Birkett, M. A., Di-Ilio, V. and Pickett, J. A. 2021. Effects of Nicotine and Tobacco-Related Products on the Feeding Behavior of the German Cockroach (Blattodea: Blattellidae). *Journal of Insect Science.* 21 (11), pp. 1-9. https://doi.org/10.1093/jisesa/ieaa147

OXFORD UNIVERSITY PRESS | Journal of Insect Science

Effects of nicotine and tobacco related products on the feeding behaviour of the German Cockroach Blattella germanica.

Journal:	*Journal of Insect Science*
Manuscript ID	JIS-2020-0182
Manuscript Type:	Research
Date Submitted by the Author:	20-May-2020
Complete List of Authors:	Di Ilio, Vincenzo; Rothamsted Research, Biointeractions and Crop Protection Birkett, Michael A.; Rothamsted Research, Biointeractions and Crop Protection Pickett, John; Cardiff University, School of Chemistry; Rothamsted Research, Biointeractions and Crop Protection
Organism Keywords:	Blattellidae
Field Keywords:	Chemical Ecology, Behavior, Feeding Behavior, Physiological & Chemical Ecology

Michael A. Birkett,
Rothamsted Research, Harpenden, AL5 2JQ
United Kingdom.
Phone: + 44 (0) 1582 763 133
Email: mike.birkett@rothamsted.ac.uk

Effects of nicotine and tobacco related products on the feeding behaviour of the German Cockroach *Blattella germanica*.

Vincenzo Di Ilio[1], Michael A. Birkett[1], John A. Pickett[1,2]

[1] Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ United Kingdom

[2] Cardiff University, Cardiff, CF10 3AT, Wales, United Kingdom

Abstract

Animal olfaction detects developmentally significant volatile organic compounds (VOCs) in their environment. This study seeks to expand these interactions by modifying the olfactory responses of insects to selected VOCs through the creation of a drug-addicted status. This will be obtained by administering drugs of human abuse, by which the drug acts as an artificial unconditioned stimulus, or reward, and a selected marker VOC as the conditioned stimulus using an olfactometric assay and feeding studies. In this study, both the drug nicotine and a Tobacco Smoke Particulate matter (TSP) extract were assayed as possible addictive compounds on the males of the German cockroach *Blattella germanica* Linnaeus. The TSP treated food was preferred over the control food and over the nicotine treated food. Surprisingly, nicotine, which is expected to be the most important addictive tobacco component, did not induce any noticeable effect on cockroaches. This is apparently due to a rapid detoxification probably in the haemolymph. Against expectations, the olfactometric assay demonstrated that cockroach males did not choose the TSP treated food by an olfactory mechanism even when attempts were made specifically to train via this modality. This discovery offers the hypothesis that the insects must eat the treated food to show a clear preference and that addiction-like mechanisms are involved due to the compounds contained in the TSP extract.

Key words: *Blattella germanica*, Nicotine, tobacco smoke.

Introduction

The olfactory system of animals can be exploited, through associative learning processes, for the detection of volatile organic compounds (VOCs) that are unconnected with the lifecycle of the animals themselves (Suckling and Sagar 2011; Schott et al. 2013). In the original model of learning, animals learn to associate an original neutral stimulus, the so-called conditioned stimulus, with a biologically active unconditioned stimulus or reward (Pavlov 1927). The reward elicits an innate response that is an unlearned physiological reflex. In classical conditioning, the innate response represents the expectation of the reward that comprises an internal representation of the reward in the absence of reinforcement by the cues and events predicting such a reward (Tolman 1959; Gil 2007). In such a system, the value of the reward associated with a stimulus is not an intrinsic property of the stimulus itself. However, animals can assign different values to the stimulus in relation to their previous experience (Schultz 2000).

The idea of this study is that addiction to psychoactive substances modifies the motivation priorities of animals, replacing innate unconditioned stimuli with induced artificial needs that can be exploited in a classical conditioning paradigm. Building on this theoretical assumption, we attempted to modify the olfactory priorities of insects creating an addicted status, obtained by administrating drugs of human abuse, using the German cockroach, *Blatella germanica* as the model insect (Kaun et al. 2012). This species was chosen because of its evolutionary and physiological features. *B germanica* lives in association with human populations in chemically complex environments, is sensitive to a huge spectrum of VOCs (Dow 1986; Bell 1990) and is able to discriminate odours in complex mixtures (Sakura et al. 2002). This species also exhibits an extremely flexible behaviour that is associated with its ecological success (Sakura and Mizunami 2001; Lent and Kwon 2004; Decker et al. 2007). In this context, our aim was to identify cues of addiction that could be used as an “artificial” unconditioned stimulus to be used in the context of a Classical Conditioning Pavlov's paradigm. Nicotine was chosen as the addictive compound because it is readily available relative to other drugs of human abuse and is recognised as a potent addictive compound among mammals (Di Matteo et al. 2007; Benowitz 2010). However, it has been reported that nicotine is weakly reinforcing and does not account alone for the addictive effects of tobacco in human subjects (Ambrose et al. 2007; Lewis et al. 2007). Many authors report that, among mammals, components of the tobacco smoke other than nicotine play a key role in the onset of addiction (Ambrose et al. 2007; Lewis et al. 2007; van Amsterdam et al. 2006; Brennan et al. 2013). Conversely, it was recently reported that nicotine is repellent at high concentrations, while enhancing the learning performance of bumblebees during pollination, thereby suggesting that the volatile nature of the alkaloid free base may contribute to the activation of olfactory sensilla (Baracchi et al. 2017; Kessler et al. 2015). In this work, both nicotine and a tobacco smoke particulate matter (TSP) extract were assayed as possible addictive substances for *B. germanica* males.

Materials and methods

Insects.

Gravid female German cockroaches, *Blattella germanica*, were purchased from i2L Research Ltd (Cardiff, United Kingdom) and maintained under constant temperature and humidity (28 ± 2 °C; RH 60 ± 5 %) with a photoperiod of 12: 12 h (L: D). Insect husbandry was carried out in 12 l airtight containers equipped with a hole in the lid covered with a light metal net. The rim of the container was painted with Fluon PTFE (Blades Biological Ltd, Cowden, UK) to avoid escapes. Water and ground dog pellets as food were provided *ad libidum* together with cardboard strips as harbourage. At emergence the first instar nymphs were collected and transferred into 1.7 l containers with water, food and cardboard harbourages and allowed to reach the adult stage. 7 day-old adult males were used for the experiments.

Chemicals.

Nicotine (99% TLC purity) from Sigma Aldrich (Gillingham, United Kingdom) was prepared as a stock solution in ethanol (50 mg/ml). Tobacco Smoke Particulate (TSP) extraction was carried out on 20 exhausted cigarette ends using ethanol (3 x 100 ml at ambient temperature), with the combined ethanolic extract being evaporated *in vacuo* to yield a dark brown residue (1.39 g on average) that was re-suspended in ethanol (10 ml).

Nicotine quantification

The amount of nicotine in the TSP extract was quantified by extraction with diethyl ether (3 times with a double volume of diethyl ether) and analysis on an Agilent 6890 GC (Agilent Technologies, UK) equipped with a cold on-column injector, a flame ionization detector (FID), and a non-polar HP-1 bonded-phase fused silica capillary column (50 × 0.32 mm i.d., film thickness 0.52 μm). The oven temperature was maintained at 30 °C for 1 min, programmed at 5 °C min^{-1} to 150 °C and held for 0.1 min, then increased at 10 °C min^{-1} to a final hold at 230 °C for 50 min. Hydrogen was the carrier gas. Results were obtained with an enhanced integrator (HP Chemstation).

Experimental food.

Nicotine and TSP were administered with food. Diets used in cockroach bioassays comprised of (i) a liquid mixture containing tap water (60%), yeast extract (32,5%), sucrose (6.5%), corn oil (0.2%) and 0.1 mg/ml potassium sorbate (0.8%) or (ii) a solid food comprising the liquid mixture described above plus 1% agar. The test food was obtained mixing either nicotine stock solution or TSP extract with the liquid mixture. The two test food mixtures were normalized at same concentration of nicotine (0.5 mg/ml). Control food, nicotine- and TSP-food administered to insects contained the same amount of ethanol.

Addiction bioassays.

Bioassays with German cockroaches were carried out in controlled environment under constant temperature and humidity (28 ± 2 °C; RH 60 ± 5 %) with a photoperiod of 12: 12 h (L: D).

Experiment n.1 – Nicotine and TSP native preference

The aim of this experiment was to define the innate preference for nicotine or TSP when administered in association with the artificial control food.

The experiment was carried out in two distinct phases. Two groups of 8 males were randomly selected among adults 7 days after the last moult and placed each in a 1l airtight container equipped with a drilled lid for ventilation. The container was provided with a water dispenser and one cardboard strip as harbourage. The first group was starved for 72 hours while the second continued fed on 0.5 g of artificial control food offered in solid form inside a 1.5 ml Eppendorf tube.

In the second phase, males were placed individually into a round 1l airtight containers and allowed to make a choice between two test solutions in liquid form administered by means of two 25 µl glass capillaries inserted through the container wall, according to a modified version of the two-choice Capillary Feeder (CaFe) assay (Ja et al. 2007). Males could imbibe the fluid from the exposed tip of the capillaries and the amount of food consumed was recorded daily.

Both the starved and the nourished adults could make only a binary choice between treated and control food. Therefore, starved males were divided in two sub-groups: the first could choose between Control food (BLA) and Nicotine treated food (NIC) while the second could choose between Control (BLA) and TSP treated food (TSP). Similarly, the nourished males were divided in two sub-groups whose choices were between BLA and NIC in the first and between BLA and TSP in the second sub-group.

The structure of the experiment is summarized in Table 1. Four replications were carried out.

Experiment n.2 – TSP and NIC conditioning

This second experiment had the objective to verify if the training of adult male cockroaches affects the choices of the individuals.

This experiment was carried out in three distinct phases. In the first phase, two sets of 12 males each were randomly selected among adults 7 days after the last moult and placed in two separate 1l airtight container equipped with a drilled lid for ventilation. Each container was provided with a water dispenser and one cardboard strip as harbourage. In the first container adults were offered 0.5 g of TSP treated food in solid form inside a 1.5 ml Eppendorf tube. In the second container males fed on 0.5 g of nicotine treated food (NIC). The amount of food consumed in phase one was recorded.

After 7 days each of the two sets was divided in three groups: the first group was starved for 72 hours, the second continued to feed on the test mixture while in the third group was re-established the control diet.

Similarly, to the Experiment 1, in phase three, males were placed individually into a round 1l airtight containers and allowed to make only a binary choice between treated and control food administered by 25 µl glass capillaries.

Males treated in the first phase with TSP treated food, and put under the three different food regimes in the second phase as described above, could only choose between control (BLA) and TSP treated food (TSP) while the individuals fed with nicotine treated food in the first phase, and separated in the three different feeding regimes during the second phase, could only chose between Control (BLA) and nicotine treated food (NIC).

The amount of food consumed by the single males in each cage was recorded daily. The structure of the experiment is summarized in Table 2. Four replications were carried out.

Experiment n.3 - Cockroach behaviour.

Olfactometric bioassays were conducted to verify the olfactory preference for the TSP or control food. Experiments comprised of three distinct phases. In the first phase, 10 males were placed in a 1l airtight container fitted with a lid containing drilled holes for internal ventilation, provided with a water dispenser and one cardboard strip as harbourage. Food was offered in solid form inside a 1.5 ml Eppendorf tube continuously for 5 days. Three treatment groups were prepared: a control group (BLA) where individuals were fed on control food, a TSP group where the males fed on TSP-food, and a STV group where no food was provided. In the second phase, all the treatment groups were starved for 48 hr. In the third phase, insects could make a choice between two olfactory stimuli presented in two-way olfactometer, which was assembled such that the insects were unable to come into contact with the odour sources kept in airtight glass vials placed upstream to the Y shaped arena. Air entering the system was purified by means of a carbon filter and then humidified by passing through distilled water. The flow rate was set at 150 ml/min. Males were kept in the olfactometer for 5 minutes. Variables recorded were: the time spent in each branch of the Y olfactometer, the first choice and the number of entries in each of the two branches per minute. The experiment was replicated four times.

Statistical analysis.

The data of the bioassays of all experiments were analysed using Student *t*-test and one -way ANOVA using SPSS for windows.

Results and Discussion

Experiment n.1 – Nicotine and TSP native preference

The consumption food was recorded in the first phase of the experiment. Non-starved insects consumed on average 4.15 ± 1.01 milligrams of food per day per individual.

The regime of starvation does not alter the food intake in the next choice phase. In fact, in the choice phase, the total amount of food consumed by the starved individuals was not statistically different from the total amount of food consumed by the nourished cockroaches (t=1.88; P=0.07; DF=61) (Figure 1).

In the choice test, data were organized to obtain fractions between the average consumption of TSP or NIC treated food as numerator and blank control food as denominator. Therefore, the value 1 represent an equal consumption of food; values of the fractions over 1 show preference for treated food over the control, while values between 0 and 1 reveal a preference for control food. TSP treated food appeared to be significantly more appetitive than control food both for the starved (t=5.62; P<0.01; DF=30) and the nourished (t=3.78; P<0.01; DF=30) male cockroaches. The figure 1 it shows that the value of the ratio between TSP and Control food are largely above the value 1 for both the nourished and the starved group indicating a highly significant preference for the tobacco extract treated food over the control food.

On the contrary the difference between the consumption of NIC treated food and control food is not statistically significant (Starved t=1.04; P=0.31; DF=28; Nourished t=0.96; P=0.34; DF=30) and in fact the value of the ratio NIC/BLA assume a value close to 1 for both the starved and the nourished cockroaches (Figure 1) indicating that there is not any significant preference for the NIC treated food over the Control.

Experiment n.2 – TSP and NIC conditioning

The experiment n.2 was aimed to evaluate the effects of training on the choices of the insects and verify the onset of a status of addiction. The experiment was therefore divided in three separate phases: a first training phase, a second stabilization phase and a third choice phase.

First phase

In the first phase, no significant differences were recorded between the consumption of TSP and NIC treated food (table 3). The male cockroaches fed with TSP or nicotine treated food consumed the same amount of food. This amount is consistently similar to the intake of control food observed in the phase 1 of the experiment 1. In fact TSP treated insects consumed on average 4.07±0.57 milligrams of food per day per individual and NIC treated cockroaches consumed on average 4.63±0.52 milligrams of food per day per individual

Second phase

During the second phase, male cockroaches treated with TSP were divided in three groups: the first was starved for three days, the second group was fed with control food and the third continued to feed on the TSP treated food.

Likewise, the insects that fed with NIC treated food during the first phase were divided in three groups, the starved group, the group in which control food was provided and a third group that continued to feed on the NIC treated food.

When total intake of food in the first and in the second phase, regardless its nature, is compared, the average individual quantities consumed by the cockroaches are not statistically different.

No significant differences were detected also between the average individual quantities of food consumed by the insects coming from the TSP and the NIC food regime in the first phase. However, considering that the average amount of food ingested per individual in first phase and in the second phase are not significantly different and considering also that the first phase lasts for 7 days while the duration of the second phase is three days, we can assume that the average individual food intake increased in the second phase, leading to hypothesize that there was a general increase of the appetite of the insects in all the treatment groups (Table 3).

Moreover, in the second phase, the statistical analysis shows that there is not any significant difference in food intake between the individuals that continued to feed on the treated foods and the ones that fed on the control mixture. This observation lead to hypothesize that, at least in the experimental conditions set, the restoration of blank food after a former treatment with either TSP or NIC did not affect the consumption of food.

A part of the insects trained on the NIC and TSP treated food were deprived of food during the stabilization phase to check for eventual withdrawal effects induced by nicotine or tobacco smoke extract that could be observed in the third phase of choice.

Third phase

Within the TSP group of training, all the insects were offered a choice between TSP treated food and Control food. The ANOVA test showed that the total amount of food consumed by the insects that were kept in the different food regimes during the second phase (starvation, feeding on Control and TSP treated food) was not statistical different (F=0.5467; P=0.5826; DF=47). This analysis led to hypothesize that, at least in the experimental conditions set, the starvation regime does not affect the feeding behaviour of the insects trained on TSP treated food.

However, in the choice test, the Student's *t*-test shows a significant preference for the glass capillaries containing the TSP treated food over the ones that contain control food despite the different food regimes during the previous phase (starved: t=2.9611; P=0.0059; DF=30; Control food: t=2.4073; P=0.0224; DF=30; TSP treated food: t=3.807; P=0.0006; DF=30) (Figure 2).

The preference was not due to the spatial arrangement of the glass capillaries inside the container because, in preliminary tests where the glass capillaries contained the same diet, no significant differences were found in the consumption of food from the two sources.

Similarly, to what described for TSP trained males, NIC trained insects in the first phase underwent either starvation or fed with control food or NIC treated food. Entering in the third phase all the insects were offered a choice between NIC treated food and Control food.

Unlikely the TSP trained males, the ANOVA test showed that the total amount of food consumed by starved insects in the choice phase is significantly higher (F= 3.9493; P= 0.0265; DF=47) than other groups of treatments in the second phase (Figure 3).

However, looking at the preference for NIC treated food over the control food the Student's *t*-test did not show any significant difference in any of the three food regime groups of the second phase (figure 4).

Since there is no significant preference for Control food and NIC treated food we can conclude that the increase in the total consumption of food in the starved group of males is due to the undernourishment in the second phase and not to the onset of an addiction-like effect.

Taken together the data show that clearly male cockroaches prefer the TSP treated food to the blank food, while no difference was detectable when nicotine treated food was offered versus the BLA food.

Moreover, males trained in the first phase with a TSP treated food always preferred the TSP treated food over the control whatever their diet in the second phase. The insects continued to express a significant preference for the TSP also when non-treated food was restored in the stabilization phase. Insects trained on TSP preferred this food over the control food when they were starved or continued to feed on TSP treated food or even when they were offered the choice to feed on control food (Figure 3). It was also found that different regimes in the stabilization phase did not interfere with the preference for TSP food in the choice phase. In fact, the statistical analysis showed no significant differences among the ratios of food consumption of the TSP trained males subjected to different food regime in the second phase.

Therefore, it is concluded that the TSP treated food was always preferred over the control no matter what was offered in the training and in the stabilization phase.

For what concern the pure nicotine trained males, no significant preference for the nicotine treated food versus the blank food was observed. Since the ratio between the values relative to the consumption from the two glass capillaries, control and nicotine treated food, are all close to one, we can assume that male cockroaches do not express any preference in the choice between these two types of food (Figure 4). This observation is the same for all the treatment groups in the stabilization phase and was confirmed by the statistical analysis that showed no statistical differences among sub-groups of the NIC training cluster. This observation is consistent with the data coming from the experiment 1 where no preference was observed between blank and nicotine-added food and suggest that, among cockroaches, nicotine is not responsible for the observed preference for TSP treated food.

Olfactometer assays

The choice tests discussed above show that *B. germanica* has a strong preference for the TSP over the control. This preference could derive from olfaction or from other mechanisms among which is the onset of an addicted status. To determine any crucial role played by olfaction in the expression of the choice, the olfactometric assay was carried out. The data revealed that there is no significant olfactory preference by cockroach males for the TSP treated food over control food. In Figure 5 it is seen that the males spent most of their time in the mixed odours branch of the Y shaped arena where they were exposed to both odours. Males deprived of food in the first phase spent less time in the mixing branch than the other treatment groups. This behaviour was probably due to the higher need to feed. In particular, starved insects spent more time in the control branch than in the TSP arm indicating that TSP was not attractive as a food when the insects made the choice only on an olfactory base (figure 5). Comparing the time spent in the two treatment arms without considering the mixing area, the statistical analysis did not show a significant preference between the control and the TSP food odour. The number of visits of the two treated arms was also not significantly different (Figure 6). This data confirm the indication that the preference observed for the TSP in the experiments 1 and 2 is due to a gustatory choice rather than an olfactory selection. This feeding response lead to hypothesize the onset of an addicted-like status that should be clarified by further experiments.

Conclusions

The aim of this study was to define the effects of tobacco-related products as addictive compounds to be used as a new “artificial” unconditioned stimulus, for a conditioned learning in the German cockroach *B. germanica* (Watanabe et al. 2008; Watanabe et al.2003). Tests were carried out using both nicotine and an ethanolic extract of Tobacco Smoke Particulate Matter (TSP) as possible addictive materials. We demonstrated that the TSP treated food, is the most preferred mixture in the experimental condition set. It is significantly preferred over the control food which in turn does not elicit a different feeding response respect to the nicotine treated food. Nicotine, that is commonly recognised as the most important tobacco addictive drug (Benowitz 2010; Ambrose et al. 2007), does not induce alone any effect on cockroaches. Instead, the TSP extract may contain compounds that either are addictive themselves or enhance the addictive properties of the nicotine. The olfactometric assay results support the conclusion that the TSP preference may elicits an addiction-like status because cockroach males do not choose TSP treated food on an olfactory basis but need to eat the treated food to express a preference. Further experiments are planned to test if TSP promotes the release of neurotransmitters and if there is a concomitant inhibition of monoamine oxidases, as reported in human subjects (Herraiz and Chaparro 2005). Finally, further experiments are necessary to identify the compounds responsible for the observed effects and what is the relationship between these molecules and the nicotine.

Acknowledgements

The authors thank Dr. Keith Chamberlain. VDI was supported by a Marie Sklodowksa-Curie Intra European Fellowship (Call: H2020-MSCA-IF-2014; Type of Action: MSCA-IF-EF-CAR; Number: 655905).

References

Ambrose, V., J.H. Miller, S.J. Dickson, S. Hampton, P. Truman, R.A. Lea, and J. Fowles. 2007. Tobacco particulate matter is more potent than nicotine at upregulating nicotinic receptors on SH-SY5Y cells. Nicotine Tob. Res. 9: 793–799.

Baracchi, D., A. Marplesm, A.J. Jenkins, A.R. Leitch, and L.Chittka. 2017. Nicotine in floral nectar pharmacologically influences bumblebee learning of floral features. Sci. Rep. 7: 1951.

Bell, W.J. 1990. Biology of the cockroach. pp. 7-12. In: Huber I., Masler E.P. and Rao B.R. (eds), Cockroaches as models for neurobiology: applications in biomedical research, Vol. 1. CRC Press, Boca Raton.

Benowitz N.L. 2010. Nicotine Addiction. NEJM. 362 (24): 2295–2303

Brennan, K.A., A. Crowther, F. Putt, V. Roper, U. Waterhouse, and P. Truman. 2013. Tobacco particulate matter self-administration in rats: differential effects of tobacco type. Addict. Biol. 20: 227-235.

Decker, S., S. McConnaughey, and T.L. Page. 2007. Circadian regulation of insect olfactory learning. PNAS. 104: 15905–15910.

Di Matteo, V., M. Pierucci, G. Di Giovanni, A. Benigno, and E. Esposito. 2007. The neurobiological bases for the pharmacotherapy of nicotine addiction. Curr. Pharm. Des. 13: 1269–1284.

Dow, J.A. 1986. Insect midgut function. Adv. In Insect Phys. 19: 187–328.

Gil, M. 2007. Learning reward expectations in honeybees. Learn. Mem. 14: 491-496.

Herraiz, T., and C. Chaparro. 2005. Human monoamine oxidase is inhibited by tobacco smoke: b-carboline alkaloids act as potent and reversible inhibitors. Biochem. Biophys. Res. Commun. 326: 378–386.

Ja, W.W., G.B. Carvalho, E.M. Mak, N.N. de la Rosa, A.Y. Fang, J.C. Liong, T. Brummel, and S. Benzer. 2007. Prandiology of Drosophila and the CAFE assay. PNAS. 104: 8253–8256.

Kaun, K.R., A.V. Devineni, and U. Heberlein. 2012. *Drosophila melanogaster* as a model to study drug addiction. Hum. Genet. 131: 959–975.

Kessler, S.C., E.J. Tiedeken, K. Simcock, S. Derveau, J. Mitchell, S. Softley, A. Radcliffe, J.C. Stout, and G.A. Wright. 2015. Bees prefer foods containing neonicotinoid pesticides. Nature. 521 (7550): 74-76.

Lent, D.D., and H.W. Kwon. 2004. Antennal movements reveal associative learning in the American cockroach *Periplaneta Americana*. J. Exp. Biol. 207: 369-375.

Lewis, A., J.H. Miller, and R.A. Lea. 2007. Monoamine oxidase and tobacco dependence. Neurotoxicology. 28: 182–195.

Pavlov, I. 1927. Conditioned Reflex and Psychiatry. International Publishers, NY.

Sakura, M., and M. Mizunami. 2001. Olfactory Learning and Memory in the Cockroach *Periplaneta Americana*. Zoolog. Sci. 18: 21-28.

Sakura, M., R. Okada, and M. Mizunami. 2002. Olfactory discrimination of structurally similar alcohols by cockroaches. J. Comp. Physiol. A. 188: 787–797.

Schott, M., C. Wehrenfennig, T. Gasch, and A. Vilcinskas. 2013. Insect Antenna-Based Biosensors for In Situ Detection of Volatiles. Adv. Biochem. Eng. Biotechnol. 136, 101–122.

Schultz, W. 2000. Multiple reward signals in the brain. Nat. Rev. Neurosci. 1: 199–207.

Suckling, D.M., and R.L. Sagar. 2011. Honeybees Apis mellifera can detect the scent of Mycobacterium tuberculosis. Tuberculosis. 91: 327-328

Tolman, E.C. 1959. Principles of purposive behaviour, pp. 92–157. In: S. Koch, (ed), Psychology: A study of a science, Vol. 2. McGraw-Hill, NY.

van Amsterdam, J., R. Talhout, W. Vleeming, and A. Opperhuizen. 2006. Contribution of monoamine oxidase (MAO) inhibition to tobacco and alcohol addiction. Life Sci. 79: 1969–1973.

Watanabe, H., Y. Kobayashi, M. Sakura, Y. Matsumoto, and M. Mizunami. 2003. Classical Olfactory Conditioning in the Cockroach *Periplaneta Americana*. Zoolog. Sci. 20: 1447-1454.

Watanabe, H., C. Sato, T. Kuramochi, H. Nishino, and M. Mizunami. 2008. Salivary conditioning with antennal gustatory unconditioned stimulus in an insect. Neurobiol. Learn. Mem. 90: 245-254.

Figures and Tables

1st Phase (3 days)	Choice Phase (5 days)			
	Choice	Label	Males/Rep	Replications
Starved	BLA vs NIC	BS-BN	4	4
	BLA vs TSP	BS-BT	4	4
Blank	BLA vs NIC	BB-BN	4	4
	BLA vs TSP	BB-BT	4	4

Table 1. Structure of the experiment 1 showing the food regimes in the first phase and in the choice phase. In the choice phase male cockroaches can freely make a binary choice of the food source. BLA (B) Artificial control food; NIC (N) Nicotine treated food; TSP (T) Tobacco Smoke Particulate matter treated food.

Training phase (7 days)	Stabilization ph. (3 days)	Choice Phase (5 days)			
		Choice	Label	Males/Rep	Replic.
Tobacco smoke particulate matter TSP (T)	Starved (S)	BLA vs TSP	TS-BT	4	4
	TSP (T)	BLA vs TSP	TT-BT	4	4
	Blank Control (B)	BLA vs TSP	TB-BT	4	4
Nicotine NIC (N)	Starved (S)	BLA vs NIC	NS-BN	4	4
	NIC (T)	BLA vs NIC	NN-BN	4	4
	Blank Control (B)	BLA vs NIC	NB-BN	4	4

Table 2. Structure of the experiment 2 showing the food regimes in the first phase of training, in the second phase of stabilization and in the choice phase. In the choice phase male cockroaches can freely make a binary choice of the food source. BLA (B) Artificial control food; NIC (N) Nicotine treated food; TSP (T) Tobacco Smoke Particulate matter treated food.

Phase 1 (7days)			Phase 2 (3 days)		
Treatment Group	Mean	SD	**Treatment Group**	Mean	SD
NIC Nicotine	32.42	3.66	**Starved**	- - -	- - -
			Control	31.04	4.27
			NIC	33.96	8.59
TSP Tobacco smoke particulate matter extract	28.46	3.96	**Starved**	- - -	- - -
			Control	30.00	4.08
			TSP	34.17	3.91

Table 3. Average ratios of food consumption in phase 1 (left) and in phase 2 (right) given in mg per individual. The standard deviations SD are indicated on the right of the means. The Student's t-test shows that the difference between the quantity of food consumed in the first phase between NIC and TSP treated food is not significantly different (t=1.4683; P=0.1924; DF=6). As regards the second phase, the difference between Control food and NIC treated food is not significantly different (t=0.6081; P=0.5654; DF=6). Likewise, also the difference in the intake of control food and TSP treated food is not significantly different (t=1.4744; P=0.1908; DF=6). In the second phase, the total food ingestion (control + NIC) of the cockroaches treated with NIC treated food in the first phase is not significantly different from the total amount of food (control + TSP) consumed by the individuals treated with TSP treated food in the first phase (t=1.1515; P=0. 8817; DF=14)

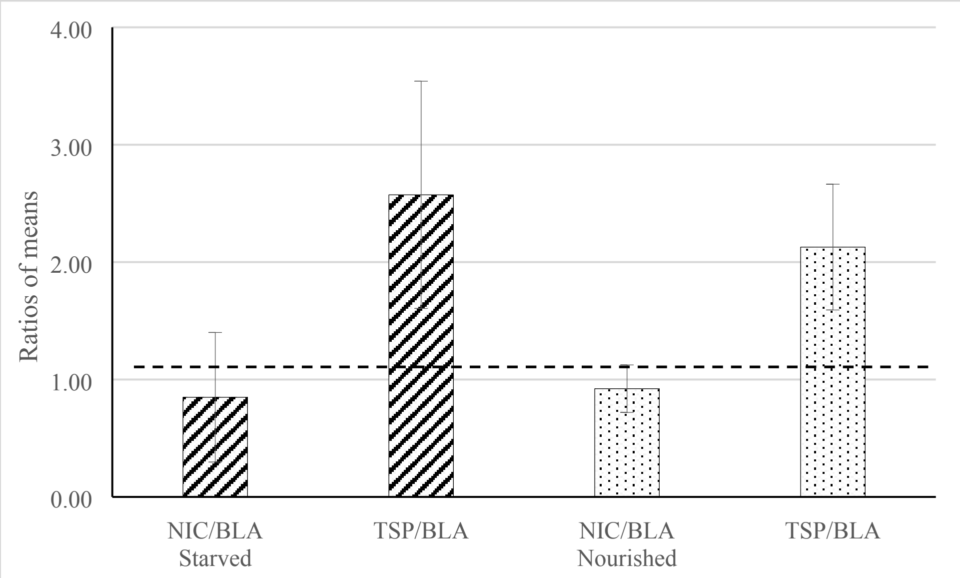

Figure 1. Average individual consumption of experimental food during the choice phase. The values are obtained by a ratio between the average consumption of TSP or NIC treated food and blank control food (BLA) in the choice phase. Therefore, the value 1 represent an equal consumption of food (dotted bold line). Ratio values over 1 show preference for treated food over the control while values between 0 and 1 reveal a preference for control food. Standard deviations are presented as error bars. The total consumption of food of starved and nourished individuals are not significantly different at P=0.05. Preference for TSP treated food over the control food is highly significant for both the starved and the nourished cockroaches. No significant difference has been detected between the average consumption of blank control food and NIC treated food at P=0.05 for both the starved and the nourished individuals.

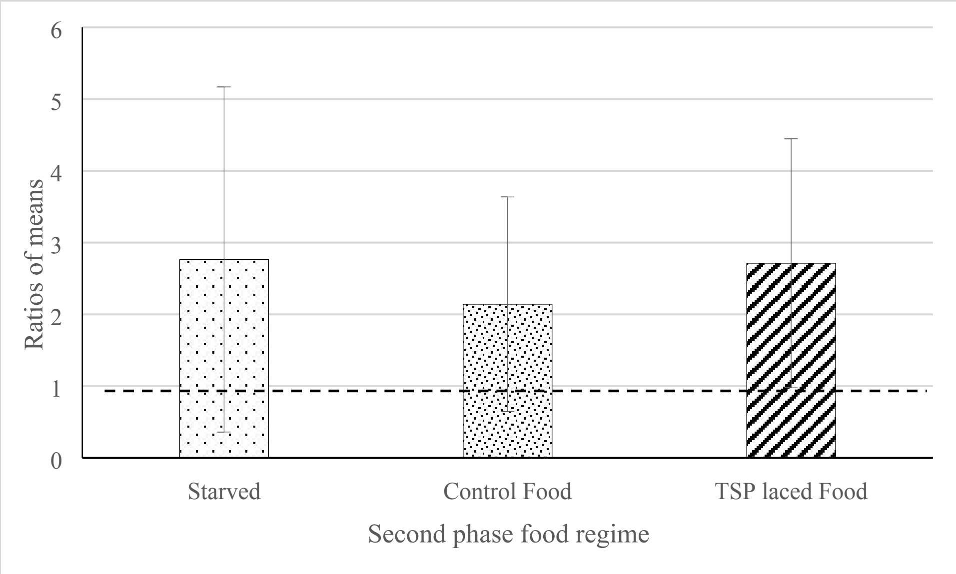

Figure 2. Average ratios of consumption of experimental food during the choice phase for the insects trained on TSP. The values are obtained by a ratio between the average consumption of TSP traced food and control food in the choice phase. Therefore, the value 1 represent an equal consumption of the two types of food (dotted bold line). Ratio values over 1 show preference for TSP food over the control.

Standard deviations are presented as error bars. The total consumption of food in the three treatment groups are not significantly different at P=0.05. Preference for TSP traced food over the control food is significant for the Starved group (t=2.9611; P=0.0059; DF=30), the Control group (t=2.4073; P=0.0224; DF=30) and the TSP group (t=3.807; P=0.0006; DF=30).

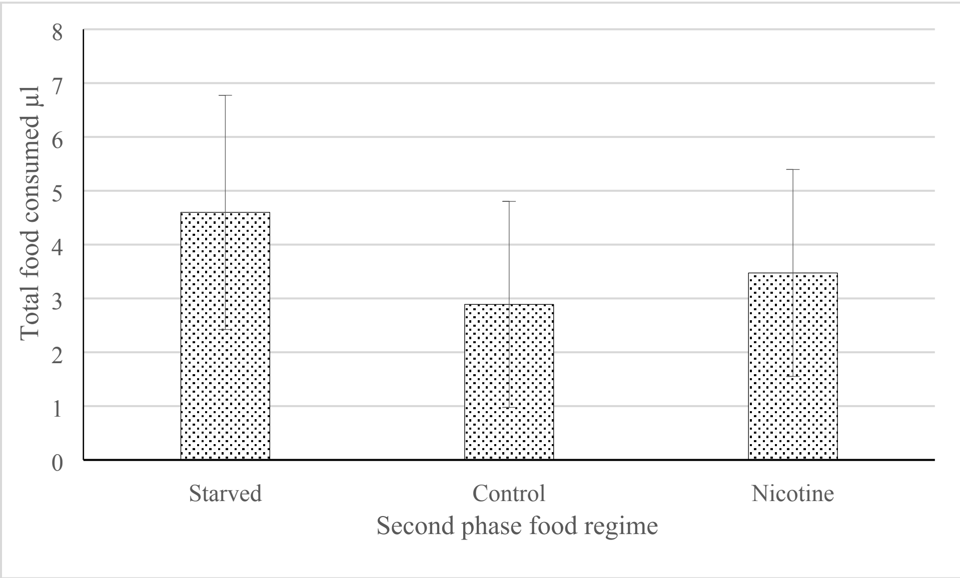

Figure 3. Average individual consumption of experimental food during the choice phase for the insects trained on NIC treated food. Standard deviations are presented as error bars. The total consumption of food in the three treatment groups are significantly different at P=0.05. Total intake of food of the Starved group is significantly higher than the other groups (F=3.9493; P= 0.0265; DF=47).

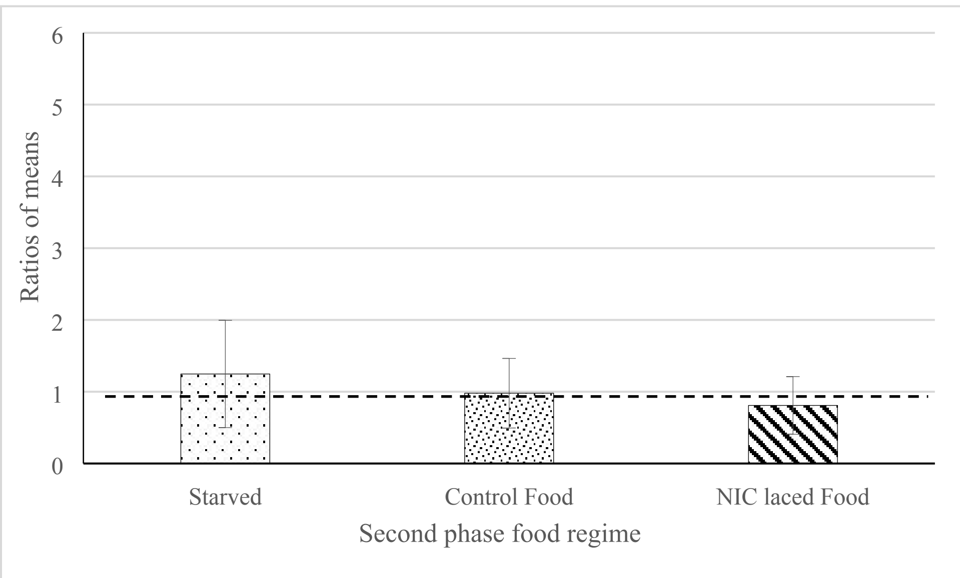

Figure 4. Average ratios of consumption of experimental food during the choice phase for the insects trained on NIC treated food. The values are obtained by a ratio between the average consumption of NIC treated food and control food in the choice phase. Therefore, the

value 1 represent an equal consumption of the two types of food (dotted bold line). Ratio values over 1 show preference for NIC treated food over the control while values between 0 and 1 reveal a preference for control food. Standard deviations are presented as error bars. Preference for NIC treated food over the control food is not statistically significant for any group of treatment.

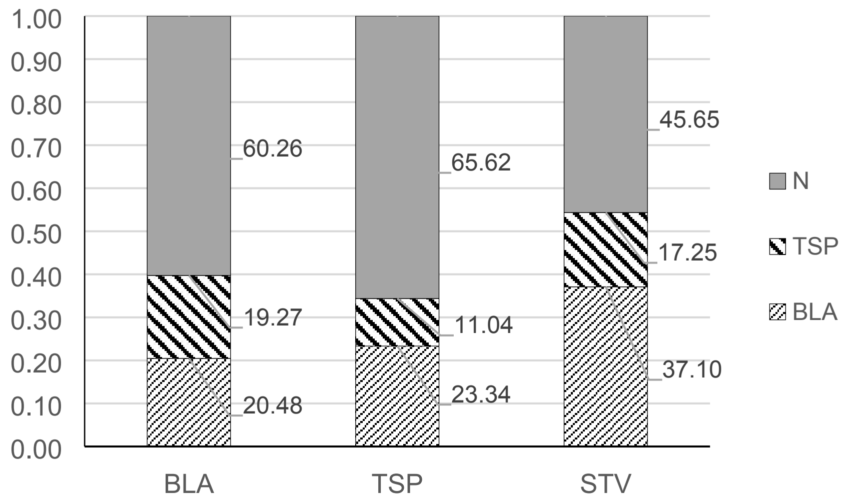

Figure 5. Olfactometer test. Time spent in each arm of the olfactometer by males trained on TSP or BLA food or deprived of food. N indicates the mixing arm of the olfactometer. Values are given as a percentage of the total time of the test. Difference in the time spent in the two treatment arms of the olfactometer is not statistically significant.

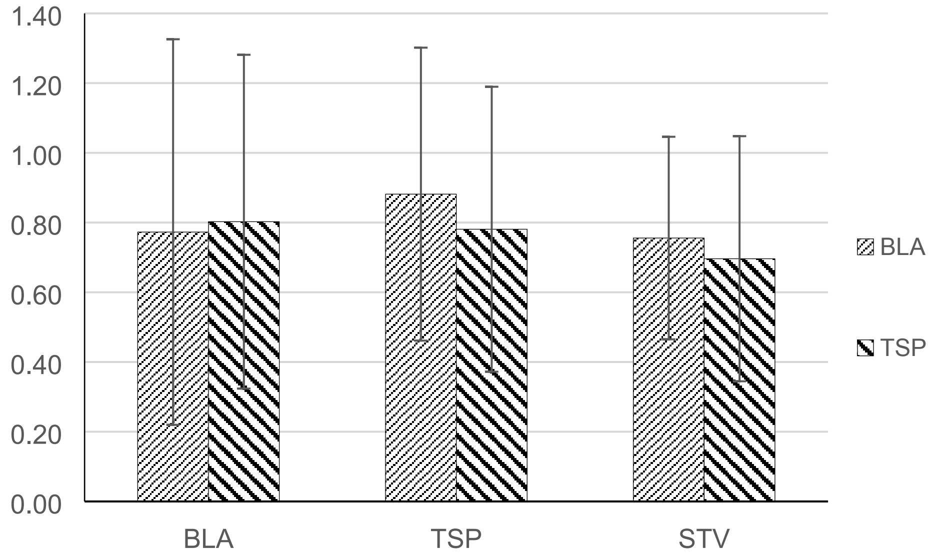

Figure 6. Olfactometer test. Number of entries per minute in each of the treatment arms of the olfactometer. Standard deviation for each group is represented as error bars. Differences are not statistically significant.

1st Phase (3 days)	Choice Phase (5 days)			
	Choice	**Label**	**Males/Rep**	**Replications**
Starved	BLA vs NIC	BS-BN	4	4
	BLA vs TSP	BS-BT	4	4
Blank	BLA vs NIC	BB-BN	4	4
	BLA vs TSP	BB-BT	4	4

Training phase (7 days)	**Stabilization ph. (3 days)**	**Choice Phase (5 days)**			
		Choice	**Label**	**Males/Rep**	**Replic.**
Tobacco smoke particulate matter TSP (T)	Starved (S)	BLA vs TSP	TS-BT	4	4
	TSP (T)	BLA vs TSP	TT-BT	4	4
	Blank Control (B)	BLA vs TSP	TB-BT	4	4
Nicotine NIC (N)	Starved (S)	BLA vs NIC	NS-BN	4	4
	NIC (T)	BLA vs NIC	NN-BN	4	4
	Blank Control (B)	BLA vs NIC	NB-BN	4	4

Phase 1 (7days)			Phase 2 (3 days)		
Treatment Group	Mean	SD	**Treatment Group**	Mean	SD
NIC Nicotine	32.42	3.66	**Starved**	- - -	- - -
			Control	31.04	4.27
			NIC	33.96	8.59
TSP Tobacco smoke particulate matter extract	28.46	3.96	**Starved**	- - -	- - -
			Control	30.00	4.08
			TSP	34.17	3.91

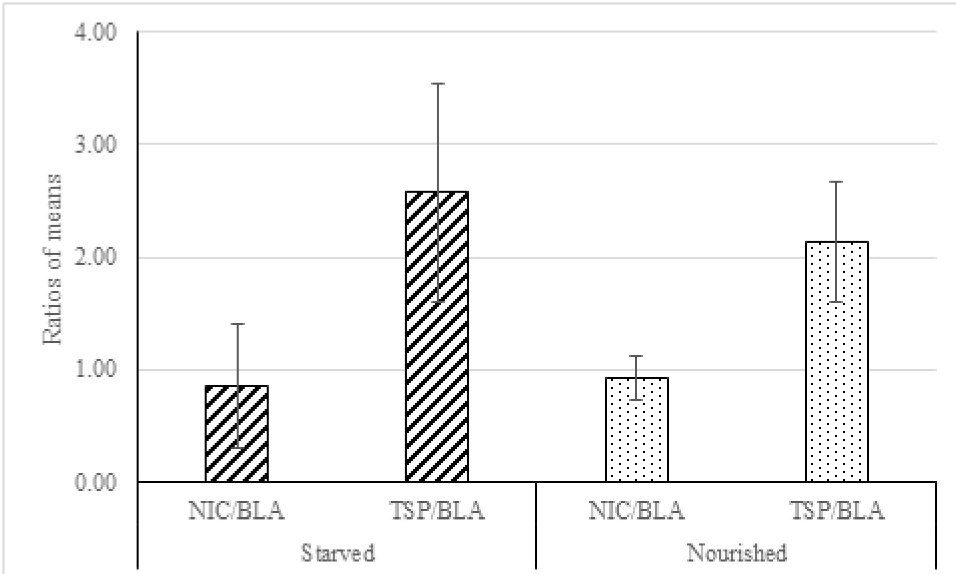

Figure 1. Average individual consumption of experimental food during the choice phase. The values are obtained by a ratio between the average consumption of TSP or NIC treated food and blank control food (BLA) in the choice phase. Therefore, the value 1 represent an equal consumption of food (dotted bold line). Ratio values over 1 show preference for treated food over the control while values between 0 and 1 reveal a preference for control food. Standard deviations are presented as error bars. The total consumption of food of starved and nourished individuals are not significantly different at P=0.05. Preference for TSP treated food over the control food is highly significant for both the starved and the nourished cockroaches. No significant difference has been detected between the average consumption of blank control food and NIC treated food at P=0.05 for both the starved and the nourished individuals.

128x136mm (96 x 96 DPI)

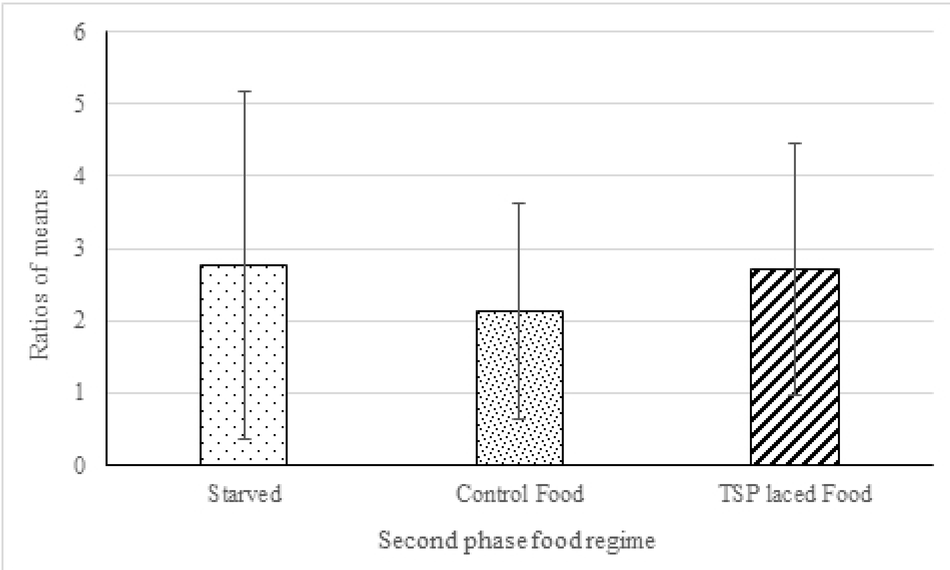

Figure 2. Average ratios of consumption of experimental food during the choice phase for the insects trained on TSP. The values are obtained by a ratio between the average consumption of TSP traced food and control food in the choice phase. Therefore, the value 1 represent an equal consumption of the two types of food (dotted bold line). Ratio values over 1 show preference for TSP food over the control.
Standard deviations are presented as error bars. The total consumption of food in the three treatment groups are not significantly different at P=0.05. Preference for TSP traced food over the control food is significant for the Starved group (t=2.9611; P=0.0059; DF=30), the Control group (t=2.4073; P=0.0224; DF=30) and the TSP group (t=3.807; P=0.0006; DF=30).

128x136mm (96 x 96 DPI)

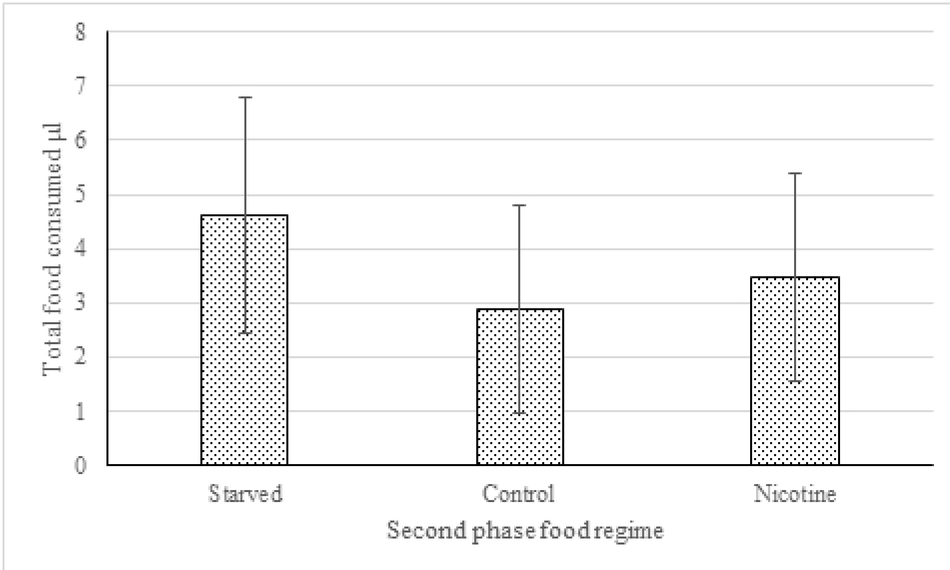

Figure 3. Average individual consumption of experimental food during the choice phase for the insects trained on NIC treated food. Standard deviations are presented as error bars. The total consumption of food in the three treatment groups are significantly different at P=0.05. Total intake of food of the Starved group is significantly higher than the other groups (F=3.9493; P= 0.0265; DF=47)

128x136mm (96 x 96 DPI)

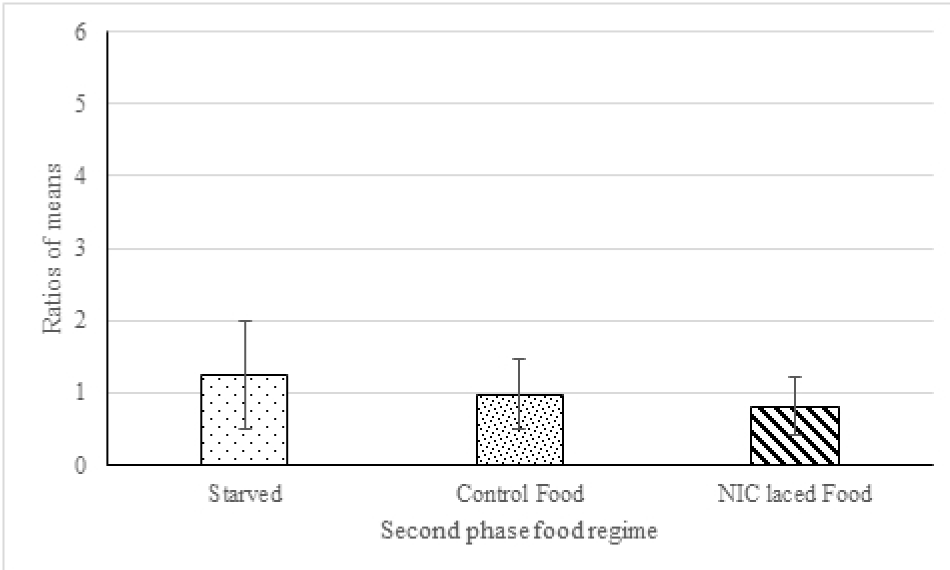

Figure 4. Average ratios of consumption of experimental food during the choice phase for the insects trained on NIC treated food. The values are obtained by a ratio between the average consumption of NIC treated food and control food in the choice phase. Therefore, the value 1 represent an equal consumption of the two types of food (dotted bold line). Ratio values over 1 show preference for NIC treated food over the control while values between 0 and 1 reveal a preference for control food. Standard deviations are presented as error bars. Preference for NIC treated food over the control food is not statistically significant for any group of treatment.

128x136mm (96 x 96 DPI)

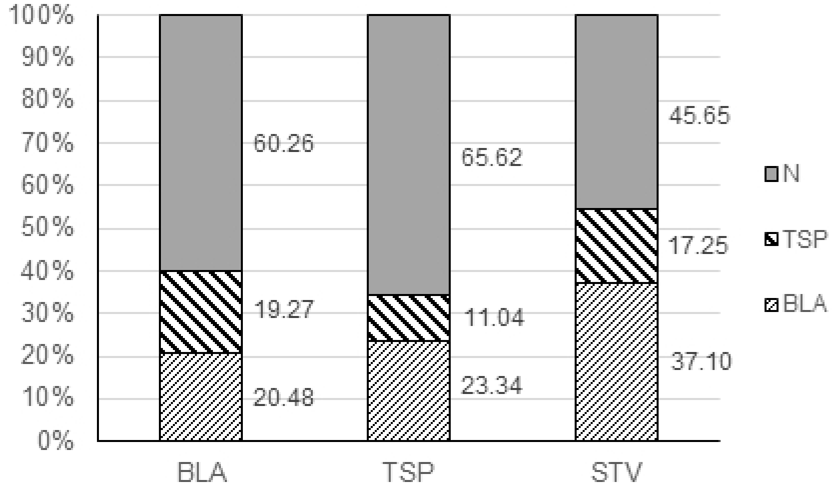

Figure 5. Olfactometer test. Time spent in each arm of the olfactometer by males trained on TSP or BLA food or deprived of food. N indicates the mixing arm of the olfactometer. Values are given as a percentage of the total time of the test. Difference in the time spent in the two treatment arms of the olfactometer is not statistically significant.

128x136mm (96 x 96 DPI)

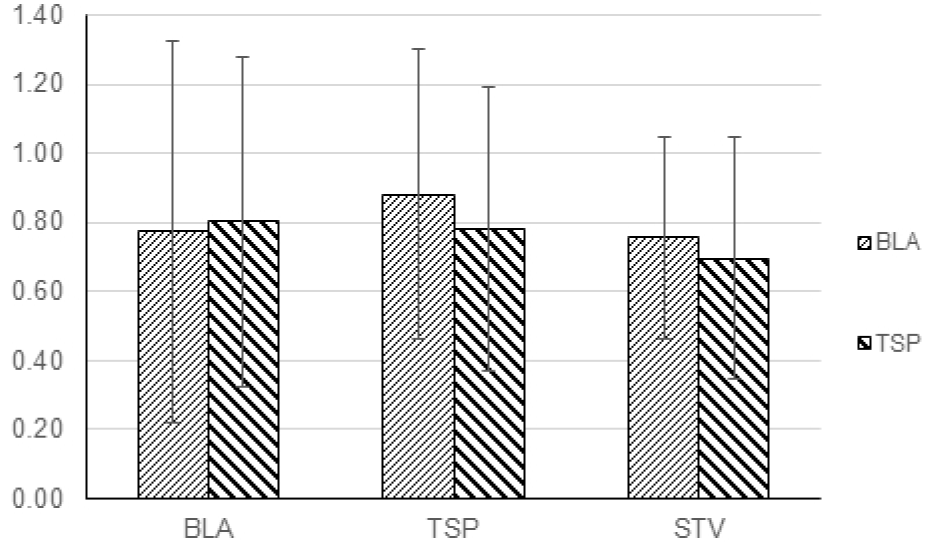

Figure 6. Olfactometer test. Number of entries per minute in each of the treatment arms of the olfactometer. Standard deviation for each group is represented as error bars. Differences are not statistically significant.

128x136mm (96 x 96 DPI)