Physiological Entomology (2007) DOI: 10.1111/j.1365-3032.2007.00583.x

Tenebrio beetle pupae show a conditioned behavioural response to pulse rotations of a geomagnetic field

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Abstract. Pupae of holometabolous insects are not motionless and insensitive developmental stages. Their behavioural display is restricted to rotations or contractions of the abdomen in a range of movements from the clearly visible to the microscopic of the order of tenths of microns. Pupae react spontaneously and surprisingly sensitively to mechanical, light or sound stimuli. In the present study, reactions of yellow mealworm beetle (*Tenebrio molitor*, L.) pupae to a geomagnetic field rotation are examined. By means of a micromechanical recording technique, peaks of abdominal contractions are monitored before and after magnetic treatment and show that spontaneous behavioural reaction to a magnetic pulse is insignificant. Nevertheless, using negative reinforcement training, a conditioned magneto-sensitive reaction is elicited. These surprising sensory and behavioural capacities of an insect pupa are discussed.

Key words. Abdomen, behaviour, conditioning, extracardiac pulsations, insects, magnetoreception, movement, pupa, *Tenebrio molitor*.

Introduction

Magnetoreception, the capacity to perceive the magnetic field of the Earth (geomagnetic field), is among the least understood animal senses. Recently, the most advanced research has been performed on vertebrate magnetoreception (Wiltschko & Wiltschko, 2006), but insect magnetosensitivity has been reported also in a number of experiments during the last two decades (Vácha, 2006).

Although insect magnetoreception as such has been proven repeatedly, the challenging problems of where the putative receptor is located in the insect's body, or by what mechanism it operates, remain to be solved. Even the fundamental question of what value this capacity may be to its owner is hard to answer satisfactorily in some cases.

Most of the published evidence of magnetoreception by insects has been acquired by monitoring behaviour in relation to the geomagnetic field either in a laboratory or in the field. In experiments designed as either conditioned or nonconditioned, the most frequently observed phenomena are locomotory activity, body positions in relation to the geomagnetic field axis, visits to the insect feeder, and the direction that the insects prefer when released in a laboratory arena or during outdoor migration.

To date, only adult insects have been investigated with positive results in accordance with the *a priori* expectations of the imago's most developed sensory capacities necessary for their active way of life and reproductive tasks. In considering preimaginal stages, neuroethological studies should benefit from the simpler nervous circuits and restricted spectrum of behaviour (Scherer *et al.*, 2003; Boyle & Cobb, 2005). However, no experimental evidence of magnetoreception abilities of preimaginal stages of insects has been published to date. A finding of such a model might extend the framework of methods necessary to explain the phenomenon of magnetoreception.

The yellow mealworm beetle (*Tenebrio molitor* L.) is one species in which magnetoreception has been found repeatedly under laboratory conditions. The magnetic compass sense of adult beetles was originally described by Arendse & Vrins (1975) and later verified by Vácha & Soukopová (2004).

In the present work, pupae of *Tenebrio* were studied. Pupae were used because, for holometabolous insects, the pupa represents a period of life when a substantial rebuilding of larval body structures into the imaginal body occurs. The metamorphic processes run as hidden events under the cuticle shield. Outwardly, the pupa displays a motionless developmental

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stage that is not much concerned about its surroundings, and possibly insufficient receptors to do so; however, this external passivity is illusory: micromechanical recordings of abdominal movements reveal regular organized patterns of pulsations changing with the development and ageing of a pupa (Sláma, 1984; Vácha, 1997). These regular contractions of intersegmental muscles are understood in connection with the vegetative metabolic events of the metamorphosing organism. They are believed to enhance both the circulation of haemolymph and tracheal ventilation, termed the extracardiac haemocoelic pulsations or the autonomic coelopulse system (Sláma, 2000).

In intervals of true rest (i.e. between bursts of large abdominal contractions of the order of tens to hundreds of microns), the opportunity arises to observe reactions of pupa to stimuli from the outside world. The movement response (e.g. to a touch to the head) is spectacular and involves surprisingly energetic abdominal rotations: the pupa uses its modest movement potential to repulse an enemy or to change its body position. Similarly, some other kinds of stimuli (e.g. light or sound) may also elicit movement (irritation) reactions, although wagging of the abdomen is not necessarily as strong as in the case of touch. Irritation reactions may occur only as a tiny abdominal contraction of the order of tenths of microns, or as a change of heart rhythm, both of which are measurable if ultrasensitive recording of abdominal movements are made. These small irritation reactions are surprisingly responsive to a variety of very weak stimuli, such as vibrations or noise. This fact complicates the present search for a magnetosensitive behavioural response. Surprisingly, sensory capacities of pupae may not be as less developed as those of adult insects as they may seem.

Pupal behaviour, compared with the more diverse behavioural repertoire of larvae and adults, is reduced to abdominal movements only, making an extraordinarily simple system that is not restrained or stressed by tethering. The pupae do not have to feed, or crawl; they do not attempt to mate or escape; and all their behavioural manifestations are concentrated into a single parameter.

The present study proceeded from the fact that a biological model with a very simple behavioural display whose sensitivity to geomagnetic field is likely but generally unexplored is disposable. A behavioural reaction of mealworm beetle pupae, tethered in a head-to-the-north position, to a short rotation of a horizontal component of the natural geomagnetic field by 60° was monitored. The working hypothesis was that if the pupa could perceive the direction of geomagnetic field, then a rotation of the horizontal magnetic component may be interpreted as a disturbing signal and may cause a detectable irritation response. In preliminary experiments, an innate spontaneous reaction of pupae to a 60° counterclockwise rotation of the horizontal component of the geomagnetic field by was tested without success. In subsequent experiments, classical conditioning by means of negative reinforcement succeeded: pupae were trained to associate a magnetic stimulus with a short irritant heat shock. After a couple of training cycles, pupae were exposed to a magnetic stimulus alone, and a conditioned irritation reaction was sought.

Materials and methods

Animal larvae

The mealworm beetle were kept on a dry nutrient medium composed of crushed wheat and dried yeasts at a temperature of 25–27 °C under an LD 12 : 12 h photoperiod. Young pupae, aged 0–6 h (determined according to the cuticle pigmentation), were selected for the recording. Recording of abdominal movements started on the next day (1-day-old pupae) and continued for 2 more days (3-day-old pupae). From 4 days onward, records were not taken because the quiescent periods between vegetative pulsations became very short. Ecdysis occurred at approximately 8 days under these conditions.

Magnetic conditions

The geomagnetic field in the basement laboratory had the parameters: horizontal component = 20 μT, vertical component = 44 μT, inclination = 65° (measured by an HMR 2300 magnetometer (Honeywell, Phoenix, Arizona) and EDIS Software (Kosice, Slovakia).

The horizontal component of the natural geomagnetic field was experimentally rotated by 60° counterclockwise using a Helmholtz coil (diameter 18 cm) fed by the D/A converter output of a personal computer. The axis of the coil made an angle of 120° with the main north–south axis. The pulse shifting the north direction by 60° was rectangular and lasted for 10 s; the current was 7.3 mA (Fig. 1).

Movement recordings

Recordings of abdominal movements were as described in detail by Vácha (1997). In brief, a pupa, fixed by its ventral

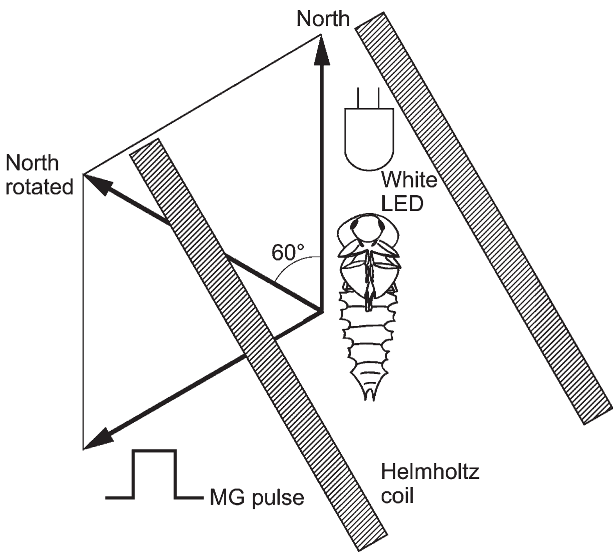

Fig. 1. Position of pupa and Helmholtz coil towards the geomagnetic field. The rectangular pulse lasting for 10 s shifted the north position by 60°. The illumination was provided by the light-emitting diode.

side to a heat bed by paper glue, had a thin wire loop glued onto the tip of its abdomen (Fig. 2), so that movements were transmitted by a thin wire attached to the movable shutter of the optoelectronic transducer with very low mechanic resistance. An electrical signal emanating from the photoelectric cell was amplified (amplifier EXT-MC, Experimetria, Hungary); and via an A/D converter, recorded onto a PC using the registration software (ScopeWin, Czech Republic). To eliminate a slow drift of the signal and to prevent the signal from running off the scale, the input into the amplifier was filtered by a simple capacity high pass filter.

Heat shock at training

An irritant heat stimulus was applied together with a magnetic pulse in the conditioned training experiments (Fig. 2). The heat bed, to which the pupa was glued, consisted of a transistor with a nonmagnetic metallic heat-dispersing face with dimensions 3 × 4 mm. The current conducted through the transistor was of rectangular shape and lasted for 15 s at the value of 50 mA. The maximum value of the heat bed temperature reached approximately 60 °C at the end of 15-s interval when the current was switched off.

Light conditions

During the experiments under light, the head of the pupa was illuminated anteriorly in the direction of the body axis by a white light-emitting diode (LED) placed 3 cm away from the pupa's head. The light intensity at the head level was 0.2 ± 0.1 W m^{-2} (IL 700, SHD 033 probe; International Light, Peabody, Massachusetts).

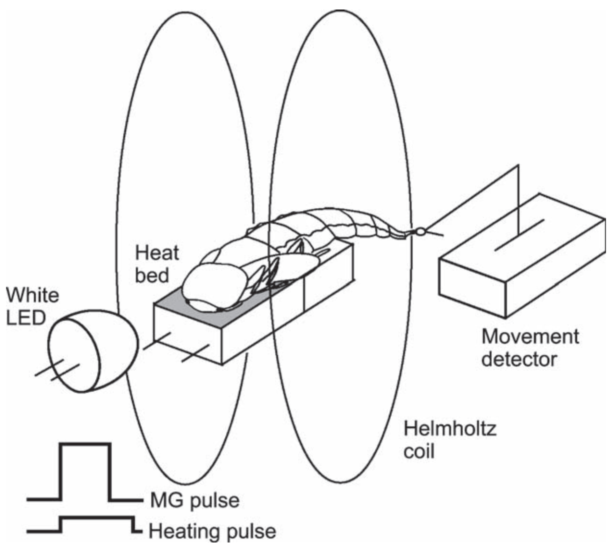

Fig. 2. Overall layout of the experiment. The pupa was fixed by glueing its ventral side to the heat bed. The abdominal contractions were transmitted to the movement detector and recorded on a personal computer. In conditioning experiments, a magnetic pulse (10 s) was applied together with a heating pulse (15 s). The temperature peaked at the moment of switching the heat pulse off.

Overall layout of the experiment

The pupa, LED, Helmholtz coil and sensor were enclosed in a wooden box of inner dimensions 30 × 30 × 50 cm, equipped with a 10-cm foam rubber layer protection against noise. The entire box was laid on a table with its board insulated from vibrations. To prevent transmition of hypothetically potential microvibrations, the Helmholtz coil was mechanically separated and insulated by the foam rubber from the holder of the pupa and sensor. All equipment in close vicinity of the pupa was made of nonferromagnetic materials. To ensure that magnetic pulses themselves did not affect the recording setup whether electrically or mechanically and caused false peaks in data, records obtained with no pupa (i.e. having only the wire fixed to the heat bed) were analysed. Such blank records did not show any biasing impact of the apparatus. The temperature inside the box were in the range 21–25 °C.

Measurements were made 24–36 h after tethering during the night to minimize disturbing sounds and vibrations caused by movement of people in the building. The experiment were managed fully automatically without any experimenter being present.

A recording lasted for 5 min (with the magnetic or heat/magnetic pulses in the middle of the recording) and each was stored as a separate file. In the case of nonconditioned layout, testing was from 02.30 h until 05.30 h and the recordings were made at intervals of 25 min, which resulted in the obtaining of seven recordings that were evaluated statistically. In the case of negative reinforcement, an experiment started at 01.00 h and consisted of a series of 12, 5-min training courses with a heat shock. The recordings followed sequentially with no intervals. At 02.00 h, the thermostimulation was switched off automatically, followed by a series of seven test recordings for statistical analysis.

Analysis of recordings and statistics

Seven 5-min recordings for each day were obtained, but those containing large vegetative pulsations occurring coincidentally during a 5-min interval were excluded (on average, 20% of the recordings). A comparison of incidence of irritation-induced abdominal contractions (see Results) during the second half of the recording (after the geomagnetic field rotation) against their incidence during the first half of the recording (before the geomagnetic field rotation) was made. The 'effective amplitude' of the peaks was chosen to be the most effective measurement, comprising their occurrence, amplitude and duration. Effective amplitude represented a total of the sample values contributing to one peak – a peak integral (the frequency of the AD converter was 100 Hz). It was not possible to calculate the effective amplitude of the full half-course because small accidental heart pulsations around the baseline would bring too much noise into the data. Therefore, the effective amplitude was calculated only from the irregular large peaks. Only peaks that reached an amplitude of 10% (and more) of the maximum amplitude of the sample were chosen. The sum of effective amplitudes from the second half of the recording was compared with the sum of effective amplitudes from the first half.

The effective amplitude values showed great interindividual variability, and they were not normally distributed. Therefore, effective amplitude from the second and the first half of each recording were compared by nonparametric pair statistics (Wilcoxon's test). The total analyses consisted of 51 recordings over 12 days performed with ten pupae and focused on a spontaneous reaction in the dark; 80 recordings during 15 days performed with nine pupae and focused on a spontaneous reaction in the light; 87 recordings during 17 days performed with 11 pupae and focused on a conditioned reaction in the dark; and 51 recordings during 14 days performed with nine pupae and focused on a conditioned reaction in the light.

Results

Figure 3(A,B) shows two typical reactions of the pupae to the heat stimulation during training. The irritation reactions to the disturbance appeared as a burst of energetic rotations of the entire abdomen exceeding the recording limits. The pupae reacted similarly to a short light pulse (Fig. 4A). If the stimulations were weaker (such as sound, vibration caused e.g. by slamming a door; Fig. 4B), the irritation abdomen contractions were recorded as an individual peak, not reaching more than an amplitude of 1 μm that faded after 10–20 s. These peaks also appeared spontaneously, however, without any apparent cause (e.g. in the first half of the recording; Fig. 5C).

The results of four experiments (light vs. darkness, conditioned vs. untrained) are shown in Fig. 6. Due to the high interindividual variability of the effective amplitude, there was no reason to calculate average values. An overview of data entering the statistical evaluation is shown as histograms (frequency of incidence) with a logarithmic axis of effective amplitude values. First, a spontaneous untrained reaction to a rotation of the geomagnetic field was tested. No significant difference in the effective amplitude peak area in the first and second half of the test was detected either in the dark (A) or in the light (B). In the subsequent series, where conditioning was involved, a significant increase in effective amplitude was found after the magnetic stimulus in both dark (C) and light (D).

All four experiments show a congruent trend in the increased activity (effective amplitude) after the magnetic treatment, although the increasing is not significant when observing untrained pupae.

Discussion

The diversity of behaviour and complexity of the nervous systems of adult insects can become an obstacle during a search for the principles of construction and functionality of the sensory systems. The present series of experiments recording the abdominal movements of a tethered pupa demonstrate clearly that a very sensitive neuroethological system is involved: a system that has its behaviour coded in a single easily measurable (non-invasively) parameter (Sláma, 2000).

Even when tethered pupae are placed in a soundproof box, they react sensitively to sounds and vibrations occurring in their surroundings. This was the reason for the selection of an automatic recording during the night hours when looking for a reaction to a weak magnetic impulse.

Generally, magnetic information represents a relatively feeble sensory stimulus compared with other sensory modalities (Wiltschko & Wiltschko, 1995), and a visible behavioural reaction is dependent on the motivation of animals to show what they perceive. However, such natural motivation is sometimes hard to achieve, especially under laboratory conditions. The 'motivation world' of a pupa is much simpler than that for adult insects, and the pupa presents an extremely restricted (and therefore relatively motivation-resistant) behavioural model. Additionally, one advantage of the conditioning experiments with pupae described in the present study is that there is no need to manipulate the animal during the interval between the training and the test because any manipulation can cause a reduction or loss of response.

Thus, it might appear easy to be an ethologist of pupae: after all, behavioural expression involves only one parameter, abdominal contractions. However, the disadvantage is that

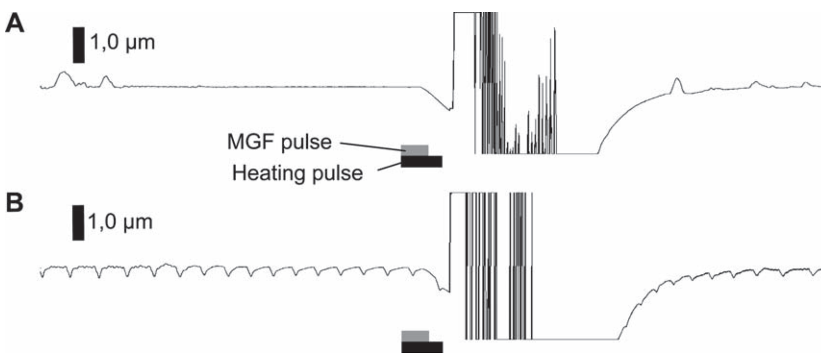

Fig. 3. Two demonstrative training recordings. The heat pulse causes rapid and energetic abdomen contractions exceeding the range of record lasting approximately 1 min (A,B). Heart activity in the form of small rhythmic contractions around the baseline is visible on the recording (B). The recordings lasted for 5 min. Scale is given as a black column on the left.

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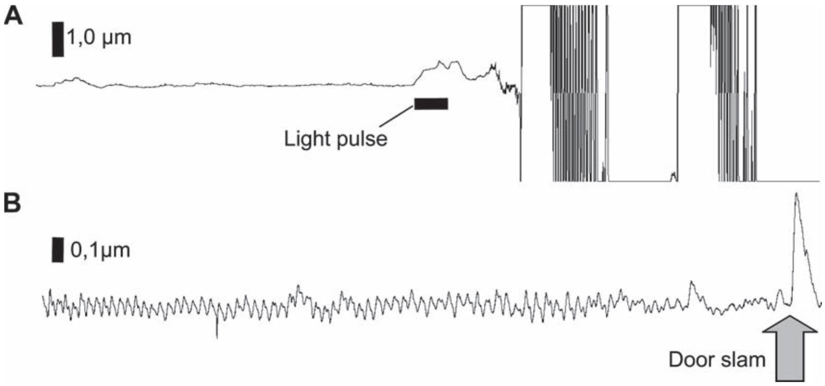

Fig. 4. The pupae react to light stimuli (A) or sounds and vibrations (B).

the expected reaction can be disguised by vegetative rhythms or contractions of unknown origin. Thus, the present measurements are limited to intervals between strong haemocoelic pulsations that shorten during ageing, making measurements practical only up to the age of 3–4 days.

During observation of pupal movements, it must be noted that a pupa is a complex organisms that does not always react the same way, and is under the influence of many other external or internal inducements. Not even in cases of strong stimuli such as light or sound can one expect a stereotypical and mechanically consistent reaction, and less so with weak stimuli of a magnetic type, when the movement reaction is sometimes spectacular and at other times not. The disturbing irritation effect is noticeable only as a statistical increase of movement activity in a time window after an irritant stimulus.

In the present study, the data obtained indicate a magnetoreceptive mechanism functioning in both light and dark. Such a result is consistent with predictions of light-independent receptive mechanisms: either induction mechanism of magnetoreception (suggested only for marine vertebrates) or magnetite transduction model (Wiltschko & Wiltschko, 2006). By contrast, previous results obtained using *Tenebrio* adults showed light-dependence of magnetoreceptive behaviour (Vácha & Soukopová, 2004). The new data could be interpreted as evidence of a motivation obstacle of adult animals in the behavioural reaction in the dark, rather than loss of functionality of the basic receptive mechanism. Nevertheless, the role of light in magnetoreceptive mechanisms and subsequent behaviour remains a completely open question of the entire magnetoreception phenomenon (Johnsen & Lohmann, 2005). The problem as to whether free and unrestrained movement of animals is necessary for successful magnetosensitive behaviour has been discussed (Wiltschko & Wiltschko, 1995). The data presented here provide the first available evidence of positive magnetosensitive behavioural reaction recorded on tethered and fully stationary animals.

The present study provides evidence that the sensory perception of insects may already be well-developed in the preimaginal stage and that it is surprisingly functional even in the pupal stage. The recording methodology employed for fine contractions of the pupal abdomen can, with careful interpretation, be used in studies of reactions to various physical or chemical stimuli. Analogous to other neuroethological models (Giurfa, 2004), developing a conditioned response to reward or punishment appears to be more effective than monitoring a spontaneous behavioural reaction.

It is as yet unknown whether, and in what form, magnetic sense conveys any adaptive value for the insect. *Tenebrio* adults in nature do not move in a radius of action greater than hundreds of meters and thus there is no migration in the usual sense. Apart from that, individuals used in the present study were bred in captivity for many generations, in constant conditions, but their sensitivity has persisted.

The demonstration that even an immature developmental stage, such as a pupa, where locomotion is zero and perception is apparently poor, has a sense for the magnetic field of

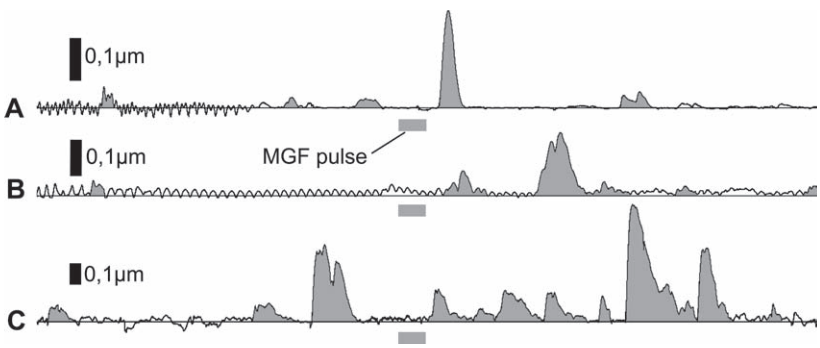

Fig. 5. Three examples of the test (A–C). Only a magnetic impulse without a heat shock is applied. The effective amplitudes of peaks (the grey-filled areas) were considered in the statistical evaluation. Only peaks larger than 10% of the highest peak were selected.

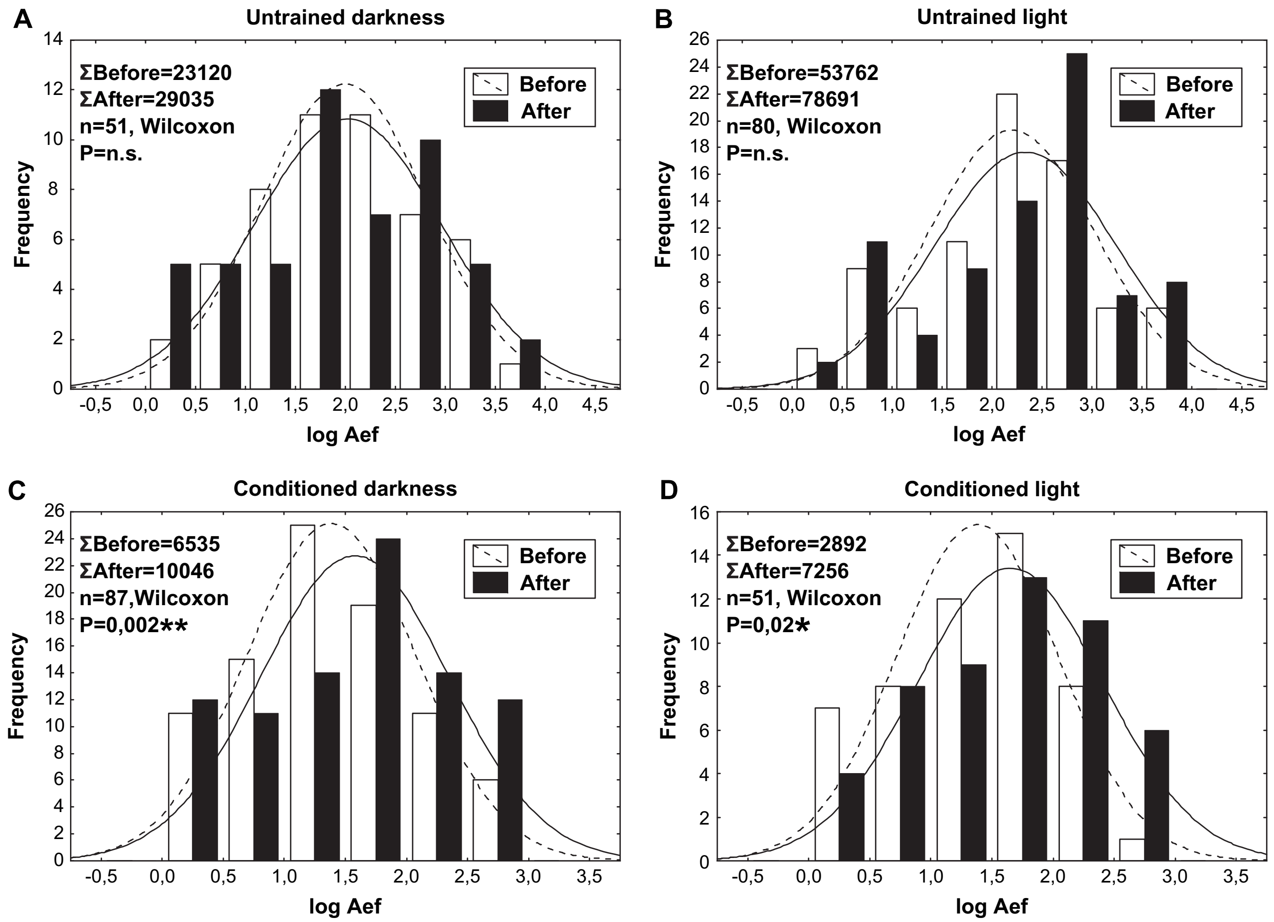

Fig. 6. Frequency histograms for effective amplitudes (Aef). The value categories are stated on the logarithmic axis, *x*, and their frequency on the *y*-axis. Values of peak areas (effective amplitudes) are shown: before (open bars) and after magnetic treatment (filled bars). The increase of effective amplitude values after the magnetic treatment is not significant during spontaneous untrained reaction in the dark (A) and light (B). During the conditioned reaction, the effective amplitude after magnetic treatment is significantly larger in both dark (C) and light (D) conditions. Curves help with the comparison of data distributions. Total sums of values, numbers of samples and significance of Wilcoxon's pair test are presented at the top left corner of each set of histograms.

the earth opens new opportunities to study the neural and molecular bases of receptive mechanisms in a simple nervous system, such as in insect larvae.

Acknowledgements

The author thanks Professor Karel Sláma for his help and scientific inspiration. The research was supported by a grant from the Czech Science Foundation (GACR 206/05/0911) and from the Ministry of Education, Youth and Sports (MSM 0021622416).

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Accepted 24 April 2007