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Behavioural phase change in the Australian plague locust, *Chortoicetes terminifera*, is triggered by tactile stimulation of the antennae

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Abstract

Density-dependent phase polyphenism is a defining characteristic of the paraphyletic group of acridid grasshoppers known as locusts. The cues and mechanisms associated with crowding that induce behavioural gregarization are best understood in the desert locust, *Schistocerca gregaria*, and involve a combination of sensory inputs from the head (visual and olfactory) and mechanostimulation of the hind legs, acting via a transient increase in serotonin in the thoracic ganglia. Since behavioural gregarization has apparently arisen independently multiple times within the Acrididae, the important question arises as to whether the same mechanisms have been recruited each time. Here we explored the roles of visual, olfactory and tactile stimulation in the induction of behavioural gregarization in the Australian plague locust, *Chortoicetes terminifera*. We show that the primary gregarizing input is tactile stimulation of the antennae, with no evidence for an effect of visual and olfactory stimulation or tactile stimulation of the hind legs. Our results show that convergent behavioural responses to crowding have evolved employing different sites of sensory input in the Australian plague locust and the desert locust.

Keywords: Phenotypic plasticity, behaviour, locust phase polyphenism, antennae, *Chortoicetes terminifera*.

1. Introduction

Locust phase polyphenism is an extreme example of phenotypic plasticity, where a range of adaptive phenotypes can arise from a single genotype via the influence of local environmental cues. Phase characteristics can include striking differences in colouration, morphology and physiology in some locust species (Pener and Simpson, 2009; Simpson and Sword, 2009), but the trait common to all locusts is the propensity to form migratory bands and swarms, as a result of density-dependent behavioural changes at the individual level (Uvarov, 1966; Roessingh et al., 1993; Gray et al., 2009). At low population densities, individuals will tend to shy away from conspecifics and exhibit a relatively sedentary life history; the solitarious phase. In contrast, individuals exposed to crowded conditions for only a few hours will begin to exhibit gregarious phase characteristics, including heightened activity levels and a tendency to group with other locusts. The transition between these two phases is critical in swarm formation, and therefore of considerable economic importance worldwide (see Sword et al., this issue).

Of all locust species, by far the best understood in terms of behavioural phase transition is the desert locust, *Schistocerca gregaria*. In this species, the interactive effect of seeing and smelling conspecifics yields a significant shift in behavioural phase (Roessingh et al., 1998), as does tactile stimulation, particularly on the outside surface of the hind femur (Simpson et al., 2001). Rogers et al. (2003) identified the

neural pathways relaying these tactile signals to the metathoracic ganglion of the CNS. A transient increase in the neuromodulator serotonin (5HT) was recently determined to mediate the initiation of behavioural gregarization (Rogers et al., 2004; Anstey et al., 2009). Levels of serotonin are substantially and transiently elevated within the metathoracic ganglion after stimulation of mechanosensory afferents from the hind femur, or by a combination of olfactory and visual stimulation. This serotonin activity is both necessary and sufficient to cause behavioural phase transition in the desert locust (Anstey et al., 2009).

In contrast to the situation for the desert locust, very little is known about the mechanism underlying behavioural phase change in other locusts. In a recent review, Pener and Simpson (2009) listed 18 locust species (plus a further 5 grasshoppers) that express some level of density-dependent phase polyphenism. These species form a paraphyletic group within the acridid grasshoppers (Song, 2005) suggesting that phase polyphenism has evolved multiple times within the family. While they all share locust-like features of their biology, there is no reason to suppose that the mechanisms employed in each case are the same: convergent evolution may yield similar phenotypes via different means, even among closely related species (Arendt and Reznick, 2008).

The Australian plague locust (*Chortoicetes terminifera*) is of considerable importance to Australian agriculture, and was recently shown to express strong behavioural phase polyphenism (Gray et al., 2009) while not exhibiting the range of density-dependent colour and morphological phenotypes for which other locust species are well known. Furthermore, as a member of the Oedipodinae it is phylogenetically distant from the desert locust (Cyrtacanthacridinae), allowing us to test whether the mode of behavioural gregarization is conserved among locusts, or has

independently evolved via more than one mechanism. In this study, we aimed to determine the effects of visual, olfactory and tactile stimuli on behavioural phase change in the Australian plague locust.

2. Materials and methods

2.1. Insects

Chortoicetes terminifera rearing protocols were as previously described (Gray et al., 2009), and were themselves adapted from methods developed for the desert locust (Roessingh et al. 1993; Simpson et al., 1999). Locusts were collected from wild populations in Western Australia and New South Wales, and reared under crowded conditions in the gregarious phase for multiple generations. Solitarious phase animals were acquired by removing individuals from the gregarious stock colony within two days of hatching, and subsequently rearing them in physical, visual, and olfactory isolation in a separate controlled-temperature insectary. It has previously been shown that this period of solitary rearing produced fully solitarious behavioural characteristics (Gray et al., 2009).

All experiments were performed using final-instar solitarious nymphs (2-3 days after ecdysis). All animals were tested once only in the behavioural assay. Untreated animals from the solitarious (n=123) and gregarious (n=124) cultures were used to build the logistic regression model (see 2.4. below), but were not used in any experiments, rendering the model independent of the test insects.

2.2. Stimulation protocols

We conducted the following three experiments to evaluate the effects of olfactory, visual and tactile stimulation on behavioural phase change.

1. An investigation of the single and interactive effects of visual stimuli (sight of 10 locusts) and natural olfactory stimuli (air passed over 50 locusts) (after Roessingh et al., 1998).
2. An investigation into the effect of tactile stimulation of different body regions with a paintbrush (after Simpson et al., 2001).
3. A study to determine whether tactile stimulation in experiment 2 could potential lead to secondary self-stimulation, through test insects jumping and colliding with the inside of the treatment chamber.

All experiments were performed for 6 h in the same controlled-temperature room at 30-32^{o}C.

For the initial visual/olfactory stimulation experiment, solitary-reared test locusts were placed into a clear plastic cylindrical container (6cm height × 5cm diameter), which would act as the treatment chamber for the duration of the experiment. This chamber was in turn placed into a larger, rectangular plastic container (17cm long × 11cm wide × 6cm high).

Locusts assigned to the 'visual' treatment had 10 crowd-reared conspecifics placed into the outer container surrounding them. Charcoal-filtered air was piped into the top of the inner treatment chamber via rubber tubing (at approximately 50mL min^{-1}) with a separate tube acting as an exhaust. This maintained a constant airflow through the treatment chambers, and ensured that the treatment animals remained in olfactory isolation from the surrounding visual stimulus animals. Conversely, locusts assigned to the 'olfactory' treatment had their airflow diverted through a 5L conical

flask containing 50 crowd-reared conspecifics, while opaque dividing cards prevented visual stimulation from locusts in neighbouring treatment chambers. 'Visual + olfactory' treated insects were subject to both stimuli, while control animals were subjected to charcoal-filtered air only.

For the tactile stimulus experiment, test locusts were placed into treatment chambers with the same dimensions as those used for the visual/olfactory experiment, allowing direct comparisons to be made during the analysis. Treatment chambers were adapted so that the end of each container was replaced with a plastic mesh, providing a perch on which the test animals would normally rest. A small hole was made in the chamber lid opposite, providing access to the insect with a paintbrush (Leonhardy size 2). Multiple treatment chambers were affixed to the bench on their side, and separated from each other with opaque cards to prevent visual stimulation between neighbouring test insects. For 20 s every 4 min, test locusts were subjected to tactile stimulation at a particular body region, by stroking gently and repeatedly with a paintbrush. All insects were assigned their own paintbrushes to prevent potential chemical cross-stimulation between individuals. The following five body regions were stimulated: (i) Whole head, including the face, eyes, antennae and mouthparts, (i) Antennae only, (iii) Thorax, including the pronotum and wing buds, (iv) Abdomen, and (v) Left femur. In addition, we included a control group of insects that were simply left alone in their treatment chambers for the 6 h duration of the experiment.

We observed during the second experiment that tactile stimulation frequently invoked a strong escape/avoidance response. This would typically constitute the test locust jumping, and consequently colliding with the inside of the treatment chamber. [In this

respect they were much more active than desert locusts under similar circumstances (Simpson, unpublished observations)]. We therefore needed to account for potential gregarizing effects of self-stimulation that might secondarily occur when agitated individuals collided with the inside of the treatment chamber. This was done by measuring the frequency of jumping caused by tactile stimulation at each of the five body regions touched in experiment 2. Using the same experimental conditions, 10 locusts were each stimulated at the head region for 20 s with a paintbrush, while an independent recorder noted the cumulative number of jumps elicited. The same insects were then stimulated for 20 s each at the antennae, thorax, abdomen and femur, with the number of jumps recorded in each case. This treatment regime continued for 6 h, to determine whether touching different body regions evoked more jumping than others, and whether there were differences in the amount of time required for insects to habituate to the tactile stimulus treatment.

2.3. Assaying behavioural phase state

Following 6 h of treatment, test locusts from experiments 1 and 2 were assayed for their behavioural phase state using an established automated video-tracking protocol (Gray et al., 2009). Briefly, insects were introduced into the middle of a rectangular arena (36cm long × 15cm wide × 10cm high), via a modified plastic syringe. The long walls of the arena were built from white Perspex, and treated with Fluon® (a white, Teflon-based paint) to prevent the test insects from climbing them. The end walls were constructed of clear, perforated Perspex, behind only one of which was placed a stimulus group comprising 20 crowd-reared final-instar locusts. Both ends of the arena were backlit by 25W fluorescent tubes. The arena floor was covered in fresh

white paper for each experimental day, and clear Perspex lids on the main arena and stimulus group prevented the test/stimulus locusts from escaping. The behaviour of the test insect was recorded in real time by a colour CCTV camera, for 480s after introduction into the arena. Subsequent analysis with Ethovision® 3.1 (Noldus Information Technology, 2005) provided values, for each test locust, for a set of variables measuring individual activity levels and position relative to the stimulus group of conspecifics in the arena (see next section).

2.4. Statistics

A measure of behavioural phase state was calculated for each test locust from experiments 1 and 2, using an algorithm derived from a binary logistic regression model built in SPSS (version 16.0)(Gray et al., 2009; Simpson et al., 2001). The model was constructed specifically for this experiment and tested before all other experiments began using 124 crowd-reared locusts and 123 solitarious locusts. The activity related behaviours included in the model were total distance moved (mm), mean velocity (mm s^{-1}), mean absolute angular velocity (degrees s^{-1}), and mean absolute meander (degrees mm^{-1}). The positional variables (relative to the stimulus group) included in the model were mean distance to stimulus wall (mm), time spent in stimulus third of the arena (s), time spent in centre third of the arena (s), time spent in non-stimulus third of the arena (s), and time spent climbing the non-stimulus wall (s).

The binary predictor variable (gregarious vs. solitarious phase state) was regressed against the values for this suite of parameters, for 70% of the model insects (98 solitarious, 75 gregarious). This produced an optimally fitting logistic regression model with 92.5% predictive power (93.9% of solitarious and 90.7% of gregarious

insects were correctly assigned), that retained four of the available parameters: (i) mean absolute angular velocity, (ii) mean velocity, (iii) time spent in the non-stimulus third of the arena, and (iv) time spent climbing the non-stimulus wall. This model was then validated using the parameter values for the remaining 30% of the model insects (25 solitarious, 49 gregarious) to ensure that the model was not over-fitted, and remained a robust predictor of behavioural phase when independently applied to individuals outside of the model-building cohort. It corrected classified 97.3% of this subset (96.0% of solitarious and 97.9% of gregarious insects).

The logistic regression model was used to quantify the behavioural phase state of all test locusts from experiments 1 and 2. The algorithm provided a linear predictor, *P*(greg), of the probability of a given insect being in the gregarious phase, ranging from 0.0 (indistinguishable from the model solitary insects) to 1.0 (behaving fully gregariously).

3. Results

3.1. Effects of visual, olfactory and tactile stimulation on behavioural phase state

The post-treatment behavioural phase state of all 240 locusts from experiments 1 and 2 are presented as frequency histograms of *P*(greg) in Fig. 1. Median values for *P*(greg) after 6 h of each treatment were as follows: antennae-tickled, 0.9759; head-tickled, 0.6598; thorax-tickled, 0.3623; abdomen-tickled, 0.7813; femur-tickled, 0.1813; visual stimulus only, 0.2257; olfactory stimulus only, 0.0638; visual and olfactory stimuli together, 0.1359; untreated controls, 0.1214.

An analysis of variance of all treatments in experiments 1 and 2, using rank-normalized values of *P*(greg) as the dependent variable, was highly significant ($F_{(8, 231)} = 3.322$, $p = 0.001$). Post-hoc testing was performed using Dunnett's 1-tailed test, which compared each treatment to the control group (n = 49) with the *a priori* assumption that phase state could only shift towards gregariousness (since all treatment insects were reared in isolation). *P* values for these comparisons were as follows: antennae-tickled, 0.001 (n = 20); head-tickled, 0.040 (n = 20); thorax-tickled, 0.108 (n = 20); abdomen-tickled, 0.058 (n = 20); femur-tickled, 0.777 (n = 21); visual stimulus only, 0.600 (n = 30); olfactory stimulus only, 0.896 (n = 30); visual and olfactory stimuli together, 0.993 (n = 30).

A separate analysis of variance was performed to test for an interactive effect of visual and olfactory stimuli. Rank-normalized values for *P*(greg) were extracted from the main dataset, and used as the dependent variable. Neither the main effects nor interaction were significant ($F_{(3, 116)} = 1.279$, $p = 0.285$). In summary, only tactile stimulation of the head and especially the antennae significantly induced behavioural gregarization.

3.2. Propensity of final-instar nymphs to jump in response to tactile stimulation of different body regions

Between them, the 10 locusts used in experiment 3 jumped a total of 3155 times over 6 h. This can be subdivided by treatment thus: head-tickled, 673 jumps; antennae-tickled, 66 jumps; thorax-tickled, 845 jumps; abdomen-tickled, 1151 jumps; femur-tickled, 420 jumps. Fig. 2 highlights the gradual decrease in mean number of jumps per 20 s tickle session over 6 h, for the five treatment categories.

3.3. Controlling for self-stimulation through jumping

The relationship between jumping frequency and *P*(greg) value is presented in Fig. 3. Tactile stimulation of the antennae was strongly gregarizing, but did not elicit a strong jumping response. The head-tickling treatment included touching the antennae, but caused the insects to jump when other parts of the head (especially the eyes) were touched with the paintbrush. The other three treatments clearly show a positive relationship between degree of gregarization induced and extent to which stimulation evoked jumping, strongly implying an effect of secondary self-stimulation of the antennae. It should be noted, however, that while the R^2 value for this relationship is strongly positive at 0.9017, a formal linear regression analysis was not appropriate since there are only three data points.

4. Discussion

Behavioural phase change in the Australian plague locust is triggered by tactile stimulation of the antennae, and this appears to be the sole mechanism through which the change occurs. Visual and olfactory stimuli were ineffective, whether presented individually or together. The Australian plague locust has therefore evolved a mechanism of behavioural gregarization that employs markedly different sensory inputs from those reported in the desert locust (Roessingh et al., 1998; Simpson et al., 2001), despite the fact that the behavioural traits themselves are both qualitatively and quantitatively quite similar in the two different species (Gray et al., 2009).

Until recently, density-dependent phase polyphenism was an unproven and controversial phenomenon in the Australian plague locust, but its importance in the biology of this species is no longer in question (Hunter, 2004; Gray et al., 2009). The initial steps required for the current work were to assay the behaviour of 123 solitary-reared insects and 124 crowd-reared insects, and construct a logistic regression model to use as a predictor of phase state in subsequent treatment animals. That we attained a model with 92.5% predictive power is in clear support of the findings of Gray et al. (2009) that the Australian plague locust shows strong behavioural phase change.

Until now, the over-arching paradigm in locust phase research has been based on findings in the desert locust, in which behavioural gregarization is caused by mechanosensory stimulation of the hind legs, in conjunction with the interactive effect of seeing and smelling conspecifics (Roessingh et al., 1998; Hägele and Simpson, 2000; Rogers et al., 2003; Anstey et al., 2009). Our results quite clearly indicate that this is not the case in the Australian plague locust and raise the tantalizing prospect of different mechanisms in other species as well. In contrast to the stimuli that induce behavioural gregarization in the desert locust, visual and olfactory stimuli, and tactile stimulation of the femur, actually yielded the lowest degree of behavioural gregarization of all treatments for the Australian plague locust (for visual stimulus only, median value for P(greg) was 0.2257; olfactory stimulus only, 0.0638; visual and olfactory stimuli together, 0.1359; femur-tickled, 0.1813) (Fig. 1).

Tactile stimulation of the antennae caused the greatest degree of behavioural gregarization. As might be expected therefore, tactile stimulation of the head was the only other treatment to cause a significant shift towards the gregarious state. Antennae were stimulated as part of the head-tickling treatment, though these insects would have necessarily received a lower amount of antennal tactile stimulus relative to the

antennae-only animals. This lower degree of direct antennal stimulation was not overcome by the increased jumping evoked by touching the head, with associated possibility for self-stimulation of antennae. Other than for antennal stimulation, jumping frequency correlated positively with an increase in behavioural gregarization (Fig. 3) and was most likely due to insects stimulating their own antennae, by colliding with the inside of their treatment chamber when they jumped.

The abdomen-tickled cohort is worthy of comment in the context of jumping. This group had a median *P*(greg) score of 0.7813, and was approaching significance when compared with the control group (one-tailed $p = 0.058$). However, this treatment also evoked by far the strongest aversion response to stimulation, with each of the test locusts jumping, on average, a total of 115 times over the 6 h experiment duration in response to being touched on the abdomen (see Fig. 2 and Fig. 3). This particularly strong aversion could be partly explained by recent insights made by Bazazi et al. (2008), who showed that marching behaviour in migratory bands of desert locust nymphs is driven by cannibalistic interactions. Individuals that remain stationary for too long will eventually succumb to hungry conspecifics following close behind, and are thus forced to remain moving once a member of a migratory band. The abdomen was particularly susceptible to cannibal attack. Denervating the abdomen resulted in an increased rate of cannibalism and a reduced tendency to march, even though insects were behaviourally normal except for a failure to respond to contact from behind (Bazazi et al., 2008).

Although mechanostimulation of the antennae is the most likely explanation of our results, two alternative possibilities for a gregarizing effect of jumping and antennal stimulation should be mentioned, however unlikely. The first possibility is that proprioception associated with jumping perhaps played some role. Rogers et al.

(2003) reported for the desert locust that stimulation of proprioceptors associated with the hind coxa was involved in behavioural gregarization, but only if paired with touch of the outer surface of the femur. Hence, inducing vigorous hind leg-kicking movements by puffing acetic acid onto the leg without physical contact had no behaviourally gregarizing effect. Femur mechanostimulation with a paintbrush in the current paper would have also stimulated these proprioceptors and, since this treatment was not gregarizing, we argue that this avenue can be ruled out as a potential mechanism of gregarization.

There is also a theoretical possibility that chemical self-stimulation through repeatedly using the same paintbrush one the same individual throughout the experiment may have had an influence on gregarization in the present experiments (see Hägele and Simpson, 2000). This would require that there are sufficient effective compounds on the antennal cuticle of one insect to powerfully evoke phase change in that same insect, since that is the only region that was stimulated repeatedly in the antennal stimulation treatment.

Despite the clear differences in the nature and location of stimuli inducing behavioural phase change between the Australian plague locust and the desert locust, there are common features between the two species. Both locust species gregarize as a result of tactile stimulation, and employ cuticular mechanoreceptors in the initial perception of conspecifics (Rogers et al., 2003; Anstey et al., 2009). Similarly, both species employ sensors on the head, although the desert locust appears to use the antennae not for tactile recognition (Simpson et al., 2001) but for olfactory perception of other locusts in conjunction with associated visual stimuli (Roessingh et al., 1998). Previous reports that removing antennae rendered already gregarious desert locust nymphs behaviourally solitarious (Gillett, 1983; Heifetz et al., 1996), are explicable in

terms of a reduction in the chemosensory inputs required to sustain the gregarious state (see Heifetz et al., 1996; Pener and Simpson, 2009, pg 204).

A critical next step will be to determine whether the suite of neuromodulators and neurotransmitters that play a role in desert locust phase change (Rogers et al., 2004) are conserved in their functions in the Australian plague locust, even though the site of mechanosensory stimulation for behavioural gregarization differs. In the desert locust, a transient increase in thoracic serotonin was recently discovered to be both necessary for behavioural phase change to occur and sufficient to induce it. The pulse of thoracic serotonin was induced both by gregarizing sensory inputs from the hind leg (tactile stimulation) and from the head (a combination of sight and smell of other locusts) (Anstey et al., 2009). It is possible that mechanosensory inputs from the antennae are similarly stimulating a release of serotonin in the thorax in the Australian plague locust, and that from that point onwards the underlying neural mechanisms of phase change are the same as in the desert locust. Thus, the effect of serotonin and its site of action should be the focus of future work in the Australian species.

Given that we now know that more than one mechanistic pathway to gregarization exists, what sources of stimulation induce behavioural gregarization in other locust species? The migratory locust, *Locusta migratoria*, should be considered a strong candidate for future analysis; as another member of the Oedipodinae, it might be expected to share more similarities in its behavioural phase acquisition with the Australian plague locust than it does with the desert locust. Stimuli experiments similar to the work presented here, in conjunction with phylogenetic comparisons (Song and Wenzel, 2008) will yield a clearer picture of the patterns, processes and mechanisms at work across taxa in the evolution of locust behavioural phase change.

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Figure Captions

Fig. 1. Frequency histograms showing the percentage of final-instar initially-solitarious Australian plague locusts falling into different categories of behavioural phase state [*P*(greg)] after 6h of stimulus treatment. 'Head', 'antennae', 'thorax', 'abdomen' and 'femur' all represent treatment groups subject to tactile stimulation with a paintbrush at each of those body regions. Visual treatment was the sight of 10 conspecifics; olfactory treatment was the smell of 50 conspecifics. Control animals were not subject to any stimuli. Arrows indicate median values of *P*(greg) in each case (reported in main text).

Fig. 2. Graph showing decrease in insect response to tactile stimulation at 5 body regions, over a 6-h period. Fitted lines for each treatment are (A) abdomen, (B) thorax, (C) head, (D) femur, (E) antennae.

Fig. 3. Graph showing the relationship between the mean number of jumps per insect evoked by tactile stimulation, and corresponding median *P*(greg) values for stimulation of 5 body regions with a paintbrush (from experiment 2). The fitted line of 'femur', 'thorax' and 'abdomen' data points shows a positive correlation between number of insect jumps and induced level of gregariousness. Note that 'head' is an outlier to this line, while 'antennae' is an extreme outlier.

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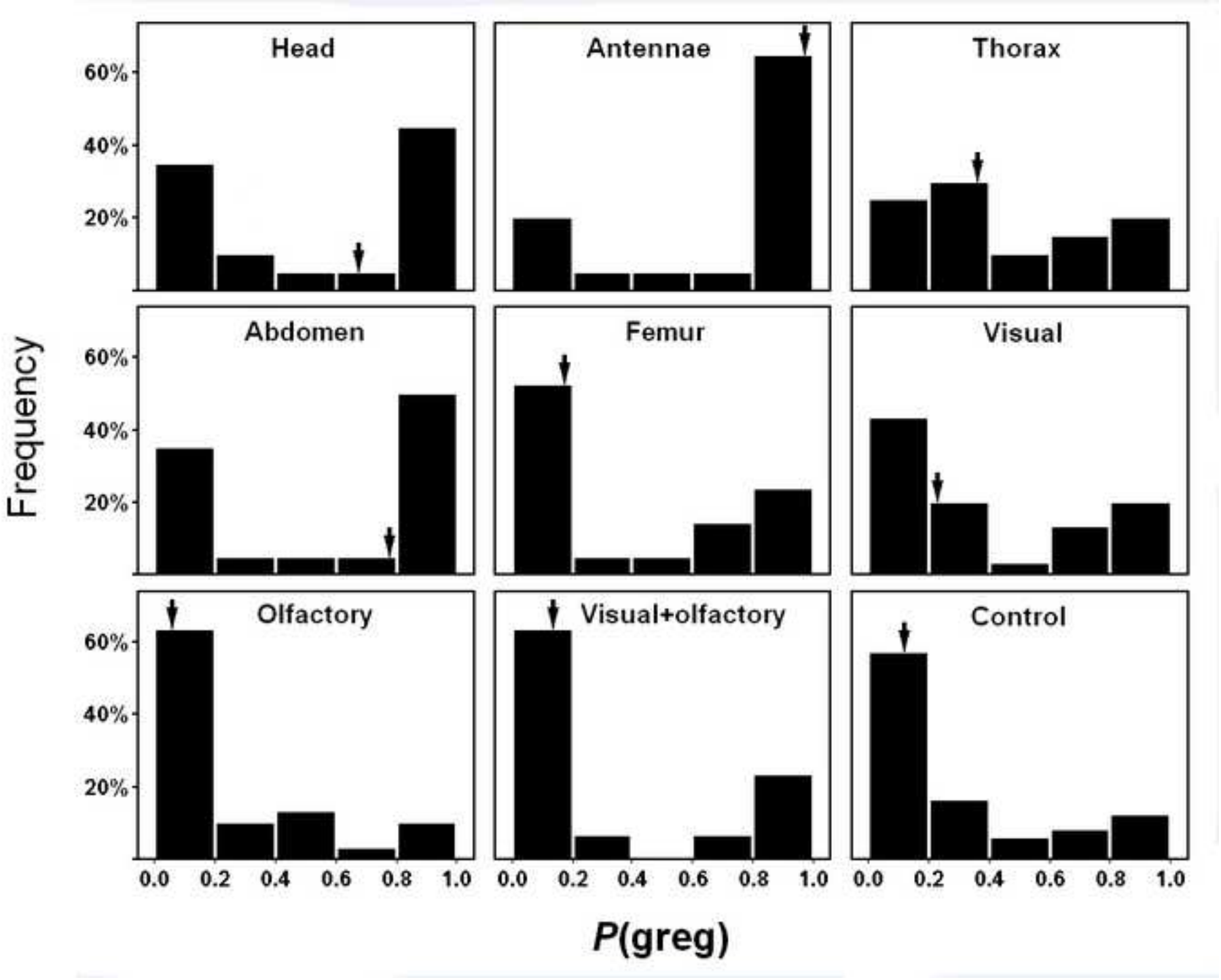

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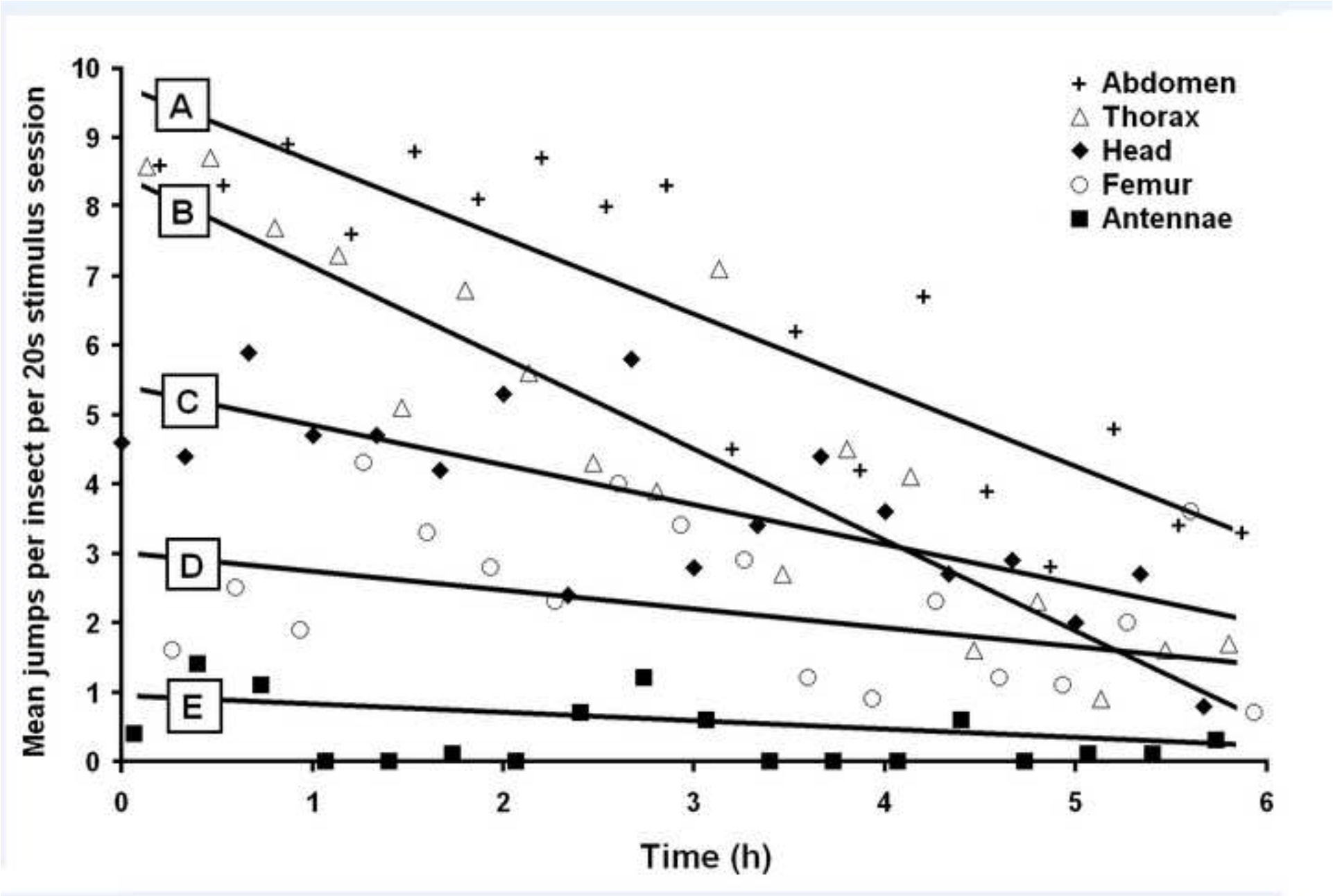

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