ASCAROSIDE-INDUCED NEURONAL REPROGRAMMING

OF THE GERMLINE IN THE NEMATODE

AUANEMA FREIBURGENSIS

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A THESIS SUBMITTED FOR THE DEGREE OF PHD

THE UNIVERSITY OF WARWICK, SCHOOL OF LIFE SCIENCES

NOVEMBER 2017
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ACKNOWLEDGEMENT

First and foremost, I would like to thank and express my deep gratitude to my advisor Dr. Andre Pires da Silva. His support and patience, together with his enthusiasm for science and deep knowledge, have been fundamental during my Ph.D. experience. I have learnt from him not only how to perform experiments and delivery scientific talks, but especially determination and the ability to think critically.

I would like to thank my panel advisors, Dr. Brian Thomas and Dr. Daniel Hebenstreit, for their valuable guidance and their support throughout my project.

I am extremely grateful to my lab mates, past and present, not only for experimental help and scientific support, but especially for always providing a joyful environment to be working in.

Thanks to all my friends. All the pages of this thesis would not be enough to say thanks to them one by one, because this journey would not have been the same without them!

Last, but not least, I cannot express enough gratitude towards my family. My parents, for teaching me that happiness is in simple things, for supporting my choices and believing in me, and for always reminding me “There is no night so long that it prevents the sun from rising”. My sisters, for being always on my side. My grandmother, for being the best example of courage and determination.
“But all science would be superfluous if the outward appearance and the essence of things directly coincided.”

Karl Marx
Abstract

This thesis examines the response of the trioecious nematode *Auanema freiburgensis* to environmental cues leading to an increase of hermaphrodite sex-phenotype in the offspring. It is shown that *A. freiburgensis* hermaphrodite mothers produce a higher fraction of hermaphrodite progeny if exposed to crowded conditions, and by sampling secretions (supernatant) of dense *A. freiburgensis* cultures this shift can be induced *in vitro* with individual worms. The signal from the supernatant is sensed by an amphid neuron that is exposed to the environment, which is revealed by laser ablation of the neuron, after which the supernatant effect disappears. With the aim to understand how the signal is passed to the progeny, RNAseq (for small RNAs) and histone-methylation immunostaining were conducted. In these experiments, it was found that small RNAs that potentially silence genes involved in epigenetic DNA modification are regulated upon supernatant exposure of the mother nematode, and the immunostaining revealed that this correlates with a change in histone modification of the developing oocytes in the pre-meiotic tip of the mother’s gonad, hence affecting the progeny germline. The results from this study suggest that crowding cues sensed by an *A. freiburgensis* hermaphrodite mother are translated into an epigenetic information passed to the offspring, leading to a larger fraction of hermaphrodite progeny. Every hermaphrodite goes through a starvation-resistant larval stage named dauer. Thus, environmental signals received by the mother indicating imminent overcrowding, and thus lack of food, may be of adaptive value. While the mechanism of epigenetic signal transmission in *A. freiburgensis* is not finally revealed, the results presented here are in accordance with descriptions of “maternal effects” and “intergenerational inheritance”.
Introduction

Organisms adapt to an environment by natural selection, for which information needs to pass through several generations. In Mendelian genetics, a core concept is built on the notion that inheritance occurs through transmission of functional units (termed alleles), which combine into a phenotype of an organism. A centerpiece in Biology for over a century was the notion that these alleles are subject to evolution, which occurs by stochastic genetic mutation, selection, and drift. Adaptation hence implies natural selection following environmental constraints, so that random mutations are selected either positively (if conferring a fitness advantage) or negatively (disadvantage or no effect), to increase the overall fitness. In this concept, the developmental history of an individual has no effect on hereditary variations. Strictly related with this is the idea of genetic determinism, in whose conceptual borders a potential role of the environment as driving force in transgenerational phenotypic plasticity was dismissed. Consistent with this, environmental stimuli experienced by an organism during its lifetime are considered to have a minor effect in sculpting the phenotype of subsequent generations. This leads to a slowly-evolving kind of inheritance, impervious to environmental influences, defined as “hard inheritance”.

However, the environment experienced by an organism during its life is not something static, but conditions and challenges change dynamically. Lamarck envisioned a mechanism for the transformation of species through which traits acquired during the life of an organism (adaptive characteristics) can be transmitted to ensuing generations. He suggested that the use or the not-use of an organ, for instance, had an immediate effect with regard to improving or removing this particular organ (amplification or atrophy), and this acquired phenotype was then subsequently expressed by following generations, suggesting a goal oriented
evolution and development.\textsuperscript{2,5} Lamarck firmly supported this idea, and he was quoted with:\textsuperscript{5}

\begin{quote}
... the law of nature by which new individuals receive all that has been acquired in organization during the lifetime of their parents is so true, so striking, so much attested by the facts, that there is no observer who has been unable to convince himself of its reality”.
\end{quote}

Lamarck postulated the presence of a fluid which, by moving across generations, could sculpt the phenotype of organisms.\textsuperscript{5} Darwin also endorsed a belief in the inheritance of acquired characters, and considered the effects of use and disuse of parts and called the tiny heredity particles “gemmules”, in which context he was cited with:\textsuperscript{5}

\begin{quote}
In the cases in which the organization has been modified by changed conditions, the increased use or disuse of parts, or any other cause, the gemmules cast off from the modified units of the body will be themselves modified, and, when sufficiently multiplied, will be developed into new and changed structures”.
\end{quote}

The modern synthesis of evolutionary genetics at the beginning of the 20\textsuperscript{th} century rejected the core concept of inheritance of acquired characters and dispensed with the notion of a role for environment in modulating inheritance.\textsuperscript{2} As pointed out by the German evolutionary biologist August Weismann, whose idea was central to the modern synthesis of genetics, information cannot flow from soma to germ cells, which in animals are isolated from the rest of the body, preventing somatic information from being inherited.

August Weismann performed a simple experiment: he repeatedly cut off the tails of mice for five generations. Even after so many generations he never found mice that were born with a missing tail or with a rudimentary tail. The implications of his finding were fundamental, namely that phenotypes acquired by the somatic (body) cells cannot be inherited. Instead, only the germ cells (germline) contain heritable information. Also Weismann, who envisioned this theory, was aware that some environmental stimuli, for instance the fact that temperature can determine the colour of butterflies’ wings, can lead to phenotype changes.\textsuperscript{6} Weismann supported the idea
that those changes induced by the environment were due to ‘parallel induction’, events in which environmental factors can affect the germ cells.

The concept that information cannot pass from the soma to the germline, known as the Weismann barrier, has been the dogma in Biology ever since his initial report in 1889. However, during the 20th century, an increasing number of evidences indicating that information can flow from somatic tissues to the germ line and across generations challenged the dogma, giving weight to the idea that additional layers of information can also be transmitted. In order to define events that go beyond the gene, Waddington in 1942 coined the term epigenetics, from the Greek prefix epi-(ἐπι- "over, outside of, around") and “genetics”. In 1957 Waddington published his famous drawing of the epigenetic landscape, which represents a powerful metaphor in developmental biology. Waddington likens the process of cellular differentiation to a ball, which represents a pluripotent cell, rolling on top of a hill through a changing potential surface. Each valley or path of the hill will eventually lead to one of a number of distinct, discrete, differentiated cell fates.

Subsequently, in parallel with a deepening understanding of the underlying molecular mechanism, the term “epigenetic” received a narrower meaning than originally assigned and became synonymous of epigenetic inheritance, defined as: “The study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence”. However the original and the currently widely accepted definition both encompass the notion that environmental conditions shape alternative pathways of development.

Epigenetics and its underlying mechanisms (DNA methylation, histone modification, transmission of small RNAs) brought up to the surface the old debate about transmission of acquired traits, leading to the rise of the concept of inheritance of epigenetic variations. In this regard, several groundbreaking studies suggested that Lamarckian evolution, more than simply being a myth, could represent a reality, and reveal the mysterious nature of Darwin's gemmules and the Lamarckian fluid.

A compelling example for epigenetics as described by Waddington, with environmental factors influencing development, is found in honeybees (Apis mellifera), where nutritional input determines the phenotype of genetically identical
individuals. In the wild, female larvae can undergo two different developmental trajectories. If larvae are fed an exclusive diet of food called "royal jelly", they develop into fertile queens, which differ in physiology, morphology, and behaviour from worker bees, fed with other food. It has been reported that epigenetic mechanisms, particularly a down-regulation of a DNA methyltransferase which occurs during larval development, act as bridge between the environment and the alternative developmental fates, hence integrating intrinsic signals and environmental factors.

If epigenetic modifications occur in the germline upon environmental factors, these can act as a vector in order to transmit the information of the environmental exposure from preceding to succeeding generations. Therefore, transgenerational inheritance contributes to give an equilibrium between robustness and flexibility, essential for survival. In fact, epigenetic transmission of environmental signals over generations confers the ability to future generations to face and cope with challenging environmental conditions which might be present in a volatile and fluctuating natural environment. Whether these phenotypic modifications can even be translated into modifications in the genetic sequence, is still subject of discussion.

**Epigenetic transmission of environmental signals across generations**

Recognizing that non-DNA variations can be transmitted between generations widens the notion of heredity and broadens the widely accepted idea of a gene-centered evolution. Consistent with this, recent evidence indicates that the environment experienced by parental generations can lead to phenotypic variation in the offspring. Although many examples are known for the parental influence on the phenotype of the progeny, which represent examples of maternal or paternal effects, the mechanisms by which this is achieved are poorly understood. Some of these examples of paternal effects, however, involve epigenetic modifications and also fit within the conceptual border of transgenerational epigenetic effects.

In dynamic environmental conditions, mothers may play a role in shaping the phenotype of offspring, regardless the offspring's genotype. This phenomenon, also called adaptive transgenerational phenotypic plasticity, provides organisms the
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possibility to react and cope with different environmental conditions by modulating their phenotype.\textsuperscript{18}

The implications of these findings to human health are considerable, i.e. epidemiological studies in humans suggest that environmental factors (e.g. nutrition) can influence the propensity to develop diseases in subsequent generations.\textsuperscript{19-22} Two cohort studies, the “Dutch Hunger Winter Families Study” in the western Netherlands\textsuperscript{19} and the “Överkalix cohort” in Sweden,\textsuperscript{20-22} support the notion that environmental cues can influence phenotype of future generations in a non-genetic manner also in humans.\textsuperscript{20,22}

“The Dutch Famine Birth Cohort Study” indicated that susceptibility to disease may be related to malnutritioned ancestors.\textsuperscript{19} During the Dutch Hunger Winter, at the end of World War II, a blockade imposed by the Nazis led food rations to drop to 500 kcal/day. Females whose mothers were exposed to those starving conditions during pregnancy were more susceptible to develop health problems such as diabetes, cardiovascular disease, obesity and microalbuminuria, compared to the unexposed siblings. This increase in metabolic diseases has not been observed only in the first generation, but persisted in the second-generation, suggesting that famine experienced by mothers triggers pathways which have an impact on the phenotype of several subsequent generations.

Epidemiological data collected in Överkalix, in northeast Sweden, from the late 1800s through to the early 1900s utilizing historical records from three-generation groups of families, showed a link between grandparental diet and grandchild’s growth and metabolic phenotype.\textsuperscript{20-22} It has been reported that overnutrition for the paternal grandfather during his slow growth period (SGP), i.e. the time before the start of puberty (which was set approximately between the ages of 9-12 for boys and 8–10 years for girls) influenced the grandson’s longevity negatively; on the other hand, shortage of food for the grandfather resulted in healthier grandsons.

However, the concept that stimuli experienced by the soma can be transmitted to the germline in humans is still controversial. Some of the reasons for the debate include: the lack of evidence as being a mechanism with adaptive significance; no evidence for epigenetic changes in the germline; few studies take into account confounding
factors such as the uterine environment across generations.\textsuperscript{23} It is therefore very difficult to prove causation in human studies, due in part to their long generation time and to the fact that these studies have been limited mostly to the investigation of inherited traits in the F1 generation.\textsuperscript{8} Furthermore, it is not trivial to infer soma to germline transmission because the phenotype of the progeny might be influenced by maternal care and social behavior, resulting in a misleading interpretation of how the information is transferred from one generation to the next.\textsuperscript{11}

Examples for non-genetic mechanisms of inheritance are found for a wide variety of traits.\textsuperscript{18,24-27} In insects, many maternal effects have evolved as a kind of phenotypic plasticity.\textsuperscript{18} For instance, environmental factors such as temperature, photoperiod, or host availability sensed by an ovipositing female influence offspring development, determining the probability to enter a diapausal (dormant) state. The adaptive significance of this environmentally modulated phenotypic plasticity is evident: maternal decision to induce diapause in the next generation, upon environmental cues, which is based on nuclear genome loci, represents a protective mechanism to allow offspring to deal with unfavourable environmental conditions, such as during cold seasons.\textsuperscript{18}

Another example of an adaptive maternal effect is provided by the increase in size of Northern American red squirrels. Environmental cues, specifically population density, to which mothers are receptive, influence growth rates of the offspring. When mothers were exposed to high-density conditions, offspring developed faster and survived their first winter at a larger fraction than the control group.\textsuperscript{25} A similar study showed that increased population pressure experienced by the dung beetle \textit{Onthophagus taurus} mothers led to an increase of horn size in offspring, preparing them for a competitive environment they are likely to face.\textsuperscript{24}

Other environmental stimuli, such as predation, may determine changes to F1 phenotype. Studies on defensive responses of the water flea, \textit{Daphnia cucullata}, showed that exposure of mothers to chemical cues, indicating the presence of the predator \textit{Chaoborus}, led to the development of a helmet, which confers resistance and reduced vulnerability. This phenotype was visible also in the offspring, even if they had never been exposed to the predator-related signal molecules.\textsuperscript{28} However, the relevance of these findings are tempered by the fact that \textit{Daphnia} reproduce mainly
Introduction

parthenogenetically, and since only the F1 was investigated it is possible that the
effect resulted from maternal provision to the eggs while still in the maternal ovary.

Maternal effects can also influence the behaviour of the offspring, e.g. in
antipredator response. In this context, *Gryllus pennsylvanicus*, the fall field cricket
common in North America, represents an example of an adaptive transgenerational
maternal effect. When gravid crickets were exposed to the cues of a wolf spider
*Hogna helluo*, the progeny showed higher antipredator immobility when confronted
with the same predators, compared to offspring of non-exposed mothers.

In conclusion, these are interesting examples where actual experiences of the
parental generation are passed to the progeny, e.g. as a warning for a potentially
dangerous environment which they may likely face during their life.

**Molecular mechanisms involved in environment-induced epigenetic inheritance**

Transmission of non-genetic information across generations has been cast into two
broad non-exclusive classes, transgenerational epigenetic “effects” and
transgenerational epigenetic “inheritance.” Epigenetic “effects” refer to the
transmission of non-genetic information from parent to offspring, whose underlying
mechanisms are not yet determined. Parental effects, soft inheritance, non-Mendelian
inheritance, and fetal programming, fit within the border of epigenetic “effects”
because, even if they represent different phenomena, transmission of non-genetic
information certainly occurs. On the other hand, transgenerational epigenetic
“inheritance” implies the transmission of epigenetic modification through meiosis, an
essential step in order to affect inheritance.

The meiotic transmission of epigenetic states has been seen as controversial. The
argument is strictly related to mechanisms involved in germline reprogramming, a
cornerstone in developmental biology. Germline reprogramming, which implies
erasure and remodeling of epigenetic patterns during mammalian development,
occurs in both the female and male germline during gametogenesis and soon after
fertilisation. This epigenetic reprogramming is required in order to guarantee the
totipotency of the embryo and a proper initiation of embryonic gene expression by
the erasure of acquired epigenetic marks.
Introduction

At some loci the epigenetic features are not erased, they represent part of a normal developmental pathway, such as genomic imprinting and paramutations.\(^1\) Imprinting\(^31\) is defined as a parent-specific epigenetic, heritable mark which regulates gene expression. It is commonly established by histone modifications, chromatin organisation and small RNA mediated modifications. Paramutations\(^32\) are changes in allele expression caused by changes in the expression of regulatory machinery, rather than by mutations of the actual gene sequence. Several mechanisms leading to a changed allele expression, which in part are only working models, are associated with the term paramutation, as this concept is insufficiently investigated in terms of its mechanism. For instance, expression changes can be induced by changes in regulatory methylations of the target gene (on histones and/or nucleotides), or small RNA mediated regulation of gene expression (activating or repressing, further introduced below). In worms, the latter is discussed, and the origin of small RNAs is thought to be from gene clusters (i.e. Piwi interacting RNA (piRNA) genes) that initiate the formation of regulatory small RNAs (introduced in detail below). As the piRNA genes are chromosome located, paramutation effects are heritable and can be reinstated in ensuing generations. Notably, with the allele expression change being mediated by diffusible molecules such as small RNAs, the original paramutation is trans-acting on both paramutated, and non-paramutated homologous alleles. It has been suggested that paramutations may be initiated by environmental stimuli, so that chromatin modification by methylation as well as regulatory small RNA expression could be potential molecular carriers of heritable epigenetic marks across generations.\(^8,32-34\)

According to the number of following generations that express the acquired trait subsequent to an environmental exposure of the parental generations, transmission of environmental-induced acquired traits have been classified in three different models: intergenerational, multigenerational, and transgenerational. It has been suggested that in order to be considered “transgenerational” and not “intergenerational”, the effect need to last more than 2 generations, so that it is possible to rule out a potential effect of in utero exposure to specific environmental, nutritional, or hormonal factors of the embryo and its germline.\(^11\) Therefore, only F3 offspring can be considered completely not exposed to the initial trigger and can be regarded as of transgenerational epigenetic inheritance. Mutigenerational effects occur when F1 and
Introduction

F2 generations showed the phenotype. When inheritance occurs e.g. through the paternal lineage of females prior to pregnancy, effects in F2 generations can be also considered as transgenerational.\(^8,15,35\)

Mechanisms underlying environment-induced epigenetic inheritance encompass different epigenetic changes which may allow organisms to transmit specific ancestral environment information to ensuing generations.\(^8\)

In the context of transgenerational epigenetic inheritance, covalent modification of nucleotides plays a critical role.\(^8\) In mammalian cells, methylation of single DNA residues, and particularly methylation at position 5 of cytosine (5mC), has been linked to the transmission of epigenetic memories over generations.\(^36\)

Consistent with this, several studies reported that heritable modification in DNA methylation at specific loci occurred as a consequence from a high-fat diet and maternal stress. For instance, individuals whose parents experienced famine during the Dutch ‘Hunger Winter’, showed lower levels of methylation at the imprinted insulin-like growth factor II (IGF2) locus, compared to individuals whose parents were unexposed.\(^37\)

5mC, a hallmark of gene repression in mammals, is rare in the model organisms Drosophila melanogaster and Caenorhabditis elegans.\(^8\) However, recently, other forms of DNA modifications, such as methylation at the sixth position of adenine (6mA), have emerged as a novel mechanism underlying epigenetic regulations in these species.\(^38\)

Epigenetic modification at the nucleosomal level, and the subsequent change of the underlying genetic (DNA) information accessibility, represents another relevant layer of epigenetic modifications.\(^2\) N-terminal tails of histone proteins can be chemically modified via addition of a methyl-, acetyl- or phosphoryl-group, or via covalent attachment of proteins, such as Small Ubiquitin-like Modifier (or SUMO) or ubiquitin proteins.\(^39\)

In species where DNA methylation is rare, such as C. elegans, D. melanogaster and Schizosaccharomyces pombe, addition of methyl-groups to histone proteins has been reported to be involved in the mechanism underlying transgenerational inheritance of
acquired traits.\textsuperscript{8} In \textit{C. elegans} for instance, deficiencies of the histone H3 lysine 4 trimethylation (H3K4me3) complex (composed of ASH-2, WDR-5 and the histone methyltransferase SET-2, regulating lifespan) in the parental generation have been implicated in epigenetic memory of longevity in descendants.\textsuperscript{40} It has been shown that longevity could be inherited in a transgenerational epigenetic manner: genetically wild-type F3 descendants from P0 parents with a deletion of \textit{wdr-5}, which encodes part of the trimethylation complex (see above), showed a 20% extension of lifespan when compared to descendants from wild type parents. This phenotype was still present in the wildtype F4 descendants of a \textit{wdr-5} mutant parent (crossed with wildtype), but not in the F5 descendants, suggesting that the parental H3K4me3 status may influence the gene expression of descendants for several generations. Histone modifications also play a role in epigenetic memory in species where DNA methylation constitutes one of the major epigenetic regulations, such as plants.\textsuperscript{8}

\textit{C. elegans} provides more evidence that histone marks function as stable epigenetic memory. For instance, a mutant was investigated which had a transposon insertion in the gene \textit{spr-5} (in the conserved amine oxidase catalytic domain), the ortholog of Lysine-specific histone demethylase 1A (KDM1A) (also known as lysine (K)-specific demethylase 1A (LSD1)) which demethylates the histone 3 lysine 4 dimethyl mark (H3K4me2).\textsuperscript{41} This mutation lead to a decreased demethylation activity. From a parental P0 generation with \textit{spr-5} mutation, the brood size decreased from the first to the twentieth offspring generation, at which sterility was observed. Hence, defects in resetting H3K4me2 marks at selected germline genes in the primordial germ cells, and the inappropriate retention of H3K4me2, caused a progressive transgenerational sterility, suggesting a mechanism required for epigenetic reprogramming between generations.

In mammals, the extent to which environment-induced histone marks are involved in the inheritance of acquired phenotypes is still a matter of debate.\textsuperscript{8} In humans and mice, for instance, a potential epigenetic role of sperm chromatin in regards to embryo development has been ruled out since late in the haploid phase of spermatogenesis canonical histones are mostly cleared and replaced with protamines, small nuclear proteins involved in normal sperm function.\textsuperscript{42} However, it has been found that in the haploid genome of mature spermatozoa a certain fraction of...
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histones (approximately 10 % in humans and 1 – 2 % in mice) are retained, suggesting that histone modifications may represent a relevant mechanism that modulate inherited information, acting as epigenetic memory across generations. In addition to DNA methylation and histone modifications, small RNAs may be involved in shaping the traits of progeny across multiple generations. Different classes of small non-coding RNAs (sncRNAs), such as microRNAs (miRNAs), endogenous small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs), act within the RNA interference (RNAi) pathways, in which small RNAs neutralise targeted mRNA molecules. sncRNAs were reported to play a critical role in the transmission of acquired phenotypes as they can be passed to the germline, which is additionally the case for transfer RNA (tRNA) fragments. It has been shown in *C. elegans* that environmental stimuli, such as starvation or temperature stress, correlate with a change in small RNA expression over several generations. While it is not well understood how the stimuli are initially translated into a small RNA expression change, this is a clear example of cross-generational transmission of environmental information mediated via small RNAs. For tRNAs, it was shown in a mouse model that a parental exposure to diet rich in fat led to an alteration in expression profiles of a subset of sperm transfer RNA-derived small RNAs (tsRNAs), which might be associated with metabolic disorders in the offspring. Sperm tsRNAs can therefore also function as epigenetic carriers of metabolic disorders induced by a fat rich diet.

In *C. elegans*, transgenerational inheritance of anti-viral small RNAs (viRNAs) results in an antiviral response in following generations. An effective strategy against viruses is based on the process of RNA interference (RNAi) by production of targeted viRNAs, a gene-silencing mechanism that has been well characterized in *C. elegans*. Transgenerational inheritance of viRNAs was investigated by using an established model, a transgenic worm that allows replication of the Flock House virus (FHV). FHV is a plus-strand RNA nodavirus with a wide host specificity. By using this transgenic system, it is possible to measure viral expression and silencing through a GFP signal. Infection with the Flock Virus led to the production of viRNAs, from the viral genome as template, which in turn silenced the virus. It was shown that viRNAs and hence these antiviral effects were transmitted transgenerationally for 50 generations in a non-Mendelian way, without the original
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template, and even in progeny worms which had lost the ability to produce small RNAs *de novo* (e.g. due to mutations). To remove the original viral template after infection (i.e. genomic integration), genetic crosses in this well-defined transgenic nematode model were employed, and it could be shown that the antiviral response was still active in the absence of a template, confirming transgenerational transmittance which occurred *via* sperm. Therefore, information of the exposure of the parental generation to specific environmental factors which might constitute a biological challenge is memorised over following generations, providing an adaptive benefit.

In the context of transgenerational epigenetic inheritance, a compelling feature concerns the mechanisms underpinning the transient behaviour of the acquired phenotypes and the associated decay of the epigenetic effect, which *vice versa* constrain the underlying mechanism of signal transmission. The persistence of transmitted phenotypes for only a limited number of generations might be explained by the assumption of the existence of an active breaking mechanism which leads to an abrupt decline of the level of an inherited molecule, and to a subsequent reversion of the phenotype. On the other hand, the level of inherited molecules can decrease slowly over time until they reach a lower threshold, although it is not yet fully understood how this is achieved. For instance, in the case of small RNA, a potential dilution of the silencing factors over multiple generations can explain the mechanism underlying the transience of the acquired phenotypes.\(^{15}\)

**Environmental information can be passed by non-genetic inheritance**

Several lines of evidence indicated that different kinds of environmental factors, such as stress, diet, and environmental toxins, can lead to epigenetic modifications and effect subsequent generations.\(^{15}\) Even the memory of a specific odor during early development can affect future generations. Transgenerational inheritance of olfactory imprinting was reported in *C. elegans*.\(^{51}\) Exposure of worms to odorants early in development (L1 larval stage) led to olfactory imprints which shaped some behavioural responses during adulthood. For instance, adult worms, upon encountering the imprinted odorants which they experienced during their larval stage, showed an improved propensity to migrate towards the specific chemical, perhaps by retaining a memory of past experiences which they associate with a
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benign environment. Interestingly, this olfactory imprint can be inherited by the following generation. Moreover, the exposure to specific odors for five consecutive ancestral generations resulted in a stable inheritance of the olfactory imprinting through 40 generations, suggesting that, while a transient inheritance implies the presence of a mechanism which acts in order to reset the memory of the acquired phenotype, a stable inheritance, which arises as a consequence of environmental changes, may involve a process of “genetic assimilation”.51

To a certain extent, a similar observation was reported in a mouse model. Before conception, male mice were subjected to olfactory fear conditioning, obtained by combining an odor (acetophenone) with mild shocks. It was observed that the offspring of the conditioned mice (F1 and F2 generations) showed a heightened reactivity to the specific odor, but not to other stimuli.52 Moreover, this startle reaction upon the conditioned odor, together with modification at a neuronal level (specifically in the glomerular areas), was observed in the subsequent generations also after cross-fostering and in vitro fertilization (IVF), suggesting that the above mentioned features, rather than being socially transmitted from the parental generation, were inherited transgenerationally in a non-Mendelian manner and that transmission of the memory occurred via gametes.

Diet represents another important environmental factor, which can lead to particular phenotypic outcomes. Several studies have shown a strict relation between parental diet and the risk of chronic disease in ensuing generations.53 For instance, in one study, male mice were subjected to a diet low in protein, and subsequently mated with females fed with a normal diet. Male and female offspring showed increased hepatic expression of several genes which play a role in lipid metabolism and cholesterol synthesis, compared to the offspring of males fed a balanced diet. Moreover, further indication that the epigenome of sperm can be influenced by the paternal diet came from the observation that epigenetic modifications (DNA methylation and histone modifications) occurred in males fed a low-protein diet. Although the specific changes responsible for the transmission of a metabolic phenotype to following generations remain to be determined, it has been suggested that the diet may influence the content of sperm and seminal fluid (e.g., RNA, chromatin, metabolites) and these modifications can be passed to the next generation.7
Stress factors were also reported to play a role in sculpting an offspring phenotype. In *Drosophila melanogaster*, some environmental stressors, such as osmotic stress and heat shock, can trigger a mechanism which influences the structure and organisation of the heterochromatin, and these changes are transmitted to following generations in a non-Mendelian manner. Upon heat shock or osmotic stress, phosphorylation and activation of a transcription factor, drosophila activation transcription factor 2 (dATF-2) involved in heterochromatic nucleation, induce a defective heterochromatin state which was reported to be transmitted to subsequent generations through the germline.

Moreover, exposure of a gestating female rat to vinclozolin, a common dicarboximide fungicide which disrupts endocrine signaling pathways, and to methoxychlor, an insecticide which acts also as an endocrine disruptor, has a consequence in the subsequent generations. Specifically, male F1 progeny showed a reduction in the number of their sperm and a reduced spermatogenic capacity, with a subsequent increase of defects in reproduction over at least four generations. However, since exposure to chemicals can induce mutations, it needs to be considered that a role of genetic factors in the context of transgenerational inheritance upon toxicants is difficult to assess.

*Auanema freiburgensis* as model system for epigenetic transmission

In the present study, a case of intergenerational adaptive response induced in anticipation of a changing environment is presented. Environmental factors experienced by the parental generation influence sex determination in the next generation, providing evidence for phenotypic plasticity across generations that is triggered when local environments can be anticipated. This provides a means for the mother to adjust the phenotype of the offspring to enhance their success in that environment.

The study was conducted using a nematode system, the free-living trioecious species, *Auanema freiburgensis*. The phylum Nematoda is an excellent group for studying soma to germline inheritance given that many nematodes are oviparous and do not show maternal or social behavior. Moreover, they have a short generation time, small
size, and a large number of progeny, allowing the control of environmental and genetic factors.

_Auanema freiburgensis_ was isolated from a dung pile in Freiburg, Germany, in August 2003 by W. Sudhaus. Molecular phylogenetic analyses place _Auanema freiburgensis_ in a new described genus, _Auanema_.

In populations of _Auanema_ nematodes, three sexes coexist (male, female and hermaphrodite), see also Figure I-1), representing an example of a mixed breeding system. In animals, this mating system is rare, and the presence of males, females and hermaphrodites within the same population has been viewed as being a transient state in an evolutionary context. In Nematoda, a trioecious mating system evolved only three independent times.

The three sexes are produced by both outcrossing and selfing. Sex determination between males and non-males is mediated by an XX:OX mechanism, in which XX animals develop into females and hermaphrodites and XO animals become males. The mechanism of hermaphrodite versus female sex determination is largely unknown. Experimentally, worms of different sexes can easily be distinguished: males have a specialised behaviour and morphological structures (particularly size, mating organs, tail shape), and hermaphrodites can be distinguished from females by their ability to reproduce in the absence of a male partner by producing sperm which is stored in the spermatheca and used to self-fertilise oocytes.

After hatching, _A. freiburgensis_ females and males enter into a series of feeding larval stages (L1-L4) after which they moult to the reproductive adult stage. In hermaphrodite development, an additional, non-feeding (dauer larva) stage is always adopted after the L2 stage. Hence, in _A. freiburgensis_, XX larvae that pass through the dauer stage always become hermaphrodites, whereas XX non-dauer larvae develop into females. This is different from _C. elegans_, where dauer formation is facultative and only occurs under conditions which are unfavourable for growth, including food shortage, high temperature or exposure to hazardous chemicals.
Figure I-1. *A. freiburgensis* exhibits three different sexes (male, female and hermaphrodite).
Note that females and hermaphrodites are morphologically mostly identical (and both develop an equivalent gonad for oocyte production), though hermaphrodites also produce sperm which are stored in the spermatheca. The male worms develop a different type of gonad for sperm-production. Note that the worm schemes are not to scale; the male worms are smaller than their female/hermaphrodite counterparts.

The production of hermaphrodites might be related to the lifestyle of this free-living nematode. *A. freiburgensis* feeds on bacteria that grow in rotting dung. Because *A. freiburgensis* reproduces rapidly, habitats become frequently unfavourable in terms of food-supply, and non-feeding dauer larvae could easier persist during this condition. *A. freiburgensis* dauer can easily be recognized by their nictation behavior, in which they stand on their tails and wave their bodies. This increases their chances of attaching to a phoretic carrier and migrate to a more favourable environment. Since *A. freiburgensis* dauers develop into selfing hermaphrodites, colonizer individuals assure propagation in the new habitats even in the absence of conspecific males.
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Scope of this thesis

The aim of this thesis is to identify environmental conditions that lead to an increase of hermaphrodite offspring in *Auanema freiburgensis*. Given that *A. freiburgensis* females and hermaphrodites are genetically identical, the focus of analysis was to determine the relative abundance of female/hermaphrodite in the offspring of single hermaphrodite mothers after exposure to different cues.

It is revealed that crowded conditions and certain pheromones (ascarosides) experienced by the hermaphrodite mother increase the fraction of hermaphrodite progeny. The developmental dauer stage, which is obligatory in hermaphrodites, might be advantageous to survive in unfavourable (crowded) conditions. A supernatant collection protocol is established to imitate the crowding cues, and this supernatant is efficient to induce a shift in progeny sex-phenotype towards hermaphrodites in individual hermaphrodite mothers.

In order to identify the signal perception, laser ablation is employed to deliberately deactivate neuron cells with subsequent testing of the supernatant effect. It is shown that ablation of a single neuron pair (ASH on left and right side of the worm) that is exposed to the environment is sufficient to render *A. freiburgensis* insensitive to the supernatant effect. However, other neurons appear to be involved also.

Attempts to reveal the mechanism of the soma-to-germline information transmission resulting in the shift in sex-phenotype of the progeny were made by analysing potential (small) silencing RNAs with RNAseq, and testing for alterations in histone modification by immunostaining. It is found that supernatant exposure induces changes in small RNA expression and the histone-modification of oocytes developing in the pre-meiotic tip, suggesting an involvement of intergenerational inheritance by an epigenetic mechanism.
Chapter 1 – Supernatant-mediated signal transmission

“Obviously mononchs (predatory nematodes) hunt by the aid of some sense other than sight...picture these ferocious little mononchs engaged in a ruthless chase in the midst of stygian darkness. We may imagine them taking up the scent of the various small animals upon which they feed... pursuing them with relentless zeal that knows no limit but repletion.”

—Nathan A. Cobb, founder of Nematology (1917)

Introduction

Inter-individual chemical communication

Chemical senses are, in terms of evolution, amongst the oldest senses to have evolved, allowing organisms to identify food, dangers, enemies, potential mating partners, or other members of the same species.61

Production of a chemical profile, given off as volatile molecules, occurs in all animals. All organisms, including bacteria, can distinguish environmental chemical information. Moreover, across the animal kingdom, animals can detect chemosensory information released from other organisms. In fact, ancient and widespread chemical senses mediate social interaction, and they play a critical role in detection of potential food resources and predators.62

Chemical interaction between organisms occurs through molecules that have been defined as “semiochemicals” (from the Greek σημεῖον (‘semeion’), a signal or mark).62-64 In the context of semiochemical communication, two different broad classes can be distinguished, as communication can occur within a species (intraspecific) or between individuals of different species (interspecific).64

Whittaker introduced the term “allelochemical” to define chemicals involved in interspecific interactions.64 Chemical interactions can carry benefits and be
advantageous to the emitter of a specific signal, to the receiver, or both. According to the benefits and costs involved in the chemical interaction, allelochemic interactions are further subdivided into four subgroups: allomones (cues produced by an organism that can be detrimental to the receiver), kairomones (chemical cues that determine a favorable response in the receiver, not in the emitter), synomones (cues favorable to both the receiver and the emitter), and apnuemones (substance produced by nonliving material, that can either have a favorable response in a recipient species or be detrimental to a different species associated with the nonliving material).\textsuperscript{62,64}

Pheromones, on the other hand, elicit intraspecific responses. Pheromones are further sub classified into alarm pheromones (warning or protective signals), epidietic pheromones (regulate population density), and sex pheromones (response between or within sexes).\textsuperscript{62,64,65} The portmanteau word "pheromone" was proposed by Peter Karlson and Martin Lüscher in 1959, based on the Greek φερω (pheroo, 'to transfer') and ὁρμων (hormone, 'impetus').\textsuperscript{66,67} To distinguish hormones from pheromones, Karlson and Lüscher specified pheromones as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process".\textsuperscript{67} The German biochemist Adolf Butenandt and his first chemical identification of a pheromone, bombykol (see Figure 1-1), the sex pheromone of the silk moth \textit{Bombyx mori}, marks the start of modern pheromone research, by showing that chemical signals between animals exist and can be identified.\textsuperscript{68} The identification has been conducted by the following four criteria, the equivalent of “Koch’s postulates”: (1) initial isolation of an effect induced by (a) pheromone(s), (2) identification and (3) synthesis of the molecule, and (4) a bioassay in order to confirm the activity of the synthesized molecules.\textsuperscript{69}

![Figure 1-1. Structure of the \textit{Bombyx mori} sex-pheromone bombykol.](image)
The structure was drawn according to PubChem database entry CID 445128 using the freeware program ACD ChemSketch (ACD/Labs).
When the term pheromone was first coined in 1959, the idea of inter-individual communication and environmental sensing was not entirely new. As reviewed elsewhere,\textsuperscript{69,70} Francois Rabelais reported in 1565 that the ancient Greeks were aware of some form of information transmission from the observation that female dogs in heat attract males, which, as known now, is mediated by secretion of chemical signals. Furthermore, it is reviewed that Charles Darwin suspected in his \textit{The Descent of Man, and Selection in Relation to Sex} (1871) the smells of animals to be of relevance for sexual selection.

Electrophysiology was applied to investigate Butenandt’s silk-moth extracts for activity. Schneider, pioneer of modern olfactory research, developed the electroantennography (EAG) as a method to investigate the sense of smell of the silk moth \textit{Bombyx mori} as model organisms.\textsuperscript{70} For EAG, electrodes (metal wires) are inserted into a moth antenna, and the electrical signal is measured while applying different odorant signals. This technique is still today used as a standard to screen for insect pheromones, and insects (including \textit{B. mori}), which sense many signals by their antenna, are seen as excellent model organisms for pheromone research. Most prominently, research on \textit{B. mori} using EAG revealed that bombykol, stimulates the antenna of the male moth, and even a small quantity of bombykol is detected.\textsuperscript{69,70}

After the identification of bombykol, a huge variety of compounds acting as pheromones has been found; identification, synthesis and the study of the biological effects of new compounds represent an ongoing challenge for interdisciplinary research in biological chemistry.\textsuperscript{62,71,72}

Challenges in identification and characterization of new compounds are related to the fact that pheromones, often low molecular weight organic compounds, rather than being a single molecule, act as species-specific mixture of compounds in precise ratios. This blend of pheromones can mediate a variety of effects, according to the context and the receiver.\textsuperscript{69} Moreover, pheromones have been defined as “anonymous” molecules: a pheromone mediates the same message in all the individuals within a species of the same physiological state or of the same type (for example, females, males), without considering from which individual the pheromone was released.\textsuperscript{62,73} However, there is variation in the amount of pheromone produced per individual, and also over time in the same individual.\textsuperscript{74} Pheromones are still
anonymous even in the case of, for instance, some male mouse pheromones that are released only by dominant male territory holders. They indicate, for example, the presence of a dominant male mouse, rather than a particular individual.62,74

While the pheromones of insects, fish, and mammals, have been well studied, little is known for some other taxa. For instance, although chemical communication by crustaceans and crabs apparently involves a considerable use of pheromones, only few of them have been chemically characterized.62,75 At a similarly early stage is the study on chemical cues in birds. Studies on avian communication so far have focused on vocal and visual cues, not chemical ones.76 It has been shown though that birds are excellent smellers and odors influence a variety of behavioral reactions such as food orientation, food searching, but also breeding.76-78

Advanced analytical techniques are now available which have enabled progress in pheromone identification.69 For the first pheromone characterization, 500,000 female silk moths (B. mori) were required for identification of bombykol (only around 12 milligram of solid extract were obtained), while today this large number would not be necessary as analytical devices have become more sensitive. For example, honeybee brains were studied upon pheromone exposure with microarray genomics, a high-throughput transcriptomics, to identify changes in gene expression patterns, and pheromone-release was analyzed in real time for fighting wasps.79

Nematode communication

Nematodes, the most abundant multicellular animals on earth, inhabit a broad range of environments.80 These animals, successful in adapting to nearly every ecosystem, are found to inhabit volcanic ash, deserts, ocean trenches, sulfurous sediment, human lymphatic ducts, whale placenta, pig intestines, plant roots, arctic ice, and many other diverse ecosystems.81-83 Nematodes include many parasitic (plant, vertebrate, invertebrate) and free-living (sediment, fresh, marine, brackish, or estuarine water) species.84 Presumably as a consequence of their parasitic and widespread free-living occurrence, nematodes are able to communicate not only with nematodes of the same and different species, but also with a broad variety of organisms, such as plants, microbes, insects, and other animals.80
The first studies of communication between nematodes in the 1960s aimed to understand mating dynamics. In 1964, Greet observed that opposite genders of the free-living nematode *Panagrolaimus rigidus* are able to attract one another from a distance. Males and females of *P. rigidus* were placed on opposite sides of an agar plate, separated by a cellophane barrier, which allowed small molecules to move from one side to the other, but not the worms. Greet found that males and females congregated at the barrier. Same-gender populations did not exhibit the same behaviour. Henceforward, the presence of sex-pheromones, released from one or both sexes, has been reported for at least 37 more species, including free-living nematodes and plant- and animal-parasitic species.

Chemical cues are involved in the regulation of further nematode behaviors including aggregation, the tendency of individuals to gather in the same circumscribed place of the environment; chemotaxis, the movement of individuals in response to another worm or metabolite; leaving, the propensity of some worms to move away from a food source e.g. because of the absence of worms of opposite gender (search for mating partners); holding, the tendency of individuals to stay in the location where another individual or metabolite is present; mating and social behaviour.

### Dauer pheromone

Research on nematode communication is mostly conducted in the small, transparent *C. elegans*, the first multicellular organism for which the whole genome had been sequenced and the only organism for which its connectome (neuronal "wiring diagram") has been completed. *C. elegans* is a good model system for investigating the role of pheromones in modulating a variety of behaviours and developmental decisions. In fact, the *C. elegans* life cycle is regulated by pheromones, which allow the plasticity of its development pathway at a distinct point. In this regard, a clear example is the alternative stress-induced dauer diapause stage.

In a good growth environment for *C. elegans*, with abundant food supply, optimal temperature (ca. 20 °C) and a low population density, the development of *C. elegans* from egg to adult progresses through four larval stages L1-L4. In less favourable
conditions though, including elevated temperature, food shortage and competition in dense populations, dauers are formed as an alternative L3 stage.\textsuperscript{95}

The suggestion that dauer formation can be induced by small molecules found in supernatants of liquid cultures was made by Golden and Riddle.\textsuperscript{93} They indicated that “pheromone is present in very small amounts, and that it may be a hydroxylated, short-chain fatty acid or a mixture of closely related compounds” which is responsible for the transition into the dauer stage.\textsuperscript{66,93,96} Evidence that the promoting dauer signals must be worm-derived came from the identification of a mutant strain, \textit{daf-22}, defective in producing dauer-inducing activity.\textsuperscript{96,97}

For more than twenty years its chemical identity remained enigmatic. In 2005, the first \textit{C. elegans} pheromone was identified: a pheromone inducing dauer formation, called “daumone” (dauer pheromone).\textsuperscript{66,96,98}

This compound was isolated by ethyl acetate extraction of a large volume of liquid culture (300 L fermenter) with subsequent separation of the extracted fraction by liquid chromatography. The chromatography fractions were tested for dauer inducing activity and, after sufficient purification, the chemical structure of the daumone was revealed by Nuclear Magnetic Resonance (NMR) spectroscopy and mass spectrometry (MS).\textsuperscript{66,98} The identified pheromone (chemical name (\textsuperscript{-})-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid (see Figure 1.2) acts by modulating the population density-dependent development of dauer larvae.\textsuperscript{98}

The daumone belongs to a class of pheromones termed ascaroside and is nowadays also referred to as ascaroside ascr\#1. The term “ascaroside” was introduced almost 100 years before the study in \textit{C. elegans} pheromone biology, for a type of glyco-lipid discovered in the eggs of \textit{Ascaris} species (parasitic nematodes known as large intestinal roundworms).\textsuperscript{66,96} Long-chain ascarosides were first identified in the nematode \textit{Parascaris equorum} (a horse parasite)\textsuperscript{99} and later characterized in \textit{Ascaris lumbricoides} (a human parasite) and \textit{Ascaris suum} (a pig parasite).\textsuperscript{100-102} These long-chain ascarosides form a protective inner layer in \textit{Ascaris} eggs that confers impermeability and resilience.\textsuperscript{66,96} In comparison with \textit{C. elegans} ascarosides, the ascarosides found in \textit{Ascaris} and \textit{Parascaris} usually have a longer fatty acid chain, and were reported to act only as protective compounds.\textsuperscript{66,103,104} Ascaroside-like
molecules have only been found in nematodes. However, ascarylose, a sugar compound to which the ascaroside lipid chains are often attached (see Biosynthesis section below), have been identified in some gram-negative bacteria.

After identification of the first daumone, using a similar approach two additional ascarosides with dauer-inducing activity were identified, 5-O-ascarylosyl-5R-hydroxy-2-hexanone, which has a six-carbon side chain bearing a ketone functionality (“C6”, ascr#2) and an ascaroside derivative of 8R-hydroxy-2E-nonenoi acid, a nine-carbon α,β-unsaturated carboxylic acid side chain (“C9”, ascr#3) (Figure 1-2). These have the ascarylose moiety as ascr#1, differing in the length of the lipid side chain. It was reported that ascr#2 and ascr#3 are roughly two orders of magnitude more potent that ascr#1 at inducing dauer, and that there is a mild synergistic effect when employed as a mixture. Interestingly, it has been reported that cultivation and environmental conditions determine the molecular composition of the dauer pheromone: investigation of the temperature dependence of dauer induction showed that ascr#1 is present at lower relative concentration in extracts obtained from liquid cultures growing at 20 °C, than from cultures at 25 °C.

![Figure 1-2](image-url)

**Figure 1-2.** Structure of the ascarosides ascr#1 (A), ascr#2 (B) and ascr#3 (C).
The structures were drawn with ACD ChemSketch (ACD/Labs) according to PubChem entries CID 11471380, 16066475 and 16066476, respectively.

Ascaroside icas#9 was identified as dauer-inducing ascaroside in a later study. icas#9 (Figure 1-3) is similar in structure to ascr#1-3 in that it contains an ascarylose core and a lipid side chain; however, it bears an indole carboxy moiety bound to the ascarylose. Furthermore, it is distinct in its activity profile since it induces dauer formation at low nanomolar concentrations but its activity declines at higher concentrations.
Ascaroside ascr#5 (also: “C3”, Figure 1-3), bearing a three-carbon side chain (and hence being structurally more similar to ascr#1-3), was found to be involved in dauer formation, however, not on its own, but only when it was tested in combination with ascr#2 and #3.\textsuperscript{109} Hence, this ascaroside only exerts an effect as a synergist to enhance dauer induction.

Figure 1-3. Structure of ascarosides icas#9 (A), icas#7 (B), ascr#5 (C) and ascr#8 (D).
The structures were drawn with ACD ChemSketch (ACD/Labs) according to PubChem entries CID 44205804, 86289730, 44202063 and 86289660.

The fact that some ascarosides do not show dauer induction activity individually, but only have an effect in combination with other ascarosides, makes their physiological identification difficult. An extraction method as described above for daumone (ascr#1), with chromatographic separation and testing the chromatography-fractions for biological activity, is difficult in these cases, since combinatorial tests of fractions would be necessary. To overcome this obstacle, 2-dimensional NMR spectroscopy-based comparative metabolomics was used for comparison of the \textit{C. elegans} wild type metabolome with the one of the \textit{daf-22} (dauer formation defective) mutant\textsuperscript{103}

# 26
which was found to be unable to produce short fatty acid chains required for some ascarosides. With this approach it is possible to determine multiple compounds in parallel. If an appropriate ‘loss of function’ mutant is available, the associated metabolite differences can be inferred. From the wild type/daf-22 comparison, several additional ascarosides were identified, including another dauer-inducing ascaroside ascr#8. This ascaroside is distinct from the above described ascr#1-3 and icas#9 since it contains a p-aminobenzoic acid moiety forming an amide with a seven-carbon α,β-unsaturated fatty acid (Figure 1-3). Nevertheless, it is active in dauer-induction as individual compound.

Therefore, more than 20 years after the initial discovery, it became clear that the dauer-inducing pheromonal cue, rather than being composed of only one or two signaling molecules, is a complex and versatile signaling compound mixture, in which each component has an effect in synergy with others and in which the concentration of each compound in the mixture is relevant to determine the specific biological effect. Pheromone molecules elicit different responses dependent on the context (and not exclusively based on an individual molecule’s activity), so overlapping subsets of ascarosides play an important role not only in dauer formation, but they also influence a variety of behaviours, such as avoidance, male attraction to hermaphrodites, hermaphrodite attraction to males, hermaphrodite aggregation and foraging suppression. Interestingly, many ascarosides, while inducing dauer formation in C. elegans larvae, also induce avoidance reactions in adults. Moreover, isolation of sex pheromones in C. elegans revealed that a blend of two major dauer pheromone constituents (ascr#2 and ascr#3), produced by hermaphrodites, exert a synergistic effect to mediate mate finding and male-attracting activity. Therefore, the same common set of ascarosides is involved in modulating two different behavioural responses: male attraction and dauer formation. While the attraction of males is signaled by low concentrations (femtomolar to picomolar), the developmental pathway of dauer formation is induced at higher concentrations.

The complexity and synergistic structure- and concentration-dependent differential activity of ascaroside signaling suggested that a large and diverse receptor repertoire, with different affinities for different compounds, may be involved in dauer pheromone perception. However, why the pheromonal signals in C. elegans,
such as dauer pheromone and sex pheromone, consist of several ascaroside compounds, is still unresolved. In regards to the dauer pheromone, it has been speculated that the different compounds, rather than simply providing information about population density, are informative about the status of conspecifics, like their metabolic status, influencing dauer decision.\endnote{119}

**Nomenclature**

Different naming conventions have been followed to categorize ascarosides. Since they contain fatty acid-like side chains of different lengths, ascarosides were initially named based on the length of the fatty acid chains.\endnote{104} For example, ascaroside ascr\#2, which contains a hexacarbon lipid chain, is also referred to as “C6”. For other ascarosides, names referring to their function are common, such as ascarosides ascr\#1, ascr\#2 and ascr\#3,\endnote{106,120} which are also called “daumone-1”, “daumone-2” and “daumone-3” since they act as dauer inducing pheromones.

A more systematic naming convention is attempted by the SMID database project, which assigns identifiers to small molecules (as ascarosides) discovered in *C. elegans*.\endnote{121} SMIDs (Small Molecul IDentifier) contain four letters, a hash and a number (e.g. ascr\#3, icas\#7) to make these pheromones searchable in the database. This kind of systematic naming appears appropriate given that more than 100 different pheromone compounds have been discovered, featuring a large chemical variety through modifications which would make a structure-based (unambiguous) naming convention complicated.

**Chemical structure**

The ascarosides have a modular structure, and consist always of a fatty-acid-derived side chain attached to an ascarylose sugar head group (compare Figures 1-2 and 1-3). Chemical modifications are found at both the terminus of the fatty acid chain (e.g. methylketones and *p*-aminobenzoylation), and on the ascarylose head group (e.g. by indole-3-carboxylation, *p*-hydroxybenzoylation or *p*-aminobenzoylation, glycosylation, 2-methyl-2-butenoylation).\endnote{96,122}
Two types of connection of the fatty acid side chain to the ascarylose sugar have been reported.\textsuperscript{92,109,123} Most commonly in dauer pheromones, the fatty acid chain is bound to the sugar \textit{via} its (ω-1) carbon, so that the terminal carbon of the lipid chain resides as a methyl side group (e.g. Figure 1-2, A-C, and Figure 1-3, A, B and D). Alternative connections have been noted \textit{via} the (terminal) ω-carbon (e.g. Figure 1-3, C). Based on this feature the terminology of (ω-1)- and ω-ascarosides is in use.

It has been reported that just one of the dauer pheromone compounds is an ω-ascaroside, asc-ωC3 (ascr#5; C3; see section “Dauer pheromone” in this introduction and Figure 1-3, C), and this ascaroside acts in synergy with the other dauer pheromones in order to induce dauer.\textsuperscript{92,109,123}

Ascarosides produced by \textit{C. elegans} vary in fatty acid chain length, unsaturation and side chain functionality. It was shown that many non-natural, synthesised ascarosides, although some of them had a highly similar structure to the natural dauer pheromone compounds, did not show any dauer-inducing activity.\textsuperscript{122} For example, ascr#3 (Figure 1-2, C) and ascr#7 (Figure 1-3, B) have a similar lipid attached to an ascarylose. Both have a carbon-carbon double bond feature adjacent to the terminal carboxylate group, though the lipid chain of ascr#7 is by 2 carbons shorter. In addition, ascr#7 contains an indole group attached to the ascarylose, which however does not affect the lipid chain. Even though the lipid is this similar, ascr#3 is a good attractant for males, while ascr#7 does so only to a little extent.\textsuperscript{115} In a systematic screen of closely related (synthetic) ascaroside structures, it was found that already small structural changes caused large differences in the ascaroside’s ability to induce dauer formation in \textit{C. elegans}.\textsuperscript{122} It was suggested that this might be due to the specificity of the receptor proteins involved, which in ascaroside sensing typically belong to the class of G protein coupled receptors (GPCRs). It is conceivable that this specificity has developed to avoid crossreactions to ascarosides released by other nematode species which inhabit the same environment, given that ascarosides are well conserved among nematodes.\textsuperscript{122,124}

**Biosynthesis**

Biosynthesis of ascarosides and the biosynthetic pathways involved in the specific assembly of the ascarylose sugar, fatty-acid side chain, and peripheral building
blocks (see section above: “Chemical structure”) are only poorly understood.\textsuperscript{112,115} The biosynthetic pathway of the ascarylose sugar moiety in \textit{C. elegans} is not completely determined.\textsuperscript{96} Some enzymes have been identified and show an interesting homology to genes from bacterial ascarylose-biosynthesis pathways. Peripheral building blocks are chemically versatile and derived from different biosynthetic pathways, which are not covered here as these features share little canonical structure. Progress has been made in understanding the fatty acid biosynthesis which, while leading to a similar compound class, offers wide variability through different lipid chain lengths. The fatty acid side chains derive from peroxisomal $\beta$-oxidation cycles required to shorten the side chains of the ascarosides by two carbons per cycle. In this catabolic process, four peroxisomal enzymes, an acyl-CoA oxidase (ACOX-1), enoyl-CoA hydratase (MAOC-1), (3R)-hydroxyacyl-CoA dehydrogenase (DHS-28), and 3-ketoacyl-CoA thiolase (DAF-22) are involved.\textsuperscript{42,104} Sequence analysis revealed that homologues of these enzymes were found in mammals, which participate in peroxisomal $\beta$-oxidation of long-chain fatty acids, branched-chain fatty acids and acid intermediates.\textsuperscript{42,96,125}

The enzyme encoded by the gene \textit{daf-22}, the \textit{C. elegans} ortholog of human sterol carrier protein SCPx, catalyzes the final step in peroxisomal fatty acid $\beta$-oxidation reactions.\textsuperscript{56,104} \textit{daf-22} mutants do not form dauer larvae in unfavourable conditions, since they are defective in pheromone production.\textsuperscript{97} The \textit{dhs-28} gene encodes a protein with a strong sequence similarity to the human D-bifunctional protein, and biosynthetically acts just upstream of SCPx.\textsuperscript{104} It was found that both \textit{daf-22} and \textit{dhs-28} mutants generate an accumulation of ascarosides with long-chain fatty acid side chains, arrested at different steps in the $\beta$-oxidation process.\textsuperscript{104} These ascarosides with long side-chains are considered to be intermediate products generated during the biosynthesis of dauer pheromones. Accumulation of the long-chain ascarosides was found both inside the worms, and in the culture media. However, whether the worms were able to actively secrete/excrete these long-chain ascarosides, or if these intermediate products were released after death and lysis of the worm, is still uncertain.\textsuperscript{104} For the intermediate compounds released from \textit{daf-22} mutants, no pheromone activity was found, but interestingly, dauer-inducing activity was reported after extended incubation of worm-culture extracts, raising the possibility of further enzymatic or nonenzymatic processing.\textsuperscript{104}
The tissue location of ascaroside processing by DAF-22 was assessed by fusing the protein to Green Fluorescent Protein (GFP). It was found by fluorescence microscopy that the gene is expressed in the intestine, hypodermis, and body wall muscle. Transgenic expression of *daf-22* exclusively in the intestine (controlled by an intestine-specific expression promotor), although not sufficient to restore all the dauer-inducing activity of the medium supernatant conditioned by growth of the transgenic *C. elegans* strain, was sufficient to recover the production of ascarosides ascr#2, ascr#3 and ascr#5, suggesting that also natively they are primarily produced in intestinal tissue. However, it was highlighted by the authors that since only some ascarosides were screened for, it is possible that additional, not yet identified ascaroside pheromones require *daf-22* expression also in other of the tissues where it is natively expressed (see above).

While progress has been made in the investigation of the ascaroside biosynthetic pathway and functional analysis, little is known about the mechanism that allows ascarosides to be released and transported from their site of biosynthesis. Ascarosides present in the excretomes (liquid culture supernatant) of *C. elegans* were compared with those from worm body metabolomes (worm pellet extract) in order to shed light on the presence of non-excreted ascaroside derivatives. Excretome and metabolome showed a different ascaroside profile, suggesting that ascarosides are differentially released, putting emphasis on the theory that *C. elegans* strictly regulate excreted molecules, with a preferential excretion ascarosides with unsaturated fatty acid side chains.

Shedding light on the ascaroside biosynthetic pathway, and understanding how environmental factors can modulate pheromone production, will potentially provide insight into chemical communication in *C. elegans*, and in other nematode species that use ascarosides as pheromone. Moreover, this will provide information on the response of nematodes to a changing environment and their ability to adapt to potentially fluctuating situations.

**Biosynthesis Regulation**

The production of different mixtures of ascarosides in *C. elegans* depends on genetics and different environmental conditions such as temperature, food,
population density; factors like sex and larval stages of the worm also influence production of specific signaling molecules.\textsuperscript{42,112,119} This signaling via such complex and nuanced collections of chemical cues requires a sophisticated regulation to allow nematodes to transmit and communicate different information to conspecifics and to improve the survival of other individuals in the population.\textsuperscript{42}

In adults, increased food availability results in a decreased production of short chain (\(\omega-1\))-ascarosides, such as the dauer pheromones ascr\#2 and icas\#9, giving weight to the theory that favorable conditions and availability of food leads to a less potent dauer pheromone production.\textsuperscript{42,119} However, production of the potent dauer pheromone ascr\#5 increased upon availability of food and well-fed conditions in larval stage worms. It has been speculated that favourable conditions and the presence of food trigger the production of this potent dauer pheromone component, as a mechanism for the worm to allow some of its progeny to enter into dauer, hence making use of the resistant dauer stage in some part of the population constitutively, which provides a survival benefit.\textsuperscript{42,119} Therefore, elucidating when production of ascarosides occurs and under which environmental conditions, and examining which enzymes are involved in pheromone biosynthetic pathways and how they are regulated, are key factors in order to shed light on ascaroside functions.\textsuperscript{119}

A correlation has been reported between ascaroside production and transcriptional regulation of Acyl-CoA oxidases (ACOXs).\textsuperscript{119} ACOXs are involved in the introduction of oxygen to Acyl-CoA and catalyze the first step of the \(\beta\)-oxidation cycle. Additionally, the initial oxidation by ACOX can lead to the synthesis of a double bond (\(\Delta\), unsaturated fatty acids) between the \(\alpha\) and \(\beta\) carbon (adjacent to the terminal to carbonyl group of the fatty acid).\textsuperscript{119} In \textit{C. elegans}, three ACOXs have been shown to be involved in ascaroside production.\textsuperscript{42,119} For instance, the expression of ACOX-1.3, involved in the production of shorter chain (\(\omega-1\))-ascarosides such as the dauer pheromone ascr\#2, decreased in the case of good food availability, which explains the reduced production of shorter chain (\(\omega-1\))-ascarosides in favourable conditions. On the other hand, the expression of ACOX-1.2, which catalyzes the first step in the \(\beta\)-oxidation cycle in order to produce the dauer pheromone ascr\#5, increased upon good food availability.\textsuperscript{119}
Characterization of ascaroside profiles from exudates in 19 evolutionarily divergent nematode species, including free-living and parasitic nematode species, by high performance liquid chromatography/mass spectrometry (HPLC-MS) revealed that ascarosides represent a conserved family of signaling molecules amongst nematodes and correlate with phylogeny, ecological niche and lifestyle.\(^\text{127}\)

The conserved nature of ascaroside production evokes the analogy to bacterial quorum sensing, in which acyl homoserine lactones (AHLs) are produced and sensed by several species of gram-negative bacteria.\(^\text{127,128}\) AHLs are constituted of a small invariable core structure, the homoserine lactone, attached to a fatty-acid-derived side chain presenting species-specific chemical variations.\(^\text{127,129}\) Ascaroside, based on the dideoxy-sugar ascarylose attached to a fatty-acid-derived side chain featuring modifications, presents a similar structural organization. Moreover, several nematode species are able to produce and respond to a complex mixture of ascarosides, similar to what has been observed in the bacterial AHL signaling. The similarity between the two systems could shed light on biochemical communication interactions.\(^\text{127}\) Quorum sensing coordinates several bacterial behaviours, such as biofilm formation, bioluminescence, mobility and antibiotic production.\(^\text{130}\) In likewise manner, ascarosides influence several *C. elegans* behaviours, such as decision to enter into dauer, repulsion, aggregation, mate finding and olfactory plasticity. Moreover, *C. elegans* modulates its ascaroside profiles according to changes in environments, such as growth and environmental perturbations.\(^\text{126,127}\)

**Hermaphrodite-induction in *A. freiburgensis***

In this chapter, the investigation of the effects of ascarosides on the production of progeny from hermaphrodite mothers of nematode *A. freiburgensis* is presented. Hermaphrodite progeny are determined to enter a dauer stage. While the nematode *C. elegans* only has two sexes (male and hermaphrodite)\(^\text{131}\), *A. freiburgensis* populations include male, female and hermaphrodite adults.\(^\text{56}\)

It is shown that the *C. elegans* dauer inducing pheromone ascr#3 also acts on *A. freiburgensis*, though in a different way. If a hermaphrodite mother worm of *A. freiburgensis* is exposed to ascr#3, its progeny shows a shift to more hermaphrodite worms over female worms. As female and hermaphrodite worms have the same
genetic karyotype, this result can be interpreted as a pheromone-induced developmental plasticity.

It is furthermore shown by culture-supernatant assays that *A. freiburgensis* itself generates and secretes a signal shifting the sex phenotype of the progeny. Attempts were made to isolate the responsible molecule and progress in this is summarised.

**Materials and Methods**

In this chapter, mother nematodes *A. freiburgensis* were exposed to different compounds and conditions, and their progeny were examined for sex phenotype. Only female and hermaphrodite progeny were considered, since they are genetically equal (same number of chromosomes and XX karyotype), while males (X0 karyotype) were ignored or removed as soon as they could be identified from their morphology. In a standard experiment, at least 96 eggs laid by the mother were collected and grown individually on bacteria-seeded agar within a 96-well plate before sex was determined. In other experiments, eggs were put on a larger petri dish agar plate (6 cm), males were removed at an early stage of development, and hermaphrodites were distinguished from females by their obligatory larval dauer stage. Additionally, the ability to lay eggs (only present in hermaphrodites) was considered. The details of agar plate growth are described below.

**Strain and culture**

*Auanema freiburgensis* was isolated from horse dung in Freiburg, Germany, in August 2003 by W. Sudhaus. The strain was cultured at 20 °C on standard Nematode Growth Medium (NGM) plates seeded with the *Escherichia coli* OP50 strain, as for *C. elegans*. Microbial contamination was prevented by using 25 µg/mL nystatin and 50 µg/mL streptomycin in the NGM medium.

**Isolation of dauers**

*C. elegans* dauers are resistant to 1% sodium dodecyl sulfate (SDS), and the same applies for *Auanema* dauers. By treating a plate with SDS, only the non-dauers will be killed. First, worms were resuspended in 2 ml of sterile water for each plate,
transferred to 15 ml tubes and sedimented by centrifugation at 3500 rpm for 3 minutes. After discarding the supernatant, 10 ml of 1% SDS was added to each tube and mixed well. Worms were incubated with SDS at room temperature for 30 minutes, after which they were sedimented by centrifugation at 3500 rpm for 3 minutes. 10 ml of water was added to each tube, and worms were resuspended and centrifuged at 3500 rpm for 3 minutes. Dauers were transferred to a freshly seeded 6 cm plate and left to crawl out of the carcasses and debris. Alternatively, dauers were isolated from an overcrowded plate, in which they stand on their tails and so are easily identifiable, by picking.

**Sexing progeny of selfing parents**

In order to determine the sex of the F1 from selfing hermaphrodites, the self-progeny of *A. freiburgensis* animals was collected throughout their lifespan. The hermaphrodite mothers were selected by first isolating dauers, which in *A. freiburgensis* always develop into hermaphrodites. Each dauer was placed on a 6 cm seeded NGM plate and kept at 20 °C to develop into an adult. Eggs laid by the hermaphrodite mother were collected and placed onto 96-well plates. The three sexes were identified based on several traits including: the ability to produce offspring in isolation and dauer formation as obligatory larval stage for hermaphrodites, lack of offspring for isolated females, and tail morphology for males.

**Dauer-inducing supernatant (Protocol-10dS)**

Worms were grown on 6 cm plates for almost 5 days (20 °C). Three of these 6 cm plates were washed into a flask using 10 ml S medium (3 ml per plate). To the same flask were added 5 ml S medium, 4 µl Nystatin (50 mg/ml in Dimethyl sulfoxide (DMSO), stored at -20 °C), 10 µl streptomycin (20 mg/ml in H₂O, sterile filtered, stored at 4 °C) and 250 µl 20 % w/v OP50-1 suspension in S medium (see paragraph below for preparation). This liquid culture was incubated at 22 °C and 180 rpm for 10 days. After spinning the worms and bacteria down at 5000 rpm for 15 minutes, the supernatant was poured into 15 ml falcon and frozen as soon as possible at -20 °C.
Chapter 1 – Supernatant-mediated signal transmission

In order to prepare the 20 % w/v OP50-1 in S medium, 2 bottles (800 ml) of LB medium were poured into a 3000 ml Erlenmeyer flask, and the flask was inoculated with one colony of *Escherichia coli* strain OP50-1. This culture was incubated overnight at 37 °C and 180 rpm. The overnight culture was transferred into falcon tubes and centrifuged at 5000 rpm for 10 min. The supernatant was discarded (carefully poured out). The tube with the OP50-1 pellet was weighed and stored at -20 °C (2.5 ml S medium was added for 0.5 g *Escherichia coli* OP50-1 strain, in order to get 20 % w/v OP50-1 in S medium).

**Dauer-inducing supernatant (Protocol-3wM9)**

Plates of *A. freiburgensis* on NGM medium (with *Escherichia coli* OP50-1) were grown for ca. 6 days (20 °C) until a high population density was reached (usually considered > 1,000 worms/cm^2^), and worms were washed off with M9 medium (10 ml, supplemented with 25 µg/mL nystatin) into a 1,500 ml flask. This procedure was repeated with several plates over 3 weeks, until 1,000 ml of liquid culture was produced. The liquid culture was during that time incubated on a rotary shaker at 22 °C and 100 rpm. Contamination was prevented by adding 25 µg/ml nystatin to the medium. Subsequently, the conditioned medium was centrifuged to sediment worms and bacteria, and the supernatant was collected and lyophilized.

**Assays with supernatant**

To gain insight into whether mother hermaphrodites respond to supernatant signals, they were cultured either in isolation or with supernatant (prepared as described above). 1 % SDS treatment was performed to obtain dauers and they were placed on 6 cm seeded plates for 24 hours, to grow to the L4 larval stage.

Each L4 hermaphrodite was moved to a 6 cm plate containing lyophilized supernatant powder (50 mg), applied by dissolving the powder in 200 µl OP50-1 medium used for seeding, and grown overnight. Non-hatched eggs that were laid during the night were collected and were washed with 200-300 µl of M9. Each egg was moved to a single well of a 96-microtiter plate (100 µl NGM media). Sex of the offspring was analyzed as described in previous sections above. Each supernatant exposure experiment was done side by side with an individual control (L4
hermaphrodite on plate, without mixing supernatant powder in the OP50-1 seeding medium).

To address the question whether L1 larvae can respond to supernatant, the following experiment was performed. Dauers were placed on a 6 cm seeded plate for 24 hours, well separated from each other so that they grew as isolated larvae. After development, each L4 hermaphrodite was transferred to one well of a 24-well plate (1.5 ml NGM media) and left to lay eggs. The offspring (at L1 stage) were removed and placed onto single wells of a 96-well plate (each well containing 100 µl NGM and 10 µl of supernatant). Sex of the offspring was scored.

**Supernatant fractionation by HPLC**

Supernatant (3 weeks incubation, 3wM9 – 1st batch, 2nd batch and 3rd batch) fractionation was done by collaborators (Frank C. Schroeder, Cornell University, Ithaca, USA) by high pressure liquid chromatography (HPLC). One successful fractionation was achieved with a C18Aq RediSepRf High Performance Gold column (Teledyne Isco) and a flow rate of 40 ml/min. Solvents were: A = water + 0.5 % v/v acetic acid; B = acetonitrile + 0.5 % v/v acetic acid. A gradient elution was used as follows (only the fraction (%) of solvent B is shown, fraction of solvent A = 100 – fraction solvent B): 0 min – 0 % B; 10 min – 0 % B; 41 min – 40 % B; 53.5 min – 70 % B; 60 min – 100 % B; 65 min – 100 % B. Fractions were collected in subsequent time windows (each minute). The solvent was evaporated (vacuum, speedvac) and the dried fractions subjected to an activity assay (see below).

**Activity assay for fractions from chromatographic supernatant fractionation**

To identify which fractions were active in changing the offspring of mother hermaphrodites, an activity assay was carried out. Dried fractions from the chromatography were dissolved in water (50-100 µl, until no insoluble residue was seen), and 30 µl were added on a 6 cm seeded agar plate, left to dry, and their activity to induce hermaphrodite-offspring was tested.

To isolate hermaphrodite mothers for the assays, a plate with high population density was treated with 1% SDS. Dauers were transferred to 6 cm seeded plates and kept at
20 °C for about 24 hours to allow them to develop into adult hermaphrodites. These hermaphrodites were transferred to seeded plates containing sample from the fractionations (as described above). Sexes of the progeny were scored as above.

**Dauer Recovery assay**

Dauers can persist in the dauer stage for extended periods in unfavourable environments, and here it was tested whether supernatant addition inhibits the recovery of dauers (into hermaphrodites) in food-rich conditions. 20 dauers were placed onto 6 cm seeded (*Escherichia coli* OP50-1) plates and exposed to supernatant (as described above) for 24 hours. After exposure to supernatant, dauers were observed to determine whether they became hermaphrodites or whether they stayed in the dauer stage for an extended period of time.

**Environmental stressors assay**

- **Temperature stress (25 °C)**

Dauers were picked from a 6 cm agar plate spotted with a ~5 mm diameter *E. coli* OP50-1 lawn, kept at 20 °C. At early L4 stage each worm was moved to a 6 cm NGM spotted (*E. coli* OP50-1) plate and incubated at 25 °C. Eggs laid at 25 °C were moved to a single well of a 96-well plate and incubated at 20 °C to allow them to develop into adults. Sexes of the progeny were determined as above.

- **Starvation**

Dauers were picked from a 6 cm agar plate spotted with a ~5 mm diameter *E. coli* OP50-1 lawn, kept at 20 °C. At early L4 stage each worm was moved to a 6 cm NGM plate, in the absence of food. Eggs laid in the absence of food were moved to a single well of a 96-well plate and incubated at 20 °C to allow them to develop into adults. Sex of the offspring was analyzed as above.

**Assays with ascaroside ascr#18**

To test if this ascaroside can influence sex determination, hermaphrodite mothers were cultured in isolation and incubated with synthetic ascr#18 (provided by Frank
C. Schroeder, Cornell University, Ithaca, USA). To isolate hermaphrodite mothers for the assays, a plate with high population density was treated with 1 % SDS. Dauers were transferred to 6 cm seeded (E. coli OP50-1) plates and kept at 20 °C for 24 hours to allow them to develop into adult hermaphrodites. The hermaphrodites, which were used as mothers, were transferred to a seeded plate containing asc#18. Different concentrations of asc#18 were tested (100 nM, 10 nM, 1 µM, 5 µM, 10 µM, 20 µM, 60 µM). Sex of the offspring was scored as described above. Controls contained no asc#18, but water as solvent control.

**Assays with ascaroside asc#2 and asc#3**

Hermaphrodite mothers were cultured in isolation and incubated with synthetic asc#2 and asc#3 (provided by Frank C. Schroeder, Cornell University, Ithaca, USA). To isolate hermaphrodite mothers for the assays, a plate with high population density was treated with 1% SDS. Dauers were transferred to 6 cm seeded plates and kept at 20 °C for 24 hours to allow them to develop into adult hermaphrodites. The hermaphrodites that were used as mothers were transferred to a seeded (Escherichia coli OP50-1) plate containing asc#2 or asc#3. asc#2 was tested at the concentration of 100 µM. Different concentrations of asc#3 were tested (33 µM, 66 µM, 130 µM). Sex of the offspring was scored as described above. Controls contained no ascaroside, but water as solvent control.

**Results and Discussion**

**Hermaphrodites with and without supernatant**

To test if the age of hermaphrodite mothers influences the production of XX karyotype progeny (i.e. female and hermaphrodite), the sex ratios of the progeny of hermaphrodite mothers (N = 2) during their entire life was determined. It was found that, when cultured in isolation or non-crowed condition, they produced almost exclusively female progeny irrespective of their age (Day 1 – 98 % ± 2 female, total progeny = 95; Day 2 – 100 % female, total progeny = 188; Day 3 – 99 % ± 1 female, total progeny = 202; Day 4 – 100 % female, total progeny = 35, see Figure 1-4 and Table 1-1).
Figure 1-4. Effect of mother age on the sex of the progeny.

The eggs from 2 hermaphrodites were collected on four subsequent days, and the sex of progeny determined. Numbers between hyphens identify the total number of progeny counted per timestep. An Unpaired Welch T-test for the difference in hermaphrodite offspring against Day 1 offspring was carried out and confirmed that there was no significant difference (all $p > 0.1$).
Table 1-1. Data for the effect of mother age on the sex of the progeny, see Figure 1-4.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermaphrodite mother 1</td>
<td>35 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>58 (97 %)</td>
<td>2 (3 %)</td>
</tr>
<tr>
<td><strong>DAY 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermaphrodite mother 1</td>
<td>91 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>97 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>DAY 3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermaphrodite mother 1</td>
<td>84 (99 %)</td>
<td>1 (1 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>117 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>DAY 4</strong></td>
<td></td>
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<tr>
<td>Hermaphrodite mother 1</td>
<td>32 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>3 (100 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

To investigate whether in *A. freiburgensis* the environment experienced by the parental generation determines the sexual fate of the offspring, mothers (N = 4) were transferred to plates containing supernatant derived from liquid culture (3 weeks incubation, 3wM9 – 1st batch). The F1 progeny developed mostly into hermaphrodites (85 % ± 10 hermaphrodite, total progeny = 131), whereas mothers kept in isolation (N = 3) produced mostly female offspring (97 % ± 1 female, total progeny = 141, p < 0.01, see Figure 1-5 and Table 1-2). When the F1 from exposed mothers were grown further on the supernatant-free NGM, it was regularly observed (by microscopy inspection as general impression of population) that in the F2 only a negligible number of dauers (as larval stage of hermaphrodites) formed, hence indicating that the supernatant exposure of the mother did not affect the progeny of the F1 (which was hence not further, in detail, investigated).
Figure 1-5. Effect of supernatant (3wM9 – 1st batch)-exposure on the sex of the progeny.
Supernatant was prepared from 3-week-old culture. The increase in hermaphrodites was tested by an Unpaired Welch T-test against the non-exposed control, and the statistical significance is indicated (*** p < 0.01). Numbers between hyphens identify the total number of progeny counted per condition.

Table 1-2. Data for effect of supernatant (3wM9 – 1st batch)-exposure on sex of progeny (Figure 1-5).

<table>
<thead>
<tr>
<th># number (% of progeny)</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>59 (95 %)</td>
<td>3 (5 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>40 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>37 (97 %)</td>
<td>1 (3 %)</td>
</tr>
<tr>
<td><strong>SUPERNATANT – 3wM9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>3 (11 %)</td>
<td>24 (89 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>5 (19 %)</td>
<td>22 (81 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>1 (3 %)</td>
<td>36 (97 %)</td>
</tr>
<tr>
<td>Test 4</td>
<td>11 (28 %)</td>
<td>29 (73 %)</td>
</tr>
</tbody>
</table>
The effect of liquid worm culture obtained after 10 days incubation (10dS; ca. half of the 3 weeks (21 days) incubation time) was tested (see Figure 1-6 and Table 1-3). Mother hermaphrodites exposed either to dried supernatant (as powder) (N = 9) or to liquid culture supernatant (150 µl), applied on a 6 cm diameter agar plate, produced mostly female offspring (90 % ± 8 female, total progeny = 479 and 91 % ± 3 female, total progeny = 328, respectively), as did the non-exposed mothers kept in isolation (N = 4) (83 % ± 22 female, total progeny = 87, p > 0.1). Hence, no effect on the progeny was observed, suggesting that perhaps a quite strong “crowding” signal is needed to induce the sex-shift in *A. freiburgensis* progeny.

**Figure 1-6.** Effect of supernatant(10dS)-exposure of the mother on the sex of the progeny.

Supernatant was prepared from 10 day old cultures. The increase in hermaphrodites was tested by an Unpaired Welch T-test against the non-exposed control, but no statistical difference (at least p < 0.1) was observed. The treatments were (compare x-axis label): Addition of 50 mg dried supernatant (S – Powder), addition of 150 µl culture supernatant (S – Liquid). All amounts refer to supernatant-assays on plates with 6 cm diameter. Numbers between hyphens identify the total number of progeny counted per condition. An Unpaired Welch T-test for the difference in hermaphrodite offspring against the control was carried out and confirmed that there was no significant difference (all p > 0.1).
Table 1-3. Data for the effect of supernatant(10dS)-exposure of the mother on the sex of the progeny, see Figure 1-6.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>33 (94 %)</td>
<td>2 (6 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>25 (93 %)</td>
<td>2 (7 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>20 (95 %)</td>
<td>1 (5 %)</td>
</tr>
<tr>
<td>Control 4</td>
<td>2 (50 %)</td>
<td>2 (50 %)</td>
</tr>
<tr>
<td><strong>SUPERNATANT POWDER – 10dS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>96 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>91 (99 %)</td>
<td>1 (1 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>59 (95 %)</td>
<td>3 (5 %)</td>
</tr>
<tr>
<td>Test 4</td>
<td>57 (90 %)</td>
<td>6 (10 %)</td>
</tr>
<tr>
<td>Test 5</td>
<td>39 (87 %)</td>
<td>6 (13 %)</td>
</tr>
<tr>
<td>Test 6</td>
<td>35 (95 %)</td>
<td>2 (5 %)</td>
</tr>
<tr>
<td>Test 7</td>
<td>20 (87 %)</td>
<td>3 (13 %)</td>
</tr>
<tr>
<td>Test 8</td>
<td>23 (77 %)</td>
<td>7 (23 %)</td>
</tr>
<tr>
<td>Test 9</td>
<td>26 (84 %)</td>
<td>5 (16 %)</td>
</tr>
<tr>
<td><strong>SUPERNATANT LIQUID – 10dS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>63 (91 %)</td>
<td>6 (9 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>56 (97 %)</td>
<td>2 (3 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>67 (92 %)</td>
<td>6 (8 %)</td>
</tr>
<tr>
<td>Test 4</td>
<td>57 (92 %)</td>
<td>5 (8 %)</td>
</tr>
<tr>
<td>Test 5</td>
<td>58 (88 %)</td>
<td>8 (12 %)</td>
</tr>
</tbody>
</table>

Supernatant acts in a reversible way

To determine if the supernatant affects sex determination in a reversible manner, hermaphrodite mothers (N = 14) were first left to lay eggs for 24 hours on one plate lacking supernatant (see Figure 1-7). As expected, progeny developed into females (99 % ± 2 female, total progeny = 386). When the same individual mothers (N = 14)
were transferred to a plate with supernatant (3wM9 -1st batch), offspring were mostly hermaphrodite (82 % ± 12 hermaphrodite, total progeny = 415). After 24 hours of exposure to supernatant, mothers (N = 9; 5 died) were rinsed with buffer and placed onto a new plate without supernatant, and progeny developed into females again (83% ± 9 female, total progeny = 364). The non-exposed controls (N = 5), which were also moved to three different NGM plates each 24 hours and left to lay eggs, produced female offspring (98 % ± 2 female, total progeny = 280, see Figure 1-7 and Table 1-4).

**Figure 1-7.** Reversibility of supernatant effect.

The same hermaphrodite mothers were cultured on plates without (“-“) and with (“+”) supernatant, in an alternating way (daily moving on different plate; result shown in barplot from left to right). The progeny sex-phenotype was analysed, and the percent fraction of hermaphrodites at the different stages compared against a control grown without supernatant. An Unpaired Welch T-test (for the fraction of hermaphrodites in the progeny) against the non-exposed control was used and the p-thresholds are indicated in the plot (*** p < 0.01, no indicator for p > 0.1). Numbers between hyphens identify the total number of progeny counted per condition.
Table 1-4. Data for reversibility of supernatant effect, see Figure 1-7.

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>99 (98 %)</td>
<td>2 (2 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>47 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>49 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Control 4</td>
<td>37 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 5</td>
<td>43 (96 %)</td>
<td>2 (4 %)</td>
</tr>
<tr>
<td><strong>BEFORE SUPERNATANT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermaphrodite mother 1</td>
<td>41 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>27 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 3</td>
<td>52 (96 %)</td>
<td>2 (4 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 4</td>
<td>16 (94 %)</td>
<td>1 (6 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 5</td>
<td>13 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 6</td>
<td>26 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 7</td>
<td>21 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 8</td>
<td>19 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 9</td>
<td>39 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 10</td>
<td>20 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 11</td>
<td>24 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 12</td>
<td>49 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 13</td>
<td>21 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 14</td>
<td>13 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>ON SUPERNATANT – 3wM9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hermaphrodite mother 1</td>
<td>6 (27 %)</td>
<td>16 (73 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 2</td>
<td>2 (4 %)</td>
<td>48 (96 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 3</td>
<td>6 (19 %)</td>
<td>25 (81 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 4</td>
<td>5 (24 %)</td>
<td>16 (76 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 5</td>
<td>5 (10 %)</td>
<td>46 (90 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 6</td>
<td>0 (0 %)</td>
<td>23 (100 %)</td>
</tr>
<tr>
<td>Hermaphrodite mother 7</td>
<td>28 (35 %)</td>
<td>51 (65 %)</td>
</tr>
</tbody>
</table>
These results indicate that environmental conditions (supernatant exposure) sensed by the parental generation (hermaphrodite mother) influence the sex of the progeny, suggesting the presence of signaling molecules in the supernatant that are perceived by the mothers and trigger a pathway that subsequently affects the developmental trajectory of their offspring.

5 out of 14 hermaphrodite mothers died during this experiment, and no results from the “after supernatant” phase could be obtained.
Dauers on supernatant

*C. elegans* dauer are known to recover into hermaphrodites when the conditions are favourable (e.g. non-crowded, optimal temperature, availability of food). However, it has been shown that active molecules present in worm-conditioned medium (culture supernatant) inhibit this recovery\(^\text{106}\). To understand if this is the case also in *A. freiburgensis*, 20 dauers (*A. freiburgensis*) were moved onto a food-rich seeded (*E. coli*) spotted plate containing 50 mg dried supernatant (3wM9 – 1\(^\text{st}\) batch, see Materials and Methods). It was observed that all 20 dauers became hermaphrodites, suggesting that in *A. freiburgensis* supernatant molecules do not inhibit recovery of the dauer.

Assays with ascr\#18

Previous experiments in the research group suggested that synthesised ascaroside ascr\#18 is the active compound present in the conditioned medium and plays an important role at inducing hermaphrodite formation in the F1 generation (information provided by Andre Pires da Silva). In the experiments presented in this thesis, however, the effects were relatively weak. Several trials were done to test the activity of this chemical. These were compared statistically (Unpaired Welch T-test) against mothers without treatment (N = 11) which produced mostly female progeny (97 % ± 6 female, total progeny = 529, see Figure 1-8 and Table 1-5).

- Mothers exposed to different concentrations of ascr\#18 produced mostly female offspring (note that the concentrations of ascr\#18 refer to the volume of the agar plate on which the test was conducted, 24-well plates with 1.5 ml agar per well):
  - 10 nM ascr\#18 N = 2, 100 % female (p > 0.1), total progeny = 76
  - 100 nM N = 3, 87 % ± 16 female (p > 0.1), progeny = 153
  - 1 \(\mu\)M N = 1 (no t-test possible), 94 % female, progeny = 95
  - 5 \(\mu\)M N = 2, 89 % ± 5 female (p > 0.1), progeny = 59
  - 10 \(\mu\)M N = 6, 85 % ± 23 female (p > 0.1), progeny = 290
  - 20 \(\mu\)M N = 1 (no t-test possible), 89 % female, progeny = 46
  - 60 \(\mu\)M N = 1 (no t-test possible), 83 % female, progeny = 42
• The effect of culturing in constrained environment in addition of the ascaroside supplementation (wells of 96-well plate, 100 µl of agar, supplemented with ascr#18 at 10 µM) was tested. The small well of the 96-well plates could increase “crowding signals” from additional secretions which, working in synergy with the ascr#18, could induce hermaphrodite formation. Two mothers (N = 2) were tested, and offspring developed mostly into female (89 % ± 15 female, (p > 0.1), total progeny = 113).

• To check if the SDS treatment used to select hermaphrodites (only the dauers, which become hermaphrodites, are resistant to SDS) could influence and interfere with these assays, mother hermaphrodites (N = 2) were either selected by picking dauers or by SDS treatment (standard protocol for dauer-isolation, see Materials and Methods) and exposed to ascr#18 (10 µM), in a single well of a 96 well plate. Offspring developed primarily into females (95 % ± 6 female, (p > 0.1), total progeny = 72).
Figure 1-8. Effect of ascaroside ascr#18 and different treatments on the sex phenotype of progeny. Mother hermaphrodites were exposed at different concentrations of ascr#18 in the range 0.01 – 60 µM, as indicated by the x-labels of the figure. Note that the concentrations refer to the volume of the agar plate on which the test was conducted (usually in 24-well plates with 1.5 ml agar per well). Additionally, the effect of culturing in constrained environment (wells of 96-well plate, 100 µl of agar, supplemented with ascr#18 at 10 µM) was tested, and this condition was furthermore used to test the effect of a SDS-treatment protocol (standard protocol for dauer-isolation, see Materials and Methods). Numbers between hyphens identify the total number of progeny counted per condition. An Unpaired Welch T-test (for the fraction of hermaphrodites in the progeny) against the non-exposed control was used, but no statistical indicators in the plot are shown since either it applied that p > 0.1, or the number of observations was insufficient (for the experiments with 1, 20 and 60 µM ascr#18).

Table 1-5. Data for the effect of ascr#18, see Figure 1-8.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>37 (97 %)</td>
<td>1 (3 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>59 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>26 (100 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>
# Chapter 1 – Supernatant-mediated signal transmission

| Control 4 | 39 (100 %) | 0 (0 %) |
| Control 5 | 100 (100 %) | 0 (0 %) |
| Control 6 | 38 (100 %) | 0 (0 %) |
| Control 7 | 30 (79 %) | 8 (21 %) |
| Control 8 | 28 (100 %) | 0 (0 %) |
| Control 9 | 59 (97 %) | 2 (3 %) |
| Control 10 | 48 (98 %) | 1 (2 %) |
| Control 11 | 53 (100 %) | 0 (0 %) |

**0.01 µM ascr#18**

| Test 1 | 32 (100 %) | 0 (0 %) |
| Test 2 | 44 (100 %) | 0 (0 %) |

**0.1 µM ascr#18**

| Test 1 | 34 (69 %) | 15 (31 %) |
| Test 2 | 53 (95 %) | 3 (5 %) |
| Test 3 | 47 (98 %) | 1 (2 %) |

**1 µM ascr#18**

| Test 1 | 89 (94 %) | 6 (6 %) |

**5 µM ascr#18**

| Test 1 | 12 (86 %) | 2 (14 %) |
| Test 2 | 42 (93 %) | 3 (7 %) |

**10 µM ascr#18**

| Test 1 | 45 (92 %) | 4 (8 %) |
| Test 2 | 20 (38 %) | 32 (62 %) |
| Test 3 | 69 (100 %) | 0 (0 %) |
| Test 4 | 35 (100 %) | 0 (0 %) |
| Test 5 | 40 (89 %) | 5 (11 %) |
| Test 6 | 37 (93 %) | 3 (8 %) |

**20 µM ascr#18**

| Test 1 | 41 (89 %) | 5 (11 %) |
Since this compound did not show activity, supernatant from *A. freiburgensis* was fractionated in order to shed light on other putative components involved in intergenerational plasticity.

### L1s and supernatant

To test if earlier developmental stages (larvae) are sensitive to the supernatant and high population density conditions, and to investigate whether in *A. freiburgensis* L1 larvae can also respond to crowding conditions as *C. elegans* L1s, eggs derived from mothers cultured in isolation were left to hatch and undergo larval development in plates with supernatant (3w9M – 2nd batch) until adulthood. Here, it was expected that they mature into mostly females, as they were collected as F1 generation from an isolated hermaphrodite mother which grew in non-crowded environment (see Materials and Methods). 89 % ± 2 (N = 18) of these L1s exposed to supernatant developed into females, indicating that larvae do not respond to crowding conditions (see Figure 1-9 and Table 1-6). For the control, eggs from mothers kept in isolation were left to hatch and undergo larval development in plates without supernatant. In these conditions, eggs developed mostly into females also (82 % ± 1, N = 94). These results are in accordance with a hypothesis that environmental factors experienced by hermaphrodite mothers of *A. freiburgensis* influence sex determination in the following generation, and it is not the offspring itself which senses crowding cues.
**Figure 1-9.** Exposure of L1 larvae to supernatant.
L1 larvae were sampled and grown on plates with and without supernatant supplement. After reaching the L4 stage, they were grown isolated in 96-well plates and the sex was determined. Numbers between hyphens identify the total number of progeny counted per condition.

**Table 1-6.** Data for exposure of L1s to supernatant, see Figure 1-9.

<table>
<thead>
<tr>
<th># number (%) of adults</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL – L1 grown without supernatant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>42 (82 %)</td>
<td>9 (18 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>35 (81 %)</td>
<td>8 (19 %)</td>
</tr>
<tr>
<td>SUPERNATANT – exposure of L1 to supernatant 3wM9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>9 (90 %)</td>
<td>1 (10 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>7 (88 %)</td>
<td>1 (12 %)</td>
</tr>
</tbody>
</table>
Chromatographic supernatant fractionation

In order to identify the putative active molecules responsible for induction of dauer formation in the next generation, supernatant (3wM9 – 1st batch, 2nd batch, 3rd batch) was fractionated by HPLC.

![Graph showing percentage of progeny as a function of number of fractions](image)

**Figure 1-10.** First chromatographic separation attempt.

Fractions from the chromatography were pooled as indicated in the x-axis label, and their activity to induce hermaphrodite offspring was tested. From the offspring, the numbers of females and hermaphrodites were determined and are shown as percent of total offspring (females plus hermaphrodites). Two to three worms were tested per fraction pool. Numbers between hyphens identify the total number of progeny counted per condition. An Unpaired Welch T-test (for the fraction of hermaphrodites in the progeny) for fraction-exposed samples against the non-exposed control was carried out, but as no statistical significance was found (with at least p < 0.1), due to large standard deviations in the fraction samples, no indicators are shown.

In a first chromatography run (3wM9 – 1st batch), fractions were collected and tested in pools of several fractions (Figure 1-10, Table 1-7). Mother hermaphrodites were exposed to fractions and progeny were scored. These were compared against mothers
without treatment (N = 8) which produced mostly female progeny (97 % ± 6 female, total progeny = 251).

- Fraction 1 – 7 N = 3, 40 % ± 46 female (p > 0.1), total progeny = 129
- Fraction 8 – 16 N = 3, 75 % ± 20 female (p > 0.1), progeny = 79
- Fraction 17 – 26 N = 2, 98 % ± 3 female (p > 0.1), progeny = 34
- Fraction 27 – 33 N = 2, 99 % ± 2 female (p > 0.1), progeny = 62
- Fraction 34 – 43 N = 3, 96 % ± 5 female (p > 0.1), progeny = 195
- Fraction 44 – 53 N = 2, 97 % ± 4 female (p > 0.1), progeny = 125
- Fraction 54 – 64 N = 2, 90 % ± 9 female (p > 0.1), progeny = 112
- Fraction 65 – 78 N = 2, 70 % ± 25 female (p > 0.1), progeny = 90

Activity was found in pooled fractions 1-7, 8-16 and 65-78, which however was not statistically significant (p > 0.1), due to considerable standard deviations. Hence, with this initial success of identifying activity in HPLC fractions, separation and assay were repeated on another batch of supernatant (see below).

Table 1-7. Data for first chromatographic separation attempt, see Figure 1-10.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>34 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>35 (97 %)</td>
<td>1 (3 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>19 (95 %)</td>
<td>1 (5 %)</td>
</tr>
<tr>
<td>Control 4</td>
<td>16 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 5</td>
<td>32 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 6</td>
<td>59 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 7</td>
<td>23 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 8</td>
<td>25 (81 %)</td>
<td>6 (19 %)</td>
</tr>
<tr>
<td>FRACTIONS 1 – 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>38 (93 %)</td>
<td>3 (7 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>2 (5 %)</td>
<td>36 (95 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>11 (22 %)</td>
<td>39 (78 %)</td>
</tr>
<tr>
<td>Fractions 8 – 16</td>
<td>Test 1</td>
<td>Test 2</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>27 (75 %)</td>
<td>24 (96 %)</td>
</tr>
<tr>
<td></td>
<td>9 (25 %)</td>
<td>1 (4 %)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 17 – 26</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13 (100 %)</td>
<td>0 (0 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (95 %)</td>
<td>1 (5 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 27 – 33</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27 (100 %)</td>
<td>0 (0 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34 (97 %)</td>
<td>1 (3 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 34 – 43</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 (90 %)</td>
<td>8 (10 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 (100 %)</td>
<td>0 (0 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>75 (97 %)</td>
<td>2 (3 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 44 – 53</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68 (100 %)</td>
<td>0 (0 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>54 (95 %)</td>
<td>3 (5 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 54 – 64</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 (84 %)</td>
<td>9 (16 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>53 (96 %)</td>
<td>2 (4 %)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fractions 65 – 78</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 (52 %)</td>
<td>28 (48 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 (88 %)</td>
<td>4 (12 %)</td>
<td></td>
</tr>
</tbody>
</table>
A new chromatographic separation (3wM9 – 2\textsuperscript{nd} batch) was done, aiming at a better separation, and the fractions from this run were tested again. A first test was done by exposing mothers to pooled fractions (Figure 1-11 and Table 1-8). These mothers where compared to the non-exposed mother (N = 1) and the pooled fractions 13-28 showed activity.

**Figure 1-11.** Second chromatographic separation attempt – activity test part I.

Fractions from the chromatography were pooled as indicated in the x-axis label, and their activity to induce hermaphrodite offspring was tested. From the offspring, the numbers of females and hermaphrodites were determined and are shown as percent of total offspring (females plus hermaphrodites). One worm was tested per fraction pool. Numbers between hyphens identify the total number of progeny counted per condition. No t-test assessment was carried out due to the low replicate number in this experiment.
Table 1-8. Data for second chromatographic separation attempt – activity test part I, see Figure 1-11.

<table>
<thead>
<tr>
<th># number (% of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>68 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 3 – 4</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>76 (99 %)</td>
<td>1 (1 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>113 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 5 – 12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>86 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 13 – 28</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>33 (48 %)</td>
<td>36 (52 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 29 – 38</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>39 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 39 – 48</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>43 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 49 – 58</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>47 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 59 – 68</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>67 (99 %)</td>
<td>1 (1 %)</td>
</tr>
</tbody>
</table>

Subsequently, smaller pools were tested (Figure 1-12 and Table 1-9), and activity was only found in the pool of fractions 24-26. A non-exposed mother (N = 1) produced offspring which developed into female (97 % female).

- Fraction 13 – 17 N = 1, 98 % female (no t-test possible), progeny = 42
- Fraction 18 – 20 N = 1, 100 % female (no t-test possible), progeny = 47
- Fraction 21 – 23 N = 1, 98 % female (no t-test possible), progeny = 45
- Fraction 24 – 26 N = 1, 20 % female (no t-test possible), progeny = 40
- Fraction 27 – 28 N = 1, 100 % female (no t-test possible), progeny = 38
Figure 1-12. Second chromatographic separation attempt – activity test part II.

To refine the result from Figure 1-11, smaller pools of fractions (as indicated by the x-label) were tested for activity to induce hermaphrodite offspring. From the offspring, the numbers of females and hermaphrodites were determined and are shown as percent of total offspring (females plus hermaphrodites). One worm was tested per fraction pool. Numbers between hyphens identify the total number of progeny counted per condition. No t-test assessment was carried out due to the low replicate number in this experiment.
Table 1-9. Data for second chromatographic separation attempt – activity test part II, see Figure 1-12.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>37 (97 %)</td>
<td>1 (3 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 13 – 17</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>41 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 18 – 20</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>47 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 21 – 23</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>44 (98 %)</td>
<td>1 (2 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 24 – 26</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>8 (20 %)</td>
<td>32 (80 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 27 – 28</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>38 (100 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

Finally, hermaphrodite mothers exposed to a double-fraction 24-25 (N = 1) and single fraction 26 (N = 1) were found to be active in inducing hermaphrodites in the next generation (for double fraction 24-25, 50 % hermaphrodite, total progeny = 81; for fraction 26, 55 % hermaphrodite, total progeny = 82, see Figure 1-13 and Table 1-11). A mixture of these fractions showed the highest activity at inducing hermaphrodite progeny. Offspring from a mother exposed to fractions 24 -25/ 26 developed mostly into hermaphrodite (90 % hermaphrodite, total progeny = 96). A control mother (N = 1) assayed in parallel produced only females (100 % female, total progeny = 66).
Figure 1-13. Second chromatographic separation attempt – activity test part III.

To refine the result from Figure 1-12, fractions were tested as one double-fraction 24-25 (received as such from the cooperation partner) and a single fraction 26. For comparison, a mixture of these fractions was tested, representing a repeat of the pooled fraction test in Figure 1-12. From the offspring, the numbers of females and hermaphrodites were determined and are shown as percent of total offspring (females plus hermaphrodites). One worm was tested per fraction pool. Note that for the individual testing, 40 µl of water-reconstituted fraction were added per agar seeded plate (6 cm diameter) for the exposure test, while for the mixed fraction 24-25/26 from each fraction 30 µl were mixed together, and a total of 60 µl was used for the plate (again 6 cm diameter). Numbers between hyphens identify the total number of progeny counted per condition. No t-test assessment was carried out due to the low replicate number in this experiment.
Table 1-10. Data for second chromatographic separation attempt – activity test part III, see Figure 1-13.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>66 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 24 – 25</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>40 (49 %)</td>
<td>41 (51 %)</td>
</tr>
<tr>
<td><strong>FRACTION 26</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>37 (45 %)</td>
<td>45 (55 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 24 – 25 / 26</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>10 (10 %)</td>
<td>86 (90 %)</td>
</tr>
</tbody>
</table>

As an additional experiment repeat, a third attempt was undertaken (3wM9 – 3rd batch) (Figure 1-14 and Table 1-11). Here, a pooled fraction (82-106) showed activity. Mother hermaphrodites were exposed to fractions and progeny were scored, and these were compared against mothers without treatment (N = 2) which produced mostly female progeny (98 % ± 2 female, total progeny = 97).

- Fraction 82 – 106 N = 1 (no t-test possible), 61 % female, progeny = 33
- Fraction 107 – 115 N = 1 (no t-test possible), 96 % female, progeny = 74
- Fraction 116 - 121 N = 1 (no t-test possible), 91 % female, progeny = 45
- Fraction 122 – 130 N = 1 (no t-test possible), 88 % female, progeny = 42
- Fraction 131 – 139 N = 1 (no t-test possible), 92 % female, progeny = 64
- Fraction 140 - 148 N = 1 (no t-test possible), 96 % female, progeny = 56
- Fraction 149 - 157 N = 1 (no t-test possible), 94 % female, progeny = 65

Due to time-constraints, no further improvement could be undertaken for the final isolation of the active compound. Mass spectrometric analysis of the different fractions are pending.
Figure 1-14. Third chromatographic separation attempt.
Fractions from the chromatography were pooled as indicated in the x-axis label, and their activity to induce hermaphrodite offspring was tested. From the offspring, the numbers of females and hermaphrodites were determined and are shown as percent of total offspring (females plus hermaphrodites). One worm was tested per fraction pool. Numbers between hyphens identify the total number of progeny counted per condition. No t-test assessment was carried out due to the low replicate number in this experiment.

Table 1-11. Data for third chromatographic separation attempt, see Figure 1-14.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>63 (97 %)</td>
<td>2 (3 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>32 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 82 – 106</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>20 (61 %)</td>
<td>13 (39 %)</td>
</tr>
<tr>
<td><strong>FRACTIONS 107 – 115</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>71 (96 %)</td>
<td>3 (4 %)</td>
</tr>
</tbody>
</table>
Chapter 1 – Supernatant-mediated signal transmission

<table>
<thead>
<tr>
<th>FRACTIONS 116 – 121</th>
<th>Test 1</th>
<th>41 (91 %)</th>
<th>4 (9 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRACTIONS 122 – 130</td>
<td>Test 1</td>
<td>37 (88 %)</td>
<td>5 (12 %)</td>
</tr>
<tr>
<td>FRACTIONS 131 – 139</td>
<td>Test 1</td>
<td>59 (92 %)</td>
<td>5 (8 %)</td>
</tr>
<tr>
<td>FRACTIONS 140 – 148</td>
<td>Test 1</td>
<td>54 (96 %)</td>
<td>2 (4 %)</td>
</tr>
<tr>
<td>FRACTIONS 149 – 157</td>
<td>Test 1</td>
<td>61 (49 %)</td>
<td>4 (6 %)</td>
</tr>
</tbody>
</table>

**Effect of ascr#2**

In parallel with fractionation of supernatant by HPLC, aimed at shedding light on the putative molecule(s) active at influencing sex determination in *A. freiburgensis*, the effect of further ascarosides, which are known to induce dauer formation within the same generation in *C. elegans*, ascarosides ascr#2 and ascr#3, were tested.\(^{106}\) Exposure of mother hermaphrodites of *A. freiburgensis* (N = 1) with synthetic ascaroside ascr#2 resulted in the production of female progeny (100 % female, total progeny = 35), as did the control mother not exposed to ascr#2 (100 % female, total progeny = 32, see Figure 1-15 and Table 1-12). Hence, a potential role for this molecule as active compound sensed by the parental generation and influencing sex of the progeny was ruled out.
Chapter 1 – Supernatant-mediated signal transmission

Figure 1-15. Effect of ascr#2 on *A. freiburgensis* progeny.
This ascaroside was tested at 100 µM concentration. Numbers between hyphens identify the total number of progeny counted per condition. No t-test assessment was carried out due to the low replicate number in this experiment.

Table 1-12. Data for the effect of ascr#2 on *A. freiburgensis* progeny, see Figure 1-15.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>32 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>100 nM ascr#2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>25 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>10 (100 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>
Chapter 1 – Supernatant-mediated signal transmission

Effect of asc#3

Exposure of hermaphrodite mothers to asc#3 resulted in the production of hermaphrodite progeny (see Figure 1-16 and Table 1-13), in a concentration dependent manner. First identified in *C. elegans* as a small-molecule signal which controls dauer entry, asc#3 in *A. freiburgensis* played a role in the mechanism of intergenerational plasticity, influencing the developmental pathways which promote dauer formation (therefore hermaphrodites) in the next generation. asc#3 was tested at three concentrations, of which 2 showed an effect on the F1 progeny. At 33 µM (N = 1), 52 % were hermaphrodite (total progeny = 44), and at 66 µM (N = 2), 53 % ± 31 were hermaphrodite (total progeny = 284), compared to non-exposed controls (N = 2) with only 1 % ± 1.5 hermaphrodite (total progeny = 173) (note that for the 33 µM sample, insufficient replicate numbers did not support a t-test, while it applied that p > 0.1 for the 66 µM test). The highest tested asc#3 concentration of 130 µM did not induce dauer formation in the next generation (N = 1, 9 % hermaphrodite, total progeny = 230, insufficient replicate number for t-test). As an additional statistical assessment, it was tested (Unpaired Welch T-test) whether the hermaphrodite progeny in asc#3 exposed samples (pooled result for 33, 66 and 130 µM) would be significantly different from the non-exposed controls (N_total = 4, 42 % ± 29 hermaphrodite, total progeny = 558). Taken together, the significance was p < 0.1, but not < 0.05 (Figure 1-16). However, as seen from the data of the individual concentrations, the result could be reproduced at two concentrations with a total of 3 mother hermaphrodites.
Figure 1-16. Effect of ascr#3 on *A. freiburgensis* progeny.

This ascaroside was tested at three concentrations (33 – 130 µM with respect to total agar plate volume of 9 ml). Since only a limited number of worms was tested at each concentration, the barplot shows additionally the combined progeny results at the different concentrations, summarized under the label “ascr#3”. From this combined result, the change in hermaphrodite fraction of progeny was statistically compared against the non-exposed control. An Unpaired Welch T-test against the control was used and the p-thresholds are indicated in the plot (* p < 0.10). Numbers between hyphens identify the total number of progeny counted per condition.

Table 1-13. Data for the effect of ascr#3 on *A. freiburgensis* progeny, see Figure 1-16.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>32 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>138 (98 %)</td>
<td>3 (2 %)</td>
</tr>
<tr>
<td>“ascr#3”</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Combined all results from dataset shown below.*

| 33 nM ascr#3            |          |               |
### Environmental stressors (temperature, starvation)

<table>
<thead>
<tr>
<th>Stress Factor</th>
<th>Control</th>
<th>Starvation</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hermaphrodite</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

**Figure 1-17.** Effect of stress to the mother on the progeny sex phenotype.

L4 hermaphrodites were exposed to starvation or high temperature (25 °C, the normal growth temperature is 20 °C). Numbers of tested worms were: 1 (control), 3 (starvation), 2 (temperature). Numbers between hyphens identify the total number of progeny counted per condition. No t-test was carried out due to insufficient replicate numbers for the control in this experiment (note though that the control result with higher fraction of female progeny was reproduced multiple times in separate experiments, see other results in this chapter).
Table 1-14. Data for the effect of stress to the mother on the sex of the progeny, see Figure 1-17.

<table>
<thead>
<tr>
<th></th>
<th>Female (%)</th>
<th>Hermaphrodite (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>64 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>STARVATION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>48 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>43 (91 %)</td>
<td>4 (9 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>51 (100 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td><strong>TEMPERATURE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>89 (97 %)</td>
<td>3 (3 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>73 (99 %)</td>
<td>1 (1 %)</td>
</tr>
</tbody>
</table>

The decision to enter the alternative developmental stage of dauer depends on environmental signals such as low food supply, high temperature, and a population density cue. According to the results presented in this thesis, in *A. freiburgensis*, the decision to become dauer is established across generations instead of within the same generation (compare Figures 1-5 and 1-7) and high population density is a key factor influencing sex determination (compare results with different supernatant protocols in Figures 1-5 and 1-6).

In order to investigate if this polyphenic trait in the next generation can also be influenced by other factors like high temperature and low food availability, parental generation nematodes (mother hermaphrodites) were exposed (at early L4 stage) to environmental stressors such as 25 °C and starvation. The assumption behind these experiments was that stresses could favour hermaphrodite offspring, which can sustain periods of stress in the more resistant dauer stage.

Exposure of young hermaphrodites either to starvation (*N* = 3) or 25 °C (*N* = 2) did not influence sex determination in the next generation (with starvation, 97 ± 3 female, total progeny = 146; with temperature stress 98 % ± 2 female, total progeny = 166). These mothers were compared to a control mother (*N* = 1) which was cultured at normal condition and produced mostly female progeny (100 % female,
total progeny = 64, no t-test due to insufficient number of control samples). This data suggests that high population density experienced by the mother is the critical factor to induce an adaptive response and a polyphenic trait in the next generation.

Supernatant from JU1782

![Supernatant from JU1782](image)

**Figure 1-18.** JU1782 supernatant tested on JU1782 hermaphrodites (positive control).

One worm was tested for the control, and two worms with supernatant exposure. For supernatant preparation, the standard (3 weeks (3wM9)) protocol developed for *A. freiburgensis* was also used for JU1782. The exposure bioassay was also performed as for *A. freiburgensis*. Numbers between hyphens identify the total number of progeny counted per condition. No t-test was carried out due to the insufficiently low number of control replicates.

To investigate whether a similar supernatant-mediated signalling occurs in other nematodes, the 3-weeks supernatant protocol (3wM9) was tested in another isolate, the free-living trioecious nematode *Auanema sp.* JU1782 (henceforth JU1782). It was observed that, similarly to *A. freiburgensis*, when cultured in isolation or not crowded condition, JU1782 hermaphrodites (N = 1) produced primarily female progeny (82 % female, total progeny = 28), while exposure of hermaphrodite
mothers to the supernatant generated from JU1782 cultures (N = 2) induced hermaphrodites in the next generation (94 % hermaphrodite, total progeny = 127) (see Figure 1-18 and Table 1-15).

Table 1-15. Data for JU1782 supernatant tested on JU1782 hermaphrodites (positive control), see Figure 1-18.

<table>
<thead>
<tr>
<th># number (%) of progeny</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>23 (82 %)</td>
<td>5 (18 %)</td>
</tr>
<tr>
<td>JU1782 supernatant tested on JU1782 hermaphrodites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>3 (5 %)</td>
<td>57 (95 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>5 (7 %)</td>
<td>62 (93 %)</td>
</tr>
</tbody>
</table>

**Conclusion**

The free-living trioecious species, *Auanema freiburgensis*, exhibits three different genders (male, females and hermaphrodites) and it represents an example of a mixed breeding system. The three sexes are produced by both outcrossing and selfing. Somatic sex determination is mediated by an XX:XO mechanism, in which XX animals develop into females and hermaphrodites and XO animals become males. However, the mechanism of hermaphrodite versus female sex determination is largely unknown. As adults, hermaphrodites can be distinguished by their ability to reproduce in the absence of a male partner by producing sperm that is used to self-fertilize oocytes. The question that has fueled this study aimed to understand why and how genetically identical individuals can become different during their life and develop into females or hermaphrodites.

This study showed that *A. freiburgensis* hermaphrodite mothers, when cultured in isolation or at low population density, produce mostly female offspring. However, when these mothers are exposed to chemicals produced by conspecific nematodes, and in high population density condition, they produce progeny that go through a non-feeding larval stage (dauer) that later become selfing hermaphrodites. To expose...
the parental generation to crowded cues, *A. freiburgensis* was cultured in liquid media for 3 weeks, the supernatant was then collected, freeze dried, and the resulting powder was found to induce hermaphrodite F1 (progeny) when hermaphrodite mothers were exposed to this supernatant powder. A culture obtained after only 10 days incubation (ca. half of the 3 weeks - 21 days) showed no effect on the sex of progeny, suggesting perhaps that a quite strong “crowding” signal is needed to induce the sex-shift in *A. freiburgensis* progeny. Exposure of larval stage worms to crowding cues from the supernatant did not have any effect on sex phenotype in the adult stage.

Therefore, *A. freiburgensis* represents a case of cross-generational polyphenism in which the sex of the progeny depends on environmental cues experienced by the parental generation; in other words, genetically identical individuals can become females or hermaphrodites depending on factors sensed by the mother. *A. freiburgensis* is a unique model to study cross-generational polyphenisms because, contrary to other nematodes, this plasticity is established across generations instead of within the same generation.

Interestingly, the mother reacts to supernatant in a reversible manner; when a hermaphrodite mother is placed in a plate with no supernatant, she produces only non-dauer progeny. However, when placed in a plate with supernatant, the mother produces mostly dauer progeny. After washing the mother and placing in a new clean plate, she resumes the production of non-dauer, suggesting that the mechanism regulating dauer formation acts quickly, providing the offspring with survival advantages to deal with environmental perturbations (see summary in Figure 1-19).

This study is in agreement with recent evidence that suggests that parental environmental conditions can affect the phenotypes of progeny. In fluctuating environments, mothers may influence the phenotype of their offspring, independent of the offspring’s genotype. Hence, if local environments are to some extent predictable across generations, this phenomenon, also called adaptive transgenerational phenotypic plasticity, may ameliorate risks in order to increase offspring fitness in specific environments.
To address this question further, and to thoroughly understand if other kinds of conditions experienced by the parental generation of *A. freiburgensis* can be transmitted to the next generation influencing sex determination, hermaphrodite mothers were exposed to different environmental stressors, such as high temperature or starvation. However, these kinds of stressful conditions did not have any influence on the sex of the next generation and did not trigger the pathways that induce dauer formation, suggesting that high population density experienced by the mother is the most critical factor for induction of an adaptive response in the next generation leading to a different sex phenotype.

To shed light on the putative molecule present in the supernatant, the *A. freiburgensis* supernatant was fractioned by chromatography (HPLC). After separation, fractions which induce the F1 hermaphrodite phenotype when mother hermaphrodites were exposed to the fraction were identified in activity tests. A mass
spectrometric (MS) or nuclear magnetic resonance (NMR) analysis to identify the chemical cues contained in the fractions similar to previous studies is ongoing. This kind of analysis is not trivial since even after HPLC fractionation, multiple compounds can be present in a fraction, and structure-analysis by NMR requires large amounts of compound (at least 50-100 µg which can sometimes need separation of supernatant of several litres of culture).\textsuperscript{92} At this stage, it is unclear whether the fractions from HPLC actually contained single, or multiple compounds requiring further separation and purification. However, having shown here that elution fractions from HPLC show activity, it is clear that the crowding cue survives the HPLC treatment and binds to the employed HPLC column, setting the stage for further separation attempts in order to purify single compounds for activity testing and structure determination.

It is presented in this thesis that in \textit{A. freiburgensis} ascr\#3, identified in \textit{C. elegans} as a small-molecule signal which controls dauer entry,\textsuperscript{96} plays a role in the mechanism of adaptive transgenerational phenotypic plasticity, influencing the developmental pathways which promote dauer formation (therefore hermaphrodites) in the next generation. It has been reported that chemical communication and ascaroside signaling are widely conserved in nematodes,\textsuperscript{127} which is in agreement with the ascr\#3 activity in \textit{A. freiburgensis}.

Similar to other ascarosides, such as icas\#9,\textsuperscript{42} the response to ascr\#3 in \textit{A. freiburgensis} is concentration dependent. High concentration of the compound did not show activity at inducing dauer formation in the next generation, suggesting a different mode of receptor activation. Moreover, while the effect of the exposure of the parental generation to supernatant resulted in a strong and fast effect and a high percentage of hermaphrodite progeny, ascr\#3 shows a different profile. It was observed that, while the parental generation is in contact with the synthesized chemical, the first 15-20 eggs develop into females, with a delay in the production of hermaphrodite progeny. One hypothesis could be that different ascarosides are present in the supernatant obtained from liquid culture worms, acting in synergy and being involved in the mechanism which regulates this kind of phenotypic plasticity. Similar to this hypothesis, the \textit{C. elegans} dauer pheromone (to which ascr\#3 belongs) is a complex and versatile signaling compound mixture, in which each component
acts in synergy with others and in which the concentration of each compound in the mixture is relevant to determine the specific biological effect.\textsuperscript{96}

Therefore, \textit{A. freiburgensis} may provide a good model for how environmental information experienced by the maternal soma can be transmitted across a generation. In \textit{A. freiburgensis}, the adaptive significance of the production of dauer in crowded conditions, signaled by chemical cues, is probably linked to the lifestyle of this free-living nematode. Because \textit{A. freiburgensis} reproduces very quickly, its habitat may become highly populated and food runs out rapidly. Ascarosides such as (or similar to) ascr\#3 are probably sensed as a stress-signaling mechanism to induce the development of dauer, which are morphologically, physiologically and behaviourally adapted for dispersal.\textsuperscript{133} The development of dauers into self-fertilizing adults may be adaptative, because it allows the reproduction of even a single colonizing individual in the absence of a mating partner.

Having established that crowding cues (supernatant) and particular ascarosides (ascr\#3) can induce \textit{A. freiburgensis} to shift to a larger fraction of hermaphrodites in the offspring of an exposed hermaphrodite mother, the identification of signal receiving cells (i.e. neurons) and attempts to understand the neuronal response to these cues are presented in Chapters 2 and 3 of this thesis.
Chapter 2 – Neuron signaling in *Auanema freiburgensis*.

“You, your joys and your sorrows, your memories and ambitions, your sense of personal identity and free will, are in fact no more than the behavior of a vast assembly of nerve cells and their associated molecules.”

*Francis Crick*

**Introduction**

**How do nematodes sense the environment? An overview on nematode chemosensation**

Chemosensation, defined as the sensing of chemical stimuli, and specifically olfaction (detection of volatile chemicals), allows nematodes to thrive by leading them to food sources, warning them of harmful chemicals and alerting them to high population density. In chemosensation, neuronal receptors recognize specific small molecules and elicit the associated signaling transduction pathways, which transform the chemical signaling into a neuronal response. Despite its compact nervous system, the nematode *C. elegans* is able to sense and respond to hundreds of different chemicals, making it an excellent model for studies of chemosensation.

In *C. elegans*, the nervous system represents the most complex tissue. Neurons constitute almost a third of all the cells in the body (302 out of 959 in the adult hermaphrodite). Each neuron differs from others by several properties, such as position, connectivity and morphology. This outstanding variety of neuron types has led to group neurons in 118 classes: 39 classes of sensory neurons, 27 classes of motoneurons, and the remainder are classified as interneurons. However, this represents a simplification, since several sensory neurons and motoneurons can function as interneurons.

The structure of the nervous system has been characterized in details by electron microscopic reconstruction, making *C. elegans* the only metazoan to have its
connectome (neuronal "wiring diagram") fully characterized down to the synaptic level. In fact, the high-resolution section electron micrographs allowed the identification of all the synapses (6393 chemical synapses, 890 electrical junctions, and 1410 neuromuscular junctions), the mapping of all the connections, and the morphology of each neuron. A single mammalian hippocampal pyramidal neuron can make the same number of chemical synapses as found in C. elegans.

Some of these neurons are situated along the ventral cord and in tail ganglia. In males, specific neurons are found in the copulatory tail. However, the majority of the neurons are located in the head around a central neuropil called the nerve ring, where they are furthermore organized in groups to form a simple epithelial sense organ, known as sensilla. Each sensillum is composed of one or more neuronal cells with ciliated endings and two non-neuronal cells: a socket cell (Amso), which connects the sensillum to the hypodermis by adherens junctions, and a sheath cell (Amsh), which surrounds the ending of the neurons.

Two bilaterally symmetric large sensillia, the amphids, are open to the outside through little pockets on the cuticle at the sides of the lips. Eight different neurons send their ciliated endings through this opening, while another four are enclosed with the sheath cells. Amphid neurons belong to bilaterally symmetric pairs, with the right and left members of each pair showing a similar structure. Two sets of sensory organs are involved in chemosensation: two bilaterally symmetric phasmds, in the tails, characterized by a simple organization with just two ciliated endings exposed to the external environment, and six inner labial sensory organs in the head, each containing the endings of two types of sensory neurons. Due to their structure, amphids represent the main chemoreceptive organ in C. elegans, allowing a group of nerve endings to communicate and to be exposed to the environment.

The name of the amphid neurons are: AWA, AWB, AWC, AFD, ASE, ADF, ASG, ASH, ASI, ASJ, ASK and ADL. Amphid neurons were initially named by using the letter from “A” to “L”; now they are referred to by three letters, the first being “A” for amphids. The second letter refers to the number of endings. For instance, most amphid neurons have a single sensory ending (ASE, ASG, ASH, ASI, ASJ, ASK), whereas others have dual (ADF, ADL) ciliated sensory endings exposed to the external environment. AWA, AWB and AWC have winged endings, while
AFD is the only one with 'finger-like' ciliated endings. Since in *C. elegans* there are 12 neurons in the amphidial complex, each neuron is assigned a third letter, from A to L, to uniquely identity each neuron.¹⁹¹,¹⁴⁰

By killing specific neurons with a laser microbeam and a subsequent phenotypic and behavioral analysis of animals in which defined neurons were killed, it was possible to define the specific role of the amphid neurons responsible for chemosensory and thermosensory behavior.¹⁴² In *C. elegans*, for instance, laser ablation of the AFD neurons makes most animals athermotactic, suggesting that this pair of neurons plays a role in thermotaxis behavior.¹⁴³ AFDs, the major thermosensory neurons, are involved in the discrimination of small differences of temperature (as small as 0.05 °C) and in the mechanism of memory of the previous culture temperature.¹⁴⁴-¹⁴⁶ It has been shown that, when *C. elegans* was moved to a thermal gradient after growing at a uniform temperature, it preferentially migrated and moved isothermally around this growth temperature.¹⁴⁴

Beside the temperature, other chemical stimuli influence the behaviour of nematodes. Chemotaxis represents a complex mechanism through which sensory information is translated into movement in a specific direction.⁸⁷ ASE neurons in *C. elegans* are fundamental for chemotaxis to water soluble attractants including Na⁺, Cl⁻, cAMP, biotin and lysine. After laser ablation of ASE neurons, ADF, ASG, ASI, ASK, and ASJ contribute to a weak residual response. This apparent functional redundancy among different chemosensory neurons may be a strategic way to increase the versatility and fidelity of the response.¹⁴⁷ The nervous system of most animals is largely bilateral symmetric, for instance, insects compare their left and right antenna in order to respond to chemical stimuli.¹⁴⁸ In *C. elegans* laterality in ASE neurons’ capacities has been reported, perhaps as a mechanism to enhance further the fidelity and robustness of the chemotaxis response.¹⁴⁸,¹⁴⁹

*C. elegans* can discriminate between water soluble chemicals, sensed by the above-mentioned neurons, and volatile cues, detected by AWA, AWB and AWC.⁸⁷,¹⁵⁰ AWA and AWC, rather than being directly exposed to the environment, have sensory cilia that are enclosed with the sheath cell. Odours can either diffuse through the cuticle or they can be transported to the cilia by the sensory endings through the sheath.¹⁴⁰ AWC neurons can sense at least five synthetic odorant (butanone, isoamyl
alcohol, 2,3-pentanedione, 2,4,5-trimethylthiazole and benzaldehyde), while AWA can sense at least three (diacetyl, pyrazine, and 2,4,5-trimethylthiazole). Like the ASE neurons, the left AWC and the right AWC neurons are not functionally redundant, and this functional asymmetry has been reported to be relevant for the detection of different molecules. An interesting question is how nematodes distinguish between attractive or repulsive odours. It has been shown that these neurons are mostly associated with a specific behavioural response; while AWA and AWC are preferentially linked to attraction, AWB is associated with repulsion.

Nematodes are able to avoid a variety of noxious stimuli, such as high osmolarity, heavy metals such as Cu$^{2+}$ and Cd$^{2+}$, bitter alkaloids such as quinine, acid pH, and some organic odours. The presence of chemical repellents causes an escape response, which leads to a rapid change of locomotion. This avoidance response is mediated by ASH neurons. The ASH neurons are also involved in responses that mediate withdrawal from light touch to the nose. By responding to both chemical and mechanical stimuli, ASHs play an important role as polymodal nociceptors, similar to the polymodal nociceptive neurons which mediate the sensation of pain in vertebrates. Studies in vivo have shed light on the nature of polymodal sensory responses. By expressing the calcium indicator protein cameleon in ASH, an increase in ASH Ca$^{2+}$ was observed upon stimulation with osmotic shock, chemical cues, and nose touch. Ca$^{2+}$ influxes in ASH enter through voltage-activated calcium channels, causing a rapid and direct depolarization. Avoidance responses mediated by ASH act through the transient receptor potential vanilloid (TRPV)-related channel proteins OSM-9 and OCR-2. This was confirmed by the finding that the heterologous expression of the mammalian transient receptor potential vanilloid receptor 1, TRPV1/VR1 (a non-specific, calcium-permeable ionotropic cation channel, which is activated by protons as well as capsaicin) in C. elegans led to an induction of a rapid “artificial” avoidance behavior after administration of the TRPV1 agonist capsaicin.

Nervous systems process complex sensory information in order to respond to environmental stimuli, influencing at the same time developmental decisions of nematodes, such as the regulation of the alternative dauer larva stage formation. The decision to enter into dauer and to recover from this larval arrest stage is regulated by...
sensory cells involved in the sensation of dauer pheromone or food, the two major inputs that drive this developmental process. Chemosensory neurons can promote or repress dauer formation. In unfavourable conditions, neurons which mediate dauer repression promote an active repression of the pathways that induce dauer formation. On the other hand, in favourable conditions, neurons which promote dauer formation are inactive. Ablation of dauer-repressing neurons results in a dauer formation–constitutive (Daf-c) phenotype, promoting dauer formation even under non-inducing conditions, whereas ablation of dauer-promoting neurons results in a dauer formation–defective (Daf-d) phenotype, a failure to form dauers under dauer-inducing conditions.

Defects in amphid morphology affect the ability of larvae to enter into, or exit from dauer stage. When the all of the amphid cells ADF, ASG, ASI and ASJ were killed by laser ablation in single animals at L1 stage, 81% (n = 37) of the larvae became dauer in the absence of dauer pheromone. The dauers never recovered when exposed to food, indicating that these amphid neurons inhibit dauer formation in intact animals and are involved in the mechanism of dauer larva recovery. However, when ADF, ASG and ASI were ablated (with ASJ intact), they went through dauer only transiently and afterwards recovered. Therefore, since ablation of ADF, ASI, and ASG results in a constitutive entry into the dauer stage, this suggests that these neurons can sense environmental cues (pheromone and food) that induce dauer formation while ASJ chemosensory neurons are involved in the mechanism of dauer recovery. In contrast to the dauer-inhibiting neurons ADF, ASI, and ASG, the amphid neurons ASK and ASJ promote dauer formation.

Different sensory cells regulate the mechanisms that induce dauer formation. The fact that amphid neurons act in parallel could refine the critical and crucial decision that animals have to undertake to pass to the dauer stage during their development. Failure in dauer formation under unfavourable situations can lead to death or starvation. At the same time, forming a dauer in favourable conditions would lead to an unnecessary process which requires time and energy, so it would imply a reproductive disadvantage. Therefore, this redundancy can increase and refine fidelity and robustness of the response, especially in face of fluctuating and unpredictable environmental situations. The presence of sensory cells with
overlapping functions can be critical for mediating and combining responses to different factors such as pheromones, temperature and food. Integration of multiple cues and factors can indeed lead to a much more sophisticated response. Remarkably, the sensory neuroanatomy between different nematode species is very similar, as comparative studies revealed.\textsuperscript{163} For instance, electron microscopy reconstruction of the amphids of the free-living nematode \textit{C. elegans} and several parasitic nematodes such as \textit{Haemonchus contortus} and \textit{Ancylostoma caninum} has shown a perfect correlation and a remarkable similarity of the amphid structure. Surprisingly, there is also a perfect conservation of the neuroanatomy of \textit{C. elegans} even with more distantly related nematode \textit{Strongyloides stercoralis}.

\textbf{Ascaroside receptors}

Chemoreceptor proteins, such as G protein-coupled receptors (GPCRs), guanylyl cyclases (GCs), and cyclic nucleotide-gated ion channels (CNGs), mediate the first step in the transduction of environmental chemical stimuli.\textsuperscript{87,118,164-166} In nematodes, G protein-coupled receptors (GPCRs) are encoded by a large family of genes.\textsuperscript{87,96} The \textit{C. elegans} genome encodes for 1,699 genes of which 1276 were described as intact genes and 423 as pseudogenes. Many of these genes are expressed only or principally in chemosensory neurons.\textsuperscript{167} Receptors from the G-protein-coupled receptor family have been reported to be required for dauer induction and ascaroside perception. Two G-protein alpha subunits, encoded by the genes \textit{gpa-2} and \textit{gpa-3}, are involved in signal transduction in response to specific ascarosides. Overexpression of these genes promotes dauer formation, while \textit{gpa-2} and \textit{gpa-3} mutants show a dauer formation–defective (\textit{Daf-d}) phenotype.\textsuperscript{168}

A genetic screen was used to identify the first dauer pheromone GPCRs, encoded by the genes \textit{srbc-64} and \textit{srbc-66}. These GPCRs localize to the sensory cilia of ASK neurons and mediate responses to ascarosides via the two G-protein alpha subunits, GPA-2 and GPA-3.\textsuperscript{96,123} Loss-of-function mutations of \textit{srbc-64} and \textit{srbc-66} (single and double mutants) in worms resulted in defects to form dauer and respond to synthetic ascosides ascr\#1, ascr\#2, and ascr\#3, and to less extent to ascr\#5. However, high concentrations of ascr\#1, ascr\#2, and ascr\#3 restored the ability to induce dauer formation in \textit{srbc-64} and \textit{srbc-66} single and double mutants, giving weight to the theory that the complexity and versatility of ascaroside signaling is
associated to a large and diverse ascaroside receptor repertoire. In order to confirm this, it has been reported that genetic ablation of ASK neurons (by heterologous mouse caspase expression under an ASK-specific promotor) results in defects at inducing dauer formation mediated by ascr#2, and these defects were stronger than those observed for srbc-64, srbc-66 double mutants, suggesting that additional GPCRs are present in the ASK chemosensory neurons, mediating perception of the ascarosides. The expression of srbc-64 and srbc-66 has not been found in ASI neurons,\textsuperscript{123} although these chemosensory neurons play a major role in dauer formation.\textsuperscript{161} ASI neurons produce a transforming growth factor (TGF-beta) related peptide, encoded by the gene daf-7, which acts as neuroendocrine signal to repress dauer formation.\textsuperscript{161,169} Mutation of daf-7 results in dauer formation under non-inducing conditions. In wild type animals, dauer-inducing pheromone inhibits daf-7 expression, promoting dauer formation. After restoring favorable conditions, in low population density and availability of food, reactivation of daf-7 expression mediates recovery from the dauer state.\textsuperscript{87,169} It has been suggested that an unknown signaling pathway allows a communication between ASK and ASI neurons, in order to mediate the critical decision to enter into dauer.\textsuperscript{123} ASI neurons may integrate different environmental factors such as pheromone, food, and temperature, in order to regulate a sophisticated and complex decision between two alternative developmental trajectories, one leading to arrest in the stress-resistant dauer larva stage and the other to rapid reproduction.

After the identification of first dauer pheromone GPCRs in ASK chemosensory neurons, subsequent studies showed evidence of the presence of additional chemoreceptors in ASIs which mediate ascaroside signaling. These studies began with the observation that two laboratory strains of \textit{C. elegans}, LSJ2 and CC1, held in liquid axenic media culture for almost 50 years and 4 years, respectively, acquired multigenic resistance to pheromone-induced dauer formation obtained from wild-type worms. In both strains, active components of dauer pheromone such as ascr#2, ascr#3, ascr#5, and icas#9 did not induce dauer formation.\textsuperscript{96,170} Generation of recombinant inbred lines (RILs) from these resistant mutants allowed the identification of two GPCR genes, \textit{serpentine receptor class g (srg)}-36 and -37. These receptors are expressed in the ASI neurons and have as ligand the above
mentioned ascarosides. Similarly, in the related nematode *Caenorhabditis briggsae*, partial deletion of a gene paralogous to the *C. elegans srg-36* and *srg-37* conferred resistance to the dauer pheromone in high density cultures.¹⁷⁰

Other G-protein–coupled receptors, encoded by *daf-37* and *daf-38* act cooperatively to mediate ascaroside perception.⁹⁶,¹⁷¹ The receptor DAF-37 is involved in the perception of ascr#2, cooperatively with DAF-38, which can perceive ascr#2 and other ascarosides. By targeting the same GPCR (DAF-37) in two different neurons, ascr#2 can induce different phenotypes. ascr#2 can play a role at inducing dauer by binding to the DAF-37 receptor in ASI neurons or, by targeting the same receptor in the ASK neurons, can mediate hermaphrodite repulsion. Moreover, the identification of these receptors, in parallel with photo-affinity-labeling studies, lead to the first evidence of a specific and direct binding between an ascaroside and its receptor, ascr#2 and DAF-37.

These studies show that ascaroside signaling is a highly sophisticated and complex system, in which several components act in synergy, and in which neurons express a large and diverse receptor repertoire with redundant and overlapping functions, with at least three different families of GPCR being involved.⁹⁶

### Laser ablation

The function of specific chemosensory neurons that mediate responses to different chemicals can be studied by eliminating them through laser (light amplification by stimulated emission of radiation) ablation.¹⁴²,¹⁷² This approach had a great impact in the field of developmental biology, since by removing one single cell it is possible to investigate the development of other cells and study signaling and inductive interactions between cells.¹⁷³ Laser ablation of a single cell is possible by three dimensional focusing of a laser beam on a particular target. Damage to the cell and adjacent structures can be visualized through the microscope during and after the operation.¹⁷³

A clear and unambiguous identification of cells is a crucial and challenging step for any experiment involving laser ablation. In *C. elegans*, neuronal nuclei for instance can be identified usually from morphological features, such as a small round shape,
the absence of prominent nucleoli, and the presence of a punctate nucleoplasm. Moreover, amphid neurons (ASI, ADL, ASK, AWB, ASH, and ASJ) and the phasmid neurons (PHA and PHB) can be stained with lipophilic fluorescent dyes such as 5-isothiocyanato-fluorescein (FITC), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO). The presence of fluorescence in cells is a convenient way to learn the position of particular cells in animals.

For the laser ablation, pulsed lasers are commonly used, and these can be distinguished based on several parameters: the center wavelength, the duration of a pulse, the peak power, the average power, repetition rate, and the energy per pulse. These factors establish the extent of the tissue damage caused by focused, pulsed laser beams. According to the pulse duration, it is possible to place lasers in two categories:

- Nanosecond lasers:

The first lasers used to kill C. elegans cells and most common nowadays for cell killing, these nitrogen or diode lasers produce violet/blue light with pulse durations in the nanosecond range and pulse energies up to several mega Joules.

- Femtosecond lasers:

Titanium-sapphire lasers produce near-infrared pulses, durations in the range of ~100–200 femtoseconds and pulse energies up to 50 nano Joules.

Damage induced by both nanosecond and femtosecond lasers is caused by a process known as optical breakdown or laser-induced plasma formation. At the focus of the laser beam, electrons in the material are accelerated by the electric field, which can cause liberation of the electrons. This generates a subsequent production of localized plasma that can break chemical bonds and potentially vaporize water and other tissue components, with a further thermal and mechanical disruption of the tissue.

After laser killing, detailed analysis which includes a simultaneous observation of Nomarski images and fluorescent images of these cells, is a critical step in verifying the damage. Some features, such as disappearance or change of refractive index in the nucleus, a ruptured basement membrane and morphology change of the cell, can
be sufficient to confirm that damage has occurred. If a fluorescent marker or dye is used, a substantial photobleaching/disruption is typically observed. However, loss of fluorescence is not always an indication of cell killing, since this can be merely a consequence of photobleaching.

**Chemosensation in *A. freiburgensis***

In this chapter, an investigation of the role of amphid neurons in the nematode *A. freiburgensis*, involved in the transmission of environmental information, is presented. The overall aim of this chapter is to shed light on the mechanisms which regulate chemosensation in *A. freiburgensis*, to determine how hermaphrodite mothers sense the environment and how this information can reprogram the germline, influencing sex determination in the next generation. As initial hypothesis, it is assumed that ascarosides present in the supernatant trigger at least one of the types of amphid neurons to secrete a signal that is relayed to the germline. This signal would induce changes in the germline of *A. freiburgensis* to specify dauer formation in the following generation.

In this chapter, the anatomic structure of chemosensory neurons in *A. freiburgensis* was investigated. Since the anatomy is invariant between individuals of the same nematode species and the number of neurons is essentially conserved between many nematode species,\(^{163}\) it was possible to proceed to the identification of amphid neurons in the nematode *A. freiburgensis*. The subsequent identification of chemosensory neurons provided a basis for laser microbeam ablation studies, to determine whether amphids mediate ascaroside sensing and to identify the specific neuron(s) mediating the cross-generational effect.

**Materials and Methods**

**DiI staining**

The red fluorescent, lipophilic, cationic indocarbocyanine dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Molecular Probes), was used to identify the amphid neurons. A stock dye solution containing 2 mg/ml (2 mM) DiI in dimethyl formamide was stored at -20 °C in a tube wrapped in aluminum foil to
Chapter 2 – Neuron signaling in Auanema freiburgensis.

protect it from light. Hermaphrodites at L4 stage were washed from plates using M9\textsuperscript{132} buffer and collected by centrifugation (3 minutes at 3,500 rpm). A washing step was carried out by suspending the worms in 1 mL M9 and sedimenting them again by centrifugation. Afterwards, worms were resuspended in 1 mL of M9 containing 8 µl DiI stock solution (1:125 final dilution) and incubated on a slow shaker at 20 °C for at least 3 hours in the dark. After the incubation, worms were washed twice with M9 buffer (see above) to remove residual dye. Worms were then moved to a fresh NGM media\textsuperscript{132} plate and were left to crawl on the bacterial lawn for at least 1 hour to allow clearance of ingested dye from their digestive tracts.

The identification and ablation of amphids

A drop of melted agarose was placed on a slide and flattened into a pad. Two slides containing spacers were used as guides for flattening the agarose, so the thickness of the agarose pad was equal to that of the spacer layer. A drop of M9 buffer containing 0.1 M sodium azide (an inhibitor of mitochondrial respiration, used here as anesthetic during imaging and surgery) was placed on top of the agarose pad (note that the latter served as soft support to later close the microscope slide without harming the worm). Worms were picked and placed in the drop of anesthetic solution (M9 with 0.1 M sodium azide), and a coverslip was placed on the slide. Care was taken to avoid creating bubbles next to the worms, as air-water interfaces can interfere with imaging and laser surgery.

Amphid neurons were identified according to the position of the cells viewed under Nomarski optics (Zeiss Axio Observer.Z1), using the appropriate filters (DiI gives red fluorescence). For ablation, a nanosecond diode laser-pumped pulsed dye laser (Micropoint laser ablation system, Andor) was used. According to the supplier, this laser unit generates a laser beam with peak wavelength of 435 nm, pulse energy up to 50 µJ, peak power 12 kW (average power 750 µW), peak duration 3-5 ns, pulse repetition rate 0-15 Hz.

To rescue worms after ablation, the coverslip was removed from the slide very gently and 200 µl of M9 were used to move the worm to a seeded (\textit{Escherichia coli} OP50-1) NGM\textsuperscript{132} plate (25 µg/mL nystatin, 50 µg/mL streptomycin). Since worms were
very dehydrated at this point, they were allowed to recover for 2 or 3 hours before proceeding with subsequent biological assays.

For the biological assay, each ablated L4 hermaphrodite was moved to a 6 cm plate containing freeze-dried supernatant powder (50 mg; supernatant 3w9M – 2nd batch) dissolved in 200 µL of an overnight culture of *E. coli* OP50-1 in LB medium\(^{176}\) supplemented with 50 µg/mL streptomycin. Non-hatched eggs that were laid during the night were collected and were washed with 200-300 µl of M9 buffer. Each egg was moved to a single well of a 96-well microtiter plate (100 µL NGM media per well). Sex of the F1 offspring was identified as follows: for isolated worms, the occurrence of F2 offspring indicated hermaphrodites, its absence females or males, and the latter were distinguished by the specific tail morphology of males. The procedure for mock ablations was identical (sodium azide treatment, fluorescent illumination, time in slide), except that no neuron was ablated.

**Results**

**Identification of amphid neurons**

Identification of the specific neurons mediating the cross-generational effect is essential in order to understand how environmental cues are detected by hermaphrodite mothers and how this information can reprogram the germline, influencing sex determination of the progeny. As a first step forward this goal, a comparative approach was used to identify a potential correlation in the structure of amphid sensilla between *A. freiburgensis* and *C. elegans*, in which these neurons have been well-characterized.\(^{141,174,177,178}\)

Through this approach it has been demonstrated that the chemosensory organ in *A. freiburgensis*, similar to other nematodes,\(^{163}\) consists of a collection of amphid neurons whose cell bodies are situated anterior to the pharyngeal bulb and have axons that join with the nerve ring (Figures 2-1 and 2-2). The neuronal dendrites reach the anterior end of the animal.

While in *C. elegans* the dye can enter and fill 6 types of amphids (ASI, ASK, ADL, AWB, ASH and ASJ) and all amphids show relatively strong dye uptake,\(^{163}\) in *A.
freiburgensis only three amphid neurons could be visualized. Based on their relative position and shape, these stained neurons were identified by comparison with *C. elegans* and other nematodes as ADL, ASK and ASH, while neurons AWB, ASI and ASJ were stained only very weakly compared with *C. elegans*.

**Figure 2-1.** Identification of amphid neurons ASH, ASK and ADL.
Shown is an overlay of brightfield and fluorescence image (DiI stained neurons; red fluorescence filter) on the left, and only the fluorescence channel on the right. The locations of the neurons ASH, ASK and ADL are indicated in the left figure by arrows.

**Figure 2-2.** Localization of amphid neurons ASH, ASK and ADL.
Amphids were stained with DiI and DIC images are overlaid with DiI fluorescence images (red fluorescence filter). The left figure shows fluorescent ASH, and the right figure shows ASK (upper fluorescent spot) and ADL (lower).
Although the mechanism of dye uptake is not known, most likely the exposed ciliated endings play a direct role in dye uptake.\textsuperscript{179} It has been suggested that while the differential dye uptake between different species doesn’t constitute a sufficient criterion to establish a relation of functional equivalency between neurons, it can, however, reflect the conserved characteristics of chemosensory neurons presenting dendritic extensions which are exposed to the environment.\textsuperscript{163}

Beyond these discrepancies and differences due to a differential dye uptake between the amphid neurons in \textit{C. elegans} and \textit{A. freiburgensis}, this comparative study also showed some conservation of neuroanatomy between the two species. In particular, an important feature of the neuronal arrangement in \textit{C. elegans} is the presence of three neurons on both the right and the left side (ASI, ADL, and ASK) which form one dorsal triplet on each side. The dorsal triplet amphid neurons ASI, ADL, and ASK in \textit{A. freiburgensis} showed the same feature and were found in an equivalent position.

**Ablation of neurons ASH, ASK, ADL in combinations**

Identification of the amphid cell bodies’ localisations in \textit{A. freiburgensis} provided a basis for laser microbeam ablation studies to test whether amphid neurons mediate ascaroside sensing and to determine the specific neuron(s) mediating the cross-generational effect. Since the amphids are bilaterally symmetrical in terms of localisation, morphology and DiI uptake (fluorescence staining), in this study (if not differently stated) the corresponding neurons in both amphids were killed.

Laser ablation of a single neuronal cell was performed by three dimensional focusing of the laser beam on the particular target. Damage induced by the laser to the specific neuronal cell was visualised through the microscope during and after the operation. To confirm that a damage had occurred, features such as difference in the morphology of the cell before and after ablation and the change of refractive index in the nucleus were taken in consideration. Loss of fluorescence of the ablated cell was not considered sufficient to assume that a damage was induced, since this can be merely due to a photobleaching mechanism.
Chapter 2 – Neuron signaling in Auanema freiburgensis.

Figure 2-3. Effect of supernatant after ablation of amphid neurons (combination or individual).

In a first experiment (A), amphids ASH, ASK and ADL were ablated on either both (left of figure) or only one lateral location (right of figure). Thereafter (B), individual neuron pairs were ablated as indicated in the figure. For all experiments, both the control and ablated worms were tested with *A. freiburgensis* supernatant. Since not all ablations were done in parallel, each ablation experiment received its own parallel control samples and is hence shown in a separate panel. The fraction of hermaphrodite progeny was subjected to statistical analysis: An Unpaired Welch T-test for the ablated worms against the respective control was used and the p-thresholds are indicated in the plot (**** p < 0.01, ** p < 0.05, * p < 0.1, no indicator if p > 0.1). Numbers between hyphens identify the total number of progeny counted per condition.
In this study, priority was given to ablation microsurgery of all three pairs of neurons (ASH, ASK, ADL) that can be easily stained by fluorescent dye applied in the medium. Since exposed sensory cilia mediate chemosensation, the above mentioned neurons were ablated in hermaphrodites at L4 larval stage. Afterwards, each ablated L4 hermaphrodite was exposed to supernatant (see 3 weeks supernatant preparation in Chapter 1 of this thesis), in order to test its ability to sense this environmental cue in the absence of those neurons. Note that the supernatant exposure of non-ablated hermaphrodite mothers increases the fraction of hermaphrodites in their F1 progeny (e.g. see Figure 1-5 in Chapter 1 of this thesis).

Table 2-1. Progeny counts from neuron ablation experiments.
The data shown here is used in Figure 2-3.

<table>
<thead>
<tr>
<th># number (% of progeny)</th>
<th>Female</th>
<th>Hermaphrodite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL – for experiment ablation ASH + ASK + ADL both sides</strong> (Figure 2-3, A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>6 (12 %)</td>
<td>45 (88 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>16 (22 %)</td>
<td>56 (78 %)</td>
</tr>
<tr>
<td><strong>ABLATION – for experiment ablation ASH + ASK + ADL both sides</strong> (Figure 2-3, A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>55 (95 %)</td>
<td>3 (5 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>60 (83 %)</td>
<td>12 (17 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>64 (70 %)</td>
<td>28 (30 %)</td>
</tr>
<tr>
<td><strong>CONTROL – for experiment ablation ASH + ASK + ADL one side</strong> (Figure 2-3, A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>6 (12 %)</td>
<td>45 (88 %)</td>
</tr>
<tr>
<td>Control 2</td>
<td>16 (22 %)</td>
<td>56 (78 %)</td>
</tr>
<tr>
<td>Control 3</td>
<td>7 (8 %)</td>
<td>86 (92 %)</td>
</tr>
<tr>
<td><strong>ABLATION – for experiment ablation ASH + ASK + ADL one side</strong> (Figure 2-3, A)</td>
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<td></td>
</tr>
<tr>
<td>Test 1</td>
<td>64 (97 %)</td>
<td>2 (3 %)</td>
</tr>
<tr>
<td>Test 2</td>
<td>33 (80 %)</td>
<td>8 (20 %)</td>
</tr>
<tr>
<td>Test 3</td>
<td>62 (87 %)</td>
<td>9 (13 %)</td>
</tr>
<tr>
<td>Test 4</td>
<td>34 (48 %)</td>
<td>37 (52 %)</td>
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</table>
## Chapter 2 – Neuron signaling in Auanema freiburgensis.

<table>
<thead>
<tr>
<th>Test</th>
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<th>ABLATION – for experiment ablation ASH (Figure 2-3, B)</th>
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<tr>
<td>5</td>
<td>25 (40 %)</td>
<td>38 (60 %)</td>
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<tr>
<td>6</td>
<td>13 (24 %)</td>
<td>42 (76 %)</td>
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<td>7</td>
<td>11 (14 %)</td>
<td>67 (86 %)</td>
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<td><strong>ABLATION – for experiment ablation ASH (Figure 2-3, B)</strong></td>
</tr>
<tr>
<td></td>
<td>Control 1</td>
<td>Test 1</td>
</tr>
<tr>
<td></td>
<td>6 (12 %)</td>
<td>13 (93 %)</td>
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<tr>
<td></td>
<td>16 (22 %)</td>
<td>79 (89 %)</td>
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<td>7 (8 %)</td>
<td>116 (91 %)</td>
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<td>7 (5 %)</td>
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<td>8 (7 %)</td>
<td>24 (27 %)</td>
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<td>5 (6 %)</td>
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<td>5 (7 %)</td>
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<td>74 (82 %)</td>
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<td><strong>ABLATION – for experiment ablation ASK (Figure 2-3, B)</strong></td>
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<td></td>
<td>Control 1</td>
<td>Test 1</td>
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<td>8 (7 %)</td>
<td>46 (46 %)</td>
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<tr>
<td></td>
<td>5 (6 %)</td>
<td>4 (3 %)</td>
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<tr>
<td></td>
<td></td>
<td>52 (73 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>CONTROL – for experiment ablation ADL (Figure 2-3, B)</strong></td>
<td><strong>ABLATION – for experiment ablation ADL (Figure 2-3, B)</strong></td>
</tr>
<tr>
<td></td>
<td>Control 1</td>
<td>Test 1</td>
</tr>
<tr>
<td></td>
<td>5 (6 %)</td>
<td>8 (13 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53 (87 %)</td>
</tr>
</tbody>
</table>
Chapter 2 – Neuron signaling in Auanema freiburgensis.

Laser-ablated animals (all three neuron pairs ASH, ASK, ADL) produced a much lower fraction of hermaphrodite offspring (N = 3 animals tested, 17 % ± 13 hermaphrodite offspring, total progeny = 222) in the presence of supernatant than the non-ablated control (N = 2 controls, 83 % ± 7 hermaphrodite, total progeny = 123, p < 0.01, see Figure 2-3 (A) and Table 2-1). Having shown that the ablation of ASK, ASH and ADL neuron pairs removes the supernatant effect, it was tested whether ablation of only the neurons on one side (right or left) would be sufficient to produce the same result. Total brood of ablated hermaphrodite parents (N = 7) resulted in less hermaphrodite than female progeny (44 % ± 33 hermaphrodite, total offspring = 445, see Figure 2-3 (A) and Table 2-1), which is a significant decrease of hermaphrodite offspring when compared with the control (N = 3 control animals, 86 % ± 8 hermaphrodite, total offspring = 216, p < 0.05). However, while ablation of ASK, ASH and ADL sensory neurons on both the left and right lateral locations almost completely reverted the effect of the supernatant (see Figure 2-3 (A), left), the ablation of only one neuronal cell for each pair (i.e. only one side ablation, Figure 2-3 (A), right) had a less strong effect.

To gain insight into whether only one specific neuron type mediates the ascaroside signaling, or if the three pairs of neuronal cells work in synergy to mediate the decision to induce dauer formation in the following generation when hermaphrodite mothers are exposed to crowding cues, each pair of chemosensory neurons was ablated individually. Ablation of ASH neurons in mother hermaphrodites (N = 8) subsequently exposed to supernatant prevented them from producing dauers, resulting in a higher percentage of female progeny (82 % ± 23 female, total offspring 664) compared to the control (N = 6 control animals, 10 % ± 6 female, total offspring 553, p < 0.01, Figure 2-3 (B) and Table 2-1). Laser ablation of ASK chemosensory neurons in hermaphrodite mothers (N = 3) resulted in a higher average percentage of female progeny (41% ± 35 female, total offspring = 290) compared to the control (N = 2 control animals, 6% ± 1 female, total offspring = 209), though this difference was not significant (p > 0.1, Figure 2-3 (B) and Table 2-1). Still, this might suggest that ASK may be also involved in chemosensation and ascaroside sensing, although to a lesser extent than ASH neurons. Finally, laser ablation of ADL chemosensory neurons, which also took up the fluorescent dye, was performed in one hermaphrodite mother, followed by exposure to supernatant crowding cues. After
ablation of these neurons in the parental generation, the ability to sense the environment and to respond to ascaroside signaling appeared intact. Under this condition, the mother hermaphrodite (N = 1) produced mostly hermaphrodite progeny (87 % hermaphrodite, total offspring = 61), as did the control (N = 1 control animal, 94 % hermaphrodite, total offspring = 89, see Figure 2-3 and Table 2-1). Note that only the result from one ADL-ablated worm is shown. Since ablation generally lead to a decreased survival of the worms, and this ADL result did not indicate a reaction of ADL to the supernatant (i.e. no effect after ablation), this experiment was not pursued further.

Conclusion

The idea that genetic information flows from germline to soma, but not vice-versa, has been the dogma in Biology ever since its initial report in 1889. However, recent evidence shows that environmental signals received by somatic tissues can affect the phenotype of the following generation. Consistent with this, the findings with *A. freiburgensis* presented in this thesis show that the soma somehow instructs the germline: in fact, environmental signals received by the soma (neurons) are relayed to the germline, influencing development behavior and sex of the progeny. Mother hermaphrodites produce female progeny in favourable conditions, while the presence of chemical cues in the environment produced by conspecific animals under crowded conditions (here applied as culture supernatant extract), induces hermaphrodite progeny production. It is reasonable to assume that these chemical cues are ascaroside pheromones, based on the examination presented in Chapter 1 of this thesis, and signal transmission could for example involve a neuroendocrine signaling which induces reprogramming of oocytes.

Neuron ablation studies in *A. freiburgensis* showed that ASH sensory neurons mediate sensing of crowding cues, and are involved in cross-generational plasticity by directing a binary developmental decision. ASH neurons have not previously been implicated in dauer formation. In *C. elegans* they represent the main nociceptor, mediating avoidance responses from noxious stimuli. However, consistent with the role of ASH in *A. freiburgensis*, a recent study reported new functions for nociceptive neurons in mediating pheromone-induced dauer formation in *C.
Chapter 2 – Neuron signaling in Auanema freiburgensis.

Through an unbiased forward genetic screen and a subsequent identification of \textit{phd} (pheromone response-defective dauer) mutants, a previously uncharacterized role for the gene \textit{qui-1}, which encodes a WD40 domain-containing protein, was found. \textit{qui-1}, expressed in ASH nociceptive chemosensory neurons, was originally identified in a genetic screen for mutants in which the avoidance response was defective. An additional role for \textit{qui-1} in dauer formation was identified, since a null function mutation in \textit{qui-1} resulted in the \textit{phd}-mutant phenotype. Wild-type \textit{qui-1} expression in ASH nociceptive neurons partially rescued the dauer formation. Therefore, pheromone-induced dauer formation is strictly related to environmental noxious chemicals, and \textit{qui-1}-dependent activity of the ASH nociceptive neurons plays a critical role in driving this critical binary developmental decision.

However, more studies need to be done in order to determine the specific receptor expressed by ASH sensory cilia in \textit{A. freiburgensis} mediating ascaroside signaling. Most likely, in order to increase and refine fidelity and robustness of the response, individual neurons express multiple chemoreceptors which respond to ascarosides regulating the mechanism that induces dauer formation. Dauer formation is a crucial decision, which requires time and energy investment, hence the complexity of ascaroside signalling most likely reflects a large and diverse ascaroside receptor repertoire, as known from other ascaroside sensing pathways. Since integration of multiple cues and factors can indeed lead to a much more sophisticated response, the presence of functionally redundant sensory neurons can be crucial to elicit a response which can combine different factors influencing dauer formation, such as different ascarosides, temperature and food.

To determine more rigorously whether ASH neurons cooperate with other neurons in order to respond to ascaroside, the effect of ablation of ASK chemosensory neurons in mother hermaphrodites subsequently exposed to supernatant was tested. In \textit{A. freiburgensis} it is shown here that ASK chemosensory neurons are also involved in receiving the crowding cues from supernatants. Ablation of these neurons in hermaphrodite mothers, and the subsequent exposure to supernatant, resulted in a higher number of females than hermaphrodite progeny. ASK neurons are known to promote dauer formation in \textit{C. elegans}, where ablation of these chemosensory neurons lead to a dauer formation–defective \textit{(Daf-d)} phenotype, which implies a failure to form dauers under dauer-inducing conditions. The response to
ascarosides ascr#1, ascr#2, and ascr#3 is mediated by two dauer pheromone GPCRs, encoded by the genes *srbc-64* and *srbc-66*, expressed on the sensory cilia of ASK neurons.\(^{96,123}\) Since in *A. freiburgensis* ascaroside ascr#3 can act as an inducer for an increased fraction of hermaphrodite progeny (see results in Chapter 1 of this thesis), it could be hypothesised that *A. freiburgensis* expresses the same (or similar) receptors which are involved in the detection of ascr#3 in *C. elegans*. This hypothesis could be supported by the fact that the sensory neuroanatomy between these different species is similar, based on the presented morphological similarities and the fact that this is a common pattern even between distantly related nematodes.\(^{163}\)

Interestingly, this study shows that while the ablation of ASH and ASK sensory neurons at simultaneously the left and the right lateral location almost reverted the effect of supernatant, the ablation of one neuronal cell per pair (i.e. only one side) had a notably weaker effect. This could indicate a functionally bilateral symmetry of those neurons, suggesting that the same receptors with redundant and overlapping functions could be expressed in the right and left neuron of each pair. However, it would also be possible that there is a bilateral asymmetry, and e.g. different receptors are utilised on the respective sides. The observed different strength of the supernatant effect after half-sided ablation could then, for instance, arise if the bilateral signal would be integrated, hence resulting in a levelling depending on which side was ablated (i.e. the one generating the major signal or the one generating a weaker, refining signal).

Another important question to address is strictly related to the immediate effects observed regarding chemosensation of environmental cues by the nematode *A. freiburgensis* which determines the developmental trajectory and sex of the F1 generation. For instance, after ablation of all three neuron pairs ASH, ASK and ADL, as well as after ablation of only the neuron pair ASH, only a small fraction of progeny became hermaphrodite upon exposure to supernatant (17 % ± 13), while the non-ablated controls’ progeny was in clear majority hermaphrodite (83 % ± 7). This direct comparison shows that the supernatant acted immediately on the sex of the progeny produced upon the exposure to the supernatant. Since the chemosensory neurons do not connect directly to the germline, the supernatant signal must be relayed to the germline by messenger molecules. Considering that the supernatant...
signal is sensed by neurons, it would be a possibility that the relay could be mediated by common peptidergic (i.e. neuropeptides) or monoaminergic (i.e. serotonin, tyramine, octopamine etc.) signaling pathways, which are for instance known to be modulators of ASH behaviours as aversive responses. Also, small RNAs, of which some have been shown to circulate in *C. elegans*, could act as messenger.

In order to understand the actual pathway in the neuronal signal-perception by *A. freiburgensis* in response to the supernatant crowding-signals, further analyses of the specific receptor proteins and cellular responses are required. As part of this, an analysis of small RNA signaling in response to supernatant was attempted in Chapter 3 of this thesis, to evaluate the potential involvement of epigenetics in the intergenerational effect resulting from supernatant exposure.
Chapter 3 – Molecular mechanism of ascaroside-mediated signal transmission.

‘‘Who controls the past controls the future’’ (George Orwell, 1984)

Introduction

Endogenous small RNA pathways in nematodes

In nematodes, endogenous small RNA silencing pathways can be divided into three main categories: microRNAs, endogenous small interfering RNAs, and PIWI-interacting RNAs (see scheme in Figure 3-1).186 In general, small, non-coding, single-stranded RNAs exert their functions by binding to an Argonaute protein; this complex can modulate gene expression via mRNA degradation, mRNA sequestration, epigenetic changes, or by inhibiting translation.187 Throughout the Metazoa these pathways are conserved, and related pathways have been identified also in bacteria, plants and fungi.188

![Figure 3-1. Schematic representation of the production and action of small RNAs. Small RNAs are transcribed from the chromosome, and three classes of small RNAs (micro, si, pi/PIWI) are distinguished based on their length, the proteins they bind to, and the exact cellular response they elicit. By binding to corresponding mRNAs, they either directly affect this mRNA (e.g. induce degradation and inhibit translation) or have downstream effects (e.g. expression of additional small RNAs 22G), eventually causing effects on the mRNA and chromatin level. For further details see associated text and reference.186]
In nematodes, knowledge about endogenous RNA silencing pathways has emerged mostly from studies conducted in the model system C. elegans. In C. elegans 25 different Argonaute proteins have been identified. Several Argonaute proteins play a role in a specific small RNA pathway, by representing essential components of the RNA-induced silencing complex (RISC) and effectors of the gene silencing phenomenon known as RNA interference (RNAi).\textsuperscript{186} However, the exact function of many Argonautes remains unknown.

**microRNAs:**

The identification of the gene lin-4 coding for a small RNA, negatively regulating the level of the LIN-14 protein, led to the discovery of the first microRNA (miRNA) in C. elegans. These small RNAs decrease the amount of LIN-14 protein, without however decreasing the level of the corresponding mRNA. lin-4 determines lin-14 translation regulation via an antisense RNA-RNA interaction.\textsuperscript{189} The identification of a second miRNA in C. elegans, let-7, which is conserved amongst a wide variety of animal species including molluscs, vertebrates, hemichordates, ascidians, arthropods and annelids, confirmed that endogenous small RNAs can trigger transcript regulation through an antisense mechanism.\textsuperscript{190} The number of known small RNAs has since increased considerably,\textsuperscript{191} and it is now clear that miRNAs play an important role for increasing the robustness of physiological pathways during development and adulthood. C. elegans double mutants of both miRNA-specific Argonautes ALG-1 and ALG-2 showed defective development and increased lethality.\textsuperscript{186,192}

Although the functions of hundreds of small RNAs have been investigated, the mechanisms regulating their biogenesis remains unclear. Micro-RNAs, single-stranded RNAs ~22 nucleotides in length (ranging 19–25 nt), are transcribed by RNA polymerase II as long primary transcripts (pri-miRNAs), which can be several kilobases long and present local stem-loop structures.\textsuperscript{193} Maturation occurs via a cleavage which releases a small hairpin (~65 nt) that is termed precursor-miRNAs (pre-miRNA). This reaction takes place in the nucleus and is carried out by the nuclear RNase III enzyme Drosha.\textsuperscript{194} Following export from the nucleus, the RNase III enzyme Dicer is responsible for cleaving the pre-miRNA near the terminal loop, thereby releasing miRNA duplexes (~22 nt).\textsuperscript{186,191} After Dicer cleavage, the duplexes
are loaded onto one of the miRNA-specific Argonaute proteins ALG-1 or ALG-2. One strand of the duplex forms a complex with ALG-1 or ALG-2, knowns as miRNA-induced silencing complex (miRISC), while the other strand is degraded.\textsuperscript{186,191}

An important feature of the single stranded miRNA is a sequence called “seed region” (approximately six to eight nucleotides near the 5’ end) which is required for the targeting of mRNAs and allows the complementary binding between the miRNA and the mRNA, influencing gene expression \textit{via} translation inhibition, or mRNA degradation.\textsuperscript{186,195}

\textbf{endo-siRNAs:}

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, represents a family of RNA molecules acting within the RNA interference (RNAi) pathway.\textsuperscript{196} Endo-siRNAs, rather than only influencing gene expression through degradation of mRNA and by blocking the translation inhibition, play an important role in the shaping of the chromatin conformation of a genome.\textsuperscript{186,197} Although the biogenesis mechanism is not entirely understood, it is known that endo-siRNAs, rather than being independently transcribed, derive from spliced mRNA transcripts.\textsuperscript{198}

siRNAs have been classified into two categories, according to their length and the specific Argonaute (AGO) proteins with which they interact. The length of siRNAs distinguishes 26G RNAs and 22G RNAs.\textsuperscript{186,196} 26G RNAs are 26 bases in length and have a 5’ guanine. The Dicer enzyme and the RNA-dependent RNA polymerase (RdRP) RRF-3 catalyze production of siRNAs from long endogenous dsRNAs or small hairpin RNAs.\textsuperscript{191} 26G RNAs can act in oocytes and embryos, by binding to an Argonaute protein encoded by \textit{ergo-1}, or they can be required for normal sperm development, by acting in a complex with the Argonaute proteins encoded by \textit{alg-3} and \textit{alg-4}. Once the 26G RNAs are bound to Argonautes, this complex interacts with perfect complementarity to target transcripts, inducing mRNA cleavage. The complex of 26G RNA with Argonaute and the mRNA triggers vigorous production of secondary siRNAs (22G RNAs), which is achieved by the action of RdRP polymerase RRF-1.\textsuperscript{186,196}
Chapter 3 – Molecular mechanism of ascaroside-mediated signal transmission.

22G RNAs are 22 nucleotides long with a 5’ guanine and they can also be divided into two subclasses according to Argonaute (AGO) proteins with which they interact.\textsuperscript{199} WAGO (Worm ArGOnaute protein)-1 bound 22G RNAs are mostly involved in silencing transposons, pseudogenes, and cryptic loci as well as certain genes, while the CSR (Chromosome-Segregation and RNAi deficient)-1 bound 22G RNAs act in order to promote chromosome segregation.\textsuperscript{186,199} Similar to 26G RNAs, 22G RNAs typically work by cleaving the mRNA before translation, and have 100 % complementarity, hence very tight target specificity.\textsuperscript{186}

piRNAs:

PIWI-interacting RNAs (piRNA) represent a highly complex family of small RNAs. By interacting with PIWI proteins, these RNA-protein complexes play an important role in genome stability and integrity in the germline of animals by silencing of transposons.\textsuperscript{200} Mutants that completely lack piRNAs in \textit{C. elegans} show a wide variety of germ line defects.\textsuperscript{39,186}

\textit{C. elegans} piRNAs are also named 21U RNAs since they are constituted of 21 nucleotides and present a uridine at their 5’ end. RNA polymerase II transcribes ~26 nucleotide small RNAs carrying a 5’ 7-methylguanylate cap (csRNAs), which are precursors of piRNAs.\textsuperscript{186} Transcription of these precursors occurs from two large clusters located on chromosome IV and from the promoter region of protein coding genes. Once piRNAs are associated with PIWI Argonaute (PRG-1), they undergo a maturation process which includes decapping, removal of two nucleotides from the 5’ end, trimming at the 3’end and a methylation with a 2’-O-methyl group.\textsuperscript{201}

piRNAs recognize transcripts which are partially complementary. Once the complex piRNA-Argonaute with mRNA transcripts has formed, they trigger RNA-dependent RNA polymerase to produce 22G RNAs,\textsuperscript{186,202} similar to as described above for the siRNA. There is a direct relation between the amount of 22G RNAs produced and the complementarity of the piRNA to the target: more mismatches lead to less 22G RNAs.\textsuperscript{202} This feature has an additional role as genome surveillance pathway. The piRNAs monitor the presence of potentially invading, foreign nucleic acids and, in case of occurrence of exogenous transcripts, they trigger several cytoplasmic and nuclear events which lead to the silencing of those elements. The silencing response
can be enhanced by the production of the 22G RNAs, particularly if exogenous DNAs are integrated into the genome. Thus, the WAGO bound 22G RNAs and CSR-1 bound 22G mentioned above, for instance, can act in order to maintain a silent state through a chromatin remodeling mechanism. However, it has been suggested that CSR-1 bound 22G RNAs, which target germline-expressed genes, can, in rare instances, antagonize the piRNA pathway and promote the expression of germline transcripts, although it is not entirely known which chromatin mark this pathway uses in order to license germline transcripts. It has been suggested that these pathways, the WAGO bound 22G RNAs and CSR-1 bound 22G RNAs, can lead to adaptive advantages, conferring to the organisms the potential acquisition of additional and perhaps beneficial information that can be transmitted to subsequent generations.

**Non-genetic mechanisms of inheritance**

In the late 19th century, Friedrich Leopold August Weismann strongly promoted the idea that “genetic information cannot transfer from the soma to the germline”, hence preventing mutations occurring in somatic cells from being inherited. This idea was accepted for several years and became commonly known as “the second law of biology”. However, even August Weismann, who envisioned this theory, by observing for instance that temperature can affect the color of butterflies’ wings, was surely aware of the fact that several environmental factors can induce phenotypic plasticity, although only transiently without leading to an evolutionary change.

During the 20th century, the interest in this area was renewed, and emerging studies on epigenetic phenomena, such as histone modification, DNA methylation, transmission of regulatory RNAs showed that an interaction between soma and germline is possible, and that some environmental changes can have consequences in the following generations, even without any changes in the genotype. However, the mechanisms by which this is achieved are poorly understood and there are still many unresolved questions. For instance, whether these transgenerational epigenetic effects originate as a consequence of direct changes in the parental germline or whether they are initiated by modifications which occur in the parental somatic cells and subsequently are transferred to the parental germline, is mostly still an open question. In the attempt to answer these questions, the role of epigenetics is
crucial. Parental effects in fact fit within the concept of transgenerational epigenetic effects, some of which are known to involve gametic epigenetic effects.

In this context, a variety of non-coding RNAs, including small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and micro RNAs (miRNAs) play a role in transgenerational epigenetic inheritance. C. elegans has emerged as a model for investigation of pathways involved in gene regulation via small RNAs. Small RNAs, by entering the germline, could enable an organism to transfer information from somatic cells across generations, suggesting that this mechanism could be involved in the transmission of environmental effects in subsequent generations. A multigenerational inheritance of a silencing response implies as first step that the silent state is established in the parental generation; subsequently, gametes transmit the silencing signal to the next generation, and the progeny needs to re-establish or maintain the silencing state. Although the exact mechanism which allows the transfer of small RNAs from soma to the germline is still unknown, a study using fluorescently labeled dsRNAs in C. elegans has shown that double-stranded RNA can enter oocytes together with yolk via an endocytosis pathway. dsRNAs are carried in vesicles and the multi-transmembrane transporter SID-1 (Systemic Interference Deficient) plays a role in the release of acquired mobile dsRNA. An additional role of SID-1 in transmission of small RNAs between cells has been suggested, but is under debate. In the context of endogenous small RNAs, movement of dsRNA from soma to germline, or between different tissues, has not been observed.

The conditions that determine which specific inherited epigenetic “memories” would persist over generations is still not clear. Several RNAi experiments in C. elegans showed that the silencing responses lead to a hypomorphic phenotype and that the strongest effect is detected in the F1 progeny, with a reversion of the original phenotype in the F2 and subsequent generations. In this intergenerationally transmitted response, it is difficult to rule out that also the progeny is directly exposed to the initial RNAi trigger though. Because of the short generation time of a worm, the effect could be a mere consequence of a slow dilution of small RNAs.
However, examples of transgenerational RNAi with a silencing response maintained in the F2 generation and beyond have been observed. For instance, it has been reported that expression of double-stranded RNA (dsRNA) in neuronal cells and the subsequent production of dsRNA-derived mobile RNAs, led to transgenerational silencing of a gene of matching sequence in the germline. These silencing effects lasted for more than 25 generations in the absence of the original trigger. In an extreme case, in the nematode *C. elegans*, a single injection of *ceh-13* dsRNA, resulting in a small and dumpy phenotype, persisted for more than 80 generations, again without the presence of the original trigger.

This long-lasting gene silencing induced by RNA interference can be explained by taking into consideration that RNAi is a catalytic reaction (see scheme in Figure 3-2). In yeast, plants and worms it has been observed that the enzyme RNA-dependent RNA polymerase (RdRP) catalyzes the amplification of new silencing signals by synthesizing *de novo* secondary RNAs in each inheriting generation, using the mature target mRNA as a template. The amplified response, triggered by both siRNAs and piRNAs, can lead to the production of small RNAs that are 100 times more abundant than the first trigger of small RNAs. It has been reported that exogenous dsRNA, after binding with a dsRNA-binding protein (RDE-4 (*RNAi-Defective-4*)), recruits the protein Dicer to process the dsRNA into siRNAs. Primary siRNAs, by binding to the Argonaute protein RDE-1, initiate the degradation of the mRNA targets. The primary siRNA/RDE-1/mRNA complex recruits RdRP which act in order to produce secondary siRNA. The following generation can inherit and amplify secondary siRNAs, leading to propagation and transmission of silencing information to subsequent generations. RdRP is able to produce siRNAs that can be inherited over several generations by acting on mRNA in the germline. Amplified secondary RNAs have been found to be transported to the germline by three nuclear Argonautes: by the previously mentioned CSR-1 and WAGO-1 proteins and by HRDE-1 (*Heritable RNAi Deficient-1*). The HRDE-1 protein triggers a response with regard to a dsRNA-induced transgenerational silencing, and the association with 22G endo-siRNAs in the nucleus leads to gene-silencing effects in germ-cell nuclei, inducing multigenerational RNAi inheritance. Hence, amplification of primary small RNAs, by preventing their dilution over generations, can potentially enhance transgenerational RNAi effects. In support of
this concept, it has been found that RdRP is necessary for transgenerational inheritance of antiviral responses.45

Long-term gene silencing can also be related to the fact that epigenetic modifications such as chromatin modification and transmission of small RNAs across generations act together and one mechanism does not exclude the other (see scheme in Figure 3-2).47,215 Small RNAs regulate transcription in the nucleus by binding the nascent RNA messenger.187 Acting in cooperation with chromatin-remodeling proteins, small RNAs promote cytosine methylation (in plants, fission yeast, and mice) and histone modifications (in plants, fission yeast, worms, and flies).218 In C. elegans small noncoding RNAs play an important role for the establishment of repressive chromatin modifications, such as the deposition of the heterochromatic mark histone 3 lysine 9 methylation (H3K9me), which can repress transcription and increase the effect of gene silencing.187,203 Therefore, both mechanisms (i.e. RNAi and chromatin remodeling in the germline) are not mutually exclusive and they play an important role in epigenetic inheritance.47 A good example showing the reciprocity and mutualism of the interaction between RNAs and proteins involved in chromatin remodeling is represented by Schizosaccharomyces pombe, where RNAi promotes deposition of H3K9me3 marks on specific cognate genes, and on the other hand the histone mark can also induce the recruitment of the RNAi machinery, creating a lasting effect that can be maintained throughout cell division and hence several (asexual, vegetative) generations.218,221 In C. elegans, the question whether the deposition of histone 3 lysine 9 methylation (H3K9me) marks are directly inherited or need to be reestablished in the following generation is still a debated issue.47,209 A time-course experiment in C. elegans indicated that small RNAs represent the primary heritable material, since they are detectable before the chromatin marks, suggesting that inherited small RNAs can re-establish H3K9me3 marks de novo in the offspring.222 Moreover, the fact that in C. elegans small RNAs undergo a process of amplification in following generations (see above) suggests that small RNAs could be able to reconstruct de novo the heterochromatin status, by cooperating with the chromatin modification machinery.222

Both RNAi, and some chromatin modification marks play a role in the regulation of silencing effects.209,215 Consistent with this, in C. elegans it has been found that four genes, all involved in chromatin remodeling, are required for the maintenance of
silencing: *hda-4* (a class II histone deacetylase), *K03D10.3* (a histone acetyltransferase), *isw-1* (a chromatin-remodeling ATPase), and *mrg-1* (a chromo-domain protein). Evidence that histone acetylation can also be involved in RNAi inheritance comes from the observation that the treatment of *C. elegans* with the histone deacetylase inhibitor trichostatin A (TSA) can mitigate the silencing effect of RNAi.

![Figure 3-2. Sketch for soma to germline transmission of small RNAs.](image)

Primary small RNAs (white/black) from the soma are transported to the germline or initiate secondary small RNA synthesis (22G RNAs, indicated in grey) by recruiting RdRPs to an mRNA molecule, which serves as template for the synthesis of the secondary small RNAs. 22Gs RNAs are carried by Argonautes to the worm germline and enter the nucleus by the nuclear Argonaute RDE-3 (not indicated in the sketch). Small RNAs drive a transgenerational response by regulating the expression of their targets and modulating chromatin structure through histone modifications.

Hence, even if the complete picture of the silencing signal transmitted from the parental generation to their progeny is still not clear, small RNAs represent potential candidates which can convey the silencing information. The silencing effect is
transmitted through both sperm and oocytes to the next generation, but the transmission through sperm is more effective than through the oocyte, even though the sperm cells are considerably smaller (ca. 1 % of volume of the oocyte).\textsuperscript{209,214} Interestingly, it has been reported that direct injection of dsRNA in males does not trigger a silencing response in the next generation, while hermaphrodite injection does, suggesting that different mechanisms occur in males and hermaphrodites to establish the silencing trigger and only subsequently is the male germline more efficient to transmit the signal, though the mechanism of this difference is not yet known.\textsuperscript{209,223}

Another important question is the biological importance of the existence of inheritance of silencing. Environmental conditions can trigger a response in an organism, e.g. an altered gene expression pattern, by which the organism attempts to adapt to a situation to mitigate e.g. stress or other non-optimal conditions.\textsuperscript{209} Transmitting this information to the succeeding generation hence helps the offspring to prepare for these potential risks, at the cost of potentially adapting to a condition that is not present anymore. An evolutionary advantage might hence arise from a precautionary risk-mitigation, balancing potential high impact risks (i.e. food shortage, toxic stress) against the cost of unnecessarily activating a stress response mechanism (e.g. expression of proteins).\textsuperscript{209} For instance, inheritance of a small RNA-based antiviral response has been reported in \textit{C. elegans}.\textsuperscript{45} Therefore, small RNA inheritance permits the inheritance of physiologically important acquired traits. In nematodes, the presence of anti-viral small RNA (viRNAs) protect them from viruses.\textsuperscript{6} It has been shown that viRNAs mediated their effect by silencing viral genomes, and the effects were passed to the following generations. The RdRP enzyme catalyzes the amplification of the small RNAs in each generation, maintaining the persistence of the anti-viral viRNAs.\textsuperscript{45} Similar “immunity” inheritance has been observed after infecting \textit{C. elegans} with the Orsay virus, the only known virus that infects \textit{C. elegans} nematodes in the wild.\textsuperscript{6,224}

It has been shown that introduction of foreign genetic elements (viruses, transposons, transgenes) and the subsequent production of small RNAs elicits an inheritance response that can last for several generations.\textsuperscript{184,215} Similarly, endogenous small RNA signals, produced as a consequence of environmental cues, are maintained through following generations by re-amplification.\textsuperscript{44} Hence, epigenetic transmission
of responses, rather than merely being involved in the defense against exogenous nucleic acids, represent a general adaptive phenomenon.\textsuperscript{14} In this context, several studies indicate that epigenetics can represent a plausible mechanism which allows organisms to face different kinds of environmental challenges, as has been suggested to be the case for at least multicellular eukaryotic organisms.\textsuperscript{14,44}

In \textit{C. elegans}, the first study showing evidence of small RNAs induced by an endogenous response to an external cue involved a set of small RNAs triggered by starvation, which were transmitted transgenerationally.\textsuperscript{44} Adults that experienced severe starvation (6 days without any source of food) as larval stage L1 showed a drastic change in the levels of primary endogenous small RNAs (26G) and secondary small RNAs (22G). Moreover, changes in the levels of endogenous siRNAs, induced by starvation of \textit{C. elegans}, were found in the parental generation that experienced starvation as L1s, and a highly similar pool of small RNAs was identified in their F3 progeny even though all progeny generations (F1 to F3) were cultured under normal condition (i.e. presence of sufficient food supply). Within this pool of small RNAs with changed level, several were identified that aligned with genes related to food supply (vitellogenin genes (i.e. yolk formation), fat regulation, protein turnover), longevity, stress resistance and reproduction. While this may suggest that the transgenerational regulation of these genes could be a strategy to protect following generations in the case of potential food shortage, no physiological analysis of the consequences of the gene regulation was undertaken. However, lifespan analysis revealed that the offspring of the starved parental generation had indeed a longer lifespan, correlating with the change in small RNAs aligning with longevity genes. Further indications that a specific RNAi pathway is involved in this mechanism related to the fact that heritability of the siRNA trigger by starvation required the dsRNA-binding protein RDE4. Production of small RNA and the regulation of the mRNA targets was abolished in \textit{rde-4} mutants. Transgenerational inheritance of the small RNAs depended also on the germline-expressed nuclear Argonaute HRDE-1.

The observation that well-fed worms whose great-grandparents had experienced starvation lived longer than worms whose ancestors grew in non-starved conditions potentially relates to chromatin modification marks, as it has been reported that the histone H3 lysine 4 trimethylation (H3K4me3) complex, composed of ASH-2, WDR-5 and the histone methyltransferase SET-2, is involved in the regulation of \textit{C.}
\textit{Caenorhabditis elegans} lifespan.\textsuperscript{40} Perturbation of specific chromatin modifiers only in the parental generation can cause an epigenetic memory of lifespan in following generations. While there is a clear co-occurrence of altered small RNA patterns and chromatin reprogramming with starvation-caused heritance of longevity, the mechanism and hence causation chain of small RNA involvement in chromatin modification and heritability of lifespan is still not entirely clear.\textsuperscript{40,44,47}

Beyond starvation, in \textit{C. elegans} another kind of environmental stress, transient exposure to mild heat stress (25°C), induces changes in messenger RNA levels which persist for two to three generations.\textsuperscript{46} It was observed that if heat stress was applied over two consecutive generations, the level of altered transcripts (two transcripts with different alteration were tested) in the progeny increased with respect to a culture only stressed for one generation and, for one of the genes tested, it was observed that the two-generation stress was affecting the transcript level still in a progeny generation which did not show transcript-alteration in the one-generation stressed culture. Moreover, the alterations of transcripts affected particularly genes targeted by germline siRNAs, suggesting that endogenous RNAi plays an important role in order to initiate and maintain the heritable effects. No effect of temperature on transcript levels was observed in RNAi defective worms.\textsuperscript{46} Since temperature change is a stress that \textit{C. elegans} can experience in the wild, the ability to transmit information about fluctuating environmental conditions can provide a selective advantage,\textsuperscript{46} e.g. in order to react appropriately to short-term weather fluctuations that occur within longer seasonal cycles but might affect considerably organisms with short lifespan as \textit{C. elegans}.

An important question to address in the context of endogenous small RNA is how an environmental signal can elicit the production of specific small RNAs.\textsuperscript{44,47} Although the biogenesis mechanism is poorly characterized, endo-siRNAs mostly originate from mRNA transcripts.\textsuperscript{198} It has been speculated that mRNAs which are transcribed in response to an environmental condition serve as templates for the production of specific endo-siRNAs, hence the availability of cognate mRNAs determines the synthesis of endo-siRNAs.\textsuperscript{44} A bidirectional transcriptional mechanism could be involved in the production of dsRNAs (double stranded), which subsequently undergo a maturation process leading to small RNA synthesis.\textsuperscript{44,47} Another hypothesis could be that an environmental factor triggers a pathway that induces the
expression of a specific biogenesis protein which induces dsRNA production, processed afterwards into small RNA. Whether some environmental condition triggers small RNA production in the soma or in the germline is not clear. In worms, it has not been directly shown whether endogenous small RNAs transfer between cells and act systemically, as do exogenous RNAs. As reviewed elsewhere, every environmental stimulus that triggers the synthesis of small RNAs which can reach the germline or modify the expression of small RNAs which are expressed in the germline could, in theory, induce an effect which can be transmitted for several generations even in the absence of the original trigger. Inheritance of small RNA leads to a flexible epigenome able to evolve rapidly, defined as a “soft RNA genome” which, by acting together with other epigenetic features, interacts with a “hard” DNA genome. It has been suggested that the “soft” RNA genome can reflect the environmental experiences of previous generations, therefore it for example can induce transient and short-term effects in response to fluctuating conditions overlaying longer cycles (e.g. temperature, as discussed above), and the silenced (hence unused) genes could over time accumulate mutations (since the genes are silenced, no selection pressure would occur to keep the original sequence intact) which in turn can change the genome and hence lead to a stable, heritable genotype.

Moreover, environmental challenges, and the subsequent modulation of heritable small RNAs, can trigger a complex response, leading to upregulation or downregulation of several genes. As discussed before, while the association of endo-siRNAs with HRDE-1 results in gene silencing, the CSR-1 pathway can on the other hand increase transcription.

**Intergenerational inheritance in *A. freiburgensis***

The nematode *A. freiburgensis* represents a case of cross-generational polyphenism in which environmental factors experienced by the parental generation influence sex determination in the next generation, as shown in Chapters 1 and 2 of this thesis. Hence, *A. freiburgensis* provides an additional evidence that the Weismann barrier can be breached, giving weight to the theory that environmental challenges can trigger inheritance of physiological responses.
Chapter 3 – Molecular mechanism of ascaroside-mediated signal transmission.

The hypothesis is that in *A. freiburgensis*, ascaroside molecules, produced by conspecifics in conditions of crowding, reprogram the germline via the sensory neurons. Perhaps, environmental stress, such as the level of crowding, modulates the pool of transmitted small RNAs, exerting an effect on the next generation.

In this chapter, epigenetic mechanisms such as histone modification and transmission of regulatory RNAs are investigated as potential factors that enable environmental signals received by the soma (neurons) of *A. freiburgensis* mothers to relay to the germline, influencing the larval development and sex of the progeny.

Materials and Methods

**Total RNA isolation**

RNA extraction was performed from adult mother hermaphrodites and progeny at larval stage (L1s). Extractions were conducted in three to four replicate extractions, and in two different conditions: with and without exposure to supernatant.

**Adults:**

Dauer were isolated by picking and allowed to develop into larval stage 4 (L4, required ca. 24 hours). 20 worms (L4 hermaphrodite) were moved to a 6 cm NGM(+OP50-1)-plate and left overnight. For supernatant exposure samples, cultures were prepared equally except that the plate medium contained additionally 50 mg of freeze-dried supernatant powder, from the 3-weeks protocol reported in Chapter 1 of this thesis (3w9M – 2nd batch). Several plates were made in order to collect 200 worms for each replicate. After 24 hours of exposure to supernatant, 200 worms were collected by picking into 200 µl M9 using a 1.5 ml safety lock Eppendorf centrifuge tube. Samples were centrifuged at 1,100 g and the supernatant discarded. Samples were washed with 400 µl M9 buffer three times with in between centrifugation and discarding the supernatant. After the last centrifugation step, 200 µl of Trizol (Life Technologies) were added. Samples were immediately frozen at -80 ºC until the RNA extraction was done.
Progeny at L1s:

To collect progeny at larval stage 1 from hermaphrodite mothers, mothers were placed on a NGM media plate with or without supernatant (3w9M – 2nd batch) as described above for the adults. Exposure with supernatant was done for 24 hours or longer, enough to allow each mother to produce eggs (almost 100 eggs per plate). Mothers were then removed from the plates and the eggs were left to hatch for about 24 hours. Worms were collected at L1 stage by pipetting 500 µl of M9 buffer on the plates, suspending the worms by pipetting up and down, and moving the suspension into a 1.5 ml Eppendorf tube. Samples were cleaned (washing with M9 and centrifugation; see adult protocol above) and, after discarding the supernatant in the last step, Trizol was added as described above. Samples were immediately frozen at -80 ºC until the RNA extraction was done.

RNA extraction:

Worm samples from -80 ºC were thawed and subjected to 2-3 freeze-thaw cycles (dry ice and room temperature; freeze-crack method). After the final thawing, 200 µl of Trizol and 80 µl chloroform were added, samples were mixed by vortexing for 15 seconds and incubated at room temperature for 5 minutes. Afterwards, a centrifugation step was performed at 12,000 g for 15 minutes at 4 ºC. The upper aqueous phase was transferred into a new tube. 200 µl of 100 % isopropanol (1/2 volume of the originally added Trizol) were added. Samples were mixed and incubated at room temperature for 10-15 minutes. A centrifugation step was performed at 12,000 g for 10 minutes and at 4 ºC, and the supernatant was discarded. The pellet was washed with 400 µl of 70 % ethanol. A centrifugation step was performed at 7,500 g for 5 mins at 4 ºC, the supernatant was removed and the pellet was air-dried for 10 minutes to remove the residual alcohol. The pellet was dissolved in 20 µl of nuclease-free water (diethyl pyrocarbonate (DEPC) treated for RNAse deactivation). The RNA was DNAase treated by using the kit TURBO DNA-free™ Kit, Life Technologies. RNA Clean & Concentrator™ kit from Zymo Research was used to clean the samples. Quality of the RNA was determined by using UV/vis spectroscopy and checking the integrity of the rRNA bands in agarose gel electrophoresis. For storage, RNA samples were kept at -80 ºC.
Small RNA library preparation and sequencing

Preparation of the library was performed by Oded Rechavi and Sarit Anava (Tel-Aviv University, Israel).

For the library, the NEBNext Multiplex Small RNA Library Prep Set for Illumina was used according to the supplier’s protocol. This isolation kit fuses several adaptor sequences to RNA sequences, converts the resulting constructs to DNA with a reverse transcriptase, and amplifies them in a polymerase chain reaction. First, RNA sequences receive a 5’ and 3’ adapter sequence, after which a barcode and second adaptor sequence are introduced at 3’end. The terminal adaptor sequences are later used to capture the constructs in a fluidic chip exposing complementary sequences, from where the sequencing is carried out, which means that a chip-bound primer complementary to the terminal adapter regions is also used as a primer for the sequencing process. The barcoding sequences allow multiplexing of the sequencing with different samples receiving individual barcodes. Sequencing and identification of which sample a construct belonged to is then achieved by parallel sequencing of constructs on the fluidic chip (read out from fluorescence of labelled nucleotides incorporated at each step, in parallel over multiple fragments on the chip), followed by sequencing of the barcode region (to infer which sample the fragment belonged to). The initially introduced 3’ adaptor used here had the sequence 5’-TGGAATTCTCGGGTGCCAAGG-3’ (note that this sequence is different from the default adaptor in the NEBNext Multiplex Small RNA Library Prep Set used here) and was read in sequencing together with the sequence of interest (i.e. the sequence of the original small RNAs in the samples, converted to DNA with reverse transcriptase, see above), to confirm that an appropriately captured fragment had been read out.

All sequencing experiments were done for 3 or 4 replicates of RNA isolations (see above) per condition. The total RNA samples were treated with tobacco acid pyrophosphatase (Epicenter), in order to ensure 5’-monophosphate independent capturing of small RNAs. The resulting cDNAs were separated on a 4% agarose E-Gel (Invitrogen, Life Technologies), and the region of the gel containing 140–160 nt length species was excised. The rationale here was that after fusing the adaptors of the NEBNext kit (see above) to small RNA, constructs result, after reverse
transcription, in cDNA fragments of ~ 143-146 nt and 153 nt bp for miRNAs and piRNAs, respectively, so that a first separation from fragments resulting from other RNAs was achieved. cDNA was purified using the MinElute Gel Extraction kit (QIAGEN) and libraries were sequenced on an Illumina HiSeq2500.

**Small RNA-seq analysis**

Illumina fastqc output files were assessed for quality with the program “FastQC” (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The 3’ adaptor sequences (see above, sequence 5’-TGGAATTCTCGGGTGCCAAGG-3’) were clipped from all sequences by using “Cutadapt”, and insufficiently short reads (< 15 nt) were discarded. The following specifications were used: > cutadapt -m 15 --discard-untrimmed -a TGGAATTCTCGGGTGCCAAGG input.fastq > output.fastq (-m 15: discard reads which are shorter than 15 nucleotides after the adapter clipping).

Clipped reads were aligned either to the genome of *Auanema rhodensis* (not published) or the transcriptome of *Aunema freiburgensis* (not published) using “Butter”. Small RNA reads of 20-27 nucleotide in length were filtered using the tool “Samtools” and an awk one-liner script. Reads were counted using “Salmon”. The Salmon outputs are files with the raw counts of how many reads hit genes in antisense (which was selected via optional parameters for Salmon).

The R package DESeq2 was used to define differentially regulated genes using adjusted p-values (from Wald test following Benjamini-Hochberg correction) of FDR (false discovery rate) < 0.05 and log2(fold change) > 2 (increased expression) and < 2 (reduced expression). Since many low-count reads were noted in the library, different normalisation methods were compared:

- The “GLM” (generalised linear model) method, which is the default normalisation in the DESeq2 package. This method applies the same (constant) normalisation factor, estimated from the size factors by the “median of ratios” method, to all genes in a sample. Hence, this method normalises for sequencing depth differences under the assumption that the read counts of all samples follow the same (negative binomial) distribution.
- DESeq2 normalisation with an additional shrinkage of log2 fold expression changes, using the “lfcShrink” function and the “apeglm” (approximate posterior estimation for generalised
linear model) estimator, a method described for lowering the impact of low-count (and low significance) differential expression data.\textsuperscript{231}

- A quantile normalisation (function: normalize.quantiles())\textsuperscript{232} implemented in the preprocessCore R-package. By using quantile normalisation, all count read distributions are forced into the same shape, resulting in the same count number for the gene with the highest read count (average of counts of the gene across samples), the same second highest for the second highest etc. without distinguishing which gene this was. Therefore, in this selection of normalisation methods, the quantile normalisation is changing the most the relative normalised count number for a gene with respect to other genes in the same sample. Because of this, this method was included for comparison with the other methods only, but the results were only considered with care. Note that this normalisation was done prior to analysis with the DESeq2-function, and the default GLM normalisation in DESeq2 was deactivated. Since the quantile normalisation results in non-integer normalised counts, while DESeq2 only accepts integer count data as input, the quantile normalised results were rounded to the next integer, which imposes a source of deviation in the normalised data, particularly in the low-count region.

After analysis with DESeq2, annotation of differentially expressed genes was done using the Nucleotide “Basic Local Alignment Search Tool” (BLASTn),\textsuperscript{233} against the genome of \textit{C. elegans} (with cutoff at an \textit{e}-value of $10^{-10}$). Scripts for the analysis were provided by Sophie Tandonnet.

**Gonad Dissection, immunostaining, and analysis**

Dauers were isolated from a crowded plate and transferred to a 6 cm seeded NGM medium plate. After 24 hours, when dauers had developed into L4 larval stage, they were placed on a 6 cm NGM plate containing 50 mg of supernatant (3w9M – 3\textsuperscript{rd} batch). Exposure with supernatant, gonads were dissected on a slide (Superfrost microscope slide, VWR) covered with M9\textsuperscript{234} buffer. After dissection, a coverslip was put on top of the slide. Slides were moved to a frozen block in dry ice for 10 minutes. After removing the coverslip, slides were incubated with -20 °C methanol for 1 min. Slides were then fixed with a fixative solution (4 % paraformaldehyde in PBS (phosphate buffered saline), with 80 mM HEPES (pH 7.4), 0.8 mM EDTA, and 1.6 mM MgSO\textsubscript{4}) in a humid chamber for 30 minutes, at room temperature. Slides were washed two times in PBST solution (prepared by mixing 10 ml PBS 100X, 10 ml Tween-20 10%, 890 ml deionized water). Samples were blocked in PBST with 0.5 % BSA (bovine serum
albumin) for 1 hour. Slides were incubated overnight with mouse primary antibodies to H3K9me3, H3K27me3 (both provided by Hiroshi Kimura, Hokkaido University, Japan) and H3K4me3 (CMA304; from Millipore 05-1339-S), at a range of 0.2-1 µg/ml in PBST plus 0.5 % BSA. Afterwards, slides were washed three times in PBST for 5 minutes. Secondary antibody (Goat, Anti-mouse, Alexa Fluor 594, Invitrogen) was applied at 1:100 dilution in PBST, for at least 2 hours. Any excess of secondary antibody was removed by washing three times with PBST. 15-25 µl of Fluoroshield mounting medium with DAPI (4',6-diamidino-2-phenylindole, Abcam) was applied thereafter. Images were taken with a 60X objective in 2.40 µm z stack intervals (12 sections) with a Deltavision microscope (Olympus).

Results

RNAseq analysis of small RNAs

Libraries of small RNAs were prepared from hermaphrodite mothers and progeny (at larval stage L1), the mothers in both cases either exposed to supernatant (assay) or not (control). Experiments were done with three RNA isolations (i.e. independent triplicates), except for samples related to L1s on supernatant, for which 4 samples were prepared. It should be noted that worms needed to be collected from different plates (also for the exposure assay), since they had to be grown in low density to avoid crowding effects on the culture plates, so that RNA isolation was insufficient from a single plate to conduct a sequencing experiment. This pooling may represent additional variability beyond the triplicate / 4 sample preparations, since plates which were prepared in parallel and pooled together could still experience slightly different environments (e.g. growth state, but also temperature deviations in incubator positions, etc.). For the later statistical testing in differential regulation analysis (see below), this might imply that the number of replicates in the testing is chosen conservative by only counting the pooled samples (which themselves consist of multiple parallel, or replicate samples) so that the false discovery rate is also conservative and actual differentially regulated genes might be considered to be not significant. The sequencing was carried out by Oded Rechavi and Sarit Anava (Tel-Aviv University, Israel) on an Illumina HiSeq2500 instrument, and the analysis of the sequencing is presented in the following.
FastQC Reports

Paired-end sequencing reads were assessed using the program fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Quality checks of the reads, based on nucleotide distribution, length (35 – 50 nt, i.e. construct formed from small RNA and sequenced 3’-adaptor of 21 nt), and sequencing quality showed that all samples were of high quality, except two samples which, after subsequent analysis steps (see “Read Trimming” below) were removed from the analysis (see text below and Table 3-1).

Read trimming

Before proceeding to the further analysis, the adapter sequences at the 3’ were clipped from the small RNA libraries using the program “Cutadapt”. The resulting processed reads shorter than 15 nucleotides were discarded. The quality of the reads was further validated using the fastQC program. For most samples, ~90 % of the reads were retained, and ~10 % were removed by the length filter (Table 3-1). For two samples (sample numbers 4 and 3 in Table 3-1), 99 % of the reads were discarded and hence these samples were excluded from further analysis.
Table 3-1. Number of reads before and after using the program “Cutadapt”.
The column “Condition” specifies whether adult hermaphrodites (“Adult”) or L1 progeny (“L1”) were
analysed, and “ctr” and “sup” identify whether the samples refer to the control, or supernatant-
exposed treatment, respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Condition</th>
<th>Raw counts</th>
<th>After cutadapt</th>
<th>% after trimming</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Adult_ctr</td>
<td>23,294,497</td>
<td>21,005,663</td>
<td>90.2</td>
</tr>
<tr>
<td>26</td>
<td>Adult_ctr</td>
<td>21,999,957</td>
<td>19,694,037</td>
<td>89.5</td>
</tr>
<tr>
<td>27</td>
<td>Adult_ctr</td>
<td>14,400,411</td>
<td>12,380,650</td>
<td>86.0</td>
</tr>
<tr>
<td>32</td>
<td>Adult_sup</td>
<td>23,572,617</td>
<td>21,270,594</td>
<td>90.2</td>
</tr>
<tr>
<td>34</td>
<td>Adult_sup</td>
<td>22,949,525</td>
<td>20,471,406</td>
<td>89.2</td>
</tr>
<tr>
<td>4</td>
<td>Adult_sup</td>
<td>36,307,365</td>
<td>260,080</td>
<td>0.7</td>
</tr>
<tr>
<td>1</td>
<td>L1_ctr</td>
<td>5,580,042</td>
<td>4,971,780</td>
<td>89.1</td>
</tr>
<tr>
<td>2</td>
<td>L1_ctr</td>
<td>6,304,113</td>
<td>5,763,554</td>
<td>91.4</td>
</tr>
<tr>
<td>3</td>
<td>L1_ctr</td>
<td>23,616,467</td>
<td>117,040</td>
<td>0.5</td>
</tr>
<tr>
<td>35</td>
<td>L1_sup</td>
<td>21,669,823</td>
<td>19,035,034</td>
<td>87.8</td>
</tr>
<tr>
<td>36</td>
<td>L1_sup</td>
<td>9,169,420</td>
<td>8,101,651</td>
<td>88.4</td>
</tr>
<tr>
<td>37</td>
<td>L1_sup</td>
<td>13,623,163</td>
<td>11,969,495</td>
<td>87.9</td>
</tr>
<tr>
<td>38</td>
<td>L1_sup</td>
<td>12,049,927</td>
<td>10,505,581</td>
<td>87.2</td>
</tr>
</tbody>
</table>
Alignment to the genome of *Auanema rhodensis*

The genome sequencing and annotation of *Auanema freiburgensis*, the species studied in this thesis, was not yet completed (as part of the present thesis the DNA isolation was accomplished (Appendix) but the annotation of sequencing data is pending). To proceed with the RNAseq analysis of small RNAs, a mapping of the sequencing reads was instead attempted against the genomic scaffolds of *Auanema rhodensis*, a closely related species. Molecular phylogenetic analyses place *A. freiburgensis* as sister species of *A. rhodensis*. Clipped reads were aligned using the tool “Butter” (Bowtie UTilizing iTerative placEment of Repetitive small rnas), allowing up to 1 mismatch. The percentage of uniquely mapped reads and multi-mapped reads was very low (on average less than 10 %, see Table 3.2). This mode of analysis was not further investigated.
Table 3-2. Mapping of RNAseq reads from *A. freiburgensis* against the genomic scaffolds of *A. rhodensis*.

The table shows the number of reads mapped in the respective categories against the reference scaffolds, and the percentage number in brackets represents the fraction of these reads with respect to the number of input-reads (see Table 3-1, “after cutadapt”), i.e. the number of reads after running “Cutadapt”.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uniquely mapped reads</th>
<th>Multi-mapped reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult_ctr (25)</td>
<td>1,046,895 (5.0%)</td>
<td>1,429,314 (6.8%)</td>
</tr>
<tr>
<td>Adult_ctr (26)</td>
<td>1,232,140 (6.3%)</td>
<td>1,269,188 (6.4%)</td>
</tr>
<tr>
<td>Adult_ctr (27)</td>
<td>820,687 (6.6%)</td>
<td>553,607 (4.5%)</td>
</tr>
<tr>
<td>Adult_sup (32)</td>
<td>133,063 (5.3%)</td>
<td>1,278,247 (6.0%)</td>
</tr>
<tr>
<td>Adult_sup (34)</td>
<td>1,528,139 (7.5%)</td>
<td>782,886 (3.8%)</td>
</tr>
<tr>
<td>L1_ctr (1)</td>
<td>87,771 (1.8%)</td>
<td>20,887 (0.4%)</td>
</tr>
<tr>
<td>L1_ctr (2)</td>
<td>110,549 (1.9%)</td>
<td>24,390 (0.4%)</td>
</tr>
<tr>
<td>L1_sup (35)</td>
<td>1,269,467 (6.7%)</td>
<td>88,975 (1.0%)</td>
</tr>
<tr>
<td>L1_sup (36)</td>
<td>157,937 (1.9%)</td>
<td>7,097 (0.3%)</td>
</tr>
<tr>
<td>L1_sup (37)</td>
<td>359,220 (3.0%)</td>
<td>58,109 (0.5%)</td>
</tr>
<tr>
<td>L1_sup (38)</td>
<td>775,970 (7.4%)</td>
<td>110,774 (1.1%)</td>
</tr>
</tbody>
</table>
Alignment to the transcriptome *A. freiburgensis*

Clipped reads were instead aligned to the transcriptome of *A. freiburgensis* by using the same tool “Butter” as above. *De novo* transcriptome assembly was carried out by Sally Adams. The transcriptome was sequenced by paired-end RNA-seq on Illumina from a mixed population of females and hermaphrodites, at different developmental stages. The transcriptome was reported as follows: Contig N10: 4,820; Contig N20: 3,672; Contig N30: 2,948; Contig N40: 2,401; Contig N50: 1,959. Median contig length: 612; Average contig: 1,115.97. Number of sequences: 35,877. Longest sequence 21,927; Shortest sequence 224; Average length 1,353. Total number of bases 48,544,902.
Table 3-3. Mapping of RNAseq reads from *A. freiburgensis* against its transcriptome.
The table shows the number of reads mapped in the respective categories against the reference scaffolds, and the percentage number in brackets represents the fraction of these reads with respect to the number of input-reads (see Table 3-1, “after cutadapt”), i.e. the number of reads after running “Cutadapt” (see numbers in Table 3-2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uniquely mapped reads</th>
<th>Multi-mapped reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult_ctr (25)</td>
<td>4,469,805 (21.3 %)</td>
<td>8,826,025 (42.0 %)</td>
</tr>
<tr>
<td>Adult_ctr (26)</td>
<td>4,769,106 (24.2 %)</td>
<td>6,376,928 (32.4 %)</td>
</tr>
<tr>
<td>Adult_ctr (27)</td>
<td>3,067,705 (24.8 %)</td>
<td>4,419,478 (35.7 %)</td>
</tr>
<tr>
<td>Adult_sup (32)</td>
<td>4,617,755 (21.7 %)</td>
<td>8,777,803 (41.3 %)</td>
</tr>
<tr>
<td>Adult_sup (34)</td>
<td>5,794,842 (28.3 %)</td>
<td>6,668,063 (32.6 %)</td>
</tr>
<tr>
<td>L1_ctr (1)</td>
<td>2,985,037 (60.0 %)</td>
<td>392,883 (7.9 %)</td>
</tr>
<tr>
<td>L1_ctr (2)</td>
<td>3,723,244 (64.6 %)</td>
<td>423,632 (7.4 %)</td>
</tr>
<tr>
<td>L1_sup (35)</td>
<td>5,025,209 (26.4 %)</td>
<td>2,932,752 (15.4 %)</td>
</tr>
<tr>
<td>L1_sup (36)</td>
<td>3,324,557 (41.0 %)</td>
<td>431,486 (5.3 %)</td>
</tr>
<tr>
<td>L1_sup (37)</td>
<td>5,046,225 (42.2 %)</td>
<td>752,059 (6.3 %)</td>
</tr>
<tr>
<td>L1_sup (38)</td>
<td>1,555,105 (14.8 %)</td>
<td>359,672 (3.4 %)</td>
</tr>
</tbody>
</table>
Filtering reads and counting reads

Small RNA reads of 20-27 nucleotide in length were filtered using the tool “Samtools” and an awk one-line command. Quantification of reads was performed to estimate small RNA abundance and compare the expression level of small RNAs between different conditions. Since several studies have shown that small endo-RNAs usually align in the antisense orientation and almost exclusively to exons, optional command-line parameters implemented in the tool Salmon were used to count reads in antisense orientation to genes.

Normalisation of count data for differential expression analysis

The count data form the Salmon output were read into R and analysed by using the DESeq2 package. The distribution of the raw and normalised counts was compared. First, the default normalisation in DESeq2 (generalised linear method (GLE), see materials and methods) was used, and the count data distribution is compared in Figures 3-3 (adult samples) and 3-4 (L1 samples). Both the adult, and the L1 samples had a considerable number of low and zero read counts in all samples. Because of this, the boxplot distribution representation does not in all cases show the completed quartile boxes and medians since the decadic logarithm of the counts is shown. In both cases, the normalisation had only a limited effect on harmonising the distribution, as seen from both the boxplots, and the density distribution of the data as shown in the appendix (Figures A-6 and A-7). The density plots furthermore show that for the adult samples, the curve shape is different for the control and supernatant exposed samples (but similar within replicates, Figure A-6), explaining the limited success of the GLE normalisation method implemented in DESeq2. For the L1 samples (Figure A-7), it can be seen that the normalisation worked fairly well for genes with higher base means (i.e. log_{10}(base mean) > 1.5), while particularly at low base means after normalisation only some samples had entries (i.e. -1 < log_{10}(base mean) < 0.5), most likely a result from low sequencing depth and numerous zero read counts in some samples, which remain zero upon the GLE normalisation. Note that an additional shrinkage of log_{2}(fold change) (lfcShrink, see Materials and Methods) was tested, which however did not affect the count data distribution (it only shrinks after the differential regulation assessment)
and hence resulted in the same normalised count dataset and density distribution as for the GLE-method.

![Graph showing raw counts vs. DESeq2 normalised counts](image_url)

**Figure 3-3.** Count distribution for raw and DESeq2-normalised counts for the adult samples. A boxplot representation is used, showing the medians (bold horizontal lines), quartiles (box horizontal lines showing the 25 and 75 % quartiles), outliers (individual data points), and the whiskers indicate the highest data point within 1.5 times the interquartile range (75 % quartile minus 25 % quartile) beyond the boxes (i.e. the quartiles). Note that the counts are shown as \(\log_{10}\), which explains why for samples with large numbers of zero reads the quartile-boxes are not seen in the plots.
Figure 3-4. Count distribution for raw and DESeq2-normalised counts for the L1 samples. A boxplot representation is used, showing the medians (bold horizontal lines), quartiles (box horizontal lines showing the 25 and 75% quartiles), outliers (individual data points), and the whiskers indicate the highest data point within 1.5 times the interquartile range (75% quartile minus 25% quartile) beyond the boxes (i.e. the quartiles). Note that the counts are shown as log10, which explains why for samples with large numbers of zero reads the quartile-boxes are not seen in the plots.

A quantile normalisation was tested in addition, which ranks the samples from the highest to the lowest count value and gives each gene with the same rank the same count value (i.e. the average across the samples). While this method is independent of the sample’s original count distribution (i.e. by averaging genes of the same rank, it will result in the same final count distribution), it also changes the relative count level of genes within one sample to each other (i.e. implies considerable changes on the data) and was hence only used for comparison. Figures A-8 (adults) and A-9 (L1s, both in appendix) show the boxplots of the count data before and after quantile normalisation, and the density plots are shown in Figures A-6 and A-7 (respective
lower figures, both in appendix). As seen from the boxplots, the quantile normalisation aligned the genes with the highest count reads well, both for the adult (Figure A-8) and L1 (Figure A-9) samples. However, because of overall low read counts, the lower count range was not normalised well. This was also evident from the density plots (appendix Figures A-6 and A-7) which showed that normalisation resulted in the same distribution in the range above ca. $\log_{10}(\text{base mean}) > 1$, while the distributions differed below. This is explained by the fact that the quantile normalised data needed to be rounded to the next integer in order to conduct the DESeq2 analysis, and this rounding particularly changes the normalised count data in the low count region (see Materials and Methods).

Principle Component Analysis (PCA)

To assess the similarity between samples and visualize sample-to-sample distances, a principal component analysis (PCA) was performed. By reducing multidimensional data into two dimensions, PCA analysis permits determination of whether samples show higher variability in expression between experimental conditions (supernatant versus control) than between replicates in one condition.

The R package DESeq2 was used to generate PCA plots of the different conditions. Specifically, PCA graphs were obtained using the “plotPCA” function and log-transformation of the reads. PCA-plots for the comparison of supernatant-exposed and control hermaphrodites are shown in Figure 3-5, and the PCA analysis for the L1 progeny of control and supernatant-exposed mothers is shown in Figure 3-6. Note that DESeq2 uses the rlog-transformed raw count data, which implies that in case of the different normalisation methods (see Materials and Methods) in the analyses with GLE and shrunken $\log_2$(fold change) (lfcShrink), where the same raw count dataset was used, the same PCA plots were obtained. For the quantile normalisation, the count data was normalised before DESeq2 analysis, and hence the PCA analysis was carried out on this normalised dataset (Figures A-10 (adults) and A-11 (L1s)).

The x-axis (PC1) representing the dimension with the largest variability between the samples, separated the controls from the supernatant-exposed samples in both cases. The y-axis (PC2), orthogonal to the first direction, is the dimension that separates the sample set the second best. The percentage values next to the axis labels give a
measure of how much of the between sample variability is explained by the axis. With the non-normalised count data (Figures 3-5 and 3-6), the large values in PC1 are the effect of the visible separation of sample groups, while the lower PC2 values result from the fact that the sample groups overlap partly in this dimension. In conclusion, for both experiments, there is a clear separation of the control from the supernatant samples, strongly expressed by PC1. A very similar result was achieved when the samples were quantile normalised before the DESeq2-analysis (Figures A-10 and A-11).

Figure 3-5. Principle Component Analysis (PCA) of regularized log-transformed reads (adult samples).

5 adult samples (green: control, orange: supernatant) shown in the 2D plane spanned by their first two principal components. Samples show larger variability between experimental conditions than between replicates (i.e. clear separation in PC1). Analysis was done by using the DESeq2 function plotPCA(). For sample names (indicated in the figure) compare Tables 3-2 and 3-3; the indicator “salmon_quant_” identifies these samples after read filtering and counting with the program “Salmon” (see above).
Figure 3-6. Principle Component Analysis (PCA) of regularized log-transformed reads (L1 samples). 6 L1 samples (green: control, orange: supernatant) shown in the 2D plane spanned by their first two principal components. Samples show larger variability between experimental conditions than between replicates (i.e. clear separation in PC1). Analysis was done by using the DESeq2 function plotPCA(). For sample names (indicated in the figure) compare Tables 3-2 and 3-3; the indicator “salmon_quant_” identifies these samples after read filtering and counting with the program “Salmon” (see above).

Sample distance matrix

Another way to show the distances between the samples is to use a clustering image map (CIM) or heatmap, using the R package DESeq2 and the function heatmap.2 from the r CRANpkg ("gplots") package. Note that this analysis again uses the log-transformed raw count data, and hence the sample distance matrix is the same for the analysis with GLE- and shrunken log₂(fold change) normalisation (see Materials and Methods). Using the R-function dist(), the euclidian distance between the samples is calculated.
Similar to the results obtained using Principle Component Analysis (PCA), the heatmap showed that supernatant and control replicates cluster distinctly for both the adults, and the L1s (Figure 3-7 and 3-8). Similarly, while with different values, clustering was observed with the quantile normalised counts (appendix Figures A-12 (adults) and A-13 (L1s)). For the adults, the distance order between the samples was very similar, resulting in the same dendrogram representation. For the L1s, however, the order was different. For instance, while for the raw counts the most closely related supernatant samples were sup_36 and sup_37 (Figure 3-8), the most closely related with the prior quantile normalisation were samples sup_35 and sup_37 (Figure A-13). However, the distinct clustering of control and supernatant samples in both cases supported to proceed with the differential regulation analysis.
Figure 3-7. Heatmap of regularized log-transformed reads (adult samples).

This heatmap indicates the similarities in between the respective adult samples by a grey-scale and a hierarchical clustering method (i.e. similar samples are organized next to each other). The different individual samples (with colour coding orange for supernatant, and green for controls) are compared against each of the other samples, with a black square being most similar (here, this is the comparison of a sample with itself) and white the most dissimilar (grey scale indicating distance values from 0 (white, dissimilar) to 100 (black, equivalent)). The dendrogram shows the sample relation in a hierarchical manner, with the closest related samples being clustered together first. For sample names (indicated in the figure) compare Tables 3-2 and 3-3; the indicator “salmon_quant_” identifies these samples after read filtering and counting with the program “Salmon” (see above).
Figure 3-8. Heatmap of regularized log-transformed reads (L1 samples).

This heatmap indicates the similarities in between the respective L1 samples by a grey-scale and a hierarchical clustering method (i.e. similar samples are organized next to each other). The different individual samples (with colour coding orange for supernatant, and green for controls) are compared against each of the other samples, with a black square being most similar (here, this is the comparison of a sample with itself) and white the most dissimilar (greyscale indicating distance values from 0 (white, dissimilar) to 100 (black, equivalent)). The dendrogram shows the sample relation in a hierarchical manner, with the closest related samples being clustered together first. For sample names (indicated in the figure) compare Tables 3-2 and 3-3; the indicator “salmon_quant_” identifies these samples after read filtering and counting with the program “Salmon” (see above).
Volcano plot

Differentially regulated genes were visualized using a type of scatterplot, “Volcano plot”. In the plot, genes are represented in data-points, aiding identification of those genes that display large magnitude changes in expression levels that are also statistically significant.

![Volcano plot diagram]

**Figure 3-9.** Volcano plot for small RNA expression in supernatant-exposed adults *vs.* controls.

The green points indicate genes of interest that show both large-magnitude log₂(fold change) (x-axis) as well as high statistical significance (−log₁₀ of Benjamini-Hochberg corrected Wald statistical test p-value, y-axis) (false discovery rate (FDR) < 0.05 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression)).

The plot encompasses two regions of interest: points with large magnitude mean log₂(fold change) in expression, whose position is on the right or left with respect to the center of the figure, and points which show high statistical significance, towards the top. The green points represent genes of high interest, which show large-magnitude fold-changes (mean log₂(fold change), x-axis) and display high statistical
significance according to the Benjamini-Hochberg corrected Wald statistical test (−log_{10} of p-value (here equivalent to false discovery rate FDR), y-axis).

The green points indicate genes of interest that show both large-magnitude log_{2}(fold change) (x-axis) as well as high statistical significance (−log_{10} of Benjamini-Hochberg corrected Wald statistical test p-value, y-axis) (false discovery rate (FDR) < 0.5 and log_{2}(fold change) > 2 (increased expression) and < 2 (reduced expression)).

First, an analysis was evaluated in which the count data was normalised by DESeq2’s default generalized linear method (GLM). Comparing the expression of genes in adults with supernatant-exposure against non-exposed controls (Figure 3-9), differentially regulated genes with high (green) and limited (yellow) specificity were observed, and a set of genes with less specificity or change (black and red) (numbers of genes noted below in Results section). For the L1 samples (Figure 3-10), an even larger number of genes with large expression change and high (green) or limited (yellow) specificity was found, and another set of genes without notable change in
expression (black). Note that in the L1s a larger number of differentially regulated small RNAs were downregulated, rather than upregulated.

Figure 3-11. Volcano plot for small RNA expression in supernatant-exposed adults vs. controls after applying the \( \log_2(\text{fold change}) \) shrinkage method (LfcShrink).

The green points indicate genes of interest that show both large-magnitude \( \log_2(\text{fold change}) \) (x-axis) as well as high statistical significance (\( -\log_{10} \) of Benjamini-Hochberg corrected Wald statistical test p-value, y-axis) (false discovery rate (FDR) < 0.5 and \( \log_2(\text{fold change}) \) ≥ 2 (increased expression) and < 2 (reduced expression)).

Next, the samples were analysed with application of DESeq2’s \( \log_2(\text{fold change}) \) shrinkage method (LfcShrink, see Materials and Methods, Figure 3-11 (adults) and 3-12 (L1s)). As can be seen for both cases, the LfcShrink function shrunk the \( \log_2(\text{fold change}) \) of genes with high false discovery rate (FDR) closer towards zero (see particularly black and orange data points in the volcano plots). Furthermore, the \( \log_2(\text{fold change}) \) of several genes with FDR < 0.05 was shrunk (see particularly change in red data points), as the LfcShrink function with “apeglm” method,\(^{231}\) which was used here (see Materials and Methods), also attempts to shrink the \( \log_2(\text{fold} \)
change) of genes with low count numbers in the raw data. This is particularly evident from the analysis of MA-plots presented below in a later section. Note that the shrinkage also affected genes with an FDR < 0.05 and log₂(fold change) > 2, which partially fell below these thresholds after shrinkage, which can be again attributed to the shrinkage of low count genes.

![Volcano plot](image)

**Figure 3-12.** Volcano plot for small RNA expression in supernatant-exposed L1s vs. controls after applying the log₂(fold change) shrinkage method (lfcShrink).

The green points indicate genes of interest that show both large-magnitude log₂(fold-change) (x-axis) as well as high statistical significance (−log₁₀ of Benjamini-Hochberg corrected Wald statistical test p-value, y-axis) (false discovery rate (FDR) < 0.5 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression)).

The volcano plots for the quantile normalised data are shown in the appendix Figures A-14 (adults) and A-15 (L1s). The results differed from the analysis with DESeq2’s GLM normalisation and the shrunken log₂(fold change). The general shape of the volcano plots was however similar to the analysis with the GLM method, though the
position of the data points (i.e. the genes’ log₂(fold change) and associated false discovery rate) differed.

**MA plot**

An MA plot gives an overview for the gene regulation change between conditions (log ratios, M, y-axis) against the average expression of genes in all (control and treatment) individual samples (average mean, A, x-axis). Each gene is represented with a dot, a large base mean indicates an overall high expression in the respective samples, and the log₂(fold change) again indicates the differential regulation between the (supernatant) treatment samples against the non-exposed controls. Genes which show similar expression levels are shown around the horizontal line y = 0. Genes below the significance threshold of a Benjamini-Hochberg corrected Wald test p-value (false discovery rate (FDR)) < 0.05 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression) are colored in red.

The results from normalisation with DESeq2’s GLM method is presented first. Here again, from the red-labelled data points, it becomes obvious that a notable number of supernatant-treated adults (Figure 3-13) show differential regulation supported by high significance, while the number of significant, differentially regulated genes in the L1s (Figure 3-14) are even higher, with a trend of more genes having lower expression levels. In both cases though, the MA plots do not follow the typical appearance with the majority of data points being close to a log₂(fold change) around zero (this trend is expected under the assumption that the majority of genes will not be differentially regulated between a treatment and control assay). The reason for this deviation from the common MA-plot shape can either indicate indeed a large number of differentially regulated genes, or a difference in count data distribution. The latter is indicated for the adult samples from the density distribution of normalised count data shown in appendix Figure A-6 (middle). For instance, in the density distribution, all control samples had a lower density than the supernatant samples in the range with $1 < \log_{10}$(base mean) < 2, while they were on average higher in the range $0 < \log_{10}$(base mean) < 1. This was mirrored in the MA plot for the adult samples (Figure 3-13) where a cloud of genes with positive log₂(fold change) occurred centred around log₁₀(base mean) = 1, balanced by a cloud with negative log₂(fold change) starting around log₁₀(base mean) = 1.5. For the L1
samples (Figure 3-14) an atypical MA plot arose from a large number of low count genes, which resulted in a large number of data points at low base means (see also the boxplots in Figure 3-4 demonstrating large numbers of zero counts, and the count data distributions in appendix Figure A-7). As a consequence, for both the adult, and L1 samples the generalised linear normalisation method (GLM) in DESeq2 was insufficient to harmonise the count data distribution of the samples, which resulted in atypical MA plots.

**Figure 3-13.** MA plot for adult samples with DESeq2’s GLM normalisation. Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald statistical test p-value < 0.05 and \( \log_2(\text{fold change}) > 2 \) (increased expression) and \(< 2 \) (reduced expression) are colored in red. The grey horizontal line indicates \( \log_2(\text{fold change}) = 0 \).
which a large number of data points appeared not differentially regulated, in
result
Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald statistical test p-value < 0.05 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression) are colored in red. The grey horizontal line indicates log₂(fold change) = 0.

Given the shortcomings of the GLM normalisation, the log₂(fold change) shrinkage (lfcShrink) of the DESeq2-package with “approximate posterior estimation generalised linear model” (apeglm), which shrinks the differential regulation results for genes with low count in the raw data or high false discovery rate, was employed. For the adult samples (Figure 3-15), this resulted in a shrinkage of log₂(fold change) values for a large number of genes with log₁₀(base mean) < 1.5 (and several genes above). A cloud with negative log₂(fold change) and log₁₀(base mean) > 2 still resided, though was shrunken closer to zero at the lower base mean end. For the L1 samples also (Figure 3-16), a large number of genes with low base mean and low significance were shrunken towards a log₂(fold change) of zero, for which a large number of data points appeared not differentially regulated, in accordance with the underlying hypothesis (see above). However, a considerable
number of genes even with rather low $\log_{10}(\text{base mean}) < 1.5$ were not shrunken as they appeared to be significantly differentially regulated (red data points). In both cases again, the pattern of significant differentially regulated genes (red in the figures) changed due to the shrinkage-dependency on the raw read count number.

**Figure 3-15.** MA plot for adult samples with DESeq2’s GLE normalisation and shrunken $\log_2(\text{fold change})$ (ΙfcShrink function).
Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald statistical test p-value (false discovery rate (FDR)) $< 0.05$ and $\log_2(\text{fold change}) > 2$ (increased expression) and $< 2$ (reduced expression) are colored in red. The grey horizontal line indicates $\log_2(\text{fold change}) = 0$. 
Figure 3-16. MA plot for L1 samples with DESeq2’s GLE normalisation and shrunken $\log_2$(fold change) (lfcShrink function).
Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald statistical test p-value (false discovery rate (FDR)) $< 0.05$ and $\log_2$(fold change) $> 2$ (increased expression) and $< 2$ (reduced expression) are colored in red. The grey horizontal line indicates $\log_2$(fold change) $= 0$.

The MA plots for the quantile normalised analysis are shown in the appendix Figures A-16 (adults) and A-17 (L1s). For the adults (Figure A-16), this normalisation had the effect that the previous cloud with negative $\log_2$(fold change) and $\log_{10}$(base mean) $> 1.5$, which was observed in the normalisation with GLM in DESeq2 (compare Figure 3-13) had now a $\log_2$(fold change) closer to zero (note also the better aligned count data density in Figure A-6 which was in accordance with this observation). However, this cloud still appeared distinct, indicating that this population of genes was distinct irrespective of the normalisation approach. Furthermore, a number of genes with $\log_{10}$(base mean) $< 2$ was biased to positive $\log_2$-fold change, though less than in the GLM normalised data set (compare Figure 3-13). Overall, the quantile normalised adult data set appeared more symmetric.
around the log₂(fold change) of zero, though still the MA plot showed an atypical shape.

The MA plot of the quantile normalised L1 data (appendix Figure A-17) also showed an atypical appearance. As for the GLM normalised data (compare Figure 3-14), a large number of genes particularly with lower log_{10}(base mean) < 2 showed high differential regulation, which was not symmetric around the zero line, even though their values were different with respect to the GLM-normalisation. This can be attributed to the low read counts in the raw data, which resulted in considerably different density curves in the range log_{10}(base mean) < 1.

Note in addition for both quantile normalisation analyses that the normalised data needed to be rounded to the next integer in order to feed them into the DESeq2 analysis (see Materials and Methods). This could explain deviations in distribution in the low base mean region, since rounding errors can have a considerable effect. Also, since all normalisation methods employed here require that the raw count data is multiplied (or divided) by a scaling factor (a constant in GLM and log₂(fold change) shrunken analysis, and individual per gene according to its rank in the quantile method), both methods fall short for samples which have large numbers of zero read counts, which remain zero also after the normalisation. This occurred in the analysed datasets (see raw count distribution boxplots in Figures 3-3 (adults) and 3-4 (L1s) and density plots in appendix Figures A-6 (adults) and A-7 (L1s)) and can hence be considered a source of error in the low base mean region.

**Differential Expression Analysis based on the RNAseq results**

Counted reads from RNAseq were examined in order to determine the differential regulation of genes.

The “Salmon” outputs were used as an input file for the R DESeq2 package. DESeq2 analyses and reveals differential regulation of genes between different conditions. Because small RNAs were mapped to the *A. freiburgensis* transcriptome, this analysis indicates genes which are potentially silenced by small RNAs through interference. The differentially regulated genes were defined using a cutoff of adjusted p-value (Wald test, Benjamini-Hochberg corrected; false discovery rate
(FDR) < 0.05 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression).

**Table 3-4.** Summary table of genes with relation to chromatin remodeling (see discussion in text) and differential expression in either supernatant exposed adults, or L1s.

The table lists the gene names and information related to differential regulation derived from DESeq2 analysis with different normalisation methods, as indicated in the table header. For each of these methods, the table lists the genes’ base mean (log₁₀), differential expression (log₂(fold change)) and false discovery rate (FDR, negative decadic logarithm, i.e. – log₁₀; note that the threshold FDR = 0.05 equates to –log₁₀(0.05) = 1.30 and all genes with larger value are hence significant in terms of differential regulation). The gene names were derived from blasting of the transcriptome sequences to which the smallRNA library was aligned, and are used in the following text for in-depth discussion. “n.d.” indicates that a gene was not detected as significantly differentially regulated by a particular analysis method.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>DESeq2 GLM normalisation</th>
<th>DESeq2 GLM normalisation and lfcShrink</th>
<th>Quantile normalisation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log₁₀(base mean)</td>
<td>log₂(fold change)</td>
<td>log₁₀(FDR)</td>
</tr>
<tr>
<td><strong>Genes of interest and differentially regulated in adult samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tax-2</em></td>
<td>1.99</td>
<td>-2.40</td>
<td>5.81</td>
</tr>
<tr>
<td><em>sinh-1</em></td>
<td>2.30</td>
<td>-2.65</td>
<td>4.74</td>
</tr>
<tr>
<td><em>osta-1</em></td>
<td>1.75</td>
<td>-7.77</td>
<td>1.92</td>
</tr>
<tr>
<td><em>glit-1</em></td>
<td>1.30</td>
<td>-2.30</td>
<td>2.03</td>
</tr>
<tr>
<td><em>best-24</em></td>
<td>1.91</td>
<td>-2.11</td>
<td>5.68</td>
</tr>
<tr>
<td><strong>Genes of interest and differentially regulated in L1 samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aex-6</em></td>
<td>1.99</td>
<td>-3.12</td>
<td>2.82</td>
</tr>
<tr>
<td><em>his-24</em></td>
<td>1.14</td>
<td>-7.27</td>
<td>4.52</td>
</tr>
<tr>
<td><em>cec-1</em></td>
<td>0.76</td>
<td>-6.56</td>
<td>1.82</td>
</tr>
<tr>
<td><em>ergo-1</em></td>
<td>1.30</td>
<td>-9.22</td>
<td>6.12</td>
</tr>
</tbody>
</table>
Chapter 3 – Molecular mechanism of ascaroside-mediated signal transmission.

Small RNAs complementary to putative target genes were blasted against the genome of *C. elegans* to shed light on their functions. A comparison of the pool of small RNAs between mothers and progeny exposed to supernatant revealed changes of the level of six genes which occurred both in supernatant exposed hermaphrodite mothers, and in the progeny. Additional changes in gene abundance which did only occur in either the adults, or L1s, are summarized below. Only a limited number of genes indicating differentially regulated small RNAs could be identified by BLASTn, and some of these genes of special interest are presented below and summarised in Table 3-4.

Small RNA expression in adult hermaphrodites exposed to supernatant

The different normalisation approaches resulted in a different number of significantly differentially regulated genes. For the supernatant-exposed adults, these were:

- GLM normalisation in DESeq2: 43 genes differentially expressed (8 increased, 35 decreased, 10,610 unchanged)
- GLM normalisation with log2(fold change) shrinkage: 22 genes differentially expressed (7 increased, 15 decreased, 12,930 unchanged)
- Quantile normalisation: 40 genes differentially expressed (9 increased, 31 decreased, 12,011 unchanged).

Differentially regulated and identified (BLASTn) RNAs were found mostly to be complementary to genes which in *C. elegans* are expressed in neurons (discussed in detail in the following), and which hence in *A. freiburgensis* might potentially regulate the sensing of specific molecules present in the supernatant. Five differentially regulated genes were considered of particular interest due their function (as discussed in the following) which was inferred by identification of complementary genes by BLASTn. Two of these genes were found to be differentially regulated by all three normalisation methods employed, and were complementary to *tax-2* and *sinh-1* (see Table 3-4). Three additional genes were only found differentially regulated by two of the methods and not after the log2(fold change) shrinkage (complementary to *osta-1, glit-1* and *best-24*; see Table 3-4).

*tax-2* and *tax-4* encode two subunits of a cyclic nucleotide-gated channel. A genetic study in *C. elegans* indicated that the TAX-2 protein, required for normal olfaction,
chemosensation and thermosensation, is localised to developing axons and the cilia of sensory neurons.\textsuperscript{164} \textit{tax-2} and \textit{tax-4} have been reported to be involved in the developmental decision between dauer and non-dauer larva formation, either promoting or inhibiting dauer, in a context-dependent manner.\textsuperscript{236} A recent study in \textit{C. elegans} indicated that the cGMP-gated channel \textsc{TAX}2/\textsc{TAX}4 plays a role in the detection of a potential nematode alarm pheromone, perhaps a novel ascaroside or a mixture of ascaroside and non-ascaroside compounds, released from injured conspecifics.\textsuperscript{237} Interestingly, the cGMP-gated channel \textsc{TAX}2/\textsc{TAX}4 has been suggested to be required in \textit{C. elegans} for the response to the ascaroside \textsc{ascr}#3,\textsuperscript{238} and this ascaroside can induce an increase in hermaphrodite sex-phenotype in the progeny of \textit{A. freiburgensis} hermaphrodite mothers, as shown in Chapter 1 of this thesis.

\textit{sinh-1} in \textit{C. elegans} encodes a homolog of \textit{Schizosaccharomyces pombe} \textit{SIN1} (stress-activated MAP kinase interacting protein 1).\textsuperscript{239} In \textit{S. pombe}, the inactivation of the protein \textit{SIN1} led to a higher degree of sensitivity with respect to several environmental stresses (e.g., high temperature and osmotic stress).\textsuperscript{240} In \textit{C. elegans}, reduction of \textit{sinh-1} activity \textit{via} RNAi resulted in increased tolerance against high temperature and oxidative stress, enhanced stress response and enhanced dauer formation. Hence, in \textit{C. elegans sinh-1} has been suggested to be involved in the modulation of stress responses by preventing hypersensitive reactions.\textsuperscript{239}

\textit{osta-1}, expressed in the sensory neurons in \textit{C. elegans}, encodes an organic solute transporter alpha-like protein 1.\textsuperscript{241} In \textit{C. elegans}, the \textit{OSTA-1} protein, by modulating trafficking pathways, has a role in determining the morphology as well as the protein composition of sensory neurons. It has been suggested that some external and internal factors can shape the architecture of specific cilia and the protein composition of sensory neurons. An active mechanism of regulation of cilia, which implies a balance between exocytosis and endocytosis, is crucial in order to preserve a cellular homeostasis. In this context, \textit{OSTA-1} plays an important role in regulating transport pathways, contributing to modulation of the ciliary architecture of neurons upon different inputs.\textsuperscript{241}

In \textit{C. elegans}, \textit{glit-1} (gliotactin (\textit{Drosophila} neuroligin-like) homologue) encodes a transmembrane protein expressed in dopaminergic neurons, pharynx, intestine and
several cells in the head. GLIT-1 is homologous to neuroligins, transmembrane proteins on the postsynaptic membrane, which are involved in the formation and preservation of synapses between neurons. In *C. elegans*, *glit-1* has been found to confer protection against oxidative stress.\(^{242}\)

*best-24* encodes a bestrophin-like protein within the family of calcium-activated chloride channels located in the plasma membrane, and is expressed in the nervous system of *C. elegans*.\(^{243}\)

**Small RNA expression in L1s exposed to supernatant**

In the L1 progeny from supernatant-exposed mothers, the numbers of significant differentially regulated genes with the different normalisation methods were:

- GLM normalisation in DESeq2: 278 genes differentially regulated (55 increased, 223 decreased, 6258 unchanged)
- GLM normalisation with log2(fold change) shrinkage: 265 genes differentially regulated (45 increased, 220 decreased, 6615 unchanged)
- Quantile normalisation: 432 genes differentially regulated (194 increased, 238 decreased, 2858 unchanged).

Four differentially regulated genes in the L1 samples were considered of particular interest due their function (as discussed in the following) which was inferred by identification of complementary genes by BLASTn. All of these genes were found to be differentially regulated by all three normalisation methods employed and were complementary to *aex-6*, *his-24*, *cec-1* and *ergo-1* (see Table 3-4). One differentially regulated gene, *aex-6*, in *C. elegans* plays a role in the dauer pathway,\(^{244}\) consistent with the fact that, when in the presence of supernatant, *A. freiburgensis* mother hermaphrodites produce progeny which pass through the obligatory stage of dauer.

Interestingly, it was found that genes which in *C. elegans* play a role in epigenetic mechanisms are differentially regulated, for instance *his-24*.\(^{245}\) *his-24* encodes one of eight *C. elegans* H1 linker histones. Linker histones are chromatin proteins that associate with the fundamental subunit of chromatin, the nucleosome. *his-24* has been found also to play a role in the developmental modulation of germ line chromatin architecture in *C. elegans*. It has been observed that a *his-24* mutant’s (deletion) germ line displayed an increase in the level of methylation on histone 3
lysine 4 (H3K4), a mark of active chromatin, and a decrease in the level of methylation in histone 3 lysine 9 (H3K9), a hallmark of transcriptionally silenced chromatin. HIS-24 is also essential for maintenance of the histone trimethylation H3K27me3.245,246

**cec-1** encodes a nuclear protein which contains a chromodomain (**chromatin organization modifier**), a highly conserved motif encompassed of 48 amino acids. In *C. elegans*, the function of **cec-1** has not been revealed, however it has been suggested that this chromatin-associated protein may constitute a regulatory gene.247

**ergo-1** encodes an Argonaute protein which plays a role as primary Argonaute in the endo-siRNA pathway, being involved particularly in siRNA biogenesis.248,249 Most importantly, the argonaute ERGO-1 is involved in the production of small RNAs from mRNAs and the production of secondary 22G small RNAs. As such, it fulfills an important role both in establishing, and enhancing small RNA functions.

**Histone modification analysis**

In species such as *C.elegans*, *D. melanogaster* and *S. pombe*, histone methylations have been reported to have a role in the context of epigenetic inheritance.8 Methylation of lysine (K) within the histone H3 N-terminal tail has been suggested to play an important role in the inheritance of acquired phenotypes, since this modification displays a high degree of stability and at the same time the regulation of this modification occurs in a dynamic-manner.234 Histone methylations, by modulating DNA accessibility, can lead either to transcriptional activation (e.g., H3K4me3, H3K4me2, H3K36me3) or repression (e.g., H3K9me3, H3K9me2, H3K27me3).234

With the aim to shed light on the underlying mechanisms which regulate the shift in progeny sex-phenotype towards hermaphrodites upon exposure of *A. freiburgensis* parental generations to supernatant, an investigation of the chromatin status of the hermaphrodite mothers’ gonads was conducted. Antibody immunostaining was used to reveal the methylation pattern of supernatant exposed mothers’ gonads compared to those from non-exposed controls.
Using antibodies against H3K27me3, H3K9me2, H3K36me3, no differences were observed in dissected gonads, i.e. no fluorescent signal was detected in the immunostaining microscopy analysis. However, differences were observed by using an antibody against the mark H3K9me3. The histone methyltransferase (HMT) MET-2 mediates the deposition of mono- and dimethyl groups at H3K9, while the methyltransferase SET-25 is involved in the trimethylation of the same residue.²⁵⁰

Animals exposed to supernatant displayed a less strong expression of H3K9me3 in the “mitotic region” of the gonad (distal end) compared to the control (Figure 3-17). In gonads isolated from hermaphrodites grown in isolation, most (73%, n = 67) had a high level of the H3K9me3 mark. In gonads derived from animals exposed to supernatant, most (81%, n = 91) showed only low levels of H3K9me3. As in C. elegans, this mitotically active apical region, known also as pre-meiotic tip, serves as a stem cell niche, which can self-renew through mitosis and differentiate into specialised cells (gametes).²⁵²

Hence, considering that H3K9me3 represents a hallmark of transcriptionally silenced chromatin, the low level of expression in the gonad in treated animals compared to the control could indicate an activation of expression of some genes, whose identity cannot be inferred from this analysis, though this would be in accordance with a potential mechanism of intergenerational inheritance for the observed supernatant-induced shift in progeny sex-phenotype in A. freiburgensis.
Figure 3-17. H3K9me3-immunostain and DAPI-stained adult hermaphrodite (dissected) gonads. Shown are overlays and individual layers of the H3K9me3 immunostaining (red) and DAPI (4',6-diamidino-2-phenylindole) reference stain (blue). The left column shows the micrographs of a gonad from a control worm (no supernatant exposure), and the gonad of a supernatant-exposed worm is shown in the right column. Note that in the latter a lower fluorescence in the distal tip of the gonad (pre-meiotic tip) indicates a reduced expression of the H3K9me3 mark. The pre-meiotic tip (white rectangle) is the area distal to the crescent-shaped nuclei peculiar of early meiotic prophase (leptotene and zygotene) that represent the “transition zone”.

Images were recorded by Pedro Robles Naharro.
Conclusion

In this thesis, it has been shown that *A. freiburgensis* maternal prediction of the environment which the offspring will face induces a modulation in offspring phenotype (Chapter 1). Applying crowding cues from *A. freiburgensis* culture supernatant shifted the fraction of dauer entry phenotype in the offspring of hermaphroditic mothers, hence increasing their fitness to crowded conditions. When in favourable condition, mother hermaphrodites produce non-dauer larvae, which then become female. Sensation of chemical cues indicating high population density (culture supernatant) through amphid neurons (Chapter 2) triggers a mechanism which induces dauer entry in the offspring. Although many examples are known of parental influence on the phenotype of the progeny, the mechanisms by which this is achieved are poorly understood.

To shed light on the mechanisms which in *A. freiburgensis* regulates the mother’s response to environmental conditions and to understand how signals received by the soma (neurons) are relayed to the germline, determining the sex phenotype of the progeny by directing a binary developmental decision, epigenetic mechanisms were investigated in the present Chapter 3.

To understand if an endogenous response could trigger small RNA inheritance, acting as “memory” of past environmental experiences and being able to target mRNAs even in the absence of the original trigger (for offspring) represented by supernatant exposure, a RNA-seq analysis of small RNAs was performed.

It was found in this analysis that an exposure of *A. freiburgensis* hermaphrodites to supernatant crowding cues induces a differential regulation of several genes, and furthermore, small RNA level changes were present in the progeny (L1 larvae) of exposed mothers. This taken together raises the possibility that environmental information can modulate the pool of transmitted small RNAs in *A. freiburgensis*.

In order to understand which function the small RNAs with changed expression level might have, complementary partners were identified from an *A. freiburgensis* transcriptome. Since no annotated genome was available, DNA isolation with the
aim of genome sequencing was done (see Appendix 1 & 2), but while the sequencing was meanwhile successful, the annotation is still ongoing.

Instead, as a first insight, the identified transcripts were compared to the genome of *C. elegans*. Several transcripts with changed snRNA mapping in *A. freiburgensis* supernatant exposed adults were homologues of *C. elegans* genes, particularly genes which are expressed in neurons and play a role in chemosensation. Interestingly, small RNAs from *A. freiburgensis* were found that are complementary to *tax-2*, which has been suggested to play a role in *C. elegans* in the response to the ascaroside ascr#3. This correlates with the observation that *A. freiburgensis* hermaphrodite mothers react to the presence of this ascaroside, leading to a higher fraction of hermaphrodite progeny, as shown in Chapter 1 of this thesis. Moreover, for *sinh-1*, which in *C. elegans* regulates stress responses, another complementary small RNA was found, raising the possibility that this small RNA might be related to the regulation of a stress response in *A. freiburgensis*.

Progeny of mothers previously exposed to supernatant also showed a change in the pool of small RNAs, compared to the non-exposed control. These small RNAs were complementary to genes which in *C. elegans* play a role in epigenetic mechanisms. Particularly interesting was the identification of a differentially expressed small RNA which potentially regulates a gene similar to *ergo-1*, a *C. elegans* Argonaute protein that plays a central role in the pathway of endogenous small RNA and triggers the production of secondary small RNAs. These secondary small RNAs, which mediate regulation of gene expression, have been suggested to generate transgenerational effects, preventing dilution of small RNAs across generations, and the amplified secondary effectors can amplify an initial gene regulating response, even in the absence of the initial trigger.

Moreover, in *C. elegans*, small RNAs can drive chromatin modifications. Chromatin modifiers, such us the histone H3 lysine-9 (H3K9) methyltransferases SET-25 and SET-32, and the histone methylation-binding protein HPL-2 (Heterochromatin Protein 1-Like 2), have been shown to play a crucial role in long-term RNAi. Consistent with this, *A. freiburgensis* small RNAs complementary to some genes that in *C. elegans* play a role in the modulation of chromatin architecture, for instance *his-24*, were identified. A *his-24* mutant of *C. elegans*
was reported to show a decrease in the level of methylation in histone 3 lysine 9 (H3K9), a hallmark of transcriptionally silenced chromatin, in its germline.

Strikingly, changes in the H3K9me3 mark in *A. freiburgensis* gonads are presented by immunostaining examinations after exposure to supernatant, which revealed a decreased methylation in the gonad’s pre-meiotic tip. This observation correlates with the above-mentioned change in small RNA complementary to *his-24*, and supports the hypothesis of crowding cue signals being intragenerationally inherited in *A. freiburgensis* via small RNAs and histone modifications.

To further strengthen this hypothesis, future experiments could address the potential mechanism of epigenetic signal transmission in *A. freiburgensis*. Having identified the role of chromatin modifications mediated by H3K9me3 marks, an analysis of further histone marks could be done. For instance, H1 linker histones can act in synergy with H3K9me3 marks in regulating gene expression in *D. melanogaster*. There, it was found that the H3K9me3 mark is insufficient to control gene expression; after knocking down the linker histone H1, a transposon initially silenced by a piRNA escaped from the regulation, even when the level of H3K9me3 was stably maintained.253

To better understand the effect of small RNAs identified in this thesis for differential expression after supernatant exposure, the resulting levels of protein expression could be analysed, for example by immunostaining or proteomics experiments. While RNAseq analysis does not tell the effect a small RNA will have, the derived information on small RNA levels could hence advise further analysis by identifying genes and proteins of primary interest, and these analyses in turn can reveal the mechanism of small RNA signal transmission in *A. freiburgensis*. 
Appendix

Appendix 1 – Isolation of DNA for Illumina sequencing (Materials & Methods)

Isolation of clean high-quality DNA for DNA-seq

DNA extraction was performed using the Gentra Puregene Kit from Qiagen based on the manufacturer’s protocol. Some steps were changed since insufficient yield was achieved. Worms were collected as dauers by using the split plate method (Figure A-1). Worms were pelleted and stored at -80 °C until required. Dauers collected from 200 split plates gave > 30 µg of very clean DNA (~100 µl of DNA at about ~400 ng/µl concentration). These quantification measurements were conducted by Edinburgh Genomics Facility.

Collection of a clean nematode sample:

Figure A-1. Collection of clean nematode samples using split plates.
Dauer positioned in the left part (on NGM medium) migrate to the right (M9 medium). Hence, these plates can be used for collecting clean worm samples for DNA isolation, without e.g. impurities from NGM media components or nematode culture excretions.

The approach to use split plates for collection of clean nematode samples uses the migratory nature of dauers, to collect the worms in a M9 buffer bath. The nematodes were cultured on normal NGM\textsuperscript{132} media (i.e. agarose solidified) on the NGM-side of split plates until crowded. M9 buffer\textsuperscript{132} (liquid) was added to the second half of the plate. The migratory movement of dauers over the partition and into the M9 bath
allowed their collection in large numbers with a reduced chance of bacterial contamination.

Molten NGM media was poured into one side of the split plate (9 cm) until the level reached the top of the partition and left to dry on a flat surface for 24 h. The amount of NGM is crucial, it needs to reach the correct level without overflowing on the other side; on the other hand, if it is too low, the dauers will not be able to migrate from the media to the M9 buffer bath.

The NGM media side was seeded with approximately 500 µl of OP50-1 bacteria and incubated at room temperature for 24-48 hours. Nematodes were added to the OP50-1 lawn and incubated at 20˚C in sealed plastic boxes (to reduce drying out of the media). When the plates became crowded and the bacterial food was almost depleted (usually 3 to 6 days), the plates were placed on a flat, stable surface, and sterile M9 buffer was added to the empty side of the split plate until it almost reached the top of the partition. Plates were incubated at room temperature overnight. Dauers could be observed swimming in the M9 media. If only few dauers were observed in the bath, the plates were left for another 24 hours.

Dauers (with the M9 media) were moved into eight 15 ml Falcon tubes using a 5 ml Pasteur pipette until the tubes were full. Worms were sedimented by centrifugation at 1,100 g for 5 minutes, and the supernatant was removed carefully without disturbing the pellet. More worms were harvested into the same tubes. This step was repeated until all the worms were harvested and a large worm pellet was obtained. After the last centrifugation, the pellets (wet pellet, ca. 100-300 µl volume) were transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 1,100 g. Excess liquid was removed and the sample was frozen at -80 ˚C until required.

DNA extraction with Gentra Puregene Kit (Qiagen):

The frozen worm pellet (wet pellet, see above, ca. 100-300 µl) was thawed at room temperature and it was aliquoted into 50 µl aliquots in 1.5 ml Eppendorf tubes. To remove any excess liquid, sedimentation of worms was performed by a centrifugation step, 5 minutes at full speed (bench top centrifuge).
Appendix

500 μl of lysis solution and 3 μl of proteinase K, both supplied as part of the extraction kit, were added to each sample. Each tube was incubated at 65 °C for 4 hours. Temperature was decreased from 65 °C to 55 °C for 1 hour. If necessary (i.e. if the pellet was not fully transparent and/or showed flocks), more proteinase K (1.5 μl) was added and the samples were incubated for additional 30 minutes. Afterwards, 3 μl of RNase A solution (supplied as part of the extraction kit) were added into each tube. Each sample was incubated in a heatblock at 37 °C with shaking (700 rpm) for 1 hour.

The sample was cooled on ice for 5 min. 170 μl of protein precipitation buffer (part of extraction kit) were added and the tube was inverted several times to mix. Afterwards, the tube was cooled on ice. Inversion (25 times each) and cooling steps were repeated 3 times, totaling 5 minutes duration. Centrifugation of each sample was performed for 3 minutes at 15,000 g at 4 °C. Tubes were incubated again on ice for 3 minutes, before another centrifugation step.

Supernatant was collected and moved to an Eppendorf 1.5 ml tube being careful not to disturb the pellet. To reduce DNA shearing, supernatant was either poured or it was pipetted by using a cut tip. Centrifugation was repeated to pellet any carried over protein. The supernatant was transferred again to a fresh tube. Note that this additional centrifugation step gave much cleaner DNA. 500 μl of isopropanol were added, and each tube was inverted gently for 50 times. To pellet the DNA, another centrifugation step was performed, at 15,000 g for 1 minute at 4 °C.

The supernatant was discarded and the pellet was washed in 400 μl of 70 % ethanol. Supernatant was discarded again and the pellet allowed to air dry at room temperature. Afterwards, 30 μl of Qiagen Elution Buffer (EB - Tris 10 mM, pH 8.5) was added to the pellet to dissolve the DNA.

DNA quality was checked by running 1 μl of DNA on a 1 % agarose gel. The DNA gives a distinct band of high molecular weight.

RNase treatment and phenol chloroform purification of samples:

If samples showed RNA contamination, which was seen as a smear at the bottom of the agarose gel, it was removed by retreating the isolated DNA with RNase A.
Afterwards, DNA was re-purified using Phenol:Chloroform:Isoamyl Alcohol based extraction.

0.2 µl of RNase A (Gentra puregene kit) were added and samples incubated for 5 minutes at 37 °C (700 rpm). If the volume of the sample was small, nuclease free water was added to give a final volume of 500 µl. This ensured the sample was not lost during the extraction. An equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1 volume parts; pH adjusted to 7.8 with Tris/HCl) was added and samples were mixed by gentle inversion until an emulsion formed. Afterwards, a centrifugation step was performed at maximum speed (bench top centrifuge) for 10 minutes. The upper aqueous phase was transferred to a fresh 1.5 ml tube. If the interface was very 'dirty' (observed as a white substance) the Phenol:Chloroform:Isoamyl Alcohol extraction was repeated.

Otherwise, an equal volume of chloroform was added and samples mixed by inversion, followed by a centrifugation step at maximum speed for 10 minutes, and the aqueous phase was transferred to a fresh tube. The nucleic acid fraction was recovered by standard precipitation with ethanol. The volume of the DNA sample was estimated, and 1/10 volumes of sodium acetate solution pH 5.2, 3 M (i.e. the final concentration is 0.3 M) was added. Samples were mixed well by gentle inversion. Subsequently, 2 volumes of -20 °C 100 % ethanol were added, and samples were incubated at -20 °C overnight to precipitate the DNA. A centrifugation step at maximum speed (benchtop centrifuge) for 15 minutes was performed, and the supernatant was removed ensuring that the white DNA pellet was not disturbed. The pellet was washed with 1 ml of 70 % ethanol. After a centrifugation step at maximum speed for 5 minutes, the ethanol was removed, and the pellet was left to air dry. Once the pellet was dried, it was resuspended in 20 µl of Qiagen EB (10 mM Tris, pH 8.0).

DNA quality was checked by running 1 µl of DNA on a 1 % agarose gel (the observation of one distinct high molecular band at the top of the gel and no RNA should remain). DNA isolation was performed in collaboration with Sally Adams.
Appendix

Appendix 2 – Isolation of DNA for Illumina sequencing (Results)

With the aim to sequence the *A. freiburgensis* genome, DNA from worm samples was isolated. Two sets of DNA were prepared. The first set was of good quality but not of sufficient quantity (referred to as codes 10468PA0001 and 10468PA0002 in the following). For the second set, DNA was isolated from worms collected from ca. 200 split plates. Both sets of DNA were of good quality and high concentration (codes 10468PA0003 and 10468PA0004).

All DNA samples were analysed by an agarose gel electrophoresis for initial quality control, and pictures of these gels are shown in Figures A-2 to A-5 for the different preparations (see Figure captions). The electrophoresis resulted in a single, high-molecular weight band, indicating integrity of the DNA samples, i.e. no considerable degradation had occurred during the isolation process. Furthermore, little to no RNA contamination is indicated by the fact that no smear in the low molecular weight area of the gel was observed, since RNA typically shows a diffuse signal in that low molecular weight region.

For genome sequencing, a total amount of ca. 10-15 µg DNA was required. The yields of the preparations are summarized in Table A-1, from which it is obvious that the first two preparations yielded an insufficient amount of DNA, while the other two were in a good range.

The samples were sequenced by collaborators from the group from Edinburgh Genomics facility, but genome assembly is not completed (in November 2017).
Appendix

Figure A-2. Preparation of DNA sample 10468PA0001.
Two genomic DNA samples were prepared by phenol:chloroform extraction and subjected to agarose (1 %) gel electrophoresis by loading 1 µl of sample (from ~20 µl preparations, see Materials and Methods). For size comparison, 1 µl of hyperladder I marker (144 ng / µl) was added on an extra lane. Staining of DNA bands was done with the fluorescent dye Safe-Red. After passing the electrophoretic assessment, the residual volumes of sample (accordingly, each ~19 µl) were combined.

Figure A-3. Preparation of DNA sample ID 10468PA0002.
Two genomic DNA samples were prepared by phenol:chloroform extraction and subjected to agarose (1 %) gel electrophoresis by loading 1 µl of sample (from ~40 µl preparations, see Materials and Methods). For size comparison, 1 µl of hyperladder I marker (144 ng / µl) was added on an extra lane. Staining of DNA bands was done with the fluorescent dye Safe-Red. After passing the electrophoretic assessment, the residual volumes of sample (accordingly, each ~39 µl) were combined.
Figure A-4. Preparation of DNA sample ID 10468PA0003.

Two genomic DNA samples were prepared by phenol:chloroform extraction and subjected to agarose (1 %) gel electrophoresis by loading 1 µl of sample (from ~60 µl preparations, see Materials and Methods). For size comparison, 1 µl of hyperladder I marker (144 ng / µl) was added on an extra lane. Staining of DNA bands was done with the fluorescent dye Safe-Red. After passing the electrophoretic assessment, the residual volumes of sample (accordingly, each ~59 µl) were combined.

Figure A-5. Preparation of DNA sample ID 10468PA0004.

Two genomic DNA samples were prepared by phenol:chloroform extraction and subjected to agarose (1 %) gel electrophoresis by loading 1 µl of sample (from ~60 µl preparations, see Materials and Methods). For size comparison, 1 µl of hyperladder I marker (144 ng / µl) was added on an extra lane. Staining of DNA bands was done with the fluorescent dye Safe-Red. After passing the electrophoretic assessment, the residual volumes of sample (accordingly, each ~59 µl) were combined.
Table A-1. Overview of DNA samples.
Concentration, yield and DNA integrity number (DIN, assessed by Edinburgh Genomics facility; a sample quality assessment value) are shown. Note in addition the respective gel electrophoretic analyses of DNA-preparations in the Figures A-2 to A-5.

<table>
<thead>
<tr>
<th>Internal sample ID</th>
<th>Average Concentration (ng/µl)</th>
<th>Volume</th>
<th>µg yield</th>
<th>DNA integrity number (DIN)</th>
</tr>
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<tr>
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<td>9</td>
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<td>75,4</td>
<td>68</td>
<td>5,13</td>
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<tr>
<td>10468PA0004</td>
<td>447</td>
<td>105</td>
<td>46,94</td>
<td>7,9</td>
</tr>
</tbody>
</table>
Appendix 3 – RNAseq analysis with quantile normalisation

Figure A-6. Density plot of the count data distribution from RNAseq of adult samples. The density() function in R was used on the log_{10}() of the count data before and after normalisation. Shown are the density distributions for the raw data (upper figure), DESeq2-normalised data (GLM-method, middle) and Quantile-normalised data (lower; for further information on the normalisation methods see Materials and Methods in Chapter 3).
**Figure A-7.** Density plot of the count data distribution from RNAseq of L1 samples.

The `density()` function in R was used on the log10() of the count data before and after normalisation. Shown are the density distributions for the raw data (upper figure), DESeq2-normalised data (GLM-method, middle) and Quantile-normalised data (lower; for further information on the normalisation methods see Materials and Methods in Chapter 3).
Figure A-8. Count distribution for raw and quantile-normalised counts for the adult samples.
A boxplot representation is used, showing the medians (bold horizontal lines), quartiles (box horizontal lines showing the 25 and 75 % quartiles), outliers (individual data points), and the whiskers indicate the highest data point within 1.5 times the interquartile range (75 % quartile minus 25 % quartile) beyond the boxes (i.e. the quartiles). Note that the counts are shown as log_{10}, which explains why for samples with large numbers of zero reads the quartile-boxes are not seen in the plots.
Figure A-9. Count distribution for raw and quantile-normalised counts for the L1 samples.
A boxplot representation is used, showing the medians (bold horizontal lines), quartiles (box horizontal lines showing the 25 and 75 % quartiles), outliers (individual data points), and the whiskers indicate the highest data point within 1.5 times the interquartile range (75 % quartile minus 25 % quartile) beyond the boxes (i.e. the quartiles). Note that the counts are shown as log₁₀, which explains why for samples with large numbers of zero reads the quartile-boxes are not seen in the plots.
**Figure A-10.** Principle Component Analysis (PCA) of quantile-normalised, regularized log-transformed reads (adult samples).

5 adult samples (green: control, orange: supernatant) shown in the 2D plane spanned by their first two principal components. Samples show larger variability between experimental conditions than between replicates (i.e. clear separation in PC1). Analysis was done by using the DESeq2 function plotPCA().
Figure A-11. Principle Component Analysis (PCA) of quantile-normalised, regularized log-transformed reads (L1 samples).

6 L1 samples (green: control, orange: supernatant) shown in the 2D plane spanned by their first two principal components. Samples show larger variability between experimental conditions than between replicates (i.e. clear separation in PC1). Analysis was done by using the DESeq2 function plotPCA().
Figure A-12. Heatmap of regularized log-transformed reads after quantile normalisation (adult samples).

This heatmap indicates the similarities in between the respective adult samples by a grey-scale and a hierarchical clustering method (i.e. similar samples are organized next to each other). The different individual samples (with colour coding orange for supernatant, and green for controls) are compared against each of the other samples, with a black square being most similar (here, this is the comparison of a sample with itself) and white the most dissimilar (greyscale indicating distance values from 0 (white, dissimilar) to 100 (black, equivalent)). The dendrogram shows the sample relation in a hierarchical manner, with the closest related samples being clustered together first.
Figure A-13. Heatmap of regularized log-transformed reads after quantile normalisation (L1 samples).

This heatmap indicates the similarities in between the respective L1 samples by a grey-scale and a hierarchical clustering method (i.e. similar samples are organized next to each other). The different individual samples (with colour coding orange for supernatant, and green for controls) are compared against each of the other samples, with a black square being most similar (here, this is the comparison of a sample with itself) and white the most dissimilar (grayscale indicating distance values from 0 (white, dissimilar) to 100 (black, equivalent)). The dendrogram shows the sample relation in a hierarchical manner, with the closest related samples being clustered together first.
Figure A-14. Volcano plot for small RNA expression in supernatant-exposed adults vs. controls after applying a quantile normalisation.

The green points indicate genes-of-interest that show both large-magnitude log₂(fold change) (x-axis) as well as high statistical significance ($-\log_{10}$ of a Benjamini-Hochberg corrected Wald test p-value, y-axis) (false discovery rate (FDR)) < 0.5 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression).
Figure A-15. Volcano plot for small RNA expression in supernatant-exposed L1s vs. controls after applying a quantile normalisation.

The green points indicate genes-of-interest that show both large-magnitude log₂(fold change) (x-axis) as well as high statistical significance ($-\log_{10}$ of a Benjamini-Hochberg corrected Wald test p-value, y-axis) (false discovery rate (FDR)) < 0.5 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression).
Figure A-16. MA plot for adult samples with quantile normalisation. Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald test p-value (false discovery rate (FDR)) < 0.05 and log₂(fold change) > 2 (increased expression) and < 2 (reduced expression) are colored in red. The grey horizontal line indicates log₂(fold change) = 0.
Figure A-17. MA plot for L1 samples with quantile normalisation.

Small RNAs below the significance threshold of a Benjamini-Hochberg corrected Wald test p-value (false discovery rate (FDR)) < 0.05 and log2(fold change) > 2 (increased expression) and < 2 (reduced expression) are colored in red. The grey horizontal line indicates log2(fold change) = 0.
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