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Chilling temperatures trigger pupation in Raphidioptera: *Raphidia mediterranea* as a model for insect development

(Insecta, Holometabola)

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Development of Raphidioptera is highly plastic with 9 to 15 larval instars within 1 to 3 (or even more) years. Essential for successful pupation and adult eclosion is a period of low temperature of mature larvae (or pupae). However, the threshold for the length and temperature of this chilling is unknown.

This study reports on development of mature *Raphidia mediterranea* larvae after exposure to different chilling conditions. The first indication of pupation is described by the onset of significant modifications in the anatomy of the head capsule of the mature larva shortly before pupal molt. Increasing length of chilling period at 4 °C resulted in decreased period of time between end of chilling and pupation. Chilling temperatures between 0 °C and 12 °C had no influence on the length of time between end of chilling and pupation. The results highlight the significance of low winter temperature both for the present geographical distribution of Raphidioptera and synchronisation of reproduction.

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Introduction

Raphidioptera is one of the smallest orders of holometabolous Insecta with about 250 known species living exclusively in the Northern Hemisphere (H. Aspöck et al. 1991, 2012, U. Aspöck et al. 2012). They exhibit a larval period which lasts one or more years, and a short adult live of several weeks. This mode of development occurs in many hemimetabolous as well as holometabolous insect orders and includes an arrest in growth or development during unfavorable environmental conditions which was described as dormancy (Danks 1987, Müller 1992). The triggers for arresting and resuming development are complex, and no descriptive terms adequately cover the specific responses of all species (Danks 1987, 2002).

As far as we know, the postembryonic development of Raphidioptera is highly plastic in that the number of larval instars (9 to 15) and its duration (1 to 3 or more years) might differ within a species and between offspring from any individual female (H. Aspöck 2002, Gruppe & Abbt 2018). Larvae survive winter conditions in dormancy but become

immediately active as temperature increases. This corresponds perfectly to the concept of quiescence (H. Aspöck et al. 2018). However, essential for successful pupation is that the mature larvae (or pupae) must be subjected to chilling temperatures prior to the adult stage (H. Aspöck 2002), which is a requirement fulfilled during winter under natural conditions. Knowledge of postembryonic development was largely derived from rearing field collected larvae or from rearing "ab ovo" (H. Aspöck et al. 1974, 1991, H. Aspöck & U. Aspöck 2009, Gruppe & Abbt 2018). An additional outcome of the rearing is that development seems to be not influenced by light. However, detailed studies of the postembryonic development are scarce. Development of Nearctic *Agulla* species was studied by Woglum & McGregor (1958, 1959) and Kovarik et al. (1991). However, these authors failed to consider the effect of cold temperatures during the last hibernation.

H. Aspöck (2002) defined three basic modes of Raphidioptera development (Types I, II, III) that differ in the last hibernating instar and the time period between end of hibernation and emergence of the adult. Most genera belong to 'Type I' (H. Aspöck 2002) with the last hibernation as mature larva and pupation in spring followed by immediate emergence of adults. It is well documented that pupation becomes disordered if the temperature during the last hibernation does not decrease. In these pupae the general habitus remains as that of a larva but pupal characters are more or less markedly developed. This kind of disordered metamorphosis is termed metathetely (incorrectly termed "prothetely" in former publications) (H. Aspöck et al. 2018).

The general lack of research on Raphidioptera development is most probably due to the long duration of development and the fact that most species are difficult to find in the field. Recently both restrictions have been overcome with the description of a simple rearing system (H. Aspöck & U. Aspöck 2009) and the discovery of a large population of *Raphidia mediterranea* H. Aspöck, U. Aspöck & Rausch, 1977, in Austria (Rausch et al. 2016, H. Aspöck et al. 2017, Gruppe et al. 2017). Even with the described rearing system, the emergence rate of adults is about 50 % with the highest mortality at the stage of the late pupa (H. Aspöck et al. 1974). However, the onset of pupation is morphologically visible several days before mold by the appearance of the so called prepupa, which represents the immobile phase of the mature larva.

In the last years we were able to generate large stocks of Raphidioptera larvae under standardized conditions in the lab and to test the effect of different hibernation conditions by chilling groups of larvae for different periods of time and at different temperatures under controlled conditions in the lab. In particular we conducted experiments to study the response of *R. mediterranea* to artificial chilling.

Material and methods

Females of *R. mediterranea* originated from the extensively described population in Pelmberg, Austria (Rausch et al. 2016, H. Aspöck et al. 2017, Gruppe et al. 2017), and were collected in June/July 2014 and 2015. They were caged for oviposition in plastic vials according to H. Aspöck & U. Aspöck (2009). Second or third instar larvae were transferred into individual vials. Before onset of the chilling treatments in the second year all larvae were kept under identical conditions: 20 °C, 16 h light / 8 h dark and ~60 % relative humidity until October and from October to March (first hibernation) at 4 °C in constant darkness. Larvae were fed weekly with a piece of a meal worm (*Tenebrio molitor*) from a pet shop from March to October. To be on the safe side, food was offered once a month in winter. We designed two experiments to analyze: (i) the relevance of different lengths of the chilling period (CP) at a set temperature and (ii) the significance of different chilling temperatures (CT).

The CP-experiment (length of chilling period): By September 30, 2015, mature larvae, which had hatched from eggs in July 2014, were divided into batches of 32 individuals each and transferred to a climatic chamber at 4 °C (permanent darkness). After 4, 8, 12, 16 or 20 weeks larvae were kept at 20 °C under long day conditions (16 h light / 8 h dark). Thereafter larvae were fed and their development was checked once a week until appearance of the prepupa which was judged as the onset of pupation. The time period between the end of chilling and prepupa stage was termed "post-chilling period" (PCP). The experiment was terminated 27 weeks after the end of chilling. From that data, the cumulative percentage of prepupae in any week was calculated to visualize modes of development. We tested the PCP for significant differences with Kruskal-Wallis test and post hoc Tuckey-test.

The CT-experiment (temperature of chilling): By November 22, 2016, mature larvae, which had hatched from eggs in July 2015, were assigned to 10 batches of 21 individuals each. Two batches were removed and kept at −8 °C, 0 °C, +4 °C, 12 °C or 20 °C (under permanent darkness), respectively. One batch from each treatment was transferred to 20 °C (16 h light / 8 h dark) after 8 weeks, the other after 16 weeks. Thereafter all individuals were supplied with food and surveyed for prepupae once a week. The experiment was terminated seven weeks after the end of chilling (PCP). From that data, the cumulative percentage of prepupae in every week was calculated to visualize the modes of development. We tested the PCP for significant differences between different chilling temperatures with Kruskal-Wallis test.

Fig. 1. Mature larva of *Raphidia mediterranea*.
Fig. 2. Head of a mature *R. mediterranea* larva; pigmented spot covers all stemmata (arrow).
Fig. 3. Head of a mature larva resp. prepupa, pigmented spot has begun to migrate.
Fig. 4. Head of mature larva as prepupa with pigmented spot (solid arrow) distinct from stemmata (broken arrow).
Fig. 5. Fully developed prepupa of *R. mediterranea*.

Results

Prior to pupation, Raphidioptera larvae exhibit alterations in morphology leading to the so called prepupa which occurs equally in all observed individuals. Alterations are related to the head and the habitus. In mature, highly mobile larvae (Fig. 1), there is a darkly pigmented spot close to the base of the antenna, which spans the seven stemmata at the lateral margin of the head capsule (Fig. 2). As the first indication of the prepupal stage, the pigmented spot migrates caudally to the middle of the head capsule (Fig. 3). In this phase, the anterior part of the larva bends ventrally lying on lateral side and is able to turn ventrally and move. When the pigmented spot has reached its final position after approximately 24

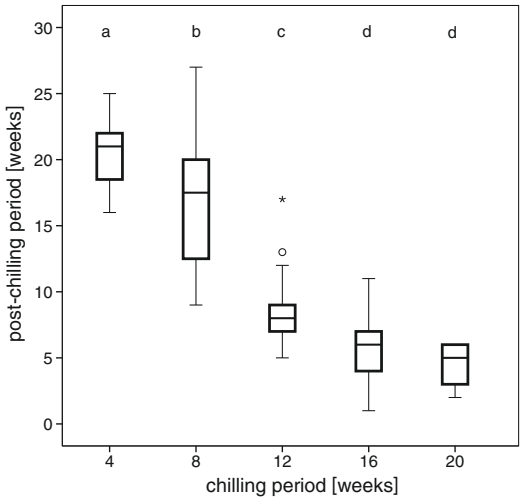

Fig. 6. Post-chilling period (in weeks) of larvae of *R. mediterranea* after 4 to 20 weeks of chilling period at 4 °C.

hours, the larva becomes immobile but remains able to twist its body when mechanically irritated (Fig. 4). In this position (Fig. 5) the prepupa rests until pupal ecdysis for one to two days.

The CP-experiment shows that chilling at 4 °C was sufficient to fulfill the requirements for pupation of *R. mediterranea* with an overall survival rate of 81.6 %. However, the length of the "post-chilling period" (PCP) decreased significantly with the length of chilling period (CP) (Fig. 6). After 4 weeks of CP, the larvae first developed into prepupae after 16 weeks of PCP, whereas after 16 weeks of CP, the first prepupae developed within one week of PCP. However, there was no significant difference in PCP between 16 and 20 weeks of cold exposure (Fig. 6). The slope of the cumulative development indicates a broad range of reaction at low CP treatments of 4 and 8 weeks, whereas at longer CP times the range of reaction is narrower (Fig. 7).

In the CT-experiment, the survival rate increased from 0 % at CT −8 °C (for both the 8-week and the 16-week CP groups) to 66.7 % (for the 8-week CP group) and 85.7 % (for the 16-week CP group) at CT 12 °C. At CT 20 °C survival rate decreased (Table 1). No significant differences in PCP were found between any CT, neither for the 8-week CP group nor the 16-week CP group (Fig. 8). The slope of the cumulative development from 0 °C to 12 °C is similar to the control at 20 °C at 8 weeks CP but barely half of the specimens reached prepupae within 7 weeks PCP (Fig. 9). In contrast, nearly all specimens of the 16-week CP treatment became prepupae after this time (Fig. 10).

Discussion

Pupation of Raphidioptera usually occurs after one or two years (H. Aspöck 2002) of larval life. We studied the response of *Raphidia mediterranea* larvae, which in general exhibit a two-year development to different temperature scenarios during their second

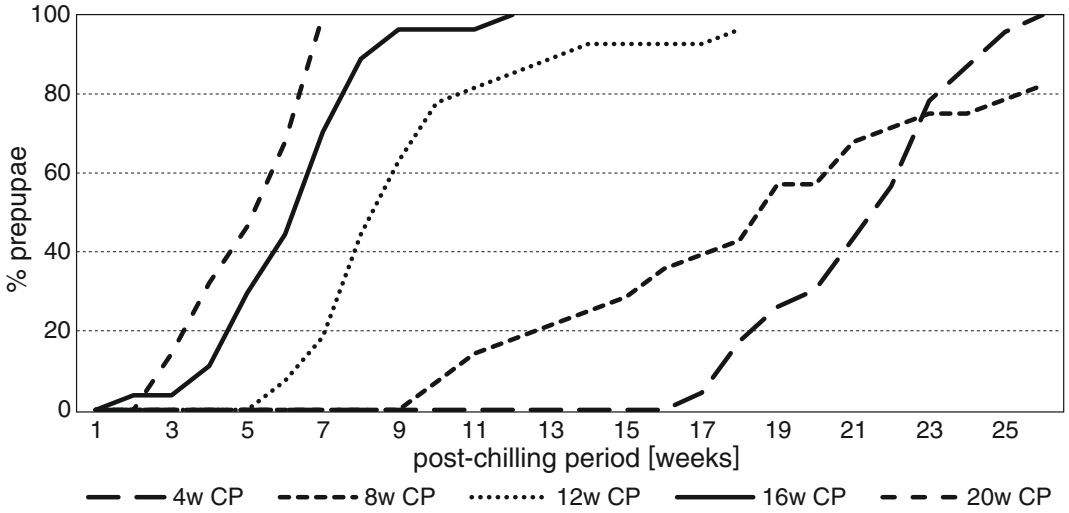

Fig. 7. Time until development of larvae of *R. mediterranea* into prepupae (post-chilling period) after 4 to 20 weeks chilling period (CP) at 4 °C.

hibernation. Timing of pupation was more strongly influenced by the duration of the length of chilling period than by the chilling temperature itself. This is the first experimental result regarding the influence of temperature on the regular development of Raphidioptera larvae (but see H. Aspöck et al. 2018, which was concerned with disordered development).

Developmental experiments with insects exhibiting a long larval period have rarely been conducted. Such species must cope with changing environmental conditions several times in their lifespan and survive unfavorable conditions in dormancy i. e. in a mode of suppressed development and growth (Danks 2002). The most important cues for timing of dormancy are photoperiod and temperature (summary in Danks 1987, Müller 1992). With regard to development, Raphidioptera are apparently not sensitive to changes in the photoperiod but to temperature since no differences were recorded in the development of larvae kept under a natural photoperiod or in permanent darkness (H. Aspöck & U. Aspöck 2009). However, the temperature during the second (last) hibernation determined normal or aberrant pupation (Woglum & McGregor 1959, H. Aspöck et al. 1991, H. Aspöck 1998, H. Aspöck & U. Aspöck 2009, U. Aspöck & H. Aspöck 2007, 2009, H. Aspöck et al. 2018). Even under optimal conditions for hibernation, the emergence rate of adults was hardly above 50 % in the lab with the highest mortality at the stage of pupa (H. Aspöck et al. 1974). To avoid this bias we chose the first signs of the prepupa, i. e. dislocation of the dark spot spanning all seven larval stemmata, to indicate metamorphosis. The prepupae of Raphidioptera have been discussed in several publications (Metzger 1960, H. Aspöck et al. 1991) but were never described in detail. We determined that the distinct change involving the larval stemmata was clear proof of the prepupal stage and pending pupation.

Chilling temperature of 4 °C fulfills the requirements of *R. mediterranea* to induce pupation. However, larvae must be kept at these conditions for 12 weeks to reach 50 % pupation within seven weeks. Shorter or longer chilling periods either prolong or shorten prepupal development. Thus, after a long chilling period, pupation will soon commence. Regarding chilling, both normal pupation and ecdysis occur after thermal treatment between 0 °C and 12 °C with no effect of the temperature itself on the post-chilling period.

Table 1. Survival rate of *Raphidia mediterranea* larvae in the CT experiment.

Chilling temperature	Chilling period 8 weeks	Chilling period 16 weeks
20 °C	38.1 %	9.5 %
12 °C	66.7 %	85.7 %
4 °C	52.4 %	61.9 %
0 °C	31.8 %	38.1 %
−8 °C	0 %	0 %

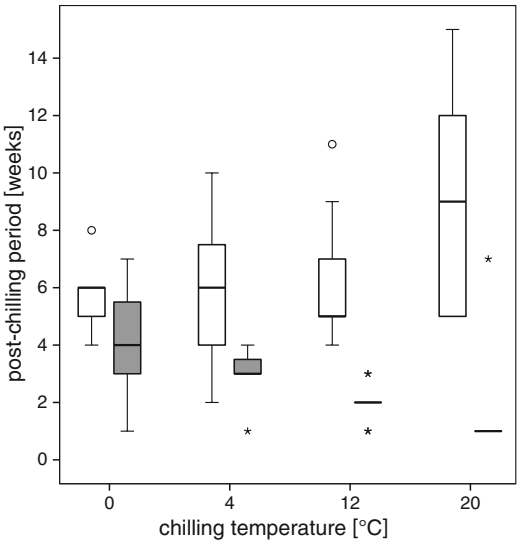

Fig. 8. Post-chilling period (in weeks) of larvae of *R. mediterranea* which were chilled for 8 weeks (white boxes) or 16 weeks (dark boxes) at different chilling temperatures.

R. mediterranea occurs in the Eastern Mediterranean region from the seashore to 1000 m a. s. l. (H. Aspöck et al. 1991) and also in continental parts of Europe (Rausch et al. 2016, H. Aspöck et al. 2017). Thus adults emerge after vastly different winter temperatures (chilling) from above 0 °C to far below 0 °C. This plasticity might be a species-specific adaptation to the distribution range of *R. mediterranea*. Complete mortality at −8 °C in our experiment might be an artefact since we directly transferred larvae to −8 °C without any period of adaptation which would normally occur under natural conditions in autumn. However, in the control treatment at 20 °C, development was especially slow and several metathetelous pupae were recorded (data not shown).

This study is the first analysis of the thermal requirements of Raphidioptera needed to resume development and ecdysis after hibernation in the last larval instar, i. e. of a species exhibiting Type I development (H. Aspöck 2002). We know that development in Raphidioptera is highly plastic and depends on environmental conditions (Woglum & McGregor 1959, H. Aspöck 1998, Gruppe & Abbt 2018). A surprising plasticity is also exhibited within single species, but this phenomenon has not yet

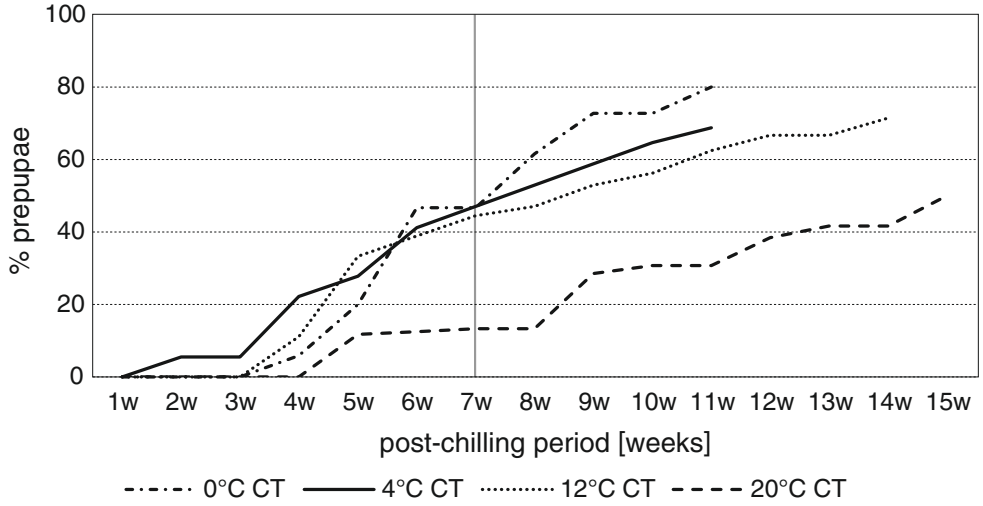

Fig. 9. Time until development of larvae of *R. mediterranea* into prepupae (post-chilling period) after chilling at different temperatures (CT) for 8 weeks (the grey line indicates the termination of the experiment for comparison to Figure 10).

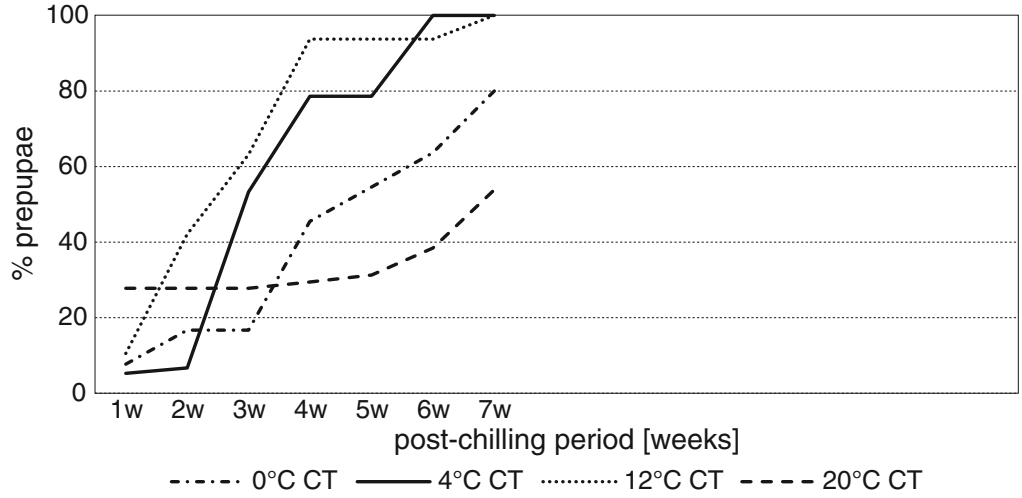

Fig. 10. Time until development of larvae of *R. mediterranea* into prepupae (post-chilling period) after chilling at different temperatures (CT) for 16 weeks.

been understood. Adults reared under the same conditions "ab ovo" emerge after one, two or three years but emergence takes place in a short time within the year (H. Aspöck et al. 1991, Gruppe & Abbt 2018). This corresponds to adult emergence in natural habitats. Adults can be found in a relatively short, species-specific period of time of two to several weeks (H. Aspöck et al. 1991). Trees and even tree crowns which serve as the habitat of bark dwelling Raphidioptera exhibit strong microclimatic gradients (Geiger 1965, Parker 1995, Shaw 2004, Gruppe et al. 2008) that lead to different thermal winter conditions even within a small spatial range. Consequently, we hypothesize that the advantage of the described mode of dormancy for Raphidioptera, aside from protecting damageable stages against adversity, is the synchronization of adult emergence and reproduction (Danks 1987, Jenkins et al. 2001, Régnière et al. 2012). Further studies are needed to demonstrate the effects of chilling conditions on the development of species originating from other climatic regions.

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Contribution of authors

A.G., H.A. and U.A. wrote the text and supervised V.A. who wrote her MSc-thesis on this topic; V.A. performed some of the experiments and of the data analysis as part of her MSc-thesis.

References

Aspöck, H. 1998. Distribution and biogeography of the order Raphidioptera: updated facts and a new hypothesis. Pp. 33–44 in: Panelius, S. (ed.). Neuropterology 1997. Proceedings of the Sixth International Symposium on Neuropterology, Helsinki, Finland, 13–16 July 1997. Acta Zoologica Fennica 209.

–– 2002. Biology of Raphidioptera: a review of present knowledge. Pp. 35–50 in: Sziráki, G. (ed.). Neuropterology 2000. Acta Zoologica Academiae Scientiarum Hungaricae 48, Supplement 2.

–– & Aspöck, U. 2009. Raphidioptera – Kamelhalsfliegen. Ein Überblick zum Einstieg. Entomologica Austriaca 16: 53–72.

–– , Abbt, V., Aspöck, U. & Gruppe, A. 2018. The phenomenon of metathetely, formerly known as prothetely, in Raphidioptera (Insecta: Holometabola: Neuropterida). Entomologia Generalis 37 (3–4): 197–230.

–– , Aspöck, U., Gruppe, A., Sittenthaler, M. & Haring, E. 2017. Anthropogenic dispersal of a snakefly (Insecta, Neuropterida) – a singular phenomenon or a model case in Raphidioptera? Deutsche Entomologische Zeitschrift 64 (2): 123–131.

–– , Aspöck, U. & Rausch, H. 1991. Die Raphidiopteren der Erde. Eine monographische Darstellung der Systematik, Taxonomie, Biologie, Ökologie und Chorologie der rezenten Raphidiopteren der Erde, mit einer zusammenfassenden Übersicht der fossilen Raphidiopteren (Insecta: Neuropteroidea). Krefeld (Goecke & Evers).

–– , Liu, X-Y. & Aspöck, U. 2012. The family Inocelliidae (Neuropterida: Raphidioptera). A review of present knowledge. Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 18: 565–573.

–– , Rausch, H. & Aspöck, U. 1974. Untersuchungen über die Ökologie der Raphidiopteren Mitteleuropas (Insecta, Neuropteroidea). Zeitschrift für Angewandte Entomologie 76: 1–30.

Aspöck, U. & Aspöck, H. 2007. Verbliebene Vielfalt vergangener Blüte. Zur Evolution, Phylogenie und Biodiversität der Neuropterida (Insecta: Endopterygota). Denisia 20: 451–516.

–– & Aspöck, H. 2009. Raphidioptera (Snakeflies). Pp. 864–866 in: Resh, V. H. & Cardé, R. T. (eds). Encyclopedia of insects. 2nd ed., Amsterdam (Academic Press, Elsevier).

–– , Haring, E. & Aspöck, H. 2012. Biogeographical implications of a molecular phylogeny of the Raphidiidae (Raphidioptera). Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie 18: 575–582.

Danks, H. V. 1987. Insect dormancy: an ecological perspective. Monograph Series No 1, Ottawa, (Biological Survey of Canada).

–– 2002. The range of insect dormancy responses. European Journal of Entomology 99: 127–142.

Geiger, R. 1965. The climate near the ground. Cambridge, MA (Harvard University Press).

Gruppe, A. & Abbt, V. 2018. Larval biology of *Mongoloraphidia sororcula* (H. Aspöck & U. Aspöck, 1966) (Insecta: Raphidioptera: Raphidiidae). Spixiana 41 (1): 27–32.

–– , Aspöck, H. & Aspöck, U. 2017. Das Strohdach des Mittermayr Hofs in Pelmberg (Oberösterreich) als Kinderstube und Puppenwiege einer mediterranen Kamelhalsfliege (Insecta: Neuropterida: Raphidioptera: Raphidiidae). Linzer Biologische Beiträge 49 (1): 577–583.

–– , Goßner, M., Engel, K. & Simon, U. 2008. Vertical and horizontal distribution of arthropods in temperate forests: from ground level to the canopy. Pp. 383–405 in: Floren, A. & Schmidl, J. (eds). Canopy arthropod research in Central Europe. Nürnberg (Bioform).

Jenkins, J. L., Powell, J. A., Logan, J. A. & Bentz, B. J. 2001. Low seasonal temperatures promote life cycle synchronisation. Bulletin of Mathematical Biology 63: 573–595.

Kovarik, P. W., Burke, H. R. & Agnew, C. W. 1991. Development and behavior of a snakefly, *Raphidia bicolor* Albarda (Neuroptera: Raphidiidae). Southwest Entomologist 16 (4): 353–364.

Metzger, R. 1960. Die Kamelhalsfliegen. Neue Brehm-Bücherei 254, Wittenberg (Ziemsen).

Müller, H. J. 1992. Dormanz bei Arthropoden. Jena (Gustav Fischer).

Parker, G. G. 1995. Structure and microclimate of forest canopies. Pp. 73–106 in: Lowman, M. D. & Nadkarni, N. M. (eds). Forest canopies. 1st ed., San Diego (Academic Press).

Rausch, H., Aspöck, H. & Aspöck, U. 2016. Rätselhaftes Massenauftreten einer mediterranen Kamelhalsfliege im Mühlviertel, Oberösterreich (Insecta: Neuropterida: Raphidioptera: Raphidiidae). Linzer Biologische Beiträge 48(1): 523–534.

Régnière, J., Powell, J., Bentz, B. & Nealis, V. 2012. Effects of temperature on development, survival and reproduction of insects: experimental design, data analysis and modeling. Journal of Insect Physiology 58: 634–647.

Shaw, D. C. 2004. Vertical organization of canopy biota. Pp. 73–101 in: Lowman, M. D. & Rinker, H. B. (eds). Forest canopies. 2nd ed., Amsterdam (Elsevier Academic Press).

Woglum, R. S. & McGregor, E. A. 1958. Observations on the life history and morphology of *Agulla bractea* Carp. (Neuroptera: Raphidiodea: Raphidiidae). Annals of the Entomological Society of America 51: 129–141.

– – & McGregor, E. A. 1959. Observations on the life history and morphology of *Agulla astuta* (Banks) (Neuroptera: Raphidiodea: Raphidiidae). Annals of the Entomological Society of America 52: 489–502.