Heat production in carrion biofilm formed by communally breeding carrion beetles

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Abstract

Insects regulate their body temperature mostly behaviorally, by changing posture or microhabitat. These strategies may be ineffective in some habitats, for example on carrion. Carrion beetles create a biofilm-like matrix by applying to cadaver surface anal or oral exudates. We tested the hypothesis that biofilm formed by communally breeding *Necrodes littoralis* L. beetles (Silphidae) produces heat, that enhances beetle fitness. We demonstrated that heat produced in the biofilm is larger than in meat decomposing without insects. Beetles regularly warmed up in the biofilm. Moreover, we provide evidence that biofilm formation by adult beetles has deferred thermal effects for larval microhabitat. We found an increase in heat production of a biofilm and a decrease in development time and mortality of larvae, after adult beetles applied their exudates on meat. Behavioral strategy revealed here for *N. littoralis* is basically a new form of thermoregulation and parental care in insects.

Keywords

Insect thermoregulation; Animal behavior; Parental care; Carrion ecology; Insect-microbe interactions; Resource competition

Temperature is a key component of animal environment. Insects usually use external heat to regulate their body temperature (1, 2). By changing body orientation or selecting microhabitat with specific thermal characteristics, insects may maintain their body temperature within thermal optima (3, 4). These mechanisms may be ineffective at certain developmental stages (e.g. larvae) and in some microhabitats (e.g. carrion or dung), indicating that other thermal options may be important for some insect ectotherms.

Carrion is an example of a "bonanza" resource, i.e. very rich but at the same time scattered and ephemeral (5). There is severe competition between microbes, insects, and vertebrates over carrion resources (6-12). Insects, e.g. blow flies (Calliphoridae) or carrion beetles (Silphidae) use carrion for breeding and their larvae are main carrion reducers in terrestrial environments (11, 13, 14). Necrophagous larvae usually feed in aggregations (15). Larval aggregates on carrion may have much higher inner temperature than ambient air (by 10-30°C), an effect originally discovered in aggregations of larval blow flies (4, 16-20). Heat in these aggregates was hypothesized to derive from microbial activity (21, 22), larval exothermic digestive processes (19) or larval frenetic movements (20, 23). However, the specific mechanisms involved are not known.

Carrion beetles, in particular burying beetles (Silphidae: *Nicrophorus*), create a biofilm-like matrix on cadavers by applying to its surface anal and oral exudates (24). In case of *Nicrophorus* the behavior was hypothesized to moisturize carrion (25), facilitate digestion (25-27), suppress microbial competitors (24, 28-31), deter insect competitors by reducing carrion-originating attractants (25, 32, 33), support larval aggregation (25) or development (28) or seed mutualistic microbes and transmit them to offspring (26, 34-36). Exudates of several adult or larval silphid beetles were found to contain antimicrobial compounds (37-41). Moreover, some supposedly mutualistic microbes (e.g. *Yarrowia* yeasts) were abundantly identified in carrion beetle guts and carrion biofilm (24, 26, 36, 42, 43). Therefore, the biofilm-like matrix on carrion may be viewed as a result of carrion manipulation by beetles. Seeding or replanting beneficial microbes and weeding harmful components of carrion microbiome are key manifestations of this strategy, benefiting beetles in several ways (26, 33, 35). Here we test the hypothesis that the biofilm, formed on carrion by communally breeding *Necrodes* beetles (Silphidae) produces heat, that enhances larval fitness.

Necrodes beetles colonize large vertebrate cadavers, where its larvae feed on carrion tissues and under favorable conditions may reduce them into dry remains (44, 45). Beetles start visiting carrion after it becomes bloated (under summer temperatures usually 4-8 days after death) and after some time many of them (even hundreds) may be present on a cadaver

(44, 46-48). Females lay eggs in a nearby soil and larvae abundantly colonize carrion usually during late decomposition (44, 46, 47). *Necrodes* beetles are members of Silphinae and, in contrast to Nicrophorinae, they colonize large carrion, breed communally and reveal no parental care (46, 49, 50).

We demonstrate that a biofilm-like matrix formed on meat by adult or larval *Necrodes littoralis* L. (Silphidae) produces heat, which is larger than heat emitted by meat decomposing without insects. Beetles regularly warm up in the matrix. Consequently, smearing carrion with exudates may be categorized as a novel mechanism for insect thermoregulation. Moreover, when adult beetles applied exudates, heat produced in a biofilm formed by feeding larvae was larger, resulting in a decrease of larval development time and mortality. Carrion manipulation by adult beetles had deferred thermal effects, that benefited beetle offspring, and this is basically a new form of parental care among carrion insects.

Results

Heat production in carrion biofilm formed by *Necrodes littoralis*.

To test if biofilm produced by carrion beetles (Fig. 1) generates heat, we monitored with thermal imaging camera conditions in colonies of adult and larval *N. littoralis* subsequently feeding on a meat resource (M+A+L) and in colonies of larval beetles only (M+L), and compared them against thermal conditions in equivalent colonies but without beetles (M). We found that adult and larval beetles smeared meat with anal exudates, forming greasy, biofilm-like coating on meat and surrounding soil, the surface of which increased with colony age (Fig. 1). The biofilm had a higher temperature than the background (Fig. 2) and beetles, particularly larvae, were regularly warming up on its surface (Fig. 3).

By quantifying the average temperature of biofilm-covered surface (meat or soil) and meat without beetle-derived biofilm (M), we found that heat emission in the biofilm enlarged with colony age and was significantly larger than heat produced in meat alone (rmANOVA for the average heat production in the pre-larval phase, $F_{1,27}$=122, N=30, P<0.001, Fig. 4a, b). Heat of the biofilm during larval feeding phase significantly increased after adult beetles prepared the meat (rmANOVA for the average heat production in the larval feeding phase, $F_{1,27}$=154.8, N=30, P<0.001, Fig. 4c). Moreover, heat production in larval feeding phase became larger, when we extended the pre-larval phase (rmANOVA for the average heat production in the larval feeding phase, $F_{2,27}$=24, N=30, P<0.001, Fig. 4a, c).

To test the effect of environmental temperature on heat emission in a biofilm, we compared thermal conditions across larval colonies (M+L) kept under different rearing temperatures, using paired containers with meat (M) as a reference. The comparison revealed that heat production of a biofilm formed by larvae enlarged with an increase in rearing temperature (rmANOVA for the average heat production, $F_{3,35}$=59.4, N=39, P<0.001, Fig. 5a, b) and was significantly larger than in meat alone at all rearing temperatures, apart from the lowest 16°C (rmANOVA for the average heat production, $F_{1,35}$=87.5, N=39, P<0.001, Fig. 5b).

Carrion biofilm, larval fitness and parental care in *Necrodes littoralis*.

To test if biofilm formed on meat by adult beetles affects fitness of larvae, we compared larval and pupal development times, larval mortality and postfeeding larval mass across colonies with or without meat prepared by adult beetles (M+A+L and M+L trials). Larvae developed significantly shorter after adult beetles prepared the meat (rmANOVA for the average larval development time, $F_{1,27}$=15, N=30, P<0.001, Fig. 6a), differences in the

pupal development time were insignificant (rmANOVA for the average pupal development time, $F_{1,27}$=0.8, N=30, P=0.39, Fig. 6b). Larval mortality was significantly lower in colonies with meat prepared by adult beetles (rmANOVA for larval mortality, $F_{1,27}$=8.3, N=30, P<0.01), differences between M+A+L and M+L trials were the largest in case of the shortest pre-larval phase (Fig. 7a). Increase in the duration of pre-larval phase enlarged larval mortality (rmANOVA for larval mortality, $F_{1,27}$=3.5, N=30, P=0.045, Fig. 7a). Postfeeding larval mass was significantly larger when adult beetles prepared the meat, but only for the shortest pre-larval phase (Fisher LSD post-hoc test, P<0.001, Fig. 7b).

Discussion

We demonstrated that *Necrodes* beetles form a biofilm-like matrix on carrion in which heat is produced and beetle larvae warm up while feeding. This thermoregulation strategy probably involves microbial activity. Carrion biofilm is a mixture of microbes, their metabolites, and compounds released into the matrix directly by beetles or through carrion digestion (24, 26). Microbes, e.g. bacteria involved in composting (51), are known to produce heat as a by-product of their metabolic processes. Biotic component of carrion biofilm may similarly produce heat. Heat may however also be generated directly through beetle actions, for example in exothermal digestion of carrion by beetle exudates (19). Anal exudates of adult *Necrodes* beetles are mixtures of secretory defensive discharges with enteric matter (i.e. excretions) (52). They have antimicrobial effects (37) and, similarly to other carrion beetles (24, 26, 36, 42, 43), probably contain mutualistic microbes. Exudates may however also contain abiotic vectors of heat-generating processes, e.g. enzymes involved in exothermal carrion digestion.

Heat produced in a biofilm formed by adult and larval carrion beetles may benefit them in several, non-mutually exclusive ways. By increasing larval development rate, heat may reduce the time that larvae spend on carrion. Current results support this interpretation. When we compared treatments with and without application of exudates by adult beetles, we found that heat production during larval feeding phase was larger and larval development times were shorter after application of exudates by adult beetles. Shorter larval development times were found also for *Nicrophorus mexicanus* (53) and *Nicrophorus vespilloides* (28) in a full care or prehatch care conditions (i.e. with exudates application by adult beetles) as compared to the no care conditions, and for *Nicrophorus orbicollis* (54) in the biparental care conditions (i.e. more exudates) as compared to the uniparental care conditions. Although temperature of a biofilm was not measured in these studies, and we do not even know if there is any heat production in the biofilm formed by *Nicrophorus* beetles, it is tempting to hypothesize that decrease in larval development time resulted from larger heat production of the biofilm after application of exudates by adult burying beetles.

Benefits of beetles from heating carrion may be less obvious. Heat produced in the biofilm may be an external and social mechanism of immune response to entomopathogenic microbes (55-58). Recent studies of *Nicrophorus* beetles highlighted the importance of beetle-microbe interactions, by indicating that smearing carrion with beetle exudates may simply suppress their microbial competitors (24, 26, 28, 29, 31). Similarly, anal exudates of *Necrodes surinamensis* were found to have a negative effect on bacterial survival (37). Although

previous studies linked the suppression with the production of antimicrobial compounds (24, 26, 29, 38, 39, 41), the heat of the biofilm may act synergistically with these compounds. There is evidence, from other insect groups, that heat may act in this way. A wax moth larvae *Galleria mellonella* (Lepidoptera: Pyralidae), colonizing dead or weak honeybee colonies, were found to elevate temperature when aggregated inside beehives (4, 59). A recent study showed substantial mortality of *Galleria* larvae after infection with *Metarhizium* fungi at 24°C, whereas at 34°C a 10-fold higher dose of the fungus was necessary to reach similar mortality rate, indicating that temperature elevation by communally feeding *Galleria* larvae suppresses entomopathogenic fungi (60). From this point of view, larger heat production in the biofilm formed by larvae after meat preparation by adult *Necrodes* beetles, in association with lower larval mortality in such colonies, suggest that heat of the biofilm may indeed suppress microbial competitors, favoring beetle survival on carrion. This interpretation is also supported by an increase in larval mortality after the extension of the pre-larval phase. The longer this phase, the more time microbes have to multiply and produce harmful metabolites, and the larger is the probability that beetle survival will be affected.

Heat in a carrion biofilm may also support the growth and activity of mutualistic microbes. Duarte et al. (35) indicated that *Nicrophorus* beetles may seed carrion with their inner microbes or replant microbes from carcass gut to cadaver surface. Recent analyses of carrion beetle microbiomes recurrently reported the abundant presence of *Yarrowia* yeasts in beetle guts and in carrion biofilm, suggesting that there is a mutualistic link between the yeasts and carrion beetles (24, 26, 36, 42, 43). Microbial mutualists are probably associated also with *Necrodes* beetles, and the heat in a biofilm may support their growth or activity.

Pukowski (25) suggested that smearing carrion with anal exudates by *Nicrophorus* beetles may favor larval aggregation. Larvae of *Necrodes littoralis* consistently respond to heat in their environment, they aggregate in hot spots and respond to changes in hot spot location by relocating themselves (15). Accordingly, heat produced in a biofilm may stimulate larvae to group in a suitable feeding site on carrion.

An increase in the heat emission of a biofilm formed by larvae after meat preparation by adult beetles, in association with a decrease of larval development time and mortality, is essentially a new form of parental care among carrion beetles. This is also the first demonstration of parental care among Silphinae beetles. Highly elaborated parental care occurs in *Nicrophorus* beetles (Nicrophorinae), with concealment and preparation of small carrion, provisioning of predigested food for young larvae and brood or carrion defense against competitors (14, 25). Full parental care of *Nicrophorus* beetles results in higher larval

survival and mass, compared to broods without care (28, 61). Simple parental care, i.e. clearing carrion of fly larvae and brood guarding against staphylinid predators was also described in the case of *Ptomascopus* beetles (Nicrophorinae) (62, 63). Our findings suggest that simple forms of parental care may be prevalent among silphid beetles. In particular, carrion manipulation by adult beetles with deferred larval benefits may be more common.

Necrodes are communally breeding beetles, abundantly colonizing large vertebrate cadavers (64, 65). Communal breeding in carrion beetles is linked to large cadaver use (66, 67). Difficulties in outcompeting microbial, insect or large vertebrate competitors on such resources, must have favored the evolution of communal strategies for efficient use of large carrion by insects. Manipulation of thermal conditions in carrion microhabitat by communal carrion beetles is an example of such a strategy. Similar thermoregulation strategies may be commonly used on carrion and other "bonanza" resources by insects. This opens up new research opportunities for these, still poorly understood, microhabitats.

Methods

Beetle colony maintenance and general rearing protocols

Laboratory colony was established using adult *Necrodes littoralis* beetles sampled from pig carcasses in alder forest of Biedrusko military range (52°31'N, 16°54'E; Western Poland). Beetles were kept in insect containers (20-30 insects in a container, sex ratio about 1:1) on a humid soil, at room temperature (20-23°C) and humidity (50-60%). Three to five containers were usually maintained at the same time in the laboratory. Colonies were provided with fresh pork meat *ad libitum*.

All experiments were performed in rearing containers with a volume of 1.5 l (initial experiments and experiment 1) or 3.5 l (experiment 2) with about 5 cm layer of humid soil. On one side of a container meat was placed, and on the opposite side, we put wet cotton wool to maintain high humidity and water for the beetles (Fig. 2). To prevent meat from drying out and to mimic skin cover below which *N. littoralis* usually feed and breed on carrion, we covered the setup with aluminum foil.

Thermal imaging and temperature quantification in a biofilm-covered surface

We monitored temperature conditions inside beetle colonies using thermal imaging camera Testo 885-2 with 30° x 23° infrared lens (Testo, Germany), mounted to a tripod while making images. Images were made in a room temperature and humidity (20-23°C, 50-60%), with containers taken outside of the temperature chamber and images made within no more than 2 minutes.

Based on initial tests with pork meat, all temperature measurements were made with the emissivity set on 0.8 and reflected temperature set on 17°C. Heat production of the biofilm-covered surface was defined as a difference between the average temperature of the surface and the background surface temperature. To obtain background temperature, we measured the average temperature of a rectangular soil area located in the upper, meat-opposite side of a container, for the first three days starting from the establishment of a colony. During these initial days, biofilm temperature was usually only a little higher than background temperature, so heat production in the biofilm had negligible effects on the background temperature. Therefore, by choosing the most distant part of a container and by quantifying average soil surface temperature there, we obtained the best measure of the background surface temperature. As a reference against which heat production in the biofilm was quantified, we used grand mean calculated from average background surface temperatures measured during the first three days in each container. Because biofilm-covered

surface enlarged with colony age, and at some time the biofilm covered also soil surrounding meat, it was impossible to quantify heat production always in the same place and within the same surface area. Instead, we quantified the average temperature in a largest possible, circular or ellipsoidal area, depending on the shape of the surface covered with biofilm. We used for this purpose the average temperature calculation tools from IRSoft 4.5 software (Testo, Germany).

Initial experiments

To determine the optimal number of beetle larvae and the optimal quantity of meat, we performed several initial trials. By comparing heat production in larval colonies of different abundance (10, 20, 30, 40, 50 and 100 larvae), we found that normal growth and clear heat production were already present in colonies of 40 larvae (Fig. 8). Accordingly, we used this number in main experiments. During initial trials, we also tested setups with different quantity of meat and decided to use 2 g per larva in Experiment 1 and 3.5 g per larva in Experiment 2. In Experiment 2 we studied effects of heat production on larval fitness, and to maintain optimal growth of larvae, we decided to provide them with more meat than we used to study heat production only (Experiment 1).

Environmental temperature and heat production in carrion biofilm (Experiment 1)

To test the effect of environmental rearing temperature on biofilm heat production, we compared heat emission of carrion biofilm across larval colonies reared under constant temperatures of 16, 18, 20 and 23 °C. Heat production in insect colonies (M+L containers, 40 larvae; 80-85g of pork) was compared against heat production in paired containers with meat only (M containers, 80-85g of pork), with 10 pairs of containers (i.e. replicates) studied in each temperature (in 20 °C nine replicates were done). To establish experimental colonies we sampled at random 40 freshly hatched first instar larvae from our main colony. Containers were kept in temperature chambers (ST 1/1 BASIC or +, POL EKO, Poland). Two temperatures were studied at the same time. Experiments in 18 and 23 °C started on 28 January 2019, and in 16 and 20 °C on 19 March 2019. To have similar temperature conditions within replicate pairs of containers (one container with larvae, the other with meat), we kept them on the same shelf in a chamber. Once a day colonies were taken out of a chamber to make thermal images. Results were analyzed using ANOVA for repeated measure designs in Statistica 13 (TIBCO Software Inc., US) with environmental temperature as an independent variable, container type within the pair (M+L or M) as a repeated measure variable and

average heat production in a biofilm (i.e. heat production averaged across measurement days) as a dependent variable. No outliers were detected. Fisher LSD test was used for post-hoc pairwise comparisons.

Heat production in carrion biofilm and its developmental consequences in different beetle colonies (Experiment 2)

Our main experiment involved comparison of thermal conditions and their developmental consequences between paired colonies: of adult and larval beetles subsequently present on meat (M+A+L) and of larval beetles only (M+L). Because we also wanted to test, if length of the pre-larval phase affects larval fitness, in the experiment we used three durations of the phase (3, 4 and 5 days). Each treatment was replicated 10 times. Pairs of containers (one with adult beetles and larvae, the other with larvae) were treated as replicates. In case of a single pair, insect colonies were established using larvae hatched at the same time (sampled at random from a single rearing container), meat from the same piece of pork and soil from the same package. Moreover, replicate pairs of containers were kept on the same shelf in a chamber. From the other side, there were differences in the origin of meat and soil, age and generation of adult beetles, generation of larvae, temperature chambers used and time in which replicates were made between different batches of replicates. Therefore, biotic and abiotic confounding factors, if present, were largely similar in containers forming a replicate pair. They were however less similar between containers from different replicate pairs. This experimental design enabled us to capture large extent of natural variation and at the same time keep our main treatment robust. Containers were kept in temperature chambers at 23 °C (ST 1/1 BASIC or +, POL EKO, Poland). Beetles were provided with 145-150g of pork. In M+A+L containers 10 adult beetles (sampled at random from our main colony, sex ratio 1:1) were kept for the duration of 3, 4 or 5 days, depending on the treatment. Afterward, meat prepared by adult beetles was transferred to a new container and 40, sampled at random, freshly hatched first instar larvae were added. We had to transfer meat to new containers, because adult beetles usually oviposited during the pre-larval phase and it was difficult to control number of larvae in the containers. In M+L containers meat was decomposing without insects for the duration of 3, 4 or 5 days, depending on the treatment, and then was transferred to a new container. Afterward, 40 freshly hatched first instar larvae were sampled at random from our main colony and were added to a container. Experiments started on 3 March 2019 (3 replicates), 15 March 2019 (7 replicates), 8 May 2019 (10 replicates) and on 5 August 2019 (10 replicates). Once a day colonies were taken out of a chamber to make thermal images.

After larvae stopped feeding and began to bury themselves, we counted and weighed them (laboratory scale AS 82/220.R2, Radwag, Poland). Then, they were transferred to soil-filled Petri dishes (3 larvae per dish), where we monitored postfeeding and pupal development to capture pupation and eclosion times. Results were analyzed using ANOVA for repeated measure designs in Statistica 13 (TIBCO Software Inc., US), with duration of the pre-larval phase as an independent variable and container type within the pair (M+A+L or M+L) as a repeated measure variable. Average heat production in the pre-larval phase or the larval feeding phase (i.e. heat production in a biofilm averaged across measurement days of the phase), average (per colony) larval and pupal development times, larval mortality and average (per colony) mass of postfeeding larvae were used as dependent variables in separate analyses. No outliers were detected. Fisher LSD test was used for post-hoc pairwise comparisons.

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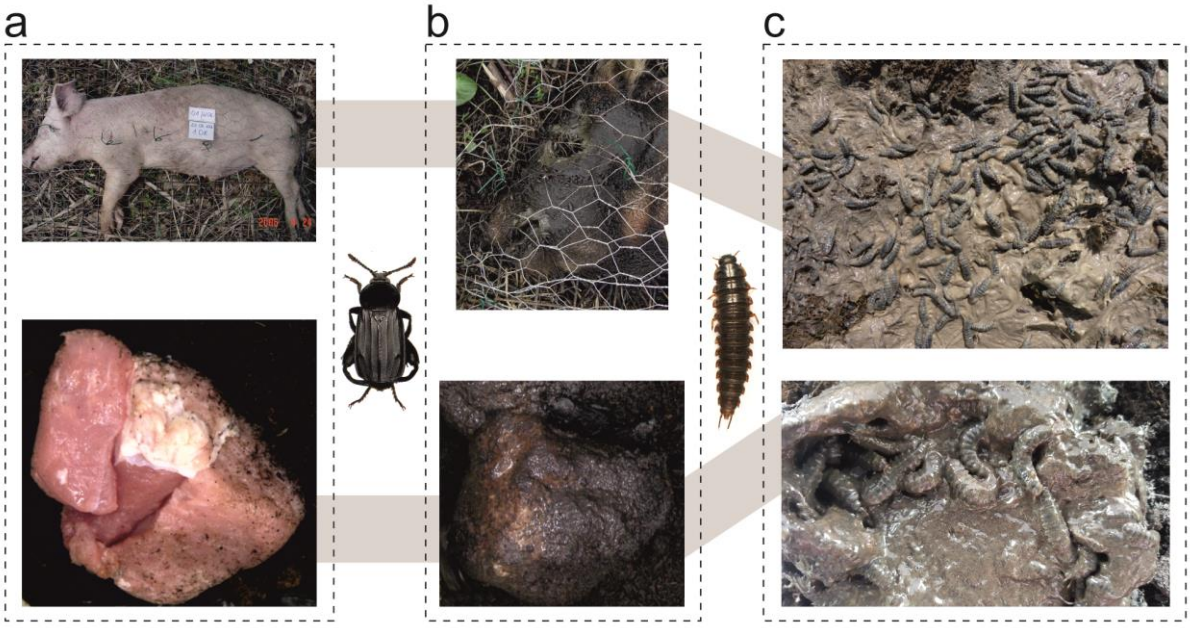

Fig. 1

Biofilm-like matrix formed by adult or larval *Necrodes littoralis* beetles on pig cadaver under field conditions (top panel) and on meat in the laboratory (bottom panel). a – fresh resources, b – biofilm created by adult beetles, c – biofilm formed by larvae.

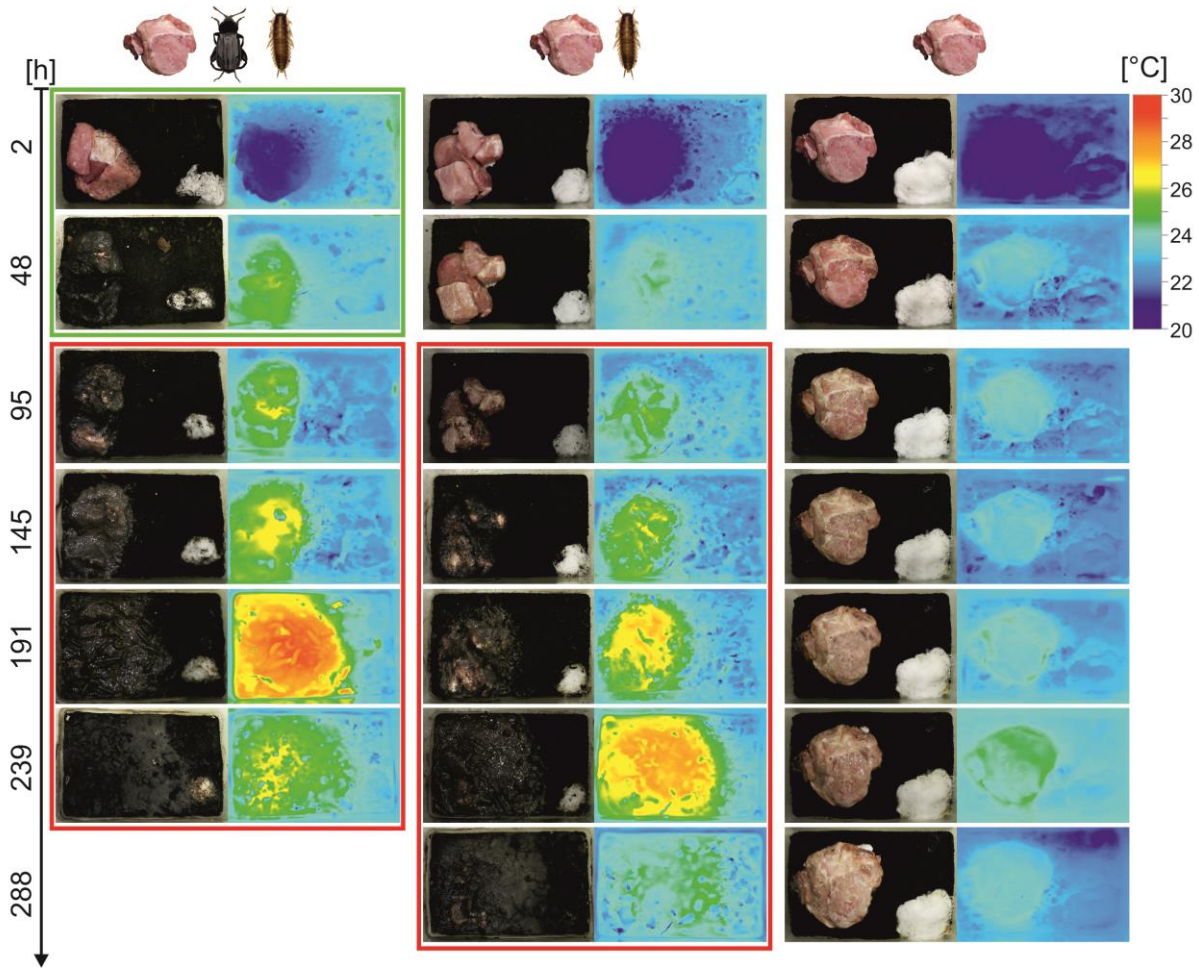

Fig. 2

Heat production of a biofilm in colonies with adult and larval *Necrodes littoralis* (left column), with larval beetles only (middle column) and without beetles (right column). The green frame includes pictures of adult beetle colonies, red frame pictures of larval beetle colonies. Pictures without frame show meat decomposing without beetles.

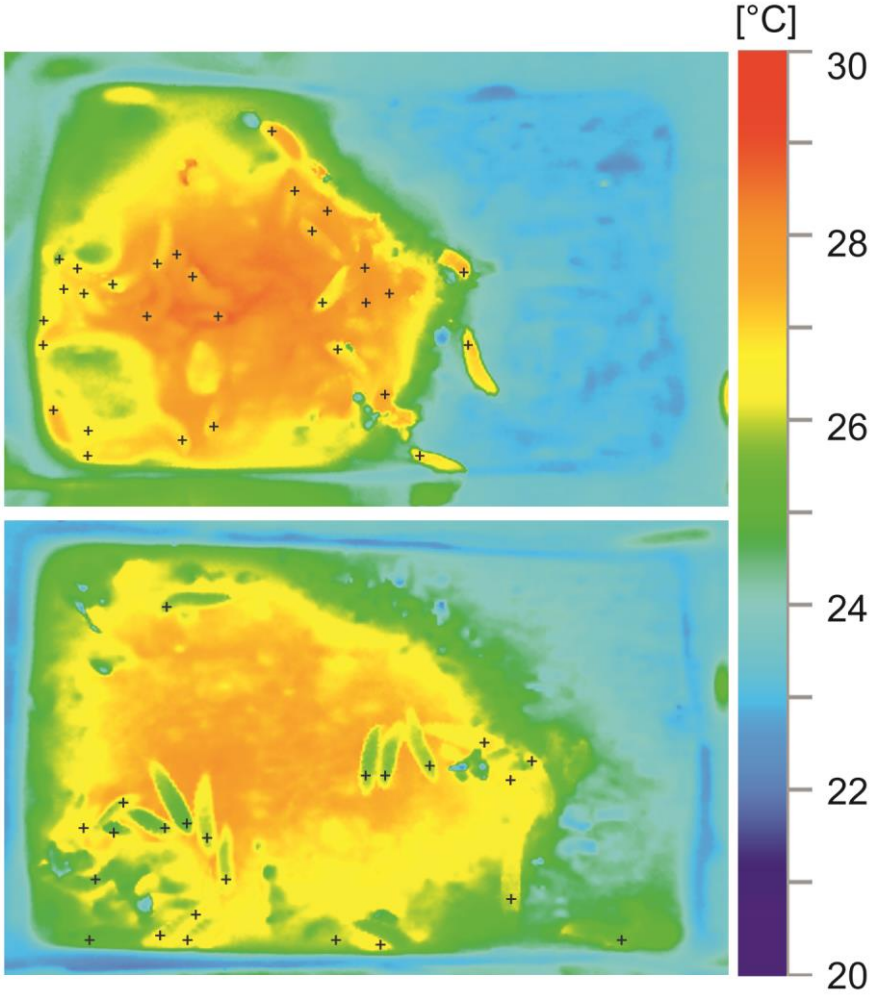

Fig. 3

Larval *Necrodes littoralis* warming up in the biofilm. Insects are marked with "+".

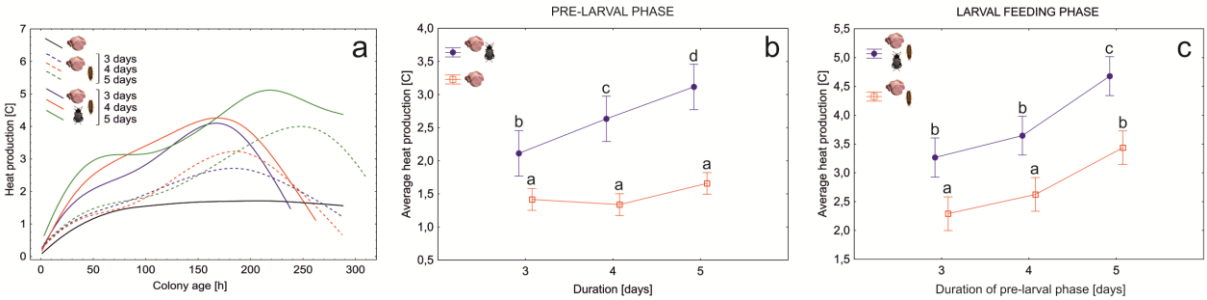

Fig. 4

Longitudinal thermal profiles (a), differences in the average heat production of a biofilm during pre-larval phase (b) and larval feeding phase (c) between *Necrodes littoralis* colonies differing in the presence of adult beetles and in the length of pre-larval phase. All colonies were reared at constant 23°C. As a no-beetle reference in (a), we used results for 23°C from environmental temperature experiment. Thermal profiles were fitted to the data using the distance-weighted least-squares smoothing procedure. Symbols – means, whiskers – 95% confidence intervals, different letters denote significant differences in pairwise comparisons.

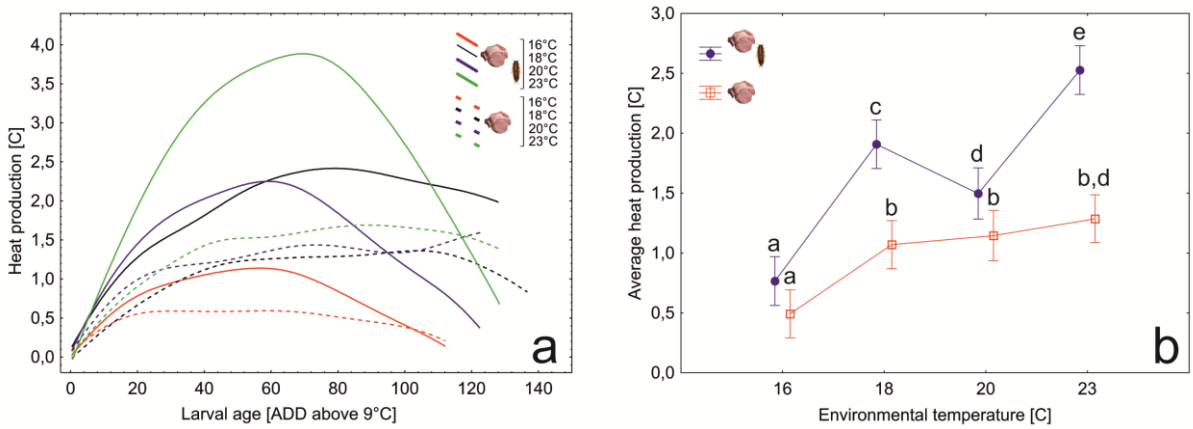

Fig. 5

Longitudinal thermal profiles (a) and differences in the average heat production (b) of a biofilm between colonies of larval *Necrodes littoralis* reared under different constant environmental temperatures, compared against thermal profiles and heat production in meat decomposing without beetles. Thermal profiles were fitted to the data using the distance-weighted least-squares smoothing procedure. ADD – accumulated degree-days, symbols – means, whiskers – 95% confidence intervals, different letters denote significant differences in pairwise comparisons.

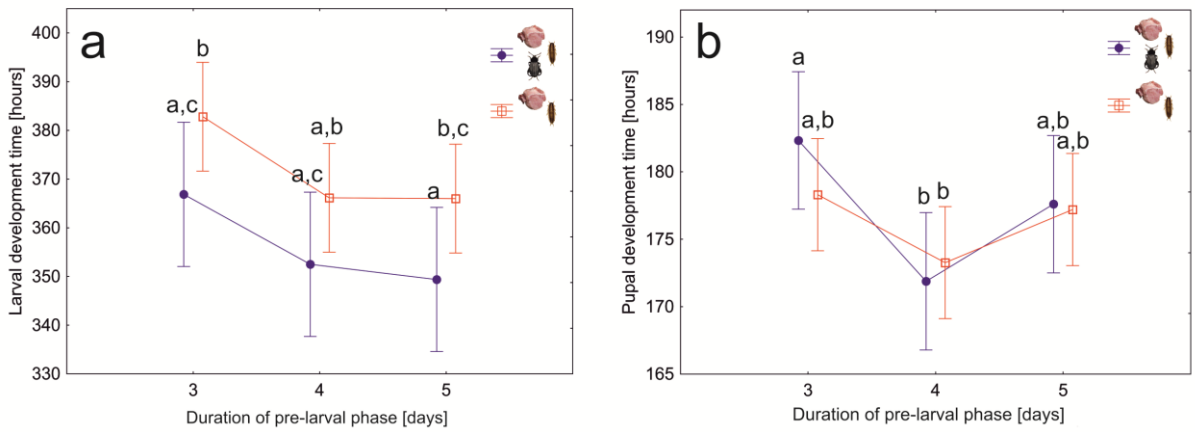

Fig. 6

Average larval (a) and pupal (b) development times for *Necrodes littoralis* colonies differing in the presence of adult beetles during the pre-larval phase and in the duration of this phase. Symbols – means, whiskers – 95% confidence intervals, different letters denote significant differences in pairwise comparisons.

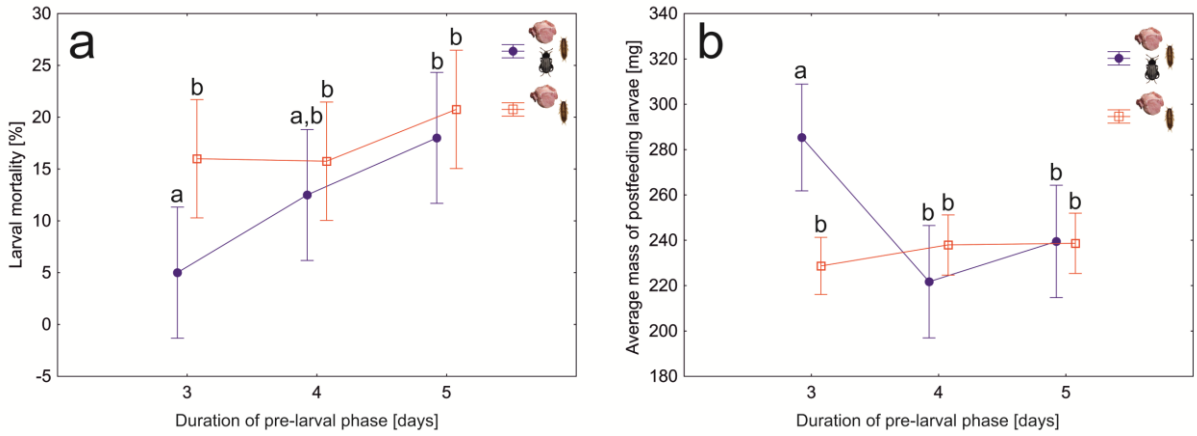

Fig. 7

Larval mortality (a) and average mass of postfeeding larvae (b) for *Necrodes littoralis* colonies differing in the presence of adult beetles during the pre-larval phase and in the duration of this phase. Symbols – means, whiskers – 95% confidence intervals, different letters denote significant differences in pairwise comparisons.

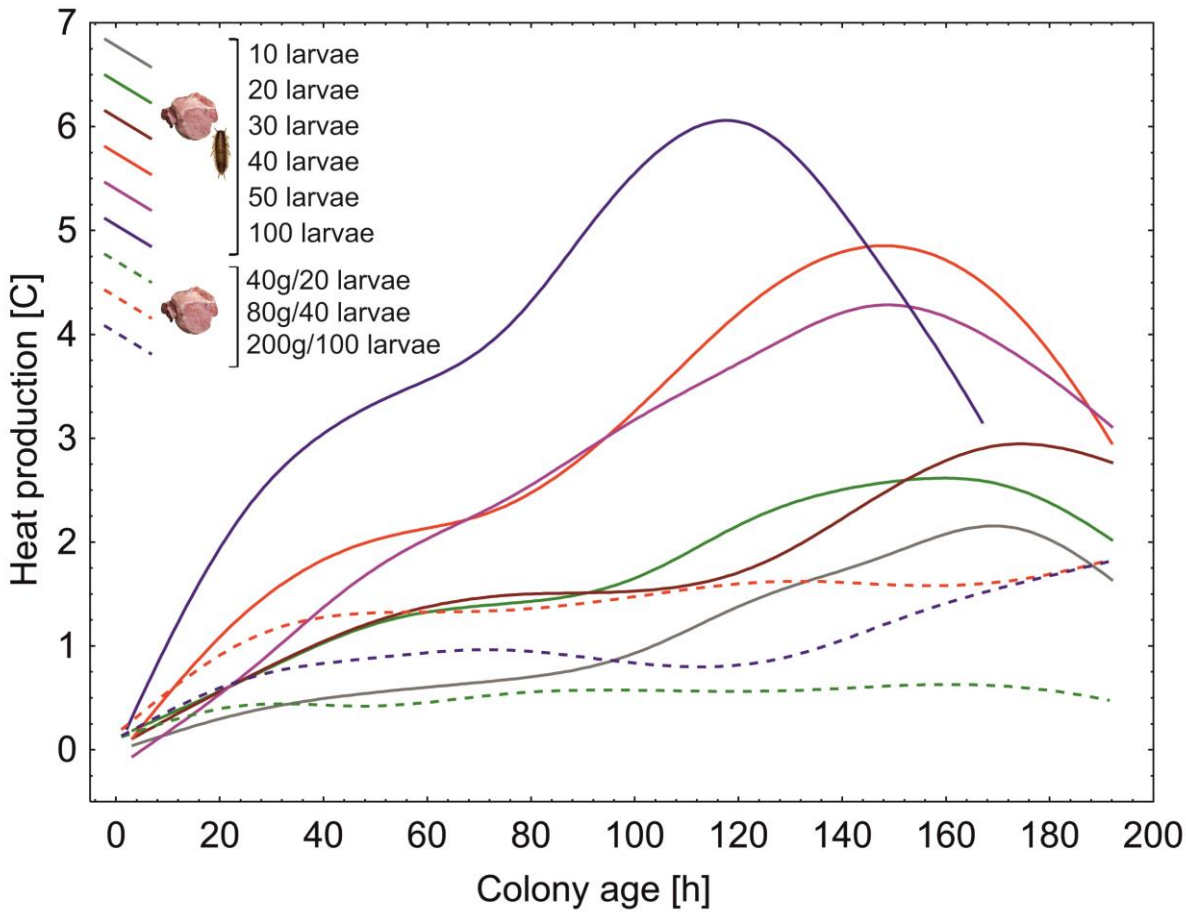

Fig. 8

Longitudinal thermal profiles of carrion biofilm in *Necrodes littoralis* larval colonies (solid lines) differing in the number of larvae and control colonies (dashed lines) differing in the quantity of meat. Colonies were reared at room temperature (20-23°C). Thermal profiles were fitted to the data using the distance-weighted least-squares smoothing procedure.