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Proteomic analysis of hemolymph during immune response of *Drosophila melanogaster* larvae by UPLC-MS.

Bachelor's Thesis

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ANNOTATION

The aim of this study was the identification of proteins in larval hemolymph of *Drosophila melanogaster* which are allocated to immune response after parasitoid egg infection of *Leptopilina boulardi*. The proteins were determined by means of UPLC-MS.

DECLARATION

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Barbara Jetzinger

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ABSTRACT

Diverse mechanisms did evolve to defend the organism during immune challenge. Several of them are evolutionary preserved and therefore found in various species. *Drosophila melanogaster* is a preferred model organism to study immunity due to the good practical handling and short generation times. During this study, the proteome in hemolymph of immune challenged *Drosophila melanogaster* larvae was examined qualitatively and quantitatively by UPLC-MS. The immune response was induced by infection of the parasitoid *Leptopilina boulardi*. The proteome was examined by comparison to the proteome of uninfected larval hemolymph. 19 different *Drosophila* proteins have been found to be up- or downregulated during wasp infection. Turandot A and C, CG18607, and IMPP are examples for proteins known to be involved in immune and stress-related responses. Other proteins found are related to metabolism (e.g. Ecdysone-inducible gene L2, FASN1) revealing further aspects of immune challenge. Proteins with unknown function were also identified showing that this procedure could be used to identify new proteins important during immune challenge. Some proteins which have been related to wasp parasitoidism in previous studies could not be identified. This suggests very specific immune responses in the hosts as well as limitations in the analysis. Restrictions in the method were caused by inoperative standards and the small number of biological replicates.

LIST OF ABBREVIATIONS

AcN	Acetonitrile
All-pep	relative quantification using all peptides
AMP	Antimicrobial peptide
ATP	Adenosine triphosphate
DIM	*Drosophila* immune-induced molecule
FA	Formic acid
FOXO	Forkhead box class O
Hi-N	relative quantification using high-N
HSP	Heat-shock-protein
Imd	Immune deficiency pathway
ImpL2	Ecdysone inducible gene L2
INPP4 (A, B)	Inositol polyphosphate 4-phosphatase (A, B)
JAK-STAT	Janus Kinase – Signal Transducer and Activator of Transcription
JNK	Jun N-terminal kinase pathway
LPS	Lipopolysaccharide
PBS	Phosphate buffered saline
PI	Phosphoinositide
PO	Phenol oxidase
PPO (1, 2, 3)	Prophenol oxidase (1, 2, 3)
RNI	Reactive intermediate of nitrogen
ROI	Reactive intermediate of oxygen
ROS	Reactive oxygen species
Serpin	Serine protease inhibitor
STAGE	Stop and go extraction
TAK1	Transforming growth factor β-activated kinase-1
TEP (I, II)	Thioester-containing protein (I, II)
TNF	Tumor necrosis factor
Tot (A, C)	Turandot (A, C)
UPLC	Ultra Performance Liquid Chromatography
VLP	Virus like particle

TABLE OF CONTENT

1. INTRODUCTION

The species *Drosophila melanogaster* is one of the most important model organisms in biological research. This development is due to many mechanistic similarities in insects and mammals. A genomic comparison from 2001 showed that for 77% of the 929 studied human disease genes corresponding sequences in *Drosophila* could be found (Reiter, Potocki, Chien, Gribskov & Bier, 2001). This and further benefits like good practical handling and short generation times led to enormous activities in research and therefore to the elaboration of a broad spectrum of examination methods (Hultmark, 2003). The progress of modern techniques allows to get access to the enormous proteomic information of organisms. The detection of very small amounts of proteins is possible, the corresponding databases for identification grow every day. Consequently, a further aspect is the knowledge about the purpose of the detected proteins, this also includes mechanisms and pathways in immune response. This study is an attempt to contribute to that research field. In this project, the proteome of *Drosophila melanogaster* during immune response induced by the parasitoid *Leptopilina boulardi* was examined.

1.1. The organisms *Drosophila melanogaster* and *Leptopilina boulardi*

Drosophila melanogaster is a holometabolic insect and therefore undergoes different developmental stages (Kück et al., 2005). Three days after pairing, the female fruit fly lays eggs at the breeding ground. Three larval instars are followed by a pupa state. The last stage is reached when about 10 days after oviposition adult flies eclose from the pupae. After about 4 hours, the flies are able to mate (Greenspan, 1997).

During this study, an immune response was induced in second and third instar larvae. This was triggered by the parasitoid wasp *Leptopilina boulardi*. Parasitoidy is a form of parasitism, the special characteristic is the ultimately death of the host caused by the demanding parasitoid. The parasitoidy sets in, when the wasp infects a *Drosophila* larva at the stage of second to early third instar (Lee et al., 2009; Lemaitre & Hoffmann, 2007). The female wasp injects one or more eggs into the host body cavity. In the beginning, eggs float freely in the hemocoel until they are bounded by fatty tissues or enclosed at the ends of the larval body. A wasp embryo undergoes 5 larval stages, followed by a pupal ecdysis, meconium formation to finally reach the state of a pharate adult. The duration from time of oviposition till rise of an adult is around 16 days (Kopelman & Chabora, 1986).

1.2. Host defense in *Drosophila melanogaster*

In pathogen defense of vertebrates, the immune system consists of two parts. Innate immunity describes an unadaptable system gained through inheritance, in this, recognition of extraneous molecules is very specific and limited. The second part is the adaptive immune system, it is the system element which is expandable, that means the host's ability to adapt to the invasion and develop specific receptors in response. Whilst invertebrates do not possess parts similarly to the vertebrate's adaptive immune system, the innate immune system is the basis in defense of invertebrates.

Key molecules in *Drosophila* immune response are antimicrobial peptides (AMPs), reactive intermediates of oxygen (ROIs) and nitrogen (RNIs), and stress-related proteins. These substances are crucial parts in mechanisms of different signaling cascades or are the resulting products of these pathways. Also, differentiation and extensive production of hemocytes is induced, which are needed for phagocytosis, encapsulation, and melanization processes (Nappi & Ottaviani, 2000). A further aspect of the immune response is the energy mobilization towards immune active cells (Bajgar et al., 2015). Various proteins involved in these mechanisms are upregulated during wasp immune challenge (Schlenke, Morales, Govind, & Clark, 2007a). In the following, the different parts of the *Drosophila* immune response are described.

1.2.1. Epithelial immunity

Epithelial immunity serves as first measure in fight against pathogens. The first barrier is a hard cuticle on the outside containing chitin and barriers between the cells, like adherens junctions and septate junctions. (Lemaitre & Hoffmann, 2007; Bier & Guichard, 2012).

1.2.2. Systemic immune response

1.2.2.1. AMPs and DIMs

Antimicrobial peptides (AMPs) are part of the systemic immune response produced in the insect fat body. These immune peptides are released into the hemolymph mainly in response to microbial and fungal attacks. Important examples are drosocin, cepropin, attacin, defensin, drosomycin, and metchnikowin (Lemaitre & Hoffmann, 2007).

Different proteins can be summed up as Drosophila immune-induced molecules (DIMs). About 30 DIMs are identified, some of them correspond to AMPs, like DIM 9 and 11 which

are derivatives of drosocin. Others are not connected to AMPs, a gene encoded at Turandot A (TotA), is the DIM 31 (AAK64523). The upregulation of DIMs is initiated when the health risk increases, therefore they belong to the group of stress-related proteins. Studies showed that an unhospitable environment like keeping at high temperatures, UV-irradiation, but also bacterial infection can trigger expression of these proteins. Known representatives of this group are also the heat-shock-proteins (HSPs) and other members of the Turandot (Tot) family. The release mechanisms and functions of stress-related proteins are not fully studied (Ekengren & Hultmark, 2001; Levy et al., 2004; Uttenweiler-Joseph et al., 1998).

1.2.2.2. ROIs and RNIs

Reactive intermediates of oxygen (ROIs) and nitrogen (RNIs) have use as cytotoxic molecules as well as mediators of AMP production. They are produced by phagocytic cells and during melanotic encapsulation processes (Nappi & Ottaviani, 2000). The production of reactive intermediates is, amongst others, dependent on nitric oxide (Nappi, Poirié, & Carton, 2009).

1.2.3. Cellular response

Phagocytosis, encapsulation, and melanization are important mechanisms on cellular stage, with the task of immobilization and killing of pathogens. Melanotic encapsulation is a key mechanism in defense against *L. boulardi*. It can be recognized as dark discoloration of the targeted tissue due to the synthesis of brown or black melanin. Essential for the defense reactions are the hemocytes. The *Drosophila* hemolymph contains thousands of these blood cells, which are produced in the embryonic cells, later in the lymph gland. Three types of hemocytes are known to play role in immune defense: plasmatocytes, crystal cells, and lamellocytes.

Plasmatocytes make up the majority of the mature larval hemocytes and monitor permanently the immune status. They act in phagocytosis of dead cells and invading microorganisms.

Crystal cells make up about 5% of the hemocytes in larvae. They produce the enzyme precursors PPO1 and PPO2, which are important for mediation of the melanization processes. The release of the precursors is caused by lysis of the crystal cells.

The occurrence of lamellocytes in larvae is minimal under normal conditions, differentiation is initiated as immune response and controlled by the lymph gland (Carton, Poirié, & Nappi, 2008). They mainly serve in encapsulation and neutralization of bigger objects which can’t be phagocytosed. The lamellocytes form a multilayer at the targeted object. Lamellocytes are also the production location of PPO3, a proenzyme needed in pathogen melanization.

Subsequently, the pathogen is killed, possible cytotoxic molecules are reactive oxygen species (ROS) or intermediates of the melanin production (Lemaitre & Hoffmann, 2007; Tang, 2009).

1.2.4. Adaptations in energy management

For enabling an effective immune defense, it is necessary to quickly provide energy where immune response takes place. The energy mobilization for the demanding defense mechanisms is done at the expense of other basic processes like growth, development, and storage. Under normal conditions, the main mechanism for ATP production is oxidative phosphorylation. Activation of immune cells leads to a switch to aerobic glycolysis which is less effective in terms of ATP generation per glucose molecule, but it is much faster in contrast. Overall this leads to a higher production of ATP in these cells. This metabolic change is known as the 'Warburg effect'. The shift in energy supply is caused by extracellular adenosine excreted from the immune cells (Bajgar et al, 2015; Cheng, Joosten, & Netea, 2014).
Also, other factors are relevant in initiation of metabolic changes. The ecdysone-inducible gene L2 (ImpL2) and the FOXO transcription factor are mediators of shifts in the energy management due to their interactions with insulin signaling (Mattila, Bremer, Ahonen, Kostiainen, & Puig, 2009; Kwon et al., 2015). Also, some signaling pathways (see 1.2.5 Recognition and signaling pathways) are related to metabolic regulations during immune challenge: The Toll pathway is associated to insulin downregulation, the Imd cascade is involved in control of metabolic gene expression in response to bacterial infection (Shokal, Kopydlowski, Harsh, & Eleftherianos, 2018).

1.2.5. Recognition and signaling pathways

1.2.5.1. Recognition molecules

Proinflammatory cytokines, endogenous pattern recognition receptors like PGN recognition proteins, members of the Nimrod family or the Dscam molecule are needed to start the immune signaling cascades (Vlisidou & Wood, 2015). The members of the thioester-containing protein (TEP) family are possible molecules for opsonization (Lemaitre & Hoffmann, 2007). A study suggests that Drosophila lectins serve as pathogen markers and lead the hemocytes to the targeted spots as they coat the surface of foreign tissues (Ao, Ling, & Yu, 2007).

1.2.5.2. Immune signaling pathways

When the host immune system recognizes damaged tissues or exogenous molecules, different signaling cascades are induced dependent on the origin of immune response. These cascades are evolutionary preserved and therefore part of the innate immune system.

1.2.5.2.1. Rel/NF-κB group pathways

The proteins Relish, Dorsal and Dif belong to different classes of the Rel/NF-κB group, the transcription factors of Toll and Imd signaling. The Toll pathway has tasks in Drosophila development, but also in humoral and cellular immune responses. Activation of the Toll pathway results in production of AMPs, further it is contributing to the regulation of hemocyte development. It has been shown, that lamellocyte differentiation can be provoked by mutations of Cactus, an inhibitory κB protein, or constantly present Dorsal, a protein in Toll pathway. The Imd pathway regulates the production of most of the Drosophila AMPs. Due to similarities it is often related to human TNFR and TLR signaling pathways (Myllymäki, Valanne, & Rämet, 2014; Valanne, Wang, & Rämet, 2011).

1.2.5.2.2. JAK-STAT pathway

The JAK-STAT pathway is functioning in preservation of prohemocytes in the lymph gland. In case of a wasp infection, the JAK-STAT signaling is stopped in lymph glands, which leads to differentiation of lamellocytes from the precursor prohemocytes. Furthermore, the pathway is induced in the fat body as immune response. In Drosophila, the initial cascade impulse is given by binding of the cytokines unpaired (Upd, Upd2, and Upd3) to the extracellular domain of the receptor domeless/dome. A protein of the JAK family (Hopscotch/Hop) binds to the receptor on the inside of the cell mediating alterations of itself and of the receptor, which enables the formation of a STAT docking site. This induces the dimerization of STATs (Stat92E/Marelle). After the STAT dimer transport to the nucleus, DNA binding starts transcription of the targeted genetic information (Morin-Poulard, Vincent, & Crozatier, 2013; Aaronson & Horvath, 2002).

1.2.5.2.3. MAPK group pathways

The JNK and P38 pathways are members of the MAPK group. The JNK signaling cascade can be activated by infection, lipopolysaccharides (LPS), but also by inflammatory cytokines like tumor necrosis factor (TNF). Eiger is a TNF superfamily ligand which can initiate the JNK

pathway. The lysis of the crystal cells is mediated by the JNK pathway and Eiger (Bidla, Dushay, & Theopold, 2007; Igaki, et al., 2002; Sluss et al., 1996). The p38 signaling is shown to be involved in proliferation and differentiation of immune cells (Nebreda & Porras, 2000).

1.2.5.2.4. PO pathway

The key cascade for production of melanin is the phenol oxidase (PO) pathway. PO is catalytically enhancing oxidation of monophenols to diphenols and quinones. The sources of PO are mainly hemocytes but also other tissues like the black cells in epithelia. Regulation of this pathway is achieved by serine protease inhibitors (Serpins) (Tang, 2009).

1.2.5.2.5. FOXO transcription factor

The Forkhead box class O (FOXO) transcription factor mediates stress-induced responses as well as metabolic and ageing processes. FOXO can initiate AMP production independently from immune system activation in contrast to the previously mentioned pathways. Its activation is triggered by energy deficiencies and stress. Also, it is tightly interrelated with the insulin signaling pathway suggesting a role in the metabolic switch during immune response (Becker et al., 2010; Mattila et al., 2009). Interestingly, FOXO is also described as a longevity gene, which is connected to the interaction with insulin signaling (Hwangbo, Gersham, Tu, Palmer, & Tatar, 2004).

The pathways themselves are not isolated systems but interfere with each other. A study from Kim et al. (2007) shows that AP-1 and STAT, important molecules in JNK and JAK-STAT pathways, suppress NF-κB induced transcription. Also, the transforming growth factor β-activated kinase-1 (TAK1) is important in the Imd pathway and in the JNK signaling cascade (Silverman et al., 2003).

1.3. Survival strategies of *Leptopilina boulardi*

As the parasitoid wants to ensure the survival of the offspring, different supporting strategies have evolved. Some of these mechanisms are studied. A very basic method of *L. boulardi* is shielding the egg's surface from hemocytes by adhesion to host tissues. Furthermore, Virus like particles (VLPs) are secreted to the Drosophila hemocoel during oviposition. These particles are very specific for each *Leptopilina* strain regarding shape and function. Although a resulting apoptosis of hemocytes is studied in other strains, effects of *L. boulardi* VLPs are

not known. The protein LbGAP is injected into the host hemocoel, there it penetrates the lamellocytes and distracts their encapsulation ability. Another mechanism to weaken the *Drosophila* immune system is to attack the PO cascade with the serpin SPNy (Poirié, Carton, & Dubuffet, 2009).

2. AIMS

- Protein identification and quantification in *D. melanogaster* larval hemolymph by comparison to known D. melanogaster and *L. boulardi* proteomes. Hemolymph analysis of infected and uninfected larvae.
- Determination of significant deviations in concentration of proteins.

3. MATERIALS AND METHODS

3.1. Culture and infection

The strain w^{1118}, which carries a mutation inducing a phenotype with white eyes, was used in the experiments. Female and male flies were kept together in embryo collection cages on agar plates for two days. They were fed on cornmeal medium. The agar plates were exchanged for two hours to collect eggs laid in this time range. The eggs were kept at room temperature (22°C). After one day, the developmental stage changed from egg to larva. 72 hours after oviposition, about 100 larvae were collected from each plate and transferred to fresh agar plates. These were connected to embryo collection cages containing the female, fertilized wasps in an equal amount. The wasps and larvae were separated after 10 to 20 minutes. During this time, the wasps injected their eggs into the drosophila larvae. The grade of infection was determined by examination of about 10 larvae under the microscope. Ideally, an infection between 4 and 10 eggs/larvae was achieved. For the control group, the same methods were applied except for the infection step.

3.2. Hemolymph collection

24 hours after wasp infection, the larvae were collected and washed twice with PBS buffer (1x). The larvae were gathered on a cooled slide covered with paraffin foil. Then, the hemolymph was released by laceration of the larvae using two forceps and collected with a pipette. The samples were centrifuged (5.000RPM for 5min). The hemocytes did gather in the in the pellet whereas the hemolymph remained in the supernatant. The hemolymph was further used in the experiments. The samples were denatured using heat (75°C for 15min) and stored at -20°C until analysis or used immediately for analysis.

3.3. Digestion

After thawing, the samples were further processed by spinning in the centrifuge (2.000RPM for 1min), and sediment and supernatant were separated. The supernatant was diluted 1:10 with 100mM ammonia bicarbonate. As standards, enolase and alcohol dehydrogenase (origin from yeast) were admixed in a concentration of 50fmol/µL. The solutions were digested with proteomic grade trypsin in a concentration of 100ng/µL for 12 hours at 37°C. 1% FA-solution was added to lower the pH, as a result the trypsin digestion was stopped. Both sample parts were spun (10.000RPM for 1min), the supernatant solutions were used for analysis.

3.4. Peptide isolation and desalting

Extraction tips were used in order to concentrate the sample and to ease the removal of salts. For this, Stop and Go Extraction (STAGE) tips were used. Details regarding this method are described by Rabbsilber, Ishihama and Mann (2003). Empore ™ C18 reversed-phase extraction disks from 3M Company (MN, USA) were used as matrix material. The matrix of the tip was conditioned using a mixture of 80% AcN and 1% FA followed by centrifuge step (2.000RPM for 1min). AcN was used to create a receptive matrix, FA lowered the hydrophobicity of the solution to keep the disk as the place of highest hydrophobicity. This conditioning was done twice. The tip was equilibrated with 1% FA-solution and spun (2.000RPM for 1min), the step was redone a second time. The sample solutions were pipetted into the prepared extraction tips, then the tips were centrifuged (2.000RPM for 2min). 50µL of 1% FA was added for removal of ions followed by a spin (2.000RPM for 2min). This washing step was done three times. To extract the peptides from the disks, the tips were washed with a mixture of 80% AcN and 1% FA and spun (2.000RPM for 1 min), a rerun was done. The collected solutions were inserted in the vacuum evaporator for 30min to remove the solvents. The samples were resuspended in 20µL of a 3% AcN and 0.1% FA solution. 1µL of the sample solution was injected in the analysis device for measurement.

3.5. Analysis

A reversed-phase Ultra Performance Liquid Chromatography (UPLC) was done using the nanoACQUITY UPLC system from Waters Corporation (MA, USA). The ESI-Q-TOF MS were performed with an ESI Q-Tof Premier MS from Waters Corporation (MA, USA).

The sample was injected into a 180um x 20mm trap column packed with 5um BEH C-18 beads. Trapping was done for 1 minute, then the trap column was connected to a 75um x 150mm analytical column packed with 1.7um BEH C-18 beads. Chromatography was performed at a flow rate of 400nL/min with a linear gradient of 3% to 40% AcN with 0.1% FA for 35 minutes at 35°C. This was directly followed by the ESI-Q-TOF MS analysis.

Data independent MS^E Identity mode was applied for data acquisition. Precursor ion spectra were generated at a collision energy of 5V, fragment ion spectra were obtained at a collision energy voltage ramp of 20-35V in alternating 1 second scans. Peptide spectra and fragment spectra were acquired with 2ppm and 5ppm tolerance, respectively.

The output data were compared to two protein databases, namely NCBI and UniProt, for the organisms *Drosophila melanogaster* and *Leptopilina boulardi*. The identification was done with PLGS 3.0 software followed by quantification with the software program Progenesis®

QI for proteomics. Acetyl N-terminal, Deamidation N and Q, Carbamidomethyl C and Oxidation M were set as variable modifications. Identification of 3 consecutive y- or b-ions was required for a positive peptide match.

Quantification of the standards, enolase and alcohol dehydrogenase, was not possible due to degradation. Therefore, relative quantification was applied on the data. Two different quantification methods, particularly relative quantification using Hi-N (Hi-N) and using all peptides (all-pep), were used for quantification.

In Hi-N quantification, the N most abundant peptides of a protein are used for quantification. In our case, N was defined as 3. In case of conflicts in the most abundant peptides, e.g. when a peptide can be assigned to more than one protein, first a Hi-N analysis is done with unique peptides only. Then, the conflicting peptides are set in relation to this for reliable quantification (Waters Corporation., n.d.-a).

In the all-pep approach, the protein abundances are determined by use of all peptides. This method does also include all conflicting peptides which leads to a higher number of proteins identified. Therefore, it will show more different proteins but also bears a higher risk of wrong results (Waters Corporation., n.d.-b).

4. RESULTS

It is important to stress that this work represented the first use of proteomic analysis in the laboratory and the methodology had to be optimized. After various attempts and optimization, only two biological replicates of sufficient quality could be used for evaluations at the end making this work rather a preliminary but nevertheless promising attempt for hemolymph proteome. The biological replicates were separated in supernatant and sediment (see 3.2 Hemolymph preparation). Three technical replicates were made from these samples each. The results were given in normalized abundances. In case both quantification methods did give results, the values from Hi-N quantification were used because of the higher accuracy of this method.

4.1. *Drosophila melanogaster* proteome analysis

Due to the relative quantification, the protein amounts in pellets and supernatants could not be summed up. Hence the concentrations in supernatants and pellets were compared separately. Two different approaches were established for analysis of the data: One describing proteins significantly changed during immune response, the other one showing trends of proteins possibly involved.

4.1.1. Proteins showing significant changes

Proteins with significant differences (determined by the comparison of the three technical replicates in uninfected and infected samples) either in supernatant or pellet are shown in Table 1. Proteins with zero occurrences in supernatant or pellet in either infected and uninfected sample but with the occurrence in the other treatment are also shown (see Table 1, [1,2]). The NCBI (105.592 protein entries) and Uniprot (42.535 protein entries) databases were used for matching. The unpaired 2-tailed t-test was used for comparison of protein concentrations in supernatant and sediment of infected and uninfected hemolymph. P values below 0.05 were considered as statistically significant. There are sometimes different values detected in experiment 1 and experiment 2, which could be explained by a difference in sample preparation (frozen samples in exp. 1 and fresh samples in exp. 2 were used). Nevertheless, proteins with similar changes are presented in Table 1.

Table 1. Proteins significantly upregulated during parasitoidic wasp infection.

				FOLD CHANGE			
				Exp. 1		Exp. 2	
Accession nb.	**Protein**	**Gene**	**Quant.**	**Sup.**	**Sed.**	**Sup.**	**Sed.**
A0ZWT1	Thiolester containing protein II	TepII	Hi-N, all-pep[1]	32.6[3]	2.1	0[4]	6.3
A1ZBU8	Uncharacterized protein	CG18067	Hi-N, all-pep[1]	1.2	2.0	4.2	3.3
Q8IN43	Protein Turandot C	TotC	Hi-N, all-pep[1]	8.1	6.8	147.9	10.9
Q8IN44	Protein Turandot A	TotA	Hi-N, all-pep[1]	10.8	40.1	65.8	115.5
Q8ML70	Immune-induced peptides	IMPPP	Hi-N, all-pep[1]	5.5	3.3	25.2	3.2
A9YHN6	CG33249-PA (Fragment)	CG33249	all-pep[2]	33.7[3]	26.6[3]	183.9[3]	11.2[3]
M9PEL7	Ecdysone-inducible gene L2	ImpL2	all-pep[2]	4.6	3.2	26.1	7.4
Q8SX76	LD24646p	pch2	all-pep[2]	4.4	1.3	15.2	1.8
Q9VLU4	Serine protease inhibitor 28Dc	Spn28Dc	all-pep[2]	6.4	1.3	4.3	1.6

The table shows the fold changes of the normalized abundances in experiment 1 (Exp. 1) and experiment 2 (Exp. 2) separated by supernatant (Sup.) and sediment (Sed.). [1] Numbers from Hi-N quantification are displayed. [2] These proteins could not be found using the Hi-N approach. [3] Zero abundances were detected in uninfected samples. The value given is the mean of the abundance in the infected sample. [4] In both infected and uninfected sample no abundance could be determined.

4.1.2. Proteins showing trends

Furthermore, proteins were characterized which did not show significant changes but notable trends. This method could identify new proteins possibly involved in immune response. The results are presented in Table 2.

Table 2. Proteins that show expression trends during parasitoidic wasp infection.

				FOLD CHANGE			
				Exp. 1		Exp. 2	
Accession nb.	**Protein**	**Gene**	**Quant.**	**Sup.**	**Sed.**	**Sup.**	**Sed.**
B7YZJ4	CG4975	CG4975	Hi-N, all-pep[1]	15.9[4]	1.5	151.4	6.9
Q6IL18	HDC10707	CG43085	Hi-N, all-pep[1]	0.5	1.8	0.6	2.7
Q7KML1	BcDNA.GH07626	FASN1	Hi-N[2]	0.5	0.7	0.5	0.7
Q9NFV8	TEPI protein	TEPI	Hi-N[2]	1.4	2.7	1.3	1.2
Q9VJD7	RH50269p	SPH93	Hi-N[2]	63.1[4]	2.3	1.3[4]	61.1
A0A0B4LH29	Uncharacterized protein	CG44245	all-pep[3]	1.3	1.6	4.6	1.5
A1Z9L0	LD05087p	Rcd1	all-pep[3]	1.7	1.3	5.3	2.7
A9UNB7	LP13860p	CG30410	all-pep[3]	1.9	1.1	2.0	1.0
G3LSS7	Lectin-24A	CG3410	all-pep[3]	1.6	1.2	3.1	2.1
Q8SZA3	RE09574p	CG15651	all-pep[3]	1.5	1.8	5.8	1.6

The table shows the fold changes of the normalized abundances in experiment 1 (Exp. 1) and experiment 2 (Exp. 2) separated by supernatant (Sup.) and sediment (Sed.). [1] Numbers from Hi-N quantification are displayed. [2] Values from all-pep quantification do not resemble this trend. [3] These proteins could not be found using the Hi-N approach. [4] Zero abundances were detected in uninfected samples. The value given is the mean of the abundance in the infected sample.

The raw data of the proteins given in Table 1 and Table 2 are presented in Table 3 in the appendix. 200-300 protein groups could be identified.

4.2. *Leptopilina boulardi* protein analysis

As basis served the proteome of NCBI (215 protein entries) and Uniprot (117 protein entries) databases. The comparison to the databases showed 17 protein groups which were determined in the hemolymph in very low concentrations. Contrary to the expectations, the proteins were found not only in hemolymph of infected but also of uninfected larvae.

5. DISCUSSION

In general, it must be pointed out, that the small number of biological replicates does not allow valid information about the proteome during immune response. So, this work serves as preliminary study to elucidate possible immune responsive proteins. Nevertheless, the analysis identified several proteins, which are known to be involved in immune response, validating thus our approach. In addition, the analysis also discovered novel proteins with uncharacterized functions but at least in some cases there are hints from high-throughput expression data available in FlyBase that these proteins might be associated with immune or stress responses. Therefore, our approach could be used for identification of new, previously unidentified proteins associated with immune response. These results must be of course verified due to a limited number of biological replicates in this work.

5.1. Immune related proteins of *Drosophila melanogaster*

5.1.1. Proteins significantly changed

5.1.1.1. Thiolester containing protein II_ isoform A (Fragment) (TepII)

The Thiolester containing protein (TEP) family has the six members TEPI-VI. Studies suggest a role in opsonization of targets of phagocytic cells or expression of protease inhibitors (Lemaitre & Hoffmann, 2007).

Larval plasmatocytes secret TEPII proteins to the hemolymph. There, TEPII acts in phagocytosis of E. coli, other bacteria tested don't show an involvement of TEPII in phagocytosis. Another finding is that TEPII is likely to be involved in Imd signaling as well as the JAK-STAT pathway of adults during wounding or specific bacterial infestations. Also, JNK signaling needs TEPII transcription when the immune system is challenged by *P. asymbiotica* (Shokal et al., 2017). Interestingly, a further study indicated other roles in metabolism and melanization: During immune response to *Photorhabdus* infection, the hemolymph trehalose concentration of TEPII loss-of-function mutants was found to be upregulated as well as nitric oxide (Shokal, Kopydlowski, Harsh, & Eleftherianos, 2018). The result from Schlenke et al. (2007b) shows an opposing direction during wasp infection with *Leptopilina* strain Lb17: TepII was overexpressed in the first 5h after infection, while a downregulation took place 24h after infection. This result could point to the role as opsonine.

5.1.1.2. Uncharacterized protein (CG18067)

The protein encoded in the gene CG18067 was found to be upregulated as response to cold shock. Its overexpression lead to faster chill coma recovery (Vermeulen et al., 2013). Analysis by modENCODE showed upregulation during infection of *Sindbis* virus (FlyBase, 2018a). It is likely that expression is initiated by the Toll pathway (Busse et al., 2007). These results lead to the assumption that the protein is stress-induced, which would explain the overexpression during wasp parasitoidism. The upregulation could not be confirmed by a preceding study (Schlenke et al., 2007b). This protein is also part of the female reproduction proteins in adult flies, this indicates versatile functions of CG18067 (Sirot et al., 2014).

5.1.1.3. Turandot A (TotA) and Turandot C (TotC)

The expression of the Turandot (Tot) family proteins is initiated under stressful conditions like heat shock, exposure to UV light, septic injuries, or bacterial infections. The TotA protein is the best studied member of the Tot family. As response to extreme stress, its production is upregulated in the fat body and secreted into the hemolymph. Its effect in stress response is not completely known, one function as protective protein against high temperatures has been described. The production is initiated by the JAK-STAT signaling pathway in the fat body, also the expression of upd3 in hemocytes is needed. Production of TotC is increased as response to cold exposure, in adult state its initiated by the male courtship song. TotC is also regulated by JAK-STAT and it is probable that the whole Tot family proteins are controlled by this pathway. Preceding studies suggest that Tot proteins are regulated by the MAPK Kinase Mekk1 which is likely to play a role in the p38 pathway. (Agaisse & Perrimon, 2004; Brun et al., 2006; Ekengren & Hultmark, 2001; Zhang, Marshall, Westwood, Clark, & Sinclair, 2011; Zhong et al, 2013). An overexpression of these two Tot proteins in response to wasp infection has been described by Schlenke et al. (2007b). The upregulation also seems plausible: The oviposition of the parasitoid egg causes the penetration of the epithelium, a damage which could trigger a stress response. Also, parasitoidism itself could trigger Tot production.

5.1.1.4. Immune-induced peptides (IMPPP)

The IMPPP is the precursor of the four Drosophila immune-induced molecules (DIMs) DIM 10, DIM 12, DIM 13, and DIM 24 (UniProt-Consortium, 2018). Furin-like sites are the cleavage sites resulting in the four DIMs. Fragments of these molecules are identified as

further immune-induced molecules, DIM5, DIM 6, and DIM 8 are parts of DIM 12 and DIM 13. The DIMs belonging to this family are active against inflammation (Levy et al., 2004). Hence, transcription during wasp infection appears to be reasonable. Studies of the proteome of microbial challenged adult flies showed peaks in transcription 24h after infection. A further study described a significant upregulation 5h after infection with *L. boulardi*, interestingly the expression decreased subsequently (Schlenke et al., 2007b). This confirms our result partly.

5.1.1.5. CG33249-PA (Fragment) (CG33249)

CG33249 is also known as CG42271 (NCBI, 2018). CG42271 is involved in phosphoinositide (PI) signaling, PIs are, inter alia, responsible in cytoskeletal function and ligand – receptor binding at cell surfaces (Balakrishnan, Basu, & Raghu, 2014). There are two orthologous proteins of CG42271 known in human, the inositol polyphosphate 4-phosphatases INPP4A and INPP4B. INPP4B was found to be related to survival of breast cancer patients, which indicates a role as tumor suppressor (Gewinner et al., 2009). Another study on Drosophila suggests that CG33249 is connected to the FOXO protein: When FOXO was overexpressed, CG33249 was upregulated in fat bodies of female adults (Hoffmann, 2013). FOXO itself is assumed to have a function in tumor suppression (Greer & Brunet, 2005) besides its task fields in stress response and metabolism. The interaction with FOXO suggests that an overexpression CG33249 could be stress induced, this would explain our results. More detailed insights in the mechanism of tumor suppression could maybe also reveal interesting aspects regarding the function during immune challenge.

5.1.1.6. Ecdysone-inducible gene L2 (ImpL2)

ImpL2 is described as an antagonist of insulin/IGF signaling causing elevated trehalose concentrations in hemolymph and degradation of glycogen and triglyceride storages. Insulin/IGF signaling itself is interrelated with the FOXO transcription factor, overexpression of ImpL2 leads to upregulation of FOXO. Studies from 2015 showed that ImpL2 is secreted by cancer tumors and triggers a cachexia-like process with the clinical picture of organ wasting of tissues like adipose or muscle tissues. The wasting is induced by changes in metabolism mediated by ImpL2 (Figueroa-Clarevega & Bilder, 2015; Kwon et al., 2015). Šokčevičová (2017) showed that ImpL2 upregulation is not only induced by cancerous disease but also during immune response. Šokčevičová described that ImpL2 is secreted by activated immune cells to provide energy for immune processes. Furthermore, she demonstrated the essential

role in the metabolic change: Flies lacking ImpL2 showed less resistance to bacterial infection. Our findings comply with the role in metabolism during immune defense.

5.1.1.7. LD24646p (pch2)

The *Drosophila* Pachytene checkpoint 2 gene (pch2) is active during the pachytene phase in meiosis. It encodes an AAA+ATPase causing a decrease in pachytene process in case of deficiencies, further on it mediates crossing overs between chromosome pairs (Joyce & McKim, 2011). Qian, Xu, & Niklason (2015) described another role examining the gene pch-2, a homolog in the nematode *Caenorhabditis elegans*, and the human homolog TRIP13. The study suggested that upregulation of TRIP13 in human fibroblasts leads to enhanced resistance against UV-radiation and oxidative stress. Also, in C. elegans this function in stress response could be demonstrated, furthermore it has been shown that an increased expression causes an extended lifespan in the nematode. These findings describe a function as stress-related protein, a competence closely linked to immune response. Therefore, upregulation during wasp infection is reasonable.

5.1.1.8. Serine protease inhibitor 28Dc (Spn28Dc)

A key element in melanization is the PO pathway. Enzymes essential in this cascade are the serine proteases which convert the proenzyme prophenol oxidase (PPO) to active PO. Proteins from the *ser*ine *p*rotease *in*hibitor group (serpins) control the melanization process. It has been suggested that Spn28D is a down regulator of PO activity in the hemolymph and tracheal organs. This seems to be contraindicated, knowing that melanization of the parasitoid egg is the most important defense mechanism. But it appears that the PO activity needs close monitoring due to the lethal effect of excessive PO activation. Downregulation of Spn28D leads to spontaneous melanization of organs in the hemocoel and tissues which are exposed to air. Therefore, this protein has a protective function during immune response. Also, it is likely that Spn28D in the hemolymph sources primary from the crystal cells, the main origin of PO in hemolymph (Scherfer et al., 2008). In our experiments, the protein serine protease inhibitor 28Dc was upregulated during wasp infection which mirrors the function in melanization.

5.1.2. Proteins showing trends

5.1.2.1. CG4975 (CG4975)

The role of CG4975 is not known. modENCODE shows low expression rates during stress response, higher values are related to *Sindbis* virus infection (FlyBase, 2018b). The human ortholog Atxn10 is a receptor in CD8 cells, which are a crucial part of the immune system (Han, Gopalakrishnan, Yu, & Wang, 2017), a function in *Drosophila* immune response to *L. boulardi* infection has not been shown up to now.

5.1.2.2. HDC10707 (CG43085)

HDC10707 is a protein with unknown function. An elevated expression of the gene CG43085 in response to *A. tabida* infection was found by Salazar-Jaramillo et al. (2017). Interestingly, in our experiments the protein HDC10707 showed a decreased level in supernatant and an upregulation in sediment. This suggests an alteration of the chemical properties upon infection.

5.1.2.3. BcDNA.GH07626 (FASN1)

It is described as fatty acid synthase and is part of the sugar metabolism (Garrido et al., 2015). Upregulation was described in stress response and *Sindbis* virus infection (FlyBase, 2018c). In contrast, the protein was downregulated during our experiments. Further studies could elucidate the role in metabolism in immune challenge.

5.1.2.4. TEPI protein (TEPI)

The TEPI protein belongs to the TEP family, a group of immune related proteins. The expression of TEPI is regulated by the JAK-STAT pathway, and it was identified as opsonin during phagocytosis of microorganisms. (Dostálová, Rommelaere, Poidevin, & Lemaitre, 2017). An upregulation induced after *L. boulardi* infection has been shown by Schlenke et al. (2007b). Our results are in accordance to the previous studies.

5.1.2.5. RH50269p (SPH93)

SPH93 belongs to the group of serine proteases, which are related to the PO cascade. Studies have shown that SPH93 is upregulated during microbial challenge and fungal infection (De

Gregorio, Spellman, Rubin, & Lemaitre, 2001). The upregulation during wasp infection could therefore support the role as immune-induced protein.

5.1.2.6. Uncharacterized protein (CG44245)

The function of the protein has not been elucidated. Data from the modENCODE project show upregulation during various stress responses as well as during *Sindbis* virus infection (FlyBase, 2018d). Thus, a role in immune response is possible.

5.1.2.7. LD05087p (Rcd1, CG8233)

Roles of the gene CG8233 were found in transcription (Raja et al., 2010) and cell division (Dobbelaere et al., 2008). Moderate expression during stress response and *Sindbis* virus infection are described by modENCODE (FlyBase, 2018e). The link to wasp infection remains unclear.

5.1.2.8. LP13860p (CG3041)

The function of CG30410 is described as pentose-phosphate shunt that regulates the NADPH levels (Wang et al., 2012). Upregulation as response to *Sindbis* virus infection and as stress response was characterized by modENCODE (FlyBase, 2018f), a specific role in immune system is not known.

5.1.2.9. Lectin-24A (CG3410)

Keebaugh and Schlenke (2012) showed that Lectin-24A is upregulated during wasp infection. They suggest a role in melanotic encapsulation and further in wasp egg recognition. Our results confirm their findings.

5.1.2.10. RE09574p (CG15651)

CG15651 is described as Fukutin-related protein and linked to glycosylation (Yamamoto-Hino et al., 2015). During embryogenesis, CG15651 is expressed in the brain and ventral nerve chord, and is connected to synaptic transmission (Tomancak et al., 2007). A connection to immune response was not examined yet.

5.1.3. Comparison to previous studies

Some identified proteins reflect the results of preceding studies. Various proteins induced during wasp infection (Schlenke et al., 2007b) and other immune challenges (UPLEM, 2013) could not be determined during this analysis. This shows that immune response is very specific to the invading organism. This was also described from Schlenke et al. (2007a), where two different pathogens of the *Leptopilina* family provoked particular immune responses in *Drosophila* larvae. A further explanation for proteins not found during our analysis are the limitations in protein quantification.

5.2. Immune related proteins of *Leptopilina boulardi*

The comparison to the *L. boulardi* proteome showed small occurrences of proteins in both infected and uninfected samples. This result could be explained by the limitations of the analysis: Wasp proteins can only occur from oviposition or from release of the egg. Hence, it is expected that the hemolymph contains very little amounts of *Leptopilina* proteins in comparison to the naturally predominant *Drosophila* proteome. This makes it likely that *Drosophila* proteins were assigned to wasp proteins and consequently lead to a false positive result. In general, it must be considered that the evaluation is based on the availability of a broad database. The databases of *L. boulardi* proteome contain relatively few protein entries compared to the proteome of the model organism *D. melanogaster*. Especially in this setup with very few wasp proteins to be expected, a meaningful outcome depends on the size of the database.

6. CONCLUSION

The analysis of *D. melanogaster* hemolymph using reversed-phase UPLC-MS can give interesting insights in the *Drosophila* proteome during immune challenge. Due to the limitation in experiment repeats, this study does not give solid evidence for immune proteins but serves as framework to show trends and possible links to be further examined. 19 *Drosophila* proteins are related to the immune reaction caused by parastoidism of *L. boulardi*. Some known stress and immune-related proteins like Tots, TEP, and IMPP could be identified demonstrating that the proteomic approach was successfully established in the laboratory. Other proteins involved in carbohydrate metabolism show further aspects of *Drosophila* immune response. Interestingly, the upregulation of various proteins known to be involved during wasp infection could not be confirmed. This could be caused by restrictions in the analysis, a very specific host response, as well as different setups. Unfortunately, quantification was limited due to problems with the added standards. The applied relative quantification bears different risks: The Hi-N method possibly misses proteins due to its restrictions, the all-pep approach, on the contrary, produces more imprecise values. This suggests that more immune-induced proteins could be found by absolute quantification. Furthermore, the method could be extended by examination under different conditions like testing other time intervals or investigating certain mutation strains. Also, analysis of the released proteins from hemocyte incubated ex vivo would be very interesting since it would possibly show proteins specifically released from activated immune cells - the hemocytes. In all cases, the role of proteins verified by additional biological replicates, will have to be subjected to functional studies, using for example genetic tools.

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8. APPENDIX

Table 3. Concentrations of significantly changed or trending proteins of *D. melanogaster* hemolymph evaluated during the 2 experiments.

					NORMALIZED ABUNDANCES							
					Control 1		Control 2		Infected 1		Infected 2	
Accession Number	Pept. Count	Protein	Gene	Quant.	Sup.	Sed.	Sup.	Sed.	Sup.	Sed.	Sup.	Sed.
A0ZWT1	26	Thiolester containing protein II	TepII	Hi-N, all-pep	0.0	55.0	0.0	9.9	30.0	120.8	0.0	62.6
					0.0	56.8	0.0	8.9	33.4	105.4	0.0	58.3
					0.0	47.5	0.0	7.9	34.5	107.5	0.0	48.7
A1ZBU8	2	Uncharacterized protein	CG18067	Hi-N, all-pep	73.7	19.0	63.6	11.8	78.3	46.3	116.2	40.1
					59.9	25.1	49.8	7.5	86.0	32.3	208.7	33.2
					63.4	16.2	29.3	14.9	78.1	42.8	275.7	40.6
B7YZJ4	1	CG4975	CG4975	Hi-N	0.0	7.0	0.7	1.0	14.5	9.1	15.4	4.8
					0.0	11.8	0.0	0.4	17.1	18.0	29.6	2.7
					0.0	4.8	0.0	1.1	16.1	8.2	60.4	9.4
Q6IL18	4	HDC10707	CG43085	Hi-N	585.2	89.3	735.3	82.4	361.3	146.2	363.5	160.6
					591.0	86.8	542.5	85.7	246.9	132.8	246.5	269.3
					640.2	71.9	811.2	87.5	255.8	168.7	608.2	256.8
Q7KML1	21	BcDNA.GH07626	FASN1	Hi-N	204.3	198.5	190.2	86.8	174.9	125.9	115.9	66.5
					208.3	136.6	125.3	102.4	61.5	144.8	35.5	56.9
					247.8	218.3	293.6	82.4	82.2	132.3	163.2	59.3
Q8IN43	14	Protein Turandot C	TotC	Hi-N, all-pep	71.3	40.2	19.0	22.8	391.0	261.1	2168.2	256.3
					59.7	38.3	15.6	22.8	588.4	229.3	2693.6	231.6
					56.2	29.9	16.8	23.2	530.8	251.3	2736.7	261.4
Q8IN44	24	Protein Turandot A	TotA	Hi-N, all-pep	121.5	12.4	74.6	1.4	942.3	541.1	5281.7	161.4
					101.0	12.4	52.4	2.0	1279.9	507.2	3829.1	155.5
					93.0	12.9	83.9	0.9	1181.2	458.9	4767.5	175.7
Q8ML70	7	Immune-induced peptides	IMPPP	Hi-N, all-pep	84.6	45.6	37.3	18.4	397.2	167.0	531.1	55.7
					99.5	49.0	59.5	21.6	549.5	159.2	1493.8	63.7
					53.8	51.8	38.9	16.2	359.1	160.7	1400.4	61.0
Q9NFV8	12	TEP1 protein	Tep1	Hi-N	151.5	37.5	89.0	28.7	168.3	118.7	82.3	39.5
					120.7	43.3	54.9	29.4	200.1	105.4	87.7	37.8
					100.3	39.5	71.1	34.2	163.4	104.7	104.3	33.2
Q9VJD7	2	RH50269p	SPH93	Hi-N	0.0	51.5	0.0	0.4	53.3	131.9	0.0	71.7
					0.0	57.1	0.0	0.7	75.0	123.4	1.3	61.2
					0.0	52.7	0.0	2.0	61.1	116.9	2.5	59.3

NORMALIZED ABUNDANCES

Accession Number	Pept. Count	Protein	Gene	Quant.	Control 1		Control 2		Infected 1		Infected 2	
					Sup.	Sed.	Sup.	Sed.	Sup.	Sed.	Sup.	Sed.
					962.6	265.0	763.8	100.2	1056.6	453.1	2951.6	212.7
A0A0B4LH29	3	Uncharacterized protein	CG44245	all-pep	952.2	284.5	690.2	107.7	1407.9	444.6	3255.8	146.1
					975.8	278.4	867.7	115.3	1286.0	464.9	4470.5	111.6
					976.4	481.2	718.6	199.6	1242.6	724.9	3088.8	1031.3
A1Z9L0	2	LD05087p	Rcd1	all-pep	815.7	564.0	615.6	192.1	1674.7	689.9	3394.0	357.0
					797.3	524.6	723.0	224.9	1503.3	700.1	4425.8	306.4
					133.1	250.0	212.9	206.1	248.6	314.3	437.1	188.0
A9UNB7	1	LP13860p	CG30410	all-pep	137.0	282.5	212.6	183.0	264.4	269.7	388.7	186.4
					148.8	219.4	232.4	199.5	294.6	264.9	508.1	241.1
					0.0	0.0	0.0	0.0	30.2	30.3	146.5	13.8
A9YHN6	1	CG33249-PA (Fragment)	CG33249	all-pep	0.0	0.0	0.0	0.0	37.7	24.3	183.8	11.1
					0.0	0.0	0.0	0.0	33.2	25.2	221.5	8.8
					471.1	328.5	582.0	159.4	849.2	388.3	826.8	296.5
G3LSS7	2	Lectin-24A	CG3410	all-pep	518.1	300.1	430.2	202.7	712.1	331.2	1629.7	376.9
					430.9	270.4	484.5	160.3	687.2	363.7	2213.0	399.7
					179.5	82.8	161.2	57.4	1089.4	291.5	3999.5	280.6
M9PEL7	1	Ecdysone-inducible gene L2	ImpL2	all-pep	207.2	97.0	148.9	77.3	442.0	228.0	1476.7	547.2
					189.4	67.0	113.8	59.0	1136.8	269.4	5591.4	603.4
					209.5	664.6	264.7	291.5	1277.9	948.8	2432.2	600.6
Q8SX76	2	LD24646p	pch2	all-pep	226.1	699.9	374.5	328.6	782.5	848.5	6152.7	486.3
					274.3	635.2	336.5	283.4	1096.6	843.4	6262.9	515.1
					888.6	198.8	585.4	57.7	989.9	409.0	2736.2	145.2
Q8SZA3	1	RE09574p	CG15651	all-pep	737.6	235.9	534.7	59.7	1308.5	388.3	3201.5	98.6
					738.9	221.7	646.5	69.0	1211.7	413.4	4285.9	58.2
					123.1	866.9	240.9	339.2	1008.1	1242.6	783.5	716.6
Q9VLU4	4	Serine protease inhibitor 28Dc	Spn28Dc	all-pep	118.0	1010.2	197.6	442.8	642.5	1160.0	1143.9	706.2
					151.5	719.2	309.3	445.2	880.6	1057.3	1276.9	563.3

The results are given in normalized abundances determined by alignment to the *D. melanogaster* proteome of the Uniprot database. Each sample is separated in supernatant (Sup.) and sediment (Sed.) showing the results of the three technical replicates.