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International Journal for Parasitology 33 (2003) 435–443
www.parasitology-online.com

Biochemical and histological changes in the brain of the cricket *Nemobius sylvestris* infected by the manipulative parasite *Paragordius tricuspidatus* (Nematomorpha)

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Received 28 November 2002; received in revised form 8 January 2003; accepted 17 January 2003

Abstract

Hairworms (nematomorpha) alter the behaviour of their insect hosts, making them commit 'suicide' by jumping into an aquatic environment required by the adult parasite for the continuation of its life cycle. To explore the physiological and neuronal basis of this behavioural manipulation, we first performed a biochemical study to quantify different neurotransmitters or neuromodulators (monoamines and amino acids) in the brain of crickets (*Nemobius sylvestris*) uninfected and infected by the hairworm *Paragordius tricuspidatus*. We also analysed several polyamines and amino-acids having no known neuromodulatory function. The presence/absence of the parasite explained the largest part of the variation in compound concentrations, with infected individuals displaying on average lower concentrations than uninfected individuals. However, for three amino acids (taurine, valine and tyrosine), a significant part of the variation was also correlated with the manipulative process. In order to compare neurogenesis between infected and uninfected crickets, we also performed a histological study on mushroom bodies in the cricket's brain. The mitotic index exhibited a two-fold increase in infected crickets as compared with uninfected crickets. This is the first study to document changes in the brain of insects infected by nematomorphs.

Keywords: Histology; Host-manipulation; Nematomorpha; Neurobiology; Orthoptera

1. Introduction

Changes in the behaviour of parasitised animals have been found in a broad range of host–parasite systems (see Combes, 1991; Poulin, 1998; Moore, 2002 for reviews). The simplest explanation for these changes is that they are non-adaptive coincidental side effects of infections (Poulin, 1995). In several situations, however, changes in host behaviour are exactly what would be expected if the host were to start acting in a way that benefits the parasite enhancing, for instance, the parasite's survival or its probability of transmission (Curtis, 1987; Brodeur and McNeil, 1989; Maitland, 1994; Thomas and Poulin, 1998; Lafferty, 1999; Berdoy et al., 2000; Eberhard, 2000; Hurd et al., 2001, see Moore, 2002 for a review). In this respect, the manipulation hypothesis stipulates that behavioural changes in infected hosts are the sophisticated products of parasite evolution aimed at host manipulation rather than accidental side effects (Barnard and Behnke, 1990; Berdoy et al., 2000). Although there are numerous studies suggesting that parasites truly manipulate the behaviour of their hosts, neural mechanisms underlying these ethological changes are only starting to be unraveled (Helluy and Holmes, 1990; Thompson and Kavaliers, 1994; Adamo, 1997; Beckage, 1997; Adamo and Shoemaker, 2000; Overli et al., 2001, Helluy and Thomas, 2003).

A recently documented case of a parasite's effect on host behaviour is found in orthoptera harbouring hairworms

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0020-7519/03/$30.00
doi:10.1016/S0020-7519(03)00014-6

(nematomorpha). Adult nematomorphs are free-living in aquatic environments and gather to mate in tight masses ('gordian knot'). Unlike adults, juveniles are parasitic in arthropods (mainly terrestrial insects). Hosts become infected with hairworms when they ingest parasitic larvae (directly or indirectly through a paratenic host, see Hanelt and Janovy, 1999; Schmidt-Rhaesa, 1997, 2001). During their development, nematomorphs grow from a microscopic larva to a large worm whose size exceeds the length of the host by a considerable amount. Worms are only ready to emerge once they reach this stage. In accordance with several anecdotal reports, Thomas et al. (2002) provided evidence that insects parasitised by hairworms commit 'suicide' by jumping into an aquatic environment that is needed by an adult worm for the continuation of its life cycle. This behaviour occurs in at least nine species of orthoptera harbouring hairworms in the south of France (Thomas et al., 2002). Nematomorphs apparently modulate the behaviour of their host with precise timing and in very subtle ways. Indeed, this behavioural change only appears once the parasite is mature (F.T., unpublished data) and involves the sudden appearance of a behaviour originally not present in the host's repertoire (i.e. entering water). From the parasite's perspective, this behavioural change is likely to be adaptive as it ensures the adult worm to be released in an appropriate location for reproduction. Therefore, we can safely assume that infected crickets are manipulated by hairworms. The aim of this study was to explore in more detail the physiological and neuronal basis of this behavioural manipulation, and these results are reported here.

2. Materials and methods

2.1. Sampling

As in Thomas et al. (2002), infected crickets, *N. sylvestris* Bosc (Orthoptera: Gryllidae), were captured at night (between 10 p.m. and 1 a.m.) around a private swimming pool ($15 \times 10\ m^2$) located in Avènes les Bains (Southern France, 70 km north of Montpellier). This swimming pool was located near a forest largely criss-crossed by small streams in which adult nematomorphs, *P. tricuspidatus* Dufour (Nematomorpha: Gordioidea) were commonly found during the summer. Between this swimming pool and the forest, a concrete area 5 m wide allowed the direct observation and capture of infected crickets arriving from the forest in the direction of the swimming pool (Thomas et al., 2002). We also captured uninfected individuals in the forest around the swimming pool. Captures were made every night during July 2001. Our sampling procedure distinguished five categories of crickets (all were nymphs). The first category was called 'night parasitised' crickets and corresponded to manipulated crickets, ie. infected individuals captured between 10 p.m. and 1 a.m. near the edge of the swimming pool just before they jumped into water (Thomas et al., 2002). As a control for this category, we also collected uninfected crickets at night in the nearby forest; we called this category 'night uninfected' crickets. Third, in order to obtain crickets harbouring a mature worm without being manipulated, we captured manipulated crickets (i.e. 'night parasitised' category) and kept them until the following day after in a terrarium containing wood and leaves from their natural habitat. We dissected (see Section 2.2) these crickets between 1 and 3 p.m., that is to say at a period of the day for which no behavioural change is observed in natural conditions (at least for *N. sylvestris*, F.T., unpublished data); we called this third category 'day parasitised' crickets. As a control for this category, we also collected uninfected crickets at night and as before, kept them until the next day before being dissected between 1 and 3 p.m.; we called this fourth category 'day uninfected' crickets. Finally, we considered a last category of crickets corresponding to individuals that have released their worm. For this, infected insects arriving at the swimming pool were visually followed on the concrete area without disturbing them until they entered the swimming pool itself. Just after the emergence of the worm, the cricket was placed in a dry opaque plastic tumbler for 1 h. After this delay, most crickets were vigorous and were dissected. We called this fifth category 'suicided' crickets.

2.2. Brain dissection

Insects were cold anaesthetised at 0°C for 15 min before dissection. The cerebral ganglia (brain plus sub-oesophageal ganglion) were dissected out in saline, corpora cardiaca and corpora allata, as well as frontal ganglion and any trace of fat body were carefully removed. Internal fat body was extirpated under saline containing: Hepes 10 mM, NaCl 150 mM, KCl 12 mM, $MgCl_2$ 3 mM, glucose 40 mM, $CaCl_2$ 15 mM, adjusted to pH 7.2 and 390 mosmol. Tissue samples were cleaned of haemolymph by rinsing with iced saline.

2.3. Biochemical analysis

Brains were rapidly sonicated in 70 μl of ice-cold 0.4 N $HClO_4$ (Merck), and then centrifuged at 10,000 *g* for 10 min at 4°C. The supernatants were collected and stored at −80°C until further analysis. NaOH (50 μl of 0.1 N) were added to the pellets for protein quantification according to the method of Bradford. Bovine serum albumin was used as standard.

2.3.1. Polyamines

Tissue extracts and standards (putrescine, spermidine, spermine and diaminoheptane as an internal standard, (DAH)) were dansylated according to the procedure described by Seiler (1970) and were then treated using the protocol adapted from Besson et al. (1986). Dansylation

proceeded in glass vials by mixing 40 μl of $HClO_4$ extracts, 10 μl of DAH , and 100 μl of 0.3 M Na_2CO_3 (Merck). The reaction was initiated by adding 200 μl of freshly prepared dansyl chloride solution (5 mg/ml) in acetone (SDS, spectrosol grade) and was allowed to proceed overnight in the dark at room temperature. After dansylation, each sample was diluted with 700 μl H_2O, vortexed and applied to a Waters Sep-Pak C18 cartridge. After washing with 4 ml of 20% methanol, the polyamine containing fraction was eluted with 2 ml of 100% methanol. The separation and quantification of polyamines were performed by reverse phase high performance chromatography (RP-HPLC) using a Waters system composed of two models 510 pumps, a Wisp 700 autosampler and a NEC APC4 data module recorder integrator. A Merck F 1050 fluorescence spectrophotometer detected fluorescence (350 nm excitation and 495 nm emission). The separations were performed on a RP18 Merck Lichrocart (25×4 mm^2, 5 μm) precolumn and a RP18, 100 CH Merck column (125×4 mm^2; spherical packing 5 μm). The solvent system was an acetonitrile/water gradient at a flow rate of 1 ml/min of acetonitrile 60% in water for 7 min, then acetonitrile 90% for 10 min and a 98% acetonitrile purge for 5 min. The column was reequilibrated to initial 60% acetonitrile conditions for 10 min between two successive injections. For each determination, 100 μl of dansylated samples were injected onto the column equilibrated in 60% acetonitrile. The major polyamines were identified by their retention times compared to those of standard polyamines. Peak areas were automatically measured by the integrator and evaluated according to the calibration method. Mixed polyamine standards from 10 to 70 pmol were reacted and chromatographed to establish linear standard curves which was used to estimate the absolute amounts of polyamines. The absolute limit of detection per injection was 1 pmol for dansylated Spd and dansylated Spm, and 7 pmol for dansylated Put. Two blank injections were routinely run between calibrations and sample analysis.

2.3.2. Monoamines

Monoamines and catabolites were separated by HPLC and detected with the electrochemical system. The mobile phase consisted of 0.1 M sodium acetate, 0.17 mM octyl sulphate, 8% methanol, 0.7 mM EDTA, pH 4.5 and was delivered through a LC-10ADvp Shimadzu pump at constant flow of 1 ml/min into a C-18 column (ODS2, 150×4.6 mm^2, Waters Spherisorb) maintained at 25°C. Samples of 60 μl were injected and analysed using a Coulochem II, ESA detector. The potential of the reference electrode was set at −50 mV and the working electrode at +280 mV. Data were computed with Waters Millenium software (via a Waters bus SAT/IN module) linked to a PC system; compound identification and peak quantification were achieved by comparison with standard solutions.

2.3.3. Amino acids

All samples were analysed for amino acid contents using HPLC along with a fluorimetric detection after precolumn derivatisation with *o*-phtaldialdehyde: 10 μl of sample were mixed with 10 μl of an *o*-phtaldialdehyde solution (*o*-phtaldialdehyde 20 mg; ethanol 100% 750 μl; borate buffer 1 M pH10 750 μl; mercaptoethanol 25 μl; water 2.25 ml) for 1 min. The 20 μl were then injected into a C-18 column (Spherisorb 5 mm ODS2 150×4.6 mm^2) maintained at 35°C. The mobile phase consisted of 75 mM potassium acetate (pH 5.8) in methanol (v/v; 1/7). Elution was performed with a methanol gradient ranging from 25 to 60%. Fluorimetric detection of derivatised amino acids was performed at 345 nm. Data were computed with Waters Millenium software (via a Waters bus SAT/IN module) linked to a PC system. Amino acid concentrations in the sample were calculated with reference to a standard mixture containing 20 different amino acids and injected after every five samples.

2.4. Histology

Cerebral ganglia were quickly dissected out in saline, then fixed for 6 h in Carnoy's fixative (ethanol 100% :chloroform :acetic acid, in 6:3:1). After three 24 h washes in 95% ethanol and three 24 h washes in 1-butanol, tissues were embedded in paraffin and cut in 6 μm serial sections. Sections were deparaffinised, rehydrated and treated for DNA staining according to the method of Feulgen–Rossenbeck: DNA was hydrolysed using 6 N HCl for 1 h and stained with Schiff's reagent (1 h) at room temperature. Sections were counterstained in 0.4% indigo carmine in a saturated solution of picric acid, dehydrated and mounted in DePeX (Merck). Mitoses were counted on serial sections of mushroom bodies.

2.5. Statistical analysis

We pooled males and females to avoid low sample sizes. Differences in the amount of neuroactive compounds between the five categories of crickets could be due to at least four independent factors. The first source of variation considered was the variable day/night as some crickets were dissected during the day (i.e 'day parasitised' and 'day uninfected' categories) while others were dissected at night (i.e 'night uninfected', 'night parasitised' and 'suicided' categories). 'Day parasitised' and 'day uninfected' crickets were coded 0 for this factor and 'night uninfected', 'night parasitised' and 'suicided' crickets were coded 1. A second source of variation is due to non-specific effects of infection, which are probably non-negligible given the huge size of the parasite. We coded 1 all crickets belonging to an infected category (ie. 'day parasitised', 'night parasitised' and 'suicided') and 0 those belonging to an uninfected category (i.e. 'night uninfected' and 'day uninfected'). A third cause of variation was directly related to the manipulative process

(as a cause or a consequence). Manipulated crickets (i.e. 'night parasitised') were coded 1, while individuals from other categories were coded 0. Finally, another source of variation considered was due to the effects of emergence, since several changes occurred during (e.g. host haemorrhage) and after (e.g. the host is not manipulated anymore, F.T. unpublished data) this event. Individuals from the 'suicided' category were coded 1 while individuals from other categories were coded 0.

The contribution of these four variables to the amounts of neuroactive compounds between the five categories of crickets was estimated by a multiple-regression procedure with all independent variables kept in the final model (Draper and Smith, 1981). Data were log transformed to avoid heteroscedasticity. To deal with the large number of amino acids (15), we employed a principal components analysis (PCA; Jongman et al., 1995; Sheldon and Meffe, 1995, Oberdorff et al., 1998; Guégan et al., 2001). By reducing dimensionality and eliminating multicolinearity (James and McCulloch, 1990), PCA forms linear, independent combinations of the original source variables. We retained two principal components, both with eigenvalues greater than one. These two principal components were used as dependent variables in multiple linear regressions (see Guégan et al., 2001). Statistical analysis were performed using S-PLUS 2000 Professional Release 2© (MathSoft, Inc.). Mann–Whitney *U*-test were used in testing for differences in neurogenesis data.

3. Results

3.1. Polyamines

We were unable to detect putrescine in our samples. There were substantial differences of spermidine levels between the five categories of crickets (Fig. 1). Mean quantities (± SE) for uninfected crickets dissected during the day (i.e. 'day uninfected' crickets) were 1.214 ± 0.091 pmol/μg. The multiple regression revealed that the greatest part of this variation was due to the non-specific effects of infection, and not by the manipulative process itself (Table 1A). There was also a slightly significant day/night effect (Table 1A). Levels of spermine were slightly higher than those of spermidine (Fig. 1). Mean quantities (± SE) for 'day uninfected' crickets were 2.814 ± 0.216 pmol/μg. As for spermidine, there was a slightly significant day/night effect (Table 1B). However, there was no significant difference between infected and uninfected crickets, suggesting no non-specific effects nor a manipulation (Table 1B).

3.2. Monoamines

We were unable to detect serotonin in our samples. For dopamine, no obvious variations were observed among the different experimental groups (Fig. 1). Mean quantities (± SE) for 'day uninfected' crickets were 31.785 ± 4.043 pmol/μg. The multiple regression only revealed a slightly significant day/night effect for explaining dopamine variation (Table 1C).

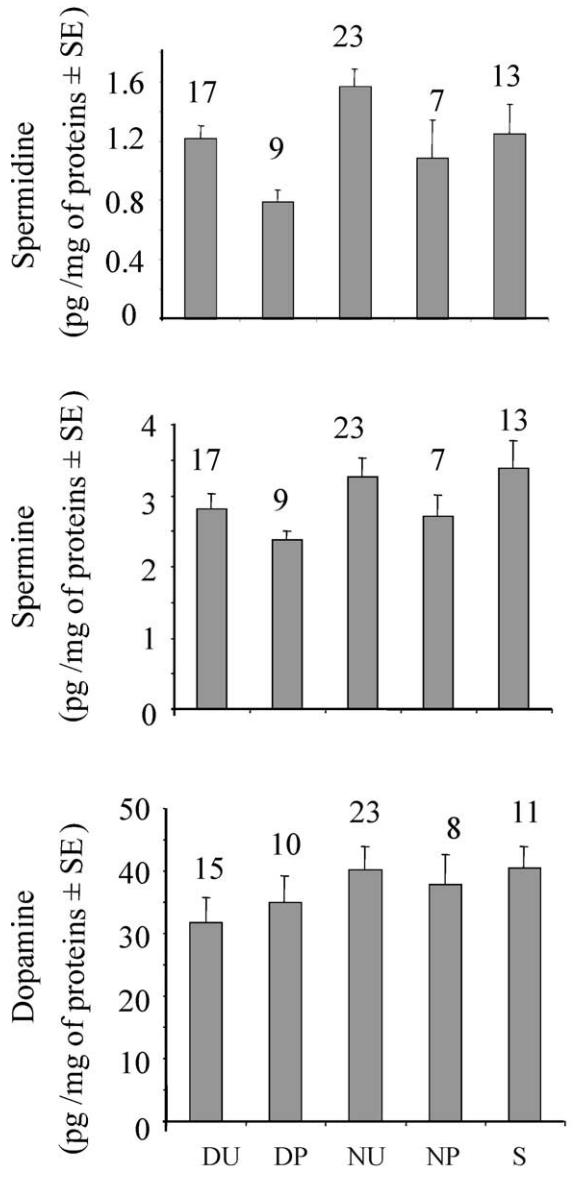

Fig. 1. Mean quantities of spermidine, spermine and dopamine among the five categories of crickets. Sample sizes are indicated above each bar

3.3. Amino-acids

Differences of amino acids levels between the five categories of crickets are presented in Fig. 2. The three principal components of the multivariate analysis performed on the 15 amino acids explained 74% of the overall variability across the five categories of crickets (Table 2). F1 combines all variables with negative loads and is alike a size factor with big individuals (i.e. high amino acid contents) having a low F1 score, and small individual (i.e. low amino

Table 1
Summary statistics of multiple regression analysis for explaining (A) spermidine variation, (B) spermine variation and (C) dopamine variation

Variable	Coefficient	Deviance	df	Residual deviance	*F*-ratio	*P*
A.						
Intercept			68	11.788		
Day/night	0.126	0.923	67	10.865	6.954	0.011
Non-specific troubles	−0.210	2.254	66	8.611	16.976	0.0001
Emergence effect	0.064	0.115	65	8.496	0.864	0.356
Manipulation	−0.007	0.0005	64	8.496	0.004	0.951
B.						
Intercept			68	6.938		
Day/night	0.067	0.462	67	6.475	4.784	0.032
Non-specific troubles	−0.068	0.069	66	6.406	0.718	0.40
Emergence effect	0.089	0.22	65	6.186	2.273	0.136
Manipulation	−0.008	0.0008	64	6.185	0.008	0.928
C.						
Intercept			66	12.659		
Day/night	0.127	0.761	65	11.898	4.019	0.049
Non-specific troubles	0.067	0.095	64	11.804	0.50	0.482
Emergence effect	−0.027	0.007	63	11.797	0.036	0.85
Manipulation	−0.070	0.059	62	11.738	0.31	0.58

acid contents) having a high F1 score (Fig. 3). F2 contrasts taurine to several other amino acids (valine and tyrosine notably) and is alike a shape factor (Fig. 3). Individuals with a high taurine level relative to valine and tyrosine have a high F2 score. The PCA of the amino-acid contents being based on log transformed values, F2 is alike the log of the ratio taurine/(valine × tyrosine). The multiple regression revealed that F1 is mainly explained by non-specific effects of the infection (Table 3A), while F2 is explained both by non-specific effect and by the manipulation (Table 3B). There were no significant results when attempting to explain F3 (analyses not shown).

The biochemical analysis thus revealed that most of the variation in the titers of neuroactive compounds was correlated with the infection status itself (i.e. infected versus uninfected). However, for three amino-acids (taurine, valine and tyrosine) a significant part of the variation was also correlated with the manipulative process.

3.4. Neurogenesis

Neuroblasts were easily distinguishable on histological sections stained with Feulgen–Rossenbeck colouration: they were grouped at the apex of the mushroom body cortex and presented a larger cell body than the surrounding interneurons. Metaphase and anaphase stages could be observed among the cluster of neuroblasts, revealing their proliferative activity (Fig. 4). The mitotic index, measured by counting all the cells in M phase in the cricket mushroom bodies, exhibited a two-fold increased in infected crickets as compared to uninfected ones (mean ± SE, 22.1 ± 3.4, $n = 10$ versus 11.9 ± 1.1, $n = 22$; Mann–Whitney *U*-test, $Z = 2.73$, $P = 0.006$).

4. Discussion

This is the first study to document physiological changes in the brain of insects infected by nematomorphs. Our biochemical analysis revealed that a first category of changes in the titers of neuroactive compounds was correlated with the infection status itself, without the need to invoke manipulation (e.g. spermidine and most amino-acids). Given the huge size of the worm relative to its host, it is reasonable to assume that crickets infected may experience several non-specific effects and/or stress

Table 2
Principal component characteristics for 15 amino-acids from five categories of crickets analysed

Principal components	Total	Percentage of explanation	Cumulative percentage
F1	8.18	54.51	54.51
F2	1.51	10.10	64.61
F3	1.45	9.67	74.28

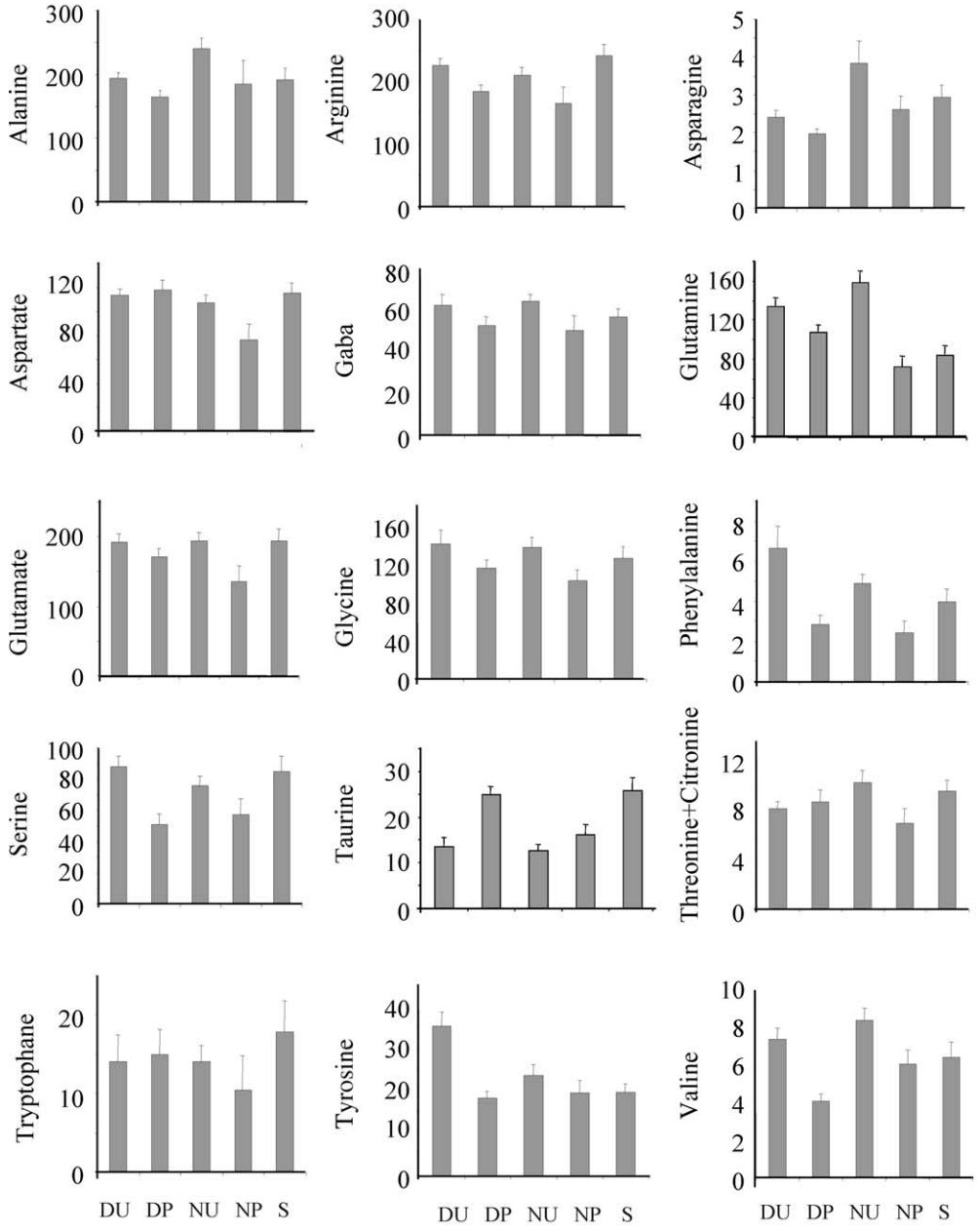

Fig. 2. Mean quantities (pmol/μg of proteins ± SE) of amino-acids among the five categories of crickets. Sample sizes : DU (‘day uninfected’, 15), DP (‘day parasitised’, 10), NU (‘night uninfected’, 24), NP (‘night parasitised’, 8) and S (‘suicided’, 13).

response. To simplify the terminology, we assume that changes seen during the day are non-specific and that changes observed in ‘suicidal’ crickets at night are associated with the manipulation. However, some of the differences observed during the day could be associated with the manipulative process but expressed behaviourally only at night. Conversely, differential titers of biochemical substances observed at night could reflect non-specific or manipulative changes. Infected individuals displayed on average lower concentrations than uninfected individuals for spermidine and most amino-acids (Fig. 3), suggesting a possible competition between the host and the parasite for nutrients sensu lato. We cannot exclude the hypothesis according to which this global depletion in infected individuals results from infected individuals spending more time searching for water than searching for food. The fact that the same tendency is observed for amino-acids having no neuronal function, supports the hypothesis according to which these changes are probably non-specific effects.

A second category of changes was correlated with the manipulative process. This concerns especially taurine, valine and tyrosine. However, we cannot determine from this study whether these changes are causes or consequences of the manipulative process. Infected crickets during the day (‘day parasitised’ category) displayed the highest concen-

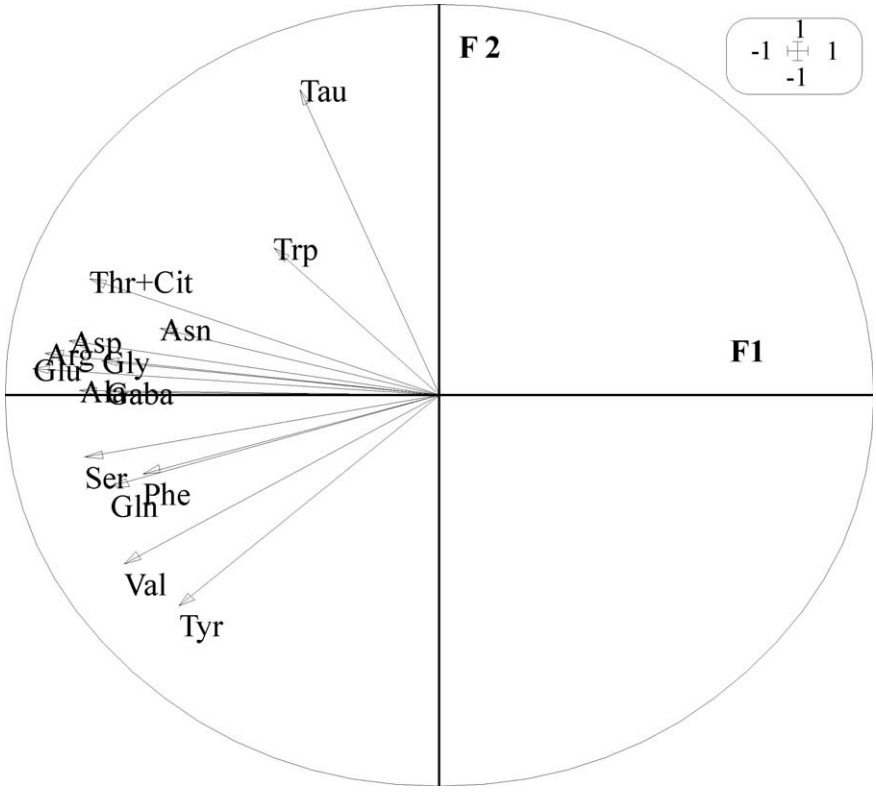

Fig. 3. Correlation circle (projection of the initial variables in the factors space, F1 and F2) Abbreviations: Tau, taurine; Trp, tryptophan; Gly, glycine; Glu, glutamate; Asp, aspartate; Gln, glutamine; Phe, phenylalanine; Arg, arginine; Asn, asparagine; Ser, serine; Thr, threonine; Cit, citronine, Val, valine; Gaba, gaba; Tyr, tyrosine; Ala, alanine.

trations of taurine. Taurine is considered as an important neurotransmitter in insects: it is one of the most abundant free amino acids in insect brains, and it participates in neurotransmission of mushroom bodies (Bicker, 1992; Sinakevitch et al., 2001). Several neurophysiological experiments suggest that taurine plays an excitation–reducing, neuromodulatory role in the insect nervous system (Witton et al., 1988; Dubas, 1991). Furthermore, taurine also regulates many biological phenomema including brain osmoprotection (Schaffer et al., 2000). We might speculate that, as suggested long ago (e.g. Blunck, 1922), hairworms cause thirst in its host during the day in order to convince it to move to the water at night. Tyrosine is a precursor of dopamine and octopamine, but as no obvious changes in dopamine content were observed in the different experimental groups, its role, as well as that of valine, in the process of manipulation is more difficult to explain at the present time. Further studies are needed in order to determine the exact function of taurine, valine and tyrosine in these processes and their potential to alter the behaviour of infected hosts.

Finally, the histological study revealed that neurogenesis

Table 3
Summary statistics of multiple regression analysis for explaining (A) F1 variation and (B) F2 variation

Variable	Coefficient	Deviance	df	Residual deviance	*F*-ratio	*P*
A.						
Intercept			69	564.174		
Day/night	0.022	0.045	68	564.129	0.006	0.937
Non-specific troubles	−0.976	62.526	67	501.603	8.719	0.004
Emergence effect	0.544	27.911	66	473.692	3.892	0.053
Manipulation	−0.793	7.549	65	466.143	1.053	0.309
B.						
Intercept			69	104.508		
Day/night	0.272	0.422	68	104.086	0.519	0.473
Non-specific troubles	1.146	41.089	67	62.997	50.521	<0.00001
Emergence effect	−0.383	0.747	66	62.250	0.918	0.342
Manipulation	−0.884	9.385	65	52.865	11.54	0.001

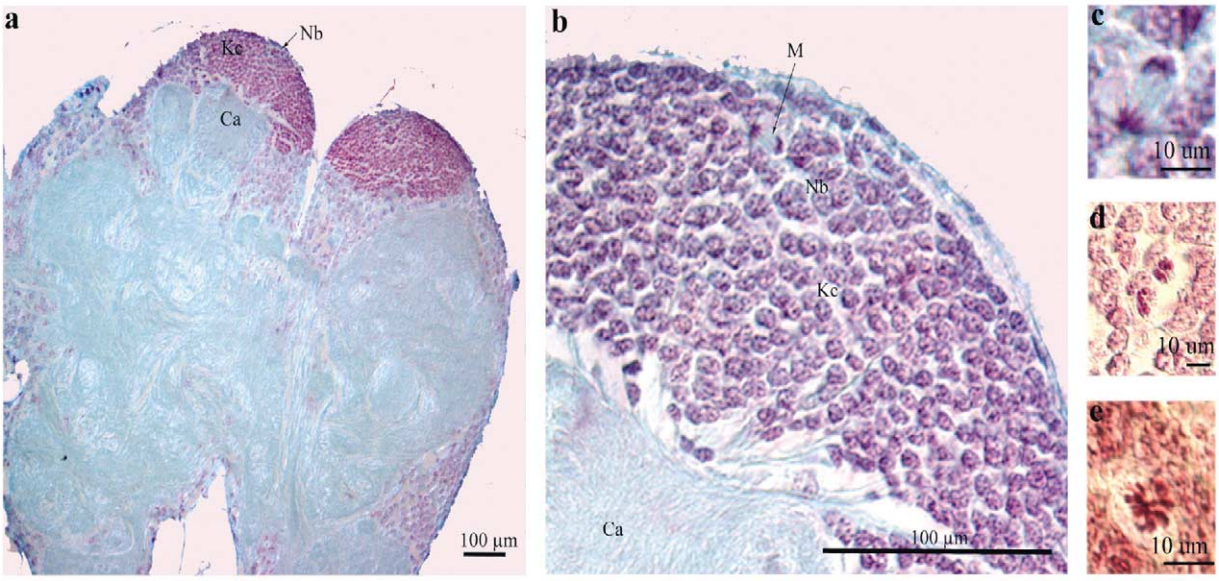

Fig. 4. Histological sections of *Nemobius sylvestris* mushroom bodies treated by the nuclear coloration of Feulgen–Rossenbeck. (a) Frontal section of the whole brain showing the position and structure of the mushroom bodies. (b) The mushroom body cortex is shown enlarged, allowing the visualization of the group of neuroblasts at the apex of the structure. A figure of mitosis is indicated by the arrow. c,d,e: pictures of anaphase and metaphases among the neuroblasts. Nb, neuroblasts; Ca, calyx; Kc, Kenyon cells; M, mitosis.

is twice as high in the brain of infected crickets compared to uninfected individuals. Adult neurogenesis has been thouroughly studied in different species of gryllidae; *Acheta domesticus*, *Gryllomorpha dalmatina*, *Gryllus bimaculatus* (Cayre et al., 1996). The cell fate of newborn cells has been determined by combination of a marker of cell proliferation (5-bromo-3′deoxyurine) and antibodies raised against glial cells. The authors concluded that unlike cockroaches where gliogenesis is observed, mushroom body progenitor cells essentially give rise to new interneurons in crickets. Knowing that taurine (at least in mammals) has been shown to stimulate neurogenesis (Chen et al., 1998), we might be tempted to establish a link between this enhanced neurogenesis and the fact that infected crickets displayed a higher concentration of taurine. However, additional experiments are needed to confirm this hypothesis. How increased neurogenesis could induce behavioural manipulation is an interesting question. We speculate that an abnormal neuronal production might interfere with neural circuitry, and, as mushroom bodies, where neurogenesis takes place, are the main sensory integrative centres of the insect brain, the analysis of environmental cues by the cricket might be perturbed, leading to aberrant behavioural responses. This hypothesis is supported by a recent study showing that neurogenesis in the hippocampus of adult rodents represents a cellular mechanism for periodic clearance of outdated memory traces, and suggesting that uncontrolled addition of new neurons into existing circuits may potentially disrupt central nervous system function (Feng et al., 2001).

Acknowledgements

We thank Mr Jean Louis Lafaurie for his cooperation during the field study, Charles Manu for his technical assistance during the sampling, Jean-Dominique Lebreton for statistical advises, Shelley Adamo, Simone Helluy, Andreas Schmidt-Rhaesa, François Renaud and Phil Agnew for interesting comments and suggestions on an earlier version of the paper. Frédéric Thomas is supported by a grant from the Ministère de la Recherche (France), ACI ‘Jeunes Chercheurs’.

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