

## Caffeine administration alters the behaviour and development of *Galleria mellonella* larvae



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### ABSTRACT

The effect of feeding caffeine on the behaviour and neural proteome of *Galleria mellonella* larvae was assessed. Caffeine was administered to larvae by force feeding and the metabolites theobromine and theophylline were subsequently detected by RP-HPLC analysis. Administration of caffeine to larvae resulted in reduced movement and a reduction in the formation of pupae. The production of the muscle relaxant theophylline may contribute to the reduction in larval movement. Analysis of the changes in proteome of the brain and surrounding tissues of caffeine fed larvae revealed an increase in the abundance of immune related proteins such as immune-related Hdd1 (6.28 fold increase) and hemolin (1.68 fold increase), ATPase associated proteins such as H<sup>+</sup> transporting ATP synthase O subunit isoform 1 (1.87 fold increase) and H<sup>+</sup> transporting ATP synthase delta subunit (1.53 fold increase) and proteins indicative of brain trauma such as troponin T transcript variant B, partial (1.55 fold increase). Proteins involved in development and protein degradation such as SUMO-activating enzyme subunit 1 (3.08 fold decrease) and chitin deacetylase, partial (3.67 fold decrease) were decreased in abundance. The results presented here indicate that caffeine is metabolised in a similar way in *G. mellonella* larvae to that in mammals and results in a variety of behavioural and developmental alterations. Utilisation of insects for studying the effects of caffeine and other neuroactive compounds may offer new insights into their mode of action and reduce the need to use mammals for this type of analysis.

### 1. Introduction

Caffeine (1,3,7-trimethylxanthine) is the most widely used central nervous system stimulant in the world and along with its metabolites, theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine), is found in a wide range of plants (Porciúncula et al., 2013). A third metabolite paraxanthine (1,7-dimethylxanthine) is not found in food, but is the main metabolite of caffeine *in vivo* (Aresta et al., 2005). Over 60 plant species contain caffeine with the most widely used being coffee (*Coffea arabica*), kola nuts (*Cola acuminata*), tea (*Thea sinensis*), and chocolate (Cocoa bean) (Vanderveen et al., 2001); and it is also available in the synthetic form. Caffeine competitively binds to adenosine receptors allowing it to act as an antagonist with inverse agonist activity (Rivera-Oliver and Díaz-Ríos, 2014). Caffeine affects cAMP signalling both by binding to adenosine receptors and by competitively inhibiting the activity of phosphodiesterases which function to degrade cAMP (Vanderveen et al., 2001). Caffeine

can also bind to intracellular calcium-channel ryanodine receptors resulting in the release of intracellular Ca<sup>2+</sup>. Caffeine can stimulate dopaminergic activity by removing the negative modulatory effects of adenosine at dopamine receptors (Ferré, 2008). In addition to the effect caffeine has on adenosine receptors, studies have also suggested that paraxanthine, the primary metabolite of caffeine in humans, produces increased locomotive activity as well as a phosphodiesterase inhibitory effect which in turn results in increased extracellular levels of dopamine (Orrú et al., 2013). The consumption of a low to moderate doses of caffeine is generally regarded as safe (daily intake of no > 400 mg in healthy adults) however the consumption of higher doses by vulnerable individuals can result in an increased risk of negative health consequences such as cardiovascular and perinatal complications (Meredith et al., 2013). The most dominant route of consumption is by oral intake but injection of caffeine or caffeine citrate is also available.

The effect of caffeine on physiology and behaviour of animals is widely studied in invertebrates as well as vertebrates (Capiotti et al.,

**Abbreviations:** FDR, False Discovery Rates; TFA, trifluoroacetic acid; PBS, phosphate buffered saline; IAA, iodoacetic acid; DTT, dithiothreitol; LFQ, label free quantification; ANOVA, analysis of variance; SE, standard error; RP-HPLC, reverse phase high powered liquid chromatography; EC<sub>50</sub>, half maximal effective concentration; MS, mass spec; FIMTrack, frustrated total internal reflection-based imaging method

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2011; Chen et al., 2008; Cruz et al., 2017; Mustard, 2014; Wu et al., 2009). In *Apis mellifera* caffeine modulates learning and memory; it specifically affects performance during acquisition but not the processes involved in the formation of long term memory (Mustard et al., 2012). In *Drosophila* chronic administration of caffeine reduced and fragmented sleep and also lengthened the circadian period (Wu et al., 2009). Administration of caffeine to Zebra fish (*Danio rerio*) embryos at concentrations above 300 ppm proved lethal, but lower concentrations resulted in shorter body length and reduced tactile sensitivity (Chen et al., 2008). This latter effect was due to the misalignment of muscle fibres and motor neurone defects in treated embryos. Zebra fish embryos also showed reduced mobility when exposed to caffeine possibly as a result of decreased expression of adenosine receptors (Cruz et al., 2017).

The aim of the work presented here was to characterize the effect of caffeine on the behaviour of insects as this might give an insight into the effect of the compound in mammals. The immune system of insects bears structural and functional similarities to the innate immune response of mammals (Browne et al., 2013). As a consequence of these similarities, insects have been utilised as models for studying the virulence of human pathogens (Junqueira, 2012) and for assessing the efficacy of novel anti-microbial drugs (Dolan et al., 2016) and give results comparable to those that can be obtained using mammals (Tsai et al., 2016; Cook and McArthur, 2013; Junqueira, 2012). Larvae of the Greater Wax Moth (*Galleria mellonella*) are inexpensive to purchase and house, easy to inoculate, and statistically robust data can be generated quickly (Browne and Kavanagh, 2013). *G. mellonella* larvae are widely used as an *in vivo* screening system and there are no ethical or legal issues surrounding their use (Kavanagh and Reeves, 2004). Larvae can be administered compounds by force feeding (Mukherjee et al., 2013b) or by intra-hemocoel injection (Cotter et al., 2000) and their response can be monitored by assessing changes in the density of circulating immune cells (hemocytes) (Bergin et al., 2003), alterations in hemolymph protein and/or gene expression (Maguire et al., 2017) and the activity of antioxidant enzymes (Büyükgüzel et al., 2013). The development of an insect based system for measuring the relative toxicity and mode of action of caffeine could contribute to reducing the need to use mammals for this type of testing and give a greater insight into its mode of action.

## 2. Materials and methods

### 2.1. *G. mellonella* larvae

*G. mellonella* larvae of the sixth developmental stage were obtained from the Meal Worm Company (Sheffield, England). Larvae were stored in wood shavings in the dark at 15 °C prior to use and only larvae weighing  $0.22 \pm 0.03$  g were used within two weeks of receipt.

### 2.2. Administration of caffeine to *G. mellonella* larvae by force feeding route

Caffeine was dissolved in PBS (NaCl 8.0 g/l; KCl 0.2 g/l; Na<sub>2</sub>HPO<sub>4</sub> 1.15 g/l; KH<sub>2</sub>PO<sub>4</sub> 0.5 g/l; pH 7.3; all chemicals were purchased from Sigma-Aldrich if not specified) and diluted to 0.1 M or 0.08 M. In order to administer compound by the feeding route, a blunted Myjector syringe was gently inserted into the mouth of larvae to force-feed 20 µl of the relevant caffeine solution (Mukherjee et al., 2013b); controls received 20 µl of PBS only.

### 2.3. Extraction of caffeine and metabolites from *G. mellonella* hemolymph

Larvae force fed 0.08 M caffeine were bled into a micro-centrifuge tube and the hemolymph was immediately centrifuged at 500 × g for 5 min at 4 °C to pellet hemocytes. Cell free hemolymph was centrifuged at 20,000 × g for 30 min. Hemolymph was diluted in extraction buffer

(CH<sub>3</sub>OH: H<sub>2</sub>O: CH<sub>3</sub>COOH ratio 9:0.9:0.1) and mixed thoroughly. The contents of the micro centrifuge tubes were centrifuged at 20,000 × g for 30 min at 4 °C and supernatant stored at 20 °C overnight and subsequently centrifuged again at 20,000 × g for 10 min at 4 °C. Samples were lyophilized until dryness. Lyophilized samples were re-suspended in apyrogenic water supplemented with trifluoroacetic acid (TFA; 0.1% v/v).

### 2.4. RP-HPLC analysis of caffeine and metabolites in hemolymph

Samples extracted from cell free hemolymph for RP-HPLC (reverse phase high-performance liquid chromatography) analysis were loaded in a 20 µl volume on a C-18 Shimadzu HPLC column with diode array detection of 273 nm. Samples were maintained at 4 °C in thermally controlled sample tray. The elution profile was 5 min of Buffer B (HPLC grade acetonitrile supplemented with 0.1% (v/v) TFA) at 5% followed by a linear gradient mobile phase with Buffer B to 100% acetonitrile for 24 min. The column was eluted fully with 100% Buffer B for 3 min and was re-equilibrated with 95% Buffer A (HPLC grade water supplemented with 0.1% (v/v) TFA), 5% Buffer B for 15 min prior to further analysis. The retention times of caffeine, theobromine and theophylline were 13.8 min, 9.5 min and 11.9 min respectively. Caffeine standard curve was constructed using concentrations from 200 to 2 µg/ml. Theophylline and theobromine standards were from 20 to 1 µg/ml.

### 2.5. Assessment of larval movement

Larvae force fed caffeine (n = 20) were placed on their dorsal surface and the time for each to return to their normal orientation was measured at 0, 1, 4, 24, 48 and 72 h. More detailed information about larval movement was gained via FIMTrack table method (Kunc et al., 2017) which used FTIR (frustrated total internal reflection) of infrared light in acrylic glass. On the boundary between glass and larva the light was reflected down and captured by camera beneath the FIMTrack table (Risse et al., 2013). Ten larvae (5 caffeine treated and 5 PBS treated as the control) were placed into square arena made from paper (28 × 28 cm), and behaviour response was recorded for 10 min. Experiment was repeated independently on two separate occasions. Images were captured via DMK 31AU03 camera (IMAGINGSOURCE) in a dark room without any additional light source except the built-in infrared light, which was generated by the FIMTrack. The size of the images was 1024 × 768 pixels. Images were captured with a frequency of 1 FPS (frame per second) for 600 s. The scale factor was 24 pixels/cm.

Images were processed by FIMTrack v2 Windows (× 86) software (downloaded from <http://fim.uni-muenster.de/>). All tracks were manually verified so that data for each track belonged to a given larval trajectory. Data gathered from software were processed and visualized in Prism 6 (USA GraphPad).

### 2.6. Analysis of effect of caffeine on pupation

Larvae force fed 0.1 M caffeine and PBS fed larvae, were placed in sterile nine cm Petri dishes lined with Whatman filter paper containing some wood shavings and incubated at 30 °C. Larval pupation was recorded every 24 h over a twelve day period. All experiments were performed on three independent occasions.

### 2.7. Label free quantitative proteomics of larval heads

Label free shotgun quantitative proteomics was performed on brains and surrounding tissue from larvae fed 0.1 M caffeine after 24 h incubation, in total five healthy larvae per treatment and controls (n = 3) were used. Protein (75 µg) was reduced with dithiothreitol (DTT; 200 mM), alkylated with iodoacetamide (IAA; 1 M) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin: protein ratio of

1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland) and 1 µg of peptide mix was eluted onto a QExactive (ThermoFisher Scientific, U.S.A.) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID), using a 120 min reverse phase gradient at a flow rate of 250 nl/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution mass spectrometer (MS) scan (300–2000 Da) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; <http://maxquant.org/>) (Cox et al., 2011) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella* (Vogel et al., 2011). The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and *N*-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. LFQ intensities were log2-transformed and ANOVA of significance and *t*-tests between the brain proteomes of control and caffeine treated larvae was performed using a *p*-value of 0.05 and significance was determined using FDR correction (Benjamini-Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least three of the four replicates in the second group (referred to as qualitatively differentially abundant proteins).

## 2.8. Statistics

Analysis of changes in the levels of caffeine and its metabolites and larval pupation are expressed as the mean ± SE and were performed by One-way ANOVA using GraphPad Prism version 5.00 for Windows 8, GraphPad Software, San Diego, California USA, ([www.graphpad.com](http://www.graphpad.com)). The assessment of larval repositioning is expressed as the mean time (seconds) ± SE. In case of FIMTrack method a Two-way ANOVA incorporated in GraphPad Prism version 6.00 for Windows 10, GraphPad Software, San Diego, California USA, ([www.graphpad.com](http://www.graphpad.com)), was used to determine statistical significance.

## 3. Results

### 3.1. Analysis of caffeine metabolism in *G. mellonella* larvae

Larvae were force fed 0.08 M caffeine as described and hemolymph was extracted at 0, 4, 24 and 48 h. Caffeine and its associated metabolites were extracted from hemolymph at each time point, samples were loaded on an Agilent C-18 column and quantified as described by RP-HPLC. The caffeine level in larvae at *t* = 0 was 150.89 ± 36.33 µg/ml but this decreased to 73.52 ± 0.34 µg/ml at 24 h (*p* < 0.01) and 75.66 ± 12.66 µg/ml at 48 h (*p* < 0.01) (Fig. 1). At *t* = 0 theobromine and theophylline were not detected (Fig. 1) but 4 h post administration of caffeine the concentration of theobromine was 4.06 ± 0.45 µg/ml and theophylline was 18.94 ± 2.17 µg/ml. The theobromine concentration increased at 24 and 48 h (*p* < 0.001) but the concentration of theophylline declined up to 48 h (*p* < 0.05).

### 3.2. Effect of caffeine on larval movement

Larvae were administered a 0.1 M caffeine by force feeding and the effect on movement was monitored as described. Larvae were placed on their dorsal surface and the time to reposition correctly was recorded (Fig. 2). Control larvae took 3 ± 0 s to reposition however larvae administered caffeine at time = 1 h took 254 ± 251 s to reposition. This increased to 319 ± 247.61 s at 4 h and to 309 ± 299 s at 24 h. The time to reposition declined at 48 h to 126 ± 243 and to 35.53 ± 136 s at 72 h. Significant increases were observed between larvae force fed PBS and larvae incubated for 4 (*p* < 0.001) and 24 h (*p* < 0.001) post-caffeine administration. Significant increases were also observed between larvae incubated for 1 h and 72 h (*p* < 0.05).

For more detailed analysis, larvae were placed on the FIMTrack table surface, images were acquired and distance travelled (Fig. 3a) and velocity (Fig. 3b) were recorded. Distance travelled and velocity of larvae force fed with caffeine were lower at all time points. Significant decreases (*p* < 0.01) in velocity and distance travelled were observed at 1, 4, 24 and 48 h after administration of caffeine compared with control larvae. The movement of larvae is represented in Fig. 3c which shows the tracks of larvae administered caffeine at the different time points.

### 3.3. Effect of caffeine on formation of pupae

The rate of pupae formation was recorded from days 0–12 in larvae administered 0.1 M caffeine by force feeding. No mortality was observed during experiments. There was a significant decrease (*p* < 0.001) in the ability of caffeine treated larvae to form pupae (Fig. 4). After 12 days control larvae showed 90% pupation while caffeine treated larvae showed 32.5% pupation. Pupation in control larvae commenced at day 4 whereas pupation commenced at day 6 in larvae force fed caffeine. In addition to a delay in the commencement of pupation, the rate of pupation was also decreased in treated larvae. Larvae that were force fed caffeine had an average pupation rate of 0.54 larvae per day over a twelve day period, however control larvae had an average pupation rate of 1.5 larvae per day over the same period.

### 3.4. Effect of caffeine on larval brain proteome

Label free quantitative proteomics was performed on the brain and surrounding tissue of larvae administered 0.1 M caffeine for 24 h (Fig. 5). In total 480 peptides were identified representing 471 proteins with two or more peptides. Seventeen proteins (7 proteins imputed and 10 proteins non-imputed) were significantly increased (*p* < 0.05) in abundance in larvae administered caffeine compared to controls (Table 1). The protein showing the highest increase in abundance in larvae exposed to caffeine was larval cuticle protein 1 at a fold increase of 2.76 (*p* < 0.05), while the proteins highest in abundance with an imputed value were immune-related Hdd1 (6.28 fold increase), AAEL003067-PA (3.11 fold increase), GG11101 (2.58 fold increase) and ras protein (1.91).

Fifteen proteins (10 proteins imputed and 5 proteins non-imputed) were significantly decreased in abundance in larvae administered caffeine compared to controls (Table 2). These proteins included uncharacterized protein Dvir\_GJ22788, isoform A (4.23 fold decrease), C-type lectin 21 precursor (2.25 fold decrease) and chemosensory protein (1.54 fold decrease), while the proteins lowest in abundance with an imputed value were proteasome beta-subunit (4.86 fold decrease), chitin deacetylase, partial (3.67 fold decrease), putative neuropeptide precursor protein precursor (3.14 fold decrease), AAEL003067-PA (3.11 fold decrease) and GG11101 (2.58 fold decrease).

## 4. Discussion

Caffeine is the most widely consumed stimulant in the world and is

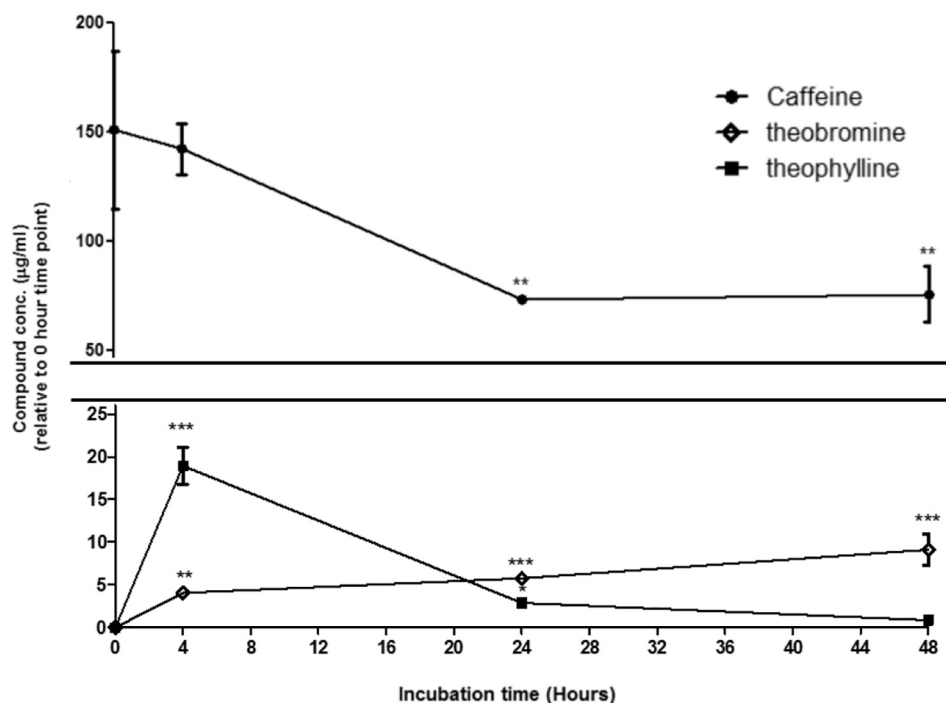


Fig. 1. Analysis of the metabolism of caffeine in *G. mellonella* larvae. The presence of caffeine, theobromine and theophylline in hemolymph were detected by RP-HPLC (\*:p < 0.05) (\*\*:p < 0.01) (\*\*\*:p < 0.001). All values are the mean ± SE, n = 15.

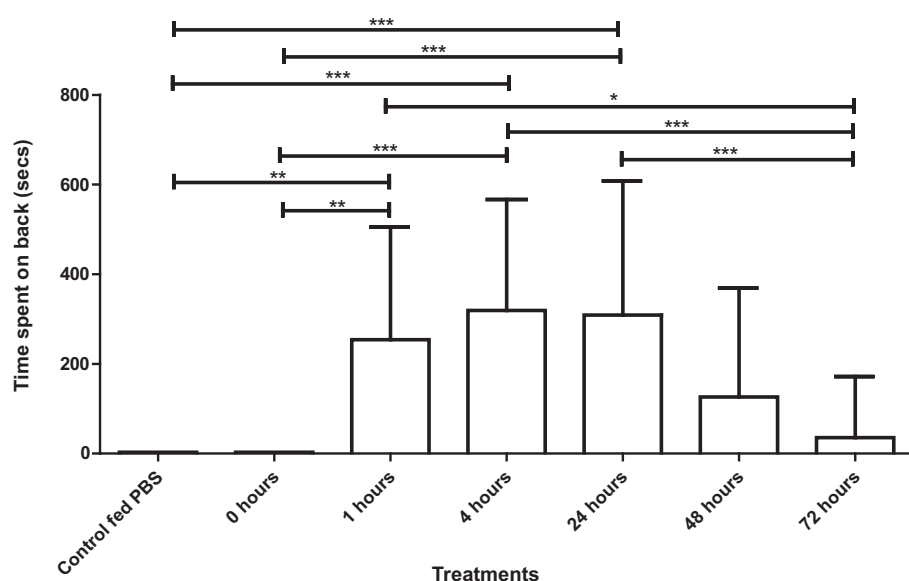


Fig. 2. Effect of caffeine on ability of *G. mellonella* larvae to move from the ventral to dorsal surface. Larvae were positioned on their ventral surface after administration of caffeine and the time to reposition correctly was recorded (\*:p < 0.05) (\*\*\*:p < 0.001). All values are the mean ± SE, n = 20.

ingested in a variety of beverages and food stuffs. It has a variety of physiological effects in humans including increasing alertness and performance, and reducing fatigue (Smith, 2002). Various histological, anatomical and physiological functions in insects share similarities with the mammalian gastrointestinal tract and microbes found in the midgut of *G. mellonella* larvae resemble those found in the intestinal microvilli of mammals (Mukherjee et al., 2013b). The fat body of insects functions in xenobiotic metabolism in a similar way to the liver of mammals and contains a number of cytochrome P450 and sulfo-, glutathione- or glucose- conjugation enzymes which are involved in drug metabolism (Büyükgüzel et al., 2013). As a result of these similarities insects have been employed to measure the acute and chronic toxicity of a range of food additives (Maguire et al., 2016; Sarikaya and Çakir, 2005). For example, *Drosophila* treated with metyrapone, an inhibitor of cytochrome P450s enzyme, showed dramatically decreased caffeine metabolism suggesting the highly conserved enzymes cytochrome P450s are

involved in the metabolism of caffeine in both mammals and insects (Coelho et al., 2015). The results presented here indicate that caffeine is metabolised in *G. mellonella* larvae in a similar manner to that in mammals and that the caffeine metabolites theobromine and theophylline were detected four hours post administration of caffeine.

Larvae incubated for 4 h following administration of caffeine showed a significant reduction in their ability to move and reposition correctly, FIMTrack software also revealed a significant decrease (p < 0.001) in distance travelled and velocity of larvae force fed caffeine following 4 h incubation compared to control larvae. This inhibition of movement corresponded to a peak in theophylline concentration of 18.94 µg/ml. The ability to move and reposition correctly gradually recovered by 48 h corresponding to a theophylline concentration of 0.87 µg/ml. Theophylline is a muscle relaxant in mammals and in human airways the EC<sub>50</sub> for airway smooth muscle relaxation by theophylline is approximately 1.5 × 10<sup>-4</sup> M (Barnes,

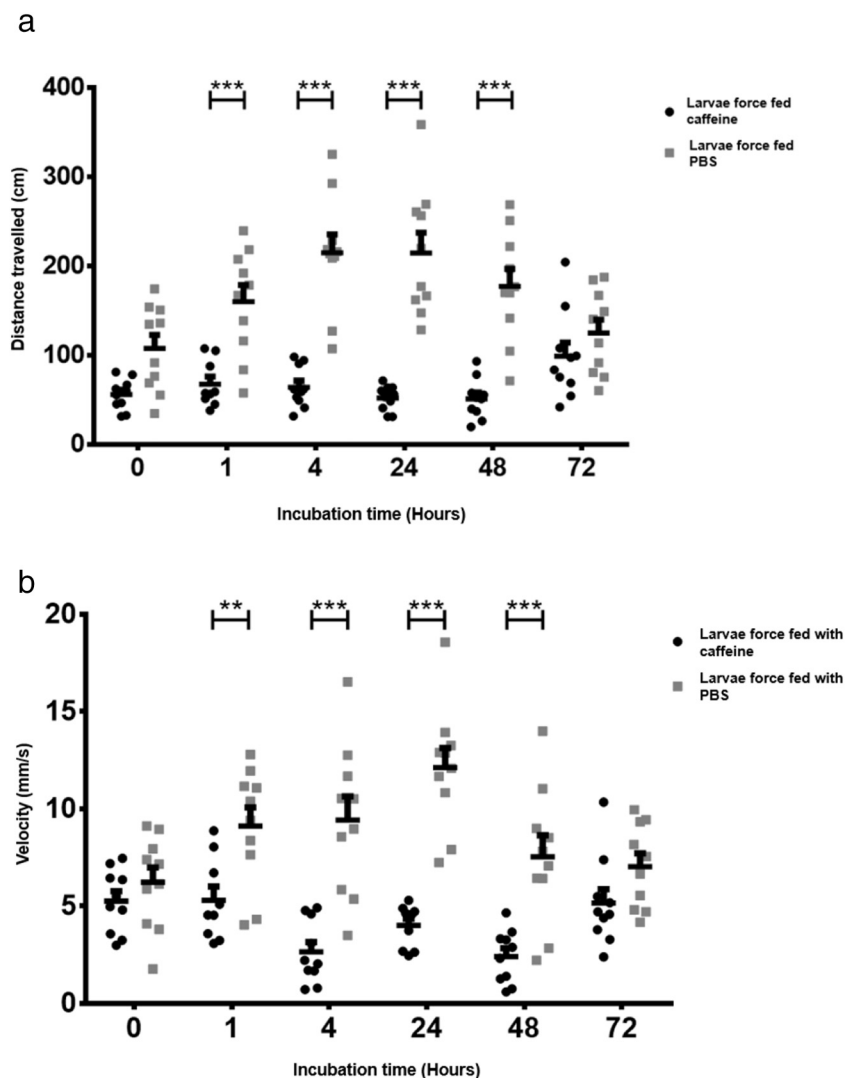


Fig. 3. Effect of caffeine on movement distance (a) and velocity (b) of *G. mellonella* larvae. Each dot represents individual larva and the line is mean  $\pm$  SE, n = 10 (\*\*:p < 0.01) (\*\*\*:p < 0.001). (c) Examples of individual tracks of *G. mellonella* larvae in different time points - five caffeine force fed larvae (yellow) and five control larvae (red) are presented on each image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2010). The presence of theophylline in the hemolymph of larvae force fed caffeine after 4 h incubation may indicate that it is acting as a muscle relaxant in larvae. Previous work on Zebra fish embryos has demonstrated administration of caffeine also reduced mobility (Chen et al., 2008).

Both the rate of pupation and commencement of pupation were reduced in larvae force fed caffeine compared with control larvae. Zebra fish embryos administered caffeine displayed developmental alterations including reduced body length, reduced tactile responses and muscle fibre formation (Chen et al., 2008). Developmental changes are also seen in the human foetus where caffeine doses of  $\geq 300$  mg per day during pregnancy were associated with reductions in infant birth weight that may be especially detrimental to premature or low-birth-weight infants (Hinds et al., 1996).

Proteomic analysis revealed the increased abundance of troponin T transcript variant B, partial (1.55 fold) (p < 0.05) in larvae challenged with caffeine. Elevated serum troponin is frequently observed after traumatic brain injury in patients (Salim et al., 2008). The increase in abundance of troponin T transcript variant B, partial in *G. mellonella* larvae would suggest that caffeine is having an adverse effect on the brain. A number of immune related proteins were increased in abundance in larvae administered caffeine including immune-related Hdd1 (6.28 fold) and hemolin (1.68 fold). The brain's immune system, which consists mainly of astrocytes, microglia and infiltrating immune cells is activated in response to pathophysiological events such as ischemia,

trauma, inflammation and infection (Haskó et al., 2005). Ischemia, head injury, seizure activity and inflammation induce rapid increases in extracellular adenosine concentrations to 30–100-times that of the resting concentration (Von Lubitz, 1999). Adenosine interacts with specific G-protein-coupled receptors on astrocytes, microglia and infiltrating immune cells to regulate the function of the immune system in the brain (Haskó et al., 2005). This suggests that caffeine is inducing brain injury in the larvae and thereby leading to increased abundance of troponin and various immune related proteins.

H<sup>+</sup> transporting ATP synthase O subunit isoform 1 (1.87 fold) and H<sup>+</sup> transporting ATP synthase delta subunit (1.53 fold) were also increased in abundance in larvae that had received caffeine. Blayney et al. (1978) studied the effects of caffeine on calcium transport by sub-cellular organelles isolated from rabbit myocardium and reported that caffeine increased myofibrillar basic and calcium-activated ATPase activity.

In *G. mellonella* larvae treated with caffeine there was a decrease in the abundance of proteins associated with cellular protein degradation (e.g. proteasome beta-subunit (4.86 fold), effete, isoform A (3.21)). The reduced formation of pupae in caffeine administered larvae may be explained by the decrease of SUMO-activating enzyme subunit 1 (3.08 fold). Smt3 is the only homologue to SUMO in *Drosophila melanogaster* and plays a role in the regulation of ecdysteroid levels during post-embryonic development (Talamillo et al., 2008). The SUMO homologue causes an ecdysteroid peak in the prothoracic glands which is required



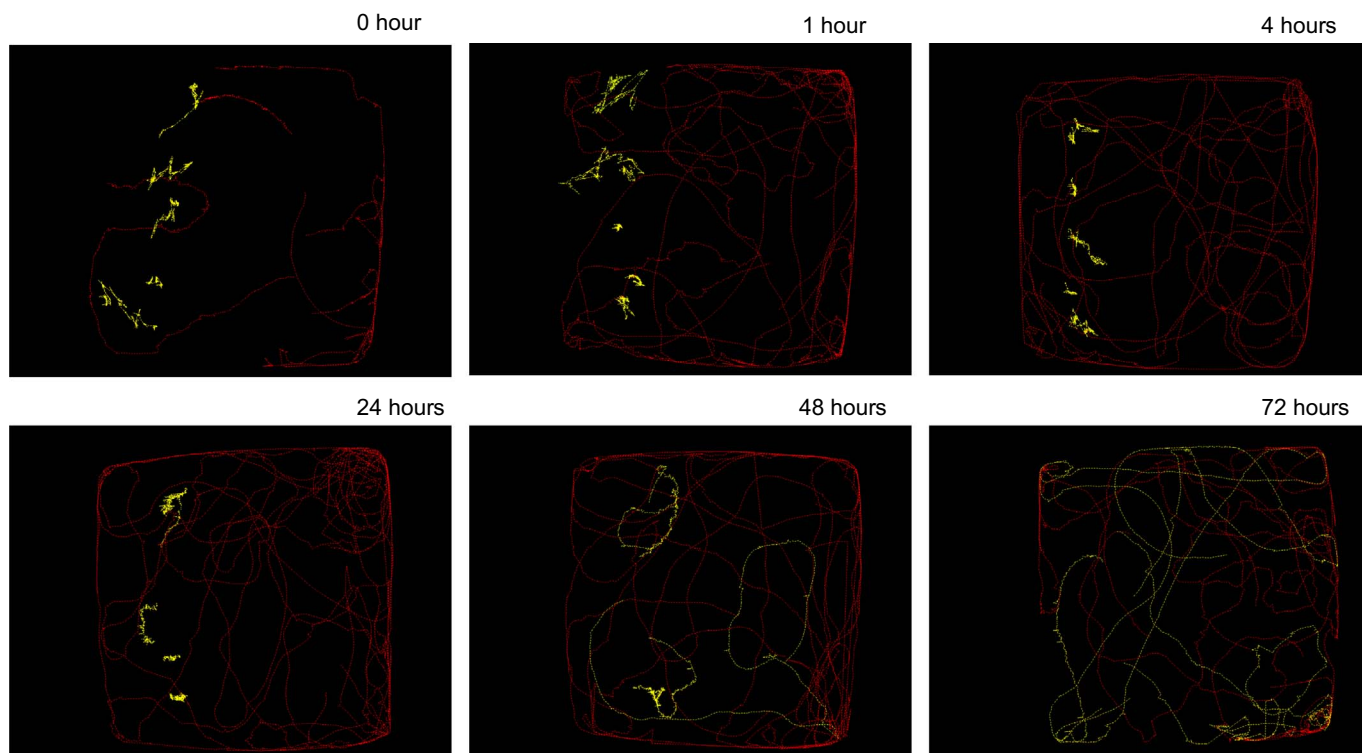


Fig. 3. (continued)

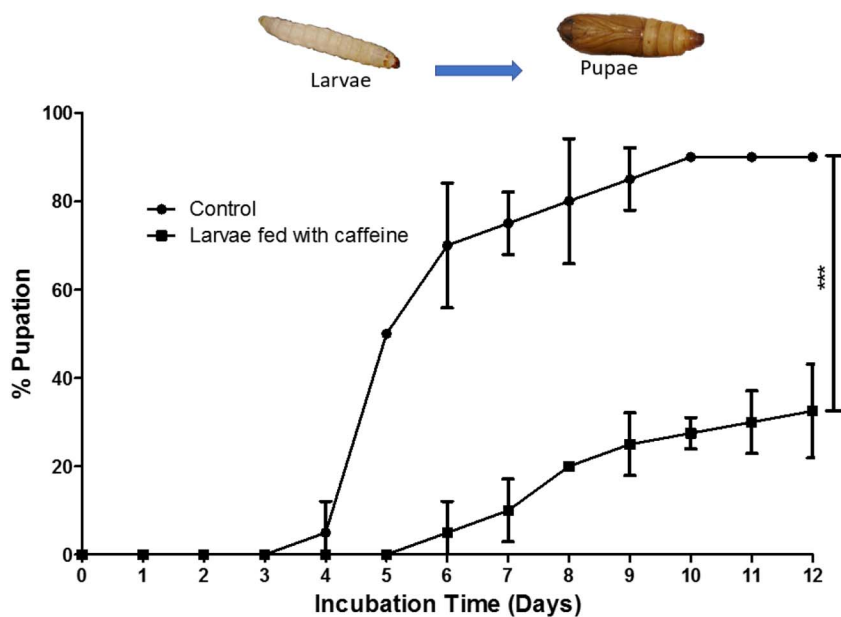


Fig. 4. Caffeine administration inhibits pupation in *G. mellonella* larvae. The formation of pupae was recorded from days 0–12 (\*\*\*:p < 0.001). All values are the mean ± SE, n = 20.

for the larval to pupal transition (Talamillo et al., 2013). Proteomic analysis also revealed decreases in cuticle proteins such as chitin deacetylase, partial (3.67 fold decrease). Chitin deacetylases are mainly expressed in the integument and play critical roles in molting, cuticle degradation, and new cuticle formation (Han et al., 2015).

The results presented here indicate that caffeine is metabolised in *G. mellonella* larvae producing theobromine and theophylline. Caffeine administration results in reduced larval movement possibly due to the presence of the muscle relaxant theophylline. Proteomic analysis revealed decreased abundance of a range of proteins associated with development including SUMO-activating enzyme subunit 1 and chitin deacetylase, partial which may play a role in reducing pupation. *G.*

*mellonella* larvae are now widely used as an *in vivo* model for assessing the virulence of microbial pathogens and for determining the activity of antimicrobial drugs. Their use has recently been extended as a model for assessing the toxicity of food preservatives (Maguire et al., 2016; Maguire et al., 2017) and for studying neural pathologies associated with *Listeria* infection (Mukherjee et al., 2013a). The results presented here show a strong correlation with the effect of caffeine on Zebra fish embryos and offer the possibility of utilising larvae as a model to study the *in vivo* activity of caffeine and related neuroactive compounds.

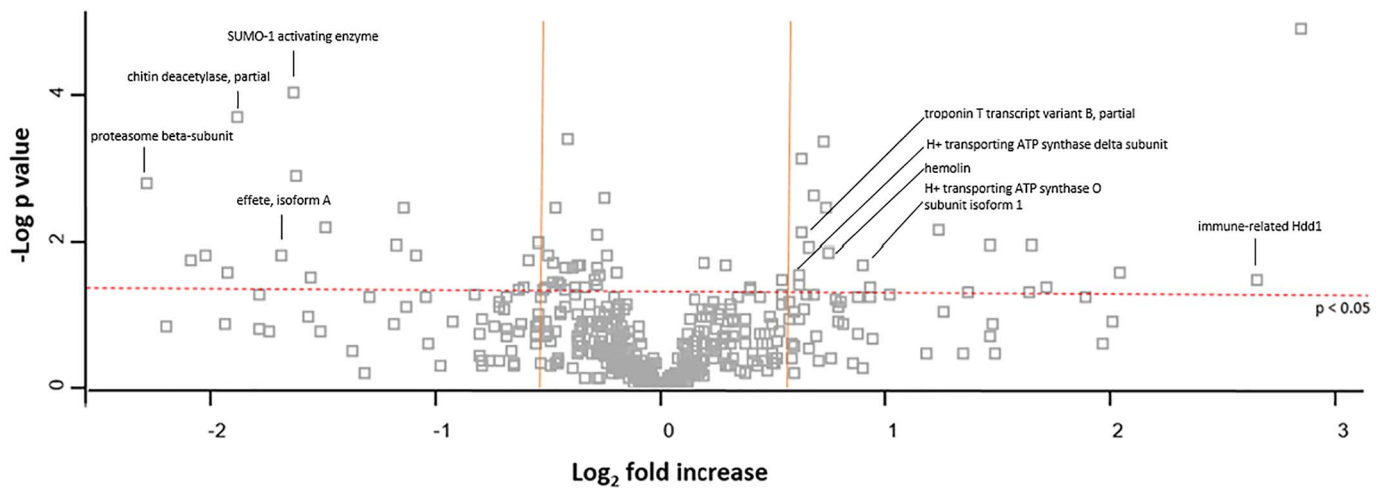


Fig. 5. Volcano plot showing proteins altered in abundance in *G. mellonella* larvae treated with caffeine. Proteins above the line are statistically significant ( $p < 0.05$ ) and those to the right and left of the vertical lines indicate fold changes  $> 1.5$  fold positive and 1.5 fold negative in the caffeine treated larvae and control larvae.

Table 1

Relative fold changes of proteins increased in abundance in *G. mellonella* larvae administered caffeine and the number of matched peptides, sequence coverage, PEP and overall intensity.

Protein Name (* = imputed protein)	Peptides	Sequence coverage %	PEP	Mean LFQ intensity	Fold difference
*PREDICTED: adenyl cyclase-associated protein 1 isoform $\times 2$ [ <i>Tribolium castaneum</i> ]	2	8.9	1.71E – 13	2.42E + 09	7.19
*Immune-related Hdd1 [ <i>Hyphantria cunea</i> ]	10	15.7	2.45E – 140	7.12E + 09	6.28
*PREDICTED: protein CREG1 [ <i>Tribolium castaneum</i> ]	4	20.6	2.99E – 59	1.83E + 09	4.11
*AAEL003067-PA [ <i>Aedes aegypti</i> ]	5	16.2	4.81E – 71	7.58E + 08	3.11
RecName: Full = Larval cuticle protein 1; Flags: Precursor	4	19	1.38E – 108	4.87E + 09	2.76
*GG11101 [ <i>Drosophila erecta</i> ]	6	20.3	2.22E – 34	7.91E + 08	2.58
Myofilin variant A [ <i>Bombyx mandarina</i> ]	6	24.3	1.09E – 59	8.96E + 09	2.35
*Ras protein [ <i>Bombyx mori</i> ]	3	4.3	1.20E – 17	1.40E + 09	1.91
H + transporting ATP synthase O subunit isoform 1 [ <i>Bombyx mori</i> ]	12	42.7	4.65E – 99	9.55E + 09	1.87
Hemolin [ <i>Heliothis virescens</i> ]	3	47.9	1.55E – 83	8.13E + 09	1.68
*Hypothetical protein, partial [ <i>Bombyx mori</i> ]	2	16.7	2.42E – 84	1.07E + 09	1.66
AGAP008115-PA [ <i>Anopheles gambiae</i> str. PEST]	4	11.7	5.25E – 145	8.39E + 09	1.65
Bifunctional purine biosynthesis protein, putative [ <i>Pediculus humanus corporis</i> ]	10	27.4	1.70E – 77	3.93E + 09	1.61
Troponin T transcript variant B, partial [ <i>Bombyx mandarina</i> ]	6	25.6	0	3.96E + 10	1.55
Heat shock 70 kD protein cognate precursor [ <i>Bombyx mori</i> ]	3	76.2	1.52E – 69	6.44E + 09	1.54
Heat shock cognate 70 protein [ <i>Spodoptera frugiperda</i> ]	4	14.8	1.89E – 13	4.72E + 09	1.54
H + transporting ATP synthase delta subunit [ <i>Bombyx mori</i> ]	7	41.8	7.98E – 106	9.07E + 09	1.53

Table 2

Relative fold change of proteins decreased in abundance in *G. mellonella* larvae administered caffeine and the number of matched peptides, sequence coverage, PEP and overall intensity.

Protein name (* = imputed protein)	Peptides	Sequence coverage %	PEP	Mean LFQ intensity	Fold difference
*Proteasome beta-subunit [ <i>Bombyx mori</i> ]	8	41.7	3.23E – 50	2.24E + 09	– 4.86
uncharacterized protein Dvir_GJ22788, isoform A [ <i>Drosophila virilis</i> ]	13	28.6	6.71E – 182	1.18E + 10	– 4.23
*PREDICTED: probable isocitrate dehydrogenase [NAD] subunit beta, mitochondrial [ <i>Tribolium castaneum</i> ]	2	14.9	1.66E – 45	1.61E + 09	– 3.78
*Chitin deacetylase, partial [ <i>Helicoverpa armigera</i> ]	5	15	3.93E – 37	9.53E + 08	– 3.67
*Effete, isoform A [ <i>Drosophila melanogaster</i> ]	4	10.5	2.65E – 31	1.71E + 09	– 3.21
*SUMO-1 activating enzyme [ <i>Bombyx mori</i> ]	8	32.4	9.40E – 72	1.82E + 09	– 3.08
*Chitin binding protein [ <i>Papilio xuthus</i> ]	6	9.1	1.64E – 37	1.39E + 09	– 3.08
*PREDICTED: similar to multiple inositol polyphosphate phosphatase [ <i>Nasonia vitripennis</i> ]	6	32.1	5.68E – 81	1.19E + 09	– 2.93
*Acyl-coenzyme A dehydrogenase [ <i>Bombyx mori</i> ]	5	7.9	5.19E – 21	1.06E + 09	– 2.80
C-type lectin 21 precursor [ <i>Bombyx mori</i> ]	9	38.1	1.89E – 73	5.00E + 09	– 2.25
*PREDICTED: protein BCCIP homologue [ <i>Nasonia vitripennis</i> ]	3	15.7	1.90E – 18	7.25E + 08	– 2.20
*Similar to CG3590-PA [ <i>Papilio xuthus</i> ]	8	25.4	1.54E – 73	1.52E + 09	– 2.12
Chemosensory protein [ <i>Papilio xuthus</i> ]	6	16.7	1.87E – 206	5.50E + 10	– 1.54
Unknown [ <i>Picea sitchensis</i> ]	10	36.8	3.52E – 178	2.34E + 10	– 1.52
RecName: Full = Inducible serine protease inhibitor 2; Short = ISPI-2	3	9.8	2.87E – 87	3.17E + 10	– 1.50

## Transparency document

The Transparency document associated with this article can be found, in online version.

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