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Running Head: Vitellogenin as potential biomarker for insecticide exposure

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Abstract

The insect yolk precursor vitellogenin is a lipoglycoprotein synthesised and stored in the fat body and secreted into the hemolymph. In honey bees, vitellogenin displays crucial functions in hormone signalling, behavioural transition of nurse bees to foragers, stress resistance and longevity in workers. Plant protection products (PPPs) such as neonicotinoids, pyrethroids and organophosphate alter transcriptional expression of vitellogenin. To assess PPP-induced alterations on protein level, we developed a rabbit polyclonal vitellogenin antibody. After characterization, we assessed its specificity and vitellogenin levels in different tissues of worker bees. The vitellogenin antibody recognized full-length 180 kDa vitellogenin and the lighter fragment of 150 kDa in fat body, hemolymph and brain. In hemolymph, a band of approximately 75 kDa was detected. Subsequent mass spectrometry analysis (LC-MS/MS) confirmed the 180 and 150 kDa band as vitellogenin. Subsequently, we evaluated vitellogenin expression in brain, fat body and hemolymph upon 24 h exposure of bees to 3 ng/bee to the neonicotinoid clothianidin. Full length vitellogenin was up-regulated threefold in the fat body and the 150 kDa fragment in the brain of exposed honey bees, while no alteration occurred in the hemolymph. Up-regulation of the vitellogenin protein by the neonicotinoid clothianidin goes in line with the previously shown induction of its transcript. We conclude that vitellogenin might serve as a potential biomarker for neonicotinoid exposure in bees.

Key words: Honey bees; vitellogenin; neonicotinoids; clothianidin; antibody characterization

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INTRODUCTION

Honey bees are wild or semi-domesticated insects that live in colonies with complex division of labor (Winston, 1987). Bee colonies consist of one egg-laying queen, the female worker caste, which includes nurse bees and foragers, and the male drones. In contrast to queens and drones, the life-histories of workers can be very plastic during adulthood - individuals change from one social care task to another (Winston and Fergusson, 1985). In summer, workers typically progress from nurse bees with in-hive tasks to foragers. In autumn, when brood production decreases, bees develop into so-called winter bees (Maurizio 1950). Besides physiological specialization enabling bees to perform different tasks, the three major worker types (nurse, forager and winter bee) differ markedly in life-span. Foragers, one type of worker bee, typically die within two weeks and are the shortest-lived individuals, whereas bees continuing nursing can have life-spans longer than 50 days. The longest-lived workers, winter bees, survive from late summer to next spring. Only the queen lives longer, surviving 2 to 3 years, and in some cases up to 5 years (Dukas 2008, Münch et al. 2013).

The transition of nurse bees to foragers is hormonally regulated by juvenile hormone and vitellogenin. During the life course of workers, vitellogenin levels in the hemolymph and fat body drop, and these reduced concentrations influence several aspects of the life history of bees (Münch and Amdam, 2010). Levels of vitellogenin in hemolymph and fat body are highest in the long-living winter bees (up to 60-90 μg/μL hemolymph), and lowest in short-living foragers (0-5 μg/μL hemolymph) (Seehuus et al. 2006).

The phospholipoglycoprotein (Wheeler and Kawooya 1990) vitellogenin is a female-specific egg-yolk precursor (Spieth et al. 1991), synthesized by most oviparous animals including insects for transfer into oocytes, where it serves as nutrition for embryos. Besides the full length 180 kDa protein, vitellogenin was reported to occur as a 150 kDa fragment (Seehuus et al. 2007) and a 40 kDa fragment (Havukainen et al. 2010) in honey bees. Vitellogenin

is also found in the non-reproductive worker castes of bees. It is produced in the fat body (a tissue functionally homologous to the white adipose tissue and liver of vertebrates) in the abdomen (Snodgrass, 1956). Vitellogenin is generally released from the fat body, circulates in the hemolymph and is taken up by the ovaries through receptor-mediated endocytosis (Amdam et al., 2003; Guidugli et al. 2005). Honey bee workers typically do not import vitellogenin to the ovaries, but vitellogenin can be taken up for example by the hypopharyngeal glands in the head of nurse bees that synthesize food jelly for young larvae, other workers and the queen (Amdam et al., 2003). Vitellogenin also shields cells from oxidative damage and protects both workers and queens from oxidative stress (Seehuus et al. 2006; Corona et al. 2007; Havukainen et al. 2013).

Several factors such as pathogens and plant protection products (PPPs, pesticides) can alter vitellogenin levels in honey bees. Infection of young nurse bees with *Nosema ceranae* decreased the expression of vitellogenin transcript (Antúnez et al. 2009) whereas infection of honey bee larvae caused increased vitellogenin titers in young adults (BenVau and Nieh 2017). Some PPPs, including neonicotinoids, the organophosphate chlorpyriphos and the pyrethroid cypermethrin led to increased levels of the *vitellogenin* transcript in the brain of mixed-aged honey bee workers exposed in the period between beginning of May and end of June (Christen et al. 2016, Christen and Fent 2017). In contrast, the organophosphate malathion and chlorantraniliprole decreased the *vitellogenin* transcript (Christen et al. 2017).

The aim of our present study is to assess whether the induction of vitellogenin mRNA upon exposure to the neonicotinoid clothianidin is paralleled on the protein level. First, we generated and characterised an *Apis mellifera* vitellogenin antibody and evaluated its presence in different tissues. Second, we analysed vitellogenin protein levels in different tissues upon exposure to the neonicotinoid clothianidin and compared them with mRNA levels. We detected tissue-specific expression patterns of vitellogenin proteins. Our study demonstrates that previously reported alterations in vitellogenin mRNA are accompanied by changes in protein

levels and may result in physiological consequences, including disturbed transition of worker bees and decreased life-span.

MATERIALS AND METHODS

Chemicals

Clothianidin (purity > 99%), dimethyl sulfoxide (DMSO), dithiothreitol (BioXtra grade) and iodoacetamide (HPLC grade) were from by Sigma–Aldrich (Buchs, Switzerland). DMSO was used to prepare clothianidin solutions. Stock solutions were diluted into 20% sucrose solution to obtain final DMSO concentrations of 0.1%. Acetonitrile (LC-MS Grade) and formic acid (LC-MS grade, Honeywell-Fluka) were from Fisher Scientific (Reinach, Switzerland).

Antibody generation

The generation of a polyclonal *Apis mellifera* vitellogenin antibody was performed by Davids Biotechnology (Regensburg, Germany). In brief, 15 mg of two peptides (peptide 1: KGKHIGKSGKVDVINAAKE, located at N-terminus, and peptide 2: EKNEAAMKLKKRIEKGANPD, located at C-terminus) were synthesized by solid phase synthesis. Peptide sequences were chosen by the company based on prediction programs. The quality of the peptides was analysed by HPLC and mass spectrometry. Peptide purity was > 80%. Before immunization, a short KLH sequence was conjugated to the peptides. Two New Zealand white rabbits were immunized with a mixture of the two peptides (concentration of each peptide: 10 mg/mL) at day 1, 14, 28, 42 and 56. At day 63, the final bleed was taken from each rabbit (40-90 mL final serum) and the polyclonal antibody was purified by affinity purification revealing two polyclonal vitellogenin antibodies with concentrations between 0.78 and 1.17 mg/mL.

Experimental design of laboratory exposures

Mixed aged adult forager honey bees (*Apis mellifera carnica*) were collected from frames from an outdoor colony located in an area without farming activity and pesticide application in the Black Forest (Germany, GPS: N 47.7667, E 7.8333) between May and August 2017.

The colony was infested with *Varroa destructor* and hence treated with formic acid in summer 2016 and oxalic acid in winter 2016. The experimental procedure was identical as described previously (Christen et al. 2016). Ten bees were placed in one PET bottle. Bees were fed either with sucrose solution containing 0.1% DMSO (solvent control, four bottles) or with 3 ng/bee clothianidin (four bottles) for 24 h. The selection of clothianidin concentration and exposure time is based on our previous study where the strongest induction of *vitellogenin* transcript was found after 24 h exposure to 3 ng/bee clothianidin (Christen et al. 2016). Per bottle, 3 bees were pooled to one hemolymph, brain, and fat body sample.

Hemolymph collection

Hemolymph collection was done according to Rutz and Lüscher (1974) and Randolt et al., (2008) with some slide modifications. In brief, hemolymph from frozen unexposed and exposed bees was collected from the dorsal part of the bees by using a sterile 10 µL micro tip. Individual bees were fixated between a pair of tweezers and the intersegmental membrane was slit slightly with the micro tip between the fourth and fifth tergite of the honeybee abdomen. Emerging clear hemolymph was collected with the micro tip which had been pre-wetted with PTU (N-Phenylthiourea, Sigma P7629) and protease inhibitor cocktail solution (Roche complete 04693124001) to prevent immediate melanisation and protein degradation. Turbid hemolymph was discarded. The hemolymph (5–10 µL per bee) was transferred into an Eppendorf tube containing an ice-cold mixture of 10% PTU and protease inhibitor cocktail. The samples were stored on ice during collection. The volume was adjusted to 60 - 80 µL with ice- cold PBS pH 7.4 and stored at –20°C until further analyses. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher) according to the manufacturer's protocol.

Fat body collection

To obtain abdominal fat body tissue, the frozen bee was briefly adjusted to room temperature. The thorax was cut off and the abdomen was fixated with needles to styrofoam. By gripping

the tip of the last body segment, the bee was slightly stretched and cut open on one side of the abdomen, using fine-tipped scissors. The sternum was again fixated with needles, all unwanted body tissue was carefully removed before collecting fat body tissue layer, lining the tergum wall. The tissue was collected into ice-cold 2x concentrated lysis buffer (100 mM Tris pH 7.4, 300 mM NaCl, 30 mM EDTA, 0.2% Triton, Duong et al. (2004)) with addition of protease inhibitor cocktail (Roche cOmplete 04693124001). The samples were stored on ice during collection. For tissue disruption the collected fat bodies were ground using a small glass bar as pestle before lysing on ice for 30 min with two intermediate vortexing steps. Lysis was finally enhanced by sonication on ice by 1-3 cycles with 10 second bursts with 30 second cooling intervals (amplitude 100%, cycle 1 in a Branson Sonifier 450 W). Cellular debris was removed by centrifugation at 13000 rpm at 4°C for 10 min. The supernatant was carefully collected and stored at –20°C until further analyses. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher) according to the manufacturer's protocol.

Honey stomach collection

To obtain honey stomach, the frozen bee was briefly thawed at room temperature. The thorax was cut off and the abdomen fixated between thumb and forefinger. Carefully, the honey stomach was removed from the front part of the abdomen using forceps. The tissue was collected into ice-cold 2x concentrated lysis buffer (100 mM Tris pH 7.4, 300 mM NaCl, 30 mM EDTA, 0.2% Triton, Duong et al. (2004)) with addition of protease inhibitor cocktail (Roche cOmplete 04693124001). The samples were stored on ice during collection. For tissue disruption, the collected honey stomach were ground using a small glass bar as pestle before lysing on ice for 30 min with two intermediate vortexing steps. Lysis was finally enhanced by sonication on ice by 1-3 cycles with 10 second bursts with 30 second cooling intervals (amplitude 100%, cycle 1 in a Branson Sonifier 450 W). Cellular debris was removed by centrifugation at

13000 rpm at 4°C for 10 min. The supernatant was carefully collected and stored at –20°C until further analyses. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher) according to the manufacturer's protocol.

Brain collection

The brain of frozen bees was removed in total by opening the cranium using a scalpel and forceps, as described before (Christen et al. 2016). The tissue was collected into ice-cold 2x concentrated lysis buffer (100 mM Tris pH 7.4, 300 mM NaCl, 30 mM EDTA, 0.2% Triton; Duong et al. 2004)) with addition of protease inhibitor cocktail (Roche cOmplete 04693124001). The samples were stored on ice during collection. For tissue disruption the collected brains were ground using a small glass bar as pestle before lysing on ice for 30 min with two intermediate vortexing steps. Lysis was finally enhanced by sonication on ice by 1-3 cycles with 10 second bursts with 30 second cooling intervals (amplitude 100%, cycle 1 in a Branson Sonifier 450 W). Cellular debris was removed by centrifugation at 13000 rpm at 4°C for 10 min. The supernatant was carefully collected and stored at –20°C until further analyses. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher) according to the manufacturer's protocol.

Analysis of antibody binding by dot blot

Dot blot analysis was run according to the protocols on the Licor home page (https://www.licor.com/bio/applications/quantitative_western_blots/protocol.htm) with slight modifications. BSA (1 µL of 2 mg/mL BSA in PBS) as negative control, secondary antibody solution (IRDye® 800CW Goat anti-Rabbit IgG, 1:500 in Licor blocking buffer diluted 1:1 with PBS-T) as positive control, hemolymph, fat body and brain lysate were spotted separately on a nitrocellulose membrane (Amersham™ Protran® Western blotting membranes,

0.45 µm). After drying, the membrane was incubated in Licor Blocking buffer (927-40100) for 1h at room temperature with agitation. After addition of 0.2% Tween-20, the membrane was incubated with the polyclonal antibody against vitellogenin in a 1:7000 dilution (Davids Biotechnologie Regensburg) for 1 h at room temperature with agitation. After three washes with PBS-T (0.2% Tween 20) the membrane was incubated for 45 min at room temperature in secondary antibody solution (IRDye® 800CW Goat anti-Rabbit IgG (H + L),1:40.000 in Licor blocking buffer diluted 1:1 with PBS-T). After 3 washes with PBS-T (0.2% Tween 20) the vitellogenin specific signals were acquired at 800 nm using the Licor Odyssey® CLx Imaging System.

SDS Polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was run according to the protocols on the Licor home page (https://www.licor.com/bio/applications/quantitative_western_blots/protocol.htm) with slight modifications. In brief, one-dimensional gel electrophoresis was carried out in vertical polyacrylamide gels (10.1 x 7.3 x 0.1 cm) containing 0.1% SDS with a 4% stacking gel on top of the separating gel. Samples were diluted with 4x concentrated Orange G sample buffer for Licor blots (250 mM Tris-HCl, pH 6.8, 12% SDS, 50% Glycerol, 6% 2-mercaptoethanol, 0.2% Orange G), heated for 5 min at 95°C and subjected to electrophoresis at constant voltage (140 V) for 20 min and 190 V until the dye front has run out. Two types of one-dimensional gels were run (10% and 7.5% polyacrylamide/ 0.1% SDS gels) for the separation of proteins in the range of 30–200 kDa, with the later found to be superior for separation of the two vitellogenin forms.

Protein extraction and digestion

The extracted proteins were resolved on one‐dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a pre-cast gradient gel (4 - 20% polyacrylamide,

Mini-Protean Tgx, Bio-Rad Laboratories, Switzerland). The gel was stained with Coomassie Blue G-250 (Bio-Safe Coomassie Stain, Bio-Rad Laboratories, Switzerland). The visualized gel bands were then cut into 5 slices per lane and further cut up into smaller pieces. The In-gel digestion was done according to Shevchenko et al. (2006). Briefly, the gel pieces were dehydrated and washed. Disulfide bonds were reduced with 10 mM dithiothreitol, followed by an alkylation step using 55 mM iodoacetamide. The samples were digested with trypsin (Sequencing Grade modified trypsin, Promega, Switzerland) at 37°C overnight. The resulting peptides were extracted using 100 µL of 5% formic acid/ acetonitrile (1:2, v/v) followed by a second extraction step using 20 µL 1% formic acid/ acetonitrile (1:1, v/v). Both supernatants were collected into a 250 µL vial and dried to dryness with a vacuum centrifuge (GeneVac EZ-2 Plus, SP Scientific, UK). The peptide mixture was re-dissolved with 30 µL of 0.1% formic acid and analysed by liquid chromatography coupled to a tandem mass spectrometer.

LC-MS/MS analysis

An Agilent 1290 Infinity LC system connected to an Agilent 6540 quadrupole time-of-flight mass spectrometer (Agilent Technologies, Switzerland) was used. The peptide mixtures (12 µL) were injected onto a reversed phase C18 column (2.1 x 150 mm, 2.7 micron, 120 Å pore size, Agilent Technologies, Switzerland). The column flow was set to 400 µL/min with a mobile phase composed of solvent A (water) and B (acetonitrile with 0.1% formic acid). Initially, 2.5% of mobile phase B was held constant for 1 min, followed by a linear gradient from 2.5% – 35% B over 90 min, and a final increase over 2 minutes to 95% B where it was held for 2 min. Re-equilibration of the initial column condition was performed within 6 minutes. Column temperature was held constant at 45°C through the run. The jet stream electrospray source was operated in positive mode with following parameter settings: nebulizer pressure 35 psig, nozzle voltage 0 V, sheath gas flow 12 L/min, sheath gas temperature 250°C, drying gas flow 10 L/min, drying gas temperature 250°C, capillary voltage 4000 V and fragmentor

voltage of 175 V. The instrument state mass range was set to 1700 m/z with 4 GHz digitalization rate, which leads to a mass resolution of 40 000 (measured at m/z 1521). Mass spectra were acquired in profile mode over a m/z range of 100 - 1700 by 1 Hz in scan and 3 Hz in MS/MS mode. The auto-MS/MS experiment was selected using the 20 most abundant precursor ions per cycle with activated precursor abundance-based scan speed. The quadrupole precursor ion isolation width was set to medium (~ 4 m/z) with a dynamic collision energy based as a function of the m/z value (slop = 3, offset = 2). Active exclusion of a precursor mass was one repeat and release after 15 seconds. The system was controlled with MassHunter Data Acquisition B.08.01 service pack 1 (Agilent Technologies, USA).

Protein identification

The raw data files were processed with Mascot Distiller 2.7 (Matrix Science, UK) to generate a peak list. A database search to identify proteins was conducted using an in-house Mascot Server 2.6 (Matrix Science, UK) against the *Apis mellifera* sequence database (15323 protein sequences of *Apis mellifera*, downloaded in November 2018 from Uniprot.org) and sequences for common protein contaminants (cRAP, downloaded from thegpm.org). The search parameters were: enzyme trypsin, maximum missed cleavages 2, fixed modification carbamidomethylation of cysteine and variable modification oxidation of methionine. Peptide mass tolerance window was set to 7 ppm and fragment ion mass tolerance to 0.70 Da. The received search results were sent to the Scaffold software 4.7.3 (Proteome Software, USA). The loaded results were searched against a second search engine X! Tandem (thegpm.org, version Cyclon 2010.12.01.1) to increase confidence. The Local FDR algorithm with 95 % probability was used for validating peptide identifications. The Protein Prophet algorithm with 99.0 % probability was used for protein identification, and contained at least 2 identified peptides (Nesvizhskii et al. 2003).

Vitellogenin identification by Western Blot

Immunoblots were performed according to the Licor protocols (https://www.licor.com/bio/applications/quantitative_western_blots/protocol.html) with slight modifications. For immunoblots, size-separated proteins were electrophoretically transferred to nitrocellulose membrane (Amersham™ Protran® Western blotting membranes, 0.45 µm: GE10600002 or 0.2 µm: GE10600004) using either Bjerrum Schafer-Nielsen Buffer with SDS (48 mM Tris, 39 mM glycine, 20% methanol (pH 9.2), 0.1% SDS) with constant current of 140 mA for 1h 30 min (OWL Device) or Pierce 1-Step Transfer Buffer with constant high current of 1.3-2.5 A for 12 min (Pierce™ Power Blotter).To control the transfer efficiency, the membrane was briefly rinsed with water after transfer, and incubated for 5 min in Revert Total Protein Stain, rinsed twice for 30 sec in washing solution (Licor: 926-11015) before acquiring the Revert total stain signal in the 700 nm channel using the Licor Odyssey® CLx Imaging System. The membranes were then incubated in Licor Blocking buffer (927-40100) for 1h at room temperature or overnight at 4°C with agitation. After addition of 0.2% Tween-20, the membranes were incubated with the polyclonal antibody against vitellogenin at a 1:7000 dilution (Davids Biotechnologie Regensburg) for 1 h at room temperature or overnight at 4°C with agitation. After 3 washes with PBS-T (0.2% Tween 20) the membranes were incubated for 45 min at room temperature in secondary antibody solution (IRDye® 800CW Goat anti-Rabbit IgG (H + L), 1:40.000 in Licor blocking buffer diluted 1:1 with PBS-T). After 3 washes with PBS-T (0.2% Tween 20) the vitellogenin specific signals were acquired in the 800 nm channel. The detected fluorescence signals were quantified using NIH software Image J (Christen et al. 2007). The intensity of vitellogenin bands was normalized against the intensity of the uncharacterized *Apis mellifera* protein, which is also detected by the antibody. Although not specified, this protein was well suited as a loading control, due to the fact that this protein is stably expressed in all analysed tissues. Moreover, there was a lack of available commercial antibodies, which are specific for honey bees and antibodies against proteins of other species may lack cross-reactivity.

Data processing and statistical analysis

One-way ANOVA and Bonferroni's multiple comparison test were applied to compare means of exposed and unexposed samples. Data are shown as means ± standard error of means. Statistically significant limits were: one asterisk at $0.05 > p > 0.01$, two asterisks at $0.01 > p > 0.001$ and three asterisks at $0.001 > p > 0.0001$.

RESULTS

Characterisation of vitellogenin antibody

Two rabbits were immunized using custom-synthesized peptides according to the vitellogenin peptide sequences. One peptide was based on the sequence located at the C-terminus and one based at the N-terminus (Fig. 1A). After 63 days of immunization, the final bleed was taken from each rabbit and the polyclonal antibody was purified by affinity purification revealing two polyclonal vitellogenin antibodies. Initially, the binding of the generated antibodies to honey bee vitellogenin from different tissues was analysed by dot blot analysis with a mixture of the two polyclonal vitellogenin antibodies. Figure 1B shows that the vitellogenin antibody mixture clearly detected vitellogenin in the hemolymph, fat body and brain tissue (Fig. 1B).

To further analyse the exact binding properties of the vitellogenin antibody, Western blot analysis was performed. The vitellogenin antibody detected the full length vitellogenin in fat body, hemolymph and brain (Fig. 2). According to our molecular size marker used, the size of the detected band was slightly lower than 180 kDa, representing the full length vitellogenin. In addition, the antibody recognized a lighter vitellogenin protein with a size slightly below 150 kDa in the hemolymph and fat body (Fig. 2A). However, in the brain, this smaller vitellogenin band was only detectable when overexposing the membrane (data not shown). In hemolymph, a band of approximately 75 kDa was detected in addition to the full-length and the lighter vitellogenin. In fat body, hemolymph and brain, an approximately 200 kDa band was detected in addition to 180 kDa vitellogenin (Fig. 2). No signal was detected in honey

stomach lysates (Fig. 2A). Mass spectroscopy was performed to identify exactly the detected bands. The vitellogenin antibody recognized the following proteins: uncharacterized protein at 200 kDa and vitellogenin at approximately 180 and 150 kDa (Fig. 2C). In hemolymph, an additional band was detected. Mass spectroscopy demonstrated that this band belongs to 6-phosphofructokinase, alpha-glucosidase, aconitate hydratase and transferrin. Which of these proteins was recognized by the antibody remains un-identified. Details of the mass spectroscopy analysis are presented in Table 1.

Induction of vitellogenin by clothianidin

As a proof of concept, we determined whether clothianidin leads to induction of vitellogenin. We previously showed that exposure of honey bee workers to clothianidin and additional neonicotinoids led to induction of *vitellogenin* mRNA in the brain (Christen et al. 2016). Here we extended our analysis onto the protein level and aimed to assess the induction of the vitellogenin protein. By Western Blot analysis we quantified vitellogenin in the hemolymph, brain and fat body of bees exposed to 3 ng/bee clothianidin for 24 h. In the hemolymph, vitellogenin levels (full-length and smaller vitellogenin) did not differ between solvent control and clothianidin exposed bees (Fig. 3A). In the brain, exposed bees showed no alteration of the full-length vitellogenin, but the smaller vitellogenin was significantly increased (Figs. 3B, C). In the fat body, a three-fold increase in the amount of full-length vitellogenin occurred in clothianidin exposed bees compared to control bees (Fig. 3D, E). These data indicate that clothianidin led to differential induction of vitellogenin protein in different tissues.

DISCUSSION

Vitellogenin influences hormone signalling, behavioural transition, stress resistance and longevity in honey bee workers (Amdam et al. 2004b, Nelson et al. 2007, Seehuus et al. 2006). Exposure of honey bees to PPPs including neonicotinoids, pyrethroids and organphosphates

altered the expression of vitellogenin on mRNA level in the brain (Christen et al. 2016; Christen and Fent 2017) but the induction on the protein level remained elusive. In our present work, we generated a polyclonal antibody against honey bee vitellogenin, confirmed it by GC-MS/MS determination, and analysed vitellogenin protein expression patterns in hemolymph, fat body, honey stomach and brain. We found cross-reactivity with three proteins that represent full-length and processed vitellogenin. We observed similar changes in vitellogenin protein levels in response to clothianidin as were reported previously for vitellogenin mRNA expression (Christen et al. 2016).

Vitellogenin in different tissues

Vitellogenins represent a multigene superfamily together with insect apolipophorins. Honey bee vitellogenin was described as a 180 kDa monomeric phospholipoglycoprotein (Wheeler and Kawooya 1990). In addition to the 180 kDa full size vitellogenin, which was detected in the hemolymph and in the fat body of workers, a 150 kDa fragment was found in the ovaries of queens (Seehuss et al. 2007), in the hypopharyngeal glands of workers (Amdam et al. 2003) and during vitellogenin purification from queen hemolymph (Wheeler and Kawooya 1990). Therefore, lower molecular weight products could represent a degradation product (Wheeler and Kawooya 1990) or processed vitellogenin (Havukainen et al. 2011). The fat body is the major source of vitellogenin, which is then secreted into the hemolymph (Raikhel and Dhadialla 1992; Nilsen et al. 2011). Vitellogenin from the fat body was proposed to be cleaved from the 180 kDa full length vitellogenin into a 150 kDa C-terminal fragment and a 40 kDa N-terminal fragment (Havukainen et al. 2011) in abdominal fat body tissue. In addition, in fresh, ion-exchange purified hemolymph only full length vitellogenin is detectable (Havukainen et al. 2011). Only with time, a 150 kDa degradation product is formed. In non-purified hemolymph, bands of 70-75 kDa occurred in Western Blots in addition to the full length vitellogenin (Havukainen et al. 2011). In addition to the full-length 180 kDa and the

smaller 150 kDa vitellogenin, a 200 kDa band was detected in fat body, hemolymph and brain lysates. This band represents an uncharacterised honey bee protein, which shows cross-reactivity with the vitellogenin antibodies.

In the fat body, hemolymph and in the brain of workers, we detected full length vitellogenin slightly below 180 kDa (Fig. 2). Corona et al. (2007) localized the vitellogenin mRNA not in the brain of bees themselves but in fat cells tightly bound to the brain. As these cells cannot be separated during dissection, our analysis covers the brain and these fat cells together. In addition to the full length vitellogenin, we also detected a band slightly below 150 kDa vitellogenin in the fat body and in the hemolymph (Fig. 2). According to Havukainen et al. (2011) this band likely represents the processed vitellogenin in the fat body and a degradation product in the hemolymph. The detected vitellogenin bands were both slightly below the published sizes of 180 and 150 kDa (Havukainen et al. 2011), potentially due to anomalous migration of the protein standard or samples as a result of incomplete denaturation or binding of detergent to the protein (Dolnik and Gurske 2011). In the brain, the lighter vitellogenin band is almost not detectable. As the fat body is the major source of vitellogenin synthesis and the hemolymph is the distributor of vitellogenin in the honey bee body, the occurrence of both vitellogenin forms in these tissues makes sense. In the brain and associated fat cells and in the hypopharyngal glands, the lighter vitellogenin form is only present in nurse bees, which are responsible for the larval feeding (Amdam et al., 2003b).

In addition to full length and processed vitellogenin, we detected a band at 70 kDa in the hemolymph (Fig. 2). Havukainen et al. (2011) used ion exchange purified hemolymph to detect different vitellogenin bands of 40, 150 and 180 kDa. However, in raw hemolymph extract a band around 70-75 kDa was detected, which is similar to the 70 kDa band detected in our present work (Fig. 2). This protein may represent a degradation product of the full length or 150 kDa vitellogenin. Both the full-length and the 150 kDa fragments are post-translationally modified by phosphorylation and glycosylation (Havukainen et al. 2011). The loss of

phosphorylation or glycosylation during our protein extraction may explain the smaller size of the full length and processed vitellogenin detected in our work (Fig 2). The functionality of the processed vitellogenin is not currently understood. We could not detect the 40 kDa fragment because it maps between residues 53 to 294 of the N-terminus of the known vitellogenin protein sequence. However, the N-terminal peptide used to immunize rabbits in the present study maps between residues 409 to 427. Therefore, the generated vitellogenin antibody is unable to detect the 40 kDa fragment. In contrast to most insects, where vitellogenin expression is tissue-specific and vitellogenin receptors have only been detected in ovaries (Chen et al. 2004; Tufail and Takeda 2005, 2007; Ciudad et al. 2006), in honeybees vitellogenin receptors were observed in head, fat body and ovaries of worker bees (Guidugli-Lazzarini et al. 2008). This may be related to the multiple regulatory function of vitellogenin in the social life of honey bees.

Vitellogenin induction by clothianidin

We previously observed that different classes of pesticides altered the expression of vitellogenin mRNA. The four neonicotinoids acetamiprid, clothianidin, imidacloprid and thiamethoxam induced the vitellogenin transcript in the brain at environmentally relevant concentrations (Christen et al. 2016). Alterations in vitellogenin expression in the brain of workers was also observed after exposure to the organophosphates chlorpyrifos and malathion, to the pyrethroid cypermethrin and to the ryanodine receptor activator chlorantraniliprole (Christen and Fent 2017). In our present study, we evaluated whether clothianidin not only leads to induction of the transcript but the protein as well. Indeed, we observed a 3-fold induction of full-length vitellogenin protein in the fat body and of the lighter vitellogenin in honey bee brain (Fig. 3B and C). These data complement our previous data that showed a fourfold induction of vitellogenin mRNA by clothianidin and other neonicotinoids (Christen et al. 2016). The

lighter vitellogenin form has been found in the hypopharyngal glands of workers, where vitellogenin constituents are processed into food secretions for larval feeding (Amdam et al. 2003a). This lighter vitellogenin is not found in the unexposed honey bee brain (Fig. 2B, 3B). As nurse bees show higher vitellogenin titers and are responsible for the feeding of the larvae (Amdam et al. 2003a, b), the lighter vitellogenin in the brain may be a marker of nurse bees. The exposure of foragers to clothianidin induced the light vitellogenin in the foragers, suggesting the presence of a "nurse-bee"-like phenotype.

Vitellogenin plays such an essential role in hormone signalling and in transition of nurse bees, which have high vitellogenin levels, to foragers, which have lower vitellogenin levels. Vitellogenin is also involved in stress management by protecting against oxidative stress and regulation of life-span; long-lived queens show high vitellogenin levels (Amdam et al. 2004b, Nelson et al. 2007, Seehuus et al. 2006). On this basis, we conclude that altered levels of vitellogenin may have significant physiological consequences in exposed worker bees. Further studies are needed to link vitellogenin alteration to physiological outcomes and to demonstrate whether vitellogenin may serve as a biomarker candidate for the exposure of honey bees to neonicotinoids and other pesticides. It should be noted that possibly other factors may also affect vitellogenin protein levels and that they may fluctuate or become increased in a compensatory manner, or induction may be transient. More detailed investigation should thus devoted to such questions before vitellogenin can be regarded as a biomarker for pesticide exposure. The advancement of our newly developed antibody is the fact that it represents a good tool to analyse in detail the physiological role of vitellogenin in honey bees and to analyse the potential effects of plant protection products on vitellogenin expression and function.

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Figure 1: A: Protein sequence of vitellogenin (https://www.ncbi.nlm.nih.gov/nuccore/NM_001011578, accession number: NM_001011578). Position of the two peptides used for immunization is marked in violet. B: Dot blot analysis: hemolymph, fat body and brain of adult honey bees were processed as described and 1 µL was spotted on a nitrocellulose membrane, following by incubation with vitellogenin antibody. Proteins were detected by IR-fluorescence.

Figure 2: Expression of vitellogenin in different body tissues of honey bees processed as described. (A) Vitellogenin in fat body (FS), honey stomach (HS), hemolymph (HL). Shown are all cross-reactive bands. (B) Vitellogenin in brain samples. Equal amounts of lysates (20 µg) were loaded in each lane. Numbers to the left give molecular weights in kilodaltons as given by the protein ladder M (L2020 UPBBio). Full-length vitellogenin is represented by the band below 180 kDa, lighter vitellogenin is presented by the band below 140 kDa (both marked with arrows), possible degradation product of vitellogenin visible at 75 kDa, * marks uncharacterized *Apis mellifera* protein, which is detected by the anti-vitellogenin antibody and used as loading control. Proteins were detected by IR-fluorescence. C: Analysis of protein bands in hemolymph by mass spectroscopy. Data shown of the proteins, which were detected by the vitellogenin antibody.

Figure 3: Expression of vitellogenin in different body tissues of control bees and bees exposed to clothianidin. Hemolymph (A, n=4), brain (B, n=3) and fat body (D, n=4) of control honey bees (solvent control) and honey bees exposed to 3 ng/bee clothianidin for 24 h were processed as described. Equal amounts of lysates (20 µg) were loaded in each lane. Numbers to the left give molecular weights in kilodaltons as given by the protein ladder M (L2020 UPBBio). Full-length vitellogenin is the band below 180 kDa, processed vitellogenin is the band below 135 kDa (both marked with arrows), * marks uncharacterized *Apis mellifera* protein, which is detected by the anti-vitellogenin antibody and used as loading control. Proteins were

detected by IR-fluorescence. The intensity of the fluorescent signals was quantified using Image J software of full length and lighter vitellogenin in brain sample (C) and of full length vitellogenin in hemolymph sample (E).

Table 1: Data mass spectroscopy (GC/MS-MS)

Sample	Protein name	Accession number	Protein molecular weight	Protein identification probability	Exclusive unique peptide count	Total spectrum count	Percentage sequence coverage	Mascot score
1	Uncharacterized protein OS=*Apis mellifera*	A0A088AS56_APIME	367,625.3	100	135	218	47.4	3503
2	Protein families 1 Vitellogenin OS=*Apis mellifera*	A0A088ADL8_APIME	200,959.8	100	5	104	45.5	1419
2	Protein families 1 Vitellogenin OS=*Apis mellifera*	VIT_APIME	201,051.9	100	2	99	41.4	1269
3	Protein families 1 Vitellogenin OS=*Apis mellifera*	A0A088ADL8_APIME	200,959.8	100	4	56	31.9	768
3	Protein families 1 Vitellogenin OS=*Apis mellifera*	VIT_APIME	201,051.9	100	2	54	29.3	707
4	Pyruvate carboxylase OS=*Apis mellifera*	A0A087ZNH6_APIME	131,882.5	100	8	8	9.7	98
4	Protein families 1 Vitellogenin OS=*Apis mellifera*	A0A088ADL8_APIME	200,959.8	100	4	45	25.9	644
4	Protein families 1 Vitellogenin OS=*Apis mellifera*	VIT_APIME	201,051.9	100	2	43	23.3	587
Triple	Uncharacterized protein OS=*Apis mellifera*	A0A088A3B7_APIME	87,207.6	100	3	3	6.49	92
Triple	6-phosphofructokinase OS=*Apis mellifera*	A0A088AHG6_APIME	98,518.8	100	2	2	2.93	69
Triple	Uncharacterized	A0A087Z	71,772.3	100	4	4	7.75	50

	protein OS=*Apis mellifera*	Q27_APIME						
Triple	Alpha-glucosidase OS=*Apis mellifera*	A0A0B4J2P5_APIME	66,596.7	100	7	7	13.1	105
Triple	Uncharacterized protein OS=*Apis mellifera*	A0A087ZTY7_APIME	75,404.6	100	3	3	6.11	42
Triple	Alpha-glucosidase; Uncharacterized protein OS=*Apis mellifera*	Q25BT8_APIME	67,359.4	100	4	4	7.99	80
Triple	Aconitate hydra-tase, mitochondrial OS=*Apis mellifera*	A0A087ZXK3_APIME	85,998.7	100	20	22	28.8	234
Triple	Transferrin OS=*Apis mellifera*	A0A088AFH7_APIME	78,630.7	100	30	43	49.9	760
Triple	Uncharacterized protein OS=*Apis mellifera*	A0A088AJI5_APIME	76,681.3	100	19	29	34.1	612
Triple	Uncharacterized protein OS=*Apis mellifera*	A0A088AQB0_APIME	75,709.1	100	9	10	21.8	230

Fig. 1:

A:

MLLLLTLLLFAGTVAADFQHNWQVGNEYTYLVRSRTLTSLGDLSDVHTGILIKALLTVQAKDSNVLAAKVWNGQYARVQQSMPDGWE
TEISDQMLELRDLPIGKPFQIRMKHGLIRDLIVDRDVPTWEVNILKSIVGQLQVDTQGENAVKVNSVQVPTDDEPYASFKAMEDSVGGK
CEVLYDIAPLSDFVIHRSPELVPMPTLKGDGRHMEVIKIKNFDNCDQRINYHFGMTDNSRLEPGTNKNGKFFSRSSTSRIVISESLKHFTIQ
SSVTTSKMMVSPRLYDRQNGLVLSRMNLTLAKMEKTSKPLPMVDNPESTGNLVYIYNNPFSDVEERRVSKTAMNSNQIVSDNSLSSSEE
KLKQDILNLRTDISSSSSSISSSEENDFWQPKPTLEDAPQNSLLPNFVGYKQKHIQKSGIKVDVINAAKELIFQIANELEDASNIPVHATLEK
FMILCNLMRTMNRKQISELESNMQISPNELKPNDKSQVIKQNTWTVFRDAITQTGTGPAFLTIKEWIERGTTKSMEAANIMSKLPKTVRT
PTDSYIRSFFELLQNPKVSNEQFLNTAATLSFCEMIHNAQVNKRSIHNNYPVHTFGRLTSKHDNSLYDEYIPFLERELRKAHQEKDSPRIQ
TYIMALGMIGEPKILSVFEPYLEGKQQMTVFQRTLMVGSLGKLTETNPKLARSVLYKIYLNTMESHEVRCTAVFLLMKTNPPLSMLQRM
AEFTKLDTNRQVNSAVKSTIQSLMKLKSPEWKDLAKKARSVNHLLTHHEYDYELSRGYIDEKILENQNIITHMILNYVGSEDSVIPRILYL
TWYSSNGDIKVPSTKVLAMISSVKSFMELSLRSVKDRETIISAAEKIAEELKIVPEELVPLEGNLMNNKYALKFFPFDKHILDKLPTLISN
YIEAVKEGKFMNVNMLDTYESVHSFPTETGLPFVYTFNVIKLTKTSGTVQAQINPDFAFIVNSNLRLTFSKNVQGRVGFVTPFEHRHFISG
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ETAKTNEEMWELIDTEKLTDRLPYPWTMDNERYVKVDMYMNLEGEQKDPVIFSTSFDSKVMTRPDTDSENWTPKMMAVEPTDKQAN
SKTRRQEMMREAGRGIESAKSYVVDVRVHVPGESESETVLTLAWSESNVESKGRLLGFWRVEMPRSNADYEVCIGSQIMVSPETLLSY
DEKMDQKPKMDFNVDIRYGKNCGKGERIDMNGKLRQSPRLKELVGATSIIKDCVEDMKRGNKILRTCQKAVVLSMLLDEVDISMEVPS
DALIALYSQGLFSLSEIDNLDVSLDVSNPKNAGKKKIDVRAKLNEYLDKADVIVNTPIMDAHFKDVKLSDFGFSTEDILDTADEDLLINN
VFYEDETSCMLDKTRAQTFDGKDYPLRLGPCWHAVMTTYPRINPDNHNEKLHIPKDKSVSVLSRENEAGQKEVKVLLGSDKIKFVPGT
TSQPEVFVNGEKIVVSRNKAYQKVEENEIIFEIYKMGDRFIGLTSDKFDVSLALDGERVMLKASEDYRYSVRGLCGNFDHDSTNDFVGP
KNCLFRKPEHFVASYALISNQCEGDSLNVAKSLQDHDCIRQERTQQRNVISDSESGRLDTEMSTWGYHHNVNKHCTIHRTQVKETDDKI
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B:

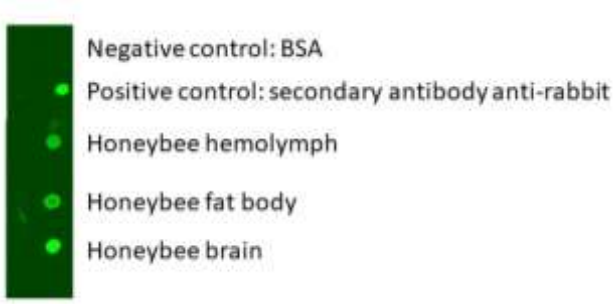
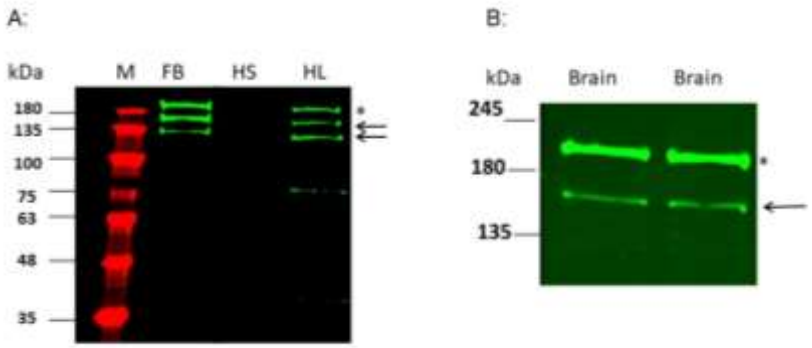


Fig. 2:



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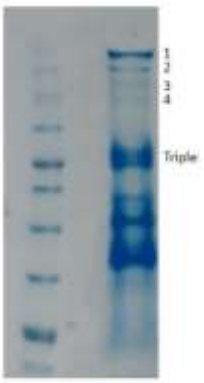

Number	Protein name	Accession number
1	Uncharacterized protein *Apis mellifera*	A0A088AS56_APIME
2	vitellogenin	A0A088ADL8_APIME
3	vitellogenin	A0A088ADL8_APIME
4	Pyruvate carboxylase	A0A087ZNH6_APIME
Triple	6-phosphofructokinase	A0A088AHG6_APIME
	Alpha-glucosidase	A0A0B4J2P5_APIME
	Aconitate hydratase, mitochondrial	A0A087ZXK3_APIME
	Transferrin	A0A088AFH7_APIME

Fig. 3:

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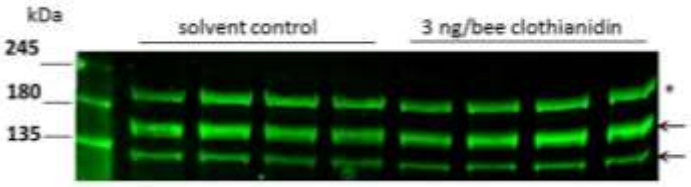

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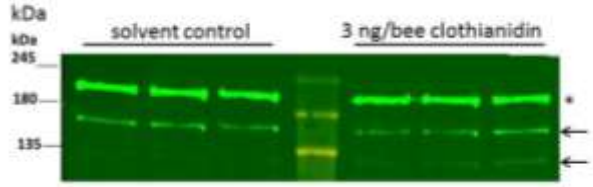

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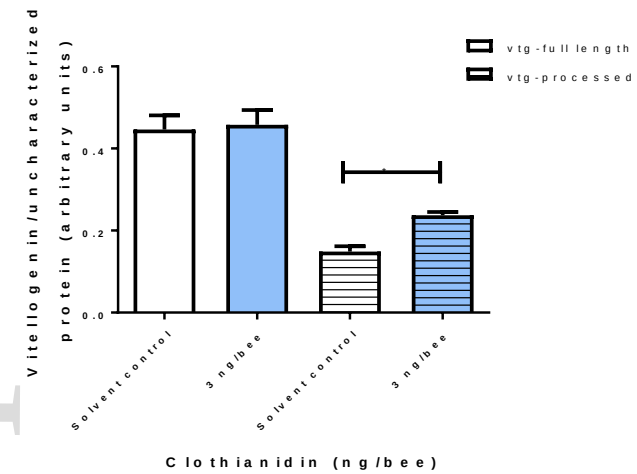

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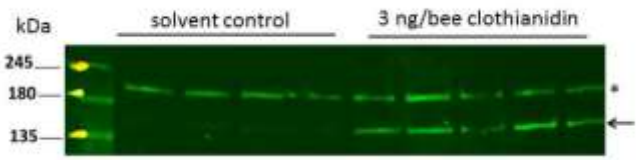

E: