1 **First indication of acetylcholine-based communication in honeybee**
2 **haemocytes and its modulation by a neonicotinoid insecticide**
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13 health, immunosuppression, immune regulation

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Abstract

There is growing concern that some managed and wild insect pollinator populations are in decline, potentially threatening biodiversity and sustainable food production on a global scale. In recent years, there has been increasing evidence that sub-lethal exposure to neurotoxic, neonicotinoid pesticides can negatively affect pollinator immunocompetence and amplify the effects of diseases, likely contributing to pollinator declines. Here we show that a range of non-neural tissues and haemocytes of the honeybee *Apis mellifera* express the nicotinic acetylcholine receptor that is the target of neonicotinoids. In addition, we demonstrate that the haemocytes, which form the cellular arm of the innate immune system, actively synthesize acetylcholine. This suggests the presence of a neural-independent acetylcholine-based immune signalling system in insects similar to that found in vertebrates. Lastly we establish that field-relevant doses of the neonicotinoid insecticide clothianidin alter this communication system. These findings provide a novel, mechanistically informed framework to understand the numerous side-effects on insects of sub-lethal pesticide exposure, including immunosuppression. They support the growing evidence for acetylcholine-based immune regulation in invertebrates that operates independently of the nervous system.

Introduction

In an attempt to meet the ever-increasing demand for pollination services, globalized pollinator-trade has led to the spread of pollinator diseases around the world, threatening managed and wild pollinator populations¹⁻³. While healthy pollinator communities are sometimes able to contain such emerging diseases, additional stressors can compromise pollinator immunity, causing lethal epidemics⁴⁻⁶. One prominent factor directly impairing pollinator immunity is their exposure to sub-lethal doses of neurotoxic pesticides such as neonicotinoids⁷⁻⁹. While the demonstrated detrimental effects of neurotoxic pesticides on pollinator behaviour^{10,11} and navigation^{12,13} are intuitive, the strong immunosuppressive effects of these neurotoxic pesticides remain unexplained¹⁴.

In vertebrates it is well established that the immune system has a close regulatory connection with the nervous system¹⁵. In particular, the ancient cholinergic signalling system based on acetylcholine (*ACh*) has been demonstrated to perform a pivotal role in maintaining homeostasis of the immune system^{16,17}. In recent years, evidence for a functionally similar *ACh*-based immune regulatory network has emerged in a handful of invertebrate model systems¹⁸⁻²⁰. In particular, haemocytes, the cellular arm of the invertebrate immune system, have been demonstrated to not only express subunits of the muscarinic (*mAChR*)²¹ and nicotinic acetylcholine receptors (*nAChR*)²², but also to directly respond to the presence of *ACh*²³. Since neonicotinoids target *nAChR* receptors with high affinity²⁴ the presence of a neural-independent, *ACh* based communication system in the innate immune system of pollinators could provide a direct mechanistic link between neonicotinoids and immunosuppression.

In this study we investigate if non-neural immune-relevant tissues (fatbody, midgut and haemocytes) of the honeybee *Apis mellifera*: 1) express *nAChR* subunits, 2) synthesize *ACh*, and 3) respond to field-realistic doses of neonicotinoids. If confirmed, this would provide a mechanistic framework to directly explain the hitherto puzzling

immunosuppressive effects of sub-lethal pesticide exposure observed in pollinators.

Results

We find that all tissues investigated express a different subset of *nAChR* subunits (Pseudo- $F_{3,23} = 7.76$, $P < 0.001$, Fig. 1A-D, Fig 2). A pairwise comparison indicates that all four tissues (fat body, haemocyte, midgut, brain) express a unique blend of subunits (all comparisons $t > 1.92$, $P < 0.006$; for details see Electronic Supplementary Material (ESM) Table S1, Fig. 1A-D, Fig. 2). A PERMANOVA using Euclidian distance yielded very similar results (see Table S1). The SIMPER analysis indicates that subunit $\alpha 7$ is the most highly expressed subunits in brain tissue separating it from all other tissues (Table S2, Fig. 1D). In contrast subunit $\alpha 9$ and $\beta 2$ are mostly expressed in the fatbody making it different from the other tissues (Table S2, Fig. 1A). Haemocytes, similarly to brain tissue, exhibit biased expression of $\alpha 2$ and $\alpha 7$ (Fig. 1B). Midgut tissue exhibits low expression levels of all subunits (Fig. 1C). The tissues also differed in terms of *ACh* expression measured as choline acetyltransferase (*ChAT*) expression ($\chi^2 = 21.96$, $P < 0.001$, Fig. 2A), with brain tissues having the highest expression compared to all other tissues (all $P < 0.001$, Fig. 2A) followed by haemocytes (all $P < 0.001$, Fig. 2A), with very little expression activity in the midgut and fatbody ($P > 0.05$, Fig. 2A). Over the 24 h experiment, bees in the clothianidin treatment group consumed on average 0.44 ± 0.16 ng of clothianidin. Treatment groups did not differ in terms of survival ($z = -0.26$, $P = 0.79$). Control and pesticide-exposed bees consumed a similar amount of sucrose solution ($W = 114$, $P = 0.95$), and we were able to extract similar amounts of haemolymph from both treatment groups ($W = 23$, $P = 0.82$). When looking at the amount of total RNA extracted from bee haemolymph we find no difference between treatment groups (totalRNA/ μ l haemolymph $W = 12$, $P = 0.23$). However, we found that *ChAT* expression was significantly increased in haemocytes of bees exposed to clothianidin ($W = 38$, $P = 0.014$, Fig. 2B).

87

88 **Discussion**

89 In this study we demonstrate the widespread expression of *nAChR* subunits in non-neural
90 and immune-relevant tissues in the honeybee *A. mellifera*. In addition we show that
91 haemocytes in *A. mellifera* actively synthesize *ACh*, which strongly suggests *ACh*-based
92 non-neural communication of the innate immune system in this important pollinator
93 species. Lastly we experimentally establish that sub-lethal field relevant doses of the
94 neonicotinoid clothianidin can influence this communication system *in vivo*.

95 Our results are in line with recent findings, which suggest the presence of non-
96 neural and immune-related *ACh* based communication in a range of invertebrate model
97 systems^{18,21,23}. Work on pest insects indicates that, similar to our findings, different
98 combinations of *nAChR* subunits are expressed in a wide range of non-neural tissues²².
99 However, the expression of these sub-units by itself does not automatically indicate the
100 presence of functional receptors, as demonstrated by Aztiria et al.²⁵. Nevertheless, the fact
101 that haemocytes can respond to the presence of *ACh* suggests that, at least in some
102 species, functional receptors must be present^{18,21}. In addition, haemocytes have been
103 shown to synthesize acetylcholine-degrading enzymes (Acetylcholineesterase) likely
104 terminating *ACh* based haemocyte excitation following pathogen exposure, thereby
105 facilitating homeostasis of the immune system¹⁹. Our results strongly support these
106 findings and indicate additionally that haemocytes are capable of actively synthesizing
107 *ACh* themselves. Taken together these lines of evidence strongly suggest that invertebrate
108 innate immune systems possess all essential components for sending, receiving and
109 terminating *ACh* based signals. It is consequently likely that, similarly to their vertebrate
110 counterparts¹⁶, the invertebrate innate immune system utilizes neural-independent *ACh*-
111 based communication.

112 In addition, we show that secondary immune-relevant tissues, the fatbody and (to a

113 lesser extent) the midgut, express *nAChR* subunits, which might be a common
114 phenomenon in insects²². It is tempting to speculate that haemocytes could utilize *ACh*
115 based signals to convey information to the fatbody and midgut and coordinate the
116 systemic immune response during infections, orchestrating the cellular and humeral arm of
117 the invertebrate innate immune system.

118 The utilization of *ACh*-based communication in the invertebrate immune system
119 relies on the presence of functional *nAChR*, receptors that are the major target of
120 neonicotinoid insecticides^{26,27}. These neurotoxins exhibit high *nAChR* affinity in
121 invertebrates, causing receptor overstimulation with lethal effects at even very low
122 doses^{24,28}. These systemic pesticides are of course aimed at pest species that eat plant
123 tissue or suck sap, but, being systemic, they migrate into both pollen and nectar, so
124 pollinators are exposed to them when visiting treated, flowering crops or contaminated
125 wildflowers^{29,30}. Once ingested, the pesticide is absorbed via the gut and passes through
126 the haemolymph on the way to its designated target: the central nervous system²⁴. In the
127 haemolymph, neonicotinoids inevitably come into contact with haemocytes, with
128 detrimental effects for haemocyte populations. It has been experimentally shown that
129 neonicotinoids drastically decrease haemocyte numbers in the haemolymph of honeybees
130 and also inhibit their ability to mount an effective immune response within 24 h of
131 exposure⁸. In molluscs, the blocking of haemocyte-based *mAChR* before pathogen
132 challenge promotes the expression of *Tumor Necrosis Factor* (TNF), which in turn results
133 in elevated haemocyte apoptosis^{21,31}. If a similar, *nAChR*-based, regulatory connection is
134 present in the haemocytes of pollinators, *nAChR* blockage by neonicotinoids could directly
135 explain their detrimental effects on haemocytes and by extension the immunosuppressive
136 effects observed in honeybees⁷.

137 While the direct effects of neonicotinoids on neuronally associated traits like
138 behaviour, memory and navigation^{12,13,32} are intuitively clear, the effects on other traits

such as reproduction have not previously been adequately explained³³. The finding that non-neural tissues can express *nAChR* could explain these counterintuitive effects by providing a mechanism for direct interaction with these tissues. It could also resolve a second puzzling phenomenon associated with neonicotinoid exposure: the susceptibility of insects to neonicotinoid exposure varies profoundly both within species (between different developmental stages)³⁴, and between species²⁴, as well as between studies using similar experimental set-ups^{33,35}. In order to understand such effects, we have to consider the *ACh* (and by extension neonicotinoid) binding properties of *nAChRs*. These characteristics are determined by their subunit composition, which can greatly alter their binding probabilities and consequently also alter their susceptibility to neonicotinoid interference by orders of magnitude^{24,36}. Work in vertebrates suggests that, in addition to species-specific and tissue-specific *nAChR* composition, environmental stimuli such as nicotine³⁷ can alter receptor composition as well. If such results also hold for pollinators, then it may be that the species, tissue, time point and condition dependent *nAChR* composition might all influence the response to neonicotinoids, giving rise to the large variation in susceptibility observed both under natural and experimental conditions^{22,33,35}.

In summary our results provide a novel, direct and mechanistically informed framework to understand the numerous unexplained and variable side effects associated with exposure of insects to sub-lethal doses of neurotoxic pesticides. In times of wild and managed pollinator decline such an analytical framework is urgently needed in order to identify, analyse and ultimately limit the side effects of pesticides.

160

161 **Methods**

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163 Bee collection

164 Foraging *Apis mellifera* worker were collected between July and September 2016 on the

campus of the University of Sussex, Brighton, UK (50°52'02.8"N 0°05'09.6"W). In all cases bees were collected between 09:00 and 11:00 in order to minimize gene expression variation caused by circadian rhythms. They were placed in 50 ml falcon tubes containing a moist cotton ball to provide them with water and to regulate relative humidity within the tube. The bees for Experiments 1 (tissue expression levels) were directly put on ice to cold anaesthetise them while the bees for Experiment 2 (clothianidin exposure) were placed in an dark incubator at 33°C and 80% relative humidity and provided with 60% sucrose water and kept at these conditions for 20 h for acclimatisation before the start of the experiment.

Experiment 1: tissue-specific expression of nAChR subunits and ChAT

Tissue & RNA extraction

After cold immobilization (~10 min) the bees were decapitated using a sterile razor blade, dissected under RNA Later (Thermo Fisher) using a sterile dissection kit and either whole brain (N = 5), fatbody (N = 7) or midgut (N = 7) was extracted (one type of tissue per bee). For haemolymph extraction, the thorax and abdomen of the bees were carefully punctured after decapitation using a sterile dissection needle and haemolymph was collected using a sterile graded glass capillary. The haemolymph of two bees was pooled (total 16 bees; N = 8) and haemocytes were collected following standard protocol³⁸. All tissues were homogenized in Trizol (ABI) using a sterile pestle and total RNA was extracted following the manufacturers instructions. The concentration and purity of RNA was determined on a Nanodrop 2000®.

Experiment 2: clothianidin exposure

Sixty two foraging *A. mellifera* workers were randomly assigned to either treatment (N = 30) or control (N = 32). Following the 20 h acclimatisation period the feeders were removed. Four hours later the treatment group was provided with new feeders containing 60%

191 sucrose solution spiked with 5 ppb clothianidin (using molecular grade acetone as solvent),
 192 while the control received sucrose solution with the same concentration of acetone only.
 193 All feeders were weighed before and after the experiment to the closest 0.001 g using a
 194 Kern PFB 300-3 scale to measure the dose (ng) of neonicotinoids that the bees had
 195 consumed. All bees had access to the feeders for 24 h after which haemolymph was
 196 collected from all surviving individuals, and samples of two bees were pooled (N = 7
 197 treatment, N = 8 control) following the procedure of Experiment 1.

198

199 RT transcription & qPCR analysis

200 100 ng of total RNA was used for reverse transcription using the Phusion RT-PCR kit
 201 (Thermo Scientific). Primers for all RT-qPCR assays of α 1-9, β 1-2, *ChAT* and the
 202 reference gene *rp49* were designed using Primer3³⁹ and published sequences available
 203 from GeneBank (See Tab.S3 for details). Primer efficiencies were measured using a
 204 dilution series of *Apis mellifera* brain cDNA (pooled subsamples of 5 individuals) covering
 205 three orders of magnitude including the cDNA concentration used in the reaction. Primer
 206 efficiencies were found to be above 91% for all primer pairs. Reaction specificity was
 207 confirmed by melting curve analysis. All analyses were performed on an ABI OneStep
 208 qPCR machine using SYBR green assays and were analysed using the OneStep software.

209

210 Gene expression analysis

211 Gene expression analysis of the *nAChR* subunits α 1-9, β 1-2, *ChAT* and *rp49* were
 212 performed in 10 μ l reactions using GoTaq® qPCR Master Mix (Promega) and 0.5 μ M of
 213 each specific primers (Sigma-Aldrich) on a StepOne™ Real-Time PCR Systems Applied
 214 Biosystems® detection system. Samples of cDNA corresponding to 2 ng total RNA in 2 μ L
 215 volumes were added and each sample analysed in technical duplicates. Each plate
 216 contained one negative control reaction for each primer pair using pooled and 1:10 diluted

RNA extracts from 5 randomly chosen individuals in order to control for gDNA contamination. The following program was used for amplification: 95°C for 2 min, followed by 40 cycles of 30 s of 95°C denaturation, 30 s annealing at 59°C and 30 s extension at 72°C following by a melting curve to ensure PCR specificity. The data used for the analysis is the target gene expression normalized to the reference gene (*rp49*) expression.

Data analysis

To compare the *nAChR* subunit expression patterns we used the programme PRIMER 6, version 6.1.13, + add-in, version 1.0.3 (PRIMER-E Ltd) to perform permutational multivariate analysis of variance (PERMANOVA) with the normalized relative expression of all 11 sub units as the response and tissue as the predictor variable. All tests were carried out using 9,999 permutations on a resemblance matrix using Chad distance estimates and the robustness of the results were tested using the Euclidian distance as an alternative estimate. We performed a SIMPER analysis to compare the expression of individual *nAChR* subunits according to tissue identity and tissue differentiation. All other tests were performed in R 3.2.4⁴⁰. Survival was analysed as the proportion of bees that died over the duration of the experiment using a GLM with binomial data distribution. The other results of Experiment 2 were analysed using non-parametric statistics (Kruskal-Wallis and Wilcoxon tests) and Bonferroni corrections in cases of multiple testing. The MDS plot was generated in PRIMER 6; all other graphs were done in R using the sciplot package⁴¹.

References

1. Wilfert, L. *et al.* Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. *Science*. **351**, 594–597 (2016).
2. Fürst, M. A., McMahon, D. P., Osborne, J. L., Paxton, R. J. & Brown, M. J. F. Disease associations between honeybees and bumblebees as a threat to wild

- 243 pollinators. *Nature* **506**, 364–366 (2014).
- 244 3. McMahon, D. P. *et al.* A sting in the spit: widespread cross-infection of multiple RNA
245 viruses across wild and managed bees. *J. Anim. Ecol.* **84**, 615–624 (2015).
- 246 4. Potts, S. G. *et al.* Global pollinator declines: Trends, impacts and drivers. *Trends in*
247 *Ecology and Evolution* **25**, 345–353 (2010).
- 248 5. Vanbergen, A. J. Threats to an ecosystem service: Pressures on pollinators.
249 *Frontiers in Ecology and the Environment* **11**, 251–259 (2013).
- 250 6. Goulson, D. *et al.* Bee declines driven by combined stress from parasites, pesticides,
251 and lack of flowers. *Science*. **347**, 1255957 (2015).
- 252 7. Di Prisco, G. *et al.* Neonicotinoid clothianidin adversely affects insect immunity and
253 promotes replication of a viral pathogen in honey bees. *Proc. Natl. Acad. Sci.* **110**,
254 18466–18471 (2013).
- 255 8. Brandt, A., Gorenflo, A., Siede, R., Meixner, M. & Büchler, R. The neonicotinoids
256 thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey
257 bees (*Apis mellifera* L.). *J. Insect Physiol.* **86**, 40–47 (2016).
- 258 9. Woodcock, B. A. *et al.* Impacts of neonicotinoid use on longterm population changes
259 in wild bees in England. *Nat. Commun.* **7**, 12459 (2016).
- 260 10. Stanley, D., Russell, A. & Morrison, S. Investigating the impacts of field-realistic
261 exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and
262 colony growth. *J. Appl.* **53**, 1440–1449 (2016).
- 263 11. Henry, M. *et al.* A common pesticide devreases foraging success and survival in
264 Honey Bees. *Science*. **336**, 348–350 (2012).
- 265 12. Fischer, J. *et al.* Neonicotinoids interfere with specific components of navigation in
266 honeybees. *PLoS One* **9**, 91364 (2014).
- 267 13. Jin, N., Klein, S., Leimig, F., Bischoff, G. & Menzel, R. The neonicotinoid clothianidin
268 interferes with navigation of the solitary bee *Osmia cornuta* in a laboratory test. *J.*
269 *Exp. Biol.* **218**, 2821–2825 (2015).
- 270 14. Sánchez-Bayo, F. *et al.* Are bee diseases linked to pesticides? - A brief review.
271 *Environment International* **89**, 7–11 (2016).
- 272 15. Sternberg, E. M. Neural regulation of innate immunity: a coordinated nonspecific
273 host response to pathogens. *Nat. Rev. Immunol.* **6**, (2006).
- 274 16. Kawashima, K., Fujii, T., Moriwaki, Y. & Misawa, H. Critical roles of acetylcholine
275 and the muscarinic and nicotinic acetylcholine receptors in the regulation of immune
276 function. *Life Sci.* **91**, 1027–1032 (2012).
- 277 17. Sternberg, E. Neuroendocrine regulation of autoimmune/inflammatory disease. *J.*

- 278 *Endocrinol.* **169**, 429–435 (2001).
- 279 18. Shi, X. *et al.* Acetylcholine modulates the immune response in Zhikong scallop
280 *Chlamys farreri*. *Fish Shellfish Immunol.* **38**, 204–210 (2014).
- 281 19. Shi, X. *et al.* The immunomodulation of acetylcholinesterase in zhikong scallop
282 *Chlamys farreri*. *PLoS One* **7**, e30828 (2012).
- 283 20. Chen, H., Zhou, Z., Wang, L. & Wang, H. An invertebrate-specific miRNA targeted
284 the ancient cholinergic neuroendocrine system of oyster. *Biol. Open* **6**, 160059
285 (2016).
- 286 21. Liu, Z. *et al.* The cholinergic immune regulation mediated by a novel muscarinic
287 acetylcholine receptor through TNF pathway in oyster *Crassostrea gigas*. *Dev.*
288 *Comp. Immunol.* **65**, 139–148 (2016).
- 289 22. Xu, G., Wu, S., Teng, Z., Yao, H. & Fang, Q. Molecular characterization and
290 expression profiles of nicotinic acetylcholine receptors in the rice striped stem borer,
291 *Chilo suppressalis* (*Lepidoptera: Crambidae*). *Insect Sci.* (2016). doi:10.1111/1744-
292 7917.12324
- 293 23. Chen, H. *et al.* The comprehensive immunomodulation of NeurimmiRs in
294 haemocytes of oyster *Crassostrea gigas* after acetylcholine and norepinephrine
295 stimulation. *BMC Genomics* **16**, 942 (2015).
- 296 24. Tomizawa, M. & Casida, J. Selective toxicity of neonicotinoids attributable to
297 specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* **48**,
298 339–364 (2003).
- 299 25. Aztiria, E. M., Sogayar, M. C. & Barrantes, F. J. Expression of a neuronal nicotinic
300 acetylcholine receptor in insect and mammalian host cell systems. *Neurochem. Res.*
301 **25**, 171–180 (2000).
- 302 26. Matsuda, K. *et al.* Neonicotinoids: Insecticides acting on insect nicotinic
303 acetylcholine receptors. *Trends in Pharmacological Sciences* **22**, 573–580 (2001).
- 304 27. Elbert, A., Haas, M., Springer, B., Thielert, W. & Nauen, R. Applied aspects of
305 neonicotinoid uses in crop protection. *Pest Manag. Sci.* **64**, 1099–1105 (2008).
- 306 28. Tomizawa, M., Lee, D. & Casida, J. Neonicotinoid insecticides: molecular features
307 conferring selectivity for insect versus mammalian nicotinic receptors. *J. Agric. Food*
308 *Chem.* **48**, 6016–6024 (2000).
- 309 29. Botías, C. *et al.* Neonicotinoid Residues in Wildflowers, a Potential Route of Chronic
310 Exposure for Bees. *Environ. Sci. Technol.* **49**, 12731–12740 (2015).
- 311 30. Goulson, D., Nicholls, E., Botías, C. & Rotheray, E. L. Bee declines driven by
312 combined stress from parasites, pesticides, and lack of flowers. *Science.* **347**,

- 1255957 (2015).
31. Liu, Z. *et al.* The simple neuroendocrine-immune regulatory network in oyster *Crassostrea gigas* mediates complex functions. *Sci. Rep.* **6**, 26396 (2016).
32. Blacquire, T., Smagghe, G., van Gestel, C. A. M. & Mommaerts, V. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**, 973–992 (2012).
33. Whitehorn, P. R., O’Connor, S., Wackers, F. L. & Goulson, D. Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production. *Science*. **336**, 351–352 (2012).
34. Grewal, P. S., Power, K. T. & Shetlar, D. J. Neonicotinoid insecticides alter diapause behavior and survival of overwintering white grubs (*Coleoptera: Scarabaeidae*). *Pest Manag. Sci.* **57**, 852–7 (2001).
35. Moffat, C. *et al.* Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Sci. Rep.* **6**, 24764 (2016).
36. Tomizawa, M. & Casida, J. Molecular recognition of neonicotinoid insecticides: the determinants of life or death. *Acc. Chem. Res.* **42**, 260–269 (2008).
37. Govind, A. P., Vezina, P. & Green, W. N. Nicotine-induced upregulation of nicotinic receptors: Underlying mechanisms and relevance to nicotine addiction. *Biochem. Pharmacol.* **78**, 756–765 (2009).
38. Negri, P., Maggi, M., Szawarski, N., Lamattina, L. & Eguaras, M. Apis mellifera haemocytes in-vitro, What type of cells are they? Functional analysis before and after pupal metamorphosis. *J. Apic. Res.* **53**, 576–589 (2014).
39. Untergasser, A., Cutcutache, I. & Koressaar, T. Primer3—new capabilities and interfaces. *Nucleic acids* (2012).
40. R Core Team. R: A language and Statistical, statistical computing. (2015). at <<https://www.r-project.org/>>
41. Morales, M. Sciplot: scientific graphing functions for factorial designs. (2011).

342 Figure 1: Relative expression of the nAChR subunits $\alpha 1-9$, $\beta 1-2$ normalized against rp49 in
 343 the four investigated tissue types: fatbody (A in red, N=7); haemocytes (B in blue, N=8);
 344 midgut (C in white, N=7); brain (D in green, N=5). There is an overall difference in the
 345 subunit expression between tissues (PERMANOVA: Pseudo- $F_{3,23} = 7.76$ $P < 0.001$). Error
 346 bars indicate SE.

347
 348 Figure 2: Multidimensional scaling (MDS) plot based on Chad distance of NACHR
 349 expression in four cell types (brain, haemocytes, fatbody and midgut) of honeybees. All
 350 groups varied significantly from each other (PERMANOVA pairwise comparison, all $P <$
 351 0.001).

352
 353 Figure 3: Mean $\pm$ s.e. relative expression of the *A. mellifera* choline transferase gene
 354 (*ChAT*) in four honeybee cell types: brain (green, B, N = 5); haemocytes (blue, H, N = 8);
 355 fatbody (red, F, N = 7); midgut (white, M, N = 7), and its role in acetylcholine synthesis.
 356 Only brain and haemocyte cells exhibit robust *ChAT* expression. Different letters indicate
 357 significant expression differences between cell types.

358
 359 Figure 4: Mean $\pm$ s.e. relative expression of the choline transferase gene (*ChAT*) in the
 360 haemocytes of honeybees treated with neonicotinoid (clothianidin; red) or control (white).

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Figure 1

Figure 2

Figure 3

388 **Figure 4**

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