Dear Editor,

We carefully revised the MS "Hard to choose for tiny pests: host-seeking behaviour in *Xenos vesparum* triungulins" following the suggestions of both referees:

- Introduction and Discussion have been shortened by omitting not relevant data on *Xenos vesparum* cycle;
- we added more information on host-seeking behaviour in other parasitoids;
- we clarified our experimental set-up by adding details on our apparatus and choice trials (see Methods);
- we omitted Carbon Dioxide trials – even if the role of CO_2 in host location may be relevant - because preliminary: limited sample size, no data about CO_2 levels on the wasp comb, experimental CO_2 emission (under 2 mL/min flow) probably too low;
- the Discussion has been re-organized according to the different topics considered (positive and negative evidences for host-location; the role of stimuli used by *Xenos* triungulins to escape from the cephalothorax; gregariousness);
- the Table has been converted to a Figure (see Fig.2).

Due to the preliminary nature of some choice trials here reported, results have been discussed with caution; therefore, we think that a Short Communication Format could be more appropriate for this paper.

With best regards

Fabio Manfredini

Hard to choose for tiny pests:
host-seeking behaviour in *Xenos vesparum* triungulins

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Abstract

The 1st instar larvae of strepsipteran parasites, commonly referred as "triungulins", are the host-seeking stage: they must locate, invade and successfully develop in the new host, in order to start their parasitic cycle. A few information are available about the behaviour of *Xenos vesparum* triungulins. They emerge in batches from the endoparasitic female infecting *Polistes dominulus*, the primary host, and reach the nest through a vector (a foraging wasp or the parasitized wasp itself). Once there, they have the possibility to penetrate into wasp immatures at different developmental stages. In this study, we performed preliminary analyses aimed to investigate which cues are important to direct triungulins movements during their brief stay on wasp nests. In laboratory conditions we selectively presented different stimuli to *Xenos* larvae: apparently, the host larva itself is attractive in an open arena, but not inside a confined space, neither epicuticular compounds of wasp larvae are able to control triungulins movements. These are more likely oriented by their gregarious behaviour, whereas light (positive phototaxy) could previously enhance their emergence via the brood canal opening in the female cephalothorax.

Key words: gregariousness, host-seeking behaviour, triungulin, infective larva, Strepsiptera, koinobiont parasitoid.

RUNNING TITLE: Host location by *Xenos* triungulins.

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Introduction

Parasites, being a significant portion of total biomass in natural ecosystems and mainly in insect societies (Hughes et al., 2008), have a great eco-ethological significance. In particular, manipulative parasites are able to redirect host relationships and to extend the range of its habitat (Lefevre et al. 2009). *Xenos vesparum* is a castrator "manipulative" parasite (Hughes 2005), which has a great impact on the small annual society of the paper wasp *Polistes dominulus*, its primary host (Beani 2006; Hughes 2005; Hughes et al. 2004b). In this host-parasite system, the association between the two insects is long-lasting and its success is based on the parasite tuning with host development. *X. vesparum* penetrates into a *P. dominulus* immature as 1st instar larva and coexists with it for weeks or months, depending on the sex of both parasite and host. The intimate association with its host forces the parasite to adapt to a changing environment: this life style is typical of insect parasitoids, a large group of insects, primarily in the orders of Hymenoptera and Diptera, whose larvae develop by feeding in / on the bodies of other arthropods (Brodeur & Boivin 2004; Strand & Pech 1995). Thus, *X. vesparum* could be reasonably linked to koinobionts parasitoids, where parasite development is finely tuned to the development of its holometabolous host, since it manages to achieve growth and metamorphosis processes (Tanaka 2006; Vinson & Iwantsch 1980).

Together with adult males, 1st instar larvae are the sole free-living developmental stages in *X. vesparum* life cycle. They are often called "triungulins" (i.e. three-clawed) for their morphological similarity to coleopteran campodeiform larvae (Kathirithamby 1989; Kathirithamby 2009; Pohl 1998), although in this species the first two pairs of legs are equipped with disc-like pulvilli, not with claws, and the last with spines (Fig. 1). They represent the host-seeking stage: they are extremely motile and resistant to environmental conditions (they may survive for 48 hours in Petri dishes without food, see Giusti et al.2007, and independently on the humidity conditions, *pers. obs.*), although relative humidity has been referred as a critical longevity factor in other species (Hassan 1939, quoted in Kathirithamby 2009). Moreover, they are particularly kin in penetrating into the host and establishing inside without any dramatic consequences neither for themselves nor for the host (Manfredini et al. 2007a).

Generally, parasitoid infective larvae are the most aggressive among immatures, capable of ensuring the parasitic success of the species by installing themselves fruitfully on the host. For this purpose, they are usually very mobile and easily exposed to physical fights with rival conspecifics (Doury et al. 1995; Giron et al. 2004); moreover, they often have a direct effect on host physiology through their bite, whereby they inject salivary glands secretions into the host, thus manipulating both cellular and humoral components of its hemolymph (Doury et al. 1997; Richards & Edwards 2002). Strepsipteran triungulins possess all the morphological features of a successful infective organism (Fig. 1): antennae, eyes, mouthparts, slender legs, tarsal expansions and long caudal appendages which allow jumping movements. Almost no information are available about their host-seeking behaviour and very few is known on the invasion process (Kathirithamby 2001; Kathirithamby et al. 2003; Maeta et al. 2001). For *Xenos* triungulins this starts once they have reached a wasp nest, either by grasping to a foraging wasp (phoretic transport) or by means of a stylopized wasp which directly visits a nest (Hughes et al. 2003; Vannini et al. 2008). The "wandering" behaviour among spring colonies, recently described (Beani & Massolo, 2007) in wasps infected by *X. vesparum* females, i.e. the source of triungulins, could be interpreted as a manipulative extension of the usual wasp range by the parasite (Lefevre et al. 2009). In the field the parasite load ranges from 1 to 4.1 per adult wasp (Vannini et al. 2008) and thus it is presumably higher at infection. After the emergence of the winged male and the extrusion of the cephalothorax of the female, a neotenic permanent endoparasite, mating occurs (Beani et al. 2005). Fertilized eggs start the first steps of segmentation process, but then stop and remain quiescent during winter diapause (*pers. obs.*). The following spring, embryogenesis will terminate to give birth to hundreds of 1st instar larvae, ready for another season of infections and usually released in groups of 10, 20 or more (*pers. obs.*). Thus, *Xenos* females may over-winter inside its host, a hibernating female wasp, whereas *Xenos* males die in summer, as well as their hosts, regardless of their putative caste (Beani 2006).

For many reasons (not just morphology), *Xenos* triungulins are quite analogous to host-seeking stages of Dipteran and Coleopteran koinobiont endoparasitoids (Castelo & Lazzari 2004; Crespo & Castelo 2008; Royer et al. 1999). According to Brodeur and Boivin (2004), in parasitoids with active larvae the host seeking stage undergoes a chain of "hierarchical steps" to achieve successful parasitism: it must locate, evaluate and penetrate the appropriate host, evade or overcome the host immune response and adapt to (or regulate) the constantly changing host environment. Host seeking larvae promptly respond to different kind of cues, generally divided in three main categories (Godfray 1994): signals from host microhabitat, which are easy to detect at a distance but give little reliable information; stimuli indirectly associated with the activity of the host (for example phonotaxy, long-distance kairomones, CO_2 emission, etc.); stimuli from the host itself, i.e. visual and chemical cues, that are the most reliable ones, often too weak for detection over long distances, due to the strong selection pressure on hosts to avoid being recognized (for a review on parasitoids exploiting chemical information, see Fatouros et al. 2008).

In this pioneering study we investigated which short-range cues are important to orient *X. vesparum* triungulins during host location on the comb: are *P. dominulus* larvae *per se* attractive for triungulins? In a series of repeated independent experiments in laboratory, we tried to recreate semi-natural choice trials, focusing on the arrival of triungulins on the nest. We registered the movements of batches of host-seeking triungulins on a neutral substrate in presence of 3rd- 4th instar wasp larvae, which were singly placed in the middle of an open arena, and successively inside a confined and screened space, in order to distinguish chemical from visual signals. In further trials, we investigated the effect of single stimuli, i.e. epicuticular compounds from wasp larvae *vs* fly larvae used as a control. Only in case of a clear-cut effect of wasp stimuli in orienting the movements of triungulins, we could apply the same technique to assess host selection, as concerns sex, developmental stage and superparasitism. Although preliminary, this study on Strepsiptera represents a good model to analyse host-seeking behaviour among immatures parasitoids, due to scanty data in literature about the ecology of active 1^{st} instar larvae (Feener & Brown 1997).

METHODS

Study animals

Hibernating clusters of *Polistes dominulus* Christ were collected in San Gimignanello (Siena, Italy) at the end of the winter. They were carefully screened to exclude parasitic infections and placed in groups of 3 individuals inside 20 x 20 x 20 cm Plexiglas cages with sugar, water and *Sarcophaga* sp. larvae *ad libitum*, to allow colonies foundation. Under fixed conditions of 15L/9D and 28±2°C, *Polistes* wasps started building first nest cells around 3 weeks after collection. These animals were the starting pool for laboratory experiments, although additional colonies were collected during the summer directly from the field. In this case, nests underwent a period of quarantine to exclude any possibility of natural infection by Strepsiptera.

Our sources of triungulins were overwintered wasps coming from the same hibernating clusters as above and parasitized by a single (rarely 2) female of *Xenos vesparum* Rossi. These females, extruding their cephalothorax through wasp abdomen, released batches of triungulins after 4 weeks at 15L/9D and 28°C. Experiments were carried out in June and July 2008, i.e. the peak of infection in wasp colonies (Hughes et al. 2003).

Choice trials in laboratory

For preliminary experiments of host location, we selected from different wasp colonies 3^{rd} or 4^{th} instar larvae, i.e. the commonest stages, easier to manipulate than 1^{st} – 2^{nd} instars and not close to pupation as 5^{th} instar. These developmental stages are easily recognizable for their relatively large size and not yet melanized (light brown) mouth apparatus. We carefully removed each wasp larva from its cell with forceps and placed it in the middle of a circular open arena (10 cm wide Petri dish, covered with filter paper which was changed at each trial). Then, with a thin needle, we collected batches of triungulins escaping from one *Xenos* cephalothorax (i.e. siblings) and we spreaded them on the paper (around 10 individuals per arena trial). Finally, we recorded their movements during first 20 minutes of activity with a video camera connected to a stereomicroscope. We excluded the nest as trial substrate to avoid a possible confounding source of chemical signals (for a review on wasp cuticular hydrocarbons, already present on the comb, see Gamboa 2004) and to better follow the movements of triungulins, able of walking and jumping on filter paper as well as on plastic substrates.

In a second set of experiments, triungulins were offered a choice in a vial between 3rd – 4th instar wasp larvae and no wasp as control: although *Polistes* nests are opened, they are often located under tiles or other shelters, thus a confined space could be appropriate to simulate a natural condition. We prepared transparent plastic tubes (1 cm wide, 7 cm long) with a small hole in the middle. The wasp larva was positioned at one extremity of the tube while the other extremity was left empty. Clusters of 20-30 triungulins were inserted into the tube with a thin needle through the small hole. A cotton membrane at 1 cm from each extremity prevented triungulins from contacting the wasp larva and a parafilm over the hole prevented triungulins from escaping. For 27 independent experiments we recorded *Xenos* larvae position in respect to target and control after 1 hr (time 1) and after 4 hrs (time 2). The central portion of the tube around the entry hole (≈ 1 cm long) was not considered, being a neutral zone. To control for a possible effect of light, we used the same transparent tubes as above (deprived of both the wasp larva and the cotton membrane) 90° oriented to the light source and we recorded triungulins prevalence in light side *vs* dark side.

Finally, an additional set of stimuli was proposed to *Xenos* triungulins in order to observe their susceptibility to wasp cuticular compounds. Single wasp larvae were placed in a glass vial and washed in 100µl heptane for 30 sec to obtain a solution of larval cuticular waxes, mainly hydrocarbons (Cotoneschi et al. 2007). We created a 4 field square arena (measuring 10 cm on each side) where one field was occupied by the stimulus (a drop of hydrocarbon solution from larval wasp cuticle), the opposite one was a positive control (a drop of hydrocarbon solution from immature *Sarcophaga* sp. fly, obtained following the same procedure as above) and the remaining fields were negative controls (no stimuli at all). Then we placed clusters of 20-30 triungulins in the middle of the arena and we recorded their distribution among 4 fields after 20 minutes. Experimental data were analysed with the computer program SPSS for Windows: we performed *chi-square test* and *Cohen's kappa coefficient test*.

Results

Preliminary 20-min experiments of host location in open arenas with diffuse lightening suggested that triungulins were attracted by the presence of living wasp immatures. When we placed batches of triungulins and one wasp larva inside the arena, they usually managed to approach the target within the first 5 minutes of activity. Typically they moved towards it not directly, but describing round trajectories and lifting their body from one side to the other, alternatively. They were able to jump, by means of their caudal setae. In 5 out of 6 trials, more than 50% of triungulins approached the larva, but only a few of them began to move on the surface of the larva, whereas the others were out of our sight under the larva or disappeared from the arena at the end of the trial.

Further choice trials between a putative host and no wasp as control (inside a tube, i.e. a confined space) showed a rather unexpected pattern (Fig. 2). First of all, we checked for positive or negative phototaxy. Light really seemed to be an attractive factor: in all 9 trials considered, after 1 hour more than 2/3 of our triungulins were positioned in the side of the tube facing the light source. For this reason, following trials were carried on by covering the tube. Among 27 trials, a significant asymmetrical distribution (χ^2 *test*) was observed in 21 trials, either after 1 hour or 4 hours and we observed a choice for the wasp larva in 13 cases at time 1 and in 14 cases at time 2. Despite the evidence of a trend to move towards the larva, the difference was not significant at both times ($\chi^2 = 1.18$ and 2.32, $df = 1$, $P_{Exact} > 0.05$). At all, in 15 trials in which triungulins were asymmetrically distributed, their position in respect to wasp larva and control at time 2 was not different compared to time 1, which means they tended to maintain the acquired position within the tube for longer time spans: the inter-rate agreement was significant ($K_{Cohen} = 0.609$, $P = 0.022$). Finally, this set of trials revealed a trend towards gregariousness for triungulins in regard to their movements within the tube: irrespectively of their choice for wasp larva or empty tube, more than 2/3 of the members of each experimental cluster were grouped in 21 trials out of 27, both at time 1 and time 2 ($\chi^2 = 8.33$, $P_{Exact} = 0.006$).

The use of single stimuli associated to the presence of the host showed that epicuticular compounds from wasp immatures did not influenced *Xenos* triungulins, once they were outside mother's cephalothorax. In 6 trials with diffuse lightening, triungulins were never clustered around spots of wasp cuticular compounds but once were significantly grouped in the opposite field with fly odorant and twice in fields without any signal (P_{Exact}<0.001): their overall distribution was random (($\chi^2 = 4.96$, $df = 3$, *ns*).

Discussion

Contrary to our expectations due to their short life-span, *X. vesparum* triungulins do not appear particularly reactive to host-associated stimuli. Nevertheless, their approaches to the wasp silhouette in an open arena suggest that they can locate the host. Their peculiar behaviour while directing themselves towards the target is noticeable: round trajectories and repeated inclinations of the body seem to respond neither to a visual nor to an acoustic stimulus (in this case we would expect a straighter displacement). On the contrary, this attitude could be interpreted as a symmetrical chemio-reception by means of antennae or further sensory organs, hypothetically placed on both sides of triungulins body. Anyhow, it is difficult to establish whether their movements are the motor pattern of the infection process or something casual: they jump on the wasp larva, explore it, sometimes stop there, sometimes leave and go behind later.

The double-choice tests in a confined space, simulating a sheltered nest, did not confirm our first expectation, i.e. that triungulins are able to exploit chemical cues to redirect their movements towards the host: they grouped at both extremities of the tube, independently on the presence/absence of the wasp larva (see Fig. 2). Then we analysed the influence of single factors, the cuticle compounds of wasp larvae first, i.e. mainly non-volatile, long-chain hydrocarbons, which were critical for nest-mate identification in wasps (Gamboa 2004) and were recently described in *P. dominulus* larvae too (Cotoneschi et al. 2007). Unfortunately, spots of epicuticular compounds did not elicit the arrestment of triungulins on the spot, as we would expect based on similar four-well arena experiments, where chemical stimuli of bee larval food influenced the movements of *Varroa destructor* (Nazzi et al., 2004), as well as in several egg parasitoids (Fatours et al., 2008). Laboratory artefacts are unlikely, due to our simple experimental set-up, nevertheless cuticular extracts need to be concentrated in future analyses.

Relatively to light as directional cue, we argue that it is probably more important for promoting triungulins' exit from mother cephalothorax than for orientating their movements once outside. We already know that blowing gently at the cephalothorax evokes the emergence of batches of 1st instar larvae within a few minutes (Kathirithamby 2009), because a light CO_2 emission might simulate the presence of a potential host (Guerenstein & Hildebrand 2008); once activated inside the mother's body, *Xenos* larvae may exploit a light cue to find the exit of the brood canal, i.e. the cephalothorax brood opening (Beani et al. 2005). Maybe they simply react to the CO_2 and light stimulus by increasing their activity without taking into account any specific direction (David Giron, *pers. comm.*). Thus, "kinesis" (a non-directional stimulus-dependent change) seems to be more appropriate than "taxis" (a directional change towards gradient stimulus intensity), nevertheless in these first trials we did not measure changes in the activity of triungulins in presence or in absence of a stimulus. As a matter of fact, during laboratory infections, clusters of triungulins normally formed at the end of the abdomen of parasitized wasps (*pers. obs.*).

It is possible that triungulins perceive the presence of the host but do not take into account any directional information, due to their direct release on the comb by a vector, a foraging wasp as well as a stylopized wasp releasing triungulins on different colonies (Beani & Massolo, 2007). If we consider that *Polistes* nests are already crowded of wasps at early summer, when most of the strepsipteran infections occur, triungulins must have a huge choice and little time to decide. Wasp larvae are very reactive, provided with mandible, and adults collaborate with them in trying to remove the annoying tiny pests from the nest. These reactions belong to the first step of insects defence process against parasitoid invasions, better known as "behavioural defence" (Bailey & Zuk 2008; Schmid-Hempel & Ebert 2003). In insect colonies, behavioural defence represents a key element of the social immune system, whereby single individuals cooperate to obtain avoidance, control or elimination of parasitic infections (Cremer et al. 2007). The annual colonies of paper wasps may be considered as a small "homeostatic fortresses" (Hughes et. al. 2008), where individuals control the disease due to both their hygienic behaviour and early desertion of the nest by parasitized inactive adults (Hughes et al, 2004 b).

The dynamic of the process is here complicated by the fact that both *Xenos* larvae and *Polistes* hosts are gregarious (Fig. 2). Regardless of the modality of transportation on wasp nest, data on parasite prevalence in naturally infected colonies (Hughes et al. 2003; Vannini et al., 2008) let us suppose that many triungulins (maybe decades) are contemporary present on the same nest, searching for a suitable *P. dominulus* larva where to penetrate. Gregariousness – here the tendency to aggregate in confined as well as in open space – promotes the displacement of batches of infective larvae through the brood canal and could have evolved to increase parasitism success when conditions are favourable. Peaks of nest infections on the field happen during the hottest hours of the day, when most foragers are outside the nest (Ortolani & Cervo 2009): this moment corresponds also to high metabolic activity of wasp larvae (thus high production of CO_2) and intense light. We cannot exclude any cooperative action between sibling parasites during the infection process (Costa & Fitzgerald, 1996): a decreased risk of predation is likely to occur in group. On the other hand, movements in group could reduce host-seeking efficiency, introducing possible mutual interference among triungulins (Royer et al. 1999); however, no competition has been observed among triungulins, as confirmed by successful superparasitism by sibling and non-sibling individuals (Vannini et al., 2008).

For many aspects, the behavioural patterns of *Xenos* infective larvae remind us of *Meloe* triungulins, even though they belong to different insect orders (Strepsiptera and Coleoptera, respectively). Also beetle 1^{st} instar larvae are gregarious, in fact they cooperate in forming aggregations (they move as a unit) and in holding onto vegetation, waiting for a foraging bee that can bring them to the nest (Hafernik & Saul-Gershenz 2000). The cooperation and chemical communication exhibited by *Meloe* triungulins for mutual benefit is so evident (sometimes they form living bridges with the aim to attract and contact the bee) that some authors suggest that it transcends aggregation behaviour to become social behaviour (Saul-Gershenz & Millar 2006). In our case, gregariousness is not such an advanced phenomenon, but it could represent anyhow an initial step in this direction. Another similarity with beetle 1^{st} instar larvae is the negative thermotaxy: *Xenos* triungulins, in fact, tend to move in the opposite direction as respect to a heat source (*pers. obs.*).

Proximate explanations for triungulins behaviour are otherwise still to be defined. A parsimonious hypothesis – based on the peculiar ecology of parasite and host – could be that, once on the nest, triungulins are more likely to find very easily a host, especially if they do not discriminate host quality. A few choice trials between large *vs* small larvae, or healthy *vs* infected ones, suggested that triungulins "overwhelmingly entered the first host they encountered" (Hughes, 2003). Based on our previous experiences, there is no evidence of any strict physiological barrier in host selection mechanisms by *X. vesparum*, differently from what reported in other host-parasitoid systems. In the laboratory we managed to successfully infect various developmental stages, naïve or already parasitized wasps (superparasitism by sibling and non-sibling triungulins is also well diffused in the field, see Vannini et al. 2008), males which are less parasitized than females in nature (Hughes et al. 2004a; Hughes et al. 2004b) and even non-primary hosts, as *P. gallicus*, in which the parasite development was interrupted (Manfredini et al. 2007b). These findings clearly show that, in our system, ecological-behavioural barriers are perhaps more important than chemical and visual signals for a perfect tuning of the parasitoid with the biology of the host.

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Figure captions

Figure 1 – Scanning electron micrographs of *Xenos vesparum* 1st instar larvae. (**A**) Dorsal view of a triungulin: note disc-pulvilli on the first two pairs of legs and spines on tarsi in the last pair (**arrowhead**). (**B**) Dorsal view of the cephalic segment with external photoreceptors well evident (**arrows**), whereas "Stemmata" (*sensu* Pohl 1998, meaning ocelli) are here not visible. (**C**) Ventral view of the cephalic segment, with mandibles (**arrow**), jaws (maxillae, **Mx**) and labium (**Lb**). (**D**) Detail of a disc-pulvillum on the first pair of legs. (**E**) Two pairs of setae on the last abdominal segments: caudal appendages (**arrowheads**) are longer and thicker.

Figure 2 – Double-choice trials by groups of triungulins (active individuals ranging from 15 to 35 for each group) moving inside a tube with one wasp larva at one extremity and no wasp as control. For both time points (**time 1** = 1 hr and **time 2** = 4 hrs) the percentage of trials is reported where triungulins were significantly grouped (*chi-square test*) towards the wasp larva or the control. Above each column is the number of observations (out of a total of 27 trials).

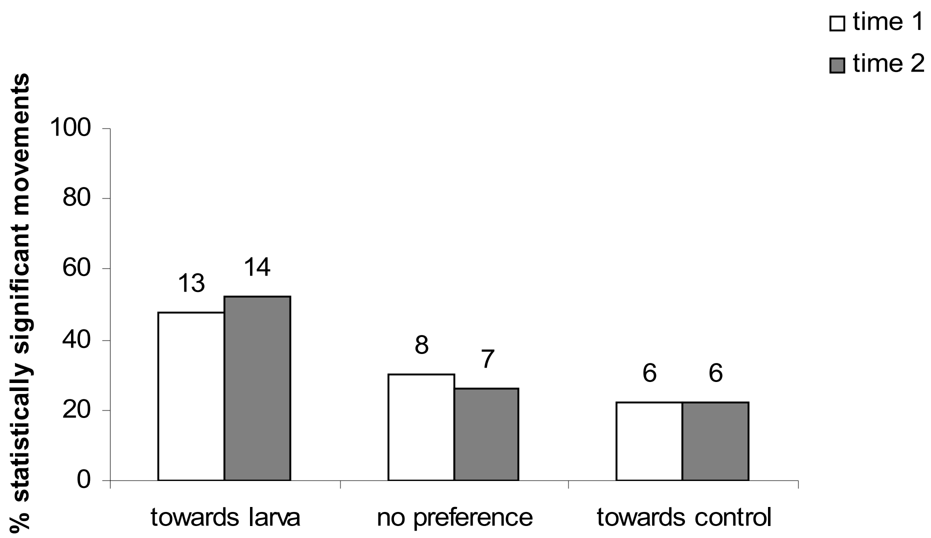
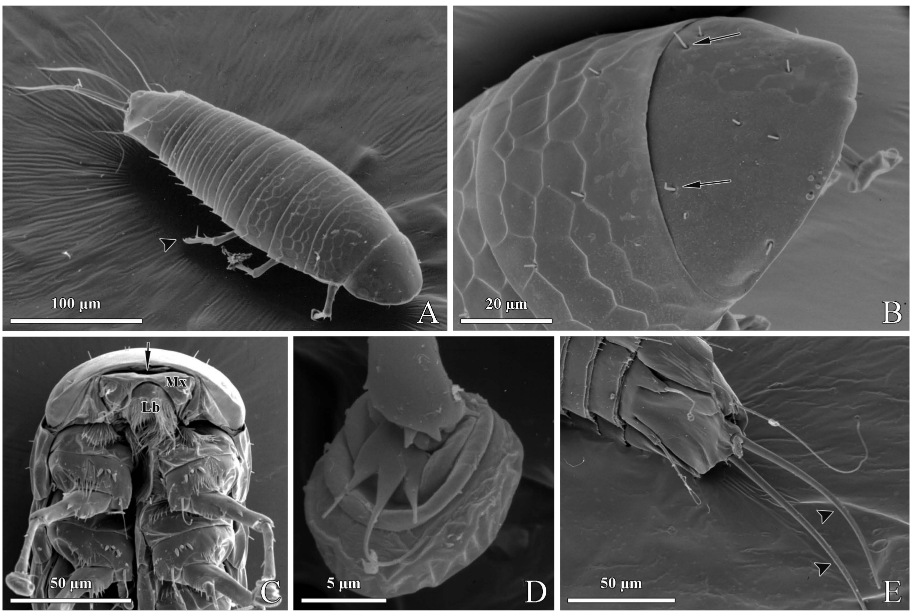

time 1
time 2
% statistically significant movements
100
80
60
40
20
0
13
14
8
7
6
6
towards larva
no preference
towards control

Figure 2



100 µm
A
20 µm
B
Mx
Lb
50 µm
C
5 µm
D
50 µm
E