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Serotonin depresses feeding behaviour in ants

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ABSTRACT

Feeding behaviour is a complex functional system that relies on external signals and the physiological state of the animal. This is also the case in ants as they vary their feeding behaviour according to food characteristics, environmental conditions and – as they are social insects – to the colony's requirements. The biogenic amine serotonin (5-HT) was shown to be involved in the control and modulation of many actions and processes related to feeding in both vertebrates and invertebrates. In this study, we investigated whether 5-HT affects nectar feeding in ants by analysing its effect on the sucking-pump activity. Furthermore, we studied 5-HT association with tissues and neuronal ganglia involved in feeding regulation. Our results show that 5-HT promotes a dose-dependent depression of sucrose feeding in *Camponotus mus* ants. Orally administered 5-HT diminished the intake rate by mainly decreasing the volume of solution taken per pump contraction, without modifying the sucrose acceptance threshold. Immunohistochemical studies all along the alimentary canal revealed 5-HT-like immunoreactive processes on the foregut (oesophagus, crop and proventriculus), while the midgut and hindgut lacked 5-HT innervation. Although the frontal and suboesophageal ganglia contained 5-HT immunoreactive cell bodies, serotonergic innervation in the sucking-pump muscles was absent. The results are discussed in the frame of a role of 5-HT in feeding control in ants.

1. Introduction

Feeding is a very complex behaviour and its regulation requires the integration of a wide range of factors. Not only do external conditions such as the season, the time of day and the quality of food determine the feeding behaviour of an animal, but also its physiological state. Biogenic amines play an important role in the control and modulation of many actions and physiological processes in both vertebrates and invertebrates. Particularly the monoamine serotonin (5-hydroxytryptamine, 5-HT), which is found in all phyla that possess nervous systems (Weiger, 1997), orchestrates diverse behaviours and processes controlling energy balance in species as disparate as nematodes and humans (Tecott, 2007). While in vertebrates it has an inhibitory effect on feeding-related activities, in some invertebrates such as annelids, molluscs and nematodes it promotes an activation of such behaviours (reviewed in Gillette, 2006; Tecott, 2007).

In insects, monoamines can act as neurotransmitters, neuromodulators or neurohormones, exerting their effects at the central or peripheral level (Bicker and Menzel, 1989; Roeder, 1999; Blenau and Baumann, 2001; Scheiner et al., 2006; Orchard, 2006). Pharmacological studies on different insects have demonstrated that the alteration of haemolymph or neuronal levels of 5-HT modifies feeding behaviour (Kaufmann et al., 2004; Neckameyer et al., 2007; Haselton et al., 2009). In the flies *Phormia regina* and *Neobellieria bullata* and the cockroach *Rhyparobia madera*, administration of 5-HT promotes a decrease in sucrose consumption (Long and Murdock, 1983; Cohen, 2001; Dacks et al., 2003). It has been suggested that this effect could be due to a decrease of the sucrose sensitivity, raising the sucrose response threshold of the insect, and consequently, diminishing its food intake (Dacks et al., 2003). The effect of 5-HT on the dynamics of ingestion and the underlying mechanism have not yet been identified for insects. Does 5-HT only affect the total amount of food consumed? Or could 5-HT rather be acting – either at a peripheral or central level – on the activity of the feeding apparatus?

Immunohistochemical studies performed in several insect species revealed that the serotonergic system is involved in the main centres that control feeding – the suboesophageal ganglion (SEG) and the frontal ganglion (FG) – as well as in the salivary gland, the mouthparts and the alimentary canal (Nässel and Elekes, 1984; Davis, 1985, 1987; Klemm et al., 1986; Nässel, 1988; Orchard et al., 1988; Lange et al., 1988; van Haeften and Schooneveld, 1992;

Abbreviations: 5-HT, serotonin; 5-HT-IR, serotonin-like immunoreactive; FG, frontal ganglion; SAT, sucrose acceptance threshold; SEG, suboesophageal ganglion.

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Ali et al., 1993; Ali, 1997; Miggiani et al., 1999; Dacks et al., 2003; Molaei and Lange, 2003; Orchard, 2006; Tsuji et al., 2007; Siju et al., 2008). This strongly suggests that 5-HT plays an important role in the control and integration processes involved in feeding modulation. However, the distribution and function of this amine seem to vary widely among taxa.

Insects achieve fluid intake by the sucking-pump; its anatomy was described for two ant species: *Lasius niger* (Janet, 1905) and *Pachycondila villosa* (Paul et al., 2002). It is composed of different sets of muscles that act by dilating, retracting and adducting the pharynx and the buccal tube; the contraction of the dilator muscles expand the pharynx generating the negative pressure that drives the fluid into the mouth. Sucking-pump activity was characterized for the nectivorous ant *Camponotus mus* (Josens et al., 2006; Falibene and Josens, 2008; Falibene et al., 2009). This ant is able to vary the velocity of sucrose solution intake depending on the sugar starvation level of the colony (Josens and Roces, 2000). Carbohydrate-starved ants reach higher intake rates by increasing pumping frequency while maintaining the volume taken per pump contraction almost constant for a given concentration (Falibene and Josens, 2008; Falibene et al., 2009).

Although many aspects of feeding behaviour and feeding regulation have been studied in ants, there is no available information about 5-HT with regard to feeding in this insect. Considering the role of the physiological state on the modulation of ingestion, the aim of the present work was

(1) to analyse the effects of this amine on feeding behaviour and sucking-pump activity, and whether the observed effects are due to changes in the sucrose perception and
(2) to study by immunohistochemical techniques the 5-HT association with the alimentary canal, the sucking-pump muscles and the major neuronal centres involved in feeding regulation.

2. Materials and methods

2.1. Insects

Six colonies of *C. mus* (Roger) composed of around 1000 workers and one or more queens were used in the experiments. The colonies were captured in Buenos Aires (34° 32′ S, 58° 26′ O) and Santiago del Estero province (27° 49′ S, 64° 03′ W), Argentina, and transported to the laboratory. Each colony was reared in an artificial nest or container consisting of a plastic box (30 × 50 × 30 cm) with its base coated with plaster and its walls painted with fluon to prevent animals from escaping. Colonies were housed in piled acrylic plates and workers had access to fresh water, honey-water and chopped insects within the container. Nests were maintained in the laboratory for one year under natural light/dark cycles and nearly constant temperature (23 ± 3 °C). Prior to the behaviour experiments, colonies were submitted to a carbohydrate starvation for a period of 10 ± 5 days. Immunohistochemical experiments were carried out at the University of Würzburg. Two *C. mus* colonies captured in the campus of the University of Buenos Aires were transported to this laboratory and were maintained there for several weeks until the experiments at 25 °C, 50% RH, a 12:12 h light/dark cycles and free access to honey–water.

2.2. Feeding behaviour experiments

2.2.1. Feeding behaviour recording

The records of the sucking-pump activity during sucrose-solution feeding were performed as described in previous works (Josens et al., 2006; Falibene and Josens, 2008; Falibene et al., 2009). Briefly, the experimental device consisted of a wooden platform (9 × 2 cm) that led to the *recording arena* which was a surface (2 × 2 cm) covered with a wet filter paper and a metallic mesh. An Eppendorf tube (0.5 ml) was placed in the centre and inserted in a hollow so that the open extreme of the tube levelled with the metallic mesh. The tube was completely filled with a 30% w/w sucrose solution until a little drop was exposed on the top, keeping no contact with the mesh, which was covered by a thin layer of conductor gel (Falibene et al., 2009). One electrode was fixed to the metallic mesh while the other one was in contact with the solution. When the ant stood on the mesh and contacted the solution with its mouthparts, the circuit closed allowing the recording of the electrical signals generated by the ant during feeding (amplified 210×; band-pass filter 0.4–17 Hz, −3 dB; sampling rate: 200 Hz). The records were observed and stored on a PC using an analogue-to-digital converter (ADC-212, Pico Technology Limited, UK).

Experiments were performed with different colonies at the same time of the day on different days. Before each assay, a group of around ten ants was allowed to feed at the experimental device to establish a pheromone trail towards the sucrose solution. Afterwards, recruited ants were individually placed on a wooden bridge (9 cm long, 0.2 cm wide) situated on a balance (Metler Toledo, resolution of 0.01 mg) in order to record the ant mass (initial weight). This bridge was then put in contact with the platform that led to the recording arena with the sugar solution. Once on it, the ant was not disturbed in order to find and drink the solution by itself. The recording began when the ant introduced its mouthparts in the drop. The volume of the offered solution was always larger than the volume a single ant could ingest in one intake. After the ant finished feeding and returned to the wooden bridge its mass was recorded again (final weight: ant plus crop load masses). After the records, ants were kept separated from the rest of their nestmates until the end of the experiment.

2.2.2. Variables and parameters of feeding behaviour

The *volume of solution ingested* (μl) was calculated for every single ant by dividing the load mass (in mg, the difference between final and initial weights) by the density of the sucrose solution obtained from tables (Wolf et al., 1984). The *feeding time* (in seconds) represents the duration of the electrical signal and coincides with the time that the ant was in contact with the drop of sucrose solution. Then, *intake rate* (μl s^{-1}) was obtained by dividing the volume of solution ingested by the feeding time. The *predominant frequency* (pump contractions per second, p s^{-1}) was defined as the frequency that presented the highest peak in the periodogram (energy × frequency) which resulted from the analysis of the entire signal (entire intake). Multiplying predominant frequency by feeding time resulted in the estimation of the *total number of pump contractions* in the entire intake. Finally, the *volume per contraction* (nl p^{-1}) was obtained by dividing the volume of solution ingested by the total number of pump contractions.

2.2.3. Drug administration

Considering that feeding behaviour in ants is modulated by their motivational state, we administered the drug orally in order to be non-invasive and minimize the manipulation of the animal. Serotonin hydrochloride (Sigma) was dissolved in a 30% or 20% w/w sucrose solution containing ascorbic acid (10 mM), which is commonly added to reduce oxidation of the biogenic amines. Solutions were prepared, stored at −10 °C (not longer than 10 days) and defrosted 5 min before being used. Depending on the experiment, ants were fed individually or in groups with the *control solution* (sucrose and ascorbic acid) or with different quantities of 5-HT added to this solution (see Section 2.2.4 for details).

2.2.4. Experimental series

2.2.4.1. Time-dependency of effects. First, we determined the necessary time to observe an effect of orally administered 5-HT on feeding behaviour in *C. mus* ants. Ants from the same colony were placed in individual flasks (3 cm diameter) and were fed with 0.25 μl of a 30% w/w control solution (*control group*) or 0.25 μl of the same solution with 5-HT in a concentration 7.5×10^{-2} M (*5-HT group*). Only the ants that took up the total of the offered solution were used for the experiment. Thereafter, each single ant was placed at a time on the wooden bridge that led to the recording arena at different times after the treatment (from 40 min until 6:30 h; administration: between 9 am and 10 am, winter). Electrical activity of the sucking-pump was recorded during intake of 30% w/w sucrose solution. In order to increase the number of records to assure a good time-resolution, we did not weigh ants before or after feeding in this assay.

With regard to the results obtained in the first experiment, we performed a second experiment using another colony. Considering that biogenic amines in previous studies were orally administered to social insects as a group (Schulz and Robinson, 2001; Barron et al., 2002; Barron and Robinson, 2005; Vander Meer et al., 2008), we decided to apply this procedure to our experimental paradigm. Ants were separated in two groups ($N = 40$); one of them was fed with 10 μl (a quantity equivalent to 0.25 μl per ant) of a 30% w/w control solution while the other was fed with 10 μl of 5-HT solution 7.5×10^{-2} M (administration: 11 am, summer). In all cases, after offering the treatment solution to each group, trophallaxis was confirmed to occur within the groups. Feeding behaviour and electrical activity of the sucking-pump were recorded from 3:30 to 5:30 h *after treatment*. Individuals were weighed before and after feeding.

2.2.4.2. Dose-dependency of effects. Ants were individually placed in flasks and orally treated with 0.25 μl of a 30% w/w control solution or a 5-HT solution 7.5×10^{-4}, 7.5×10^{-3} or 7.5×10^{-2} M. Recordings were performed approximately 4 h after treatment (administration between 10 am and 12 am; summer). Ants were weighed before and after feeding.

2.3. Control 1: locomotion activity

Locomotion was tested in order to analyse whether the effect of 5-HT on feeding behaviour was due to a change in the ant's general activity. Ants were individually treated (9–10 am; summer) with 0.25 μl of a 30% w/w control or 5-HT solution 7.5×10^{-2} M and evaluated 1 or 4 h later. For that, ants were individually placed into a circular arena (7 cm diameter) with a grid of squares (1.5 cm side) on its floor and videotaped during the first minute. Locomotion activity was evaluated by quantifying the number of grid lines crossed in 1 min.

2.4. Control 2: sucrose acceptance threshold (SAT)

Sucrose taste responsiveness was tested in order to analyse whether the effect of 5-HT on feeding behaviour observed at the time of the recordings was due to a change in the ant's sugar perception. Ants from a single colony were placed in individual flasks and were treated orally with 0.25 μl of 20% w/w control or 5-HT solution 7.5×10^{-2} M. Approximately 2 h after treatment, ants were individually placed in 1.5 ml Eppendorf tubes and anaesthetized on ice for about 2–4 min. Ants were harnessed into a bottomless pipette tip (10–100 μl). Only the head was exposed, allowing the ants to move their antennae and mouthparts freely. Ants were harnessed in groups of 30 individuals. Mounting procedure lasted around 40–50 min. Sucrose acceptance was evaluated 1 h after the last ant was harnessed, thus, SAT test was performed approximately 4 h after treatment.

2.4.1. SAT measurement

Sucrose acceptance was evaluated by quantifying the occurrence of licking behaviour in harnessed ants (Falibene and Josens, 2011). Labial palps of harnessed ants were touched with a toothpick imbibed with 0.3%, 1%, 3%, 10%, 30% or 50% (w/w) sucrose solution. These concentrations were presented to the ants in ascending order. Before the first stimulation trial and between each subsequent trial, ants were tested in the same way for their response to water. The inter stimulus interval varied between 4 and 5 min. Those ants in which palp stimulation led to extending their maxilla-labium complex were allowed to contact the solution with the protracted complex. Response was considered *positive* when the ant showed licking behaviour after contacting the solution. Response was considered *negative* when ants either did not expose the complex or exposed it but retracted it after contacting the imbibed toothpick, i.e. no licking behaviour was shown. All ants were tested until the first positive response to sucrose solution. The concentration at which an ant showed licking behaviour represents its SAT, which is an indicator of the individual responsiveness to sucrose. Ants were scored depending on the sucrose concentration that promoted its first positive response: 0.3%: 1, 1%: 2, 3%: 3, 10%: 4, 30%: 5, 50%: 6. Ants that responded to water stimulation immediately before the first positive response and ants that did not respond to any of the sucrose stimuli were eliminated from the analysis.

2.5. Immunohistochemistry

2.5.1. Alimentary canal

Major workers (head width ~2 mm) from satiated colonies were anaesthetized with CO_2 and decapitated. Heads and bodies were fixed in dental wax dishes and the alimentary canal – including foregut, midgut and hindgut – was dissected in cold fixative solution (4% Formaldehyde in phosphate-buffered saline, PBS, pH 7.2) and fixed overnight at 4 °C. Tissues were washed first with 0.1 M PBS (3×10 min) and then with PBS containing 0.2% Triton-X 100 (PBST, 2×10 min). After washing, sucking pumps and alimentary canals were pre-incubated in PBST with 2% normal goat serum (NGS; ICN Biomedicals, No. 191356, Orsay, France) for 1 h at room temperature. Then, preparations were incubated in rabbit anti-serotonin primary antibody (1:2000, DiaSorin, Stillwater, MN, Cat. No. 20080, Lot No. 051007) in PBST with 2% NGS first for 2 h at room temperature, then 2 days at 4 °C. After incubation, tissues were washed in PBS (5×10 min) and incubated in AlexaFluor 488-conjugated goat anti-rabbit secondary antibody (1:250, Molecular Probes, A-11008) in PBS with 1% NGS overnight at 4 °C and washed again (5×10 min).

2.5.2. SEG and FG

Ants were anaesthetized with CO_2 and decapitated. Heads were fixed in dental wax dishes, and whole brains (with the SEG and FG attached to it) were dissected in Ringer solution and fixed overnight at 4 °C. Tissues were washed first in PBS (3×10 min) and then in PBST (2×10 min). Afterwards, they were pre-incubated in PBST with 2% NGS for 1 h at room temperature. Whole mount preparations were incubated in rabbit anti-serotonin primary antibody (1:4000) in PBST with 2% NGS first for 2 h at room temperature, then 4 days at 4 °C. After incubation, tissues were washed in PBS (5×10 min) and incubated in AlexaFluor 568-conjugated goat anti-rabbit secondary antibody (1:250, Molecular Probes, A-21124) in PBS with 1% NGS overnight at 4 °C. Preparations were then incubated in Sytox Green (2.5 mM, Molecular Probes, S-7020) in PBST

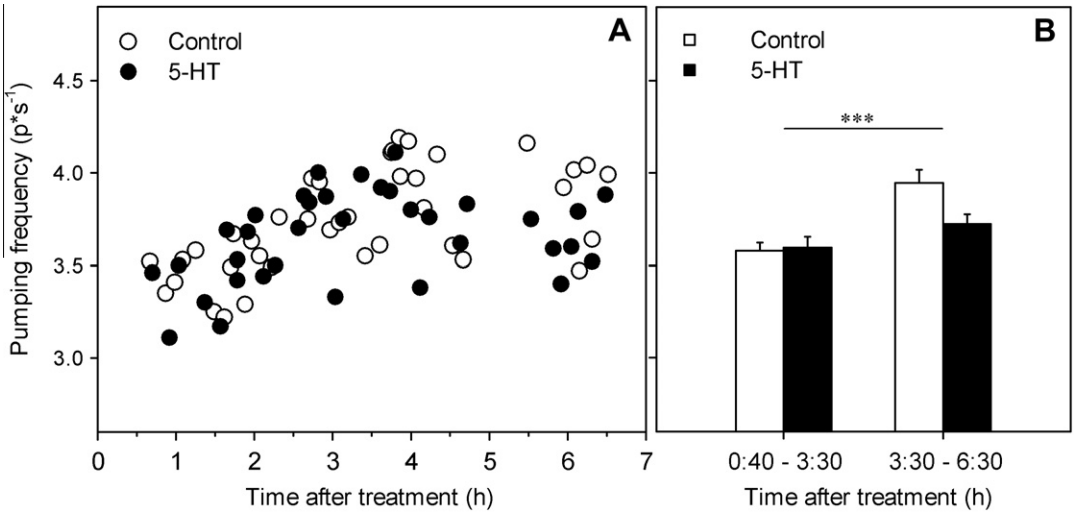

Fig. 1. Time-dependency of effects of 5-HT oral treatment. Sucking pump activity recorded during the sucrose-solution intake in ants at different times after a control (0.25 μl sucrose solution) or 5-HT treatment (0.25 μl sucrose solution with 5-HT 7.5×10^{-2} M). (A) Predominant pumping frequency in function of the time after treatment (administration time = 0 h). Each dot represents an ant. (B) Pumping frequency (mean + s.e.m.) considering two periods after administration: 0:40–3:30 h ($N_{control} = 21$, $N_{5\text{-HT}} = 20$) and 3:30–6:30 h ($N_{control} = 18$, $N_{5\text{-HT}} = 15$). $^{***}P < 0.001$.

for 3:30 h at room temperature for nuclear staining and subsequently washed in PBS (4×10 min).

2.5.3. Mounting, image view and processing

Finally, alimentary canals and brain preparations were dehydrated in an ascending ethanol series (30%, 50%, 70%, 90%, 95%, $3 \times 100\%$, 10 min each step), cleared in methylsalicylate (M-2047, Sigma Aldrich, Steinheim, Germany) and mounted in special aluminium slides with a central hole covered by coverslips from both sides. Preparations were viewed with a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany) with different HC PL APO objective lenses (10×0.4 and 20×0.7 NA imm and 40×1.25 oil). Scanning of the alimentary canals was carried out using an excitation wavelength of 488 nm and taking optical sections at 2 μm. The sucking-pump structure was visualized using cuticular autofluorescence. Scanning of the SEG and the FG was carried out using an excitation wavelength of 488 nm for Sytox Green and 568 nm for AlexaFluor 568. Series of optical sections were scanned at a distance around 1 μm. Image processing was performed with Fiji ImageJ software (ImageJ 1.44c; Wayne Rasband, National Institute of Health, USA) for image reconstruction and with AMIRA software (Mercury Computer Systems, Berlin, Germany) for volume measurements.

2.6. Statistical analysis

In time-dependency effects experiments and locomotion activity control, two-way ANOVA was used. The variables obtained in the rest of the series were analysed either by one-way ANOVA or by Kruskal–Wallis test. In dose-dependence experiments, in cases the ANOVA showed a significant result, post-hoc Fisher comparisons were applied. The significance level used was 5% in all cases.

3. Results

3.1. Feeding behaviour experiments

3.1.1. Time-dependency of effects

The sucking-pump activity recorded throughout the time after treatments did not show any differences between 5-HT and control groups until 3:30 h. From approximately 40 min to 3:30 h after treatment, pumping frequency increased with the time in both groups (Fig. 1A). From thereon, the control ants kept pumping frequencies constant until the end of the experiment (6:30 h) while 5-HT treated ants tended to pump at lower frequencies. Considering these results, we separated the 5-HT effect in two periods: between 40 min and 3:30 h and the period between 3:30 and 6:30 h after treatment (Fig. 1B). Whereas pumping frequency significantly varied between periods (*treatment*period:* $F_{1,70} = 3.72$, $p = 0.058$; *period:* $F_{1,70} = 18.39$, $p < 0.0001$; two-way ANOVA), 5-HT treatment only induced a trend towards lower frequencies compared with the control (Fig. 1A and B; *treatment:* $F_{1,70} = 2.53$, $p = 0.12$; two-way ANOVA).

Based on these results, we analysed the 5-HT effects on feeding behaviour 3:30 h after treatment in a new experiment in which the ants were treated in groups. Considering that ant size affects feeding variables, we used ants of similar size ($F_{1,16} = 0.27$, $p = 0.61$, ANOVA). Recordings showed that although the feeding time was the same for both groups ($F_{1,16} = 0.06$, $p = 0.81$, ANOVA), the volume of solution ingested by 5-HT treated ants was significantly lower than that ingested by control ants ($H_{1,N=18} = 5.07$, $p = 0.024$, Kruskal–Wallis test). This implies that 5-HT treated ants presented lower intake rates than control ants (Fig. 2A, $F_{1,16} = 6.13$, $p = 0.025$, ANOVA). In theory, a decrease in the intake rate of a certain solution may partly be due to a decrement in pumping frequency and/or in the volume per pump contraction. Pumping frequency did not vary significantly between treatments (Fig. 2B, $F_{1,16} = 0.06$, $p = 0.80$, ANOVA). This shows that intake rate reduction was mainly due to a decrease in the volume of solution taken per contraction (Fig. 2C, $F_{1,16} = 9.70$, $p = 0.007$, ANOVA). There were no significant differences in the total number of pump contractions in the whole intake ($F_{1,16} = 0.18$, $p = 0.68$, ANOVA).

In an experiment carried out with the same methodology, but under a chronic treatment (one administration per day during 6 consecutive days) with control or 5-HT solution (0.25 μl per day of 5-HT solution 7.5×10^{-2} M per ant), 5-HT did diminish the pumping frequency significantly ($F_{1,15} = 6.05$, $p = 0.027$; ANOVA) as well as the volume taken per pump contraction ($F_{1,15} = 8.39$, $p = 0.011$; ANOVA) 3:30 h after the last treatment administration ($N_{control} = 7$, $N_{5\text{-HT}} = 10$).

3.1.2. Dose-dependency of effects

Ants treated with different concentrations of 5-HT (Table 1) modified their feeding behaviour in a dose-dependent manner. Although

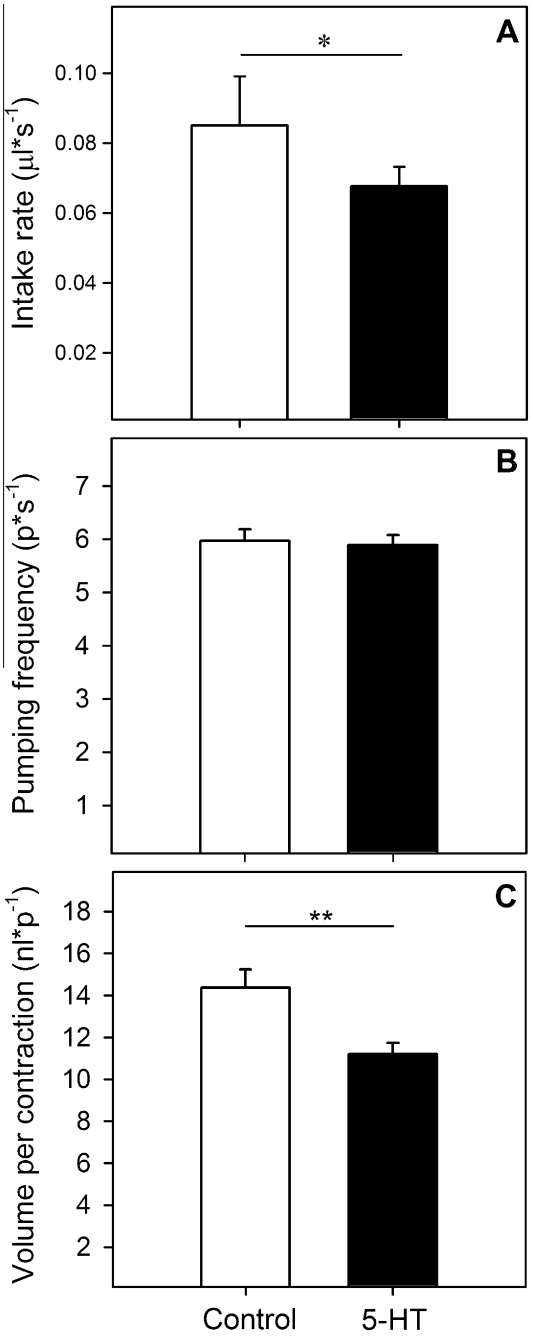

Fig. 2. Feeding behaviour of ants that had received either control ($N = 9$) or 5-HT ($N = 9$) treatment 3:30 h earlier. (A) Intake rate, (B) Pumping frequency and (C) Volume of solution taken per pump contraction. For all variables, means + s.e.m. are given. $^{*}P < 0.05$, $^{**}P < 0.01$.

ant mass did not vary significantly between treatments ($F_{3,63} = 1.11$, $p = 0.35$, ANOVA), there were slight differences in the ants' sizes between groups. Therefore, we compared ant weight-relative variables in order to avoid any bias due to these differences. Relative intake rate (nl s^{-1} mg^{-1}) varied with the concentration of 5-HT administered (Fig. 3A, $F_{3,63} = 3.32$, $p = 0.025$, ANOVA). Differences became significant beyond 5-HT 7.5×10^{-2} M (ca. 1 µg per mg ant, Table 1). In the same way as in the previous experiments, neither pumping frequency (Fig. 3B, $F_{3,62} = 1.38$, $p = 0.26$, ANOVA) nor the total number of pump contractions ($F_{3,63} = 0.60$, $p = 0.68$, ANOVA) varied, but the volume per contraction decreased in a dose-dependent manner (Fig. 3C, $F_{3,63} = 3.83$, $p = 0.014$, ANOVA). Ants treated with 5-HT 7.5×10^{-3} and 7.5×10^{-2} M took a lower volume of solution per

Table 1
5-HT concentration of the 0.25 µl of solution ingested by the ants in the dose-dependence experiment (in brackets, quantity of 5-HT ingested), ant mass of each group and amount of 5-HT ingested relative to ant weight (mean ± error).

Dose of 5-HT	Ants' mass (mg)	µg of 5-HT/mg of ant
0	3.94 ± 0.01	0 ± 0
7.5×10^{-4} M (0.04 µg)	3.85 ± 0.11	0.011 ± 0.001
7.5×10^{-3} M (0.4 µg)	4.09 ± 0.09	0.099 ± 0.002
7.5×10^{-2} M (4 µg)	4.01 ± 0.11	1.008 ± 0.028

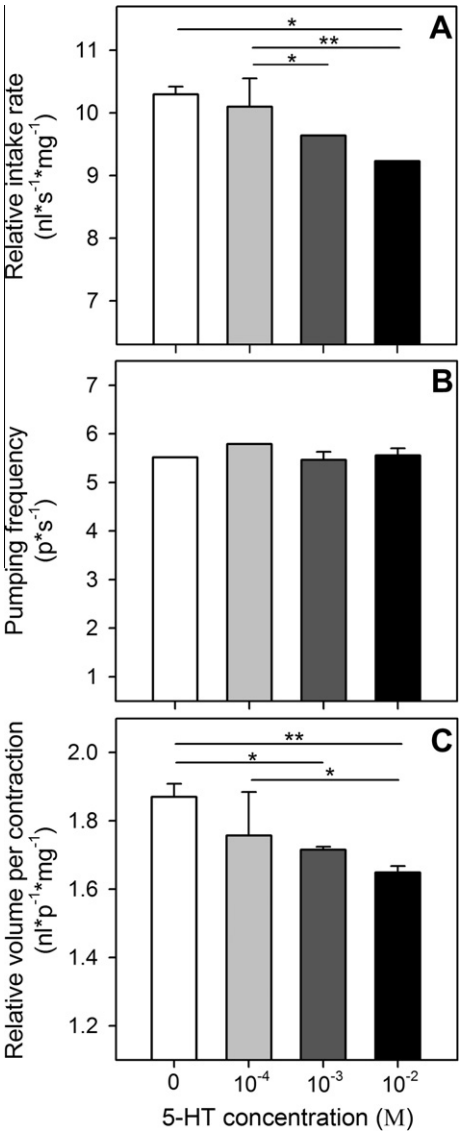

Fig. 3. Dose-dependence effects of 5-HT treatment. Feeding behaviour recorded 3:30 h after administration on ants treated with 0.25 µl of control sucrose solution ($N = 17$) or sucrose solution containing 5-HT in a concentration 7.5×10^{-4} M ($N = 17$), 7.5×10^{-3} M ($N = 17$) or 7.5×10^{-2} M ($N = 16$). (A) Intake rate (relative to ant weight), (B) Pumping frequency and (C) Volume of solution taken per pump contraction for different 5-HT concentrations. $^{*}P < 0.05$, $^{**}P < 0.01$.

pump contraction than control ants ($p = 0.03$ and $p = 0.007$, respectively; Fisher comparisons).

3.2. Control 1: locomotion activity

Oral 5-HT administration did not affect the general locomotion activity of ants neither 1 h nor 4 h after treatment (*treatment∗time*:

$F_{1,76} = 0.15$, $p = 0.70$; *treatment*: $F_{1,76} = 0.001$, $p = 0.98$; *time*: $F_{1,76} = 1.09$, $p = 0.30$; two-way ANOVA).

3.3. Control 2: SAT

Oral administration of 5-HT did not modify the SAT 4 h after treatment (Fig. 4, $H_{1,N=74} = 0.49$, $p = 0.48$, Kruskal–Wallis test). Both groups of ants responded positively to any concentration in nearly the same proportion during the testing; 65% of control- and 67% of 5-HT-treated ants. They both presented a SAT of 30% w/w (median value). As the ant mass was around 5 mg, the amount of 5-HT ingested resulted in 0.85 ± 0.05 μg per mg of ant, which is similar to the amount used in the other experiments.

3.4. Immunohistochemistry

3.4.1. Alimentary canal

Immunohistochemical analysis in whole-mount preparations of alimentary canals of *C. mus* ants ($N = 9$) revealed that 5-HT-immunoreactive (IR) processes were distributed throughout the foregut while the midgut and hindgut were completely devoid of 5-HT-IR processes (Fig. 5). Fig. 5B shows a confocal image of the sucking-pump structure obtained by cuticular autofluorescence. 5-HT-IR processes were absent in the portion of the canal inside the head (i.e. the cibarium, the pharynx and the anterior part of the oesophagus) and in the sucking-pump muscles (i.e. superior and inferior dilator muscles). 5-HT-IR was also absent in the muscles attached to the last portion of the pharynx (most likely the pharyngeal retractor described by Janet (1905) and Paul et al. (2002); Fig. 5B and C). On the contrary, 5-HT-IR processes were clearly present along all portions of the oesophagus inside the thorax (Fig. 5C and D), the crop and the proventriculus (including its bulb, Fig. 5E). Neither the midgut nor the hindgut (i.e. malpighian tubules, the intestine, the rectum and the anus) contained 5-HT-IR processes (not shown).

3.4.2. FG and SEG

Analyses of the FGs ($N = 5$) revealed between 19 and 28 (22 ± 1.58) 5-HT-IR cell bodies (Fig. 6A and B). Nuclear staining allowed identification of individual 5-HT-IR cell bodies without ambiguity. In each FG, 5-HT-IR cell bodies differed in size and staining intensities (Fig. 6C). We measured volumes between ~100 μm^3 in the smaller cells to ~1900 μm^3 in the largest ones with a mean value of 720 ± 36 μm^3 per cell. Frontal connectives, which connect the FG with the brain, also clearly showed 5-HT-IR fibres (Fig. 6A).

SEGs ($N = 8$) contained 7 pairs of 5-HT-IR cell bodies (Fig. 6D and E). These were symmetrically positioned on each side of the ganglion with a similar distribution as found in *Camponotus japonicus* ants (Tsuji et al., 2007). The cell bodies were distributed in three clusters located in the anterior (2 pairs), the medial (3 pairs) and the posterior (2 pairs) region of the SEG (Fig. 6D), volumes were 576 ± 64, 1590 ± 111 and 1155 ± 130 μm^3 for the anterior, medial and posterior groups, respectively.

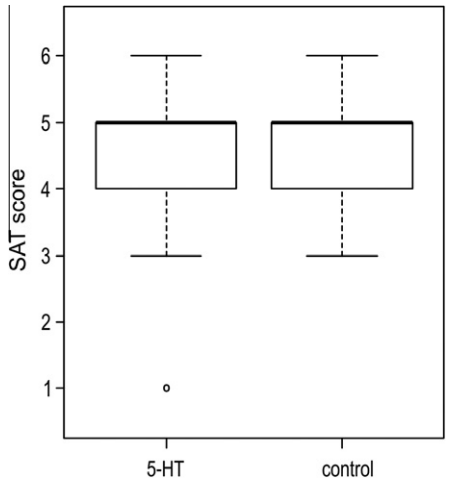

Fig. 4. SAT in harnessed ants that individually received different treatments with either sucrose solution (control; $N = 37$) or sucrose solution with 5-HT 7.5×10^{-2} M (5-HT; $N = 37$) 4 h before evaluation. Thick horizontal lines within each box represent medians, boxes show quartiles, whiskers provide the extreme values and circles indicate outliers.

4. Discussion

4.1. 5-HT and feeding behaviour

The present study indicates that 5-HT promotes a depression on nectar feeding in the ant *C. mus*. Orally administered 5-HT to starved ants reduced the volume of sucrose solution ingested, either when administered as *individual treatment* or to the *group* and shared by trophallaxis. Depressant effects of this amine on feeding have also been demonstrated in other insects. The plant-sap feeding aphid disrupts the stylet penetration behaviour when treated with exogenous 5-HT (Kaufmann et al., 2004). Insects that base their diet on sugars and proteins as *C. mus* ants show similar responses. Injection of 5-HT in the cockroach *R. madera* turns its preferences towards protein ingestion and diminishes the sucrose intake and, moreover, nymphs treated with a 5-HT antagonist overfeed on sugars (Cohen, 2001). In flies, 5-HT injection promotes a decrease in carbohydrate feeding (Long and Murdock, 1983; Dacks et al., 2003), as well as in protein feeding (Haselton et al., 2009). In addition to these results, we showed in the ant that exogenous 5-HT also affected the dynamics of ingestion and the sucking-pump activity in a dose-dependent manner. Furthermore, our recordings enabled us to discern for first time the mechanism underlying this effect: 5-HT decreased intake rate mainly by reducing the volume of solution taken per pump contraction. A single dose of 5-HT did not modify pumping frequency consistently; however, a chronic treatment with 5-HT (during 6 consecutive days) promoted a clear and significant depression in this variable.

Pumping frequency increased during the first 3 h after treatment for both 5-HT-treated and control groups, a fact that might depend on the time of the day and/or the time since the last ingestion (A. Falibene and R. Josens, pers. obs.).

Unlike in other insects, a single dose of orally administered 5-HT exerted its effects only 3:30 h after its administration in *C. mus* ants. In honeybees, measurements generally were carried out within the first hour after oral-treatment (Scheiner et al., 2002; Pankiw and Page, 2003; Spivak et al., 2003). The difference in the time required to observe an effect may be due to a difference in the metabolism of these insects. The metabolic rate of a honeybee during feeding is 125 times higher than that of a *Camponotus* worker (Blatt and Roces, 2001; Schilman and Roces, 2008). Changes in sucking-pump activity were not caused by a depressant effect on the whole muscle system, as the general activity did not vary between 5-HT treated and non treated ants. We did not detect any changes in SAT promoted by orally administered 5-HT either. Although 5-HT appears to have no effect on acceptance thresholds in *C. mus* ants 4 h after oral administration, we cannot reject the possibility that this amine has an effect on sugar perception. Previous studies performed in other insects established that acceptance threshold and gustatory neurons sensitivity are related to 5-HT levels (Brookhart et al., 1987; Blenau and Erber, 1998; Dacks et al., 2008). It is possible that the effects of this amine on SAT

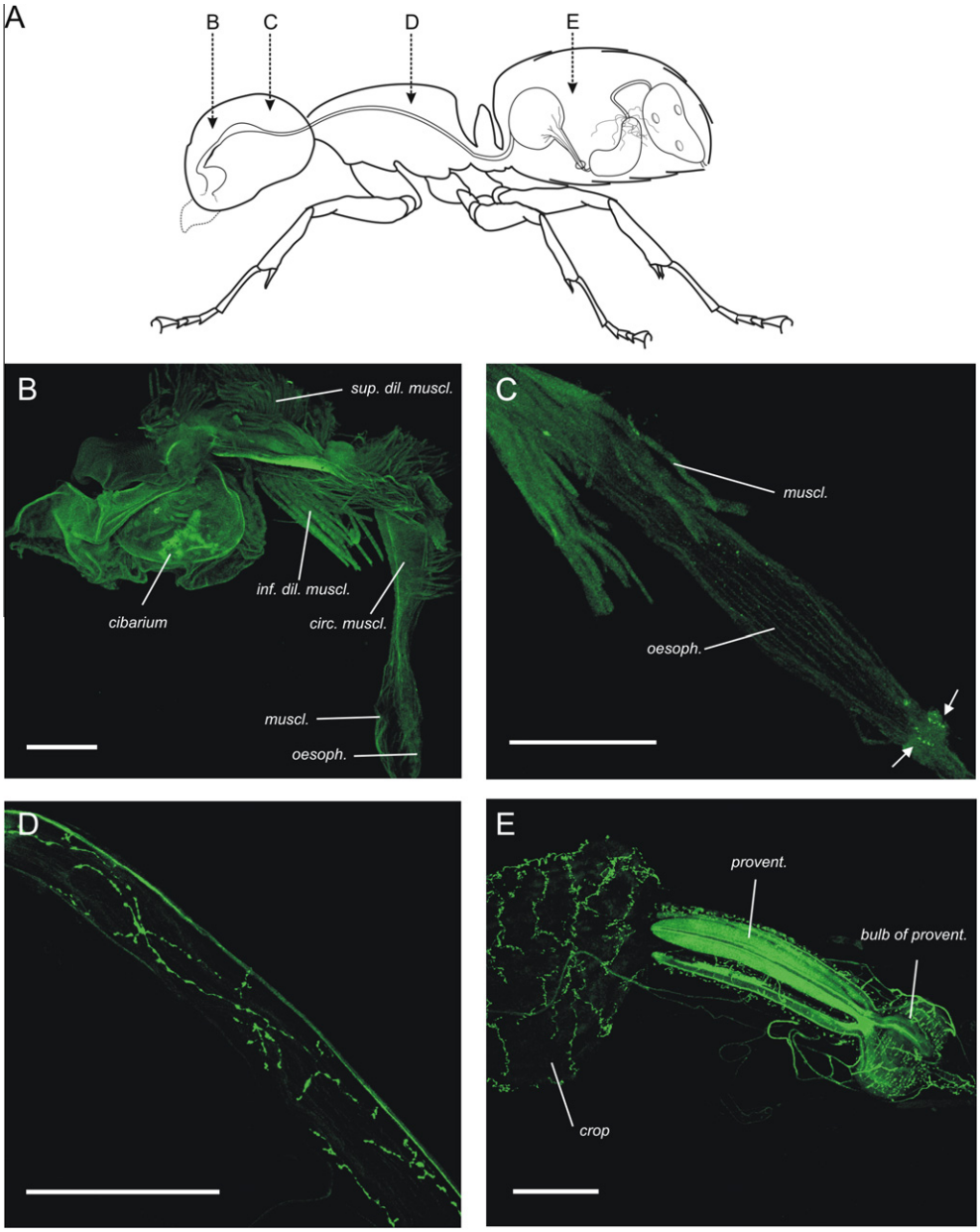

Fig. 5. 5-HT-IR processes in the alimentary canal of *Camponotus mus* ants. (A) Schematic drawing of the alimentary canal of a *Camponotus* ant (based on Hansen and Klotz, 2005). Arrows (B–E) indicate the section of the canal showed in the figures. (B) Confocal image of the sucking-pump structure using cuticular autofluorescence. Cibarium, superior dilator muscles (*sup. dil. muscl.*), inferior dilator muscles (*inf. dil. muscl.*), circular muscles at the end of the pharynx (*circ. muscl.*), muscles of the last portion of the pharynx (*muscl.*, probably pharynx retractor) and anterior part of the oesophagus (*esoph.*) are shown. (C) 5-HT-IR processes (indicated by the arrows) were only present in the portion of the oesophagus that corresponds to the neck. (D) Prominent 5-HT-IR processes in the portion of the oesophagus that lies in the thorax. (E) 5-HT-IR processes in the crop and the proventriculus. Scale bars = 200 μm.

may follow a different time course than that on sucking-pump activity. However, with this control experiment we only aimed to show that there was no effect of 5-HT on SAT at the time of recording the sucking pump activity (4 h post-treatment). Alternatively, one could argue that 5-HT may affect sucrose responsiveness, but a potential effect of the sucrose ingested during the treatment would hide a possible amine-promoted change. If this were the case, 5-HT administration by injection – as it is commonly done in insects including ants (Boulay et al., 2000) – would allow us to detect threshold changes.

Little is known about 5-HT concentration in insect haemolymph. In unfed *Rhodnius prolixus* larvae, it was reported to be about 7 nM, rising to 115 nM 5 min after the onset of food ingestion (Lange et al., 1989). In the butterfly *Pieris brassicae*, haemolymph 5-HT levels varied widely during its developmental stages and with the photoperiod, ranging between undetectable levels up to 1.6 μM in larvae and 12 μM one day after pupation (L'Helias et al., 1995; Isabel et al., 2001). 5-HT was not detected in the haemolymph of the hawk moth *Acherontia styx* larvae (detection limit: 150 pg/ml; Awad et al., 1997).

On the other hand, studies performed in bees with another biogenic amine have shown that about 1% of the total amount of drug ingested was found in the abdominal haemolymph 1 h after oral treatment (Barron et al., 2007). There is not enough information

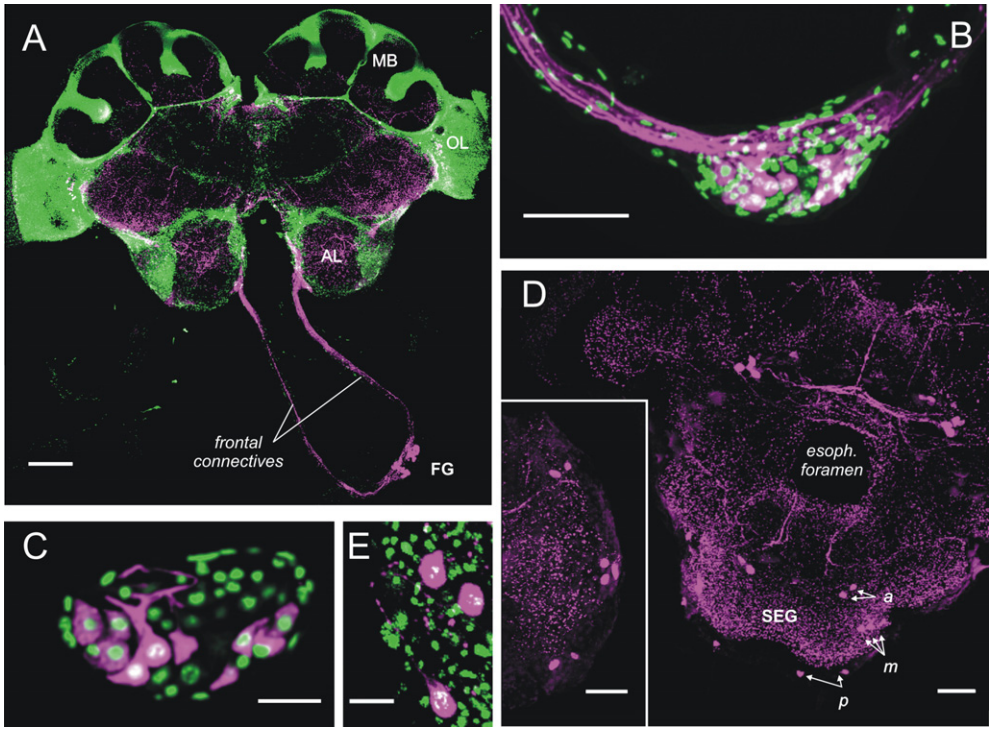

Fig. 6. 5-HT-IR in the FG and the SEG of *C. mus* ants. Confocal images with 5-HT immunoreactivity shown in magenta and nuclear staining in green. (A) Overview of the brain (two-dimensional projection of optical sections) with the FG attached by the frontal connectives. Mushrooms bodies (MB), optic lobes (OL) and antennal lobe (AL) are indicated. Scale bar = 100 μm. (B) FG with between 19 and 28 5-HT-IR cell bodies and frontal connectives showing 5-HT-IR processes. Scale bar = 50 μm. (C) Confocal image stack form the FG with 5-HT-IR cell bodies of different staining intensities. Scale bar = 20 μm. (D) Confocal image of 5-HT staining within the SEG showing 7 pairs of 5-HT-IR cell bodies symmetrically positioned on each side of the SEG. Arrows indicate the right anterior (a), medial (m) and posterior (p) groups of cells. The Inset shows a ventral view of the right side of the SEG. Scale bars = 50 μm. (E) Confocal image stack of double staining within the SEG showing the left medial cell bodies. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

available whether 5-HT acts in a similar way; however, we observed effects with 0.25 μl of 5-HT solution 7.5 mM, (e.g. 1.875 nmol of 5-HT per ant).

4.2. 5-HT and satiety

There is some evidence suggesting that 5-HT may play an important role in satiety signals in insects. In blood-feeding insects, haemolymph 5-HT levels and 5-HT-IR staining in different areas change in association with feeding events (Lange et al., 1988, 1989; Orchard et al., 1988; Siju et al., 2008). In the same way, the staining of 5-HT-IR cells of the SEG decreases after feeding in flies whose diet is based on sugar and proteins, suggesting a release of this amine as a consequence of feeding (Dacks et al., 2008). In the present work, a single dose of 5-HT diminished intake rate, as carbohydrate satiation does (Josens and Roces, 2000; Falibene et al., 2009). However, opposite to the 5-HT effect – which prompted changes in the volume of solution taken per pump contraction – feeding state causes variations in pumping frequency while the volume taken per contraction remains constant (Falibene and Josens, 2008; Falibene et al., 2009). It is worth pointing out that variation in pumping frequency was observed when 5-HT was administered daily during six consecutive days.

4.3. 5-HT association with the alimentary canal

The presence of 5-HT-IR in neuronal processes along the oesophagus is consistent across different insects (e.g. locust: *Schistocerca gregaria*, *Locusta migratoria*, crickets: *Gryllus bicamulatus*, *Acheta domesticus*; bugs: *R. prolixus* and *Oncopeltus fasciatus*; cockroaches: *Periplaneta americana*), whereas the distribution of this amine in the alimentary canal varies widely (Davis, 1985; Klemm et al., 1986; Orchard et al., 1988; Miggiani et al., 1999; Molaei and Lange, 2003). In locusts and crickets 5-HT-IR is present from the posterior pharynx in the foregut to the hindgut, but it is not in the cibarium and anterior pharynx (Klemm et al., 1986; Molaei and Lange, 2003). On the contrary, *P. americana* hindgut lacks this biogenic amine (Davis, 1985). In the present study 5-HT-IR processes in *C. mus* ants were absent in the cibarium and in the pharyngeal region -including the sucking-pump muscles -but 5-HT-IR processes were clearly associated with the rest of the foregut: the oesophagus, crop and proventriculus. These differences between insects may reflect the role of 5-HT on the control of particular components of feeding activity, and they may as well be related to differences in the nutritional requirements in different species.

In eusocial ants specialized in nectar feeding as *Camponotus* the control of the proventriculus is a key element in the balance between individual and social food distribution. The proventriculus is highly developed in *Camponotus* and regulates the flow of liquid between the crop "the social stomach" – as its contents can be shared by regurgitation among nestmates – and the midgut – where the food is digested for individual supply (Hölldobler and Wilson, 1990). In addition, carbohydrate starvation strongly affects the haemolymph sugar levels of workers (Schilman and Roces, 2008) and, on the other hand, in honeybees the level of carbohydrates is responsible for the control of the sugar transport rate through the proventriculus (Roces and Blatt, 1999; Blatt and Roces, 2002a,b). The presence of 5-HT-IR processes in the crop, proventriculus and bulb of proventriculus of *C. mus* suggests that this amine may be involved in this control.

4.4. 5-HT association with the nervous system

The SEG and FG are known to be involved in the modulation of feeding behaviour in insects. While the FG innervates sucking-pump muscles, the SEG controls the mouthparts (Miles and Booker, 1998; Rast and Bräunig, 2001; Ayali, 2004; Davis and Hildebrand, 2006) and innervates the inferior dilator muscle of the pharynx in *L. niger* ants (Janet, 1905). Furthermore, the SEG and tritocerebrum receive direct projections from gustatory and mechanosensitive sensilla in honeybees, flies and moths (Rehder, 1989; Edgecomb and Murdock, 1992; Mitchell et al., 1999; Wang et al., 2004; Jørgensen et al., 2006). Related to the presence of 5-HT, immunohistochemical studies have revealed a complex serotonergic network involved in the SEG in various insects (Davis, 1987; Nässel, 1988; van Haeften and Schooneveld, 1992; Dacks et al., 2003; Orchard, 2006; Siju et al., 2008), including ants (Tsuji et al., 2007). Our results show that *C. mus* possess a similar number and distribution of 5-HT-IR cell bodies in the SEG to the ones described for *C. japonicus* (Tsuji et al., 2007).

The FG is a principal component of the stomatogastric system in most insect taxa (rev. Ayali, 2004). This ganglion lies on the dorsal side of the pharynx and is connected to the tritocerebrum by the paired frontal connectives. The FG is the major source of foregut muscle innervation, including sucking-pump dilator muscles (Janet, 1905; Miles and Booker, 1998; Ayali, 2004; Davis and Hildebrand, 2006). The presence and number of 5-HT-IR cell bodies in the FG varies among insects (Klemm et al., 1986; Radwan et al., 1989; Davis, 1985; Nässel, 1988). For the first time in ants, we observed that the FG contains serotonergic cell bodies with differences in size and intensities of immunostaining. Despite the effect of the 5-HT on the sucking-pump activity, the close relationship between the sucking-pump and the FG and the abundance and volume of 5-HT-IR material in the FG, we did not detect direct serotonergic innervations in the sucking-pump muscles.

4.5. Possible mechanisms of feeding regulation

It is known that 5-HT acts on visceral-muscle contractions in insects. Some studies have shown an excitatory effect (Cook et al., 1969; Huddart and Oldfield, 1982; Cooper and He, 1994; Kaufmann et al., 2004; Orchard, 2006), while others showed a relaxation (Banner et al., 1987a,b; Osborne et al., 1990; Molaei and Lange, 2003). In our results, a diminution of the sucking-pump muscles tonus generated by the administered 5-HT might explain the reduction in the volume of solution taken per pump contraction. If so, this action on sucking-pump muscles would be specific (at least at the drug doses used here) since the general locomotion activity was not modified by the 5-HT administered. Exogenous 5-HT, once in the haemolymph, might act either by reaching central nervous system targets or directly on the muscles involved in feeding. Small molecules of biogenic amines would be able to pass from the gut to the haemolymph, the brain and nervous tissues, as it was shown for octopamine in honeybees (Barron et al., 2007).

As 5-HT-IR processes were not detected in the sucking-pump muscles themselves, we exclude *direct* 5-HT innervations of this structure from the FG. It is known that 5-HT -together with dopamine and octopamine- is synthesized and metabolized in the FG in big quantities and, additionally, the neurosecreted material from the FG can reach and modulate the muscles all along the alimentary canal (rev. Ayali, 2004). Biogenic amines may have their neuromodulatory effects on both the pre- and the post-synaptic site of motor neurons altering the response properties of the muscles via changes in synaptic efficacy. Even where the muscles do not receive a direct neural supply of these substances, they can be affected by their presence in the haemolymph (Chapman, 1998). Furthermore, the high amount of 5-HT-IR in the FG of *C. mus* ants suggests that 5-HT may *indirectly* affect the sucking-pump activity.

Nevertheless, the exact path 5-HT follows to prompt its effects on feeding regulation is not fully understood yet although some mechanisms have been proposed. There is some evidence showing that the foregut activity in locust may be regulated through the FG by humoral factors released as a consequence of feeding (Ayali et al., 2002). Additionally, the foregut movements in crickets are suggested to be influenced by the presence of food mediated by both nervous and humoral factors (Cooper and He, 1994). As 5-HT has been shown to be released to haemolymph after feeding in some insects, this amine could be a key component of this humoral factor, reaching either the FG or acting peripherally by reaching the alimentary canal. Alternatively, some kind of feedback from the posterior part of the foregut, which was clearly innervated by serotonergic processes, might be influencing pump contractions. Such a control mechanism of the alimentary canal muscles has previously been proposed for crickets, in which the isolated foregut increased its spontaneous activity when it remained connected to the midgut and hindgut, supporting such a feedback mechanism (Cooper and He, 1994).

In summary, we show that 5-HT (1) modulates feeding related processes in ants, (2) plays an important role in the modulation of sucking-pump activity, (3) is clearly present in the structures that regulate the balance between social and individual food consumption as well as in the nervous centres that are involved in feeding control. New insights about the relationship between the sugar starvation and the endogenous levels of 5-HT would lead us to a better understanding of satiation, feeding motivation and feeding control in insects.

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