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Functional and cellular changes after royalactin treatment in *C. elegans*

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Thesis presented in
fulfillment of the requirements
for the degree of Master of Science
in Biochemistry and Biotechnology

Academic year 2014-2015

Acknowledgements

First and foremost I would like to thank my promotor Prof. Dr. Liliane Schoofs for the opportunity to complete my thesis a the research group of Functional Genomics and Proteomics. I would also like to thank my co-promotor Liesbet Temmerman.

I would like to express my sincere gratitude to Giel Detienne for his involved supervision, his scientific insight and patience. His contribution to this thesis cannot be overestimated.

My thanks also go out to the entire research group of Functional Genomics and Proteomics. Working in this lab was honestly fun.

Finally, I should not forget to thank all those who are dear to me and supported me: my parents, friends, family and girlfriend.

Summary

Royalactin is a glycoprotein present in royal jelly, a food source reserved for honeybee larvae that are to develop into queens. Royalactin is the primary agent in queen differentiation, a process resulting in long-lived ultra-fertile queen bees. The effect of royalactin is not limited to honeybees however, as the nematode *C. elegans* shows an increased life- and healthspan when fed with royalactin. In this thesis, the functional and cellular changes after royalactin treatment in *C. elegans* are characterized further.

Several transcription factors could possibly propagate the royalactin signal once it is initiated by (direct or indirect) interaction with the EGFR. Two of these transcription factors, SKN-1 and EOR-1 (and its binding partner EOR-2) are studied through fluorescence intensity quantification of their respective targets *gst-4* and *hsp-16.2* with GFP-fused transcriptional constructs.

A wider picture of the cellular changes after royalactin treatment is achieved by performing a 2D-DIGE proteomics experiment, leading to the identification of 35 differentially expressed proteins in royalactin-fed worms when compared to untreated worms. Many of these proteins are associated with the metabolism, spiking our interest in the metabolism of royalactin-fed worms. The respiration rate and thermal activity of royalactin-fed worms are measured, but do not yield significant results (yet).

Aging and stress are often intimately linked, leading us to investigate the resistance of royalactin-fed worms against thermal, oxidative and reductive stress by conducting survival assays and monitoring the expression of mitochondrial and ER-stress associated chaperones. Overall, royalactin-fed worms survive the stress assays more successfully than their untreated counterparts and chaperone proteins are less abundant, suggesting a more effective but less severe stress response in royalactin-fed worms.

Samenvatting

Royalactine is een glycoproteïne dat aanwezig is in koningengelei, de exclusieve voeding van bijenlarven die voorbestemd zijn zich tot koninginnen te ontwikkelen. Royalactine speelt een centrale rol in het differentiatie-proces dat resulteert in lang-levende ultra-vruchtbare koninginnenbijen. Het effect van royalactin is echter niet beperkt tot honingbijen, want ook de nematode *C. elegans* vertoont een verhoogde levensduur na behandeling met royalactine. In deze thesis worden de functionele en cellulaire veranderingen na behandeling met royalactine in *C. elegans* verder uitgewerkt.

Verschillende transcriptiefactoren zouden het royalactine-signaal in de cel kunnen propageren nadat het geïnitieerd wordt door (directe of indirecte) interactie met de EGFR. Twee van deze transcriptiefactoren, SKN-1 en EOR-1 (en zijn bindingspartner EOR-2) worden bestudeerd door fluorescentie-kwantificatie van hun respectievelijke doelwit-genen *gst-4* en *hsp-16.2* met GFP-gefusioneerde transcriptionele constructen.

Een breder beeld van de cellulaire veranderingen na royalactine-behandeling werd verkregen door het uitvoeren van een 2D-DIGE proteomics experiment. Dit leidde tot de identificatie van 35 proteïnen die verschillend tot expressie kwamen in wormen die behandeld werden met royalactine. Veel van deze proteïnen zijn geassocieerd met het metabolsisme, wat onze interesse wekte in het metabolisme van royalactine-behandelde wormen. De respiratie en thermale activiteit van royalactine-behandelde wormen werden gemeten, maar leverden (nog) geen significant resultaten op.

Veroudering en stress worden vaak met elkaar gelinkt, wat ons ertoe leidde de resistentie van royalactine-behandelde wormen tegen thermale, oxidatieve en reductieve stress te onderzoeken. Dit gebeurde door het uitvoeren van overlevings-*assays* en door het bestuderen van de expressie van chaperone proteïnes die geassocieerd zijn met mitoschondriale en ER stress.
Over het algemeen zijn royalactine-behandelde wormen in staat de stress-behandelingen beter te overleven dan onbehandelde wormen en zijn hun chaperone-proteïnes minder abundant. Dit suggereest een efficiëntere en minder drastische reactie op stress in royalactine-behandelde wormen.

Abbreviations

10-HAD	10-hydroxy-2-decenoic acid
20E	20-Hydroxyecdysone
2D-DIGE	2-dimensional difference gel electrophoresis
ACN	acetonitrile (nog toepassen!)
ANOVA	analysis of variance
BCA	bicinchoninic acid
DTT	ditiothreitol
EGF	epidermal growth factor
EGFR	EGF receptor
ER	endoplasmic reticulum
FUdR	fluorodeoxyuridine
GO	gene ontology
hEGF	human EGF
HPLC	high-performance liquid chromatography
IAA	iodoacetic acid
IGF-1	insulin/insulin-like growth factor 1
InR	insulin receptor
IPG	Immobiline pH-gradient
JH	juvenile hormone
LB	Lysogeny Broth
MALDI	matrix-assisted laser desorption/ionization
MALDI-TOF	matrix-assisted laser desorption/ionization - time of flight
mEGF	mouse EGF
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MQ	Milli-Q H2O
MRJP	Major royal jelly proteins
mrjpl	mrjp-like
NGM	Nematode Growth medium
PMF	peptide mass fingerprinting
pRJ	protease-treated RJ
RJ	Royal Jelly
RNAi	RNA interference
ROI	Region Of Interest
SDS	sodium dodecyl sulfate
SDS-PA	SDS-polyacrylamide
SDS-PAGE	SDS polyacrylamide gelelectrophoresis
SEM	standard error of mean
STRING	Search tool for the retrieval of interacting genes/proteins
TAM	thermal activity monitoring
TEMED	tetramethylethylenediamine
TFA	trifluoroacetic acid
TOF	time of flight
TOR	target of rapamycin

UPR	ufolded protein response
UPR(mt)	mitochondrial ufolded protein response

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A.Literature study

1. Introduction

1.1. Anti-aging - living longer and healthier lives

Aging is a complex and seemingly inevitable process that, despite being extensively studied in the past decades, still holds many questions. The benefits that come with answers to these questions are self-evident, as about two thirds of all deaths globally and 90% of deaths in industrialized countries can be attributed to age-related diseases[1]. Examples of these diseases are cancer, type II diabetes and cardiovascular diseases.

Over the past years, many chemical compounds, dietary supplements and treatments have been heralded as "the cure for aging" by popular science. The question appears to be *when* rather than *if* we will ever substantially slow down aging. However, we still have a long way to go before anything like wholesome anti-aging therapy can be applied in humans[2]. This is because aging is not likely to be a process that can be reversed wholly by a single treatment, much less by a single "miracle molecule". This is illustrated by the recently established hallmarks of aging[3], which include epigenetic alterations, loss of proteostasis, altered intercellular communication, mitochondrial dysfunction and others. Going through these hallmarks of aging, one notices quickly that there are very little biological topics that are not in some way related to aging, be it causal or effectual. Therefore, expecting one substance to single-handedly reverse the effect of aging in its entirety seems like wishful thinking.

Still, substances that could delay the onset of age-related changes are key in furthering our understanding of how aging works. Many of these substances belong to the group of the so-called *nutraceuticals*, a term coined by Stephen DeFelice[4]. These pharmaceutical nutrients are not just healthy supplements to an established diet, but also aid in the prevention or treatment of one or more conditions or diseases[5]. In our case, this condition would be aging, and the functional decline that accompanies it.

Examples of potential anti-aging substances that have been studied in the past include rapamycin, metformin and resveratrol, all of which can extend lifespan in a significant manner in multiple model organisms[6,7]. However, the efficacy of these compounds in higher organisms should not be taken for granted. For some of these compounds (e.g. rapamycin) there is evidence of their effectiveness in higher organisms and even in humans, while others (e.g. resveratrol) remain the topic of controversies[8].

A new player in this regard is royalactin, a royal-jelly derived protein that mediates queen differentiation in honeybees. Royalactin extends lifespan in fruit flies[9] and in nematodes[10]. This protein will be the main subject of this project.
By influencing the vast web of biological interactions that is aging with specific compounds like royalactin, we can learn, discover and perhaps someday develop an effective strategy for living longer and healthier lives.

1.2. *C. elegans* - a model for aging

The nematode *C. elegans* was the first multicellular organisms to have its genome sequenced and has been used as a model for several biological research areas in the past decades, ranging from proteotoxicity[11] to drug target identification[12], environmental toxicity studies[13] and neurobiology[14]. The advantages of using this worm as a model are numerous. First of all, many aspects of its biology are well understood and provide -in many cases- a simple approach to a difficult problem. Furthermore, *C. elegans* is transparent, which provides evident advantages when observing fluorescent proteins under the microscope *in vivo*. Quantifying and locating expression of GFP-fused proteins of interest is rather easy to perform in *C. elegans*.
Whereas knocking down genes in other organisms can be a cumbersome task[15,16], in *C. elegans*, it is remarkably easy through RNA interference (RNAi). By simply feeding the worms with or soaking them in a strain of bacteria that expresses the specific dsRNA, one can knock down the corresponding gene[17]. These bacteria strains are readily available for ordering online, and are often kept in an internal library in the lab.

Our interest goes out to using *C. elegans* as a model for aging, an application that might not seem obvious to the uninitiated reader. However, *C. elegans* shows some fundamental similarities with mammals when it comes to aging. When worms age, they exhibit muscle atrophy[18], reduced agility[19] and a decline in cognitive abilities[20], just like mammals. Furthermore, simple genetic manipulations (e.g. deletion of a single gene) in *C. elegans* have shown to dramatically impact their lifespan, the most well-known being a *daf-2* mutant, which lives twice as long as wild type[21].
For these reasons, and for its aforementioned ease of use, *C. elegans* has been successfully used as a model for aging for decades[22]. *C. elegans* has been used as a model to study

complex human age-related (neurodegenerative) diseases including Alzheimer's, Parkinson's and Huntington's disease[23,24].

The short lifespan of this nematode is an added advantage in aging research; the larvae complete their development within three days and the mean lifespan of an adult worm is only two to three weeks when cultured at 20°C[11]. Consequently, studying lifespan and aging-related phenomena in *C. elegans* is relatively easy and fast.

Specifically, we would like to use *C. elegans* as a model to test the effects and mechanisms of a novel longevity-promoting honeybee protein called royalactin. The suggested involvement of well-conserved signaling pathways[9] implies that the workings of royalactin could be transferable to different organisms, including non-insect models such as *C. elegans*.

C. elegans has been used extensively in the past to study the lifespan-extending effects of several other natural substances, including blueberry polyphenols[25], *Ginkgo biloba* plant extracts[26], natural antioxidants, alpha-lipoic acid[27] and others[7].

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2. Royal jelly and royalactin

2.1. Royal Jelly - a product of the honey bee

Royal jelly (RJ) is a nutrient-rich mixture secreted by the hypopharyngeal and mandibular glands of *Apis mellifera*, the western honey bee. The ingestion of RJ by bee larvae has been known to regulate caste differentiation in honey bees for centuries[28]. Initially, all larvae, regardless of their future caste, are fed with RJ, but after three days, larvae that are destined to become worker bees are switched to a more conventional food source[29].

In contrast, larvae that are fed RJ exclusively will differentiate into queens. Queen bees have a shorter developmental time from egg to adult and a larger body size than worker bees. They also have a longer lifespan, reaching an average age of 1-2 years, whereas worker bees only live for around 3-6 weeks[30]. Queens lay approximately 1,500 eggs per day, which is in stark contrast with the mostly sterile worker caste.

It is important to note that caste differentiation occurs mainly because of this special diet of RJ and only for a small part due to genetic differences in the larvae[31]. Thus, RJ, a mixture of water (50-60%), sugar (15%), proteins (18%), lipids (3-6%) and trace amounts of minerals and vitamins[32], must contain some very special ingredients indeed.

2.2. Royal Jelly - a myriad of properties

The function of royal jelly is not limited to queen differentiation, longevity and fertility. RJ also possesses other physiological effects, many of which were already discovered decades ago[33]. RJ can have anti-tumor[34] anti-inflammatory[35] as well as vasodilative and hypotensive[36] activity.

Furthermore, several peptides and proteins that are part of RJ have a clear antibacterial or antifungal activity[37,38]. Among these are royalisin and jelleins. Royalisin is a 51-residue 5523 Da peptide with 3 intramolecular disulfide bonds that has potent antibacterial activity against Gram-positive bacteria[37]. Jelleins are a family of short peptides that have anti-bacterial activity against Gram-positive as well as Gram-negative bacteria[38].

2.3. Royal Jelly in *C. elegans*

Royal jelly has been shown to extend lifespan in fruit flies[39], mice [40] and in *C. elegans*[41].

Honda et al. examined the effect of RJ and protease-treated RJ (pRJ) on the lifespan of *C. elegans*. They observed a modest increase in average lifespan, ca. 7-9 %, in worms fed with 10 µg/mL RJ from the adult stage onward. Interestingly, pRJ, a version of RJ treated with protease N "Amano", also increased the lifespan of the worms with 7-18 %. This suggested that the protein component of RJ does not play a critical role in lifespan extension, a view that has been met with criticism in the following years. Using a microarray, the genome-wide changes in gene expression after treatment with the most active fraction of pRJ were monitored. It was found that genes encoding for several insulin-like peptides (*ins-9, ins-20* and *ins-23*) were significantly up- or downregulated in pRJ-treated worms[41], corresponding to a view where decreased insulin signaling through the DAF-16 transcription factor leads to increased lifespan (Kenyon et. al 2005). These findings were strengthened by the fact that *daf-16* deletion mutants showed a lesser (but still significant) extension of lifespan upon pRJ treatment. Furthermore, pRJ treatment facilitated DAF-16 translocation to the nucleus, a sign of decreased insulin signaling[42]. This indicated that pRJ exerts its effects both through the insulin signaling pathway and another, DAF-16 independent pathway. Honda et al attributed these effects of RJ mainly to the presence of the fatty acid 10-hydroxy-2-decenoic acid (10-HDA).

2.4. Royal Jelly - content and functional compounds

As royal jelly is a complex mixture of sugars, proteins and fatty acids, it is unlikely that RJ realizes its lifespan-promoting and other effects through one mechanism only. This is why recent studies have focused on identifying and functionally analyzing the individual components that make up royal jelly.

Several studies aimed to isolate the ingredients that gave royal jelly its properties. At first, a limited role was suspected for the proteins of RJ, suggesting more critical roles for the unique fatty acids of the mixture[43]. These fatty acids (10-HAD, 3,10-dihydroxydecanoic and sebacic acid) mediate estrogen-signaling by modulating the recruitment of estrogen receptors to their effective pS2 promoter. Although estrogen receptor recruitment plays a pivotal role in many developmental pathways, it is unlikely that the lifespan-extending effects of royal jelly are solely caused by the estrogen receptor modulating workings of these fatty acids[44,45].

Instead, the protein component of RJ was revealed to play a key role in the observed effects of RJ, specifically the so-called major royal jelly proteins.

2.5. Major Royal Jelly Proteins

There are nine major royal jelly proteins (MRJPs) which compose the bulk (82-90%) of the protein fraction of royal jelly. These proteins are produced in the hypopharyngeal glands of nursing honey bees and were first described by Schmitzova et al.[29]. Their monomeric molecular mass lies between 49 and 87 kDa. Two of these proteins, MRJP1 and MRJP2, were found to be the main allergens in RJ[46]. In the honey bee genome, which was published in 2007 [47], one can see that all MRJP encoding genes are found in a 60-kb tandem array.

MRJP-encoding genes are not only found in the *Apis mellifera* genome, but also in several other members of the *Apis* genus, and even in members of the *Bombus* genus, although their function in these species does not involve the production of RJ[48]. MRJP-encoding genes belong to the *mrjp/yellow* gene family, members of which are encountered in bacteria, fungi and insects[49,50]. Duplications of the ancestral *yellow-e3* gene have given rise to the *mrjp* genes of *Apis* genus[51]. The MRJP tandem array in *A. mellifera* is flanked by five genes encoding Yellow-family proteins. The Yellow proteins appear in many insects, where they fulfil a myriad of functions, most of which are in some way involved in the enabling of reproductive maturation.

Apart from *mrjp* and *yellow* genes, the *mrjp/yellow* family also contains several *mrjpl* (mrjp-like) genes that are present in several non-*Apis* species, such as the parasitoid wasp *Nasonia vitripennis*[52] and several ant species[51].

2.6. Royalactin in honeybees - a study in Drosophila

One of the MRJPs in particular, MRJP1, later termed royalactin, is believed to be the prime mediator of queen differentiation in honeybees[9]. It was first indicated as a marker for the freshness of RJ, as it degraded gradually over the course of storage time[53].

Royalactin is the most abundant protein in RJ. It is a 57 kDa glycoprotein and has been shown to induce queen differentiation in honeybees[9]. Because *A. mellifera* is not often used as a model system, conventional tools for studying the mechanisms behind royalactin action in the honeybee were lacking. Researchers then turned to *D. melanogaster*, an extremely well documented model organism, for the application of several tools that allowed them to study royalactin. It is important to note that, despite its reasonable evolutionary distance from *A. mellifera*, *D. melanogaster* shows many differences with the honeybee as well. *Drosophila* is not a social insect and does not show any caste differentiation whatsoever. Surprisingly however, *Drosophila* larvae which were raised on a diet enriched with royalactin showed a queen-like phenotype: faster growth, extended lifespan, larger adult size, and increased egg-laying capacity[9]. This result raised a very important question, namely how far does the effect of royalactin reach along evolutionary distances? If we can extend life- and health span of fruit flies with royalactin, then what about non-insect models?

2.7. Royalactin in other models

In 2001, Kamakura et. al already showed the effect of royalactin on rat hepatocytes[54]. Rat hepatocytes grown up in a medium enriched with royalactin (at that time still termed "the 57kDa protein in royal jelly") showed increased DNA synthesis, prolonged cell proliferation and increased protein production. They also performed these experiments with RJ that was stored at 40°C for 7 days, thereby degrading all the royalactin present in the mixture. Other bioactive components of RJ such as 10-HDA, do not degrade nearly as much with storage[53]. Treatment of the liver cells with heat-treated (royalactin-free) RJ did not show a significant increase in DNA synthesis, proliferation or protein production, suggesting that royalactin was indeed the component of RJ that caused the observed effects. The authors also noted the similarity between the effects of royalactin and epidermal growth factor (EGF), an important

growth factor that stimulates cell growth, proliferation and differentiation. They speculated that royalactin might exert an increase in hepatocyte viability through apoptosis suppression, a mechanism under the control of EGF[55].

In 2014, it was found that royalactin extends the lifespan of the nematode *C. elegans*[10]. Royalactin treated with thermolysin did not extend the lifespan of the worms, while deglycosylated and mildly heat-treated royalactin (at 37°C) did. This indicates that the N-linked oligosaccharides of the glycoprotein are not essential for its lifespan-extending effects, while the proteinaceous part is. It was also found that royalactin-treatment caused higher swimming activity in adult worms, suggesting that royalactin has a positive effect on healthspan as well.

Royalactin has been studied in several cell lines, including rat hepatocytes[54] and murine macrophages[56] but has, to our knowledge, only been studied in the following organisms *in vivo*: *C. elegans*[10], *A. mellifera* and *D. melanogaster*[9]. A recent study on rats and rat cells was also conducted but focused on the cholesterol-lowering properties of royalactin rather than lifespan-extending functions[57].

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3. A possible mechanism of royalactin action

3.1. Aging is regulated through several pathways

In modern biology, the cause of aging is regarded as a sum of accumulated damage over the course of a lifespan together with hardwired aging-regulating signaling pathways[3]. We will discuss the three most relevant of these pathways: insulin/IGF-1 signaling, TOR signaling and the EGFR pathway and their possible role in royalactin-induced lifespan extension. The interactions of these pathways are schematized briefly in figure 1.

3.2. Insulin/IGF signalling

The most well-known pathway to regulate aging is the insulin/insulin-like growth factor (IGF-1) pathway. A striking example of the involvement of this pathway in aging is found in *C. elegans*, where a single mutation in the *daf-2* gene, coding for an insulin receptor, doubles

the lifespan of the animals through translocation of the transcription factor DAF-16 to the nucleus and transcription of longevity-promoting genes[21]. Modulating and studying aging through genetic manipulation is thus very possible. The regulation of aging through the insulin/IGF-1 pathway is present in many observed species, including *D. melanogaster* and *Mus musculus*[58].

Importantly, it was found that royalactin, the protein that is the focus of this project, most likely does not exert its effect through insulin signaling in *D. melanogaster*, as insulin receptor (InR)-knockdown still induced queen-like differentiation upon feeding with royalactin[9]. The action of royalactin must thus be through a different receptor than the InR, although a more downstream role for some elements of the insulin/IGF-1 pathway cannot be excluded. As will be explained below, the EGF receptor plays a vital role in the workings of royalactin.

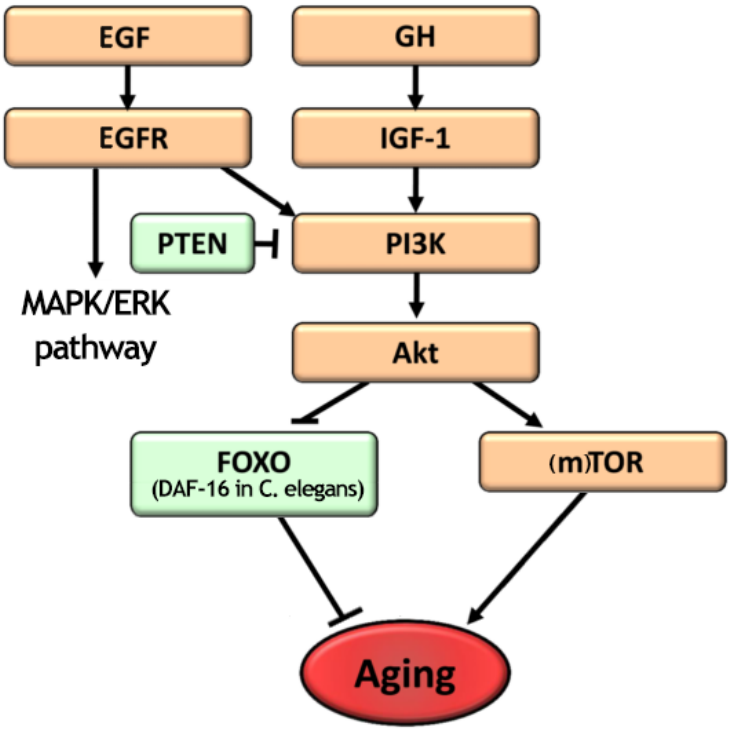

Figure 1: The different signaling pathways involved in aging and their interactions. Adapted from (López-otín et al. 2013)[3].

3.3. TOR signalling

Another player involved in the regulation of aging is the Target of rapamycin (TOR). TOR is a serine/threonine kinase that controls many aspects of growth in the cell. TOR and its

mammalian ortholog mTOR interact with various other proteins to form active complexes which exert their function on the cell.

Inhibition of TOR has been shown to increase lifespan in *D. melanogaster* and *C. elegans*[59,60]. As TOR promotes growth and development in healthy cells when copious amounts of nutrients are present in the environment, its inhibition is assumed to shift the cell's focus towards stress-resistance and survival, increasing the durability of the organism[61]. It was also shown that TOR-inhibition does not further extend lifespan in *C. elegans daf-2* mutants, which might point to similar downstream effectors of the aging-related aspects of the TOR and the insulin/IGF-1 pathways[61,62].

A third signaling pathway that influences aging is the epidermal growth factor receptor (EGFR) pathway. It will be discussed in more detail below, as it has been shown to be a key mechanism through which royalactin exerts its lifespan-extending function.

3.4. Epidermal growth factor

EGF is a naturally occurring growth factor that stimulates cellular growth, differentiation and proliferation by binding to the extracellular region of the EGFR. EGF was first detected as a substance that stimulated precocious eyelid opening and tooth eruption in mice[63]. This was caused by increased keratinisation and epidermal growth in the new-born animals, hence the name epidermal growth factor. For discovering EGF, Stanley Cohen and Rita Levi-Montalcini received the 1986 Nobel Prize in Physiology or Medicine.

Mouse epidermal growth factor (mEGF) was the first form of EGF that was described. It is a short polypeptide made up of 53 amino acid residues, connected through three intermolecular disulfide bonds[64].

Homologs of EGF were discovered in many other organisms, including humans and *C. elegans*. Human EGF (hEGF) exhibits very similar chemical, physical and functional properties to mEGF. In *C. elegans*, EGF is encoded by the *lin-3* gene while the *let-23* gene codes for the EGF receptor.

3.5. The EGFR pathway - an overview

A ligand (e. g. EGF) exerts its effect through binding of the EGFR on the cell surface[65]. Upon binding of the ligand, EGFR undergoes conformational changes which allow it to dimerize and become internalized in the cell. This dimerization activates the tyrosine kinase activity of the EGFR, which autophosporylates tyrosine residues on the receptor itself. These phosphorylated tyrosine residues act as binding sites for the growth factor receptor-bound protein 2 (GRB2) adaptor protein and others. The signal then cascades into the MAPK/ERK pathway, a well-known signaling cascade that ultimately results in the mentioned effects of EGF: cellular growth, differentiation and proliferation[66]. The workings of the EGF signaling pathway are summarized in figure 2.

Important to note is that EGF is only one of the many ligands that can bind the EGFR. Other known ligands include transforming growth factor-alpha, amphiregulin, heparin-binding EGF and betacellulin[65]. In *C. elegans* however, the only known ligand that binds to LET-23 is LIN-3.

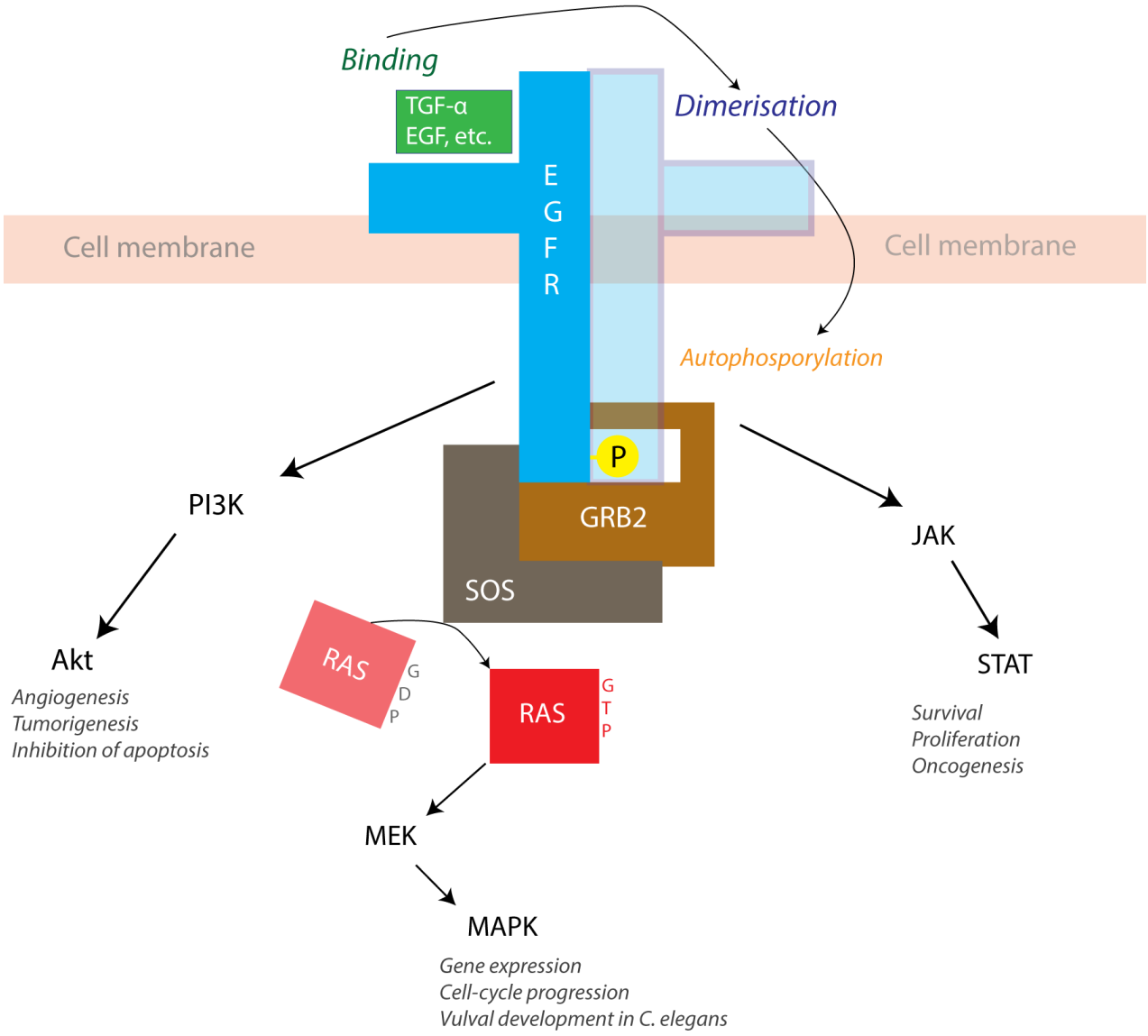

Figure 2: After ligand binding, dimerization and autophosphorylation, the EGFR signal propagates itself through multiple secondary signaling pathways, including the MAPK/ERK pathway, the JAK-STAT pathway and the PI3K-AKT-mTOR pathway.

3.6. The EGFR pathway and aging

Recent studies unexpectedly found a role for the regulation of healthy aging in *C. elegans* through the EGFR pathway[67]. Originally, Iwasa et al. were looking for secreted proteins with sequence-similarity to the extracellular domain of the insulin receptor that promoted locomotory healthspan in *C. elegans*. Two such proteins, named HPA-1 and HPA-2 for "high performance in advanced age", were found. It was found that HPA-1 and HPA-2 conferred their effects through the EGFR-pathway, rather than the much more expected insulin/IGF-1 pathway[67]. This led the EGFR-pathway to be investigated as a novel regulator of healthy aging.

It was observed that gain-of-function mutants in the *C. elegans* EGFR (LIN-23) showed an increase in median lifespan by 29% and signs of increased late-life vigor. The worms maintained muscle integrity, pharyngeal pumping (i.e. muscular contraction of the pharynx that correlates with food uptake) and swimming movement during their later life[67]. Overall, their healthspan (and lifespan) was increased considerably compared to control worms. Worms wherein the function of LIN-23 was knocked down showed the opposite phenotype: their mobility and overall survival rate in mid-to late life was lower than in control worms[67]. More specifically, Iwasa et al. showed that a certain downstream branch of the EGFR pathway was involved. A branch of the EGFR signaling pathway including the phospholipase PLC-γ and the inositol triphosphate (1,4,5) receptor IP3R were shown to be the acting part in lifespan and healthspan extension in the worms. IP3R gain-of-function mutants showed an increased health- and lifespan, while reduction-of-function mutants in IP3R reduces survival and late-life vigor, much like the gain- and reduction of function mutants did in *let-23*. Furthermore, knocking down IP3R through RNAi nullifies the lifespan-extending effects of gain-of-function mutations in *let-23*. This strongly suggests a role for EGFR signaling through IP3R in the modulation of healthy aging in *C. elegans*. HPA-1 and HPA-2 are secreted proteins that are suggested to inhibit binding of the EGF ligand to the EGF receptor[67]. This is summarized in figure 3.

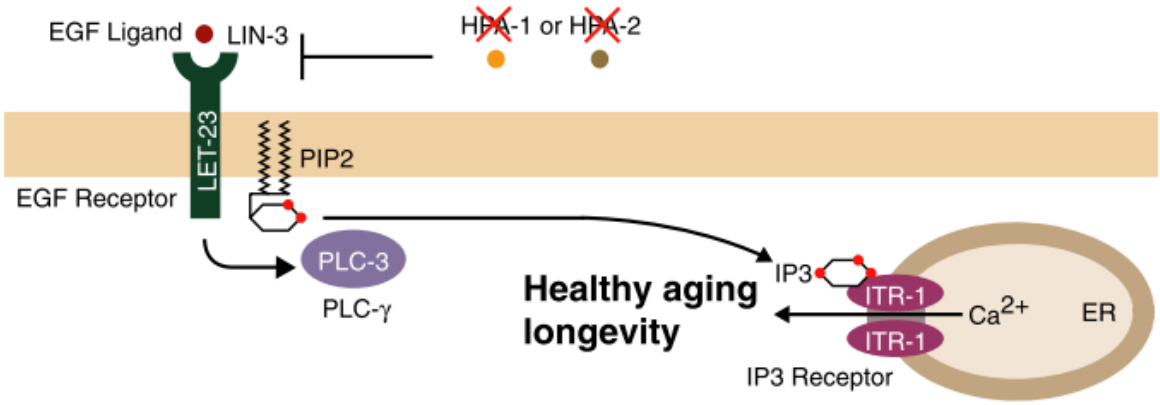

Figure 3: Binding of the EGF ligand to the EGF receptor initiates a signal that is propagated through phospholipase-gamma and ultimately results in regulating calcium homeostasis through the IP3 receptor on the ER. HPA-1 and HPA-2 are negative regulators of this system. From (Iwasa et al. 2010)[67].

IP3R regulates calcium homeostasis through release of calcium from the endoplasmic reticulum (ER). Disrupted calcium homeostasis during late life is an important controlling factor in the development of many age-related phenotypes in humans as well, such as cognitive decline[68].

A specific role for EGF in the regulation of healthy aging was found in the maintenance of healthy protein homeostasis in *C. elegans*[69], illustrated in figure 4. Through the MAPK/ERK pathway, EGF promotes the expression of the transcription factors SKN-1 and EOR-1.

SKN-1 is involved in limiting the harmful process of protein oxidation and plays a role in the restoration of redox homeostasis through the expression of a multitude of antioxidant and phase II enzymes. EOR-1, together with EOR-2, reduces the transcription of HSP-16, which regulates protein aggregation within the cell[70]. It was also shown that EGF signaling through the MAPK/ERK pathway activates the ubiquitin proteasome system, an important modulator of protein homeostasis[71]. All of these effects contribute to the maintenance of healthy protein homeostasis in aging worms.

These mechanisms are independent of the insulin/IGF-1 pathway, as mutations in the DAF-16 (FOXO-like transcription factor) or DAF-2 (insulin receptor) encoding genes did not influence health- or lifespan of *C. elegans*[69].

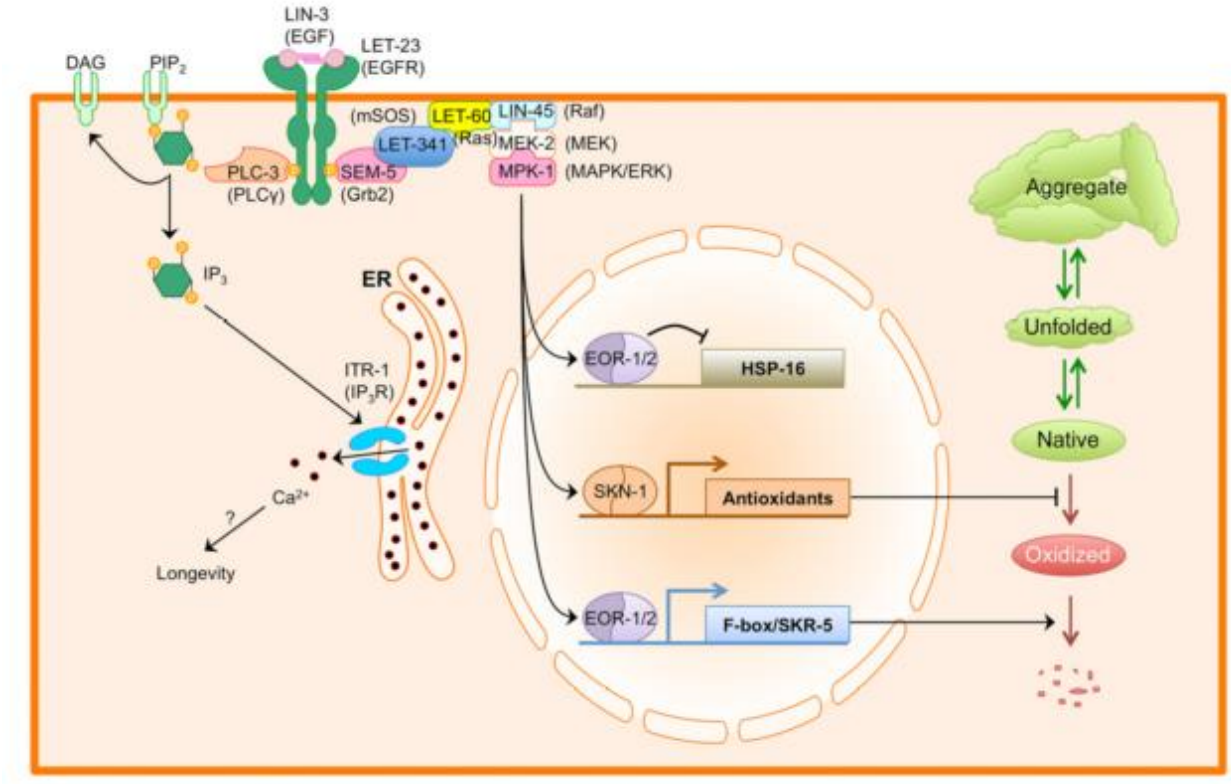

Figure 4: In adult C. elegans, EGF regulates healthy aging through maintenance of calcium homeostasis and protein homeostasis. EGF signaling through the MAPK/ERK pathway switches on the EOR-1 and SKN-1 transcription factors, which ultimately influence protein aggregation and oxidation respectively. From (Rongo et al. 2011)[69].

3.7. The EGF pathway and royalactin

After observing that royalactin acts similarly to EGF in rat hepatocytes[54,72], Kamakura knocked down the gene coding for EGFR in honey bee larvae. When rearing larvae in the presence of royalactin, *egfr* RNAi reduced adult body size and increased developmental time from egg to adult when compared to control animals that were also reared with the protein[9]. Thus, it was found that, when EGFR is knocked down, royalactin cannot induce queen differentiation in honeybees. This indicates that royalactin works in honey bees (at least partially) through the EGFR pathway described above. Upon suppressing the insulin receptor (InR), queen development proceeded normally, indicating that royalactin did not exert its effects through the insulin pathway[9].

Kamakura also showed that royalactin caused increased activity of MAPK and S6K (a ribosomal S6 kinase that regulates protein synthesis) through EGFR activation[9]. When fed with royalactin, MAPK and S6K expression increased compared to control insects, but when adding ds RNA against *egfr*, this effect was no longer observable. RNAi suppression of *TOR*, *PDK1*, *S6K* and *PI3K* showed smaller adult size compared to control queen bees.

Both royalactin and royal jelly increase the concentration of juvenile hormone (JH), vitellogenin and 20-Hydroxyecdysone (20E) in 3-day old honey bee larvae[73]. JH is an important insect hormone that regulates much of the animal's development and growth, while vitellogenin is a nutrient source for eggs. 20E is a hormone controlling molting and metamorphosis in insects.

Suppressing *egfr* with RNAi abolished the increase in JH, vitellogenin and 20E titre in 3-day old larvae[73]. Importantly, suppression of S6K did not result in any difference in JH, vitellogenin or 20E titre. Inhibition of MAPK with a specific MAPK-inhibitor PD98059 abolished high 20E titres, but not JH of vitellogenin activities.

From these results, it is possible to draw up a putative model for royalactin action in honey bees, pictured in figure 5. After (direct or indirect) interaction with the EGF receptor, the royalactin pathway splits up into at least three signaling cascades. The signal propagated by royalactin is a complex and multifaceted one that ultimately results in the phenotypical properties that make up a queen bee[73].

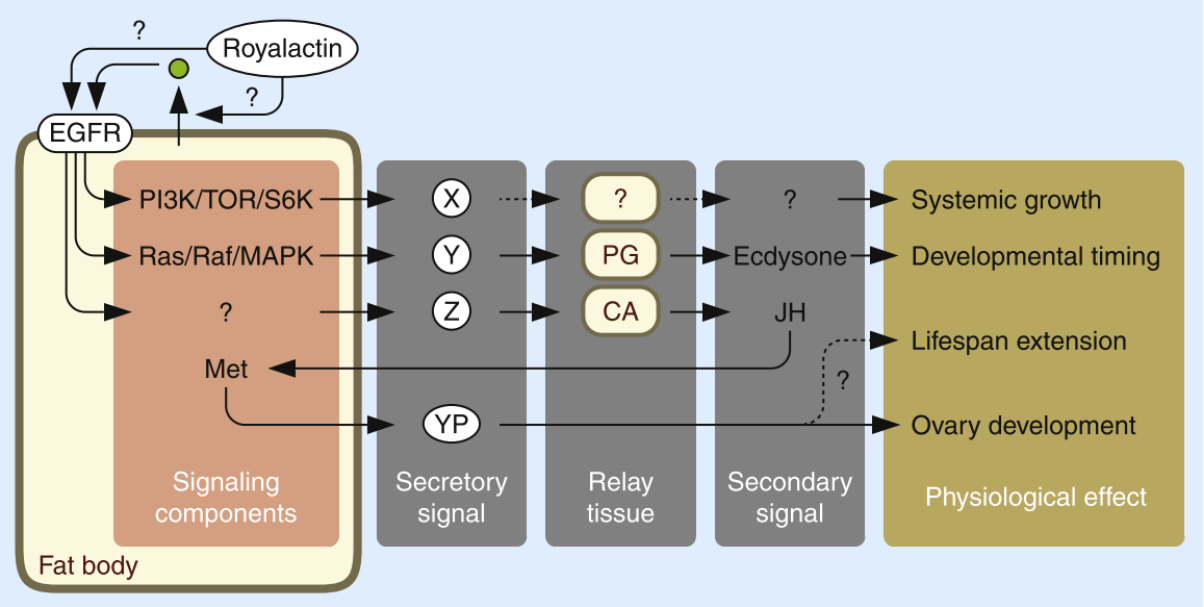

Figure 5: Signaling pathways involved in queen differentiation initiated by royalactin. Royalactin (either directly or indirectly) activates the EGFR, after which the signal splits up in at least three pathways. The signal is propagated through the unknown secretory signals X, Y and Z, which eventually result in the observed queen phenotype: enhanced systemic growth, shorter developmental timing, lifespan extension and ovary development. From (Yamanaka & O'connor, 2011)[73].

It was recently shown that royalactin exerts its lifespan-extending function through the EGFR pathway as well in *C. elegans*[10]. Knocking down *lin-3* (the EGF-gene in *C. elegans*) or *let-23* (EGFR) abolished the lifespan-extending effect, implying a central role for EGFR signaling in *C. elegans*.

3.8. Potential key molecular targets of royalactin action

The royalactin-signal initiated at the EGFR is a branched and multi-faceted signaling network, as is illustrated in figures 4 and 5. Monitoring of the expression of several key molecular targets connected to the transcription factors EOR-1, EOR-2, and SKN-1 could add to our understanding of the mode of action of royalactin in *C. elegans*.

Expression of the heat shock protein HSP-16.2 is believed to be inhibited by the transcription factor EOR-1 and its obligate binding partner EOR-2[69,71]. The levels of *hsp-16.2* expression can be observed *in vivo* through a GFP-based transcriptional reporter, i.e. a *hsp-16.2* promotor followed by *gfp*.

SKN-1 is another transcription factor that lies downstream of the receptor in the EGFR signaling pathway (see figure 4). Its expression levels can be monitored *in vivo* through a similar GFP-based transcriptional reporter with the known SKN-1 target GST-4[74,75]

4. Unresolved questions and research objectives

When it comes to royalactin action, many questions remain unanswered. We know that the EGF receptor is in some way involved. What we are still far from reaching however, is a complete picture where all the players are identified and meaningful results can be drawn for possible future applications in medicine or nutrition. In brief, we can say that there is very little that is currently known about the functional and cellular effect of royalactin in *C. elegans*.

In completing this picture, one of the first issues that needs to be addressed is the elucidation of the pathway that the royalactin signal follows downstream of the EGFR. A first step would be defining the role of the transcription factors SKN-1 and EOR-1, which can be studied through their respective targets GST-4 and HSP-16.2.

A wider picture of the cellular changes after royalactin treatment can be achieved by performing a proteomics experiment, which allows us to identify at a large number of differentially expressed proteins in royalactin-fed worms when compared to untreated worms.

Other questions that are worth researching extensively include "What is the functional part of royalactin?" With many proteins it is the case that only a small sequence of amino acid residues contains all the functional activity. This is an interesting question in itself, but becomes even more relevant with regards to possible future applications, in which the functional peptide in royalactin could be produced by genetically modified *E. coli* bacteria.

What is currently unknown as well is the effect of royalactin on *C. elegans* in matters other than life span and locomotion. What about fertility: do royalactin-reared worms produce more eggs? What about body-size? What about stress (heat, oxidative stress, reductive stress, etc.) tolerance? What about memory, learning capacity, muscle atrophy and other ageing-related capacities? In this thesis, we will try to answer some of those questions, especially the ones related to stress resistance.

To summarize, the main research objectives of this thesis are:

- to study the role of the transcription factors SKN-1 and EOR-1 in propagating the royalactin signal.
- to add to the understanding of cellular changes after royalactin treatment by identifying differential proteins through a proteomics experiment.
- to cleave royalactin and assay the bioactivity of the fragments.
- to study the resistance of royalactin-fed worms to various kinds of stress.

B. Materials and methods

1. Materials list

1.1. S-basal physiological buffer

5.85 g/L NaCl
6 g/L KH_2PO_4
1 g/L K_2HPO_4
Autoclaved before use.

1.2. M9 physiological buffer

5.8 g/L Na_2HPO_4
3.0 g/L KH_2PO4
0.5 g/L NaCl
1.0 g/L NH_4Cl
Autoclaved before use.

1.3. Phosphate buffer

108.3 g/L g KH_2PO_4
35.6 g/L K_2HPO_4
Filtered, adjusted to pH 6.

1.4. Lysogeny Broth (LB), *(E. coli* growth medium)

20 g/L LB broth (Sigma-Aldrich)
Autoclaved before use.

1.5. Soft Agar Freezing Solution

5.8 g/L NaCl
300 g glycerol
0.224 g/L NaOH
4.0 g/L agar
6.8 g/L KH_2PO_4
Autoclaved before use.

1.6. Basal Nematode growth medium (NGM)

17 g/L agar
2.9 g/L NaCl
7.5 g/L bacteriological peptone
Autoclaved and stored at 50°C before use

1.7. Hydroxylamine cleavage buffer

2M hydroxylamine.HCl
2M guanidine.HCl
0.2M K_2CO_3
Adjusted to pH 9.0 with 50% NaOH

2. General *C. elegans* techniques

2.1. C. elegans maintenance

C. elegans is cultured on solid nematode growth medium (NGM) seeded with a lawn of *E. coli* OP50 bacteria as a food source for the worms. NGM agar plates containing worms are kept in an incubator at 20°C at all times, except when handled during experiments. Two times during each week (usually on Monday and Friday), small parts of the NGM agar plates containing worms are cut out with a sterilized scalpel and transferred to a new NGM agar plate with fresh bacteria, to avoid overcrowding and starvation.

2.2. *E. coli* growth conditions

E. coli bacteria are grown by picking a single colony from a streak plate and incubating it in a falcon tube filled with liquid LB medium. The bacterial culture is then left in a shake-incubator at 37°C overnight and used to seed plates the next day.

2.3. Preparation of growth media

Basal nematode Growth Medium (NGM) is prepared in glass bottles of 250 mL, 500 mL or 1 L. For a total volume of 1 L, 17 g of agar, 2.9 g NaCl and 7.5 g of bacteriological peptones (Fluka Analytical, St. Louis, MO, USA) are dissolved in Milli-Q H_2O (MQ). After autoclaving the bottles with liquid NGM, they are stored briefly at 50°C to avoid solidification. Before pouring the agar into petri dishes, 1 mL of sterile 1M $CaCl_2$, 1mL cholesterol in ethanol (5µg/mL), 1 mL 1M $MgPO_4$ and 25 mL phosphate buffer is added. This complete medium is then left to solidify in plastic petri dishes. 100 µL of *E. coli* OP50 is then spread out over the solidified agar plates. The bacteria are left to grow to a visible layer by incubating them at 37°C for ca. 24 hours. Afterward, they are stored at 4°C until they are needed for the experiment.

Growth media for worms that are to be reared in the presence of royalactin are prepared in the same way, except that royalactin with a final concentration of 1.6 µg/(mL NGM) is added together with the phosphate buffer, cholesterol etc.

2.4. Bleaching

Bleaching is the process by which all worms are killed with a bleaching solution and the eggs are protected and retrieved to form an age-synchronous population[76].

Worms are washed off plates using S-basal buffer or M9 buffer. The worms are collected in a falcon tube, which is centrifuged at 1,100 rpm (Hettich Rotanta 46R, DJB Labcare, Buckinghamshire, UK) for 3 minutes at 4°C. Afterward, the supernatans is removed, leaving

a volume of 3.5 mL in the falcon tube. To this solution, 1.5 mL of bleaching solution (consisting of 1 mL 4% (=18°) hypochlorite mixed with 0.5 mL 5N NaOH) is added. After shaking and waiting a few minutes, it is possible to see the worms breaking open under the microscope. When more than half of the worms are disintegrated (typically between 3-6 minutes), 5 mL of a 60% sucrose solution is added to protect the eggs from further degradation. Carefully, 1.5 mL of S-basal buffer is added on top of this mixture with a pipet to form two distinct layers. The tubes are again centrifuged at 1,100 rpm for 3 minutes, after which a separation of the eggs and the dead worms in the two layers is visible (cf. sucrose density centrifugation). The eggs are located in the upper layer while the dead worms remain in the bottom layer. By carefully pipetting the eggs out and transferring them to a new tube, filled with sterile S-basal buffer, the eggs are collected. The tube is centrifuged at 1,100 rpm for 3 minutes, the supernatans is removed and new S-basal buffer is added. This process (i.e. "washing") is repeated twice, after which the eggs are left to hatch overnight in S-basal buffer or M9 buffer while rotating to start a synchronous population. By letting the worms hatch in a medium without a food source, their growth is arrested in the L1 stage. This guarantees that the population of worms is synchronous[77]. The L1 larvae are transferred to standard growth medium with a bacterial lawn as food source.

2.5. Timed egg-lay

As an alternative method to obtain synchronous *C. elegans* populations, a timed egg-lay is performed[78]. A timed-egg-lay is less time consuming than a bleach procedure and the eggs are exposed to less stress. However, a timed egg-lay can only be carried out for a relatively low amount of worms, and the resulting population is not entirely synchronous.

For every condition, ca. 60 reproductively active adults are manually picked (with a platinum wire) and divided over several NGM plates. They are left to lay eggs on the plates for 4-6 hours and then manually picked off the plates again, leaving behind a colony of eggs that are synchronously laid within the timespan of the egg-lay.

2.6. Freezing *C. elegans*

C. elegans strains that are no longer needed for ongoing experiments are frozen. Worm populations are cultured under standard conditions until they contain many *freshly starved* L1 and L2 larvae, which survive freezing best[79]. This is approximately 5 days after chunking the worms, when the bacterial food source has just been exhausted. Two to three NGM plates are used per strain. The worms are frozen in cryogenic vials containing soft agar freezing solution with glycerol (see B.1.5.). Four vials are labelled with strain info and the data on which the worms are frozen. The vials are stored in a capped and labelled Styrofoam box at -80°C in order to obtain gradual cooling.

To check whether the freezing procedure was successful, a week after storing the vials at -80°C, one vial per worm line is thawed and emptied onto one half of a seeded NGM plate. The survival of the worms is inspected through the microscope a few days later.

2.7. C. elegans strains

Several *C. elegans* strains were used in this project. They are summarized in table 1.

Table 1. A list of the *C. elegans strains* used in this project.

strain name	genotype	use	remarks
CL2166	dvIs19 [(pAF15)gst-4p::GFP::NLS] III	read-out for *gst-4* transcription	Inducible by oxidative stress
SJ4100	zcls13 [hsp-6::GFP] V	read-out for *hsp-6* transcription	marker for UPR(mt)
SJ4005	zcls4 [hsp-4::GFP; lin-15(n765)] V	read-out for *hsp-4* transcription	marker for ER stress
CL2070	dvls70 [hsp-16.2p::GFP + rol-6(su1006)]	read-out for *hsp-16.2* transcription	stress response marker
TJ356	zIs356 [daf-16p::daf-16a/b::GFP + rol-6]	read-out for *daf-16* transcription	nuclear localization
CF1553	muIs84 [(pAD76) sod-3p::GFP + rol-6]	read-out for *sod-3* transcription	Regulated by DAF-16

3. Fluorescence quantification

For several experiments, the quantification of the fluorescence intensity of GFP expressing worms is necessary. Two different methods are used to achieve this goal: a manual method in which the fluorescence of individual worms is measured after fluorescence microscopy (B.3.1.) and an automated method (B.3.2.) in which worms are deposited in a microtiter plate for instant fluorescence quantification of all the worms.

3.1. Individual method on Zeiss microscope and ImageJ

A first method of quantifying fluorescence consisted of using the software ImageJ to measure the fluorescence intensity in images of individual worms, taken with a Zeiss Axio Imager microscope (Zeiss, Oberkochen, Germany). Advantages of this technique are limited interference of eggs (since they can be excluded from the analysis) and the fact that not many worms are needed to obtain a clear signal (compared to the automated method). Taking the pictures and individually measuring the worms however, is time consuming.

3.1.1. Microscopy

Worms are grown up synchronously (as described in B.2.1.) on NGM or royalactin-containing NGM. When fully grown, the worms are rinsed of the plates with S-basal buffer and collected

in falcon tubes. They are washed with S-Basal or M9 buffer 3 times, after which the supernatans is removed.

Agarose ($2\%_{w/v}$) is melted in the microwave, after which 20 µL drops of the agarose solution are brought onto a glass microscope slide. Typically two drops per slide are applied. After flattening the drops, 3 to 10 µL of the worm solution and an equal amount of 2 mM levamisole or tetramisole (Sigma-Aldrich, , St. Louis, MO, USA) is pipetted onto the agarose drops to paralyze the worms. The slide is then covered with a cover slip and brought to the microscope.

After loading and positioning the sample, the ZEN Pro (ZEN Pro 2012 v.1.1.2.0, Zeiss) software package is used to take pictures. Both brightfield and EGFP fluorescence channels are recorded using a 5X or 10X magnification (multiplied by the built-in 10X magnification of the microscope), paying attention to the maximum intensity threshold (i.e. 255 for 8-bit images).

3.1.2. Fluorescence quantification with ImageJ

The images are analyzed with ImageJ (v.1.48)[80]. Worms are manually selected on the brightfield channel with the polygon selection tool, because their exact shape and position are easier to trace on brightfield images than on fluorescence images. These selections are saved in the Region Of Interest (ROI) Manager and applied to the fluorescence channels (see figure 6).

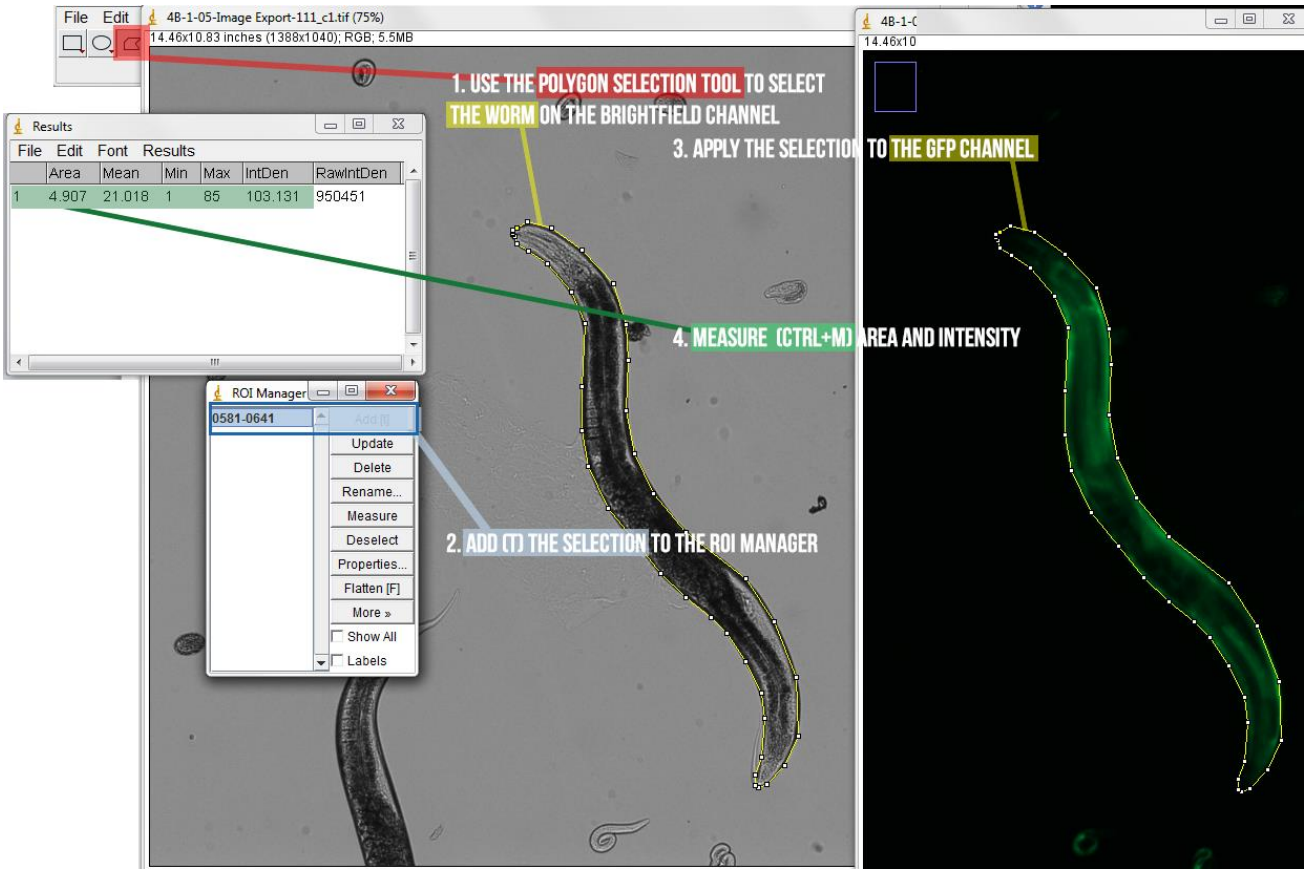

Figure 6: Workflow of fluorescence quantification with ImageJ. Worms are manually selected on the brightfield channel with the polygon selection tool. These selections are saved in the Region Of Interest (ROI) Manager and applied to the fluorescence channels. The area, integrated density and mean fluorescence are measured.

For each worm, the area, the integrated density (i.e. the total fluorescence) and mean fluorescence intensity (i.e. the integrated fluorescence divided by area) are measured. For each picture, a dark region (near the worm of interest) is selected and the mean fluorescence of this background signal is noted. The mean fluorescence of each worm is corrected for the background signal. When analyzing the pictures that are taken with 10X magnification, a single adult worm is often split over multiple pictures. In those cases, the mean fluorescence of the total worm is calculated, taking into account the differences in area. For all conditions, the average mean fluorescence intensities of the worms (minus the background signal) are calculated. Graphs representing the mean and standard error of mean (SEM) are constructed using GraphPad Prism 6 and an unpaired parametric (or unparametric when the data is not normally distributed) 2-tailed t- test assuming unequal standard deviations (or ANOVA and appropriate post-hoc test) is carried out to determine if there is a significant difference between the conditions.

3.2. Automatic method with microtiter plate

A second method of quantifying fluorescence consists of measuring the fluorescence of a large amount of worms in an automated microplate reader (Tecan Infinite® M200, Tecan,

Männedorf, Switzerland, Firmware version V_2.02_11/06_InfiniTe). By measuring a large amount of worms at once, this method provides a better representation of the entire population. The automated fluorescence quantification is also less time consuming than quantifying the fluorescence of individual worms through microscopy. Disadvantages of this method are the possible interference from fluorescence of the eggs, the need for a large amount of worms to obtain a high signal-to-noise ratio and the necessity to correct for (potential) differences in the amount of worms between different conditions.

3.2.1. Microplate measurement

When measuring the fluorescence of day 1 (or later) adults, fluorodeoxyuridine (FUdR) is added for a final concentration of 100 to 130 µM in NGM to stop the worms from laying viable eggs by inhibiting DNA synthesis, and as such minimize interference[81]. When fully grown, the worms are rinsed of the plates and washed (as described in 2.2.1) three times, after which the supernatans is removed until a volume corresponding to 8 times the volume of the visible worm pellet remains. The worms are then transferred from the falcon tubes to a 96-well microplate (Greiner 96 Flat bottom Black Polystyrol, Greiner Bio-One International AG, Kremsmünster, Austria) using a glass pipet so worms do not stick to the tip. Each well is filled with an equal volume of worm-solution. Depending on total volume, as many wells as possible are filled per condition (typically between 6 and 12). Typically, around 7,500 worms are used per condition. The final 0.5 mL of worm suspension in a falcon is not used as it contains fewer worms. At least 12 other wells are filled with S-basal to correct for the auto-fluorescence of the buffer. In order to correct the fluorescence for the total amount of protein present (see B.3.2.2.), at least 2 LoBind tubes are filled with an equal amount of the worm solution. The plate is inserted into the microplate reader and the measurement is started. To determine the optimal excitation wavelength, which can slightly differ for different GFP-variant, an excitation scan is performed for each different worm strain. For the performed experiments, excitation wavelengths of 360, 395 and 470 nm are used. The emission is always measured at 509 nm. The results are saved by the Tecan i-control software (v.1.3.3.0) and processed in Microsoft Office Excel 2010. The average background signal (i.e. autofluorescence of the S-basal buffer and the plate itself) is subtracted from all measurements. The fluorescence intensity values for all conditions are averaged and corrected for the total amount of protein present. Statistics are carried out in the same way as described for the individual method above (B.3.1.).

3.2.2. Protein concentration determination

To correct the measured fluorescence intensity in the microtiter plates for the total amount of worms present, the protein concentration in each sample was determined with a Qubit assay (Qubit® 2.0 Fluorometer, Invitrogen, Paisley, Scotland) or a bicinchoninic acid (BCA) assay[82]. Samples containing fluorescent worms are dried in the Speedvac (Savant SVC100H, Savant Instruments Inc, Farmingdale, USA). The dried worm pellet is then resuspended in 180 µL 1M NaOH and placed in a water bath at 70°C for 25 minutes. After 10 minutes and at the end of the incubation, the samples are vortexed. 1.0 mL of MQ is added to the samples after which they are centrifuged for 10 minutes at 14,000 rpm (Eppendorf Microcentrifuge 5414D, Eppendorf AG, Hamburg, Germany) to precipitate cellular debris. The supernatans is then used to measure protein concentrations with a Qubit assay[83] or BCA assay[82].
In both assays, the fluorescence or absorbance in the samples is compared to those of the protein standards. The protein concentration in each sample is calculated and used to correct the fluorescence intensities measured with the microplate reader, as described above.

4. Purification of royalactin from royal jelly

The purification of royalactin from royal jelly was done largely as described in (Detienne et al. 2010)[10].

4.1. Ultracentrifugation of royal jelly

Royal jelly is purchased from a local beekeeper's shop (Imkerij Nectar, Leuven, Belgium) and centrifuged in an Optima Le-80K ultracentrifuge (Beckman Coulter, Nyon, Switzerland) with a pre-cooled SW40 Ti 40,000 rpm swinging bucket rotor. Special attention is giving to balancing the ultracentrifuge. The royal jelly is centrifuged for 5 hours at 37,200 rpm at 6°C, after which three fractions became visible: a clear supernatans, a viscous middle-layer and a solid pellet. The supernatans is removed from the tube so that the middle layer, which contains the royalactin, can be taken out with a spatula, paying special attention that the bottom pellet is not taken out as well. This viscous middle layer is resuspended in 2 volumes MQ and left to stir for 1 hour at 20°C. The mixture is then transferred to falcons and centrifuged for 30 minutes at 28,880 g at 4°C (Sorval RC 6+ centrifuge, F13S-14X50CY rotor, Thermo Scientific, Waltham, USA), after which two fractions become visible: an opalescent micro-emulsion as the top fraction and a white sediment of protein aggregates as the bottom fraction. The top fraction is collected in ultracentrifuge tubes and centrifuged for 6 hours at 37,200 rpm at 6°C (Optima™ LE-80K ultracentrifuge, SW 40 Ti rotor). The

supernatans is then removed and the yellow sediment, enriched in royalactin, is dried in a centrifugal evaporator (Speedvac, Savant SVC100H, Savant Instruments Inc, Markham, Ontario Canada). The resulting substance is further purified through HPLC.

4.2. Purification of royalactin through HPLC

High-performance liquid chromatography (HPLC) is a technique used to separate, identify and quantify the components of a mixture based on their interaction with a solid absorbent material. Under high pressure and accompanied by a solvent, the sample mixture is injected in a column where it interacts with the absorbent material. The different components of the mixture show different affinity for the absorbent material, causing each component to pass through the column at a specific time corresponding to a specific solvent-composition.

A hydrophilic solvent (0.1% trifluoroacetic acid (TFA)) is used in combination with a hydrophobic column (Delta-pak, C18 column, 2x (40 x 100 mm)) to separate the components of the mixture obtained through ultracentrifugation. By incrementally increasing the hydrophobicity of the solvent, through the gradual increase of acetonitrile (ACN, Sigma-Aldrich) concentration (from 2% to 80% over a time span of 45 minutes), the retention time of each component is modulated; each component of the mixture will leave the column at a specific hydrophobicity of the solvent. The flow-through of the HPLC is monitored for the presence of proteins through absorbance measurement at 240 and 280 nm and collected in falcons. The first falcons are discarded as they only contain solvent and polar substances such as sugars that do not bind to the column. From previous experiments, it is known which peak in the 240 nm spectrum corresponds to royalactin[10]. The flow-through corresponding to this peak is collected in separate falcons. The royalactin-containing HPLC fractions are dried in a centrifugal evaporator and resuspended in 2.0 mL of MQ. A small volume of these fractions (corresponding to ca. 50 µg) is used to control the purity and presence of royalactin.

In order to successfully carry out the trypsinization process (see B.4.4.), it is necessary to know the total protein concentrations present in the HPLC fractions. We determined the total protein concentration in each HPLC fraction with a Qubit assay[83] by using 10 µL (or less, when the concentrations exceeded the range of the assay) of the HPLC fraction.

4.3. SDS-PAGE of HPLC fractions

In order to assess the effectiveness of the purification process so far, a small volume of each HPLC fraction (2.5 – 5 µL), is prepared for polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). Sample preparation consists of mixing variable volumes of sample (corresponding to 10 µg) with 3.75 µL of XT sample buffer and 0.75 µL of XT reducing agent and then adding MQ to a total volume of 15 µL. The samples are loaded onto a 4-12% Criterion™ XT Bis-Tris Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA). The

gel is run with XT MOPS buffer under following conditions: 70 V for the first 10 minutes, followed by 140 V for ca. 50 minutes (until the blue loading dye of the sample buffer reaches the bottom of the gel).
After electrophoresis, the gel is washed thrice with MQ on a shaking plate, and then submerged in SimplyBlue™ SafeStain solution (Thermo Fisher Scientific, Carlsbad, CA, USA) for ca. 1 hour (or until blue bands appear), after which the gel is destained with MQ and pictures of the gel are taken.

4.4. Preparation for mass spectrometry: gel-free tryptic digest

Trypsin is a serine protease that hydrolyses proteins by cleaving them at the carboxyl side of arginine and lysine residues (except when followed by a proline residue). It is often used to digest proteins into peptides for mass spectrometry analysis via peptide mass fingerprinting (PMF)[84].
A volume corresponding to 50 µg of protein is lyophilized in a centrifugal evaporator. The protein pellet is resuspended in 50 µL 30% ACN with 6.6 mM ditiothreitol (DTT, Sigma-Aldrich). DTT reduces disulfide bonds, which can hinder the digestion by trypsin. The samples are vortexed intensely and incubated at room temperature for 10 minutes, after which they are lyophilized again and resuspended in 50 µL 30% ACN with 55 mM iodoacetic acid (IAA, Sigma-Aldrich). The IAA alkylates cysteine residues to prevent the reformation of disulfide bonds. The samples are vortexed intensely and incubated for 10 minutes in the dark, after which they are lyophilized again. A working solution is made by adding a 10 µL aliquot of trypsin in trypsin resuspension buffer (50 mM acetic acid containing 1 µg of trypsin) to 140 µL of digestion buffer consisting of 50 mM NH_4HCO_3 and 50mM $CaCl_2$. To each sample, 37.5 µL of working solution is added. Samples are vortexed and incubated at 37°C overnight while shaking. Afterwards, the samples are lyophilized again in a centrifugal evaporator and are desalted and purified with ZipTips.

4.5. Preparation for MS: ZipTip desalting

ZipTips (Millipore® Ziptips C18, Sigma-Aldrich, St. Louis, MO, USA) are 10 µL pipette tips with a built-in C18 purification column. They are often used for purifying and desalting tryptic peptides[85].
To each dried peptide sample, 30 µL of a 2% ACN 0.1% TFA solution is added, after which the sample is vortexed, sonicated (Branson B5510 Ultrasonic Cleaner, Branson Ultrasonics, Danbury, CT, USA) for 1 minute and centrifuged briefly (VWR Galaxy MiniStar Microcentrifuge C1413-VWR230, VWR International, Radnor, PA, USA). The ZipTip is activated by thrice passing 10 µL of an activation solution consisting of 70% ACN 0.1% TFA through the column. The ZipTip is rinsed by thrice passing 10 µL of a rinsing solution (0.1%

TFA). Special care is given to never pass air through the column. The sample is then brought onto the column by passing it 7 times up and down the column in volumes of 10 µL. When all the sample is brought onto the column in this fashion, the column is rinsed thrice again by passing 10 µL of the rinsing solution through it. The peptides are eluted from the column by passing 5 µL of an elution solution (70% ACN, 0.1% formic acid) and collecting the eluate in a new LoBind eppendorf tube. The eluate is pipetted up and down at least 5 times to make sure all the peptides are eluted off the column. The purified peptides are then spotted on a MALDI (Matrix-assisted laser desorption/ionization) plate, for subsequent mass analysis.

4.6. Preparation for MS: spotting on MALDI plate

A matrix solution is prepared by adding a spatula tip of α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich) to a 60% ACN 0.5% TFA solution in MQ. The saturated solution is mixed, sonicated for 5 minutes and centrifuged for 10 minutes at 1,000 rpm in a bench-top centrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf AG, Hamburg, Germany). Then, 1 µL of the supernatans is spotted on a MALDI plate together with 1 µL of each purified peptide sample. 0.5 µL of a peptide standard (peptide calibration standard II, Bruker Daltonics, Billerica, MA, USA) is also spotted with 0.5 µL of the saturated matrix solution on the MALDI plate. The MALDI plate is left to dry for a few minutes, after which it is ready to be inserted into the MALDI-Time of Flight (MALDI-TOF) mass spectrometer.

4.7. Mass spectroscopy: identification of tryptic royalactin peptides with MALDI-TOF

MALDI-TOF is a technique used for the analysis of biomolecules such as peptides through ionization with a pulsed laser. The ions are accelerated in a vacuum tube inside the mass spectrometer and the time they need to reach the detector is recorded. This “time of flight” is dependent on the mass and charge of the ionized peptides. After detection, the peptides can be identified by searching a database of peptide-masses and their corresponding parent proteins.
For the identification of royalactin, an Ultraflex II MALDI-TOF mass spectrometer (Bruker Daltronics, Billerica, MA, USA) is used at the KU Leuven SyBioMa facilities on the Gasthuisberg Campus. The resulting mass spectra are visualized with FlexAnalysis 3.0 (Bruker Daltronics, Billerica, MA, USA) and searched for matching proteins. This is done by using an in-house Mascot Server (Matrix Science, Boston, MA, USA) configured to search for known *A. mellifera* proteins in the SwissProt database that match the mass spectra of the ionized peptides. The fixed carbamylation modification (due to treatment with IAA) and variable oxidation modifications are selected in the search settings. The presence of royalactin, and the absence of any detectable contaminants, in the analyzed samples is thus confirmed.

5. 2D-DIGE and proteomics

2-dimensional difference gel electrophoresis (2D-DIGE) is a major proteomics tool that can be used to separate proteins in two dimensions, usually being iso-electric point (1st dimension) and molecular weight (2nd dimension). Due to differential labeling, multiple samples can be run simultaneously so that the differences between the samples can be readily analyzed. Prior to the start of this thesis, multiple of these analytic gels labelled with CyDyes (CyDyes, GE Healthcare, Little Chalfont, Buckinghamshire, UK) were already prepared and analyzed. In order to cut out and identify the proteins inside the gels, preparative gels need to be prepared. These 2D-PAGE gels contain a large amount of protein and are dyed using deep purple staining instead of CyDyes.

For the preparative gels, whole *C. elegans* cultures were already lysed and proteins were extracted. 12 samples were pooled to obtain sufficient protein to run two preparative gels. The protein concentration of this pooled sample was determined.

5.1. 1st dimension separation by isoelectrofocusing (1st dimension)

Proteins are separated in the first dimension by their iso-electric point, i.e. the specific pH at which that protein carries no net charge. For this, non-linear Immobiline pH-gradient (IPG) strips are used (24 cm, pH 3-10, GE Healthcare). They are kept at -20 °C and need to be rehydrated before use. For each sample, 0.5 mL of Destreak rehydration solution (GE Healthcare) is mixed with 2.5 µL of IPG buffer (GE Healthcare). The mixtures are vortexed and spun down briefly. The protein samples are now loaded passively, a method that is recommended for preparative gels that contain large amounts of protein. For the first gel, 400 µg of protein is mixed with 400 µL of DeStreak mixture for a final volume of 580 µL. For the second gel, 700µg of protein is mixed with 300 µL of DeStreak mixture for a final volume of 626 µL. The volume of DeStreak mixture is lower in the second gel to prevent the rehydration volume from becoming too high, which can result in a gel that is too thick. The mixtures are left at room temperature for 1 hour and stored in a 4°C cooler for 19 hours until the start of the first dimension separation.

The loaded Destreak mixture is pipetted into each lane of an Immobiline Reswelling Tray (GE Healthcare) and spread out evenly. Each IPG strip is placed in a lane of the tray. The tray is filled with Drystrip Cover Fluid (GE Healthcare) until all the lanes are covered with fluid. This prevents evaporation of the Destreak solution. The strips are allowed to rehydrate overnight.

After rehydration, each strip is removed from the tray and placed in the manifold of the Ettan IPGPhor 3 Isoelectric Focusing System (GE Healthcare). The electrodes are placed on top of the wicks and fastened. Running of the samples takes place with the lights turned off and the blinds closed. The 1st dimension separation is run according to the manufacturer's instructions at 20°C, using 50 µA per strip.

The strips are run for a total Volt-hours (Vht) of 70,000, corresponding to 23 hours 45 minutes. Afterwards, strips are equilibrated (see below).

5.2. IPG strip equilibration

The IPG strips are saturated with SDS equilibration buffer. SDS denatures the proteins, which is necessary for the 2nd dimension separation. TrisHCl maintains the appropriate pH range for electrophoresis. Urea and glycerol improve the protein transfer from the IPG strip to the 2nd dimension.

SDS equilibration buffer
75 mg glycerol
8.375 mL TrisHCl pH 8.8
90 g ureum
5 g SDS

To 20 mL of SDS equilibration buffer, 200 mg of dithiothreitol (DTT) is added. DTT reduces the cysteine bonds in denatured proteins. For each strip, one lane of the Drystrip reswelling tray is filled with 650 µL of the DTT-enriched equilibration buffer. The strips are placed in the tray with the gel side down and left to equilibrate at room temperature for 15 minutes. Meanwhile, 900 mg IAA is added to 20 mL of SDS equilibration buffer. IAA covalently modifies the reduced sulfur groups so they do not oxidize again. A spatula tip of bromophenol blue is added to the IAA equilibration buffer to follow to progress of the electrophoresis. Next, one lane of a reswelling tray is filled with 650 µL of the IAA-enriched equilibration buffer. The IPG strips are moved from the DTT buffer to the IAA buffer, placed with the gel side down, and again left to equilibrate at room temperature for 15 minutes.

5.3. 2nd dimension separation by SDS-PAGE

Proteins are separated in their second dimension by their molecular weight using SDS-PAGE. The 12.5% SDS-polyacrylamide (SDS-PA) gels are prepared in-house. After preparation of the SDS-PA gels, the equilibrated IPG strips are loaded onto the gels.

5.3.1. Preparation of SDS-PA gels for 2D-electrophoresis

A Bind-Silane solution is prepared for making the gel adhere to the glass plates, which will carry a marker necessary for the spotpicker robot to cut out the protein spots. 15 mL of Bind-Silane solution consists of 12 mL ethanol, 300 µL acetic acid, 15 µL Bind-Silane (Sigma-Aldrich) and 2.7 mL MQ. 2 mL of this solution is spread out evenly with a Kimwipe tissue onto each glass plate. After drying the plates for a few minutes, two self-adhesive reference

markers are placed on the glass plate so that they are centered in the width of the plate. These markers provide a reference for the spotpicking robot that cuts out selected spots from the gel after separation. Each glass plate is assembled in a gel case by pairing it with another glass plate with spacers. The gel cases can now be brought into the Ettan DALTsix Gel Caster (GE Healthcare). The Gel Caster is filled according to the manufacturer's instructions.

The following solutions are prepared beneath the fume hood:

Main solution
-125 mL 30%$_{w/v}$ acrylamide/bisacrylamide (Biorad)
-75 mL Tris Base (1.5 M, pH adjusted with HCl to 8,8) (Trizma Base, Sigma)
-93.5 mL MQ
-3 mL 10%$_{w/v}$ SDS

Initiator solution
-10 mL 10%$_{w/v}$ ammonium persulfate (APS, PanReac, AppliChem, Darmstadt, Germany)

Catalyzer
-1 mL of 10%$_{v/v}$ tetramethylethylenediamine (TEMED, Sigma-Aldrich)

6 mL of the initiator solution and 830 µL of the catalyst are added to the main solution right before casting the gels. The solution is poured into the groove at the back of the Gel Caster until the solution reaches the mark on the side of the caster. A 0.1 %$_{w/v}$ SDS solution is sprayed in the top opening of the Gel Caster, after which the caster is covered with plastic foil to prevent dehydration of the gels. The gels are left to polymerize at room temperature for a minimum of 5 hours (in our case: 16 hours and 25 minutes, until the start of the 2nd dimension separation).

5.3.2. SDS-PAGE

A 10x SDS running buffer is prepared, along with a 3x and 1x dilution.

10x SDS running buffer
151,25 g Tris
720 g glycine
50 g SDS
MQ water is added for a final volume of 5L

Prior to fixing the IPG strips on the polyacrylamide gel, an SDS agar solution is prepared by adding 375 mg agarose to 75 mL 1x SDS running buffer while mixing and heating the solution. When the solution becomes transparent, the heat is lowered to 50°C.

The gel cases are taken out of the Gel Caster and the outside of the glass is cleaned with 70% ethanol.

The IPG strips are taken out of the reswelling tray and rinsed twice with 1 mL of 1x SDS running buffer. The $0.1\%_{w/v}$ SDS is decanted from the top of the Gel Caster and the IPG strips are placed on top of the gels. 1 mL of the agarose solution is pipetted on top of the strip to prevent air gaps and improve protein migration from the strips to the gel. The Ettan DALTSix Electrophoresis System (GE Healthcare) is filled with 1x SDS running buffer and the gel cases are inserted according to the manufacturer's instructions. The upper chamber is filled with 3x SDS running buffer. The electrophoresis is carried out at a temperature of 20°C and follows following steps:

- max. 8 mA/gel, 600 V, 10 W for 1 hour
- max. 12 mA/gel, 600 V, 10 W overnight

The electrophoresis is stopped when the bromophenol-blue marker reaches the bottom of the unit. The electrophoresis unit is then cooled to 10°C until the gels are removed for staining and scanning.

5.4. Deep Purple Staining

Staining of the preparative gels, from which protein spots will be cut out using an automated spot picker is carried out with the Deep Purple total protein stain (GE Healthcare). Deep Purple is a fluorescent stain that binds proteins in a very sensitive (femtomol range) way and exhibits linearity over 4 orders of magnitude[86].

The glass plate with spacers is removed from the gel case. Because of the Bind-Silane treatment the gel will stick to the other glass plate. The gels are fixed in $10\%_{v/v\ ethanol,}$ $7.5\%_{v/v}$ acetic acid (at 4°C over the weekend). Afterward, the gel is washed with a washing solution (2.94 g $NaHCO_3$, 31.8 g Na_2CO_3 in 1 L MQ water) for 30 minutes while gently shaking. Deep Purple stock solution (200x) is diluted 200 times and used to stain the gels for 1 hour while gently shaking. The gel is washed twice in 7.5 $\%_{v/v}$ acetic acid for 15 minutes. The gel is now ready to be scanned.

5.5. Scanning the gels

Preparative gels stained with Deep Purple are scanned with an Ettan DIGE Imager (GE Healthcare).

The gel is placed in the casing of the scanner with the gel side up. The casing is placed in the scanner and the Ettan DIGE software (GE Healthcare) is started up. A test run is performed

with the default settings for Deep Purple staining. ImageQuanT (GE Healthcare) is used to note down the maximum intensity observed in the test run. The exposure time for the real scan is calculated by dividing the preferred intensity (i. e. 50,000) by the maximum intensity observed in the test run. The outcome is multiplied by the default exposure time and the real scan is started.
Preparative Deep Purple stained gels are stored in 7.5% acetic acid at 4°C until spot picking can begin.

5.6. Marking differential protein spots with DeCyder

The scanned gels are analyzed with DeCyder 2D 7.0 (GE Healthcare), a software package that can visualize and quantify the spots on a 2D-DIGE gel. The program matches the spots on the preparative gels to the ones on the analytical gels. The differential intensity data can be pooled across all gels for a more robust analysis. This happens in a semi-automated fashion, but the matched spots must be checked manually and corrected. The list with x- and y-coordinates of differential spots is saved and exported to the spot picker.

5.7. Using the spot picker to cut out the selected spots

An Ettan Spot Picker (GE Healthcare) is used to cut out the differential spots that were selected with DeCyder. The list of differential spots is imported into the software accompanying the spot picker and the Deep Purple stained gel is placed into the spot picker tray. Each gel plug is automatically cut out and deposited into a microplate well.

5.8. In-gel trypsin digestion

The spots that are excised from the gel using the spot picker are digested with trypsin, in a similar fashion as described earlier (see B.4.4.). These tryptic peptides can then be identified using mass peptide fingerprinting (see B.5.9.).
Each gel plug is washed thrice by adding 150 µL of a rehydration solution (100 mM NH_4HCO_3), incubating for 10 minutes while shaking, removing the rehydration solution and adding 150 µL ACN, again incubating for 10 minutes while shaking, and removing the ACN. This washing process is repeated twice, after which the samples are dried completely in the centrifugal evaporator. Afterwards, they are stored at -20°C.
A trypsin working solution is prepared by dissolving 10 µg of trypsin in 100 µL trypsin resuspension buffer and adding 1,400 µL trypsin digestion buffer. Of this working solution, 25 µL is added to each well containing a gel plug, together with an additional 30 µL of the digestion buffer. The gel plugs are incubated at 4°C for 2 hours and 30 minutes to allow for the trypsin digestion to take place, after which more trypsin working solution is added if the gel plugs are not fully submerged. The gel plugs are incubated overnight at 37°C while shaking.

The supernatans of each digestion well is transferred to a clean LoBind collection tube and 150 µL of a first extraction solution (50 mM $NH_4HCO_{3)}$ is added to the digestion wells. The wells are incubated for 30 minutes at room temperature while shaking, after which they are spun down and the supernatans is pipetted into the corresponding collection tubes. 150 µL of a second extraction solution (50% ACN, 5% formic acid) is added to the digestion tubes. They are again incubated for 30 minutes with gentle shaking at room temperature, after which they are spun down and the supernatans is pipetted into the corresponding collection tubes. The collection tubes are dried in the SpeedVac and are purified via ZipTip, as described in B.4.5..

5.9. Identification of digested protein spots with mass-spectrometry

A Q Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) is used at the KU Leuven SyBioMa facilities on the Gasthuisberg Campus to identify the digested proteins. This mass spectrometer uses an ion trap mass analyzer to trap ions in an orbital motion and convert this movement into a mass spectrum by Fourier transform. The peptides are then fragmented into smaller fragments to improve the identification, a technique known as tandem mass spectrometry or MS/MS.
The resulting mass spectra are searched for matching proteins. This is done by performing an MS/MS ions search on an in-house Mascot Server (Matrix Science, Boston, MA, USA) that searches for known proteins in the SwissProt and NCBI databases that match the mass spectra of the ionized peptides. Following search parameters are used:

Database: Swissprot or NCBI
Taxonomy: *Caenorhabditis elegans*
Enzyme: Trypsin
Fixed modifications: Carbaminomethyl (C)
Variable modifications: Oxidation (M)
Peptide mass tolerance: ± 0.1 Da
MS/MS fragment mass tolerance: ± 0.02 Da
Peptide charge: Mr
Mass values: Monoisotopic
Instrument: ESI-FTICR

The peptide mass tolerance was decreased to up to ± 0.01 Da on several occasions to provide for a better identification.

6. Stress assays

6.1. Thermotolerance assay

To determine whether feeding worms with royalactin altered their response to heat treatment, synchronous populations of control and royalactin-fed worms are subjected to long lasting heat stress. Synchronous populations are obtained by bleaching (see B.2.4.). Adult worms are stressed in an incubator at 35°C and briefly taken out every hour or every two hours to count the surviving worms. The survival rate of the worms is assayed by observing them under a binocular microscope. If they did not move by themselves or after being gently prodded with a platinum wire, the worms are regarded as dead and discarded in a flame[87]. After each measurement, the number of dead and surviving worms is written down. The surviving worms are put back into the oven as soon as possible, and assayed again the next hour. Only one or two plates are taken out of the oven at a time to make sure the worms are in a 35°C environment most of the time. Every hour, the plates are assayed in the same order.

Survival curves for both conditions (without and with royalactin) are constructed using the built-in survival curve function of GaphPad Prism 6[88], which uses a logrank test to determine the significance of difference between survival curves.

6.2. Reductive stress assay

To determine whether feeding worms with royalactin altered their response to reductive stress, we treated synchronous populations of royalactin-fed and control worms with DTT, similar to[89]. Treatment of DTT results in ER stress as well, by accumulation of unfolded proteins in the ER. Synchronous populations are grown up by timed egg-lay (see B.2.5.). The worms are given FudR for a final concentration of 135 µM in the young adult stage (65 hours after the timed egg-lay) to stop them from laying viable eggs. Before the start of the assay, adult worms are rinsed off the plates with M9 buffer and washed twice to remove bacteria (by centrifuging at 1,100 rpm for 3 minutes discarding the supernatans and adding new M9 buffer). Afterward, 133µL of the washed worm solutions is transferred to LoBind eppendorf tubes that are filled with 1 mL of 5.5 mM DTT in M9 buffer for a final concentration of 5 mM DTT. These tubes are incubated for at least 24 hours at 20°C in a rotator.

After incubation in DTT, the tubes are spun down on a tabletop centrifuge (Eppendorf Microcentrifuge 5415D, Eppendorf AG, Hamburg, Germany) at 500 rpm for 30 seconds. The supernatans is discarded until a volume of about 150 µL, containing the stressed worms, is left over, which is mixed gently and spread out over several NGM plates. The survival of the worms is immediately assayed by microscopically observing the worms in the same way as described earlier (see B.6.1.). Dead and surviving worms are counted for both conditions. The assay was performed on day 1 and day 3 of the adult stage, taken from the same synchronous population. A 2-way ANOVA is carried out in Graphpad Prism 6.

6.3. Oxidative stress assay

To determine whether feeding worms with royalactin altered their response to oxidative stress, synchronous populations of royalactin-fed and control worms are treated with 5mM H_2O_2. Synchronous populations are grown up by timed egg-lay (see B.2.5.). Adult worms (~90 hours after timed egg-lay) were rinsed off the plates with M9 buffer and washed twice by centrifuging at 1100 rpm for 3 minutes, discarding the supernatans and adding new M9 buffer to remove bacteria. Afterward, 133µL of the washed worm solutions is transferred to LoBind eppendorf tubes that are filled with 1mL of 5.5 mM H_2O_2 in M9 buffer for a final concentration of 5 mM H_2O_2. These tubes are incubated for 1 hour at 20°C in a rotator.
After incubation in H_2O_2, the tubes are spun down on a tabletop centrifuge (Eppendorf Microcentrifuge 5415D) at 1,000 rpm for 30 seconds. The supernatans is discarded until a volume of 750 µL, containing the stressed worms, is left over. This volume containing the worms is mixed gently and spread out over several segmented NGM plates. The survival rate of the worms is immediately assayed by microscopically observing the worms in the same way as described earlier (see B.6.1.). Dead and surviving worms are counted every hour for all conditions. A 2-way ANOVA is carried out in Graphpad Prism 6.

7. Cleaving royalactin with hydroxylamine

7.1. Hydroxylamine treatment

Hydroxylamine is a reactive inorganic compound that can be used to cleave proteins at asparaginyl-glycyl bonds[90]. Hydroxylamine treatment is performed on the purified royalactin samples in order to generate multiple fragments, which can subsequently be used in bioassays to determine the bioactive part of royalactin. Under mild incubation conditions, hydroxylamine cleaves royalactin once, into a relatively short N-terminal fragment (~12 kDa) and a larger C-terminal one (~45 kDa), as illustrated in figure 7.

NILRGE**SL**N**KSL**PILHEWKFFDYDFGSDERRQDAILSGEYDYKNNYPSDIDQWHDKIFVTMLRYN

8 kDa peptide (65 AA) + sugar chains (~ 12 kDa)

+

GVPSSLNVISKKVGDGGPLLQPYPDWSFAKYDDCSGIVSASKLAIDKCDRLWVLDS**GLV**N**NTQ**PMCSPKLLTFDLTT
SQLLKQVEIPHD**VAV**N**ATT**GKGRLSSLAVQSLDCNTNSDTMVYIADEKGEGLIVYHNSDDSFHRLTSNTFDYDPKFT
KMTIDGESYTAQDGISGMALSPMTNNLYYSPVASTSLYYVNTEQFRTSDYQQNDIHYEGVQNILDTQSSAKVVSKS
GVLFFGLVGDSALGCWNEHRTLERHNIRTVAQSDETLQMIASMKIKEALPHVPIFDRYINREYILVLSNKMQKMVN
NDFNFDDVNFRIMNANVNELILNTRCENPDNDRTPFKISIHL

~ 45 kDa (incl. 2 sugar chains)

Figure 7: 2 main fragments of royalactin generated by the cleaving of hydroxyalamine. The main cleavage site is indicated in blue. Other possible cleavage sites are indicated in grey.

The protocol for hydroxylamine treatment of royalactine is optimized over several iterations (not all covered in this thesis).

Purified royalactin samples are first dried in a centrifugal evaporator and then dissolved in 1 mL hydroxylamine cleavage buffer. Cleavage buffer is made by dissolving 3.85 g of hydroxylamine hydrochloride in 12 mL of pre-cooled 4.0 M guanidine hydrochloride with 0.1M Tris (Sigma-Alrich) in an ice bath. 2 mL of 50%$_{w/v}$ NaOH is added slowly while stirring with a magnetic stir bar, followed by adding 5 mL 1 M K_2CO_3. The pH is adjusted to 9.0 by adding 50%$_{w/v}$ NaOH and continuously measuring with a pH meter (Hamilton BioTrode lab pH electrode, Sigma-Aldrich). The volume is then adjusted to 25 mL with 4.0 M guanidine hydrochloride in 0.1 M Tris.

The sample is then incubated. Different incubation periods and conditions were tried in the optimization of the protocol, namely 3h at 45°C, 3h at 37°C, 4h at 45°C, 4h at 37°C, 12h at 45°C, 12h at 37°C, 20h at 40°C and 20h at 45°C. A royalactin sample that is not treated with hydroxylamine underwent the same incubation to assess the effect of the heat treatment.

After incubation, the reactions are stopped by addition of three volumes of 2% TFA. The cleaving reaction of the hydroxylamine is then assayed by running the samples onto an SDS-PAGE gel.

7.2. Confirmation of royalactin cleavage by SDS-PAGE

To verify the hydroxylamine treatment, a gel electrophoresis experiment is performed (similar to B.4.3). Sample preparation consisted of mixing 10.5 µL of sample solution with 3.75 µL of XT sample buffer and 0.75 µL of XT reducing agent and then adding MQ H_2O to a total volume of 15 µL. The samples are loaded onto a Criterion™ XT Bis-Tris Precast Gel (Bio-Rad Laboratories, Hercules, CA, USA). The gel is run with XT MES buffer. In this case MES buffer is used instead of MOPS buffer, because bands are expected to appear at 3 and 10 kDa (i.e. cleaved royalactin fragments) and MES buffer allows for a better separation of such

small molecules[91]. The gel is run under following conditions: 70 V for the first 10 minutes, followed by 140 V for 40 minutes or until the separation is clear.
After electrophoresis, the gel is stained with SimplyBlue, after which the gel is destained with MQ and pictures of the gel are taken.

8. Metabolic measurements

These experiments were carried out in the lab of Professor Bart Braeckman (Department of Biology, Ghent University, Proeftuinstraat 86 N1, 9000 Ghent, Belgium).

8.1. Growth conditions

Worms are grown up as described earlier (see B.2.1.) in Leuven before being transported to Ghent. There they are bleached (as described in B.2.4.) and the eggs are left to hatch overnight in S-basal buffer. The buffer is removed to a volume of 500 µL and the total number of L1 worms is estimated from counting their number in 5 µL drops. About 2,000 worms are transferred to each seeded NGM agar plate. When the worms are in the L4 stage (about 48 hours after plating them out), 70 µL 44 mM FUdR is added per plate (to a final concentration of 135 µM). When worms are day 1 adults, they are rinsed of the plates with S-basal for use in respiration rate or thermal activity monitoring (TAM) experiments in 50 mL falcons. They are left to settle to the bottom of the tube after which they are washed twice by removing the medium and adding new S-basal on top. The worms are then transferred to smaller 15 mL tubes and centrifuged for 4 minutes at 4,000 rpm (Eppendorf Centrifuge 5804R). The supernatans is removed up to 4.0 mL and 8.0 mL of 60%$_{w/w}$ sucrose is added. The solution is mixed, after which 1.5 mL of S-basal is carefully pipetted on top. After 3 minutes of centrifugation at 4,000 rpm, the worms are floating in the top S-basal fraction and can be carefully pipetted into a new 15 mL falcon filled with 10 mL of S-basal buffer. They are then washed 4 times with S-basal, after which the supernatans is removed up to 2.2 mL. 900 µL of this solution will be used for respiration measurements, 2 times 100 µL will be used for protein concentration determination through BCA and 2 times 200 µL of this solution will be used for thermal activity monitoring.

8.2. Respiration rate measurement

The respiration rate of worms is measured with a StrathKelvin 929 6-Channel Oxygen System (Strathkelvin Instruments, North Lanarkshire, Scotland) and accompanying software. The instrument is first calibrated with oxygen-saturated water. Atmospheric air is run through cold water for 5 minutes. Then, 1 mL of saturated water is transferred to each cell. The sensors are

placed in the respiration cells and any air bubbles are removed by rotating the sensor. The oxygen system is calibrated for “high” using the accompanying software package.

To perform the actual respiration measurement, 900 µL of worm solution is first mixed with 4,100 µL of axenic medium[92]. 1.0 mL of worm suspension is pipetted into a respiration cell, the suspension is mixed by rotating the magnetic rod and the sensors are placed in the respiration cells. The measurement is started and allowed to register the use of oxygen for 20 minutes. All measurements are performed in technical triplicates. The slopes of oxygen-levels in all different samples are calculated using the Strathkelvin 928 Oxygen System software. These slopes are subjected to an unpaired parametric 2-tailed t-tests assuming unequal standard deviations in GraphPad Prism 6.

8.3. Thermal Activity Monitoring

Thermal activity of worms is measured with a 2277 Thermal Activity Monitor (TA instruments, New Castle, DE, USA) and accompanying software. Glass bottles are prepared for a TAM-measurement by adding a Watmann paper (ca. 1 cm^2) followed by 200 µL of worm suspension. The cap is placed on the bottle and tightened with a clam. The bottle is placed in a free slot of the Thermal Activity Monitor and measurement is started using the accompanying software. The thermal activity of the worms is measured over the span of 6 hours. A time frame of 1 hour that is relatively stable for all measurements is selected and the average heat production within that time frame is calculated. The averages are subjected to an unpaired parametric 2-tailed t-tests assuming unequal standard deviations in GraphPad Prism 6.

C.Results

1. Fluorescence quantification

1.1.GST-4

The relative expression of GST-4 is measured by observing the fluorescence intensity of a *gst-4p::*GFP transcriptional fusion construct integrated in the genome of the worms (see B.2.7.). The observed GFP-fluorescence is a measure for the *in vivo* expression of GST-4[75].

The fluorescence intensity is observed for worms reared on NGM-plates containing royalactin (1.6 µg/mL NGM) and control worms reared on normal NGM, using two independent methods. Firstly by selecting individual worms on fluorescence microscope images and measuring the intensity, secondly by automatically measuring the fluorescence intensity in a microplate reader. For detailed descriptions of both methods, see B.3.1. and B.3.2..

As shown in figure 8A, The fluorescence intensity of *gst-4p*::GFP worms is significantly higher (+13.2%; $p_{t\text{-}test} < 0.0001$) in royalactin-fed worms when compared to untreated control worms. This experiment was performed 114 hours after plating out L1 larvae, meaning that the worms were day 2 adults.

Figure 8B shows a similar result obtained using the microplate method. Fluorescence intensity of *gst-4p*::GFP is again significantly higher (+45.0%; $p_{t\text{-}test} = 0.0062$) in royalactin-fed worms. In this experiment, two positive controls (20 min incubation in 1 mM H_2O_2 or 10 mM H_2O_2) are also included. The weaker positive control (1 mM H_2O_2) did not results in a significantly ($p_{t\text{-}test} = 0.4687$) increased fluorescence intensity, when compared to untreated control worms. The stronger positive control (10 mM H_2O_2) did result in a significantly (+50.2%; $p_{t\text{-}test} = 0.0031$) increased fluorescence intensity of *gst-4p*::GFP when compared to control worms. This experiment was performed 66 hours after plating out L1 larvae, meaning that the worms were day 0 adults.

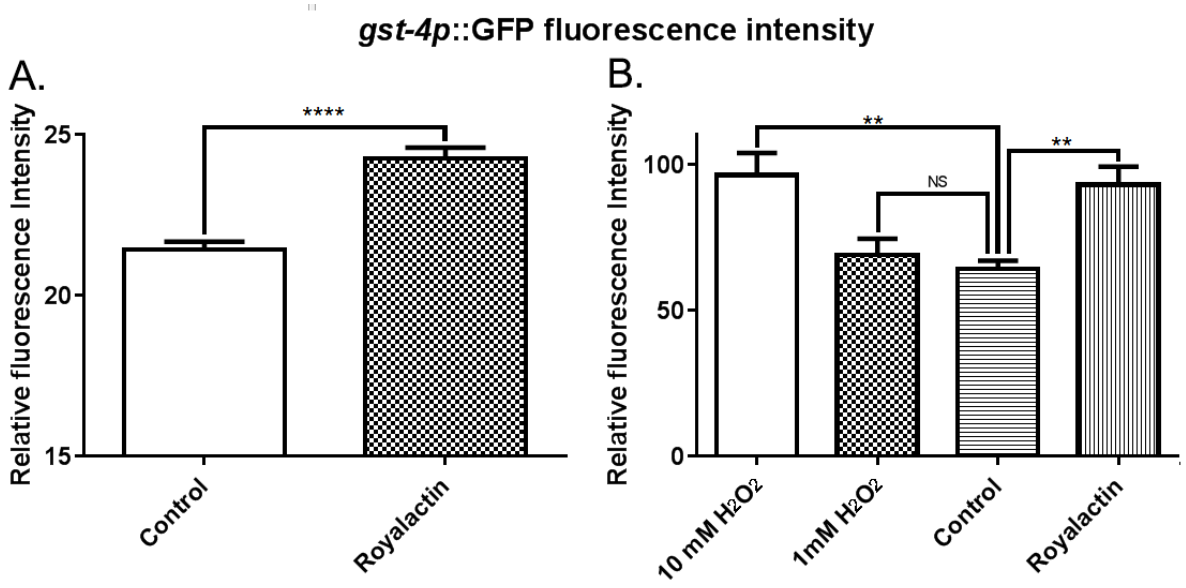

Figure 8: ***gst-4p*::GFP fluorescence intensity is higher in royalactin-fed worms** when compared to control worms, as shown in (**A.**) individual quantification of fluorescence (at day 2 of adulthood) ($n_{control}$ = 182, $n_{royalactyin}$ = 153) and (**B.**) automated quantification of fluorescence (at day 0 of adulthood) ($n_{10mMH2O2}$ = 8, $n_{1mMH2O2}$ = 8, $n_{control}$ = 5, $n_{royalactyin}$ = 5). The y-axis of each graph was adjusted for clearer representation. Error bars = SEM.

1.2.HSP-16.2

The relative expression of HSP-16.2 is measured by observing the fluorescence intensity of a *hsp-16.2p*::GFP transcriptional fusion construct integrated in the genome of the worms (see B.2.7.). The observed GFP-fluorescence is a measure for the *in vivo* expression of HSP-16.2[93].

The fluorescence intensity is observed as described above for GST-4. Two separate microplate experiments were carried out. As shown in figure 9A, the fluorescence intensity of *hsp-16.2p*::GFP is not significantly different between royalactin and control worms ($p_{t\text{-}test}$ = 0.6108). This experiment was performed 45 hours after plating out L1 larvae, meaning that the worms were in the L4/young adult stage. This experiment was repeated with a different result. As shown in figure 9B, fluorescence intensity of *gst-4p*::GFP worms is slightly but significantly (-3.7%; $p_{t\text{-}test} < 0.0001$) lower in royalactin-fed worms, compared to control worms.

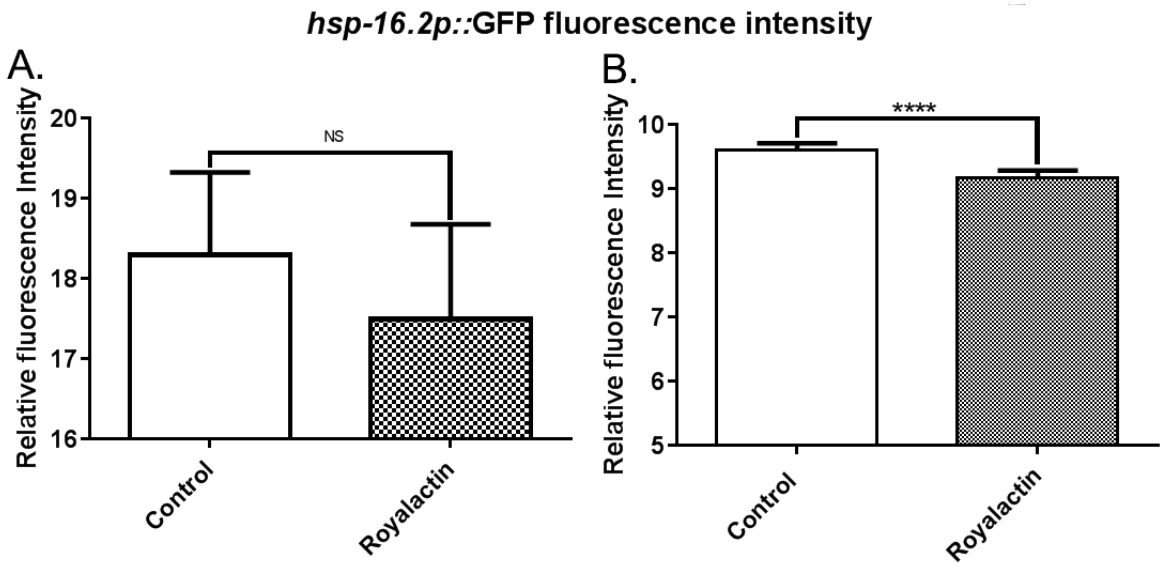

Figure 9: *hsp-16.2p*::GFP fluorescence intensity is not significantly different in royalactin-fed worms when compared to control worms in day 0 adults in the absence of a heat-shock. (**A.**) ($n_{control}$ = 112, $n_{royalactyin}$ = 67) and (**B.**) ($n_{control}$ = 143, $n_{royalactyin}$ = 228). The y-axis of each graph was adjusted for clearer representation. Error bars = SEM.

When both conditions are heat-shocked at 35°C for 2 hours, there is a larger but less significant difference between royalactin and control worms (-24.2%; $p_{t\text{-}test}$ = 0.045), as shown in figure 10, with royalactin-fed worms showing a lower fluorescence intensity. As expected due to heat stress, *hsp-16.2p*::GFP fluorescence intensity is significantly higher (+789.6%; $p_{t\text{-}test}$ < 0.0001) in the heat-shocked control when compared to the untreated control. This experiment was performed 69 hours after plating out L1 larvae, meaning that the worms were day 0 adults.

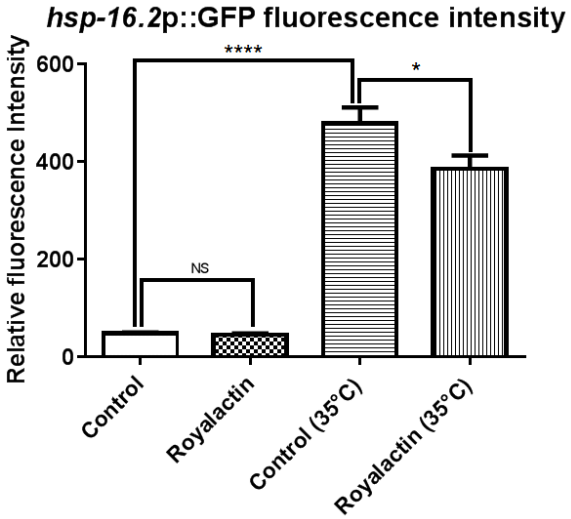

Figure 10: At 20°C, *hsp-16.2p*::GFP fluorescence intensity is not significantly different in royalactin-fed worms when compared to control worms, but **when subjecting both conditions to a 35°C heat shock, there is a small but significant difference between royalactin-fed and control worms in day 0 adults.** ($n_{control}$ = 9, $n_{royalactyin}$ = 9, $n_{rcontrolHS}$ = 9, $n_{royalactinHS}$ = 9) Error bars = SEM.

1.3.HSP-4

The expression of HSP-4, an indicator of ER stress[94], is measured in the same way as described above.

As shown in figure 11A, *hsp4*::GFP fluorescence intensity is significantly lower (-14.8%; $p_{t\text{-}test}$ = 0.0034) in royalactin-fed worms when compared to control worms. This experiment was performed 67 hours after plating out L1 larvae, meaning that the worms were day 0 adults. Figure 4B shows a similar result obtained using the microplate method. *hsp4*::GFP fluorescence intensity is again significantly lower (-29.1%; $p_{t\text{-}test}$ = 0.0134) in royalactin-fed worms. As a positive control, both royalactin-fed worms and control worms are incubated in 5 mM DTT for 2 hours. As expected, *hsp4*::GFP fluorescence intensity is significantly higher (+140.3 %; $p_{t\text{-}test}$ = 0.0002) in DTT-treated worms when compared to untreated control worms. *hsp4*::GFP fluorescence intensity is also significantly lower (-30.1%; $p_{t\text{-}test}$ = 0.015) in royalactin worms incubated in DTT when compared to control worms incubated in DTT. This experiment was performed 71 hours after plating out L1 larvae, meaning that the worms were day 0 adults.

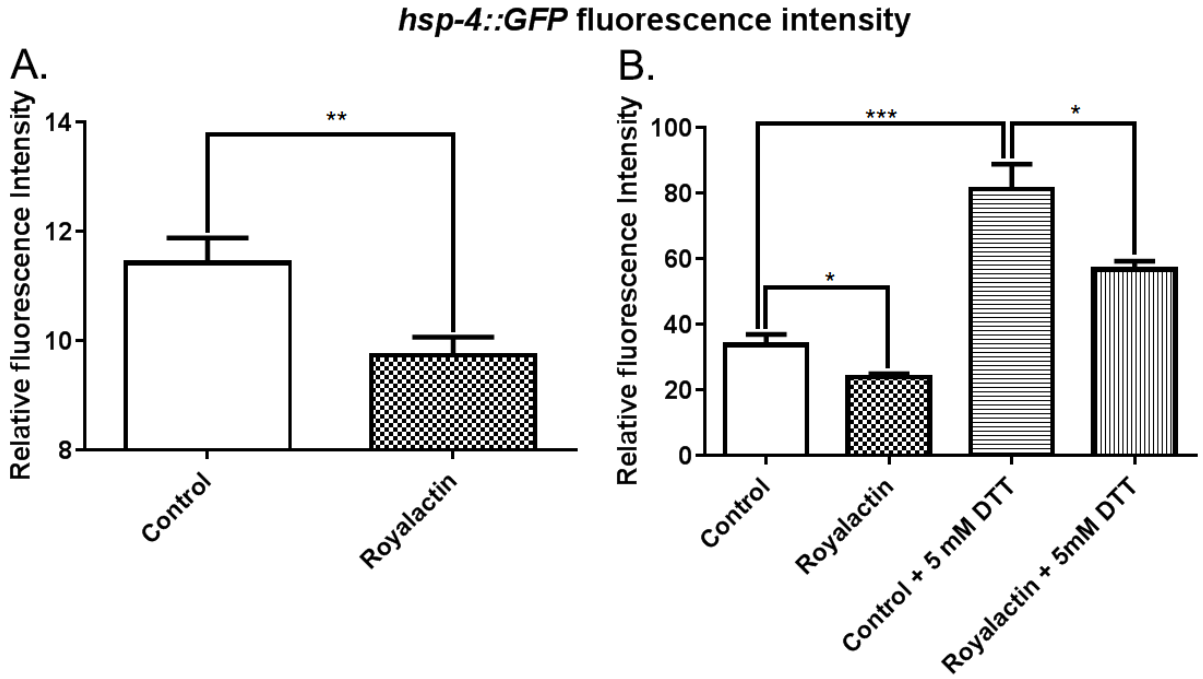

Figure 11: *hsp4*::GFP fluorescence intensity is lower in royalactin-fed worms when compared to control worms in day 0 adults, as shown by (**A.**) individual quantification of fluorescence ($n_{control}$ = 54, $n_{royalactyin}$ = 90) (**B.**) automated quantification of fluorescence. As a positive control, both royalactin-fed and control worms were incubated in 5 mM DTT for 2 hours ($n_{rcontrol}$ = 10, $n_{royalactin}$ = 7, $n_{rcontrol+5mM\ DTT}$ = 8, $n_{royalactin\ +\ 5mM\ DTT}$ = 9). The y-axis of the left graph was adjusted for clearer representation. Error bars = SEM.

1.4.HSP-6

Similar to the use of *hsp-4*::GFP to measure HSP-4 expression as an indicator of ER stress, we used a *hsp-6*::GFP strain to measure HSP-6 expression as an indicator of mitochondrial stress[95].

Two separate microplate experiments were carried out. As shown in figure 12A, *hsp6*::GFP fluorescence intensity is significantly lower (-30.3%; $p_{t\text{-}test}$ = 0.0042) in royalactin worms when compared to control worms. This microplate experiment was performed 70 hours after plating out L1 larvae, meaning that the worms were day 0 adults. In a repetition of the experiment, shown in figure 12B, worms are also incubated in 20 mM H_2O_2 for 20 minutes as a positive control for HSP-6 expression. Again, *hsp6*::GFP fluorescence intensity is significantly lower (-20.1%; $p_{t\text{-}test}$ = 0.0231) in royalactin worms when compared to control worms. *hsp6*::GFP fluorescence intensity in worms treated with 20 mM H_2O_2 is significantly higher (+35.9%; $p_{t\text{-}test}$ = 0.0406) when compared to untreated control worms. This experiment was performed 71 hours after plating out L1 larvae, meaning that the worms were day 0 adults.

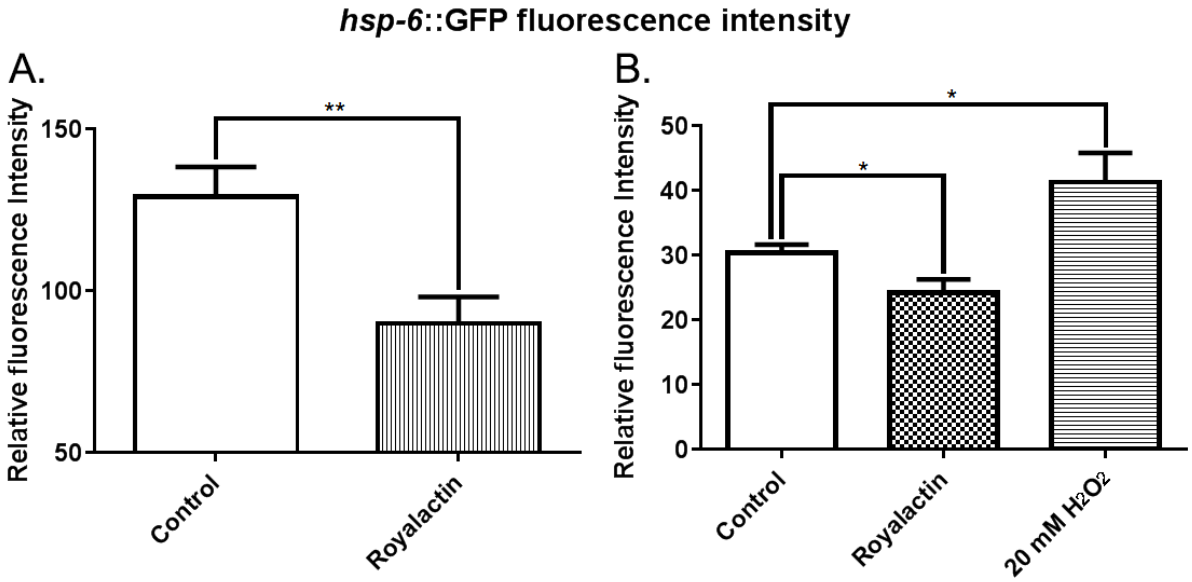

Figure 12: *hsp6*::GFP fluorescence intensity is lower in royalactin-fed worms when compared to control worms in day 0 adults. (**A.**) ($n_{control}$ = 12, $n_{royalactyin}$ = 12) and (**B.**) ($n_{control}$ = 10, $n_{royalactyin}$ = 10, $n_{20mM\ H2O2}$ = 11). In figure (**B.**) control worms are subjected to 20 mM H_2O_2 for 20 minutes as a positive control. The y-axis of the left graph was adjusted for clearer representation. Error bars = SEM.

1.5.SOD-3

The relative expression of SOD-3 is measured by observing the fluorescence intensity of a *sod-3p::*GFP transcriptional fusion construct integrated in the genome of the worms (see B.2.7.). One microplate experiment was carried out. As a positive control, worms were heat-shocked (35°C, 2h). As shown in figure 13, the fluorescence intensity of *sod-3p*::GFP is not significantly different between royalactin and control worms ($p_{t\text{-}test}$ = 0.6102). Furthermore, no significant difference is observed between the negative and positive (heat-shocked) control ($p_{t\text{-}test}$ = 0.8408).

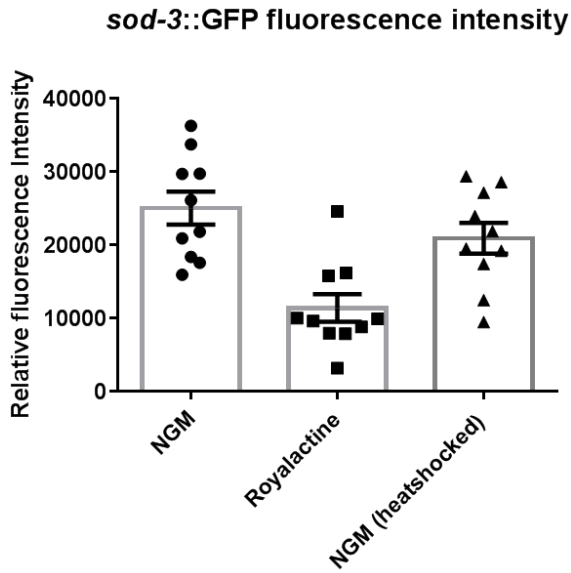

Figure 13: *sod-3p*::GFP fluorescence intensity is not significantly different in in royalactin-fed worms when compared to control worms.

1.6.EOR-1 mutant

The relative expression of GST-4 is measured in a mutant lacking a functional EOR-1 transcription factor (*eor-1(cs28)*) by observing the fluorescence intensity of a *gst-4p*::GFP transcriptional fusion construct integrated in the genome of the worms (see B.2.7.). As a positive control, worms are incubated with 10mM H_2O_2 for 20 minutes.

As shown in figure 14 the fluorescence intensity of *gst-4p*::GFP is not significantly different between royalactin and control worms ($p_{t\text{-}test}$ = 0.8693). There is a significant increase (+63.8%, $p_{t\text{-}test}$ = 0.0078) in the positive control when compared to the negative control. This experiment was performed 70 hours after plating out L1 larvae, meaning that the worms were day 0 adults.

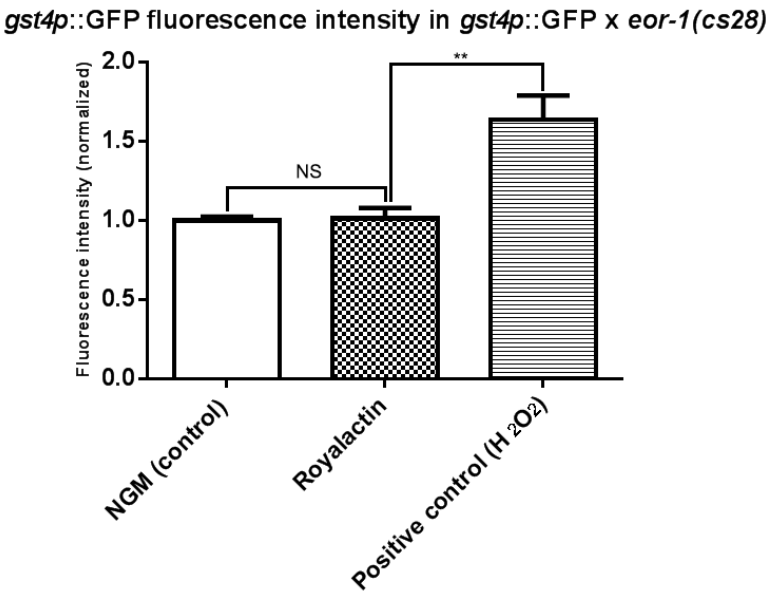

Figure 147: *gst-4p*::GFP fluorescence intensity in a mutant lacking a functional EOR-1 (*eor-1(cs28)*) is not significantly different in royalactin-fed worms. Worms were incubated in 10 mM H_2O_2 as a positive control.

2. Purification of royalactin from royal jelly

2.1. Purification of royalactin through HPLC

The purification of royalactin from royal jelly consisted of several (ultra)centrifugation steps, followed by HPLC (here performed in two separate runs). For each run, the absorbance was detected at two wavelengths: 214 nm (i.e. absorbance wavelength of peptide bonds; measured in channel 1) and 280 nm (i.e. absorbance of tryptophan and tyrosine residues; measured in channel 2). The resulting spectra are shown in figure 15. The volumes corresponding to the royalactin peaks are indicated with X1, X2 and X3. These fractions are collected, concentrated and the presence of royalactin is confirmed through SDS-PAGE (see B.2.2.) and peptide mass fingerprinting (PMF) (see B.2.3.). A second HPLC run showed a similar chromatogram. The royalactin-containing fractions of this run were labeled 2, 3, 4, 5 and 6.

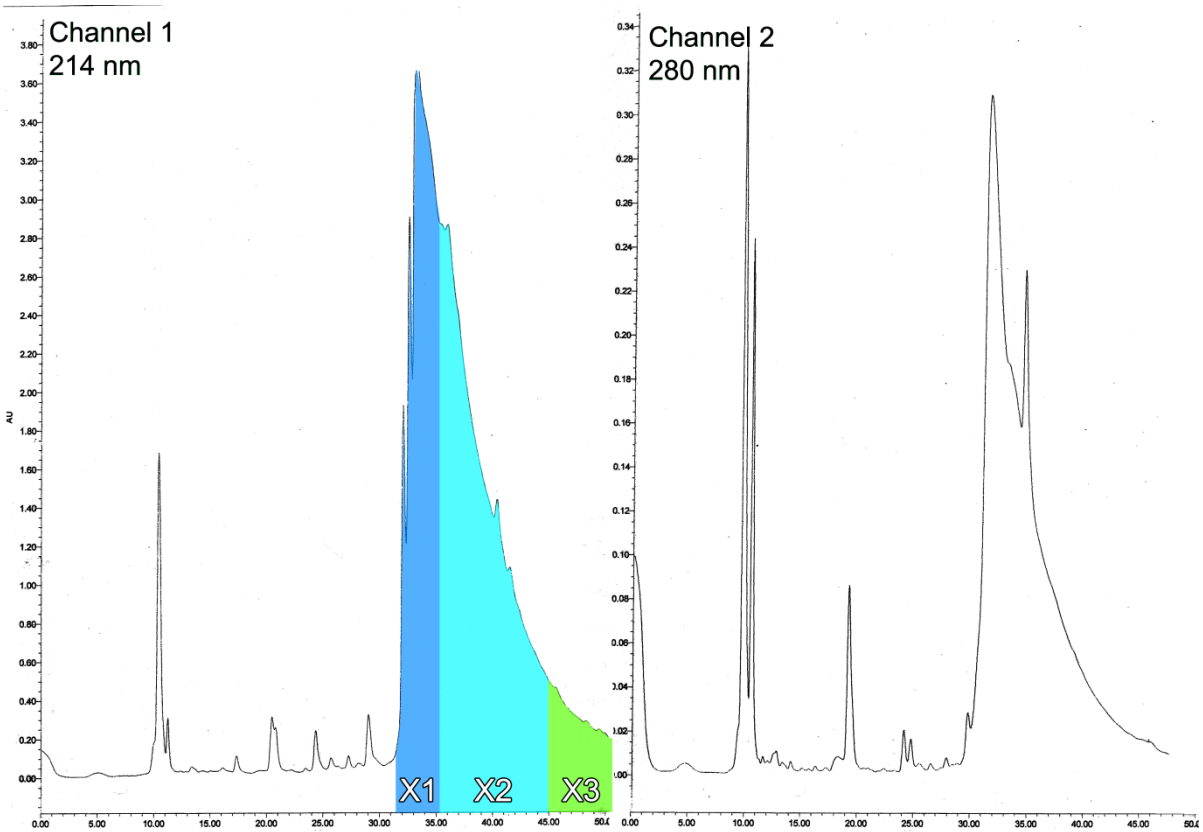

Figure 15: HPLC spectra of royal jelly purified by multiple rounds of ultracentrifugation. The large royalactin peak starts at minute 31. Three separate royalactin-containing fractions, indicated with X1, X2 and X3, are collected. A second HPLC run showed a similar chromatogram. On the y-axis the absorbance is depicted (in arbitrary units).

2.2.SDS-PAGE of HPLC fractions

The HPLC fractions collected in C.2.1. are put on an SDS-PAGE gel to evaluate the presence of royalactin, as shown in figure 16. For a detailed description, see B.4.3. The thick band above the 50 kDa mark corresponds to royalactin (~57 kDa).

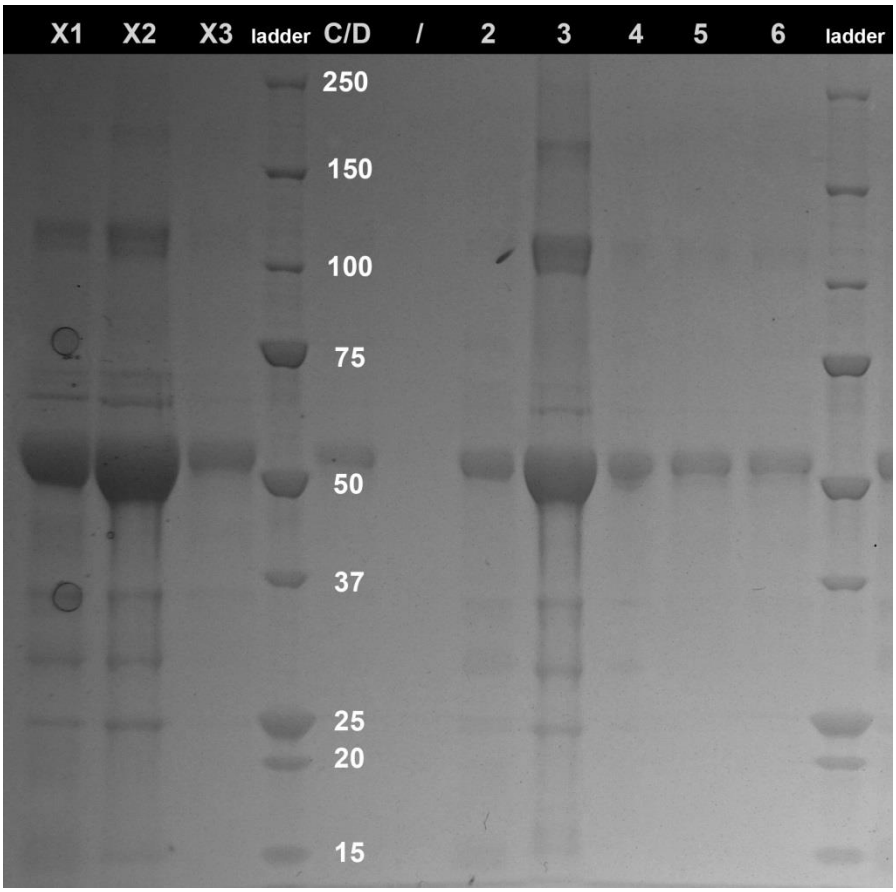

Figure 16: SDS-PAGE of the HPLC fractions of purified royalactin. Lanes X1 to X3 contain the HPLC fractions corresponding to the royalactin peak in the first run. Lanes 2 to 6 contain the HPLC fractions corresponding to the royalactin peak in the second run. The ladder is unstained Precision Plus Protein Standard (Bio-rad). The contrast of this image was increased for a clearer visualization of bands with low intensity.

2.3.Identification of tryptic royalactin peptides with peptide mass fingerprinting

The presence of royalactin (MRJP1_APIME) in several of the HPLC fractions is confirmed through mass spectrometry (gel-free PMF through MALDI-TOF). For a detailed description, see B.4.7.. Table 2 summarizes the MALDI-data.

Table 2: Identification of royalactin (MRJP1_APIME) in HPLC fractions. The E-value is a measure for the probability of observing the result by chance. A lower E-value means a lower probability of making an observation by chance. The score is a logarithmic conversion of the E-value, with scores higher than 67 being regarded as significant ($p < 0.05$). The 'queries matched' are the number of peptide-fragments found in the sample that match the identified protein. The sequence coverage is the proportion of the protein that is covered by the matched peptides.

Sample	Identified as	E-value	Score	Queries matched	Sequence coverage (%)
fraction 2	MRJP1_APIME	1.1e-07	89	13	28
fraction 2	MRJP1_APIME	8.2e-07	80	14	32
fraction 3	MRJP1_APIME	2.9e-12	135	13	26
fraction 3	MRJP1_APIME	5.8e-09	102	15	31
fraction 4	MRJP1_APIME	1.8e-14	157	14	29

3. Proteomics

In order to learn more about the downstream molecular effects of royalactin, a differential proteomics experiment was carried out. Before this thesis was started, the analytical part of the 2D-DIGE experiment was already completed, resulting in 47 differently expressed spots (i.e. protein spots on a gel that are differently expressed in control and royalactin-fed worms; $p < 0.05$). To identify the proteins located in these spots, two preparative gels with a large amount of protein are run and processed. After matching and processing the 47 spots, 41 are identified using tandem MS, yielding 35 unique proteins. Note that some proteins are identified in multiple spots (presumably due to different isoforms and/or post-translational modifications). The results are summarized in table 3.

Table 3: Identified differential proteins in royalactin-fed worms.. The mascot score is a logarithmic conversion of the E-value. The matched peaks are the number of peaks in the spectrum that are matched in the peptide database. The sequence coverage is the proportion of the protein that is covered by the matched peptides. The Decyder p-value indicates that the protein spots on the gels were significantly upregulated (**A.**) or downregulated (**B.**).

A. Upregulated proteins.

Sample name (spotpicker)	Protein name	Increase fold	Protein identifier	Gene identifier (Wormbase ID)	Mascot score	Matched Peaks	Coverage (%)	p-value Decyder
G3	Glutathione S-transferase 4	1.62	GST-4	WBGene00001752	65	5	23	0.0280
F3	Triosephosphate isomerase	1.44	TPI-1	WBGene00006601	500	18	34	0.046
E3	Dehydrogenase (SDR family)	1.42	F20G2.2	WBGene00008986	253	10	24	0.0260
B5	Actin-depolymerizing factor 1 (UNC-60)	1.40	UNC-60	WBGene00006794	1841	58	46	0.0004
B4	Aldehyde reductase	1.33	C07D8.6	WBGene00015565	1768	98	43	0.013
G4	Actin-depolymerizing factor 1 (UNC-60), isoforms a/b	1.30	UNC-60	WBGene00006794	277	21	46	0.013
F4	40S ribosomal protein SA	1.26	RPS-0	WBGene00004469	728	45	47	0.015
C4	Alcohol dehydrogenase	1.22	C30G12.2	WBGene00016274	355	10	29	0.027
D3	Probable elongation factor 1-gamma	1.21	EEF-1G	WBGene00008920	412	15	21	0.033
D5	T-complex protein 1 subunit theta	1.20	CCT-8	WBGene00021934	1228	50	43	0.022
G5	Isocitrate/isopropylmalate dehydrogenase	1.20	IDHG-1	WBGene00009440	291	8	17	0.038
B3	Isocitrate dehydrogenase	1.18	IDH-1	WBGene00010317	720	33	40	0.014
D1	Elongation factor 2	1.18	EEF-2	WBGene00001167	66	4	4	0.0015
E2	Aldehyde dehydrogenase 2, mitochondrial	1.16	ALH-1	WBGene00000107	194	9	18	0.0073
H4	T-complex protein 1 subunit zeta	1.16	CCT-6	WBGene00000381	1014	62	44	0.036
E1	Phosphoenolpyruvate carboxykinase	1.15	PCK-1	WBGene00021043	382	20	30	0.015
F2	Aldehyde dehydrogenase 2, mitochondrial	1.15	ALH-1	WBGene00000107	162	8	18	0.010
D2	Alanine aminotransferase	1.14	C32F10.8	WBGene00016333	197	9	21	0.032
H1	Intermediate filament protein ifb-2	1.13	IFB-2	WBGene00002054	254	9	17	0.038
C2	Zinc metallopeptidase	1.12	PES-9	WBGene00003982	1534	112	56	0.039
E6	Phosphoglycerate mutase	1.12	F57B10.3	WBGene00019001	507	29	26	0.015
F5	ATP synthase subunit beta, mitochondrial	1.12	ATP-2	WBGene00000229	10837	420	52	0.0098
H2	Probable succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	1.10	F47B10.1	WBGene00009812	1389	78	53	0,035

B. Downregulated proteins.

Sample name (spotpicker)	Protein name	Increase fold	Protein identifier	Gene identifier (Wormbase ID)	Mascot score	Matched Peaks	Coverage (%)	p-value Decyder
H3	Probable enoyl-CoA hydratase, mitochondrial	-1.33	ECH-6	WBGene00001155	345	12	19	0.0015
A5	UNC-87 (multiple isoforms)	-1.30	UNC-87	WBGene00006819	719	31	33	0.028
E5	Probable enoyl-CoA hydratase, mitochondrial	-1.28	ECH-6	WBGene00001155	278	11	34	0.0012
G1	Glutamate dehydrogenase	-1.26	GDH-1	WBGene00014095	440	18	32	0.047
E4	Protein kinase C substrate	-1.25	ZK1307.8	WBGene00014249	1402	54	58	0.0018
E4 - other ID	Heat shock 70 kDa protein A (HSP-1)	-1.25	HSP-1	WBGene00002005	1233	57	48	0.0018
G2	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	-1.24	DLAT-1	WBGene00009082	1243	54	39	0.038
C6	Vitellogenin 2 (also Vitellogenin 3, 4 & 5 found)	-1.23	VIT-2	WBGene00006926	553	29	19	0.013
D4	V-type proton ATPase catalytic subunit A	-1.17	VHA-13	WBGene00013025	590	28	31	0.032
D6	Actin-1 (also Actin-2, Actin-3 & Actin-4)	-1.17	ACT-1	WBGene00000063	531	19	27	0.014
H5	Troponin T, isoform A	-1.17	TNT-2	WBGene00006587	2751	159	37	0.037
A6	Glucose-6-phosphate 1-dehydrogenase	-1.16	GSPD-1	WBGene00007108	212	11	21	0.0041
B1	Glycosyl hydrolases family 31 (AAGR-3), isoform b	-1.16	AAGR-3	WBGene00009583	129	12	7	0.0430
A4	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	-1.15	ALH-8	WBGene00000114	1182	81	41	0.0033
C5	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	-1.15	ALH-8	WBGene00000114	1167	52	36	0.011
F1	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	-1.14	LET-721	WBGene00002855	1105	45	47	0.019

The proteins identified are also shown as a protein-protein interaction network, after visualizing their putative interactions with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), as can be seen in Figure 17. Each line between two proteins represents an interaction, based on co-expression data, database mining and other experimental data. On high confidence settings (0.700), 60 interactions were reported. The identified proteins form a network that is significantly enriched in interactions when compared to a random group of *C. elegans* proteins (p = 4.58e-12).

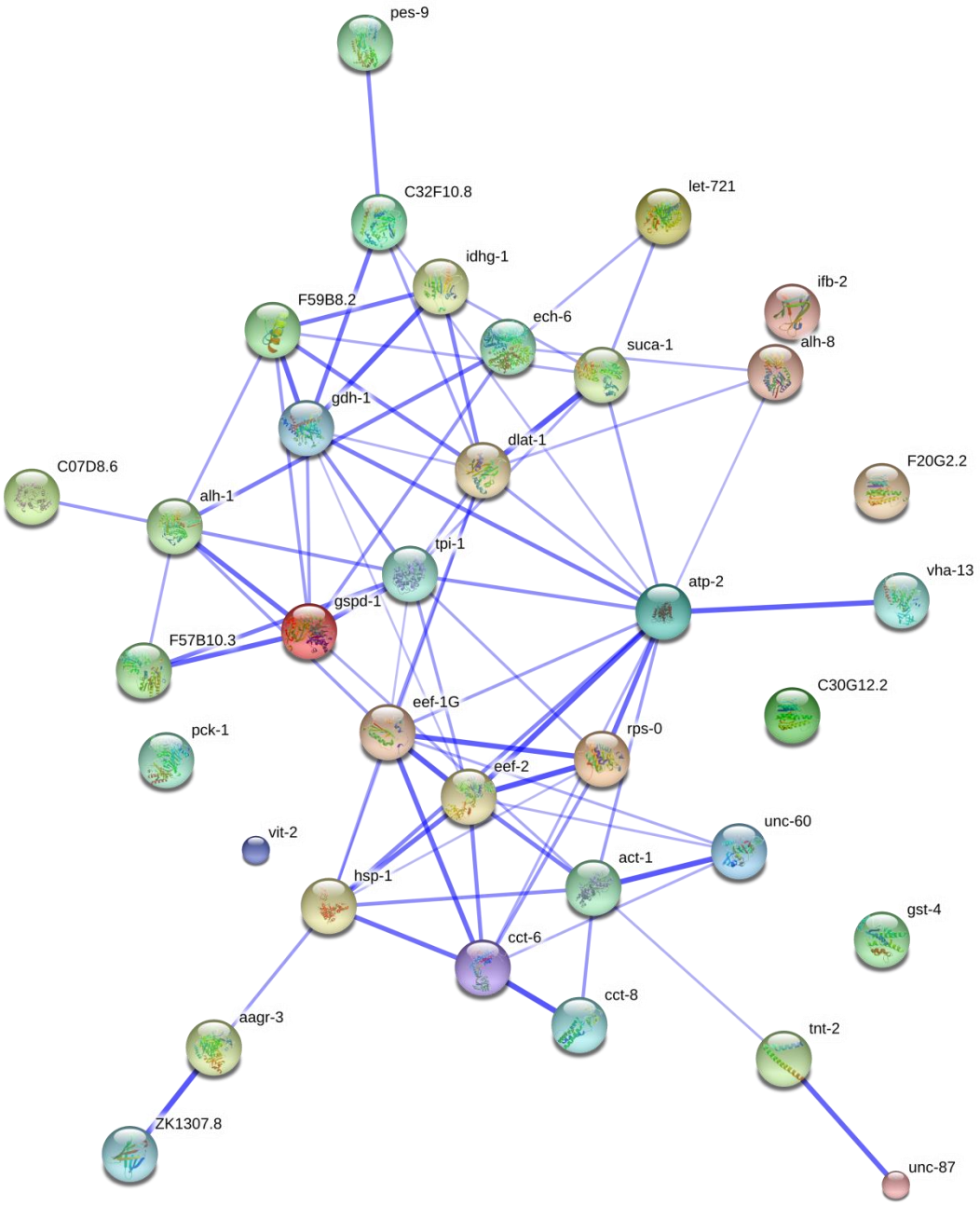

Figure 17: Visualization of proteomics data in STRING 10. (Search Tool for the Retrieval of Interacting Genes/Proteins). When entering the up/down-regulated proteins identified via MS into STRING, a network of protein associations appears. Thicker lines correspond to stronger associations. (settings; medium confidence (0.400), Active prediction methods: Co-expression, Experiments, Databases, Textmining)

4. Stress assays

4.1.Thermotolerance assay

The tolerance of royalactin-fed and control worms to heat stress is followed by subjecting the worms to a continuing temperature of 35°C and noting down their survival every hour. For a detailed description, see B.6.1.. Survival curves are constructed using the built-in survival function of GraphPad Prism 6. As shown in figure 18A, royalactin worms survive heat stress, on average, longer than control worms in a significant way ($p_{logrank} < 0.0001$). The average survival time of royalactin worms is 6.85 hours, while that of control worms is 6.02 hours, an increase of 13.8%. This experiment is repeated (shown in figure 18B) with similar results. Again, royalactin worms survive heat stress better than control worms in a significant way ($p_{logrank} = 0.0035$). This time, the average survival time of royalactin-fed worms is 8.92 hours, while that of control worms is 7.75 hours, an increase of 15.1%.

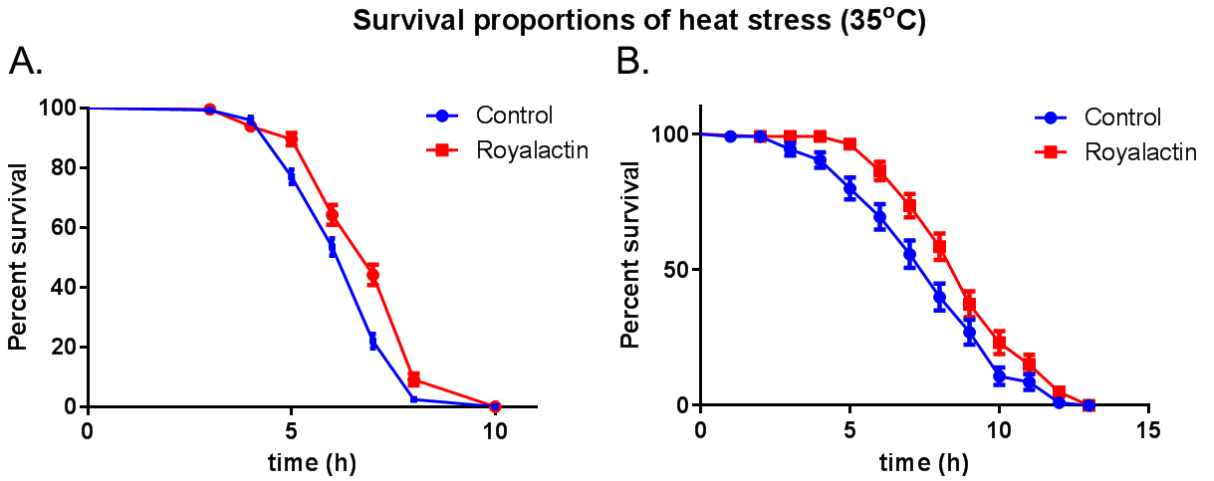

Figure 18: Royalactin-fed worms survive heat stress (35°C) longer than control worms. (**A.**) ($n_{control}$ = 290, $n_{royalactin}$ = 202) (**B.**) ($n_{control}$ = 95, $n_{royalactin}$ = 100). Error bars = SEM.

4.2.Reductive stress assay

The tolerance of royalactin-fed worms to reductive stress is followed by incubating the worms in a 5 mM DTT solution for 24 hours. Immediately thereafter, the survival of the worms is noted down. For a detailed description of this procedure, see B.6.2.. This experiment is performed for day 1 adult worms and day 3 adult worms. A two-way ANOVA is performed using GraphPad Prism 6.

Royalactin-fed day 1 adult worms survive the DTT stress assay more successfully in a significant way ($p_{t\text{-}test} = 0.0366$) when compared to control worms, as shown in figure 19. 40.54% of royalactin worms and 47.42% of control worms were dead.

Royalactin-fed day 3 adult worms also survive the DTT stress assay more successfully in a significant way ($p_{t\text{-}test}$ = 0.0261) when compared to control worms, as shown in figure 19. 5.65% of royalactin worms and 12.97% of control worms were dead.

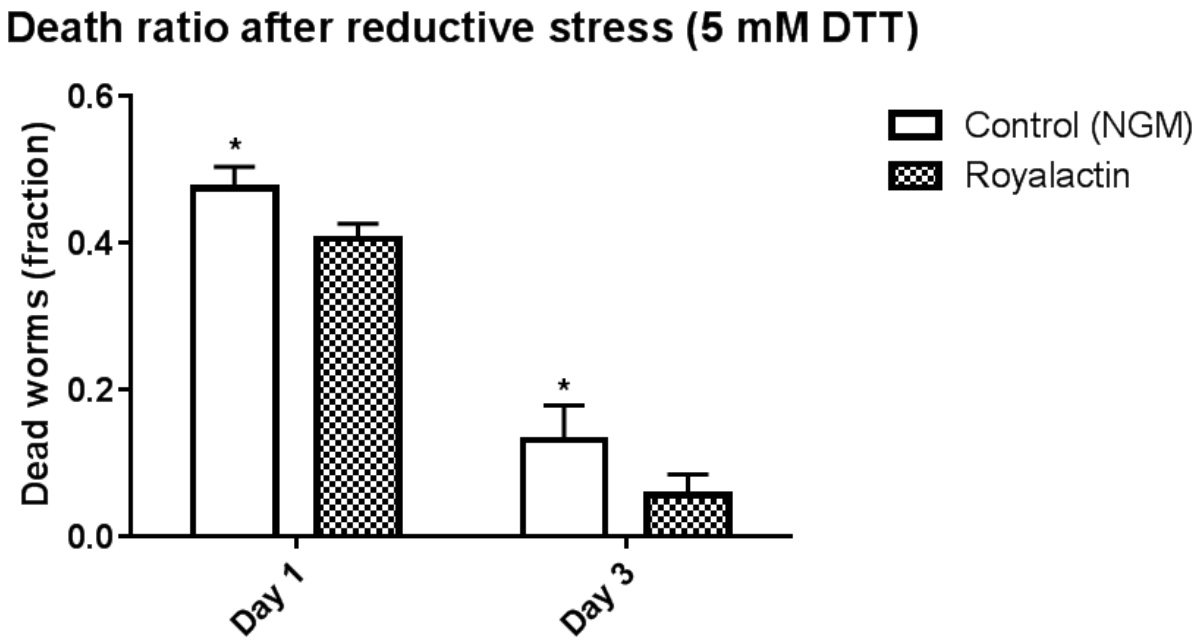

Figure 19: In both day 1 and day 3 adults, royalactin-fed worms survive 24h incubation in 5 mM DTT better than control worms. (Day 1: $n_{control}$ = 413, $n_{royalactin}$ = 276; day 3: $n_{control}$ = 508, $n_{royalactin}$ = 534.) Error bars = SEM.

4.3.Oxidative stress assay

The tolerance of royalactin-fed worms to oxidative stress is followed by incubating the worms in a 5 mM H_2O_2 solution for 1 hour and noting down their survival every hour. For a detailed description, see B.6.3.. A two-way ANOVA is performed using GraphPad Prism 6. The fraction of surviving worms at different recovery times after H_2O_2 treatment is shown in figure 20. Royalactin-fed worms survive the H_2O_2 stress assay more successfully in a significant way after 0 ($p_{t\text{-}test}$ = 0.0286), 60 ($p_{t\text{-}test}$ = 0.0052), and 120 ($p_{t\text{-}test}$ = 0.0062) minutes of recovery when compared to control worms.

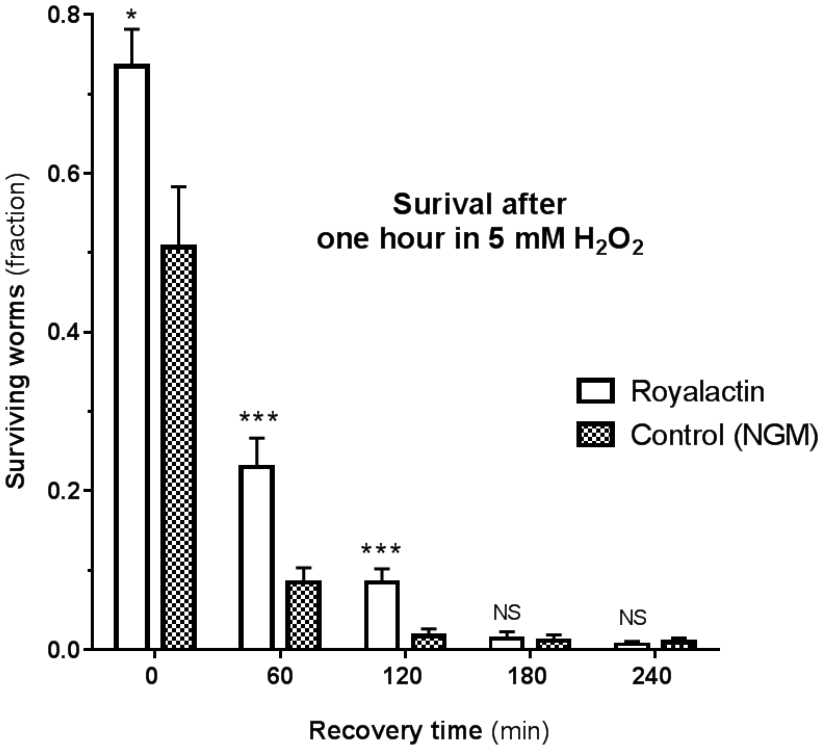

Figure 20: **Royalactin-fed worms survive incubation in 5 mM H_2O_2 for 1 hour better than control worms**. ($n_{control}$ = 304, $n_{royalactin}$ = 158). Error bars = SEM.

5. Cleaving royalactin with hydroxylamine

5.1.SDS-PAGE of cleaved royalactin

Royalactin was cleaved with hydroxylamine. The effect of hydroxylamine treatment was assayed with SDS-PAGE. The results are shown in figure 21. After 20 hours of incubation at 40°C, presumably the ~12 kDa N-terminal part of royalactin remains visibly intact. Yet, other fragments appear to be present also. Dialysis (with a molecular weight cut-off of 8 kDa) was not enough to remove these fragments (data not shown). For more details on how hydroxylamine cleaves royalactin and the used conditions, B.7.1..

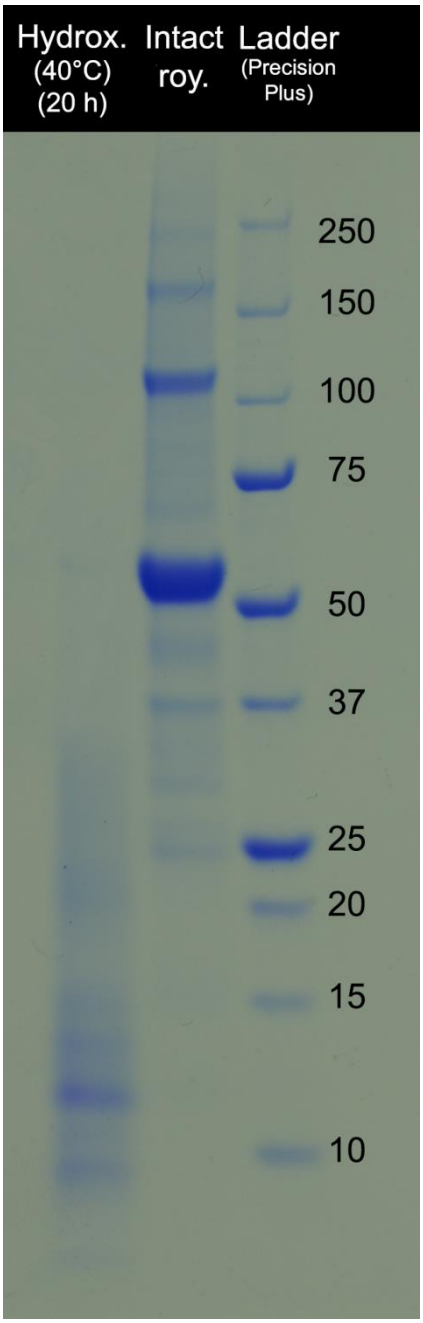

Figure 21: SDS-PAGE of royalactin cleaved with hydroxylamine. The first lane contains royalactine treated with hydroxylamine for 20 hours at 40 °C. The second lane shows intact royalactin. The third lane contains the ladder: unstained Precision Plus Protein Standard (Bio-rad).

6. Metabolic measurements

6.1. Respiration measurements

The respiration rate of royalactin and control worms is measured by monitoring oxygen levels for at least 20 minutes. The results for three biological replicates per condition (each consisting of 2-3 technical replicates) are presented in figure 22. Although we see a small increase in the mean respiration rate, no significant ($p_{t\text{-}test}$ = 0.2057) difference is observed between the respiration rate of royalactin-fed and control worms. These data are corrected for total protein content, as described in B.3.2.2..

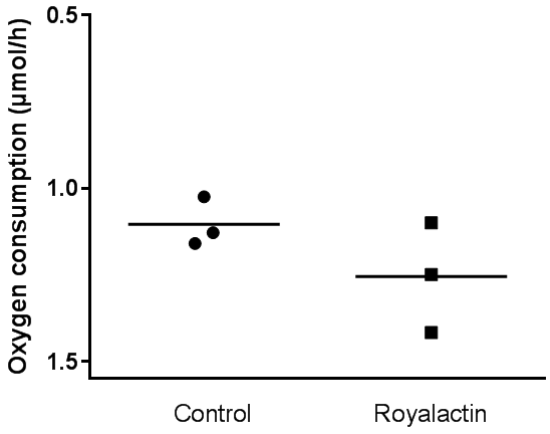

Figure 22: Oxygen consumption rate is not significantly different between royalactin-fed and control worms.

6.2. Thermal activity monitoring

The heat production (µW) of royalactin-fed worms is measured with a thermal activity monitor (TAM) over a time span of 6 hours, generating the heat production curves shown in figure 23. For a detailed description, see B.8.3.. The average heat production (shown in figure 24) is calculated over a time span of 1 hour where the heat production is relatively stable. The average heat production is not significantly ($p_{t\text{-}test}$ = 0.3493) different between royalactin-fed and control worms.

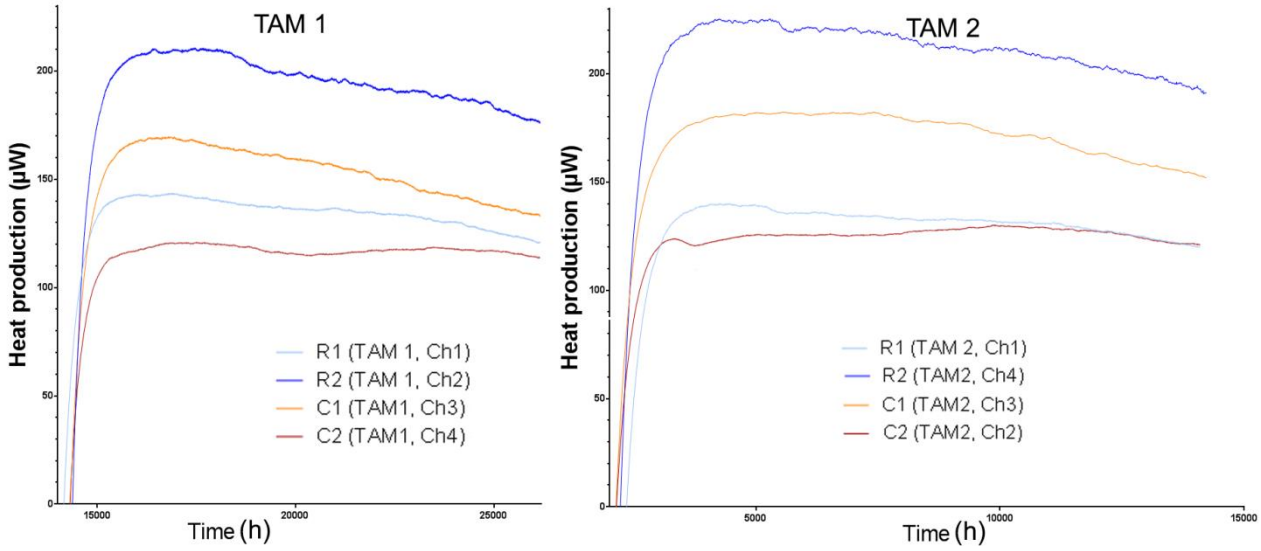

Figure 23: **Heat production curves of royalactin-fed worms and control worms**. R1 and R2 correspond to royalactin-fed worms. C1 and C2 correspond to control worms. TAM1 and TAM2 are technical replicates.

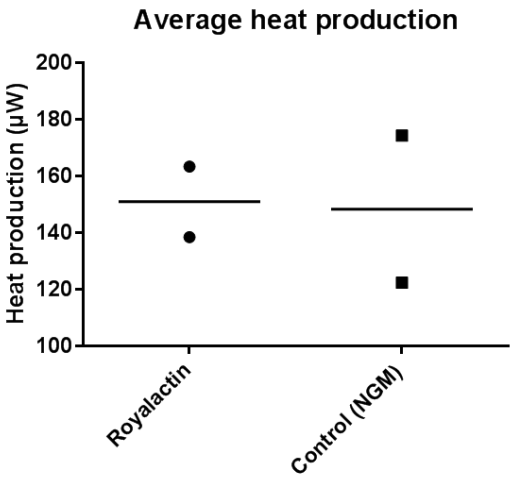

Figure 24: Average heat production is not significantly different between royalactin-fed and control worms.

D. Discussion

This thesis focused on the functional and cellular changes in *C. elegans* after royalactin treatment. Certain functional changes in royalactin-treated worms, such as an increased lifespan and locomotion[10] were already known. Still, other changes and the mechanism of royalactin-mediated signaling, downstream of the EGFR pathway, together with the specific role of certain molecular targets of royalactin remained elusive.

In this thesis, the role of the transcription factors SKN-1 and EOR-1 are studied through the expression of their respective targets GST-4 and HSP-16.2.

Royalactin-treated worms are subjected to various kinds of stress assays (heat, H_2O_2 and DTT) and the abundance of important chaperones that respond to stress (HSP-6 for mitochondrial stress, HSP-4 for ER stress) is studied in royalactin-treated worms.

A complementary but wider approach is undertaken by identifying proteins with differential abundance in royalactin-treated worms through 2D-DIGE followed by tandem MS and PMF. As many metabolic proteins are identified this way, certain metabolic parameters (i.e. respiration rate and heat production) were measured in royalactin-treated worms.

Finally, preliminary experiments were carried out to uncover the bioactive part of royalactin. The process of cleaving royalactin with hydroxylamine was optimized.

1. Following the royalactin signal

1.1. Downstream from the EGFR

The glutathione S-transferase GST-4 is a known target of the SKN-1 transcription factor[74,75], which is one of the possible propagation canals of the royalactin signal (see figure 4). Often, GST-4 transcription is used as a read-out for SKN-1 activity[75,96]. As transcription of GST-4 in royalactin worms is higher than in control worms (see C.1.1.), our results suggest that the royalactin signal, initiated at the EGFR, is propagated (at least in part) through the SKN-1 transcription factor. This is not unlikely, as SKN-1 has been shown to fulfil an essential role in oxidative stress resistance in *C. elegans*[97,98]. Furthermore, SKN-1 regulates the transcription of phase 2 antioxidant and detoxification enzymes[69], the induction of which could promote slowing down aging[99].

Taken together, our findings suggest that SKN-1 might be (in part) responsible for the observed increase in healthspan, lifespan (and stress resistance, see below) of royalactin worms.

However, our results also suggest that SKN-1 is not the only transcription factor necessary for royalactin-induced transcription of *gst-4*, as GST-4 expression does not increase after royalactin treatment in a mutant that lacks a functional EOR-1 transcription factor (see C.1.6.). This implies that EOR-1 is indeed necessary for the royalactin-dependent transcription of *gst-4*. This explanation is strengthened by a recent study's identification of a possible EOR-1 binding site on the *gst-4* promotor through a CHiP-Seq experiment[100].

Expression of the small heat shock protein HSP-16.2 is believed to be inhibited by the transcription factor EOR-1 and its obligatory binding partner EOR-2[71] (see figure 4).
A significant difference in HSP-16.2 expression is observed when subjecting both conditions (i.e. royalactin and control worms) to a heat shock (see C.1.2.). In this case, HSP-16.2 expression is slightly lower in royalactin worms, a result that can be explained by assuming that royalactin acts through EOR-1, which inhibit the expression of HSP-16.2. However, in the experiment where both royalactin and control worms are treated with heat, the observed difference is only marginally significant (-24.2%; p = 0.045), casting doubts about the biological significance of the effect. Still, it has been shown that high transcript levels of heat-shock genes (such as *hsp-16.2*) and lifespan extension are inversely related in *C. elegans*[101]. The lowered levels of *hsp-16.*2 transcription might fit in a model were lower heat-shock protein levels are associated with lifespan extension.

When royalactin-fed and control worms are not subjected to heat-shock, we did not always observe a significant difference in HSP-16.2 expression. This could mean that the royalactin-signal is not propagated through the EOR-1 transcription factor at 20°C, although this is in conflict with our earlier finding that the EOR-1 transcription factor is necessary for royalactin-induced transcription of GST-4. It possible that the difference in transcription levels of *hsp-16.2* are only apparent after heat shock treatment (which increases the signal-to-noise ratio immensely).

1.2. DAF-16 and SOD-3

The transcription of *sod-3* is regulated in *C. elegans* by DAF-16[102], a transcription factor that influences longevity through insulin/IGF-1 signaling[103]. Research has shown that the royalactin signal in *A. mellifera* most likely does not exert its effect through insulin signaling, as insulin receptor knockdown still induced queen-like differentiation upon feeding with royalactin[9].
No significant difference in SOD-3 expression was measured between royalactin-fed and control worms, suggesting that the royalactin signal does not signal through DAF-16. This

result should be nuanced however, since no significant difference was observed between negative and positive (heat-shocked) control worms either.
However, the hypothesis that royalactin works independently of DAF-16 is strengthened by the observation that DAF-16 is not localized to the nucleus in royalactin-fed worms (data not shown). Figure 25 illustrates nuclear localization of DAF-16. A comparable image would be expected after royalactin-treatment when royalactin would activate DAF-16.

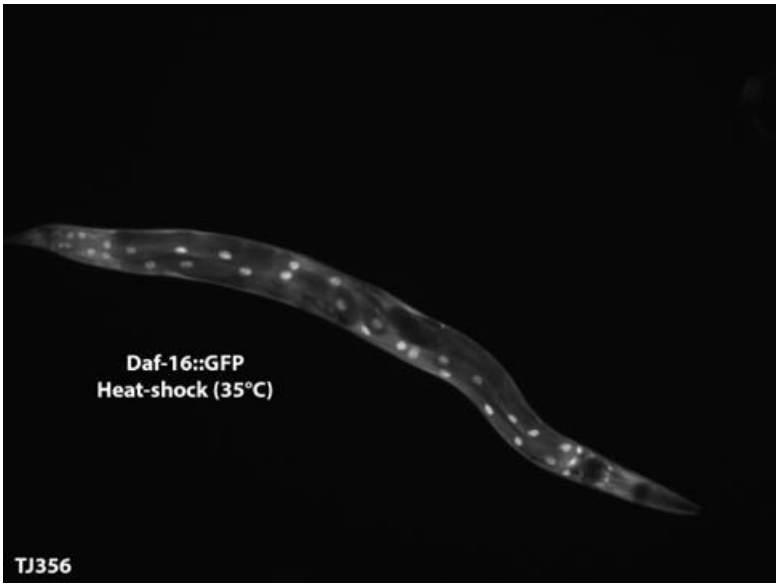

Figure 25: Nuclear localization of the DAF-16 transcription factor after a 35°C heat-shock.

2. How royalactin-fed worms deal with stress

Aging and stress, specifically oxidative stress, have been intimately linked in research for years[104]. In *C. elegans*, many long-lived single-gene mutants show an increased ability to respond to several kinds of stress[105]. Many of the experiments in this thesis were performed to address the question: do long-lived royalactin-fed worms also respond to stress differently than regular wild-type worms?

2.1.Thermotolerance

The resistance to a continuous temperature of 35°C is higher in royalactin worms when compared to control worms. Two separate experiments, performed at different time points, yield a 13.8% and a 15.1% increase in average survival time (see C.4.2.). Under the tested conditions, our results suggest that royalactin-fed worms do indeed experience a modest increase in resistance to heat stress. Whether this heat resistance is conveyed through the same pathway implied in royalactin-mediated lifespan extension, is not known.
In literature, similar effects are found after treatment with other lifespan-increasing substances in *C. elegans*. Tasco®, a commercial animal feed ingredient produced by the brown alga

Ascophyllum nodosum, has been shown to extend lifespan as well as impart thermal stress resistance in *C. elegans*[106]. Other documented examples of substances that extend lifespan as well as increase thermal stress resistance in *C. elegans* are plant adaptogens[107], quercetin (a flavonol class polyphenol)[108], fullerenol (a fullerene derivate)[109], protocatechuic acid[110] and *Acanthopanax sessiliflorus* stem extract[111].

2.2.Oxidative stress

Oxidative stress is defined as an imbalance between the production of reactive oxygen species and antioxidant defenses, in favor of the former[112,113]. The survival rate of worms treated with 5 mM H_2O_2 for 1 hour is higher in royalactin worms when compared to control worms. Our data suggest that, under the used experimental conditions, royalactin worms are more resistant to oxidative stress. A possible mechanism underlying the observed increase in stress resistance is the activation of the transcription factor SKN-1, which fulfils an essential role in oxidative stress resistance in *C. elegans*. We have shown earlier that worms treated with royalactin exhibit higher levels of GST-4 transcription (a target of SKN-1). GST-4 expression has been shown to confer oxidative stress tolerance in a dose-dependent manner[114].This experiment provides further indication that royalactin might signal through SKN-1 to impact both aging and oxidative stress resistance, but to verify this hypothesis, further experiments are needed.

Other substances that increase lifespan as well as oxidative stress resistance include quercetin[115], fullerenol[109], protocatechuic acid[110], and *Acanthopanax sessiliflorus* stem extract[111].

2.3.Mitochondrial stress

Mitochondrial stress can be a consequence of increased protein misfolding in the mitochondrion. It elicits a distinct reaction in the cell called the mitochondrial unfolded protein response (UPR^{mt}), which causes the activation of genes encoding mitochondrial chaperones that help to restore protein homeostasis[116]. One of these chaperones in *C. elegans* is HSP-6[95].

HSP-6 expression is lower in royalactin-fed worms when compared to control worms. Our data suggest that, under the used experimental conditions, royalactin causes lesser induction of the mitochondrial stress chaperone HSP-6. One explanation could be that royalactin worms generally experience less mitochondrial stress, and thus need less chaperones to maintain protein homeostasis. This also ties in with the recent observation that high transcription levels of heat-shock genes are associated with shorter lifespan in *C. elegans*[101].

2.4.Reductive and ER stress

Reductive stress is defined as the imbalance between oxidants and antioxidants in favor of the anti-oxidants, making it a direct opposite of oxidative stress[113]. It has been suggested that

reductive stress can cause a decrease in lifespan in *C. elegans*. The mechanism however remaining unclarified[117,118].

The survival rate of worms treated with 5 mM DTT, which induces reductive stress, is higher in royalactin-fed worms when compared to control worms, both in day 1 and day 3 adults. Our results suggest that, under the used experimental conditions, royalactin-fed worms are more resistant to DTT-induced reductive stress.

A different kind of stress caused by treatment with DTT is ER stress, characterized by an accumulation of unfolded proteins in the ER[119]. The observed increase in survival rate of worms treated with DTT might thus also result from an increased resistance to ER stress.

HSP-4 is a heat shock protein and *C. elegans* homologue of the mammalian BiP. HSP-4 plays a central role in the response to unfolded proteins in the endoplasmic reticulum[94].

hsp-4::GFP fluorescence intensity is lower in royalactin-fed worms when compared to control worms, indicating a lower expression of HSP-4 in royalactin worms. This effect persists when both conditions are treated with 5 mM DTT for 1 hour. Our results suggest that, in the observed conditions, royalactin causes lesser activation of ER stress chaperone proteins like HSP-4. Combined with the previous result that suggests royalactin-fed worms are more resistant to ER (and reductive) stress caused by DTT, one could speculate royalactin-fed worms are overall better equipped for maintaining their protein homeostasis: upon ER stress induction, they need to react less severely (by producing chaperones) and survive longer.

2.5.Conclusion: how do royalactin worms respond to stress?

In general, our data suggest that royalactin-fed worms have an increased capability of dealing with various kinds of stress. Royalactin-fed worms show a higher survival rate after treatment with heat, H_2O_2 and DTT. Furthermore, the chaperone proteins HSP-6 and HSP-4 are less abundant in royalactin-fed worms, suggesting a less severe compensating response to mitochondrial (HSP-6) and ER (HSP-4) stress.

3. Purification of royalactin from royal jelly

We were able to purify additional royalactin from royal jelly, using ultracentrifugation and HPLC, for use in various bioassays. The HPLC spectra depicted in figure 6 show a large peak corresponding to royalactin (personal communication, Giel Detienne), starting at minute 31.

Royalactin was present in all collected HPLC fractions, as shown in figure 7. The large band at ~57 kDa corresponds to royalactin. Fraction X1, X2 from the first run and fraction 3 from the second run, which correspond roughly to the top of the peak on the HPLC spectra, contain the largest amounts of royalactin.

PMF through MALDI-TOF confirmed the presence of royalactin in the tested fractions, as shown in table 2. Overall, we can say the purification process worked well.

4. The big picture: proteomics data

2D-DIGE followed by PMF via MS/MS resulted in the successful identification of 41 (of 47) spots, yielding 35 unique proteins. Of these proteins, 21 are upregulated (table 3A) and 14 are downregulated (table 3B) in royalactin-fed worms when compared to control worms.

Interestingly, transcription of one of the upregulated proteins identified with PMF, GST-4, was already used as a read-out for the activation of the SKN-1 transcription factor before the results of the proteomics experiments were known. These fluorescence experiments confirmed the increased transcription of GST-4 in royalactin-fed worms, while the proteomics experiment confirmed its abundance on a protein-level. This highlights the strength of taking a two-pronged approach: when the results of classical biological assays such as fluorescence intensity measurement of a transcriptional construct are in agreement with protein-level experiments such as PMF, solid conclusions can be drawn.

Analysis with the bio-informatics tool STRING shows that the identified proteins form a network, based on different kinds of interaction data (co-expression, experiments, databases and text mining). These proteins can be grouped based on their biological processes, a process known as gene ontology (GO) enrichment analysis. This type of analysis shows which biological processes occur more often than would be expected from a random group of proteins. Table 4 shows the GO enrichment analysis based on biological processes performed on the 35 identified proteins. Many of these proteins are associated with metabolic processes, development, and aging. Taken together, these data suggest that royalactin could have an influence on the general metabolic state of the worm.
It is also interesting to see that 11 of the identified proteins are associated with locomotion. It was previously reported that royalactin-fed worms showed an increased locomotory activity in mid-life[10].

Table 4: GO enrichment analysis based on biological processes, performed with STRING 10. The biological process, number of genes corresponding to this process and p-value after fakse discovery rate (FDR) correction are indicated. (settings: SLIM, FDR, IEA: allowed.)

Term	Number Of Genes	p-value (FDR)
generation of precursor metabolites and energy	6	1.99E-5
small molecule metabolic process	10	1.99E-5
anatomical structure development	20	4.63E-4
embryo development	18	4.63E-4
aging	10	6.06E-4
reproduction	16	6.96E-4
carbohydrate metabolic process	6	2.62E-3
growth	5	8.83E-3
catabolic process	7	1.06E-2
locomotion	11	1.66E-2

5. Hydroxylamine treatment: the bioactive part of royalactin

The hydroxylamine treatment of royalactin underwent many optimizations. Initially, a short incubation time with hydroxylamine was tested in order to generate a large C-terminal fragment, leaving the rest of the protein mostly intact (data not shown). This route was eventually abandoned because the fragments were hard to separate from intact royalactin through HPLC.

Royalactin was cleaved successfully after treatment with hydroxylamine at 40°C for 20 hours, which leaves the short (~12 kDa) N-terminal fragment intact and fragments most of the rest of the protein. Figure 12 shows a band at ~12 kDa, which corresponds to the N-terminal part of royalactin with intact sugar chains. While the band at 12 kDa is the most visible, other bands, corresponding to various other cleavage products, can also be seen. The optimization of this process is not fully finished.

A pilot experiment for determining the bioactivity of this N-terminal fragment was carried out (data not shown). Cleaved royalactin was added to the growth medium of *gst4p*::GFP worms in the same fashion as intact royalactin for previous experiments. The aim was to measure *gst4p*::GFP fluorescence intensity as readout for royalactin bioactivity. While this experiment did show a small but significant difference in fluorescence intensity between a negative control and worms fed with the fragmented royalactin, the intensity of worms fed with intact royalactin (as a control) was lower than that of untreated worms, casting doubt on the validity of this experiment.

6. Metabolic measurements

In our experiments, no significant difference was found in respiration rate and thermal activity between royalactin-fed and control worms. Analysis of the proteomics data however showed us that many of the up/down-regulated proteins were connected to metabolic processes, suggesting that royalactin might have an influence on the metabolism after all.
This influence might be very small or – more likely – the experiments were not performed in enough replicates as to produce statistically sound results.
In the case of the respiration measurements (see C.6.1.), there does seem to be a difference, with the oxygen consumption of royalactin worms being lower, but the used amount of replica's (n=3) is not high enough to draw statistically relevant conclusions.

7. Future research

Although the possible role of several key players (i.e. the transcription factors EOR-1 and SKN-1) was suggested, a complete model of the molecular workings of royalactin remains unachieved. Possible future research includes experiments with worms that lack a functional SKN-1 transcription factor, as was already done with EOR-1.

The output of a proteomics experiment is often extensive but –in itself- does not provide enough evidence for robust conclusions. This is why some of the most promising identifications emerging from the proteomics experiment should be confirmed with classical biological assays (e.g. fluorescence quantification of GFP-fused transcriptional constructs) in future experiments. On one hand, it is important to validate the up/down-regulation of the proteins identified through PMF, as was already done with GST-4. On the other hand, experiments can be designed to identify the differential proteins that are at the causal basis of lifespan extension and not merely correlated to its intermediary effects (e.g. a change in metabolism).

The metabolic experiments seemed like a promising route at first, but the results were in both cases non-significant. While there is of course a good chance that the metabolic rate of royalactin-treated worms does not differ (in a detectable way) from normal worms, this route of research could be pursued more extensively. Repetition of the respiration rate and thermal activity measurements with more replicates might reveal interesting observations in future research.

The hydroxylamine treatment of royalactin is functional, yet can be optimized further. Varying the buffer composition and incubation conditions might reveal a way to cleave royalactin into other interesting fragments aside from the ~12 kDa N-terminal fragment.

The N-terminal fragment can however be used for future experiments that aim to answer whether this fragment retains royalactin's bio-active function. A pilot experiment was carried out by assaying the fluorescence intensity of GST-4 (which has been shown to increase after royalactin treatment) after treatment with the N-terminal fragment. However, the result was not conclusive and this experiment should be repeated.

Although many kinds of stress were already assayed, there is still room for more. In many other life-prolonging substances, a UV stress assay is carried out to characterize the resistance of the organism against DNA damage. The exact nature of royalactin-fed worms' increased resistance could also be studied by performing an oxidative stress assay on mutants lacking the SKN-1 or EOR-1 transcription factor. This would confirm that SKN-1 and/or EOR-1 is crucial in conferring increased stress resistance on royalactin-fed worms.

Conclusions

Our results suggest that the royalactin signal results in increased expression of GST-4, probably regulated by both the SKN-1 and EOR-1 (and its binding partner EOR-2) transcription factors. A non-influence of royalactin on SOD-3 expression and lack of nuclear localization of DAF-16 suggest that the royalactin signal is propagated in DAF-16 independent fashion.

Proteomics analysis resulted in the identification of 35 unique proteins that are up/downregulated in royalactin worms. Of these proteins, many are associated with the metabolism, suggesting that royalactin has an influence on the metabolic state of the worm.
However, no significant differences were found in royalactin worms when it comes to thermal activity or respiration rate.

The hydroxylamine-treatment of royalactin is functional, yet can be optimized further.

Royalactin-fed worms were subjected to heat, oxidative and reductive stress. In all cases, it was observed that royalactin-fed worms survived the stress assay more successfully than untreated worms, an observation that could be (in part) explained by the increased expression of SKN-1 and GST-4.
Overall, royalactin-fed worms survive the stress assays more successfully than their untreated counterparts and the monitored chaperone proteins are less abundant, suggesting a more effective but less severe stress response in royalactin-fed worms.

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Addendum

Risk analysis

During laboratory work, the general safety procedures of the Health, Safety & Environment Department HSE (*Veiligheid, Gezondheid en Milieu, VGM*) were followed at all time. This includes wearing a lab coat, gloves and, if necessary, protective glasses. In this project, several dangerous chemicals were used. Although often only diluted solutions and/or small volumes were used, several safety precautions were taken.

When working with (diluted solutions of) **TFA and FA** (risk class E3), contact with skin and eyes was avoided (H314) and all actions were performed in a laminar flow to avoid inhalation (H332).
ACN (risk class E3) is highly flammable (H225) and harmful when swallowed (H302). Contact with skin or eyes leads to irritation (H312, H319) and was avoided. Care was taken to not work with ACN in the vicinity of open flames (P210).
FUdR (risk class E2) has a harmful impact on fertility. FUdR can be harmful when inhaled or swallowed (P301). A mouth mask was worn when weighing FUdR to prevent inhalation.
Preparation of a bleaching solution involves mixing of **sodium hypochlorite** and **sodium hydroxide** solutions (both risk class E3). Sodium hypochlorite is inflammable, can cause explosions (H271) and is harmful when swallowed (H302) or inhaled (H332). Care was taken not to store the substance in the vicinity of other flammable substances (P220). Contact with the skin was avoided by wearing gloves. Sodium hydroxide is corrosive (H290), harmful when swallowed (H302), can cause burns in contact with skin and is harmful to the eyes (H314). Contact with skin and eyes was avoided by working in a laminar flow and wearing gloves (P280).
Acrylamide in monomeric form is a neurotoxin that can cause genetic damage (H340), cancer (H350) and, after repeat exposure, organ damage (H372). Inhalation (H332), contact with skin (H312) or eyes (H319) is harmful and was avoided at all time by wearing gloves and working under a laminar flow (P280).
Acetic acid is flammable (H226), can cause burns after skin contact and is harmful to the eyes (H314). To avoid contact with skin and eyes, gloves were worn and all actions were performed under a laminar flow (P280).
DTT is harmful when swallowed (H302), inhaled (H335), can cause skin irritation (H315) and is harmful to the eyes (H319). To avoid contact with skin and eyes, gloves were worm and all actions were performed under a laminar flow (P280).
Methanol is highly flammable (H225) and harmful when it comes into contact with the skin (H311), when inhaled (H331) or swallowed (H301). After exposure, methanol can cause

organ damage (H370). To avoid contact with skin and eyes, gloves were worm and all actions were performed under a laminar flow (P280). The substance was kept out of the vicinity of open flames (P210).

SDS is flammable (H228), harmful when swallowed (H302) and harmful to the skin (H311). SDS can cause irritation of the skin (H315), eyes (H319) and when inhaled (H335). The inhalation of dust was prevented (P261) by wearing a mouth mask. Gloves were used when handling this substance (P280). The substance was kept out of the vicinity of open flames (P210).

Trypsin can cause irritation of the skin (H315) and eyes (H319) and is harmful when inhaled (H335). Gloves were worm when working with trypsin solutions (P280).

Hydroxylamine hydrochloride is harmful when swallowed (H302) or inhaled (H335) and can cause irritation of the skin (H315) and eyes (H319). Hydroxylamine is mutagenic for mammalian somatic cells (H340). To avoid contact with skin and eyes, gloves were worm and all actions were performed under a laminar flow (P280).

Guanidine hydrochloride is harmful when swallowed (H302) and can cause irritation of the skin (H315) and eyes (H319). Guanidine hydrochloride is a neurotoxin and repeated exposure may cause chronic respiratory irritation and organ damage (H372). Direct contact was avoided by wearing gloves and working under a laminar flow (P280).

Non-harmful genetically modified organisms (*E. coli* strain OP50) were used but do not pose a direct threat to human health. **Biological waste** was deposited in Cordi boxes for destruction immediately after use. All surfaces were cleaned with 70% ethanol after contact with biological agents.

Apart from chemical and biological hazards, precautions were also taken for several physical hazards. When performing **ultracentrifugation**, very high rotation speeds and g-forces were reached. To minimize risk, the tubes in the rotor were carefully balanced.

When **autoclaving**, special care was taken not to open the autoclave when the temperature or pressure inside was too great. When emptying the autoclave, heat-resistant gloves were used to avoid burns.

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