus* showed all symptoms of the uncoupled oxidation from oxidative phosphorylation indeed.[17] These conclusions were also confirmed by Němec,[18] who found that the hypermetabolic larvae of *D. vulpinus* maintained on a dry diet accumulated abnormal amounts of water in the body. Moreover, the hypermetabolic larvae of *G. mellonella* heated up sometimes for more than 10°C migrated to the surface of the diet to cool down and dissipate the endogenous heat into the environment.[19] It was reasonably concluded[18] that the excessive endothermic energy liberated by hypermetabolic oxidation of the dietary lipid was too large to be used in the process of oxidative phosphorylation. The overshot conversion of the nucleotides into ATP should apparently became the rate-limiting factor of intermediary metabolism.[18]

Biochemists assumed earlier that the uncoupling of oxidation from phosphorylation never existed as part of a natural physiological process.[32] Naturally, at that time, they did not take into account the possible uncoupling of respiration associated with the hypermetabolic production of water.[18,19] Moreover, the current theories on insect thermoregulation[51,52] are calculated with enhanced muscular activity without considering the hitherto little known hypermetabolic responses to JH.

According to the current theories, the temperature of insect body is determined merely by a balance between the rates of heat gain and heat loss. The heat gain is mainly ascribed to the utilization of external heat, for example, basking in the sun, or the production of internal heat through muscle contractions. The latter eventuality, known as endothermy, assumes the production of heat by synchronous contractions of the flight muscles, as observable as shivering of the thorax and wings.[20,52] It is generally expected that a small body size of insects permits a rapid heating as well as a rapid cooling. A small insect could thus warm up and initiate activity in several minutes or less and it could remain hidden while it was in torpor.[20] In the more recent analysis on thermoregulation,[20,51,52] endothermic heating of insect body by the unleashed, hypermetabolic oxidation of the nutrients remains still merely underestimated.

Our results on hypermetabolism[19] revealed that certain insect species might acquire, during the millions of years of insect evolution, the ability to heat their body by other means than a muscular activity. The vast amounts of endothermic energy, emanating as heat from the hypermetabolic insects,[17–19] represent a new phenomenon of insect thermoregulation. The fact that a small larva of the firebug (size 1 or 2 mm) can be endogenously heated for more than 2°C without increasing the muscular activity (Figs. 4 and 5) is quite amazing. We assume that modern thermovision techniques may soon open up new avenues in the study of insect thermoregulation.[19]

Honey bees, for example, urgently need water and heat when they are closed inside hives during the winter period.[31] We assume that the winter heating of bee hives could be achieved by the hypermetabolic burning of dietary sugar (honey). The selective advantage of more effective heating and the more economic production of water for the overwintering bees is obvious. Finally, the recently obtained thermovision evidence[19] that insect larvae can under certain circumstances, substantially elevate their body temperature, can affect

theories about dependence of developmental timing on the total sum of environmental temperatures.

Adipokinetic peptides versus a new lipolytic superhormone. The hemolymph and tissues of insects contain a number of carboxylesterase enzymes that hydrolyze the esters of long-chain fatty acids (lipases). Some of these proteins have been extensively investigated and are believed to have specific physiological functions like, for example, the JH esterase[44] or the extensively investigated AKHs.[53] In *P. apterus,* Kodrík et al[54] isolated and characterized an adipokinetic peptide, Pyrap-AKH, which caused partial hydrolysis of the neutral lipids yielding mono- and diglycerides. The lipid composition of *P. apterus* and its linden seed diet was investigated a long time ago by Martin[55,56] in connection with the discovery of JH activity of the "paper factor".[49] More recently, in the study of AKHs, Bártů et al[57] performed a more detailed analysis of the fatty acids present in the seeds and compared the results with the lipids extracted from the body of *P. apterus*. They found that the metabolically most important C16 and C18 fatty acids were preferentially absorbed from the seeds and stored in the fat body. The unsaturated, C18 fatty acids had the dominant role in lipid metabolism, in particular, linoleic acid (18:2).[57]

The topic related to AKH proteins and peptides is very important with respect to a hitherto unknown, lipolytic superhormone inducing the total hypermetabolic breakdown of dietary lipids into CO_2 and water.[19] Our results obtained on *P. apterus*, which corroborate previous findings on larvae of *D. vulpinus* (Coleoptera) and *G. mellonella* (Lepidoptera),[19] show no reasonable causal relationships between the JH-induced hypermetabolic hormone and numerous AKH, such as the Pyrap-AKH.[54] Our discovery of a potent lipolytic hormone, tightly related to JH, which stimulates the total breakdown of dietary lipid, seems to be a new field in the intermediary metabolism of insect lipids. A possibility that the indicated lipolytic hormone may be secreted from the JH-subordinated prothoracic gland[19] is being intensively investigated in our laboratory.

The Pyrap-AKH increases the content of lipids and proteins in the midgut, especially triglycerides and diglycerides containing the linoleic acid.[54] According to Vinokurov et al[58] the content of hydrolytic enzymes in the salivary glands of *P. apterus* was rather low after the glands ejected their content into the seed. After being pierced by the bugs, however, the seeds contained a cocktail of hydrolytic enzymes (lipase, peptidase, amylase, glucosidase). We have carefully analyzed the reported physiological functions of the AKH peptide[53,54] in *P. apterus*, in order to find possible relationships between AKH and the JH-dependent, lipolytic superhormone stimulating the total metabolic combustion of dietary lipid.[19] The difference depends on the fact that AKH stimulates partial hydrolysis of the triglycerides, while the hypermetabolic hormone causes complete metabolic breakdown followed by total β-oxidation of the free fatty acids.

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Author Contributions

Conceived and designated the experiments: KS. Analyzed the data and prepared thermographic images: JL. Wrote the first draft of the manuscript and assembled the respirographic data: KS. All authors reviewed and approved of the final manuscript.

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