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Corresponding Author: Dr. Roger Huybrechts, Dr

Corresponding Author's Institution: Laboratory of Insect Physiology and Molecular Ethology, Zoological Institute, KU Leuven

First Author: Magdalena Bil, Master

Order of Authors: Magdalena Bil, Master; Valérie Broeckx; Bart Landuyt; Roger Huybrechts, Dr

Abstract: Females of anautogenous flesh flies, *Sarcophaga crassipalpis*, need a protein meal in order to produce their first batch of eggs. This protein meal elicits an increase in midgut proteolytic activity that is under neuropeptidergic regulation. Time series of decapitation and rescue experiments of liver fed flies evidenced the need of a peptide factor released by corpora cardiaca (CC) within four hours post protein feeding in order to assure complete protein digestion. Q-Exactive quantitative differential peptidomics analysis on CC of sugar fed flies and flies five hours post protein feeding respectively, showed a unique consistent decrease in the stored amount of adipokinetic hormone (AKH) ranging between 16 up to 63%. Injection of AKH into liver fed decapitated flies as well as sugar fed intact flies resulted in dose dependent enhanced midgut proteolytic activity up to the level of intact protein fed flies. This suggests a key role of AKH in food depended reproduction

ZOOLOGICAL INSTITUTE
NAAMSESTRAAT 59
B-3000 LEUVEN (BELGIUM)

To Dr. Robert M. Does
Editor-in-Chief
General and Comparative Endocrinology

August 13, 2014

ONS KENMERK
UW KENMERK
LEUVEN,

Dear Editor,

Today we received the reviewer comments regarding our manuscript entitled “**Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*.**” by Magdalena Bil^a, Valerie Broeckx^b, Bart Landuyt^b and Roger Huybrechts^{a*} as we submitted for publication in General and Comparative Endocrinology.

According to the reviewers minor remarks and suggestions and in response to your directions we made the appropriate changes as explained below:

For Reviewer 1 the manuscript was acceptable as such and no action needed to be taken.

As reviewer 2 felt uncomfortable with some of our figures as it was hard to understand each figure due to complicated representation on different food sources and time-courses, we adopted the graphical figures by incorporating line type explanation directly on the figures. In all experimental figures (fig 3 and beyond) the broken line represent the non decapitated liver fed control situation whereas the dotted line represent the liver primed decapitated control condition and the solid line represent the liver primed decapitated experimental condition. According to directions we saved each figure file in **.tiff format**.

As in Line 336-337 reviewer 2 asked to explain "a non-protein free amino acids" for readers. We replaced the sentence by “a free amino acids meal devoid of any protein” in line 337.

3. As reviewer 2 informed about what kind of unit that was employed for enzymatic activity we adopted each figure as the relative enzymatic activity is expressed in absorption units as measured at 405 nm and normalized against the mass of the sample. Accordingly we explicitly clarified this in line 128 of M&M. We included (A_{405nm}) in the figures ordinate

We thank the reviewers for their suggestions and sincerely hope that the proposed changes suffice to make this manuscript ready for publication.

Kind regards

Prof.Dr.Roger Huybrechts

Response to reviews

According to the reviewers minor remarks and suggestions and in response to your directions we made the appropriate changes as explained below:

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Graphical Abstract (for review)

LIVER FED FLY

MIDGUT PROTEOLYTIC ACTIVITY

DECONTAMINATED LIVER FED FLY

MIDGUT PROTEOLYTIC ACTIVITY

+ CC extract:

or

+ AKH

MIDGUT PROTEOLYTIC ACTIVITY

1 Highlights

2

- 3 • Endocrine regulation of digestion in anautogenous flesh fly
- 4 • Proteolytic activity after decapitation and rescue using CC extract
- 5 • Differential peptidomics of CC prior to and post liver meal highlights AKH
- 6 • Proteolytic activity after decapitation and rescue using AKH: dose-response effect
- 7

Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*.

Magdalena Bil^a, Valérie Broeckx^b, Bart Landuyt^b, Roger Huybrechts^{a*}

^a Research group of Insect Physiology and Molecular Ethology, KU Leuven, Naamsestraat 59, B-3000 Belgium

^b Research group of Functional Genomics and Proteomics, KU Leuven, Naamsestraat 59, B-3000 Belgium

*Corresponding author:

Roger Huybrechts

Research group of Insect Physiology and Molecular Ethology

KU Leuven, Naamsestraat 59, B-3000 Belgium

Tel.: +32 16 32 39 33, Fax +32 16 32 39 02

e-mail address: Roger.Huybrechts@bio.kuleuven.be

Keywords:

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flesh fly

anaulogeny

digestion

neuropeptidomics

Abstract

Females of anaulogenous flesh flies, *Sarcophaga crassipalpis*, need a protein meal in order to produce their first batch of eggs. This protein meal elicits an increase in midgut proteolytic activity that is under neuropeptidergic regulation. Time series of decapitation and rescue experiments of liver fed flies evidenced the need of a peptide factor released by *corpora cardiaca* (CC) within four hours post protein feeding in order to assure complete protein digestion. Q-Exactive quantitative differential peptidomics analysis on CC of sugar fed flies and flies five hours post protein feeding respectively, showed a unique consistent decrease in the stored amount of adipokinetic hormone (AKH) ranging between 16 up to 63%. Injection of AKH into liver fed decapitated flies as well as sugar fed intact flies resulted in dose dependent enhanced midgut proteolytic activity up to the level of intact protein fed flies. This suggests a key role of AKH in food depended reproduction.

1. Introduction

Anautogeny is a type of reproductive strategy that requires an additional external source of proteins to start oogenesis. Anautogeny can be either an obligate or facultative characteristic and is seemingly stimulated by multiple factors. Anautogenous insects, including some species of mosquitoes and flesh flies, can turn to autogeny due to several environmental or genetic factors where the strongest determinant is the presence and abundance of food in the larval stages. Nutritional analysis of the autogenous and anautogenous strains of the mosquito *Culex tarsalis* revealed that the anautogenous strain contains much lower energy reserves and much lower amounts of total lipids, carbohydrates and proteins in their body and less free amino acids circulating in their hemolymph (Su & Mulla, 1997a; Su & Mulla, 1997b). This is likely the consequence of the expression of the genes encoding larval storage proteins (Zakharkin *et al.*, 2001).

Because of the energy deficiency in anautogenous insects, food intake and digestion plays a crucial role to obtain the nutrients that are the main source of building blocks and energy to start vitellogenesis. In insects, proteolytic digestive enzymes are synthesized and secreted by the midgut epithelial cells. In carnivorous Diptera, either hematofagous or cadaver recyclers, the major protease involved is trypsin (Borovsky *et al.*, 1996). In accordance with the specific feeding behavior of anautogenous insects, in these species, trypsin occurs in two differentially regulated forms: early trypsin that is constantly present at a relatively low level even in sugar fed insects and late trypsin that appears after the intake of a protein meal (Lu *et al.*, 2006; Muller *et al.*, 1993; Borovsky *et al.*, 1996). In anautogenous mosquitoes, late trypsin is a specific midgut protease, characteristic for adult blood feeding females only (Noriega & Wells, 1999). In mosquitoes like *Georgacraigius atropalpus*, being autogenous or anautogenous depends on the timing of the release of ovary ecdysteroidogenic hormone (OEH), which suffices by itself to stimulate both digestion and ecdysteroid production by the ovary (Gulia-Nuss *et al.*, 2012; Dhara *et al.*, 2013). OEH is a member of the neuroparsin family which is known to be absent in some *Drosophila* species (Veenstra, 2010). Remarkably, mosquito OEH is the only member of this neurohormone family known to stimulate egg development (Brown *et al.*, 1998). Due to the lack of sequenced genome data for *Sarcophaga*, it is not known if this anautogenous fly species has an OEH ortholog, and if so to what extent it is involved in regulating the process of anautogenicity. Veenstra (2010) suggested ILP's as an alternative regulatory pathway for species lacking neuroparsin like neuropeptides. Data presented in this manuscript reports the first efforts in identifying central factors involved in regulating anautogenous reproduction in the flesh fly.

As digestion is the primary event in anautogenous reproduction and since decapitation in mosquitoes is described to prevent blood meal digestion (Gulia-Nuss *et al.*, 2011) we used a similar approach, extended with differential peptidomics and rescue experiments, to identify brain and *corpora cardiaca* (CC) released neuropeptides affecting proteolytic midgut activity. So far, a plethora of neuropeptides are reported to play a role in regulating digestion in different insects. Among them are FMRFamide (Lange, 2001), FLRFamides (Lange & Orchard, 1998), proctolin (Fuse & Orchard, 1998), allatotropin (Duve *et al.*, 1999), sulfakinins (Downer *et al.*, 2007), tachykinins (Pabla & Lange, 1999; Lange, 2001), neuropeptide F (Stanek *et al.*, 2002), short neuropeptide F (Lee *et al.*, 2004), Insulin like peptide (Gulia-Nuss *et al.*, 2011)

and diuretic hormones (Ma *et al.*, 2000). In this publication we provide evidence to add one more peptide to the list of peptides regulating digestion: the adipokinetic hormone (AKH). The first AKH was isolated from the locust, *Locusta migratoria* (Stone *et al.*, 1976), and is one of the most abundant neuropeptides in most insects. It is synthesized and also stored in the neuroendocrine glands, *corpora cardiaca*. AKH usually consists of 8 to 10 amino acids and has been isolated and characterized in about 50 different arthropods (Kodrik *et al.*, 2010) but has not been reported to be found in any stoneflies. All of the AKH genes found to date share some common structural features like pyroglutamic acid at the N-terminal end, aromatic amino acids at the fourth position, a tryptophan at the eighth position and carboxamide at the C-terminal end (Weaver *et al.*, 2012;Marco *et al.*, 2011). 10 distinct forms of this neuropeptide are identified (Gade, 2009). Most insects have only one form of the AKH, like *Schistocerca gregaria* (Gade *et al.*, 1986;Gade, 2009)and *Bombus terrestris* (Lorenz *et al.*, 2001), *Lucilia Caesar* and *Neobellieria bullata* (Rahman *et al.*, 2013), but some insects have two forms, like *Periplaneta americana* (Hansen *et al.*, 2006), and *Aedes aegypti* (Kaufmann *et al.*, 2009), and some insects like *Locusta migratoria* or *Bombyx mori* even have three forms of AKH (Diederer *et al.*, 2002;Van Der Horst, 2003;Roller *et al.*, 2008). AKH is primarily involved in mobilization of stored energy carrying molecules to support all high energetic processes, as for example flying (Hansen *et al.*, 2006). Importantly, the role of AKH is not limited to metabolism only; it also participates in the immune response (Goldsworthy *et al.*, 2005), in the stress reactions (Kodrik, 2008;Kodrik *et al.*, 2005) and even in eggs development (Abdel-Latif & Hoffmann, 2007). AKH peptide is a messenger molecule that is not able to pass the cell membrane. For this reason it binds to its seven-transmembrane domain G protein-coupled receptor (GPCR) and results in an increase of the intracellular level of cyclic AMP (Gade & Auerswald, 2003).

2. Materials and methods

2.1. Insect

The anautogenous flesh fly, *Sarcophaga crassipalpis*, was reared under laboratory conditions at 24°C under a light : dark cycle of 14 : 10h according to Denlinger (1972). Only female flies of 4 days old were used for the experiments. Flies of that age already completed previtellogenic development and were fully competent to start reproduction. Up to the moment of the experiments, the adult flies were under a sugar and water diet to ensure the same level of enzymatic activity in their guts. Flies that were not used for any scientific purpose were offered a piece of cow liver to proceed normal vitellogenesis and subsequent larviposition to maintain the culture.

2.2. Enzymatic activity

Gut proteolytic activity of the flies, assured mainly by serine proteases trypsin and chymotrypsin, was measured by incubation of gut homogenates with azocasein (Bylemans *et al.*, 1994). Each datapoint results from 4 independent biological replicates whereby each individual replica consists of a pool of 3 midguts per condition. Samples were placed in 400 µl fly Ringer's solution as described by Meulemans & De Loof (1992) to provide correct physiological conditions and avoid too fast degradation. Subsequently samples were homogenized to release the enzymes to the solutions. Mixtures of 85 µl homogenized gut

solutions and 15 µl correction buffer (50mM Tris-HCl pH 7-8, 10mM CaCl₂) were incubated with 100 µl 1% azocasein for 45 min at room temperature. After stopping the reaction with 20% cold trichloroacetic acid, the amount of the digested substrate was detected by measuring absorbance at 405 nm. All results, expressed in absorbance units, were normalized against the mass of the sample. Because of the absence of a quantitative standard, the enzymatic activity measurements are relative compared to each other.

2.3. Dose response experiments

To define the active amounts of CC extract or the dose-response of AKH, flies were fed with liver for 30 minutes, decapitated and subsequently injected with different equivalents of CC extract/doses of AKH. At 4 hours post protein feeding (ppf) for each dose the enzymatic activity in the midgut of 10 individual flies was measured.

2.4. Peptides extraction

Corpora cardiaca, a part of the retrocerebral complex, were dissected in Ringer's solution. CCs were obtained from either sugar fed flies or from protein fed flies at different time points post feeding. Dissected and cleaned tissue was immediately transferred into acidic methanol (90% methanol acidified with trifluoroacetic acid (0.1% solution)). Tissue samples were sonicated, filtrated on a spin down 0.22 µm filter and dried using SpeedVac. LoBind Microcentrifuge Tubes (Eppendorf) were used to avoid sticking of the peptides to the wall of tubes. The samples were additionally prepurified on C18 ZipTip pipette tips (Millipore). The extracts meant for injection were resolubilized in Fly Ringer at a ratio of 1 CC per µl. Recipient flies were given 2 µl injections. To assure maximal response and correct comparison with the protein fed condition, the recipient flies, unless otherwise stated, were primed by a time limited protein meal and subsequent decapitation within 30 minutes.

2.5. MALDI-TOF mass spectrometry

The qualitative mass spectrometry analysis of the neuropeptides that are present in the *corpora cardiaca* was performed on a matrix assisted laser desorption/ionization time-of-flight UltraFlex II instrument (Bruker Daltonics, Germany). The samples were spotted on a stainless steel MALDI plate in a volume of 0,5 µl and covered with 0,5 µl of a α-cyano-4-hydroxycinnamic acid matrix saturated in 50% acetone, 0,1% trifluoroacetic acid. Analytes were measured in positive ion mode, reflectron detection and a m/z range from 700 to 2000. The instrument was calibrated using a peptide calibrant containing 9 standard peptides: angiotensin II, angiotensin I, substance P, bombesin, adrenocorticotrope hormone (ACTH) clip 1-17, ACTH clip 18-39, somatostatin 28, bradykinin fragment 1-7, renin substrate tetradecapeptide porcine (Calibration Standard II, Bruker Daltonics).

2.6. Quantitative ESI-MS analysis

The differential electrospray ionization mass spectrometry (ESI-MS) experiment was performed on a Q Exactive orbitrap mass spectrometer (Thermo, Fisher, Bremen, Germany). The mass spectrometer was online coupled to an Ultimate 3000 ultra high performance liquid chromatography (UHPLC) instrument (Thermo, Fisher, Bremen, Germany). The UHPLC system was equipped with a 2 µm, 100 Å Easy Spray Pepmap RSLC C18 column, 50 µm x 15 cm (Thermo, Fisher, Bremen, Germany). Before sample

separation on the analytical column, the lyophilized sample was resuspended in 10 µl of a 2% acetonitril 0.1% formic acid solution (FA). Then, 5 µl sample volume was injected and washed on a 3 µm, 100 Å, nanoviper, Acclaim Pepmap 100 C18 precolumn, 75 µm x 2 cm (Thermo Fisher, Bremen, Germany). Sample separation was performed using a 60 min gradient of 100 % H₂O, 0.1% FA (mobile phase A) and 20 % H₂O, 80 % ACN, 0.08 % FA (mobile phase B). A linear gradient of mobile phase B in mobile phase A from 4 % to 55 % in 30 min followed by a steep increase to 90 % mobile phase B in 1 min was applied at a flow rate of 300 nl/min. The Q Exactive mass spectrometer was operated in data dependent mode. All mass spectra were acquired in the positive ionization mode with an m/z scan range of 400 to 1600 thomson (Th). For each precursor spectrum, up to the ten most intense ions were selected for the generation of fragmentation spectra. A resolving power of 70,000 full width at half maximum (FWHM) was used with an automatic gain control (AGC) target of 3,000,000 ions and a maximum ion injection time (IT) of 80 ms for precursor spectra. For fragmentation spectra, a resolving power of 17,000 FWHM was used with an AGC target of 200,000 ions and a maximum IT of 560 ms. Dynamic exclusion of 10 s was applied in order to avoid repeated fragmentation of the most abundant ions. Concerning ion selection, a charge exclusion of 1⁺, 5⁺-8⁺ was applied.

For the statistical reasons, samples of both conditions were prepared in 4 biological replicas.

2.7. Database searching

The data were analyzed using the in-house MASCOT server (Matrix science, London, U.K.). Recorded tandem mass spectra were converted into mascot generic format (MGF) peak list files using Proteome Discoverer 1.4 software. For peptide identification, the Swissprot database was used and specified for *Drosophila melanogaster* taxonomy. C-terminal amidation, conversion of N-terminal glutamic acid to pyro-glutamic acid and methionine oxidation were selected as variable modifications. No digestion enzyme was specified. Peptide mass tolerance was set at 10 parts per million (ppm), fragment mass tolerance at 20 million mass units (mmu). Decoy function was used. The instrument type was specified as electrospray ionization Fourier transforms ion cyclotron resonance (ESI-FTICR).

2.8. Peptides

The synthetic equivalent of the AKH of flesh fly that was injected in the experiments was the endogenous AKH of *Drosophila melanogaster* (pQLTFSPDWa) (DmAKH). The peptide was synthesized by GL Biochem Ltd. (Shanghai). The way of purification and determination of concentration was the same as described by Caers (2012). Subsequently the purified peptide was dried and dissolved firstly in pure DMSO and then in fly Ringer's solution to obtain the particular concentration. Each fly received a dose of 100 ng AKH in 1 µl injection.

2.9. Statistical analysis

All statistical analyses were performed by use of Statistica 12 software. The differences between groups were determined by factorial ANOVA. The differences between particular time points of each group were determined by post-hoc Tukey test.

Dose response experiments were validated by T-student test by comparing all different conditions to the positive control of non-treated liver fed flies.

3. Results

3.1. Changes in enzymatic activity of midgut post protein and free amino acids feeding

Females of the anautogenous flesh flies under sugar and water diet reveal very low but constantly secreted level of serine proteases in their midguts. After the protein meal, this enzymatic activity increases 2 fold with the highest activity level between 3 and 4 hours post protein feeding (ppf) (Fig. 1). Subsequently, proteolytic activity decreases over time but still persists on a level 150%-175% of the basic sugar fed activity up to 24 hours ppf. Factorial ANOVA ($R^2=0.94$) confirms differences in enzymatic activity for different time points and different conditions. There is also an interaction between time points and conditions. The Tuckey post-hoc test confirms the differences observed between sugar fed flies and liver fed flies at each time point starting at 2 hours ppf ($p_2=0.00399$; $p_{4-24}=0.00019$). Feeding flies a defined diet of free amino acids only (IPL-41 Insect Medium, Sigma-Aldrich) does not significantly increase the midgut enzymatic activity (data not shown).

3.2. Central regulation of the midgut digestion

The protein meal seemingly elicits the increase in the digestive activity. In order to find out if this process is regulated locally or centrally, flies were first attracted and fed with cow liver for a short period of time to deliver the exogenous stimulus needed to increase digestion. By simple decapitation of the liver fed flies, it has been observed that the 'head' is needed for minimum 4 hours ppf in order to assure the complete protein digestion. Flies decapitated within 1 hour ppf show only a low level of protease activity in their midgut (Fig. 2A). The measured enzymatic activity in the midgut of these decapitated flies even dropped below the basic level of the enzymes secreted by sugar fed flies. Decapitation after 4 hours or later ppf no longer could prevent the increase in the digestive activity (fig. 2B) and accordingly flies decapitated after this time point show the normal midgut activity profile of liver fed flies. Factorial ANOVA ($R^2=0.99$) and post-hoc tests confirm the significant differences between non decapitated flies and flies decapitated within 1 hour ppf and this for each each time point starting at 2 hours ppf ($p_{2-10}=0.00014$). However there is no longer a significant difference between non decapitated flies and flies decapitated either at 4 hours ppf ($p_4=0.95732$) or at 7 hours ppf ($p_8=0.99984$; $p_{10}=1$).

3.3. Extracts of corpora cardiaca as effective elicitors of digestion

Rescue experiments using injection 2 CC equivalents per fly in liver fed decapitated flies confirmed the need for neurosecretory regulation of digestive activity in flies. Decapitated flies injected with CC derived from sugar fed flies displayed a normal proteolytic midgut activity profile (Fig.3A). Factorial ANOVA confirmed these data ($R^2=0.97$). The results of Tukey post-hoc test show that there is a significant difference between liver fed flies and decapitated liver fed flies starting at 2 hours ppf ($p_2=0.00018$; $p_4=0.000143$; $p_6=0.000143$) and also between decapitated injected liver fed flies and decapitated liver fed flies ($p_2=0.00119$; $p_4=0.000143$; $p_6=0.000143$). Additionally, the measured proteolytic activity in midguts of liver fed flies and decapitated injected liver fed flies at all 4 times point

is almost identical as expected ($p_0 = 1.00000$; $p_2 = 0.97417$; $p_4 = 0.13304$; $p_6 = 0.64916$). The same positive results were obtained using CC extracts obtained from flies dissected within a time window of 2 hours ppf. Injection of the peptidic extract prepared from CC of flies dissected after 3 hours ppf gave similar result as flies decapitated after 3 hours ppf. These pooled gut samples were also characterized by huge variation measured on each time point. Some single replicas showed very high digestive activity similar to the positive control of non decapitated liver fed flies while the other replicates presented very low proteolytic activity similarly to the negative control. CC extract obtained from the flies after 5 hours ppf was no longer able to restore the physiological level of digestion in the injected flies (Fig.3B). Factorial ANOVA ($R^2 = 0.95$) confirms our interpretation and results of Tukey post-hoc tests indicate the significant differences between liver fed flies and decapitated liver fed flies starting at 2 hours ppf ($p_2 = 0.00016$; $p_4 = 0.00013$; $p_6 = 0.00013$) and also between liver fed flies and decapitated injected liver fed flies starting at 2 hours ppf ($p_{2-6} = 0.00013$). However there is no significant difference between decapitated liver fed flies and decapitated injected liver fed flies using CC collected from flies 5 hours ppf ($p_0 = 1.00000$; $p_2 = 0.85902$; $p_4 = 1.00000$; $p_6 = 1.00000$). The same low midgut activity was observed in the group of flies injected with the peptidic CC extract collected from flies 20 hours ppf.

In order of further validating our rescue experiment we examined the dose response effect of injected CC extract. Injection of 1 CC equivalent clearly suffices to enhance the midgut proteolytic activity in decapitated liver fed females. Injection of 2 CC equivalents even elicit a higher proteolytic activity in the midguts of all individually tested flies as it reaches almost the level of liver fed non decapitated controls. Injection of 3 equivalents was still effective although less pronounced and injection of 5 equivalents was again less effective and no more pronounced as the 1 CC equivalent (Fig.4). Student T-test confirms differences between no CC versus 1, 2, 3 and 5 CC equivalents ($p_{1,2,3,5} = 0$). This test also confirmed the similarity in effect caused by either liver feeding in non decapitated flies or injection of 2 CC equivalents in liver fed decapitated flies ($p = 0.2467$).

3.4. Profiling of the neuropeptides present in corpora cardiaca of *Sarcophaga crassipalpis*

MALDI-TOF analysis obtained from a pool of 50 CCs show 18 peaks supposed to be peptides. By using the database of another flesh fly *Neobellieria bullata* (Rahman et al., 2013), known also as *Sarcophaga bullata*, it was possible to identify 9 of the peaks. The identified peptides are: 2 forms of Yamides (LPHNHGGJ-OH; LPHNHGG-OH), 4 forms of short neuropeptides F (AQRSPSLRLRFa; SPSLRLRFa; KPQRLRFa; KPQRLRFGR), 2 intermediates of AKH (pQLTFSPDWa; pQLTFSPDWGK-OH), myosuppressin (TDVDHVFLRFa) and corazonin (pQTFQYSRGWNa). The unidentified peak of m/z 1457.83 which is also present in the spectrum of *Neobellieria* could not yet be identified either.

The spectrum of *Sarcophaga crassipalpis* (Fig. 5) displays some extra peaks. Unfortunately, not all of them could be identified or came from contamination. The extra recognized peaks were the least preceded intermediate of AKH (pQLTFSPDWGKR-OH), pyrokinin (VPWTPSPRLa) and CAPA-PP-1 (SDLDSVSEGRH-OH). Some low intensity signals are Yamides and AKH with sodium or potassium adducts.

3.5. Differential peptidomics

The quantitative differential analysis of the peptides present in CC was performed by comparing *corpora cardiaca* of flies in the different digestion stages. In the first condition, samples contained a pool of 50 CCs of sugar fed flies. This extract was still fully activating digestive midgut activity and was compared with the other pool of 50 CCs of flies dissected after 5 hours ppf that was no longer stimulating digestion.

Replicates of almost no neuropeptide forms any kind of similar group of peaks among one condition as the variation in almost all biological replicas in each condition was very big. Nevertheless there was one peptide that was creating a much more regular cluster: adipokinetic hormone. It occurred in several forms of different intermediates, different isotopes and different posttranslational modifications (Table 1). All of these 16 characterized forms of AKH form higher peaks in the first condition and lower in the second one. The difference in all forms ranges between 16 to 63%. The result is clearly showing the decrease of AKH after 5 hours ppf.

3.6. *In vivo* stimulus of AKH on midgut activity

Liver primed flies were decapitated and subsequently injected with 100 ng DmAKH in DMSO/Ringer, the negative controls of decapitated liver fed flies were injected with the same mixture of DMSO and Ringer's solution. Measurement of the midgut enzymatic activity evidenced the increase in the amount of digestive proteases (Fig.5). The factorial ANOVA ($R^2=0.94$) and the results of Tukey post-hoc test show the significant difference between liver fed flies and decapitated liver fed flies starting at 2 hours ppf ($p_2=0.00058$; $p_4=0.00014$; $p_6=0.00014$) and also between decapitated liver fed flies and decapitated injected flies also starting at 2 hours ppf ($p_2=0.04406$; $p_4=0.00014$; $p_6=0.00014$). As expected proteolytic activity in midguts of liver fed flies and decapitated injected flies at 2 and 4 hours ppf was comparable ($p_2=0.74154$; $p_4=0.52873$). AKH was also injected in sugar fed non decapitated flies and here as well the digestive activity was increased to even higher values than observed in the liver fed decapitated flies as expected due to the lack of competing liver proteins in the samples (data not shown). Nonetheless, the direct incubation of the midgut or even the whole gut in medium containing different concentrations of DmAKH did so far not show any stimulating activity (data not shown).

In order to further substantiate our observation of AKH being an elicitor/enhancer of proteolytic activity in the midgut of protein fed anautogenous flesh flies we tested the dose response of injected AKH on the elicited proteolytic activity in the midgut. Flies were first fed with liver, decapitated and subsequently injected with different amounts of AKH (0 ng, 10 ng, 100 ng, 500 ng, 1 μ g) (Fig.6). Student T-test confirms differences between flies injected with 0 ng versus flies injected with 10 ng, 100 ng, 500 ng and 1 μ g ($p_{10,100,500,1}=0$) (Fig.7)

4. Discussion

It is evident that a protein meal increases digestive activity in all anautogenous insects. Flesh flies fed with the complex protein meal doubled the measured proteolytic activity in the gut. Although the

increase is considerable, the rise is not so spectacular as it is the case for mosquitoes in which the blood meal elicits the activity of trypsin and chymotrypsin to increase about 10 times (Okuda *et al.*, 2005). Anautogenous flies, *Sarcophaga crassipalpis*, did not respond to a free amino acids meal devoid of any protein, as these data repeats the findings in anautogenous *Aedes aegypti* (Gulia-Nuss *et al.*, 2011). Our results clearly suggest that the process of digestion is stimulated by a head factor most probably peptidic in nature. Altogether these data insinuate that this factor is starting to be released between 3 and 4 hours post liver meal.

The *corpora cardiaca* not only represent a major neurohaemal organ by which neurosecretion is stored and released but as well contains a glandular part that is responsible for the intrinsic synthesis of neuropeptides. Neuropeptides present in CC are: Yamides that are supposed to be a homolog to an amidated eclosion hormone (Zoephel *et al.*, 2012), short neuropeptides F with the main function of regulating feeding behavior in *Drosophila melanogaster* (Carlsson *et al.*, 2013), myosuppressin that is inhibiting midgut and hindgut contractions in *Rhodnius prolixus* (Lee *et al.*, 2012), corazonin which is involved in regulation of stress responses in *Drosophila melanogaster* (Kapan *et al.*, 2012), pyrokinin that stimulates hindgut contraction in *Tenebrio molitor* (Pawel *et al.*, 2012) and AKH. Almost all of these molecules are in some way related to feeding behavior which means that they can also be involved in the process of digestion. It is already reported that in the American cockroach, *Periplaneta americana*, short neuropeptide F inhibits protease activity (Mikani *et al.*, 2012). Not only peptides but also protein hormones can be involved in its regulation. Late trypsin activity can be directly up-regulated by OEH and by insulin-like peptide 3 (ILP-3) in blood fed mosquito, *Aedes aegypti*, and this insulin activity is synergized by free amino acids (Gulia-Nuss *et al.*, 2011). Following completion of vitellogenesis, trypsin can be transcriptionally down regulated by AeaTMOF in *Aedes* and by NebTMOF in the flesh fly, *Neobellieria bullata* (Borovsky *et al.*, 1996).

The unknown peak of 1457.89 m/z is also present in the MALDI-TOF MS spectrum of blow fly, *Protophormia terraenovae*, reared under the similar conditions; however it disappears in the peptidome of the same flies reared under the other diapause-including condition (Inosaki *et al.*, 2010).

The digestive process in all experiments was synchronized by the moment of accessibility of liver. Flies attracted by the smell of meat immediately started eating. Whereas both our decapitation and rescue experiments using extracts of CC derived from flies at different time points post liver feeding strongly suggest either a complete release or *in situ* inactivation of the CC factor responsible for stimulating digestive activity in the anautogenous flesh fly, our differential peptidomics results could not reveal the 100% differential presence of such peptide. Nevertheless there was one molecule of which the replicas formed 2 different clusters for both studied conditions: AKH. The difference in AKH amounts between CC from sugar fed and liver fed flies indeed was quite big and ranged up to 63% in some individual measurements whereas the normal release level of this peptide in locusts ranges within 2-6% (Diederer *et al.*, 2002). Although AKH did not stimulate proteolytic activity of isolated midguts *in vitro*, the evidenced *in vivo* dose-response, displaying an optimum dose of 100ng AKH per fly substantiates our finding as do the findings of AKH affecting enzymatic activity in both the midgut (Kodrik *et al.*, 2012) and salivary glands (Kodrik *et al.*, 2012) in the firebug, *Pyrrhocoris apterus*. However, since the peptidomics data of this study focused only upon CC derived peptides within the range below 2 kDa we have to believe that a follow up study exploring the higher molecular mass ranges of peptides/small proteins

present in the acidic methanolic corpora cardiaca extract might allow the additional discovery of a flesh fly specific gonadotrophin as equivalent of OEH, which so far is identified in mosquitoes only.

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537

	m/z	Charge	Average amount of condition 1	Average amount of condition 2	Sequence	Modifications
1	469.23	2	31258.77	19926.64	TFSPDWGK	-
2	581.28	2	13368794	9154103	QLTFSPDWGK	[N-term] Gln->pyro-Glu
3	864.43	1	51633.99	42410.56	LTFSPDW	[C-term] Amidated
4	650.30	1	175992.1	111939.9	FSPDW	[C-term] Amidated
5	581.28	2	197978.1	77268.87	QLTFSPDWGK	[N-term] Gln->pyro-Glu
6	751.34	1	49202.78	37677.43	TFSPDW	[C-term] Amidated
7	418.70	2	125023.6	77035.45	FSPDWGK	-
8	1161.55	1	11636519	8078390	QLTFSPDWGK	[N-term] Gln->pyro-Glu
9	488.23	2	43314.36	24171.04	QLTFSPDW	[N-term] Gln->pyro-Glu [C-term] Amidated
10	488.23	2	315236.7	261581.7	QLTFSPDW	[N-term] Gln->pyro-Glu [C-term] Amidated
11	659.33	2	2692335	1990605	QLTFSPDWGKR	[N-term] Gln->pyro-Glu
12	975.45	1	8928380	7455855	QLTFSPDW	[N-term] Gln->pyro-Glu [C-term] Amidated
13	439.89	3	13374.58	4948.895	QLTFSPDWGKR	[N-term] Gln->pyro-Glu
14	589.79	2	778142.1	562519.5	QLTFSPDWGK	-
15	497.24	2	7461.397	11327.95	QLTFSPDW	-
16	525.77	2	64779.63	51724.51	LTFSPDWGK	-